

ELECTRON TRANSPORT REACTIONS OF DENITRIFICATION.



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Abstract. Electron transport reactions of denitrification.

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A study is reported which demonstrates that electron transport to the reductase reactions of denitrification in the bacterium *Thiosphaera pantotropha* can occur aerobically. Use of Clark-type electrodes has demonstrated that the N_2O reductase enzyme of this organism is active under aerobic conditions, and that O_2 and N_2O reduction can occur simultaneously. The reduction of NO_3^- to N_2 gas, even under aerobic conditions, is shown to proceed via NO as an intermediate. It is concluded that the reaction of NO with O_2 must be sufficiently slow that it does not effectively compete with the reduction of NO to N_2O .

The ability of *T. pantotropha* to catalyse aerobic NO_3^- reduction, the first step of the aerobic denitrification process, is shown to correlate with the expression of a NO_3^- reductase enzyme that is located in the periplasm. This periplasmic enzyme is expressed, and is active, under both aerobic and anaerobic conditions. A membrane bound NO_3^- reductase is also expressed, but only under anaerobic conditions, by this organism. This latter reductase resembles the NO_3^- reductase of *Paracoccus denitrificans* in respect of both its catalytic properties and the inhibition of activity in intact cells under aerobic conditions. Mutants of *T. pantotropha* that lack the membrane bound NO_3^- reductase, and not only retain but overproduce the periplasmic enzyme, have been obtained via Tn5 mutagenesis. The periplasmic NO_3^- reductase identified in *T. pantotropha* bears catalytic and structural similarities to an enzyme previously characterised in some strains of *Rhodobacter capsulatus*.

The ability of strains of *R. capsulatus* to reduce NO to N_2O is reported together with evidence that there is a discrete NO reductase in this organism. The electron transport pathway to NO reductase has been elucidated. The first identification of a denitrifying strain of *R. capsulatus* is reported.

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To my parents.

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Identification and properties of the periplasmic respiratory nitrate reductase from an overproducing strain of *Thiosphaera pantotropha*. (manuscript in preparation) D. J. Richardson, L. C. Bell and S. J. Ferguson.

Abbreviations.

APS	ammonium persulphate
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
DAD	diaminodurene
BSA	bovine serum albumin
DDC	1,2-diphenylthiocarbazone
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
FCCP	carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone
HOQNO	2-n-heptyl-4-hydroxyquinoline-N-oxide
(I)CM	(Intra) cytoplasmic membrane
MV ²⁺ (MV ^{•+})	methylviologen oxidized (reduced) form
NAD ⁺ (N)	nicotinamide adenine dinucleotide (reduced)
PAGE	polyacrylamide electrophoresis
PES	phenazine ethosulphate
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene-diamine
TMAO	trimethylamine-N-oxide
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
Tris	tris (hydroxymethyl)aminomethane
UQ	Ubiquinone
UQH ₂	Ubiquinol

Chapter 1. General Introduction.

1.1. The importance of denitrification.

The nitrogen cycle (Figure 1.1) comprises a series of oxidation and reduction reactions, which can be divided into assimilatory and dissimilatory reactions. The assimilatory reactions occur in certain groups of eukaryotes and prokaryotes, whereas the dissimilatory reactions are restricted to prokaryotic organisms. Denitrification, a dissimilatory process, is the term used to describe the process by which NO_3^- is returned to the atmosphere as N_2 gas. As shown in Figure 1.1 this process is known to involve four distinct reduction steps.

Observations of the transformation of the oxy-anions of nitrogen to gaseous products by micro-organisms were made towards the latter half of the nineteenth century (Smith, 1867; Schloesing, 1868; Gayon and Dupetit, 1882). The term "denitrification" was first introduced by Gayon and Dupetit (1886) to describe these phenomena. "Denitrification" is now, in its strictest terms, used to describe the reduction of NO_3^- to N_2 via the intermediates of NO_2^- , NO and N_2O , although, it has sometimes been used to describe the occurrence of just some of the reactions of the overall process. Despite the large number of papers published on varying aspects of denitrification over the last hundred years, our knowledge of the underlying molecular basis of this process still remains fragmentary. It is important that progress is made to fill the gaps in this knowledge because of the widespread implications of denitrification for man and the environment.

Denitrification has long been appreciated to play an important role in agriculture by removing NO_3^- from soils. NO_3^- is important for soil fertility and for maintaining

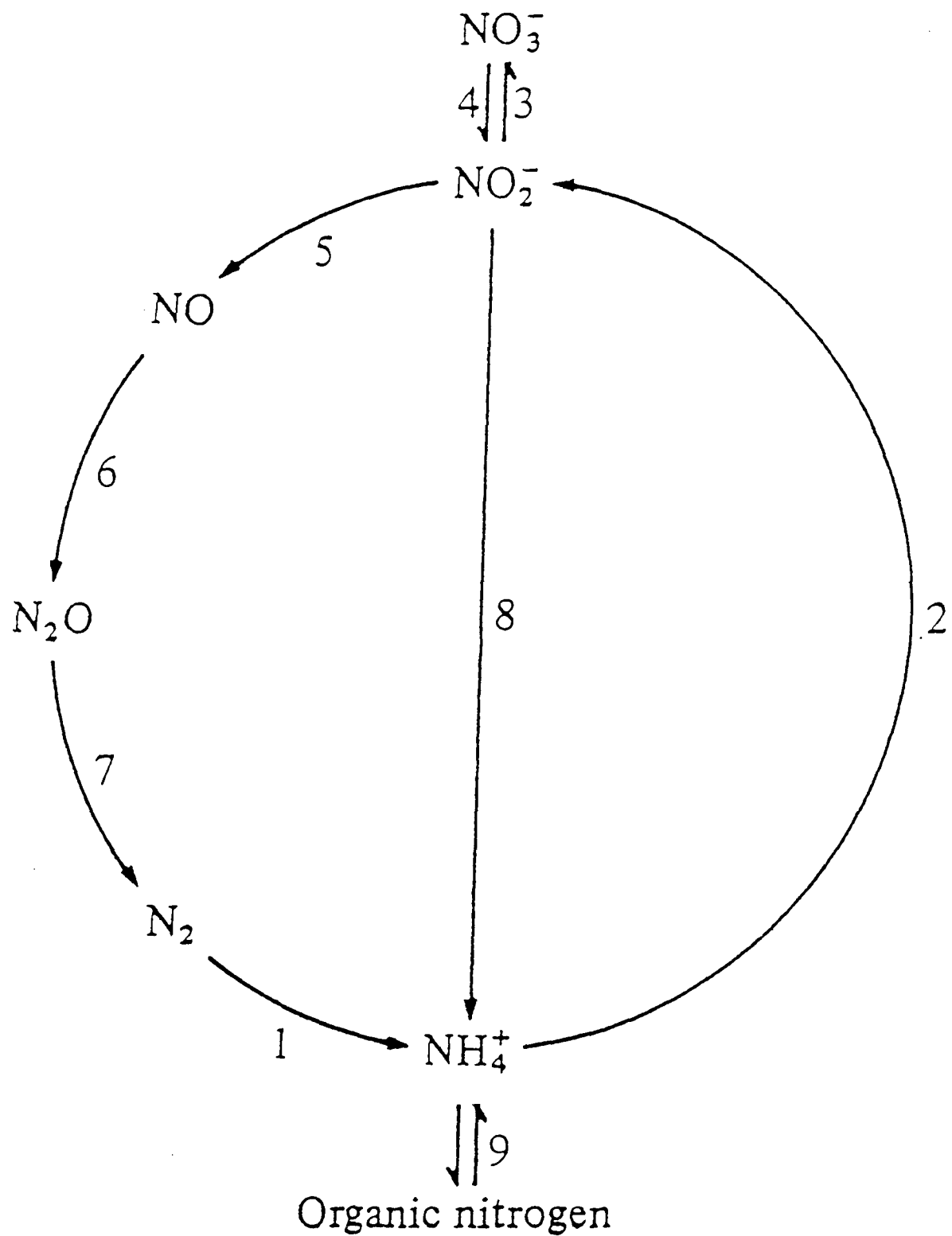


Figure 1.1. The nitrogen cycle.

Reactions are numbered as follows:-

1. Nitrogen fixation.
- 2 and 3. Nitrification.
4. Dissimilatory or assimilatory nitrate reduction.
- 4, 5, 6 and 7. The reactions of denitrification.
8. Dissimilatory or assimilatory reduction of nitrite to ammonia.
9. Ammonia assimilation.

good crop yields because NO_3^- is an important source of nitrogen for cellular biosynthesis (Freney and Simpson, 1983). This also involves reduction of NO_3^- and NO_2^- , but these assimilatory type processes are distinct from denitrification. Fertilizers, which are rich in NO_3^- , are needed to replenish the NO_3^- removed from soils by denitrification and other processes. The precise quantification of long term losses of nitrogen through denitrification in nitrogen balance studies is very difficult. It has been estimated that 10-50 % of applied fertilizer nitrogen is lost owing to denitrification in agricultural systems (Allison, 1955; Broadbent *et al.*, 1965; Focht and Stolzy, 1978). This deleterious effect of denitrification on agriculture is minimised by manipulation of the soil conditions in such a way to inhibit denitrification. The most cost effective method has been the widespread use of deep ploughing which breaks up and aerates the soil to depths of up to ten inches. Denitrification is generally regarded as a process that requires anaerobic conditions. This ploughing process greatly reduces the risk of development of soil conditions, such as anaerobiosis and waterlogging, where nitrogen losses through denitrification are serious (Freney and Simpson, 1983; The Royal Society, 1983).

Denitrification is currently exploited in various waste treatment processes. The direct addition of animal and plant residues to soil, the most primitive of disposal methods, leads to formation of zones of mineralization, nitrification (see Figure 1.1) and denitrification within the top layers of soil. For large scale treatment of municipal wastes sophisticated processes are necessary. Conditions favouring nitrification, which must precede denitrification if NH_4^+ is to be converted to N_2 (Figure 1.1), and denitrification (in general, aerobic and anaerobic conditions respectively) may be provided in different regions of the reactor, or at different times

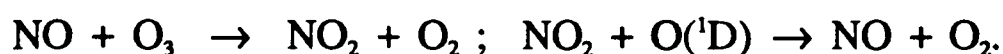
(for example, by intermittent aeration). Endogenous carbon sources are utilized to provide reductant for the denitrification reactions in such systems. Activities can be regulated by aeration, temperature control, and/or the carbon/nitrogen ratio of influent waste. The level of aeration is important in matching the relative nitrification and denitrification activities and maintaining the respective biomass populations. The removal of polluting NO_3^- and NO_2^- from water supplies is important. These ionic nitrogen-oxides, by providing a nitrogen source for growth, can promote the formation of algal blooms which causes severe O_2 depletion in even large bodies of water. This has serious consequences since growth of cyanobacterial blooms can release toxins into water supplies. The effects of such toxins range from unpleasant symptoms such as nausea or rashes in bathers to serious poisoning, in some cases leading to the death of livestock and man (Codd, 1984). Ingested NO_3^- can be reduced by enteric organisms to NO_2^- which can have deleterious effects. NO_2^- reacts with secondary and tertiary amines and amides to produce N-nitroso compounds which are known carcinogens (Magnes and Barnes, 1956; Mirvish, 1970; Magee, 1982). Haemoglobin reacts with NO_2^- to form methaemoglobin complex which has no affinity for O_2 . In young babies the presence of NO_2^- can result in a reduced capacity for transport of O_2 to the body tissues leading to the condition commonly known as "blue-baby syndrome" which can be fatal (Magee, 1977; 1982). These considerations indicate why it is necessary to minimise the dissolved NO_3^- and NO_2^- concentrations in water supplies.

It is not only NO_3^- and NO_2^- which pose threats to man and his environment, NO and N_2O also have deleterious effects. The stratospheric ozone layer, which lies above 16 km altitude, absorbs the potentially harmful ultraviolet component of solar

radiation. Upward diffusion of tropospheric N_2O into the stratosphere leads to a photochemical reaction in which NO is generated:

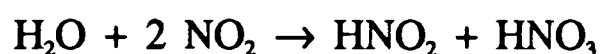


NO catalyses the destruction of ozone as follows (Johnson, 1972; Crutzen, 1970)



A great deal of concern has been expressed that extensive use of fertilizers may lead to increases in denitrification and N_2O production leading to depletion of the ozone layer; the consequences of which include global warming and an increased risk of skin cancers from increases in ultraviolet radiation. Early predictions suggested that more than 8 % of the ozone layer may be depleted (Crutzen, 1976). However, a comprehensive study between 1976 and 1980 suggested that N_2O production was increasing at a rate of 0.2 % per annum, and that this increase can be accounted for by combustion of fuels, suggesting agricultural sources are less important than had been previously thought (Weiss, 1981).

A further consequence of the reaction of NO with ozone is the formation of nitrogen dioxide (NO_2) which reacts rapidly with water to form nitric and nitrous acids :-



About 30 % of the total acid rainfall on the United Kingdom is dilute nitric acid.

The above considerations illustrate that denitrification plays important roles in many aspects of the environment. It is a process that is detrimental to agriculture. For agricultural purposes it would be advantageous to be able to inhibit the reactions

of denitrification. Conversely in other situations, for example, in the treatment of waste water, the stimulation of the denitrification process would be desirable. It is furthermore important in terms of atmospheric chemistry that the product of NO_3^- reduction is N_2 , not NO and N_2O . Therefore, it is significant that none of the reactions of denitrification are inhibited. These deleterious and beneficial consequences of the reactions of denitrification may be controlled or exploited to greater advantage if the molecular basis for these reactions is understood.

1.2 The occurrence of denitrification.

A variety of bacterial genera possess the capacity to use the nitrogen oxides instead of O_2 as respiratory electron acceptors. Jeter and Ingraham (1981) produced an extensive list of denitrifying bacteria which assigned this ability to some forty genera. However, these genera are biochemically and taxonomically diverse. Most are heterotrophs, although some grow autotrophically or photosynthetically. Denitrifying organisms express the reductases for reduction of NO_3^- to N_2 . However, some bacterial species lack one or more of these reductases and have been termed "partial denitrifiers".

Expression of the enzymes of denitrification is not exclusive of other reactions of the nitrogen cycle. Several N_2 -fixing genera including *Rhizobium*, *Rhodobacter* and *Rhodopseudomonas* also contain denitrifying species (O'Hara and Daniel, 1985; Satoh

et al., 1976; Preuß and Klemme, 1983). Some of the *Nitrosomonas* species which are autotrophic nitrifiers are also known to denitrify (Poth, 1986; Ritchie and Nicholas, 1972). The processes of NO_3^- assimilation and respiration can also simultaneously occur within a bacterial cell, although a separate NO_3^- reductase is involved in each reaction (Sias and Ingraham, 1979).

Most habitats have the potential to harbour denitrifying organisms. In a study by Gamble *et al.* (1977) denitrifiers were found in all soil types, at a wide range of temperatures, pH values, organic materials and water contents. There appears to be little correlation between these factors and the size of populations and/or the number of different isolates (Gamble *et al.*, 1977; Knowles, 1982).

1.3. The enzymes of denitrification.

Denitrification can be divided into four reduction steps (Figure 1.1), each of which is catalysed by a discrete enzyme. These reductase enzymes have been purified and characterised to varying extents from a number of sources. Each enzyme will now be discussed in detail.

1.3.1. Nitrate reductases.

Throughout the diverse range of species in which dissimilatory NO_3^- reduction has been investigated only two types of NO_3^- reductase have been identified and studied in detail, one of which is a membrane associated enzyme and the second a water-soluble periplasmic enzyme.

The membrane bound NO_3^- reductases of a number of denitrifiers including *Paracoccus halodenitrificans* (Rosso *et al.*, 1973), *Pseudomonas aeruginosa* (Carlson *et al.*, 1982), *Pseudomonas denitrificans* (Ishizuka *et al.*, 1984) and *Paracoccus denitrificans* (Forget, 1971; Calder and Lascelles, 1984; Craske and Ferguson, 1986; Ballard and Ferguson, 1988) have been identified. There is considerable variation both in the reported subunit compositions and the subunit molecular weights of the NO_3^- reductases from different sources (Hochstein and Tomlinson, 1988). This may in part reflect the methods of release from the membranes, proteolysis of the preparation, tendency for enzyme aggregation and possibly the presence of a modifying enzyme (Hochstein and Tomlinson, 1988). One subunit, the γ subunit, is particularly sensitive to heating in the presence of β -mercaptoethanol and SDS resulting in the failure to detect this subunit in many preparations (Craske and Ferguson, 1986).

The NO_3^- reductases from *P. denitrificans* and *Escherichia coli*, which is not a denitrifying organism but has the ability to reduce NO_3^- to NO_2^- which is in turn reduced to NH_3 , are remarkably similar with respect to both their catalytic and

molecular properties (Craske and Ferguson, 1986; Ingledew and Poole, 1984). The purified NO_3^- reductase from *P. denitrificans* comprises three polypeptides of molecular weight 127, 61 and 21 kDa named α , β and γ respectively (Craske and Ferguson, 1986). The α subunit contains a molybdopterin cofactor (Turner *et al.*, 1988) and is the catalytic site of NO_3^- reduction (Craske and Ferguson, 1986). In the absence of magnesium ions the α and β subunits can be released from cytoplasmic membrane vesicles into a soluble fraction, which suggests that it is the γ -subunit, an integral membrane component, that anchors these polypeptides to the membrane. These α and β polypeptides are thought to be substantially exposed to the cytoplasm of the cell. The γ polypeptide contains two *b*-type haems with mid-point potentials of +95 mV and +210 mV (Ballard and Ferguson, 1988). The nucleotide sequence of the *nar* operon, which encodes for the NO_3^- reductase of *E. coli*, has been extensively studied. This sequence indicates that the binding sites for the iron-sulphur centres, long known to be present in NO_3^- reductases, may be associated with the β -subunit (Blasco *et al.*, 1989). Electrons proceed from the electron transport chain to NO_3^- via ubiquinol (Parsonage *et al.*, 1986), but only if the γ subunit is present (Craske and Ferguson, 1986). This observation suggests that it is the *b*-type haem centres that accept electrons from ubiquinol.

Recent observations have shown that a membrane bound NO_3^- reductase similar to that found in *P. denitrificans* is present in some species of *Rhodobacter*. A membrane bound NO_3^- reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans* has been purified (Byrne and Nicholas, 1987). More recently *Rhodobacter capsulatus* strain BK5 has been shown to contain a membrane bound NO_3^- reductase, rather than a periplasmic enzyme which is found in other strains of *R. capsulatus* that express a

dissimilatory type NO_3^- reductase (Ballard *et al.*, 1990).

The presence of a second type of dissimilatory NO_3^- reductase has been reported in *R. sphaeroides* f. sp. *denitrificans* (Satoh *et al.*, 1976). This enzyme was shown to be a soluble, periplasmic enzyme (Sawada and Satoh, 1980) which exists as a complex of molybdenum-containing catalytic subunit of M_r 47 kDa, and several low M_r (13 kDa) *c*-type cytochromes (Satoh, 1981). The exact molecular composition of this complex is unknown as different preparations yielded complexes with molecular weights ranging between 60 and 112 kDa (Satoh, 1981). A periplasmic location has also been identified for the NO_3^- reductases of several strains of *R. capsulatus* (Alef and Klemme, 1979; McEwan *et al.*, 1984; Richardson *et al.*, 1990). The NO_3^- reductase purified from *R. capsulatus* strain AD2 possessed a molybdenum cofactor and was associated with a *c*-type cytochrome (Alef and Klemme, 1979). This enzyme (M_r 185 kDa) appeared to be a dimer of two identical subunits of M_r 85 kDa but it remained unclear as to whether the cytochrome formed a distinct polypeptide that escaped detection on gels. In contrast, McEwan *et al.* (1987) purified a molybdenum-containing monomeric NO_3^- reductase (M_r 90 kDa) of *R. capsulatus* strain N22DNAR⁺ which was not associated with a *c*-type cytochrome. However, more recent work has conclusively established that the NO_3^- reductase of *R. capsulatus* strain N22DNAR⁺ is associated with a *c*-type cytochrome, cytochrome *c*₅₅₂, which co-purifies with the polypeptide of M_r 90 kDa (Richardson *et al.*, 1990).

A few reports have appeared of soluble NO_3^- reductases in other bacterial genera, although the location of these reductases within the bacterial cell has not been established. The respiratory NO_3^- reductase of *Clostridium perfringens* is a soluble

non-haem iron-sulphur protein with a molecular weight of 90 kDa which functions independently of any cytochrome-containing electron transfer chain (Seki-cheba and Ishimoto, 1977; Seki-Cheba *et al.*, 1987). In *Aquaspirillum itersonii* a soluble NO_3^- reductase is co-expressed with a *c*-type cytochrome (Gauthier *et al.*, 1970). It seems probable that this NO_3^- reductase is a periplasmic enzyme, especially in light of the evidence that *c*-type cytochromes are always located in the periplasmic compartment or at the periplasmic face of the cytoplasmic membrane (Wood, 1983). In contrast to the extensive information available about the membrane bound NO_3^- reductase from *E. coli* and to a lesser extent this type of enzyme in other bacteria, almost nothing is known about the periplasmic enzyme (or enzymes).

1.3.2. Nitrite Reductases.

The dissimilatory reduction of NO_2^- is carried out by two distinct reductases. One is a metalloprotein containing copper (the copper NO_2^- reductase) and the other a haem protein that contains *c*- and *d*-type cytochromes (the cytochrome cd_1 NO_2^- reductase). In a situation that is reminiscent of the two NO_3^- reductases found within the *Rhodobacter* genera, there appears to be no rationale behind the distribution of the copper-, or cytochrome cd_1 -, enzymes among bacterial genera. Both types of NO_2^- reductases have been identified in species of genera *Pseudomonas* (Zumft *et al.*, 1987) and *Alcaligenes* (Iwaskasi and Matsubara, 1971; Katutani *et al.*, 1981a). However, a recent study of more than forty bacterial species failed to identify the presence of both types of NO_2^- reductase within a single strain of any organism (Coyne *et al.*, 1989).

The most studied NO_2^- reductase is cytochrome cd_1 . This was first identified in *P. aeruginosa* as an oxidase (Horio *et al.*, 1961) although the true physiological role as NO_2^- reductase was subsequently discovered (Yamanaka *et al.*, 1963). Cytochrome cd_1 has been purified and characterised from *P. aeruginosa* (Silvestrini *et al.*, 1979; Henry and Bessieres, 1984), *P. denitrificans* (Newton, 1969; Timkovich *et al.*, 1982), *Thiobacillus denitrificans* (Huynh *et al.*, 1982), *P. stutzeri* (Zumft *et al.*, 1979) and *Alcaligenes faecalis* IAM 1015 (Iwasaki and Matsubara, 1971). The structure of cytochrome cd_1 does not appear to vary between sources. It purifies as a dimer with a molecular weight of approximately 120 kDa, and comprises two identical subunits, each containing a *c* and a *d* haem. Despite the similarities in the structures of cytochrome cd_1 the location of these enzymes appear to vary between species. Most cytochrome cd_1 enzymes are water-soluble and periplasmic (Alefounder and Ferguson, 1980; Coyne *et al.*, 1990). However, the cytochrome cd_1 from *P. stutzeri* has a membrane associated component (Zumft and Vega, 1979), and this enzyme is entirely membrane bound in *P. halodenitrificans* (Grant and Hochstein, 1984). Nevertheless, the haems could still be exposed to the periplasmic compartment if the bulk of the polypeptide extends beyond the bilayer with a hydrophobic anchor at the N- or C-terminus.

The copper-containing NO_2^- reductases show considerable differences between sources with respect to the number and types of copper centres, molecular mass and tertiary structure of the enzyme (Masuko *et al.*, 1984). The copper-type NO_2^- reductase was initially obtained from *Ps. denitrificans* (Iwasaki *et al.*, 1963); now described as *Achromobacter xylosoxidans* (Shidara *et al.*, 1986). Subsequently, other

enzymes have been identified in *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972; Liu *et al.*, 1986), *Alcaligenes faecalis* S6 (Katutani *et al.*, 1981a), *Pseudomonas aureofaciens* (Zumft *et al.*, 1987) and *R. sphaeroides* f. sp. *denitrificans* (Sawada *et al.*, 1978; Michalski and Nicholas, 1985a).

Two copper-type NO_2^- reductases were found in the cytoplasmic fraction of *A. faecalis* S6 (Kakutani *et al.*, 1981a). The principal one was a green protein (M_r 120 kDa) composed of four subunits (M_r 30 kDa), two of which contained a type 1 copper and the others type 2 copper (Kakutani *et al.*, 1981a). The physiological electron donor to this reductase is a copper protein of M_r 12 kDa (Kakutani *et al.*, 1981b). Two subunit copper-type NO_2^- reductases are found in *A. xylosoxidans*, *P. aureofaciens* and *R. sphaeroides* f. sp. *denitrificans*. In the former two species the enzymes appear to contain only type 1 copper centres, like those found in low molecular weight electron transfer proteins such as azurin (Zumft *et al.*, 1987; Adman, 1985). The enzyme isolated from *R. sphaeroides* f.sp. *denitrificans* has a native molecular weight of 80 kDa. This enzyme comprises two nonidentical subunits with different isoelectric points and with molecular weights of 37.5 and 39.5 kDa, which contain type 1 and 2 copper respectively (Michalski and Nicholas, 1985).

The reduction product of the NO_3^- reductases is indisputably NO_2^- , whereas the product(s) formed by the catalytic action of the two types of NO_2^- reductase has been uncertain (see section 1.3.3).

1.3.3. Nitric oxide reductase.

A discrete NO reductase has only recently been established as an enzyme of the denitrification sequence. Previously there had been a great deal of controversy over the participation of NO as a freely diffusible intermediate of denitrification, but substantial evidence has now accumulated in favour of such a role for NO. As evidence against NO having such a role in denitrification, Garber and Hollocher (1981) reported that on incubation of ^{14}NO and $^{15}\text{NO}_2^-$ with cells of *P. denitrificans* a mixed isotope of N_2 (ie. $^{14}\text{N}^{15}\text{N}$), resulting from the reduction of $^{15}\text{NO}_2^-$ to ^{15}NO and then the reduction of ^{15}NO and ^{14}NO to $^{15,14}\text{N}_2\text{O}$ and onto $^{15,14}\text{N}_2$, was not formed. A similar result was observed in an analogous experiment with *P. stutzeri* (Aerssens *et al.*, 1986). Reports by Silverstrini *et al.* (1979) and Henry and Bessieres (1984) also argued against NO being an intermediate in denitrification on the basis of the observation that a preparation of cytochrome *cd₁* reduced both NO_2^- and NO to N_2O . The product of NO_2^- reduction has also been a matter of dispute. In the past, nitroxyl (HNO) (Kim and Hollocher, 1984), N_2O (Bessieres and Henry, 1984; Henry and Bessieres, 1984), NO (Yamanaka *et al.*, 1961; Matsubara and Iwasaki, 1971; Kucera *et al.*, 1987), hyponitrous acid ($\text{H}_2\text{N}_2\text{O}_2$) and oxyhyponitrite (N_2O_2^-) (Kluyver and Verhoeven, 1954) have all been proposed as the physiological product of NO_2^- reduction. All of these possible products, apart from NO, would indicate no requirement for a NO reductase. However, evidence has steadily accumulated in favour of NO as a freely diffusible intermediate of denitrification. Garber *et al.* (1982) found that anaerobically grown cells of *P. denitrificans* generated an NO-dependent proton motive force. Low concentrations of NO have been detected in

denitrifying cells by the sensitive technique of chemiluminescence (McKenny *et al.*, 1982). Shapleigh *et al.* (1987) observed that NO was released from several species of denitrifying bacteria in the presence of Triton X-100. This observation suggests that Triton X-100 may cause inhibition of NO reduction, thus increase the normally low steady state concentration of NO. Inhibition of the copper-type NO_2^- reductase activity did not inhibit NO reductase activity in cells of *A. cycloclastes* or *R. sphaeroides* f. sp. *denitrificans* (Shapleigh and Payne, 1985b). Mutants of *P. stutzeri* which lacked a functional NO_2^- reductase activity were shown to retain a wild type rate of NO reduction (Zumft *et al.*, 1988). The strongest evidence for NO as a free intermediate of dissimilatory NO_2^- reduction was obtained from experiments using extracellular traps to capture NO which is freely diffusible from denitrifying cells (Carr *et al.*, 1989; Goretski and Hollocher, 1988). The failure of the experiment described above of Garber and co-workers (1981) to isolate a mixed isotope of $^{15}\text{N}^{14}\text{N}$, is consistent with NO being an intermediate of denitrification when it is viewed in the light of the more recent evidence that NO inhibits the cytochrome cd_1 of *P. denitrificans* (Carr *et al.*, 1989).

Discrete NO reductases have recently been identified and purified from *P. denitrificans* and *P. stutzeri* (Carr and Ferguson, 1990a; Heiss *et al.*, 1989). The presence of such discrete reductases adds support to the argument that NO is an intermediate of the denitrification pathway. The NO reductase from *P. denitrificans* comprises four polypeptides of molecular masses 45, 37, 29 and 18 kDa. The 37 and 18 kDa polypeptides are associated with *b*-type and *c*-type haems respectively. The NO reductase from *P. stutzeri* also possesses a *c*- and *b*-type cytochromes, the former is associated with a polypeptide of M_r 17 kDa and the latter with a

polypeptide of M_r 38 kDa. When this protein was heated, a protein staining band of M_r 45 kDa was observed on SDS-PAGE analysis. The NO reductases from both sources appear to have molecular weights between 160-170 kDa on gel filtration. The visible absorption spectra of these two NO reductases are almost identical. However, although a certain amount of progress in understanding NO reductase has been made, a great deal more work will also be necessary to finalize the characterisation of the enzyme, or indeed to determine whether there is only ^{one} type of NO reductase found amongst denitrifying species. It will be necessary to reconstitute the fully functional enzyme into a phospholipid bilayer so it can accept electrons from its physiological donors. In contrast to the similarities of the two NO reductases described above, a separate report described a different preparation which contained NO reductase activity from *P. denitrificans*. This activity purified as large aggregates or vesicles (approximately 1000 kDa molecular mass) which lacked haem centres (Hoglen and Hollocher, 1989). It remains unresolved why preparations of the NO reductase should differ between the report of Hoglen and Hollocher (1989) and that of Carr and Ferguson (1990a). However, the higher specific activity, the presence of haem centres, the evidence for homogeneity and the similarity to the enzyme for *P. stutzeri*, all suggest that the preparation of Carr and Ferguson, (1990a) is more likely to reflect the properties of the NO reductase from *P. denitrificans*. Another, recent study reported the presence of seven or eight polypeptides (but no details of their molecular weights were given) from *R. sphaeroides* f. sp. *denitrificans* which were associated with NO reductase activity (Itoh *et al.*, 1989a). Both *b*- and *c*-type cytochromes were associated with this preparation, which had been purified from material containing the cytochrome *bc*₁ complex of this organism (Itoh *et al.*, 1989a).

1.3.4. Nitrous oxide reductase.

N_2O reductase catalyses the final step in the process of denitrification, i.e. the reduction of N_2O to N_2 . N_2O had been implicated as an intermediate in denitrification since early studies (Beijerinck and Minkman, 1910). Evidence from enzyme inhibition studies (Balderstone *et al.*, 1976; Yoshinari and Knowles, 1976) and tracer studies (St John and Hollocher, 1977) has firmly established N_2O as a free intermediate of denitrification. The reduction of N_2O is inhibited by C_2H_2 (Balderstone *et al.*, 1976), carbon monoxide, azide, cyanide and sulphide (Kristjansson and Hollocher, 1980, Sorensen *et al.*, 1980).

The N_2O reductase enzyme proved difficult to purify as in cell free extracts the reductase activity was rapidly lost, but the finding that copper was an essential metal involved in N_2O reduction paved the way for its isolation and characterisation. N_2O -dependent growth of *Alcaligenes sp.* and *P. perfectomarina* could not be sustained on copper depleted medium (Iwasaki *et al.*, 1980; Matsubara *et al.*, 1982). Cells grown anaerobically, in the presence of NO_3^- , in a copper depleted medium lacked N_2O reductase activity. Subsequently, when incubated with copper and rifampicin, such cells produced an active N_2O reductase (Matsubara *et al.*, 1982). These observations suggested that N_2O reductase contains copper and that in situations of copper deficiency a copper-deficient apoenzyme is synthesised. Zumft *et al.* (1985b) isolated several classes of transposon Tn5-induced mutants unable to use N_2O as a terminal electron acceptor. In one class of these mutants, the structural protein, designated the product of the *nosZ* gene, was present but the protein lacked copper. The *nosZ*

gene is encoded on a 2 kb region within the 8 kb *nos* region which controls N₂O respiration (Viebrock and Zumft, 1987; 1988). Mokhele *et al.* (1987) isolated mutants of *P. stutzeri*, using frameshift mutagenesis, that lacked the product of the *nosA* gene and produced an inactive, copper-free N₂O reductase. The *nosA* gene codes for another copper containing protein, NosA, of 65,000 kDa which is located in the outer membrane (Lee *et al.*, 1989). NosA has been proposed to function as a copper channel when the copper concentration is low (less than 10 µM). Copper, may at higher concentrations, enter the cell by a different route and become inserted into the N₂O reductase (Lee *et al.*, 1989; Clark *et al.*, 1989).

The purified N₂O reductases from several organisms have been shown to be multi-copper proteins (Zumft and Matsubara, 1982; McEwan *et al.*, 1985; Michalski *et al.*, 1986; Snyder and Hollocher, 1987). The properties of these enzymes appear to differ, although the extent to which these differences reflect the different methods of purification is unclear. The N₂O reductase has been characterised to a greater extent from *P. stutzeri* (formerly *P. perfectomarina*) than from other organisms. The enzyme from *P. stutzeri* exists in purple, pink, or blue forms designated forms I, II and III respectively (Coyle *et al.*, 1985). The pink and purple forms are obtained when purification takes place under aerobic and anaerobic conditions respectively. The purple form is several times more active than the pink form, particularly after alkaline activation. The inactive blue form is obtained when the enzyme is exposed to excess dithionite or ascorbate. The conversion of the purple form I to the blue form III is fully reversible, in the absence of O₂, by addition of ferricyanide (Riester *et al.*, 1989). The enzyme (M_r 120 kDa) consists of two identical subunits (M_r 74 kDa), and up to eight copper atoms have been identified per molecule of enzyme.

The EPR spectra of the purple and pink forms lack a signal characteristic of type 2 copper. The purple form of the enzyme appears to contain an unusual type 1 copper and several EPR silent copper atoms (Coyle *et al.*, 1985; Dooley *et al.*, 1987). Resonance raman spectroscopy indicates that the blue and purple forms of N_2O reductase contain the classical type 1 copper centres (Dooley *et al.*, 1987).

The N_2O reductase of *Alcaligenes xylosoxidans* has been purified as a violet protein containing five copper atoms per mole of enzyme (M_r 120 kDa) (Matsubara and Sano, 1985). This enzyme, which was purified without the exclusion of O_2 , produced an EPR spectrum characteristic of type 2 copper atoms but had lost its reductase activity. The N_2O reductase of *P. denitrificans* is extremely active if anaerobic conditions are maintained throughout its purification (Snyder and Hollocher, 1987). This enzyme (M_r 144 kDa) contained two identical subunits of M_r 70 kDa, each containing four copper atoms. Much of the copper of this enzyme was EPR silent, and the remaining copper produced a spectrum typical of an unusual type 1 copper centres. The N_2O reductases of *P. denitrificans*, *A. xylosoxidans* and *P. stutzeri* are regarded as O_2 sensitive enzymes.

N_2O reductase activity is found in the photosynthetic Rhodospirillaceae family (McEwan *et al.*, 1985). The enzyme from *R. capsulatus* N22DNAR⁺ has a single component polypeptide of M_r 76 kDa which is located in the periplasm. The N_2O reductase of *R. sphaeroides* f.sp. *denitrificans* has been purified without the exclusion of O_2 (Michalski *et al.*, 1986). The latter procedure yielded a purple enzyme (M_r 95 kDa) with four copper centres. Type 1 and type 2 copper atoms were detected by EPR analysis of this protein. Zinc and nickel (2 and 0.76 gram atoms per mole

of enzyme) were detected in this enzyme in addition to copper. Zinc has also been detected in the N_2O reductase of *P. perfectomarina* (Coyle *et al.*, 1985), but it remains uncertain whether this metal plays a role in the activity of the reductase.

The N_2O reductase has been purified to near homogeneity from the anaerobic bacterium *Wolinella succinogenes* (Teraguchi and Hollocher, 1989). This organism is taxonomically distant from denitrifying bacteria, but can grow using the dissimilatory reduction of NO_3^- and NO_2^- to NH_3 , and the reduction of N_2O to N_2 . The N_2O reductase of this organism was shown to contain a *c*-type cytochrome component. This enzyme was dimeric (M_r 162 kDa), with each subunit (M_r 88 kDa) containing three copper atoms and one iron atom (as a *c*-type cytochrome). This enzyme, in contrast to the N_2O reductases identified in other organisms, was stable in the presence of air for at least 100 h. at 4 °C and was not strongly inhibited by C_2H_2 (Teraguchi and Hollocher, 1989). To date, no other organism has been shown to possess an N_2O reductase which is associated with a cytochrome.

1.4. Bacterial electron transport processes.

The respiratory chains of bacteria reside within their cytoplasmic membranes. Each intermediate in the denitrification path is thought to serve as an acceptor of electrons from the respiratory chain of the cell. Many aspects of the organisation of electron transport chains have been best characterised for mitochondrial systems. Apart from a report that *Loxodes* can use NO_3^- as an electron acceptor (Finlay *et al.*, 1983; Finlay, 1985), mitochondrial electron transport chains differ considerably from

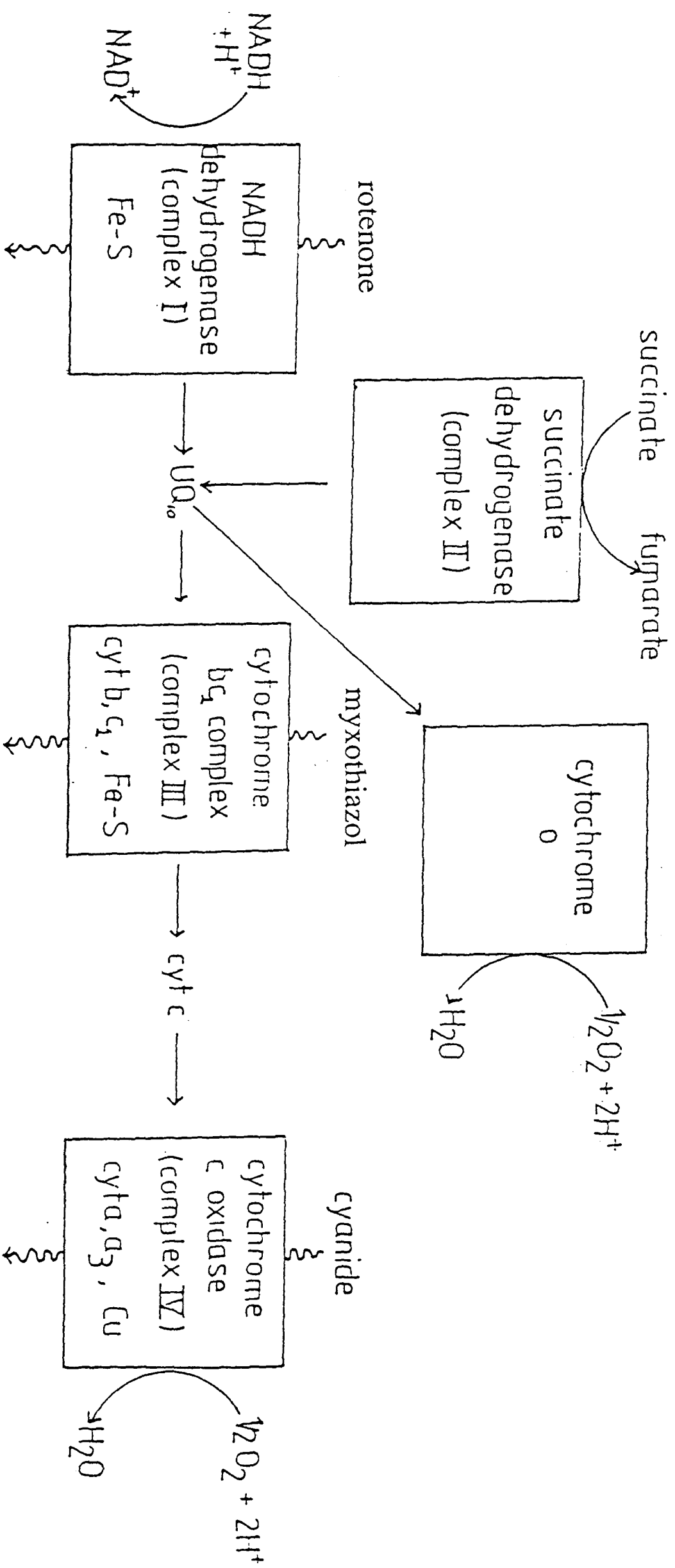


Figure 1.2. The aerobic respiratory chain of *P. denitrificans*.

The enzymes are drawn inside boxes to represent membrane bound enzyme complexes. The redox centres are known to be associated with the complexes indicated. The wavy lines indicate the sites of action of the commonly used redox inhibitors of the respiratory chain.

Abbreviations. Fe-S = iron-sulphur centres; UQ₁₀ = ubiquinone₁₀; cyt = cytochrome.

those found in micro-organisms and use O_2 as their sole electron acceptor. The mitochondrial systems are also less versatile than their microbial counterparts in terms of the number of possible electron donors that can be linked to the respiratory chain. In mitochondrial systems the possible electron donors include NADH, succinate and fatty acyl-CoA. Several electron transport components are found in a linear sequence in all types of mitochondria. These are the rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinone, antimycin- and myxothiazol-sensitive ubiquinol cytochrome *c* oxidoreductase (complex III or the cytochrome *bc*₁ complex), cytochrome *c* and cytochrome *aa*₃ oxidase (complex IV). Other components such as an alternative oxidase which accepts electrons at the ubiquinol branch point are only found in certain mitochondria (Nicholls, 1982).

In general, bacterial systems bear a superficial resemblance, but differ significantly, from the mitochondrial system described above. Furthermore, there are many differences between bacterial species (Haddock and Jones, 1977; Ingledew and Poole, 1984). Nevertheless, some bacteria, for example *P. denitrificans* and *Alcaligenes eutrophus*, bear remarkable similarity to the mitochondrial-type respiratory chains both in respect to the properties of the electron carriers and in the sensitivity of these components to inhibitors (John and Whatley, 1977; Albracht *et al*, 1980). These similarities, coupled with the similarity in the lipid compositions of aerobically grown cells of *P. denitrificans* with the inner mitochondrial membrane, have led to speculation that the inner membrane of the contemporary mitochondrion may have evolved from an ancestor related to *P. denitrificans* (John and Whatley, 1975). This is of particular interest in the present context because this organism is a denitrifier

and has the best characterised electron transport pathways to the four reductases of denitrification.

The following sections deal with the electron transport systems of organisms studied experimentally in this work.

1.5. The electron transport chain of *P. denitrificans*.

The organisation of the aerobic respiratory chain of *P. denitrificans* is well characterised, and in common with that of the mammalian mitochondrion consists of NADH dehydrogenase, succinate dehydrogenase, ubiquinone, cytochrome *bc*₁ complex, cytochrome *c*₅₅₀ and cytochrome oxidase (Figure 1.2). In addition to these components, *P. denitrificans* also possesses a constitutive alternative oxidase usually designated cytochrome *o*. This oxidase is thought to be a *b*-type cytochrome (Castor and Chance, 1959) which accepts electrons at the ubiquinol branch point of the respiratory chain (Parsonage, 1984) (Figure 1.2). However, the exact molecular nature of this alternative oxidase will only come apparent when it is purified.

P. denitrificans has the potential to use electron acceptors other than those shown in Figure 1.2. The ability to denitrify is conferred by the use of the four nitrogen-oxide intermediates of denitrification (NO_3^- , NO_2^- , NO and N_2O) as alternative electron acceptors to O_2 under anaerobic conditions. Figure 1.3 shows the pathways of electron transfer on the oxidising side of ubiquinol found in cells of this organism grown under anaerobic denitrifying conditions. The membrane bound NO_3^-

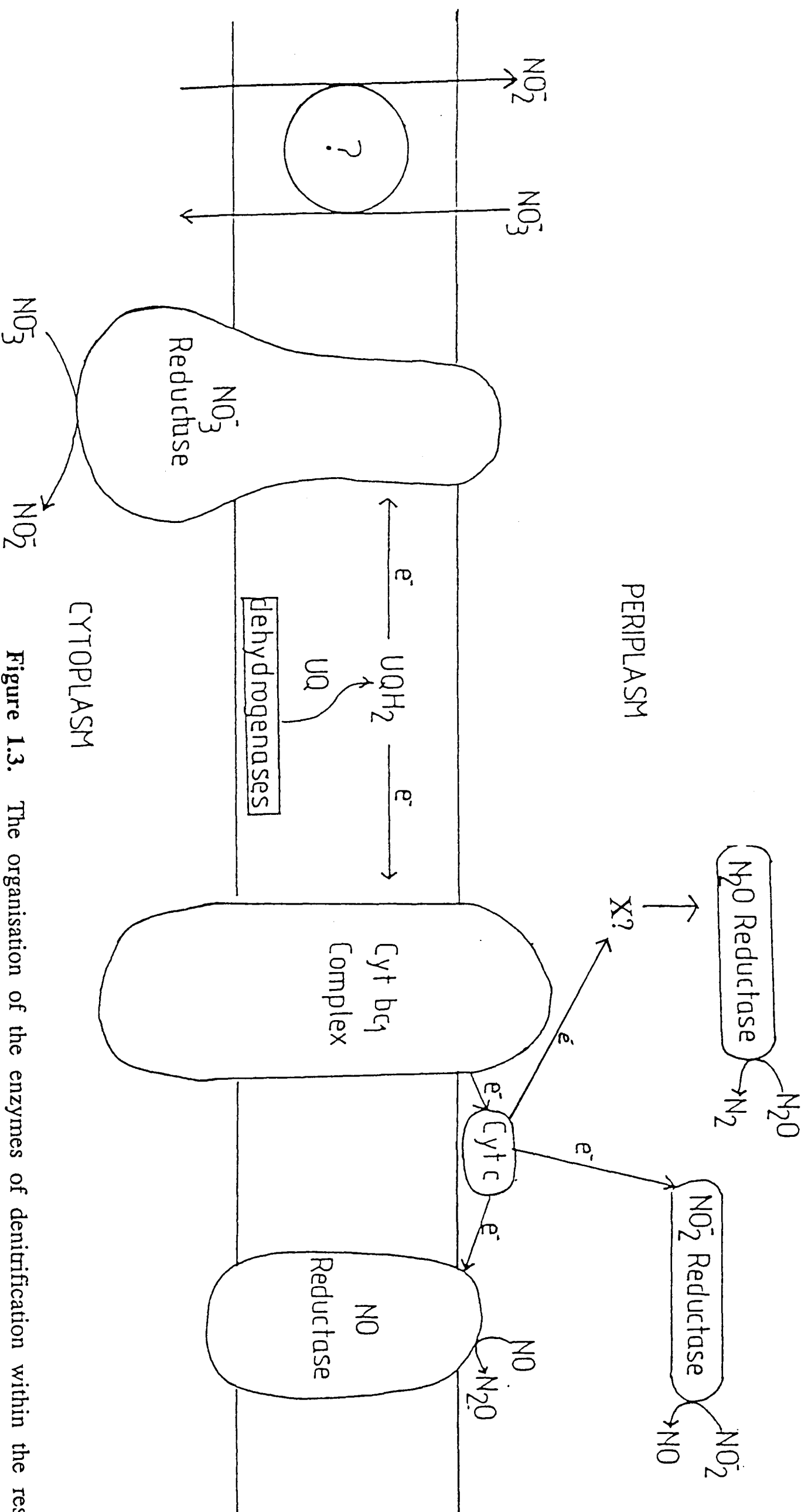


Figure 1.3. The organisation of the enzymes of denitrification within the respiratory chain of *P. denitrificans*.

Electron transport pathways to oxygen are not shown but are connected to cytochrome *c* (Cytochrome *aa₃*) or UQH_2UQ (cytochrome *o*, the alternative oxidase). A nitrate/nitrite antiport is shown tentatively.

reductase of *P. denitrificans* accepts electrons from the ubiquinol branch point of the respiratory chain (Parsonage *et al.*, 1986). Electrons are passed via the *b*-type cytochromes in the γ subunit of the enzyme to the active site of NO_3^- reduction, the α subunit (Craske and Ferguson, 1986). The cytochrome cd_1 NO_2^- reductase is a periplasmic enzyme (Alefounder and Ferguson, 1980) which has been shown in reconstitution experiments to accept electrons from cytochrome c_{550} (Timkovich *et al.*, 1982). As a previous report had shown that the cytochrome cd_1 from this organism reacts poorly with azurin (Martinkus *et al.*, 1980) it is generally considered that cytochrome c_{550} is the direct electron donor to this NO_2^- reductase. However, recent studies of a mutant in which the gene for cytochrome c_{550} has been specifically deleted indicated that an alternative donor to this enzyme must be present in such cells of *P. denitrificans* (van Spanning *et al.*, 1990). Therefore, the scheme depicted in Figure 1.3 represents a simplification of the possible electron transport processes to the reductases for the denitrification reactions. NO reduction by cells of *P. denitrificans* can be fully inhibited by either antimycin A or myxothiazol (Carr *et al.*, 1989), which suggests this membrane bound reductase accepts electrons at the level of a *c*-type cytochrome that is reduced by the cytochrome bc_1 complex (see Figure 1.3). Shapleigh and Payne (1985a) demonstrated a NO-dependent proton uptake with TMPD-ascorbate as the electron donor, in cells of *P. denitrificans*, which was suggestive of a periplasmic location of the proton consuming sites of the NO reductase. The N_2O reductase of *P. denitrificans* is located in the periplasm (Alefounder *et al.*, 1983). Electrons are known to pass via complex III to the N_2O reductase (Boogerd *et al.*, 1980; Alefounder and Ferguson, 1982). However, there is no further information about the electron transport pathway to the N_2O reductase of *P. denitrificans*, or any other organism, available. As is the case with the reduction

of NO_2^- and NO , the reduction of N_2O can proceed in a mutant which is devoid of cytochrome c_{550} (van Spanning *et al.*, 1990), indicating that cytochrome c_{550} , if it is involved, is not essential in the electron transport to N_2O .

The electron transport system of *P. denitrificans* can use electron donors other than those shown in Figure 1.2. These include methanol and methylamine which are oxidised in the periplasm, with the electrons passing via *c*-type cytochromes to the cytochrome aa_3 oxidase (Page *et al.*, 1989).

1.6. The electron transport chain of *R. capsulatus*

R. capsulatus is a photosynthetic member of the alpha-3 subgroup of purple non-sulphur bacteria. This subgroup of bacteria also encompasses non-photosynthetic members including *P. denitrificans* (Woese, 1987). The electron transport chain components in *R. capsulatus*, e.g. the cytochrome bc_1 complex and cytochrome c_2 , bear similarities to their counterparts in *P. denitrificans*. In addition to aerobic and anaerobic electron transport processes outlined for *P. denitrificans*, *R. capsulatus* possesses the capacity for photosynthetic electron transport.

The photosynthetic apparatus of *R. capsulatus* cells is located within vesicular membranes (Drews and Oelze, 1981), which are termed intracytoplasmic membranes (ICM), and thought to be contiguous with the cytoplasmic membrane (CM). The pathways of aerobic and photosynthetic electron transport share a number of redox components which are located in the plasma membrane. These electron transport

pathways are summarised schematically in Figure 1.4. As with aerobic respiration, photosynthetic electron transport results in transmembrane charge separation and the generation of ATP. Central to both pathways is the ubiquinol/ubiquinone pool. This pool can be reduced by both the reaction centre (Cramer and Crofts, 1982), which serves to transfer electrons from cytochrome c_2 with concomitant enhancement of its reducing potential, and the respiratory dehydrogenases (Baccarini-Melandri *et al.*, 1973). Ubiquinol can, in turn, reduce the cytochrome bc_1 complex or, under aerobic conditions, the alternative oxidase. The reduced cytochrome bc_1 complex can reduce cytochrome c_2 .

The roles of cytochrome c_2 and the cytochrome bc_1 complex in cyclic photosynthetic electron transport have often been considered obligatory. However, the recent construction of strains of *R. capsulatus* deleted in the *cycA* gene (coding for cytochrome c_2 ; Daldal *et al.*, 1986) and a region of the *fbc* (also designated *pet*) operon (coding for polypeptides of the cytochrome bc_1 complex; Daldal *et al.*, 1987) prompted a re-examination of these roles. The cytochrome c_2 deficient mutant, MTG4/S4, retained the capacity for photosynthetic growth at wild type rates. Growth was only significantly impaired at low light intensities. The mutant, MTCBC1, that did not express the cytochrome bc_1 complex lost the capacity for photosynthetic growth. Significant turnover rates of the electron transfer components of cyclic electron transport were not detected following single turnover excitation of the reaction centres in the latter mutants (Prince and Daldal, 1987). Thus the cytochrome bc_1 complex but not cytochrome c_2 is essential for cyclic electron transport.

Marrs and Gest (1973) isolated a number of aerobic respiratory mutants which did not express either one or both of the oxidase activities. The presence of just one of these oxidases appears sufficient for aerobic growth. Inhibition of the respiratory growth of mutant M6G (which lacks the cytochrome b_{260} oxidase) by myxothiazol indicated that the cytochrome bc_1 complex was indispensable for growth when respiration was limited to the cytochrome b_{410} oxidase (Daldal, 1988). A double mutant of *R. capsulatus*, defective in cytochrome c_2 and the cytochrome b_{260} oxidase, retained the capacity for dark aerobic chemoheterotrophic growth (Daldal, 1988). These observations demonstrate that an electron transport pathway independent of cytochrome c_2 was functional between the ubiquinol:cytochrome c_2 oxidoreductase and the cytochrome b_{410} oxidase.

In addition to the aerobic and photosynthetic electron transport pathways, *R. capsulatus* possesses the capacity for anaerobic respiration (Ferguson *et al.*, 1987). The ability of some strains of *R. capsulatus* to reduce trimethylamine N-oxide (TMAO), dimethylsulphoxide (DMSO), N_2O and NO_3^- is known (Ferguson *et al.*, 1987). However, *R. capsulatus* fails to denitrify because it lacks a NO_2^- reductase and possibly also a NO reductase. Whereas the absence of NO_2^- reductase is apparent from accumulation of NO_2^- by suspensions of cells reducing NO_3^- , there are no reports in the literature of tests for the presence of NO reductase. It is notable that partially denitrifying strains of *R. capsulatus*, able to reduce NO_3^- to NO_2^- , are unable to grow in the dark in the presence of NO_3^- as the sole electron acceptor (McEwan *et al.*, 1982). N_2O reduction under dark conditions is able to support the growth of *R. capsulatus* (McEwan *et al.*, 1985).

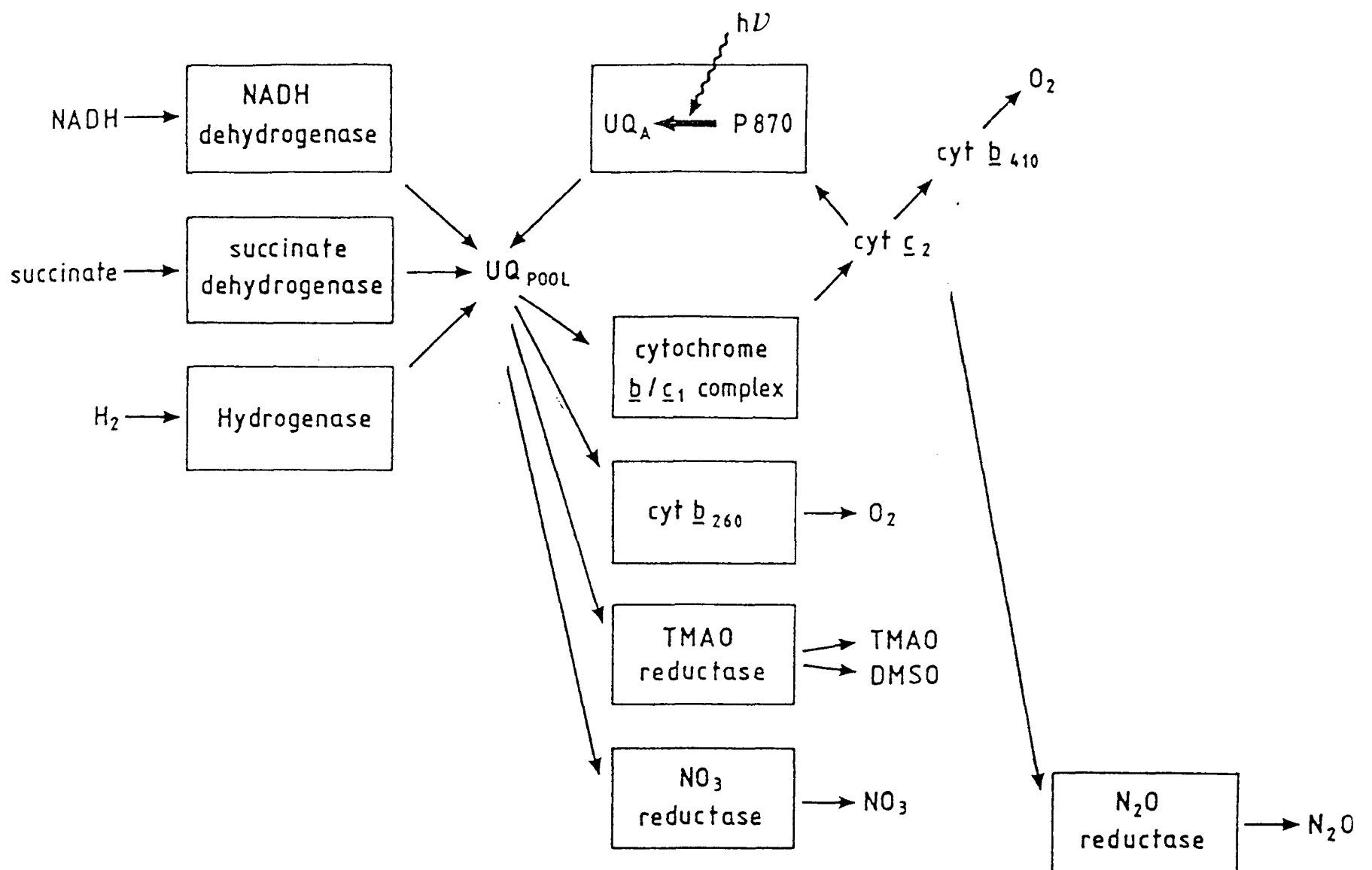


Figure 1.4. The pathways of photosynthetic, aerobic respiratory and anaerobic respiratory electron transport in *R. capsulatus*.

The NO_3^- reductase of *R. capsulatus* is a periplasmic enzyme (see section 1.3.1) in most strains, and like the membrane bound NO_3^- reductase of other organisms, it has been shown to accept electrons from the ubiquinone branch point of the chain (Figure 1.4). The electron transport pathway to N_2O was on first description suggested to be different in this organism than that found in *P. denitrificans* and other non-photosynthetic denitrifiers. This description suggested that the cytochrome bc_1 complex was not involved in this pathway and that cytochrome c' might act as the electron donor to the N_2O reductase (McEwan *et al.*, 1985). A recent re-evaluation of the components involved in electron flow to N_2O in *R. capsulatus* has concluded that electron transport to the N_2O reductase can proceed via the cytochrome bc_1 complex (see Figure 1.4) (Richardson *et al.*, 1989). However, a cytochrome bc_1 -independent pathway to N_2O reductase (not shown) is also operative in cells of *R. capsulatus* (Richardson *et al.*, 1989). In contrast, there is an obligatory requirement for cytochrome c_2 in the electron transport to N_2O in this organism. This has been established by the finding that the mutant, MTG4/S4, which specifically lacked cytochrome c_2 does not reduce N_2O despite possessing the reductase enzyme (Richardson, 1988). This point regarding the role of the cytochrome bc_1 complex and cytochrome c_2 in anaerobic respiratory electron transport processes will be further discussed in Chapter 6.

1.7. Control of the electron transport reactions of denitrification.

In *P. denitrificans* the appearance of many cytochromes in the electron transport chain appears to be controlled by the presence of O_2 . For example, the cytochrome aa_3 oxidase can be the dominant terminal oxidase when cells are grown under high levels of aeration. As the dissolved concentration of O_2 decreases, cytochrome o becomes more important and cytochrome cd_1 , which has oxidase as well as NO_2^- reductase activity, is only synthesised at very low O_2 concentrations (Sapshead and Wimpenny, 1972). The synthesis of other components involved in the electron transport reactions of denitrification in *P. denitrificans* are also sensitive to the O_2 concentration (Kucera *et al.*, 1984). Although it is well documented that many denitrifying species do not synthesise the nitrogen-oxide reductases under aerobic conditions, this control is not properly understood. It appears that the genes for these reductases are "switched on" at low dissolved O_2 tensions. This activation of gene expression can occur even in the absence of the nitrogen-oxides (Kucera *et al.*, 1984; Boublikova *et al.*, 1985). However, the nitrogen-oxides act to increase levels of enzyme induction in *P. denitrificans* (Boublikova *et al.*, 1985). A mechanism for this control of gene expression has not been elucidated in denitrifying bacteria, but it is tempting to anticipate the involvement of a FNR/type-protein. The FNR protein has been shown to control the expression of the NO_3^- reductase and several other anaerobic enzymes of *E. coli* (Bonney-Orth *et al.*, 1986; see also Chapter 4).

A second level of control is exerted at the level of enzyme activity. The presence of O_2 reversibly inhibits the electron transport reactions of denitrification. Many of the studies of such inhibition by O_2 have used *P. denitrificans* as the experimental organism (Alefounder and Ferguson, 1980; Alefounder *et al.*, 1983; 1984). For NO_3^- reduction, the inhibition has been shown to be a redox effect rather than a direct effect of molecular O_2 (Alefounder *et al.*, 1981; Kucera *et al.*, 1981). The ubiquinone/ubiquinol ratio has been proposed as a candidate for this regulatory system (Alefounder *et al.*, 1981; 1983), although any component before the site of antimycin inhibition could act as the regulatory site (Kucera *et al.*, 1981). A second interesting feature of the control of NO_3^- reduction by O_2 is that the cell lowers its permeability to NO_3^- (note from Figure 1.3 that NO_3^- is reduced on the inside of the cytoplasmic membrane) in response to the presence of dissolved O_2 concentration (Alefounder and Ferguson, 1980; Alefounder *et al.*, 1983; see section 4.1 for a more detailed discussion).

NO_2^- reduction appears to be inhibited under aerobic conditions in *P. denitrificans* by a competition mechanism for electrons within the respiratory chain (Alefounder *et al.*, 1983; Kucera *et al.*, 1986). This conclusion is based on observations that the supply of "extra" electrons to the respiratory chain, by means of TMPD/ascorbate (a non-physiological electron donating system), allows NO_2^- reduction to occur in the presence of O_2 (Alefounder *et al.*, 1983; Kucera *et al.*, 1986). Aerobic NO_2^- reduction has also been observed in cells of *P. denitrificans* which have been permeabilized or treated with an uncoupler (Kucera *et al.*, 1983; Kucera and Dadak, 1983). Under conditions of aerobic NO_2^- reduction, the electron flow to O_2 can be strongly inhibited by NO (the product of this reduction process)

thus resulting in further redistribution of electron flow from O_2 to NO_2^- (Kucera *et al.*, 1987; Carr and Ferguson 1990b).

O_2 is preferentially utilized over N_2O as an electron acceptor in whole cells of *P. denitrificans* (Alefounder *et al.*, 1983). It remains uncertain to whether this inhibition of N_2O reduction results from O_2 acting directly on the N_2O reductase of these cells, or from an overwhelming competition for electrons from the oxidase pathways. The lability of N_2O reductase in cell free extracts towards O_2 (section 1.3.4) suggests the former may be significant.

1.8. The generation of a proton electrochemical gradient from the electron transport processes of *P. denitrificans*.

The major function of membrane bound redox systems is to translocate protons and thus conserve the free energy released by a redox reaction as a protonmotive force. The cytoplasmic membranes of bacteria also contain an ATPase/ATP synthase complex which can also conserve energy by the pumping of protons driven by ATP hydrolysis. If, however, the protonmotive force generated by the redox system is sufficiently large then the ATP hydrolysis reaction is reversed in favour of ATP synthesis. This chemiosmotic mechanism of energy conservation involves the transfer of electrons within the membrane and the translocation of protons across the membrane. Under equilibrium conditions the maximum stoichiometry of proton translocation (n) per electron pair passing between a donor and acceptor is given by the relationship:

$$2 \Delta E = n \Delta p$$

where ΔE is the difference in redox potentials of the donor and acceptor couples and Δp is the protonmotive force. An important caveat to the above relationship is that all components of the segment of the electron transfer chain are defined relative to their sides of the cytoplasmic membrane (Ferguson and Sorgato, 1982).

One of the reasons often cited for the preferential use of O_2 as an electron acceptor over NO_3^- , NO_2^- or N_2O is that a higher proton translocation stoichiometry, and thus a higher growth yield, is seen when O_2 is the electron acceptor. A second, subsidiary factor, which will not be considered further here, is that NO_3^- and NO_2^- will serve as useful nitrogen sources for assimilatory reactions under conditions of nitrogen limitation (Koike and Hattori, 1975; Alefounder *et al.*, 1983).

The highest proton stoichiometries are associated with aerobic respiration when cytochrome aa_3 is the predominant oxidase. In such situations the pathway of electron flow from NADH to O_2 contains three sites of proton translocation; NADH $\rightarrow$ ubiquinone, ubiquinol $\rightarrow$ cytochrome c , cytochrome $c \rightarrow$ cytochrome aa_3 . Two sites of proton translocation are thought to be present during electron transport to O_2 via the alternative oxidase, known as cytochrome o (NADH $\rightarrow$ ubiquinone; ubiquinol $\rightarrow$ cytochrome o). Puustinen *et al.* (1989) reported observations from which they concluded that cytochrome o was a proton pump much like cytochrome aa_3 to which it is structurally related. These points will be returned to after the discussion of the proton translocation associated with the reduction of the nitrogen oxides.

The electron transport reactions of denitrification of *P. denitrificans* are now considered in terms of the proton translocation associated with them. The branch point in the respiratory chain to the membrane bound NO_3^- reductase is ubiquinol (Parsonage *et al.*, 1986). Analogy with the *E. coli* enzyme (Ingledew and Poole, 1984) indicates that 2 H^+ are translocated per electron pair flowing from ubiquinol to the cytoplasmic site of NO_3^- reduction (see Figure 1.5A). Experiments indicate that no additional protons are pumped by the membrane bound NO_3^- reductase of *E. coli* from the cytoplasm into the periplasm. It is expected that NO_3^- reduction in *P. denitrificans* is similar in terms of the stoichiometry of proton translocation. However, it is just thermodynamically feasible for the ubiquinol to NO_3^- span to pump 1 H^+ from the cytoplasm to the periplasm as each electron flows in the opposite direction. Such movement of two charges, at a typical protonmotive force of approximately +200 mV, would require that ΔE for the span ubiquinol to NO_3^- to be greater than 400 mV. As the E° for $\text{NO}_3^-/\text{NO}_2^-$ is +420 mV and that for ubiquinone/ubiquinol is +50 mV, an ΔE of +400 mV could be reached with plausible physiological concentrations of ubiquinone/ubiquinol and $\text{NO}_3^-/\text{NO}_2^-$.

In *P. denitrificans* the sites of NO_2^- , NO and N_2O reduction are located in the periplasm. Thus electrons must be moved from sites of substrate, NADH or succinate, oxidation at the cytoplasmic face of the membrane. This movement of negative charge out of the cell and the consumption of protons in the periplasm is counterproductive to the generation of a protonmotive force. Figure 1.5B shows how these periplasmic reduction reactions can be linked to the formation of a proton gradient during electron transport from ubiquinol. The electrochemical gradient develops because 4 H^+ are moved out of the cell alongside $2e^-$. Thus each reaction is

Figure 1.5. Proposed spatial organisation of, and proton translocation by, the electron transport chains from ubiquinol to nitrate, nitrite, nitric oxide and nitrous oxide.

A. For nitrate reduction electrons flow from the proposed branch point at ubiquinol via the reductase enzymes to the site of nitrate reduction in the cytoplasm.

B. For nitrite, nitric oxide and nitrous oxide electrons flow via the cytochrome bc_1 complex to the reductases. The cytochrome bc_1 complex is shown to operate by a Q-cycle mechanism (Crofts *et al.*, 1983). The Q-cycle explains the $4\text{ H}^+/2\text{e}^-$ stoichiometry for electron flow through the complex.

Closed arrows show electron transfer reactions. Open arrows show chemical transitions. Two arrows are shown to indicate the sequential passage of two electrons within the cytochrome bc_1 complex.

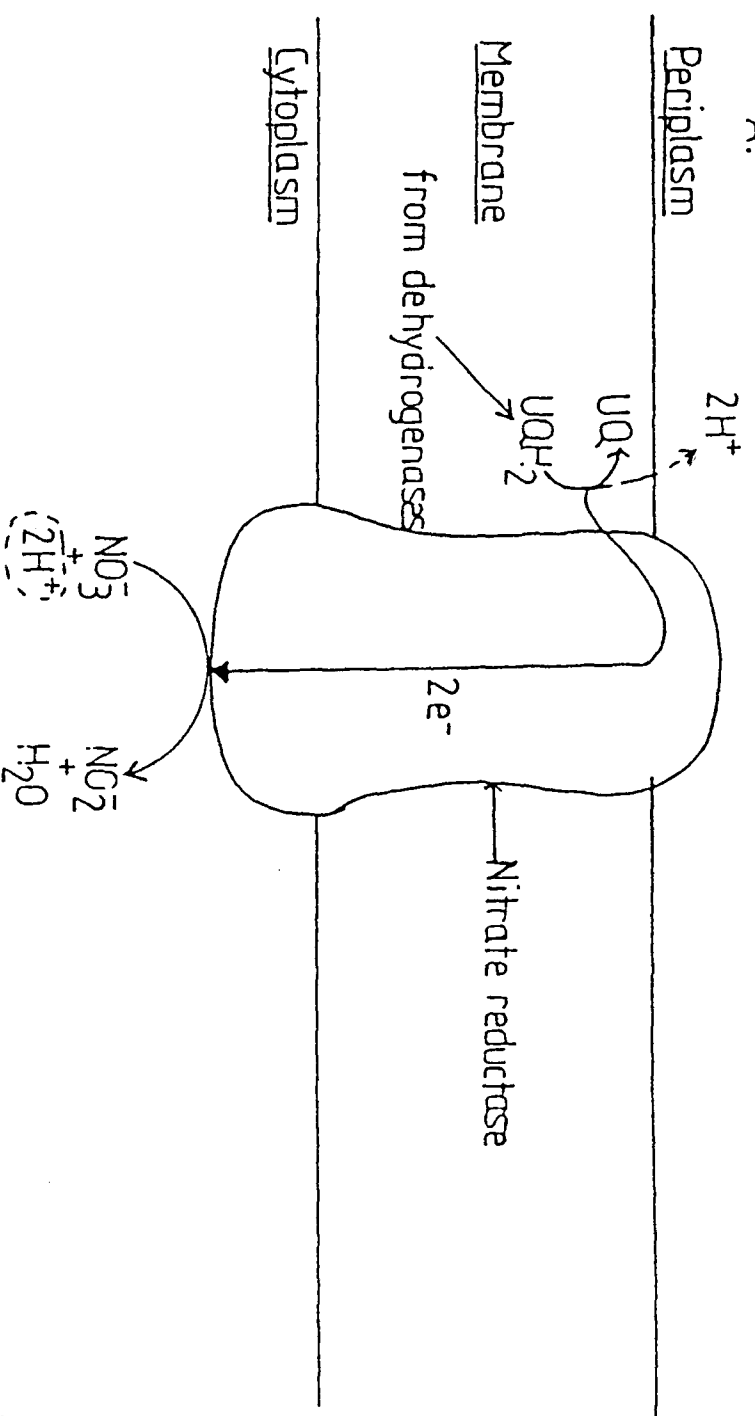
Abbreviations: UQ = ubiquinone; UQH₂ = ubiquinol;

UQH₂ = site which catalyses a quinol: ferricytochrome b : quinone oxidoreductase reaction;

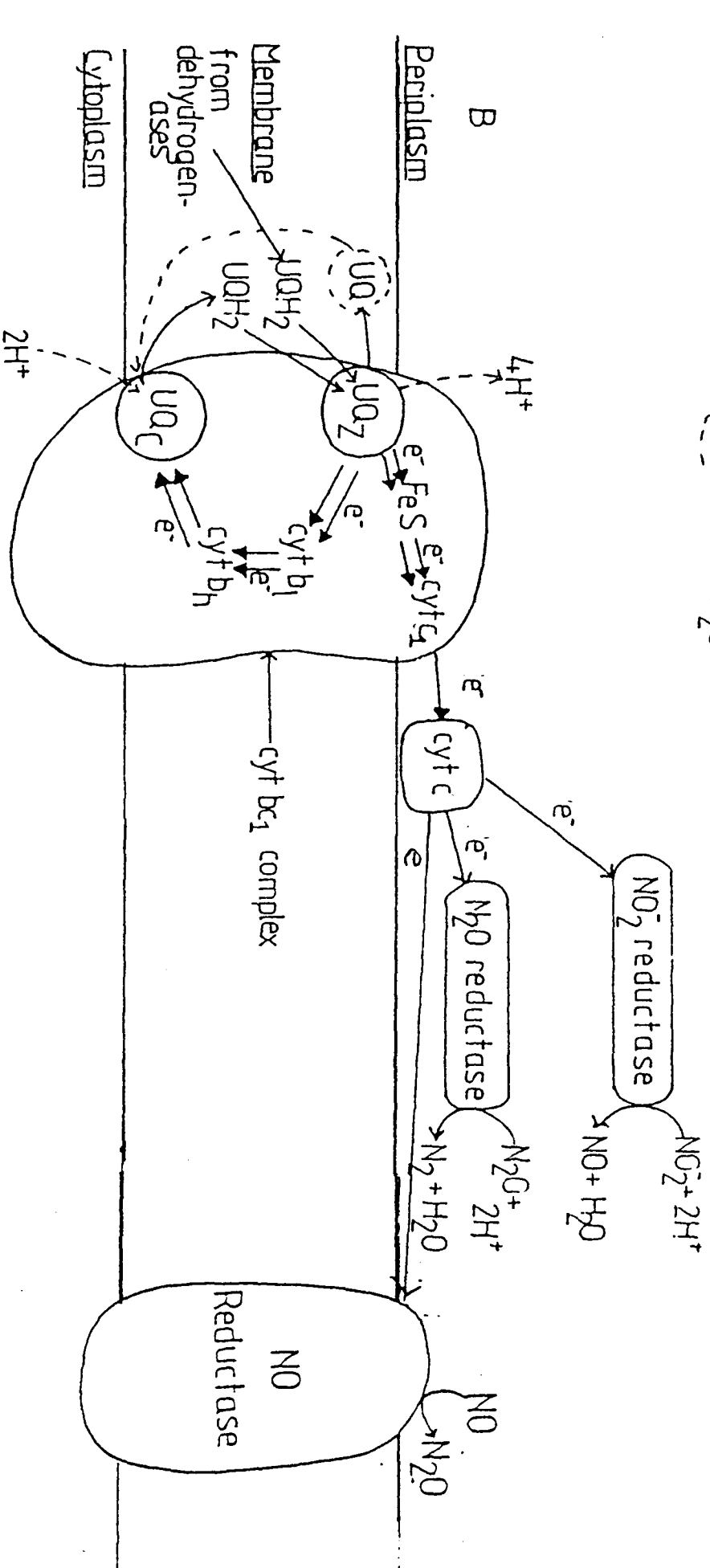
UQ_c = site which catalyses a ferrocycytochrome b : quinone oxidoreductase reaction;
cyt = cytochrome;

FeS = Rieske iron sulphur protein.

A.



B



associated with a net movement of two positive charges out of the cell (Ferguson, 1986). Hence the possible ATP yield associated with the reduction of either NO_2^- , NO or N_2O is the same as for NO_3^- despite the flow of electrons further down the electron transport chain and the fact that $\text{N}_2\text{O}/\text{N}_2$ couple is more oxidising ($E' = +1.1$ V) than the $\text{NO}_3^-/\text{NO}_2^-$ couple.

The importance of the such considerations of spatial organisation of electron flow are further illustrated by the periplasmic oxidation of methanol coupled with the reduction of NO_2^- or N_2O in *P. denitrificans*. Electron flow is entirely in the periplasm and therefore does not involve charge separation (Ferguson, 1989). Thus no ATP synthesis can result despite a large difference in redox potentials. This serves as a further example that the inspection of redox potentials is an inadequate guide to cellular energetics and that it is necessary to understand the organisational aspects of the system.

The foregoing descriptions of the proton translocation linked to the reduction of the nitrogen-oxides indicate that the stoichiometry of the process is indeed lower than that achieved when electron flow to O_2 via either oxidase in *P. denitrificans*. As higher growth yields would be seen with O_2 rather than the NO_3^- , NO_2^- , NO or N_2O as an electron acceptor this may in part explain the preferential usage of O_2 in this organism. The electron transport chains and the energetics of O_2 respiration have not been studied in so much detail in other denitrifying bacteria. Therefore, it is impossible to draw general conclusions from the example of *P. denitrificans*.

1.9. Aerobic denitrification.

Despite clear evidence that O_2 inhibits the reactions of denitrification in many organisms and the above rationale for such inhibition, there exist a number of reports demonstrating the ability of other organisms to denitrify in the presence of O_2 , a process termed "aerobic denitrification".

Breal (1892) made the first report of a culture which continued to reduce NO_3^- , in a dissimilatory process, even when provided with O_2 . This claim was met with disbelief, as have many recent claims of this ability. Many of the early reports have perhaps rightly deserved this scepticism, as many of the observations were made as the result of poor aeration or bad experimental design. For example, in the report of Breal (1892) the cells were exposed to air without any attempt to provide mixing. Such a procedure would have resulted in an O_2 gradient within the culture, and so it is likely that most, if not all, the NO_3^- reduction observed occurred in the anaerobic area of the culture. The extent of aerobiosis within a culture needs to be more clearly defined in order to collect reliable evidence in favour of the process of aerobic denitrification. The dissolved O_2 content of a culture is dependent both on the rate of O_2 transfer from the gas to the liquid phase and the rate of O_2 uptake by the organisms. However, several early reports contain enough experimental detail to establish that O_2 was present homogeneously throughout a culture medium during the

experiment. One such example is a report by Meiklejohn (1940) in which NO_3^- is clearly removed from a culture of *Pseudomonas sp.* under conditions of continuous sparging with O_2 and this removal can not be accounted for in terms of NO_3^- assimilation. This work provides the first real evidence that NO_3^- and O_2 can be simultaneously reduced.

Many recent reports demonstrate the occurrence of denitrification at low, but significant concentrations of dissolved O_2 . Meiberg *et al.* (1980) observed the induction of the denitrifying enzymes in *Hyphomicrobium X* at dissolved O_2 concentrations as high as 28 % of air saturation. Interestingly, the microaerophilic species, *Aquaspirillum magnetotacticum*, which has an absolute growth requirement for O_2 , when grown in the presence of NO_3^- at O_2 concentrations between 0.2 to 1 % air saturation reduced NO_3^- to N_2 (Bazylinski and Blakemore, 1983).

Other organisms have, however, been reported to denitrify in the presence of much higher concentrations of O_2 . Krul (1976) demonstrated, using electrodes which were sensitive to O_2 and NO_3^- , that a species of *Alcaligenes* was able to reduce NO_3^- and O_2 simultaneously. NO_3^- reductase activity is constitutive in cells of this species, as even cells grown aerobically (at dissolved O_2 concentrations of 200 % of air saturation) in the absence of NO_3^- retained the ability to reduce NO_3^- (Krul and Veeningen, 1977). Robertson and Kuenen (1983) reported the isolation of a denitrifier, *Thiosphaera pantotropha*. Aerobically grown cells of *T. pantotropha* reduced NO_3^- to a gaseous product under anaerobic conditions, without the expected 4-5 h. induction period normally required for the synthesis of the reductases of denitrification. This observation suggested that the enzymes of denitrification were

constitutive in *T. pantotropha*. Subsequent studies of this organism showed it simultaneously reduced NO_3^- and O_2 (Robertson and Kuenen, 1984; Robertson *et al.*, 1986; see also Chapter 3). NO_2^- did not accumulate in these cultures to significant extent and consequently this organism was considered to complete all the reactions of denitrification under aerobic conditions. However, firm evidence for the occurrence of all the reactions, in particular the formation of N_2 from N_2O , in the presence of O_2 of the denitrification sequence was lacking (see Chapter 3).

1.10. *Thiosphaera pantotropha*- a brief overview of this organism.

T. pantotropha was isolated, in a recent study, as a sulphide-using denitrifier (Robertson and Kuenen, 1983). This organism was capable of autotrophic and heterotrophic growth under both aerobic and anaerobic conditions. *T. pantotropha* can use a diverse range of respiratory electron donors and acceptors (Robertson and Kuenen, 1983). Comparisons with two similar species, *Thiobacillus* A2 and *P. denitrificans*, showed a fair degree of similarity in substrate utilisation. However, this isolate differed significantly from the other two and thus merited separate classification. The fact that this isolate is a coccus means that it lies outside the genus description for *Thiobacillus*. The isolate differed from *P. denitrificans* in many respects, including its ability to oxidise sulphur under anaerobic conditions, and thus could not be classified as a new isolate of *Paracoccus*. The genus of *Thiosphaera* was proposed in recognition of its sulphur metabolism. The species name *pantotropha* was designated in acknowledgement of its versatility in terms of

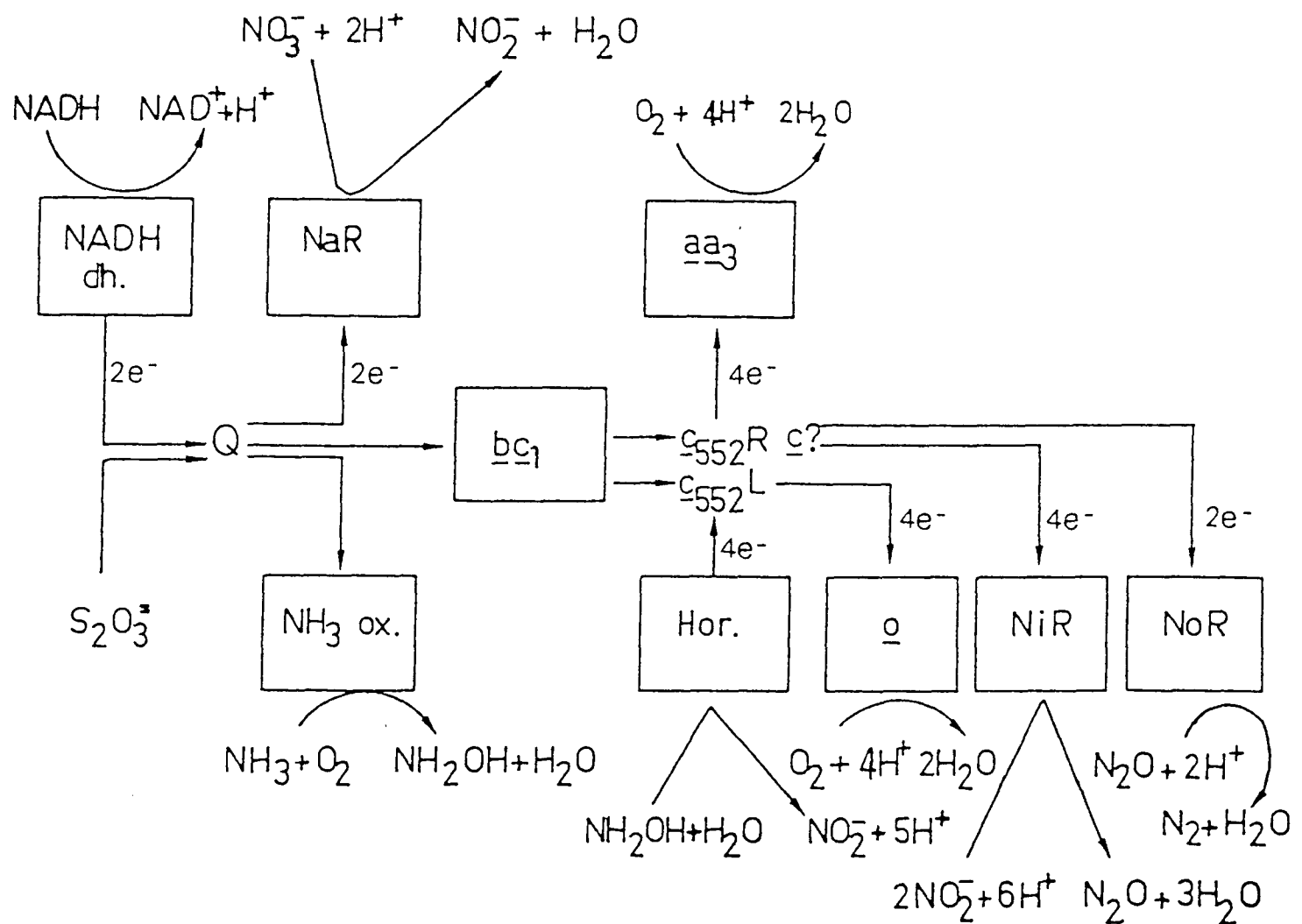


Figure 1.6. Schematic representation of the model used to describe electron transport in *T. pantotropha* (Robertson, 1988).

The cytochromes are underlined; NADH dh = dehydrogenase; NaR = nitrate reductase; NH₃ ox = ammonia monooxygenase; Hor = hydroxylamine oxidoreductase; NiR = nitrite reductase; NoR = nitrous oxide reductase; c ? indicates the presence of one or more additional c-type cytochromes; S₂O₃²⁻ = thiosulphate oxidation pathway.

substrates for growth (Greek prefix-*panto* all; Greek noun *trophos* feeder).

Central to the theme of this Thesis is the organisation of the electron transport pathways for the reactions of denitrification. Previous work has assumed that the electron transport chain of *T. pantotropha* bears a great deal of resemblance of that of *P. denitrificans* (Robertson, 1988). However, because *P. denitrificans* can not denitrify on reduced sulphur compounds, it has been considered that the cytochrome chain of *Thiobacillus denitrificans* is also of relevance (Robertson, 1988). The working model previously used to describe the potential routes for electron transfer in *T. pantotropha* is shown in Figure 1.6. If *T. pantotropha* is indeed an aerobic denitrifier, it would be extremely important to investigate, in detail, both the organisation of the electron transport pathways, and the molecular nature of the terminal reductases of the denitrification reactions in this organism. Such studies may provide an important insight into the molecular basis of aerobic denitrification.

1.11. Aims of the present work.

As described in this Chapter the basis for inhibition of three of the denitrification reactions (those of NO_3^- , NO_2^- and N_2O reduction) of *P. denitrificans* in the presence of O_2 has been described. Given that *T. pantotropha* has been reported to denitrify aerobically, the investigations presented in this work were designed (1) to investigate whether N_2O is reduced to N_2 under aerobic conditions; (2) whether NO could be formed from NO_2^- and reduced to N_2O in the presence of O_2 , despite the reactivity of

the latter with NO; (3) how the block of NO_3^- movement to its reductase under aerobic conditions, reported for *P. denitrificans* and other organisms, could be bypassed in *T. pantotropha* to allow aerobic NO_3^- reduction, which is the first step of the process of aerobic denitrification.

The recent establishment of NO as an intermediate in denitrification, and the identification of a NO reductase in several organisms, prompted the examination as to whether *R. capsulatus*, an organism already known to respire NO_3^- and N_2O , possesses a NO reductase activity.

Chapter 2. Materials and methods.

2.1. The maintenance and growth of bacterial cultures.

2.1.1. Organisms and strains studied.

Thiosphaera pantotropha strain LMD 82.5 was obtained from Dr. L. A. Robertson, Department of Microbiology, Technical University of Delft, the Netherlands. *Paracoccus denitrificans* strain NCIB 8944 was obtained from the National collection of Industrial Bacteria, Aberdeen. The strains of *R. capsulatus* used in this study and their sources are listed in Table 2.1.

2.1.2. Growth conditions.

Cells were grown in batch culture throughout the present work. All media and growth vessels were sterilised by autoclaving at 121 °C (at a pressure of 1.05 Kg.cm⁻²). Solutions of antibiotics, NaHCO₃, KNO₂, glucose, dimethylsulphoxide (DMSO) and N₂O gas were filter sterilised using millipore 0.22 µM nitrocellulose filters before aseptic addition to cold autoclaved media.

Solid medium was prepared by adding 1.6 % bacto-agar to liquid medium before autoclaving and then cooling to 48 °C prior to addition of antibiotics. Medium was then poured into plastic Petri dishes (20-25 ml per plate) and allowed to solidify.

TABLE 2.1. The strains of *R. capsulatus* used in this study.

Strain.	Characteristics.	Source or reference.
MT1131	Green mutant, DNAR ⁻ , NOSR ⁺ .	F.Daldal.
MTG4/S4	Cyt <i>c</i> ₂ deletion mutant isolated from MT1131 DNAR ⁻ ,NOSR ⁻ , Km ^r .	Daldal <i>et al</i> (1986).
MTCBC1	Cyt <i>c</i> ₁ deletion mutant isolated from MT1131 DNAR ⁻ ,NOSR ⁺ ,pho ⁻ ,Sp ^r .	Daldal <i>et al</i> (1987).
MTGS18	cyt <i>c</i> ₂ / <i>c</i> ₁ deletions isolated from MTG4/S4 DNAR ⁻ ,NOSR ⁻ ,pho ⁻ , Km ^r , Sp ^r .	"
BK5	wild type strain. DNAR ⁺ , NOSR ⁺ .	J.H.Klemme
AD2	Wild type. DNAR ⁺ , NOSR ⁺ .	"
10a.2.3	Plasmid deficient isolate from AD2. DNAR ⁻ .Rif ^r .	J. Willison (1990).
N22	Green mutant from St. Louis. DNAR ⁻ , NOSR ⁺ .	O.T.G.Jones.
N22DNAR ⁺	DNAR ⁺ strain isolated from N22	McEwan <i>et al</i> (1982).
37b4	Wild type. DNAR ⁻ ,NOSR ⁻ , Sm ^r .	H.Hudig
H123	Tn5 insertion mutant deficient in cyt <i>c</i> ' from 37b4, Sm ^r ,Km ^r , aer ⁻ .	Hudig <i>et al</i> (1986).
Z-1	Isolated from St. Louis. DNAR ⁻ ,NOSR ⁺ .	B.Marrs.
M5	Chemical mutants isolated from Z-1 deficient in <i>b</i> ₄₁₀ & <i>b</i> ₂₆₀ oxidases.aer ⁻ .	Marrs & Gest (1973).

Km^r = kanamycin resistance; Sp^r = spectinomycin resistance;
Sm^r = streptomycin resistance; Rif^r = rifampicin resistance.
DNAR = nitrate reduction. NOSR = nitrous oxide reduction.
pho = phototrophic growth. aer = aerobic growth.

Cells were stored for long periods in their growth media in the presence of 25 % (v/v) glycerol at -70 °C. For shorter periods (1-2 weeks) cultures were streaked on plates stored at 4 °C. These plates were routinely used as a check for the purity of a culture.

2.1.3a. Growth of *Thiosphaera pantotropha*.

T. pantotropha was grown at 37 °C either aerobically or anaerobically. The medium, with NH_4^+ as the nitrogen source, was similar to that described by Taylor and Hoare (1969), except that the phenol red was omitted (Table 2.2a). Sodium acetate or sodium succinate (as specified in the Chapters 3, 4 and 5) were added as the carbon source and KNO_3 was present as an electron acceptor. The trace elements were added from a stock solution which was as described by Vishniac and Santer (1957) except that it contained 2.2 g. l^{-1} (not 22 g) of ZnSO_4 .

2.1.3b. Growth of *Paracoccus denitrificans*.

P. denitrificans was grown aerobically or anaerobically at 30 °C in the medium described by Burnell *et al.* (1975), or in the growth media described in section 2.1.3a for *T. pantotropha*. The chemical composition of the medium described by Burnell *et al* (1975) is given in Table 2.2b ; the trace element solution was a modified Hoaglands solution described by Collins (1969).

Table 2.2a Composition of the growth medium of *Thiosphera pantotropha*.

The composition of media as described by Taylor and Hoare (1969) except that for some experiments (in Chapters 3, 4 and 5) sodium succinate replaced sodium acetate as the carbon source. The trace element solution is as described by Vishniac and Santer (1957) except that the ZnSO_4 concentration was modified. Quantities are given per litre of de-ionised water. The medium was adjusted to pH 7.5 with NaOH before autoclaving.

Compound	concentration	
Carbon source.	10	mM
KNO ₃	10.1	g
NaH ₂ PO ₄ .7 H ₂ O	7.9	g
K ₂ HPO ₄ .	1.5	g
NH ₄ Cl	0.3	g
MgSO ₄ .7 H ₂ O	0.1	g
Na ₂ EDTA	100	mg
ZnSO ₄ .7 H ₂ O	4.5	mg
CaCl ₂ .	11	mg
MnCl ₂ .4 H ₂ O	10	mg
FeSO ₄ .7 H ₂ O	10	mg
(NH ₄) ₆ Mo ₇ O ₂₄ .4 H ₂ O	2	mg
CuSO ₄ .5 H ₂ O	3	mg
CoCl ₂ .6 H ₂ O	3	mg

Table 2.2b. The composition of the growth medium of *P. denitrificans*.

This medium was described by Burnell *et al.* (1975).
The pH of this medium was adjusted to 6.8 by addition of H₂SO₄.
The trace element solution is described by Collins (1969).

Compound	Concentration (per litre)	
Na Succinate	13.5	g
KH ₂ PO ₄	0.7	g
(NH ₄) ₂ SO ₄	0.6	g
KNO ₃	10.1	g
Fe.Na.EDTA	17	mg
CaCl ₂	25	mg
MgCl ₂	25	mg
Trace element solution	0.1	ml

2.1.3c. Growth of *Rhodobacter capsulatus*.

R. capsulatus was grown at 30 °C in RCV medium, as described by Weaver *et al.* (1975), except the carbon source was varied according to the requirements of particular experiments. The composition of RCV medium is given in Table 2.2c.

2.1.4a. Aerobic growth.

Typically a 0.05-0.5 % inoculum of the organism was added to growth medium that occupied only one tenth of the capacity of a conical flask. Cultures were agitated at 200 *r.p.m.*.

2.1.4b. Anaerobic growth.

Cells were grown in screw-top 500 ml medical flats, or 50 ml Falcon tubes, with a minimal head space above the culture. Culture medium was not pre-gassed with argon prior to inoculation, except where otherwise indicated, since a growing bacterial culture will respire all the dissolved O₂ during the early stages of growth.

Sealed jars containing gas packs, supplied by BDH, were used to produce an atmosphere of CO₂ and H₂ in which plates were incubated under anaerobic growth conditions.

Table 2.2c. The composition of RCV medium for *R. capsulatus*.

The media was first described by Weaver *et al.* (1975). In this work, glucose or the sodium salts of succinate, butyrate or propionate were substituted as the carbon source (as indicated in the appropriate Chapter). The pH was adjusted to 6.8 with NaOH.

C o m p o u n d	C o n c e n t r a t i o n (p e r l i t r e)	
Carbon source.	30	mM
(NH ₄) ₂ SO ₄	1.00	g
KH ₂ PO ₄ .	0.68	g
K ₂ HPO ₄ .	1.14	g
MgSO ₄ . 7 H ₂ O.	120	mg
CaCl ₂ . 2H ₂ O.	75	mg
Na ₂ .EDTA.	20	mg
FeSO ₄ . 7H ₂ O.	240	mg
H ₂ BO ₃ .	700	mg
MnSO ₄ . 4H ₂ O.	400	mg
NaMoO ₄ .	188	mg
ZnSO ₄ . 7H ₂ O.	60	mg
Cu(NO ₃) ₂ .3H ₂ O.	10	mg
Thiamine hydrochloride.	1	mg

2.1.4c. Photoheterotrophic growth.

Cultures of *R. capsulatus* were grown in 500 ml medical flats, or 28 ml screw-capped McCartney bottles, (as for anaerobic growth) in front of a bank of 100 W tungsten light bulbs.

2.1.5. Nadi testing of cells.

Nadi testing establishes whether there is a functional cytochrome *c* and cytochrome oxidase present in the cells. The experimental procedure followed was as described by Marrs and Gest (1973). Freshly plated cells were flooded with a 1:1 mixture of solutions of 1 % α -naphthol in 95 % ethanol and 1 % N,N-dimethyl-p-phenylenediamine monohydrochloride (DMPD) in water. After 15 sec. plates were drained and then dried by exposure to air. Nadi⁺ phenotypes stained blue within 15 sec., whereas Nadi⁻ phenotypes showed no staining after 30 min.

2.1.6. Harvesting procedures.

After growth to the late exponential phase bacteria were harvested at 4 °C by centrifugation using either a Beckman J2-21 or a MSE bench top centrifuge. Centrifugation was at 8,000 g for 10 min. (20 min. for *R. capsulatus*). The pellet was then resuspended in a volume of a suitable buffer (see appropriate Chapters) that was one half the volume of the growth culture and then pelleted again by centrifugation. This pellet was then resuspended in a minimal volume of this buffer and stored on ice. These cells were typically used for experiments for up to 8 h. after harvesting.

2.2. Cell fractionation.

2.2.1. Preparation of spheroplasts, periplasm and cytoplasm.

Cells were harvested by centrifugation, as described in section 2.1.6, and were washed in a volume, which was half of the original growth volume, of cold 150 mM NaCl, 10 mM Tris-HCl pH 7.3 before resuspension in cold 0.5 M sucrose, 200 mM Tris-HCl, 0.5 mM EDTA (3 mM EDTA for cells of *R. capsulatus*), pH 8.0. An equal volume of cold water containing 1 mg. ml⁻¹ lysozyme was added. The total volume at this stage was one tenth of the original culture volume for most experiments. This suspension was incubated at 30 °C for 40 minutes before centrifugation at 10,000 g for 15 minutes. (The digestion of the cell wall was

monitored by observing a decrease in absorbance at 650 nm, due to the bursting of weakened cells, when 50 μ l of the suspension added to 1 ml of water). The periplasm was carefully decanted and stored for up to 12 h. on ice.

The pellet was very carefully resuspended in 200 mM sucrose, 10 mM Hepes-HCl, pH 7.3, containing a few crystals of DNase and 2 mM magnesium acetate. These spheroplasts were stored on ice. Cytoplasmic fractions were prepared by diluting the spheroplasts ten-fold into water and stirring at 4 °C for 1 h. The resulting suspension was centrifuged at 27,000 g for 40 min. at 4 °C. The cytoplasm (the supernatant) was decanted and stored on ice. The pelleted membrane vesicles were resuspended in a minimal volume of the appropriate buffer (see Results Chapters for further details).

2.2.2. Assay to determine the extent of leakage of cytoplasmic proteins during the preparation of the periplasmic fraction.

To determine the extent of release of the cytoplasmic proteins during the preparation of a periplasmic fraction, the distribution of malate dehydrogenase, a cytoplasmic protein, between the periplasmic and the cytoplasmic fractions was determined. Samples of these two cellular fractions were added to a glass cuvette which contained 100 mM potassium phosphate buffer, pH 7.5, and 0.1 mM NADH. The reaction was started by the addition of the substrate, oxalacetate, to a final concentration of 0.2 mM. The decrease in the absorbance at 340 nm was recorded using a LKB spectrophotometer.

2.2.3. The preparation of chromatophore membranes using a French press.

In addition to the method in section 2.2.1, membrane vesicles, known as chromatophores, of *R. capsulatus* were prepared using a French press. Cells were harvested and washed in 20 mM Tris-HCl, pH 8.0, before resuspension in a minimal volume of the same buffer. The cells were then passed through a precooled French press cell at 700 lb. in⁻² until they were broken. The suspension was then centrifuged at 20,000 g. to remove unbroken cells and cell debris. The supernatant was then centrifuged at 150,000 g. for 3-4 h. The pellet, containing the chromatophore membranes, was resuspended in 2-5 ml of the Tris buffer. These membranes were stored for several hours on ice, or at -20 °C for up to 5 days.

2.2.4. Identification of cytochromes in cells and cell extracts.

Cytochromes were detected in sample preparations using the Aminco DW2000 spectrophotometer in either the split beam or the dual wavelength mode. The split beam mode was used for most purposes. To obtain a baseline the two matched quartz cuvettes were filled with water and a spectrum covering the required range of experimental wavelengths was obtained and stored in the memory of an interfaced computer. To record an absolute reduced spectrum of a solution, the contents of the sample cuvette were reduced with sodium dithionite. The reference cuvette contained water. The spectrum was recorded and the stored baseline was subtracted to give the absolute reduced spectrum. A similar procedure was employed to obtain an absolute

oxidised spectrum of a sample; oxidation was achieved by the careful addition of ferricyanide to the sample. For experiments where anaerobiosis was required, the cuvettes were sealed with rubber supa-seals and the glucose oxidase system (see section 2.3.3) was employed to scavenge for O_2 . If this system interfered with a particular assay the cuvette was, instead, sparged with argon for 2 min. prior to commencing the assay. It was also necessary to seal the sample cuvette when additions of gases such as N_2O and C_2H_2 were to be made. Differences between reduced and oxidised spectra were obtained by subtraction in the computer of the spectra of the sample in one form from the spectra of the other form.

2.3. Measurement of oxidant consumption.

2.3.1. Production of saturated solutions of nitric oxide, nitrous oxide and acetylene.

Solutions of NO , N_2O and C_2H_2 were prepared at room temperature. Glass vials which contained distilled water (13 ml) were sealed with a butyl rubber septa. The vials were then sparged first with argon or nitrogen gas for 5 min. then with the required gases for a further 5 min. Samples of these saturated solutions could be withdrawn with a gas-tight Hamilton syringe. All these solutions were stored under positive pressure. Great care was required when preparing and handling solutions of NO . NO is a very reactive gas which combines with O_2 to give NO_2 , which in turn reacts rapidly in aqueous solution to form nitric and nitrous acids. Since NO_3^- and NO_2^- are intermediates in denitrification it is important to reduce any traces of these ions from a NO solution to be used in studies of denitrification. Thus, in the case

of handling the NO solution, the syringe was first flushed with an argon-sparged solution containing glucose oxidase, catalase and D-glucose to remove any traces of O_2 . N_2O and C_2H_2 do not react with O_2 so these precautions are unnecessary. These saturated solutions of NO, N_2O and C_2H_2 were assumed to be 3 mM, 24.8 mM and 45 mM respectively (Handbook of Chemical Physics).

2.3.2. The measurement of oxygen, nitric oxide and nitrous oxide.

The concentrations of O_2 , NO and N_2O in aqueous solutions were measured amperometrically using Clark-type oxygen electrodes (Clark *et al.*, 1953). The electrodes were mounted on a stirring unit at the bottom of the reaction chamber which was surrounded by a glass water jacket. The temperature of the chamber was maintained at 30 °C for all experiments through use of a circulating waterbath connected to the water jacket. The chamber was sealed by an adjustable plug which contained a narrow entry port through which additions could be made. The chamber was constantly stirred at a uniform rate by use of a small magnetic flea. All electrodes were set up in a similar manner. A piece of teflon (12 μ m thick) was placed carefully over the electrolyte, which covered the anode and cathode, to ensure no air bubbles were present. A rubber O-ring was used to secure this membrane. The electrodes all possessed silver anodes but varied in their cathodes and the electrolyte used. The oxygen electrode had a platinum cathode and was operated at -0.6 V. The NO electrode was first described by Zimmer *et al.* (1985). It possessed a gold, rather than a platinum cathode, and was operated at -0.8 V. Saturated KCl was employed as the electrolyte for both the O_2 and NO electrodes. The N_2O

electrode had a silver cathode and was operated, as described by Alefounder and Ferguson (1982), at a polarizing voltage of -1.3 V. The electrolyte, 1 M KOH, 100 mM KCl was prepared as a fresh solution each week.

All three electrodes were sensitive to O_2 , but the O_2 electrode did not respond to any significant extent to the presence of NO or N_2O . The NO and N_2O electrodes were approximately twenty and two times, respectively, more sensitive to NO than to N_2O . Therefore, whereas all three electrodes could be used to measure O_2 respiration, the N_2O electrode was used to measure both NO and N_2O reduction and the NO electrode was used to measure NO reduction without interference from N_2O .

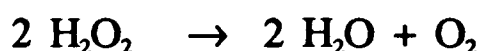
The concentration of O_2 in water at 30 °C was taken to be 236 μ M (Merck Index).

2.3.3. Production of anaerobic reaction mixtures in the chambers of Clark-type electrodes.

For much of the experimental work the contents of the electrode chamber were required to be anaerobic. The electrode was first set up containing a fully air saturated buffer at 30 °C. A glucose oxidase system, previously described by Englander *et al.* (1987), was employed to remove the O_2 . D-glucose was added to a final concentration of 16 mM, followed by the addition of catalase (25 U. ml⁻¹) and then glucose oxidase (2 U. ml⁻¹). Glucose oxidase catalyses the oxidation of D-glucose to gluconolactone with O_2 simultaneously reduced to H_2O_2 , i.e.



The H_2O_2 generated is rapidly removed by a reaction catalysed by the presence of catalase, i.e.



The gluconolactone produced is spontaneously hydrolysed to gluconic acid in the following reaction :



Thus the net result is the reduction of one molecule of O_2 for the oxidation of every two glucose molecules.

Removal of O_2 was in some experiments achieved via the respiration of cells.

2.3.4. The measurement of nitrate.

For these measurements an Orion NO_3^- electrode together with a calomel reference electrode were fitted to a 30 ml glass reaction chamber. The chamber was maintained at 30 °C via a water jacket connected to a circulating waterbath which was connected to the glass chamber. The potential developed by a NO_3^- electrode was measured against a reference electrode using a pH meter set on the mV mode. This potential was proportional to the amount of NO_3^- in the reaction medium. The reaction chamber was sealed by a rubber bung. The bung was pierced by two needles, one needle provided a gas inlet for an argon, or an air, line and the other a gas outlet. This outlet also allowed additions to be made to the chamber.

2.3.5. The measurement of nitrite.

The concentration of NO_2^- in a solution was determined using the colorimetric method described by Coleman *et al.* (1978). A 12.5 μl sample of the solution was added to a disposable plastic cuvette containing 900 μl of 1 % (w/v) sulphanilamide in 1 M HCl. The solution was mixed before the addition of 75 μl of N-(1-naphthyl)ethylenediamine dihydrochloride. The solution was mixed again and left at room temperature for 20 min. After this time the absorbance at 540 nm was read. The concentration was determined from a standard curve constructed using solutions containing known concentrations of KNO_2 .

2.3.6. Nitrite reductase assays.

The concentration of NO_2^- in a reaction vessel containing cells was measured as described above. Thus, the rate of NO_2^- reduction could be determined from such measurements over a time course. The reaction vessel was sealed and sparged with argon to ensure anaerobic conditions. Samples could be added, or withdrawn, using a Hamilton syringe. Further details of the electron donors used in this assay system are provided in the Results Chapters.

2.3.7. The methyl- and benzyl-viologen linked oxidoreductase assay for the measurement of nitrate and chlorate reductase activity.

The activities of NO_3^- or ClO_3^- reductase in whole cells, membranes or soluble extracts, could be determined by following, using visible absorption spectroscopy, the oxidant-dependent oxidation of dithionite-reduced methyl- or benzyl-viologen (MV^{2+} or BV^{2+}). The method employed was a modification of that used by Jones and Garland (1977). The sample was diluted into the reaction medium to give measurable rates of viologen oxidation. The reaction medium was added to a glass cuvette to which MV^{2+} (100 μM final concentration) and the glucose oxidase system, described in section 2.3.3, were added. The cuvette was filled completely with reaction medium to ensure that no air remained in the headspace, then sealed with a butyl-rubber septum and left at room temperature for 5 min. to become anaerobic. Aliquots of a sodium dithionite solution were titrated into the cuvette until a steady absorbance reading of 1.2 at 600 nm was obtained. The reaction was started by the injection of the oxidant into the cuvette. The resulting decrease in absorbance at 600 nm was monitored. The rate of viologen oxidation was calculated using $\epsilon_{600} = 13 \text{ mM}^{-1} \text{ cm}^{-1}$.

This assay was also employed to screen for the presence of other terminal reductase activities such as those for DMSO/TMAO reduction.

2.3.8. Measurement of carotenoid band shifts as an indication of membrane potential in *R. capsulatus*.

The generation of a membrane potential can be monitored in intact cells, and membrane vesicles, of *R. capsulatus* by recording the changes in the absorption maxima of endogenous carotenoid pigments (Jackson and Crofts, 1969) which are known to shift in response to changes in the local electrical environment. The shift has been shown to be proportional to the magnitude of the potential generated across the cytoplasmic membrane in *R. capsulatus* (Wraight *et al.*, 1978). This is conveniently measured from the difference in absorbance at 528-511 nm of brown wild type strains and at 503-511 nm for green mutant strains. The magnitude of the shift and its duration could be measured using time-based difference spectroscopy using the Aminco DW2000 spectrophotometer. A 4 ml cuvette was sealed, using a butyl-rubber septa through which injections could be made. The cuvette contained a magnetic flea and was mounted on a stirring device so that its contents could be constantly mixed. The contents of the cuvette were allowed to become anaerobic, using the glucose oxidase system described in section 2.3.3, before any measurements were made. Oxidant-dependent membrane potentials were generated by the addition of pulses of the nitrogen-oxides. The duration over which the membrane potential persisted had previously been shown to be proportional to the number of moles of the oxidant added, and thus it can be used as a measure of respiration (Richardson *et al.*, 1986). The light-induced membrane potential was measured before the measurement of respiration generated membrane potential for a given batch of cells.

This was because the light-induced membrane potential was easier to measure as it was greater in magnitude and was useful as an indicator of the approximate expected size of the oxidant generated potential (typically 2-4 X lower magnitude). The light was emitted from a 200 W lamp positioned at a 90° angle to the measuring beam and passed through a Whatman 88 A filter.

2.4. Protein purification.

2.4.1. The fractionation of enzymes in the periplasm using ammonium sulphate precipitation.

Analytical grade $(\text{NH}_4)_2\text{SO}_4$, which is low in traces of heavy metals, was ground to a fine texture using a mortar and pestle. The required quantity of salt was added slowly to the periplasm whilst stirring at 4 °C. The periplasm was stirred for a further 30 min. to allow complete equilibration before centrifugation at 20,000 g for 30 min. The supernatant was carefully decanted and the precipitate was resuspended in a minimal volume of an appropriate buffer. Both these fractions were assayed for the required enzyme activity. If necessary the supernatant was further fractionated using a higher $(\text{NH}_4)_2\text{SO}_4$ concentration.

2.4.2. Gel filtration chromatography.

Gel filtration was used to resolve proteins on the basis of their size. Pharmacia glass columns were packed with a pre-swollen Sephacryl-S200 resin (Pharmacia) which had been resuspended in the required buffer. Packing was performed at the temperature at which the column was to be utilised, commonly at 4 °C. The equilibrating buffer was pumped peristaltically at the flow rate at which the column was to be operated. Subsequently, the sample was applied to the column's surface, using a Pharmacia sample applicator to ensure even application. One column volume of buffer was pumped through the column, and the eluent was sequentially collected in one hundred appropriately sized aliquots using an automated fraction collector.

2.4.3. Ion exchange chromatography.

The columns were set up using an identical procedure to that described for gel filtration columns, but using Pharmacia ion exchange resins. The columns were inverted, as flow against gravity is recognised to result in better resolution of the protein components of a mixture (Scopes, 1987). After the protein had been loaded onto the column, one to three column volumes of buffer were washed through the column until the elution of unbound protein ceased. The eluted fractions were collected as above and assayed for the required enzyme.

2.4.4. Concentration and desalting of protein samples.

Protein samples were concentrated using an Amicon ultrafiltration cell fitted with a membrane with the appropriate molecular weight cut-off, or by placing the protein solution in a sealed dialysis bag which was coated in a thick layer of aquacide. Samples of protein were desalted using pre-packed gel filtration columns (PD10 columns, Pharmacia) according to the manufacturer's instructions.

2.5. Polyacrylamide gel electrophoresis (PAGE).

2.5.1. SDS-PAGE.

Gels were cast according to the discontinuous buffer system of Laemmli (1970). The dimensions of the resolving gel were typically 175 x 130 x 1 mm. The gels consisted of the required percentage polyacrylamide (typically 10 or 12.5 %) with a constant ratio of acrylamide to N,N' methylene bisacrylamide of 37:1. The gel was buffered with 375 mM Tris-HCl, pH 8.8, and also contained 0.05 % SDS. TEMED and freshly prepared ammonium persulphate (APS) were added to final concentrations of 0.1 % and 0.04 %, respectively, immediately prior to casting. The liquid gel mix was poured and topped with a layer of water saturated butanol and left to set. Only immediately before use was the stacking gel poured on top of the resolving gel. This stacking gel consisted of 3 % polyacrylamide, 125 mM Tris-HCl (pH 6.6), 0.1

% SDS, 0.1 % TEMED and 0.04 % APS. A 12- or 14-toothed comb was inserted into the freshly poured stacking gel and it was removed only after complete gelling of the polyacrylamide. The gel was clamped into a vertical Raven gel electrophoresis tank. The running buffer, 50 mM Tris-HCl (pH 8.3), 400 mM glycine and 0.1 % SDS, was placed in both the upper and lower reservoirs of this apparatus.

Protein samples, for gel electrophoresis, were prepared by boiling the protein with a sample buffer, at a 4:1 ratio, for 5 min. prior to use. The sample buffer comprised 627 mM Tris-HCl (pH 6.6), 5 % SDS, 20% glycerol, 10 % β -mercaptoethanol and 50 $\mu\text{g}.\text{ml}^{-1}$ bromophenol blue. The β -mercaptoethanol was omitted from the sample buffer for experiments which required non-reducing conditions. The samples (typically 50 μl) were loaded carefully into the wells in the stacking gels using a Hamilton syringe. Gels were run until the bromophenol blue dye had migrated to the bottom of the gel.

2.5.2. Gradient SDS-PAGE.

Linear gradients, from 10 to 20 % acrylamide, were formed using a gradient mixer and a peristaltic pump to pour the gels. The gels were of similar dimensions to non-gradient gels as described above.

2.5.3. Staining SDS gels for protein.

Gels were stained for protein using a Coomassie blue stain (section 2.5.3a). Where a more sensitive method was required, the Coomassie blue staining technique was modified as described by Zehr *et al.* (1989) and the gel was silver stained as described below.

2.5.3a. Coomassie blue staining.

The proteins were fixed in the gel and stained with a solution of 7.5 % acetic acid, 0.2 % (w/v) PAGE G-90 and 0.1 % naphthalene black. The gel was immersed in this solution and left for 1 h. at 70 °C. The gel was destained with a solution of 7.5 % acetic acid and 25 % methanol. Several changes of this destaining solution were necessary to reveal the protein bands. The destaining procedure was carried out at either at room temperature or at 70 °C, to accelerate the process.

2.5.3b. Combined Coomassie blue and silver staining.

The gel was placed in a solution of 10 % ethanol and 5 % acetic acid. Coomassie blue was added, from a stock solution, to give a final concentration of 0.001 %. The gel was gently shaken in this solution for 1-3 h. until the bands appeared. No destaining procedure was necessary. The gel was then ready for silver

staining when required. The gel was soaked in a 3.4 mM $K_2Cr_2O_7$ containing 3.2 mM HNO_3 for 10 min. at room temperature. The gel was washed three times in distilled water before it was immersed in a 0.012 M $AgNO_3$ solution for 30 min. The gel was rinsed twice in distilled water and then developed by immersion in a developer solution containing 0.28 M Na_2CO_3 and 0.05 % formaldehyde. Several changes of this developer solution were necessary over a five minute period. The development was stopped by placing the gel into a bath of 5 % acetic acid for 5 min. The gel could then be dried or stored in distilled water.

2.5.4. Haem staining of SDS gels.

In order to detect haem in a protein that has been subjected to SDS-PAGE, the sample needed to be prepared in the non-reducing sample buffer. The sample was not boiled prior to loading. The gel was stained for haem-dependent peroxidase activity using the method described by Thomas *et al.*, (1976). The gel was presoaked for 30 min. in 250 mM sodium acetate buffer, pH 5.0. A freshly prepared solution of 10 mM 3,3',5,5' tetra-methylbenzidine in methanol was added to this buffer in a 3:7 (v/v) ratio. The gel was incubated for at least 2 h. in the dark before H_2O_2 was added to a final concentration of 30 mM. After development the gel was photographed or a note made of the position of any bands that stained for haem.

2.5.5. Staining SDS gels for nitrate reductase activity.

The sample was prepared as described for the haem staining procedure above. Activity staining was performed according to the method of Graham *et al.* (1981). The gel was immersed in a 50 mM Tris-HCl buffer, pH 7.2, which contained 2 mM MV^{2+} and 40 mM KNO_3 . The MV^{2+} was reduced by the addition of approximately 10 mM sodium dithionite. A white band appeared against the blue gel background if NO_3^- reductase activity was present. The stain was transient, thus the position of the band was immediately recorded.

2.5.6. Western blotting of proteins from SDS gels.

An SDS gel was rinsed in blotting buffer which consisted of 25 mM Tris-HCl (pH 8.0), 192 mM glycine, 20 % (v/v) methanol and 0.02 % SDS. The gel was then equilibrated at room temperature for a further 60 min. in a fresh solution of the blotting buffer. The gel was blotted using a LKB transblotter according to the manufacturer's manual. The gel dimensions were measured so that twelve sheets of Whatman 3MM paper and one piece of nitrocellulose could be cut to an equivalent size. Once cut, these sheets were presoaked in the blotting buffer. The anode of the transblotter was equilibrated with the buffer and six sheets of the Whatman paper were placed on its surface. The nitrocellulose was positioned on top of this paper

and then gel was carefully placed onto the nitrocellulose. A further six sheets of Whatman paper were sandwiched between the gel and the cathode. The gel was blotted for 1 h. at 0.8 mA. cm⁻²

The nitrocellulose membrane was "blocked" overnight by gentle agitation in a solution of TBS-A containing 5 % bovine serum albumin (BSA). TBS-A consists of 20 mM Tris-HCl (pH 8.2), 0.9 % NaCl and 20 mM sodium azide. The membrane was washed for three times for 5 min., in TBS-A buffer containing 0.1 % BSA, before it was incubated with the primary antibody. The membrane blot was then gently shaken for 2 hours in a solution of TBS-A containing 1 % normal goat serum and an appropriate dilution of the primary antibody. Then, the nitrocellulose membrane was washed, as above, in TBS-A + 0.1 % BSA before the incubation with the secondary antibody. The secondary antibody (goat anti-rabbit) was supplied in a kit form (Auroprobe BL, Janssen). A solution of TBS-A and 0.1 % BSA which contained the secondary antibody and 5 % gelatin was agitated with the blot for 2 h. The blot was washed twice in TBS-A buffer containing 0.1 % BSA, then once in water. The bands were then enhanced by incubation with equal volumes of enhancer and initiator reagents (Intense Silver enhancement kit, Janssen) for 5-15 min.

2.6. Transposon Tn5 mutagenesis.

The donor *E. coli* strain S17-1 (Simon *et al.*, 1983a) containing the plasmid pSUP202.1::Tn5 (Simon *et al.*, 1983a) was grown aerobically overnight in LB medium (Maniatus *et al.*, 1987) containing 50 $\mu\text{g.ml}^{-1}$ kanamycin. A 1 % inoculum of this culture was placed in 100-200 ml of the same medium and grown aerobically for 3 h. (mid-exponential phase). The cells were harvested aseptically by centrifugation in Falcon tubes at 4 °C. The pelleted cells were resuspended in a minimal volume of the succinate medium described in Table 2.2a. A 100-200 ml culture of mid-exponential phase *T. pantotropha* which had been grown on minimal medium under either aerobic or anaerobic conditions was harvested and resuspended in an identical manner. Approximately equal volumes of the two cell suspensions were mixed. Aliquots of 0.4 ml of this mix were spotted upon membrane filters (millipore 0.45 μm) positioned on the surface of minimal media plates containing 0.1 % (w/v) yeast extract. These plates were incubated at 37 °C for 20-36 h. The cells were then washed off the filters using 2-3 ml of minimal medium and were stored at -70 °C until screening. To control for spontaneous resistance to kanamycin, an identical procedure was employed for *T. pantotropha* alone. Viable cell counts were performed using serial dilutions on minimal medium plates lacking kanamycin. The screening procedures are described in Chapter 5 of this Thesis.

2.7. Determination of the bacteriochlorophyll concentration.

The bacteriochlorophyll content of cells, or vesicles, of *R. capsulatus* were measured by addition of 25-50 μ l of the sample to 5 ml of acetone/methanol (7:2 v/v). The resulting suspension was mixed by vortexing for 2 min. The organic solution containing the bacteriochlorophyll was filtered using Whatman Number One size paper and the optical absorbance of the filtrate at 772 nm was recorded. The extinction coefficient for bacteriochlorophyll is $\epsilon_{772} = 75 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. (Clayton, 1963)

2.8. Protein and dry weight determinations.

The protein contents of cells, membrane vesicles, cytoplasm and periplasm, were each determined by the method of Lowry *et al.* (1951). BSA was used as a standard since the concentration could be accurately determined from the absorbance at 279 nm ($A_{279\text{nm}} = 0.667 \text{ cm}^2 \cdot \text{mg}^{-1}$, Wiolders *et al.*, 1980).

The dry weight of cells, or membrane vesicles, was determined by weighing a 0.22 μ m Whatman filter paper both before and after the following procedure. The cell suspension (5 ml) was filtered through the filter paper. This was followed by three washes of distilled water at 4°C. The filter was then dried at 50°C in an oven until no further decrease in weight was observed.

2.9. Chemical reagents.

The following reagents were obtained from:

Sigma Chemical Company Ltd., Poole, Dorset, U.K.: Lysozyme, phenazine methosulphate, phenazine ethosulphate, 3,3',5,5'-tetramethyl benzidine, diaminodurene, Hepes, Tris, methyl-viologen, benzyl-viologen, 2 n-heptyl-4-hydroxyquinoline N-oxide, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone, dimethylsulphoxide, glycine, sodium dodecyl sulphate, N,N,N',N'-tetramethyl p-phenyldiamine, kanamycin sulphate, butyric acid.

BDH Chemicals Ltd., Poole, Dorset, U.K.: Bromophenol blue, xylene cyanol FF., acrylamide, N,N'-methylene bis acrylamide, sodium D-isoascorbate, sodium dithionite, sulphanilamide, nitrous oxide, nitric oxide, argon, nitrogen, acetylene, N-(1-Naphthyl)ethylenediamine hydrochloride, ammonium sulphate, PAGE blue 83, Triton X-100 (scintillation grade), anaerocult A (CO₂ and H₂ producing packs).

Fison PLC., Loughborough, UK: Disodium diaminoethanetetra-acetic acid, hydrogen peroxide, sodium azide, potassium cyanide.

Pharmacia, Uppsala, Sweden: Sephacyl S-200, DEAE-Sepharose CL6B, DEAE Sephacel.

Boehringer Mannheim GmbH, Mannheim, Germany : Myxothiazol.

The anti-(*P. denitrificans* nitrate reductase) and the anti-(*R. capsulatus* strain N22DNAR⁺ nitrate reductase) antibodies were supplied by Anna Ballard, University of Birmingham and David Richardson, University of Oxford.

All other reagents were analytical grade.

Chapter 3. Nitric and nitrous oxides are synthesised and reduced under aerobic conditions by *Thiosphaera pantotropha*.

3.1. INTRODUCTION.

T. pantotropha was first isolated from an effluent treatment pilot plant in which it was thought that the oxidation of sulphide and denitrification were occurring (Robertson and Kuenen, 1983). Preliminary comparisons of the denitrifying abilities of *T. pantotropha* with those of *Thiobacillus versutus* (formerly *Thiobacillus* A2) indicated a difference between these two organisms. When aerobically grown cells of *T. versutus* were incubated anaerobically with NO_3^- the expected lag period, for the induction of the enzymes of denitrification, was observed before gas production was seen. However, with similar cultures of *T. pantotropha* gas evolution was immediate (Robertson and Kuenen, 1983; 1984). This was taken as evidence for the enzymes of denitrification being constitutive in this bacterium. In subsequent studies (Robertson and Kuenen, 1984) a number of other observations were made. The specific growth rate of cells of *T. pantotropha* increased during aerobic growth in the presence of NO_3^- compared to growth in the presence of O_2 (or NO_3^-) alone. However, cultures grown with both O_2 and NO_3^- , as electron acceptors, had a lower final optical density than cultures grown with O_2 alone. This is consistent with observations that less energy is conserved for the cell when a reductant is oxidised by the reactions of denitrification rather than by O_2 (Stouthamer, 1980; see also Chapter 1). When these experiments were repeated with *T. versutus* the NO_3^- had little, or no, effect on the specific growth rate or the yield of cell mass (Robertson and Kuenen, 1984). The NO_3^- was observed to disappear from the growth medium of *T. pantotropha* and only traces of NO_2^- accumulated in the medium. The possibility that NO_3^- had been reduced to NH_4^+ , in a dissimilatory process, was ruled

out since the NH_4^+ content of the medium decreased during the experiment. A third possible fate of NO_3^- , that of NO_3^- assimilation, was also excluded as NH_4^+ was not depleted during the course of the experiments. All these observations were taken as evidence that all the enzymes of denitrification were synthesised and were active under aerobic conditions (Robertson and Kuenen, 1984). However, gas production was measured manometrically without identification of the evolved gas. In the present work, direct evidence was sought for the reduction of N_2O to N_2 under aerobic conditions. At the time of the original description of aerobic denitrification by *T. pantotropha* it was not generally accepted that NO is an intermediate within the overall process of denitrification (Chapter 1). The known reactivity of NO with O_2 meant that it was important to investigate whether NO could be synthesised and reduced in the aerobic denitrification process.

3.2. RESULTS.

3.2.1. Simultaneous reduction of nitrous oxide and oxygen by cells of *T. pantotropha*.

The occurrence of "true" aerobic denitrification requires that all the reactions of denitrification must be able to occur in the presence of O_2 . The last of these reactions, the reduction of N_2O to N_2 (in characterised denitrification processes), must therefore be possible under aerobic conditions in *T. pantotropha*, if this organism is to be classified as an "aerobic denitrifier" in the strictest sense. If N_2O is to be reduced in the presence of O_2 then N_2O reductase must be able to compete with the oxidases of the cell for electrons from the respiratory chain. Thus a decrease in the rate of electron flow to either of these terminal electron acceptors (O_2 or N_2O) would be expected in the presence of the other acceptor, except if a "bottleneck" for electron transport existed in the respiratory chain after the branch point(s) to O_2 and N_2O . In that case an increase in the total electron flux would be observed when the two electron acceptors were available. This point can be clarified by a number of simple experiments using N_2O and O_2 sensitive electrodes.

A standard Clark-type O_2 electrode with a platinum cathode does not respond to the concentrations of N_2O used in these experiments when operated at polarising voltages in the region of -0.7 V. Consequently the effect of N_2O on the rate of reduction of O_2 can be readily measured. Cells, which had been grown anaerobically in the acetate medium described by Table 2.2a were added to the contents of an

electrode chamber which had been equilibrated with O_2 at 30 °C. Acetate was present as an electron donor. These cells of *T. pantotropha* respired O_2 at a linear rate (Figure 3.1a). After several minutes N_2O was introduced to the cells and the rate of O_2 reduction was diminished. Figure 3.1a shows a 57 % inhibition of the rate of O_2 reduction on introduction of the N_2O . The extent of this inhibition varied from 40-65 % with ten different batches of cells of this organism. O_2 reduction remained at a constant linear rate in experiments run on a similar time course but without addition of N_2O . When N_2O was introduced to cells in the presence of C_2H_2 no decrease in the rate of O_2 was observed. The addition of C_2H_2 reversed the inhibition by N_2O of the rate of O_2 reduction (Figure 3.1a). The effect of C_2H_2 indicates competition for electrons between O_2 and N_2O within the respiratory chain since direct inhibition of an oxidase by N_2O would not be relieved by the presence of C_2H_2 which is a specific inhibitor of the N_2O reductases in other organisms (Balderstone *et al.*, 1976). In the present work, it was shown that C_2H_2 did not inhibit the other reactions of denitrification in *T. pantotropha*. The presence of C_2H_2 alone had no effect on the rate of O_2 reduction.

A complementary experiment was performed using the N_2O electrode. This electrode is sensitive to both O_2 and N_2O , with the former being detected at a higher sensitivity. Cells of *T. pantotropha* were added to the contents of the electrode chamber which were left for several minutes until the dissolved O_2 was depleted. A pulse of N_2O was then added to the cells and was immediately reduced at a linear rate. Before all the added N_2O had been reduced, O_2 was generated within the electrode chamber by addition of H_2O_2 to the reaction medium containing 25 U. ml⁻¹ of catalase. Exogenous catalase was present at such a concentration to provide

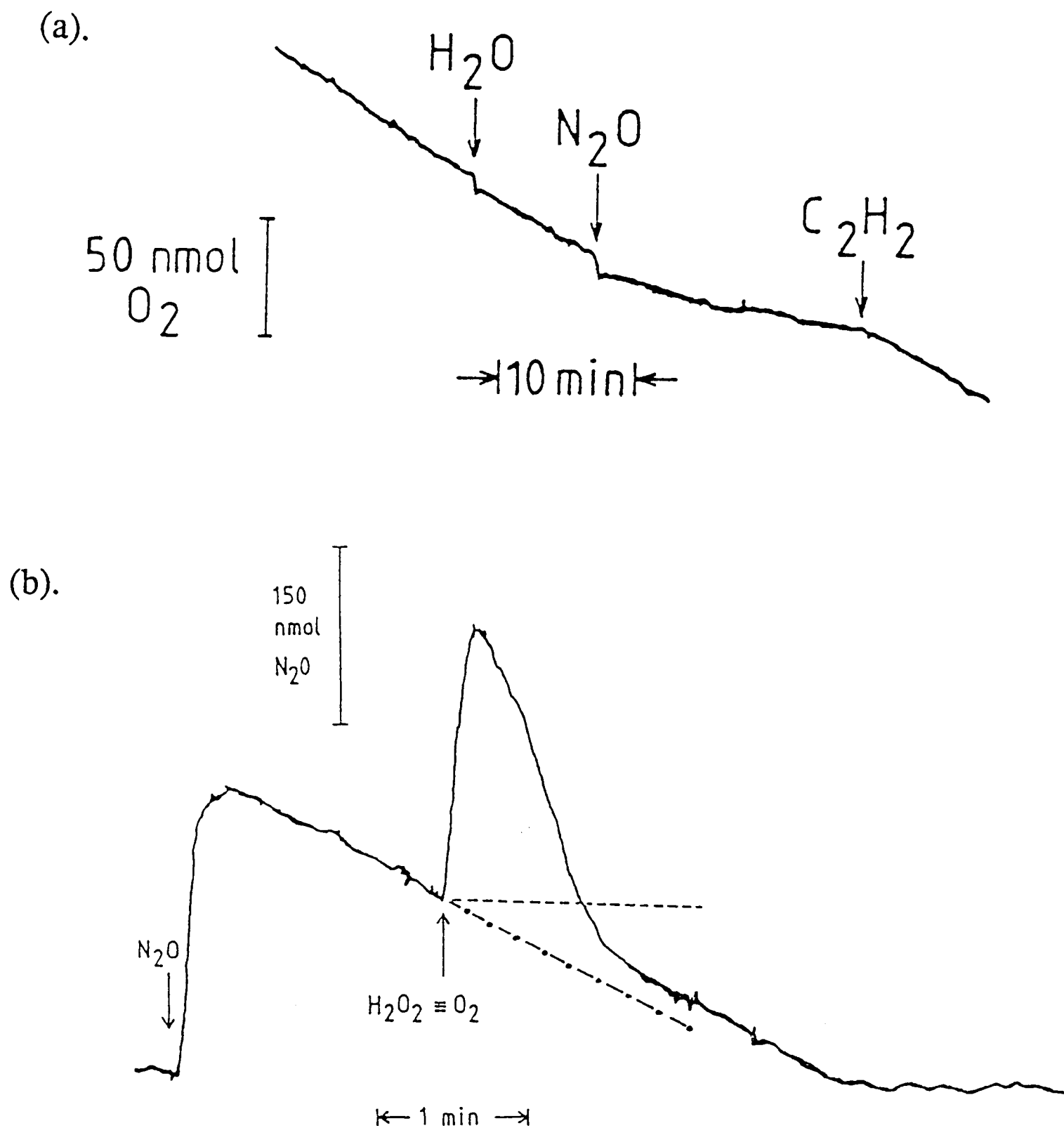


Figure 3.1. Simultaneous oxidation of nitrous oxide and oxygen by cell suspensions of *T. pantotropha*.

(a) Record of the oxygen electrode response: A 2 ml reaction mixture contained 200 mM sucrose, 10 mM Hepes/NaOH, pH 7.3, 10 mM potassium acetate, and 0.12 mg dry weight of cells. Nitrous oxide and acetylene were added as shown to give final concentrations of 0.62 mM and 1 mM respectively.

(b) Record of the nitrous oxide electrode response: The 2 ml reaction mixture had the same composition as in (a) except that 50 units of catalase was also present and the amount of cells was 0.6 mg dry weight. On exhaustion, through cell respiration, of the dissolved oxygen, nitrous oxide was added (final concentration 0.12 mM) followed by hydrogen peroxide (final concentration 25 μ M) from which oxygen was generated by catalase activity. (- - -) indicates concentration of nitrous oxide at the time of oxygen addition; (-.-.-) indicates extrapolated rate of reduction of nitrous oxide.

sufficient activity for almost immediate generation of O_2 . Thus the electron flow to any enzyme which reduced H_2O_2 had no effect on the measurements. Cytochrome *c* peroxidase, a peroxidase which reduces H_2O_2 to H_2O , has been identified in the periplasmic fraction of *P. denitrificans* (D. Hunter, personal communication) and if present in *T. pantotropha* may complicate the interpretation of the experiment, unless, as here, sufficient catalase is present to convert the H_2O_2 to O_2 . Figure 3.1b. illustrates a typical result, the presence of O_2 caused a partial inhibition of the rate of N_2O reduction. The extent of this inhibition can be calculated in the following way. The record of N_2O reduction as a function of time can be extrapolated beyond the point where H_2O_2 is added. It is evident that after reduction of the added O_2 the rate of N_2O reduction returned to its original rate (Figure 3.1b). However, on depletion of the added O_2 , the N_2O concentration was higher than would have been the case (from extrapolation) had anaerobic conditions been maintained. Thus, the rate of N_2O reduction during the period under aerobic conditions could be calculated. For the experiment illustrated by Figure 3.1b (cells were from the same batch as for Figure 3.1a) the aerobic rate of N_2O reduction was decreased to 65 % of the rate seen under anaerobic conditions; the extent of the inhibition that O_2 effects on N_2O reduction varied (between 50-70 %) for different cell batches. The specific rate of N_2O reduction, under anaerobic conditions, (90-130 nmols N_2O . min⁻¹. mg dry weight⁻¹) is greater than the specific rate of O_2 respiration, when O_2 is the sole electron acceptor, (60-100 nmols O . min⁻¹. mg dry weight⁻¹) in cells of *T. pantotropha*. Thus the greater percentage inhibition of the rate of O_2 reduction in the presence of N_2O compared to the extent of inhibition of N_2O reduction in the presence of O_2 can probably be explained by a competition mechanism between one or more oxidases and N_2O reductase for electrons.

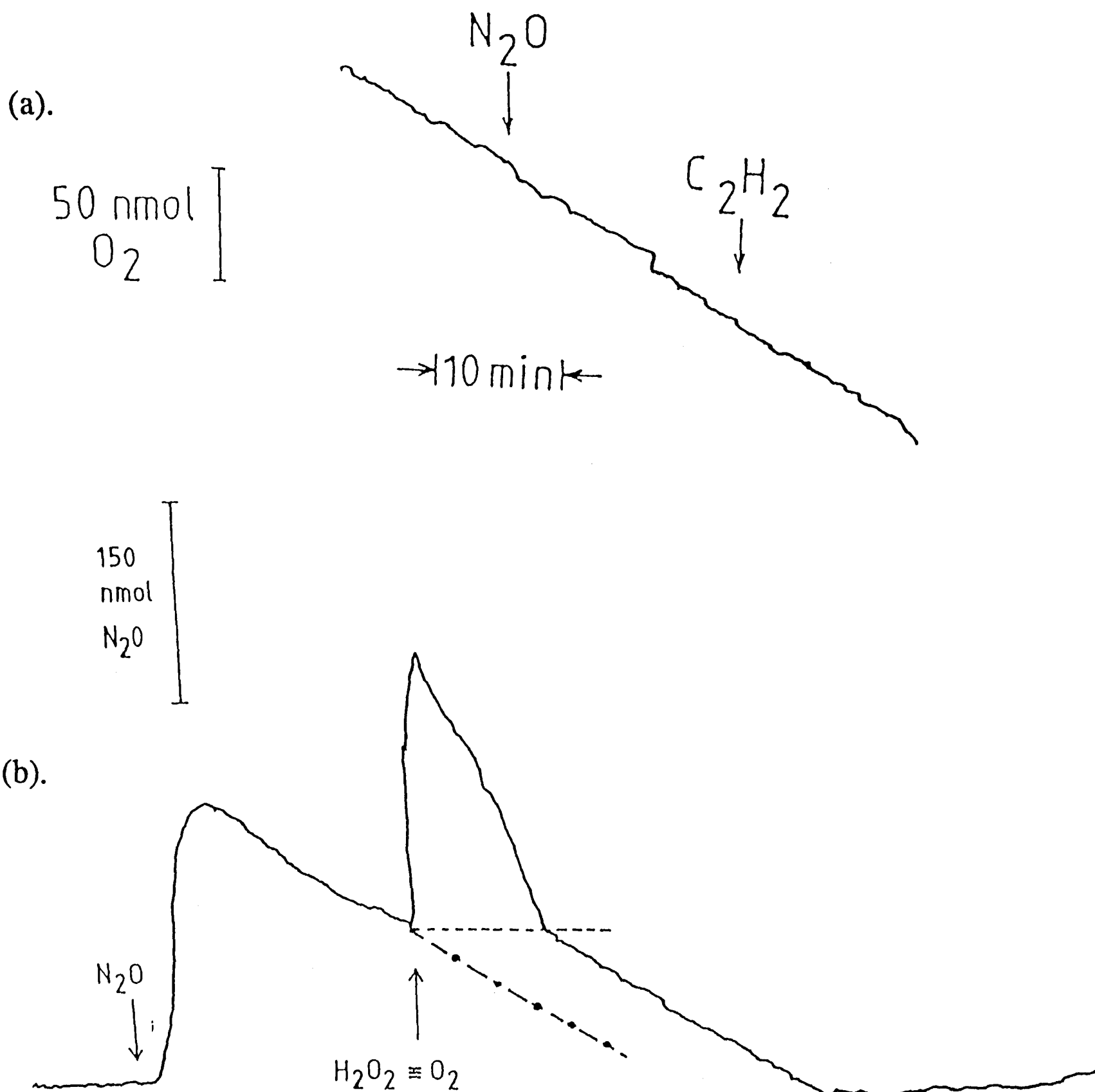


Figure 3.2. Nitrous oxide and oxygen are not simultaneously reduced by cell suspensions of *P. denitrificans*.

(a) Record of the oxygen electrode response: The 2 ml reaction volume had the same composition as the medium described for Figure 3.1a except that it contained 0.15 mg dry weight of cells (of *P. denitrificans* not *T. pantotropha*). Nitrous oxide and acetylene were added to final concentrations of 0.62 and 1 mM respectively (as indicated).

(b) Record of the nitrous oxide electrode response: The 2 ml reaction volume had the same composition as that described in Figure 3.1b but contained 0.5 mg dry weight cells. The oxygen dissolved in the medium was removed by cell respiration then nitrous oxide and hydrogen peroxide were added to the medium (as shown) to final concentrations of 0.12 mM and 25 μ M respectively.

(- - -) indicates concentration of nitrous oxide at the time of oxygen addition; (-.-.-) indicates extrapolated rate of reduction of nitrous oxide.

Figures 3.2a and 3.2b show the results obtained when the two experiments described above were repeated with *P. denitrificans*. Figure 3.2a shows that the addition of N₂O to cells of this organism reducing O₂ caused no change in the rate of O₂ respiration. This result alone does not provide evidence that N₂O respiration fails to occur alongside O₂ respiration. In agreement with the results obtained by Alefounder and Ferguson (1982), Figure 3.2b clearly shows that N₂O respiration did not occur in the presence of O₂. These observations contrast to the observations made with *T. pantotropha* (Figure 3.1b). Thus, there is a clear distinction between these two organisms in their capacities for simultaneously O₂ and N₂O reduction.

Acetate and succinate supply electrons through the dehydrogenases of the respiratory chain. The above experiments (Figures 3.1 and 3.2) were repeated for both organisms using succinate, to replace acetate as the carbon source, both in the growth medium and in the assay in the electrode chambers. The results obtained with succinate as a donor were essentially identical to those obtained with acetate in that *T. pantotropha*, but not *P. denitrificans*, reduced N₂O and O₂ simultaneously (see Alefounder and Ferguson, 1982).

Addition of ascorbate and TMPD to cells of *T. pantotropha* reducing O₂ or N₂O, in the presence of endogenous physiological reductants, leads to a substantial increase in the rate of reduction of either of these oxidants. A similar result is observed with cells of *P. denitrificans*. This increase in rate indicates that it is electron transport to cytochrome *c* (the probable branch point between the sites of O₂ and N₂O reduction) that is rate limiting in N₂O and O₂ reduction in these two organisms. The effect of

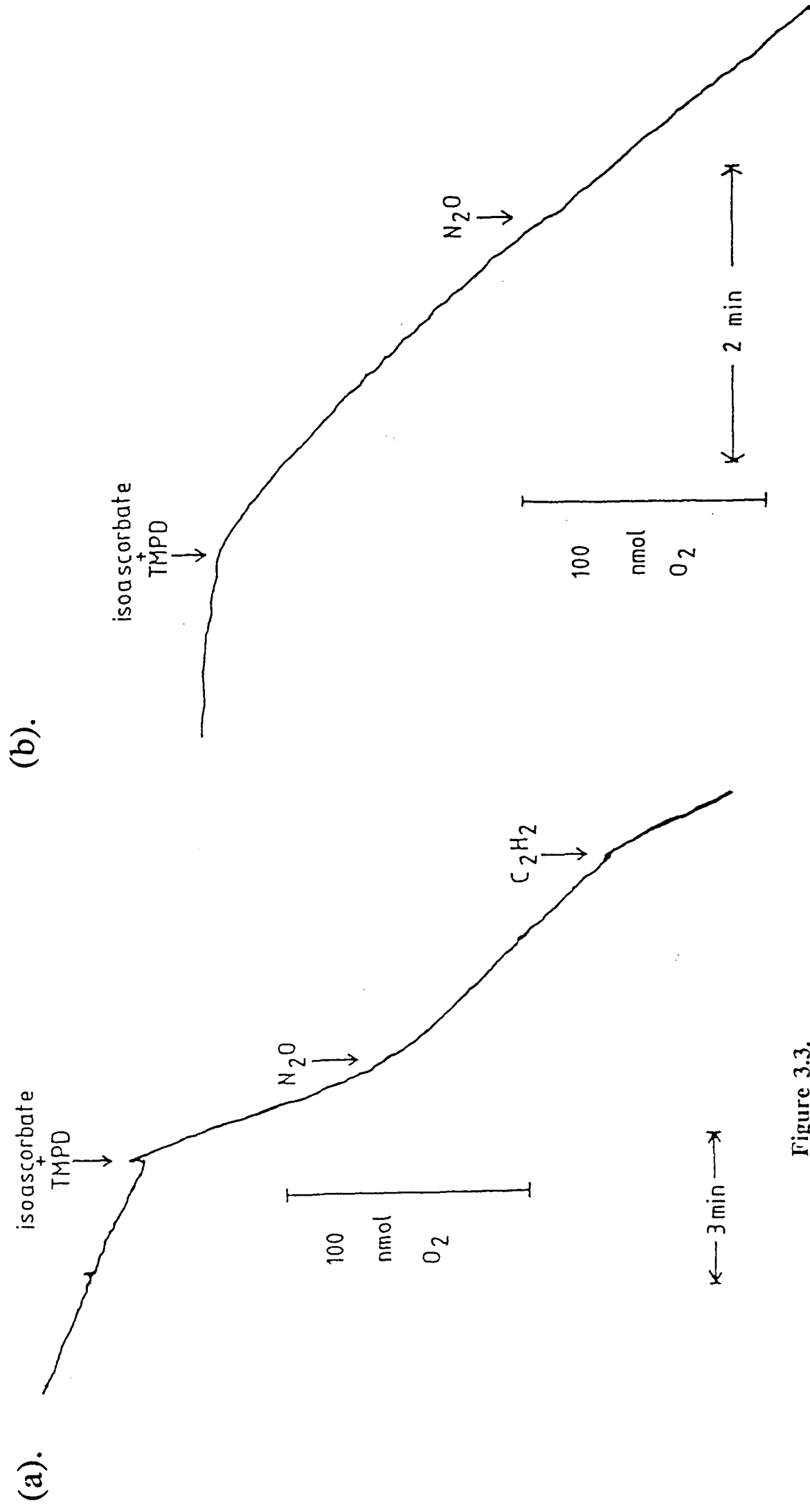


Figure 3.3.

(a) Inhibition by nitrous oxide of the reduction of oxygen by cells of *T. pantotropha* with sodium isoascorbate plus TMPD as substrate. Cells (0.53 mg dry weight) were added to a reaction chamber containing 2 ml of 200 mM sucrose, 10 mM Hepes/NaOH pH 7.3, 0.2 mM TMPD, 1 mM sodium isoascorbate. Nitrous oxide and acetylene were added as shown to concentrations of 0.6 mM and 1 mM respectively.

(b) Lack of inhibition by nitrous oxide of the rate of oxygen reduction by cells of *P. denitrificans*. Apart from the use of cells of *P. denitrificans* (0.5 mg dry weight) and the absence of acetylene the conditions were as for (a).

increasing the availability of electrons to the electron transport chains, by adding ascorbate and TMPD, on the aerobic reduction of N_2O in both organisms was studied. In these experiments, a pulse of N_2O was added to an O_2 electrode chamber containing cells which were reducing O_2 with ascorbate and TMPD as the electron donor. A typical experiment electrode trace is shown, in Figure 3.3a, for cells of *T. pantotropha*. On addition of N_2O , the rate of O_2 reduction was decreased to 36 % of its original rate (similar in magnitude to the 43 % attenuation seen with acetate as the added electron acceptor). In this batch of cells (which were typical) the rate of reduction of each oxidant alone was increased approximately seven-fold in the presence of this non-physiological electron donor. Similarly, the rate of O_2 reduction in the presence of N_2O was increased by a factor of seven in the presence of TMPD/ascorbate. Thus the N_2O reduction rate in the presence of O_2 must also increase by a factor of seven when this donor is present. As shown in the Figure 3.3a addition of C_2H_2 fully reversed the effect of N_2O as the original oxidase rate was restored.

Figure 3.3b illustrates a typical result obtained with cells of *P. denitrificans* using the same experimental approach as outlined above. In contrast to the observations made with *T. pantotropha*, no change in the rate of O_2 reduction was detected on addition of N_2O to cells of *P. denitrificans* which were respiring O_2 . The rate of O_2 reduction is increased, both in the presence and absence of N_2O , and the anaerobic rate of N_2O reduction is considerably stimulated, by the presence of ascorbate and TMPD. Thus, there can be no competition for electrons between the O_2 and N_2O reducing enzymes in the respiratory chain of *P. denitrificans*. This is strong evidence that N_2O can not be reduced in the presence of O_2 in *P. denitrificans*, even in the

presence of a non-physiological electron donor which provides electrons towards the oxidised end of the electron transport chain at the level of cytochrome c_{550} . In the case of *P. denitrificans*, the N_2O reductase and the cytochrome aa_3 , but not the alternative oxidase, known as cytochrome o can accept electrons from cytochrome c_{550} (Parsonage *et al.*, 1986). Thus the N_2O reductase would be theoretically competing with only one oxidase for electrons. The specific rate of N_2O reduction in cells of *P. denitrificans* exceeds the specific rate of O_2 reduction in this organism; this observation combined with the evidence presented in this section strongly suggests that the inhibition by O_2 of N_2O reduction in *P. denitrificans* is the result of direct inhibition of the N_2O reductase rather than a competition mechanism for electrons.

3.2.2. Reduction and synthesis of nitric oxide by *T. pantotropha*.

The product of NO_2^- reduction in cells of denitrifying bacteria has been subject to a great deal of controversy (see Chapter 1). It has recently been established that NO is the physiological product of NO_2^- reduction in the denitrification process. This conclusion has been drawn mainly from work involving trapping experiments, but these experiments have been performed under anaerobic conditions (Carr *et al.*, 1989; Goretski and Hollocher, 1988). Other evidence such as the characterisation of the NO reductase, has also been carried out, but only in bacteria which have been reported not to carry out the reactions of denitrification in the presence of O_2 (Carr and Ferguson, 1990a; Heiss *et al.*, 1989). NO is a very reactive molecule which reacts with O_2 to produce NO_2 which in turn generates NO_2^- and NO_3^- in an aqueous

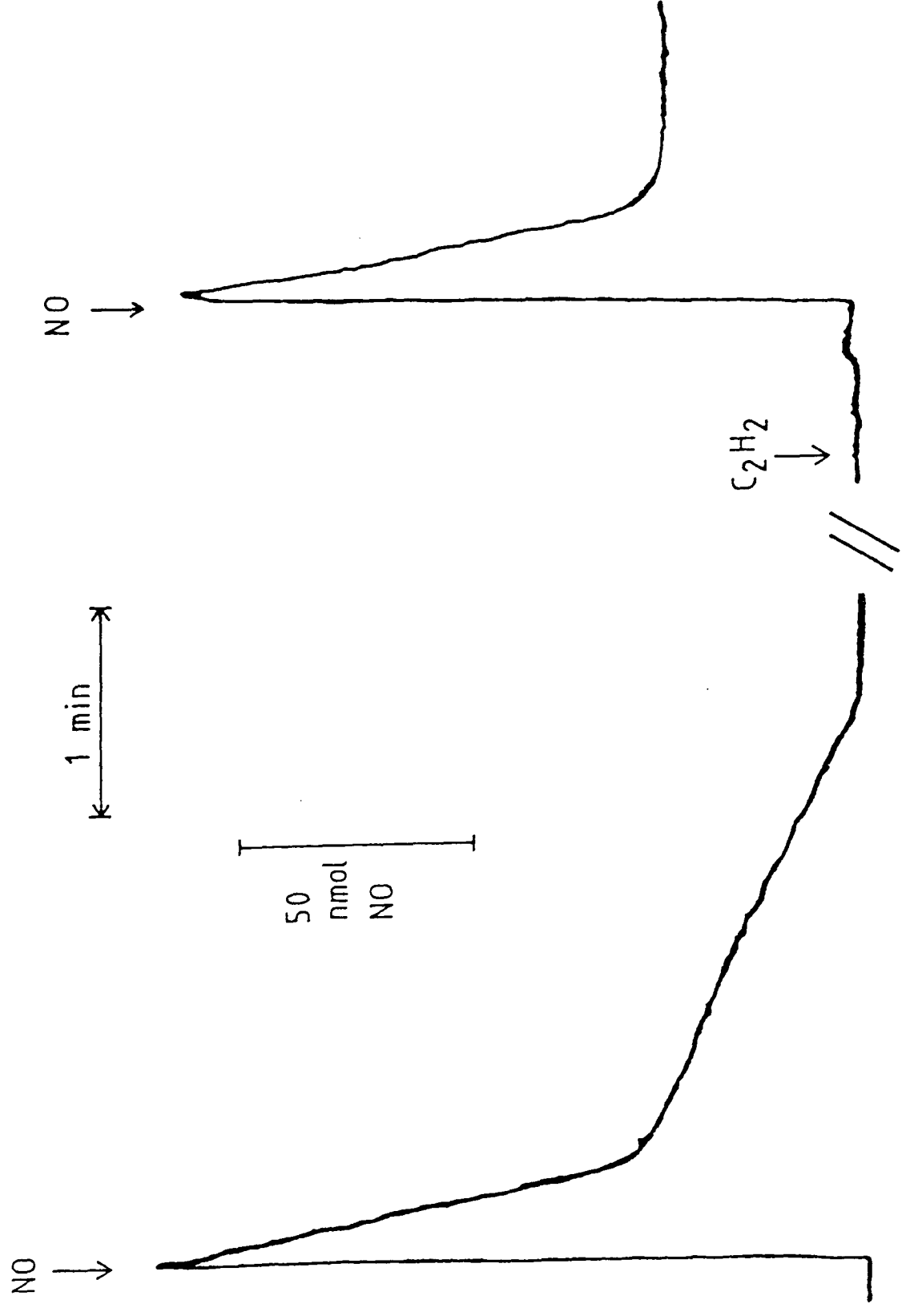


Figure 3.4. Reduction of nitric oxide to nitrous oxide by cells of *T. pantotropha*.

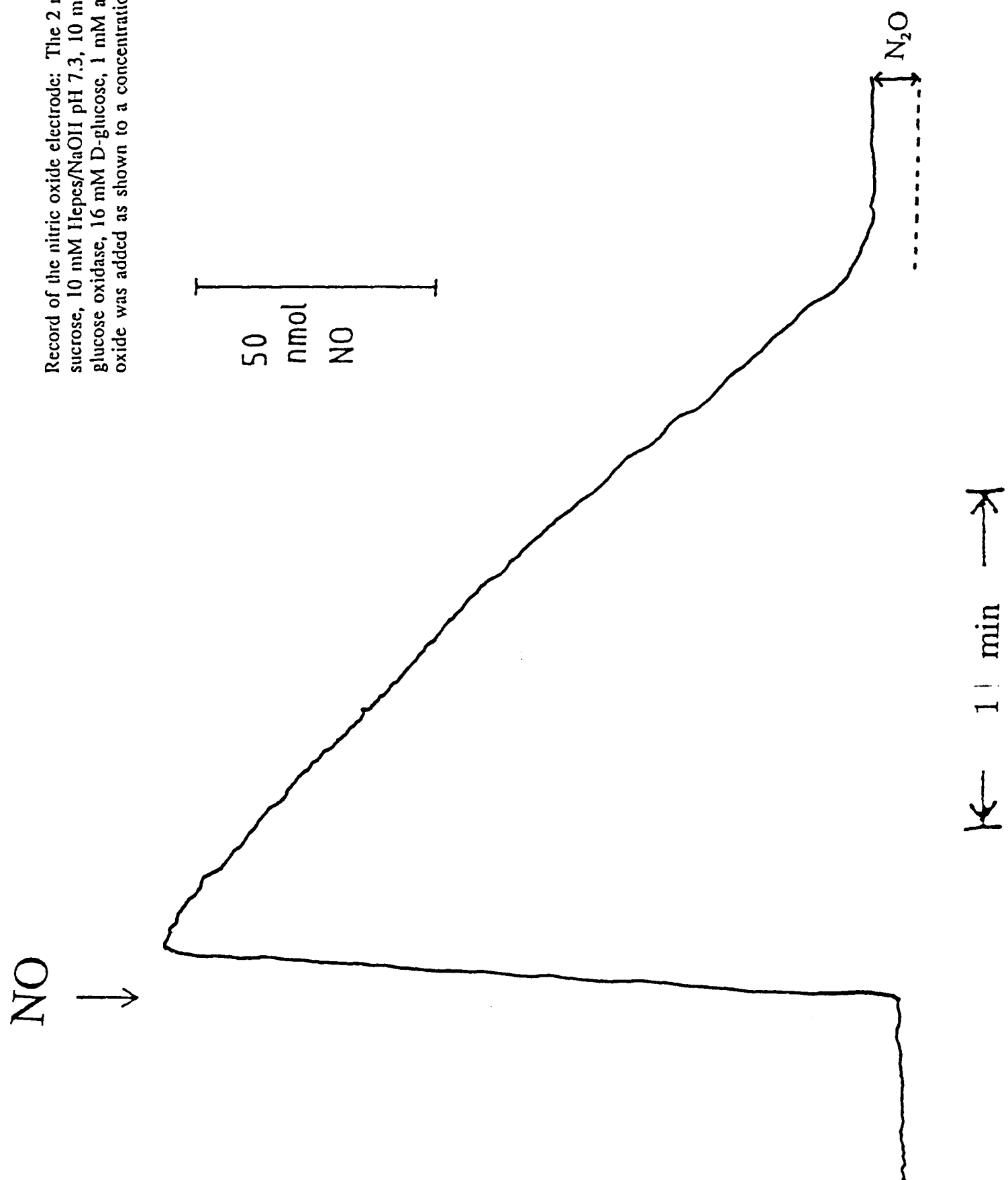
Record of the nitrous oxide electrode: Cells (150 μ g dry weight) were resuspended in 2 ml of medium containing 200 mM sucrose, 10 mM Hepes/NaOH pH 7.3, 10 mM potassium acetate, 50 units catalase, 4 units glucose oxidase and 16 mM D-glucose. When the reaction mixture had become anaerobic, nitric oxide was added to a final concentration of 75 μ M. Subsequently acetylene was introduced followed by a second addition of nitric oxide.

environment. If the rate of the reaction of NO with O_2 is faster than the reduction of NO to N_2O , then aerobic denitrification with NO as an intermediate would not be possible. Thus the question must be raised as to whether NO is an intermediate in the process of aerobic denitrification. If it is not, is NO_2^- reduced directly to N_2O , as previously and frequently suggested for denitrification in general (see Chapter 1)?

If NO is an intermediate in the process of aerobic denitrification, then bacteria which are capable of this process should possess an enzyme with NO reductase activity. Cells of *T. pantotropha* were assayed for this enzyme using the N_2O electrode which responds to both NO and N_2O . Figure 3.4 shows the events that follow the addition of NO to an anaerobic electrode chamber containing cells of *T. pantotropha*. A two phase response of the electrode was observed. When NO was added in the presence of C_2H_2 only a single phase was observed, but the final signal from the electrode was considerably displaced from the baseline. The electrode record in the absence of C_2H_2 appears identical to that reported for a similar experiment with cells of *P. denitrificans* (Carr *et al.*, 1989). The first phase corresponds to the reduction of NO to N_2O and the second phase to the reduction of N_2O to N_2 . As in *P. denitrificans* it can be seen that N_2O reduction does not occur in the presence of NO from comparing the initial amplitude of the second phase of the electrode response in the absence of C_2H_2 with the displacement in the presence of C_2H_2 . The rates of NO reduction can be calculated on an electron basis to be very similar to the rates of reduction for N_2O . Thus the inhibition of N_2O respiration by NO is likely to be due to a direct inhibition of the N_2O reductase rather than to competition for electrons. Cells of *T. pantotropha* were shown to have a high affinity for NO. The k_m for NO was estimated to be less than 10 μM from the

Figure 3.5. Reduction of nitric oxide by cells of *T. pantotropha*.

Record of the nitric oxide electrode: The 2 ml reaction medium was composed of 200 mM sucrose, 10 mM Hepes/NaOH pH 7.3, 10 mM potassium acetate, 50 units catalase, 4 units glucose oxidase, 16 mM D-glucose, 1 mM acetylene and 40 μ g dry weight of cells. Nitric oxide was added as shown to a concentration of 75 μ M.

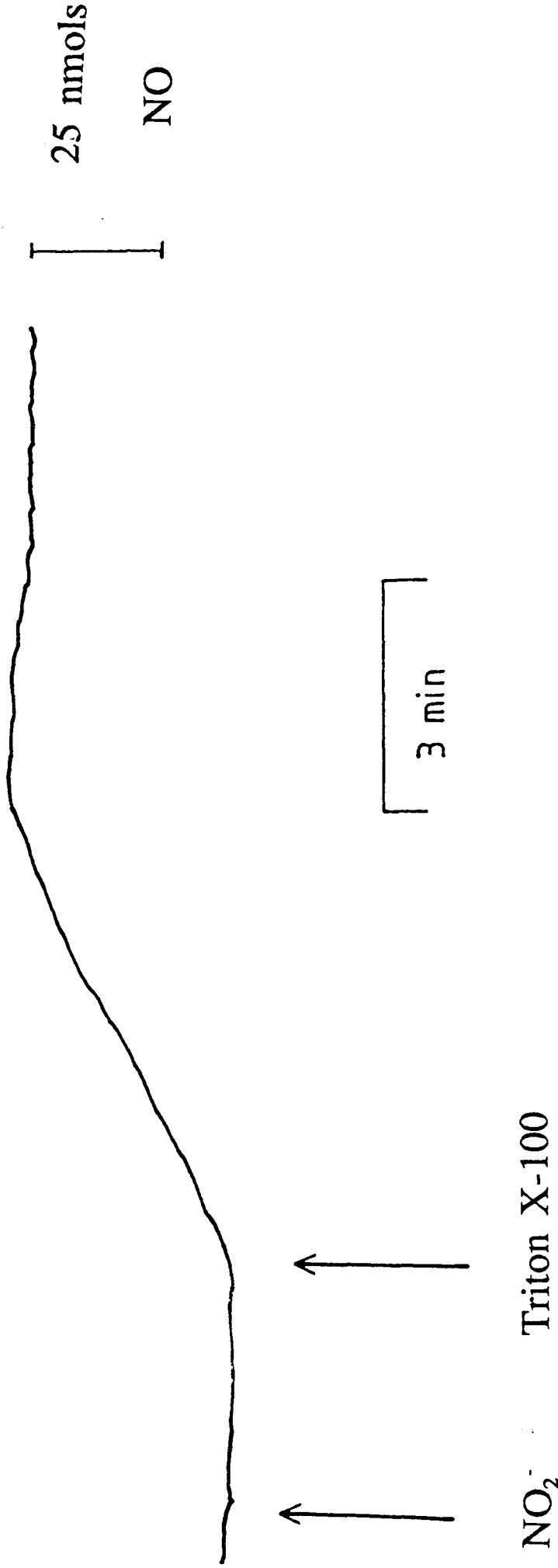


linearity of the record from the gold cathode NO electrode with which N_2O does not interfere even at low NO concentrations (Figure 3.5).

In order for a species to be a free intermediate in denitrification it needs to be a substrate for a reaction within the process and the product of that reaction needs to be another intermediate in the pathway to N_2 . NO clearly fits this criteria as it is reduced to N_2O . Furthermore, the species needs to be produced by the preceding step in the process. Thus it was necessary to determine whether NO was the product of NO_2^- reduction in cells of *T. pantotropha*. Triton X-100 has been reported to inhibit the NO reductase in cells of *P. denitrificans* (Shapleigh *et al.*, 1987). Therefore, if NO is the product of NO_2^- reduction in *T. pantotropha*, NO would be expected to accumulate in denitrifying cells of *T. pantotropha* in the presence of Triton X-100, providing that it has a similar inhibitory effect on all NO reductases. Cells of *T. pantotropha* were added to the anaerobic chamber of the NO electrode in the presence of potassium acetate. After a 5 min. period a pulse of NO_2^- was added. The baseline remained stable (Figure 3.6) until the addition of Triton X-100 to the chamber. This resulted in an upward deflection of the electrode trace for several minutes before reaching a plateau (Figure 3.6). If NO_2^- was added to cells, already in the presence of Triton X-100, the magnitude of the deflection was proportional to the amount of NO_2^- added to the cells. When the signal was calibrated against a known concentration of NO it was concluded that reduction of NO_2^- to NO followed a 1:1 stoichiometry. Therefore NO is the sole product of NO_2^- reduction, at least, under anaerobic conditions. This clearly provides evidence that cells of *T. pantotropha* possess the enzymes which allow NO to be first synthesised and then reduced in the overall scheme of denitrification.

Figure 3.6. Nitric oxide production from cells of *T. pantotrophica*.

The nitric oxide electrode record: Cells (50 μ g dry weight) were added to 2 ml 200 mM sucrose, 10 mM Hepes/NaOH pH 7.3, 10 mM potassium acetate, 50 units catalase, 4 units glucose oxidase. 16 mM D-glucose. Potassium nitrite was added to a final concentration of 50 μ M followed by Triton X 100 to 0.1 % (v/v).



3.2.3. Evidence for the role of nitric oxide as an intermediate in aerobic denitrification.

The finding that *T. pantotropha* both synthesised and reduced NO under anaerobic conditions suggested, but by no means proved, that these processes could also occur under aerobic conditions. Evidence for NO as a free intermediate in aerobic denitrification was therefore required.

For NO to be an intermediate of aerobic denitrification the lifetime of the NO molecule must be sufficient for it to diffuse from the NO_2^- reductase to the NO reductase of *T. pantotropha*. It has already been established (section 3.2.2) that the NO reductase of this organism has high affinity for its substrate ($< 10 \mu\text{M}$). In order to investigate this hypothesis an approach was devised to enable observation of trapping NO from cells reducing NO_3^- in the presence of O_2 . Haemoglobin has been used as a trap for NO in recent work (Carr *et al.*, 1989; Goretski and Hollocher, 1988) with denitrifying bacteria maintained under anaerobic conditions. Haemoglobin has a high affinity for O_2 and the visible absorption spectra of oxy- and nitrosyl-haemoglobin are very similar. Thus in experiments to establish that NO was synthesised under aerobic conditions haemoglobin would not have provided a useful trap. Consequently the ferric form of horse heart cytochrome *c* was exploited as a trap following a method introduced by Kucera *et al.* (1987). Cytochrome *c* does not bind O_2 but forms a ferric nitrosyl complex of cytochrome *c* (Fe^{3+}) on reaction with NO which can be identified by its strong absorption maxima at 563 nm. On addition of NO_3^- to cells of *T. pantotropha* which were reducing O_2 , resulted in the

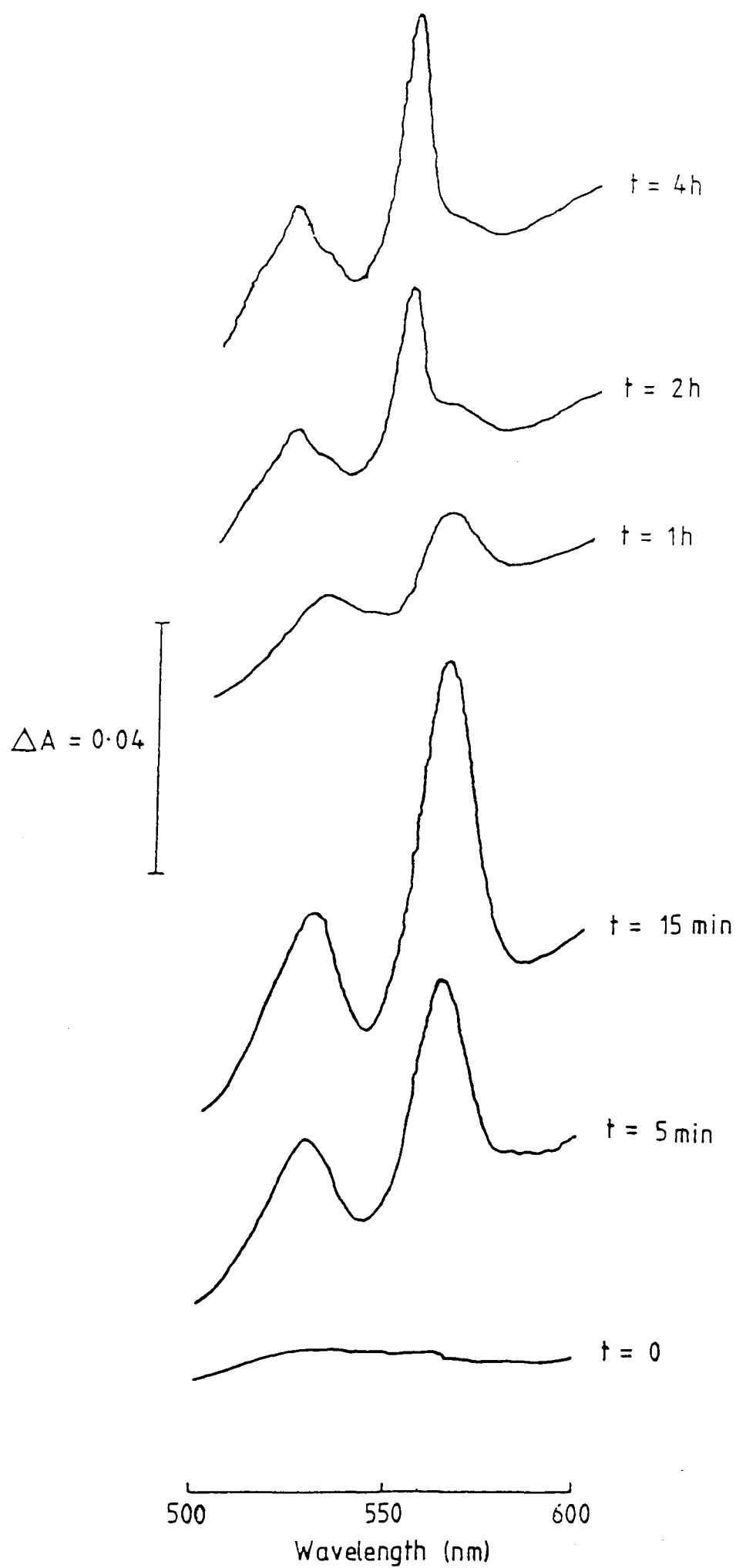


Figure 3.7. The formation of nitrosyl-ferric horse heart cytochrome *c* during the reduction of nitrate by cells of *T. pantotropha* under aerobic conditions.

Cells (33 μg dry weight) were suspended in cuvettes containing 1 ml 200 mM sucrose, 10 mM Hepes/NaOH, pH 7.3, 10 mM potassium acetate and 50 μM ferric horse heart cytochrome *c*. Potassium nitrate was added to the sample cuvette to a final concentration of 20 μM . The spectra were scanned at 1 nm. sec⁻¹ at the times shown.

cytochrome *c* in the external buffer forming the nitrosyl derivative of cytochrome *c* (Figure 3.7). The nitrosyl derivative was formed from the NO generated by the cells because no spectral changes were observed in the absence of cells when NO_3^- or NO_2^- were added to the cytochrome. Parallel measurements, made with an O_2 electrode, confirmed that the reaction mixture remained aerobic during the time course of the experiment. An identical spectrum was seen on addition of a pulse of NO to a cuvette containing ferricytochrome *c*. The cells were calculated to deplete the NO_3^- supplied in approximately 40 min. This time period corresponded with the time taken for commencement of the eventual loss of the absorption maxima at 563 nm and the appearance of a maxima at 550 nm (Figure 3.7). This is consistent with previous observations and is thought to correspond to the dissociation of the nitrosyl cation and formation of ferrous cytochrome *c* (Ehrenberg and Szezepkowski, 1960; Kucera *et al.*, 1987).

NO formation could also be detected using an identical experimental approach, but under anaerobic conditions, with both *T. pantotropha* and *P. denitrificans*. However, as predicted from reports of the inability of *P. denitrificans* to denitrify aerobically (Alefounder *et al.*, 1984), on addition of NO_3^- to cells of *P. denitrificans* reducing O_2 no formation of the nitrosyl form of cytochrome *c* was seen. These results further confirm the differences in the abilities of these two organisms to denitrify aerobically.

3.2.4. The complete denitrification pathway operates under aerobic conditions in *T. pantotropha*.

Sections 3.2.3 and 3.2.1 have conclusively demonstrated that NO is produced during the process of aerobic NO_3^- reduction by *T. pantotropha* and that this organism is capable of the aerobic reduction of N_2O . The characterisation of aerobic denitrification further required that the reduction of NO to N_2O occurred in the presence of O_2 . This appeared probable, taken that even for *P. denitrificans* there is evidence for reduction of NO under aerobic conditions (Carr and Ferguson, 1990b). Figure 3.8 shows that the addition of NO_3^- to a suspension of cells of *T. pantotropha* caused an immediate and sustained decrease in the rate of O_2 reduction. Addition of C_2H_2 substantially but not completely restored the rate of O_2 reduction. It was established that C_2H_2 inhibits N_2O reduction but has no effect on the other nitrogen-oxide or the O_2 reducing enzymes in this bacterium. Thus the interpretation of Figure 3.8 is that the reduction of NO_3^- through to N_2 diverts electrons from the oxidase system, and that presence of C_2H_2 partially alleviates this diversion by inhibiting the N_2O reductase. This acts as clear evidence for the formation of N_2O from NO as well as the reduction of N_2O in the presence of O_2 . Thus the complete sequence of denitrifying reactions occurs during aerobic NO_3^- reduction in *T. pantotropha*.

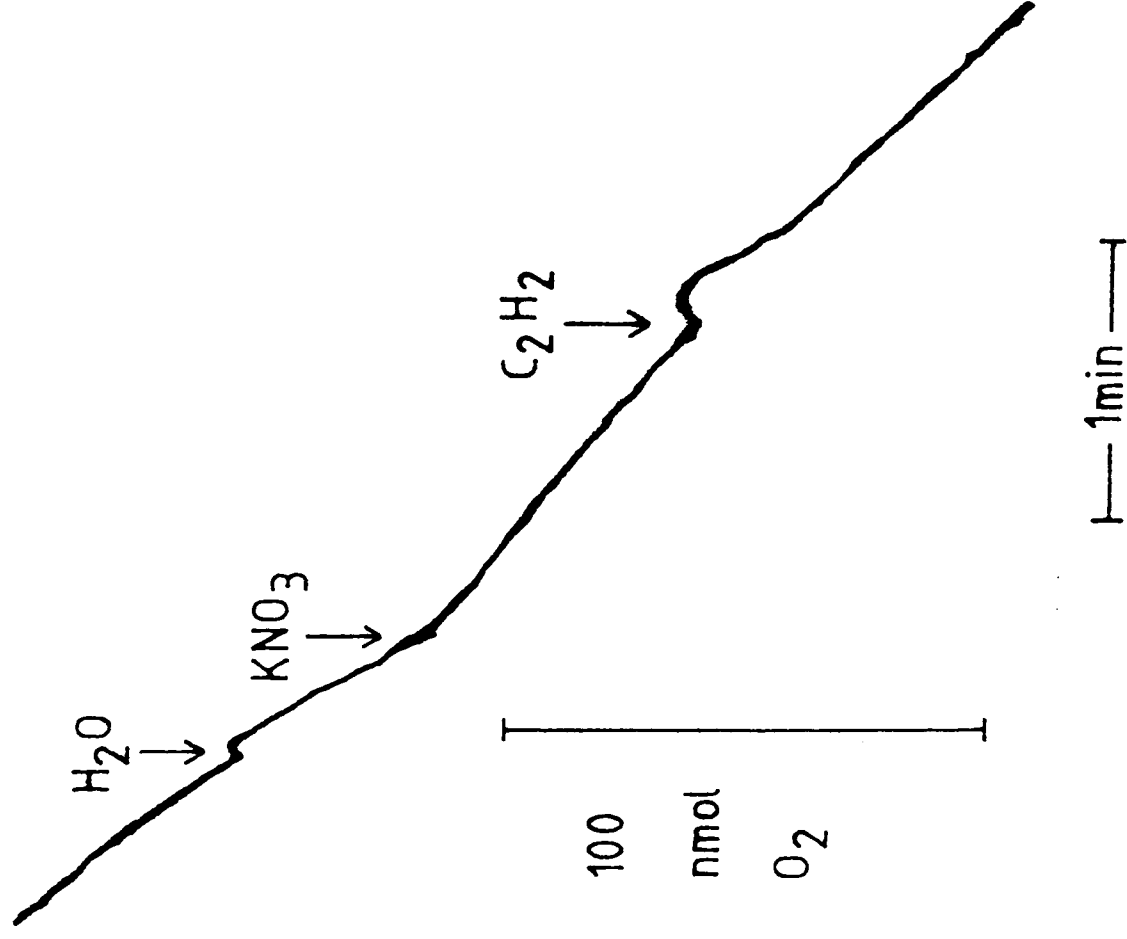


Figure 3.8. Effect of nitrate upon reduction of oxygen by cells of *T. pantotropha*.

Cells (3.6 mg dry weight) were added to a 2 ml solution containing 200 mM sucrose, 10 mM Hepes/NaOH pH 7.3, 10 mM potassium acetate in the reaction chamber of an oxygen electrode. Potassium nitrate and acetylene were added to a final concentration of 0.1 mM and 1 mM respectively.

3.2.5. The sensitivity to myxothiazol of the denitrifying and oxidase reactions in *T. pantotropha*.

Clearly during aerobic denitrification there must be distribution of electrons between the nitrogen-oxide and O₂ reducing enzymes of the cell. Such competition for electrons is a process that is not observed in non-aerobic denitrifiers such as *P. denitrificans*. It has previously been suggested that aerobic denitrification is seen in *T. pantotropha* as there is a "bottleneck" in the pathway of electron flow to O₂ via cytochrome *aa*₃ (Robertson and Kuenen, 1984; Robertson, 1988). The electron transport chain of *P. denitrificans* is one of the most studied amongst the denitrifying bacteria. The respiratory chain of *T. pantotropha* has previously been assumed to bear a basic resemblance to that of *P. denitrificans* (i.e. UQ → cyt *bc*₁ → cyt *c* → cyt *aa*₃) (Robertson, 1988; see also Chapter 1). In *P. denitrificans*, the NO₃⁻ reductase receives its electrons from ubiquinol, and the NO₂⁻, NO and N₂O reductases are supplied with electrons via the cytochrome *bc*₁ complex and one or more *c*-type cytochromes (Stouthamer, 1980; Carr *et al.*, 1989). Myxothiazol, an inhibitor of the cytochrome *bc*₁ complex, was employed to determine if the nitrogen-oxide reducing enzymes of *T. pantotropha* accept electrons at the same level of the respiratory chain as their counterparts in *P. denitrificans*. Clark-type electrode records show that a concentration of 0.5 nmol. myxothiazol per mg dry weight of cells inhibited the rate of both NO and N₂O reduction by more than 95 %. A similar concentration of myxothiazol inhibited NO₂⁻ reduction but had no effect on the rate of NO₃⁻ respiration. Similar concentrations of myxothiazol are required to inhibit electron flow through the cytochrome *bc*₁ in *P. denitrificans* (Carr *et al.*, 1989). Thus, it

appears that the basic organisation of the electron transport reactions for denitrification in *T. pantotropha* is similar to that found in *P. denitrificans*. However, caution must be exercised before concluding that these are the only electron transport pathways operative. Recent work (Richardson *et al.*, 1989; see section 1.6) with *R. capsulatus* has shown that at least two electron transport pathways to N_2O and NO can operate in the latter organism. The high percentage inhibition of NO_2^- , NO and N_2O reduction by myxothiazol observed for *T. pantotropha* suggests, however, that the capacity of any alternative electron transport pathway must be low.

O_2 reduction was inhibited maximally, by about 40 %, at the same concentration of 0.5 nmol. myxothiazol per mg dry weight of cells. This suggests that there are at least two oxidases present in cells of *T. pantotropha*, one accepting electrons via the cytochrome bc_1 complex and the second via ubiquinol. Thus the organisation of the enzymes for denitrification and O_2 reduction within the respiratory chain of *T. pantotropha* appears to resemble that of *P. denitrificans*.

3.3. DISCUSSION.

T. pantotropha has been reported to denitrify in the presence of O_2 , this is based on experimental observations which showed gas production and removal of NO_3^- , which was not included in the biomass, from aerobic cultures of this organism (Robertson and Kuenen, 1983; 1984). If the gaseous product is N_2 then this organism is able to complete all the reactions in the sequence of denitrification including the reduction of N_2O to N_2 . The inhibition by molecular O_2 of N_2O reduction in cells of non-aerobic denitrifiers is thought to arise from a direct inhibition of the N_2O reductase (Alefounder and Ferguson, 1982; Alefounder *et al.*, 1984). For example, on addition of N_2O to cells of *P. denitrificans* respiring O_2 no respiration of the N_2O was observed until the O_2 was depleted. The inhibition of N_2O reduction in cells by O_2 was fully reversible and was concluded to be a direct effect of O_2 rather than a consequence of competition for electrons (Alefounder and Ferguson, 1982). N_2O reductase has also been studied and shown to be inactivated by O_2 in cell free systems. Zumft *et al.* (1985a) observed that the N_2O reductase of *P. perfectomarina* when purified under anaerobic conditions was 2-5 times more active than the aerobically prepared enzyme. The aerobically prepared enzyme had an apparently lower Mr on gel filtration and was spectroscopically distinct from the anaerobically prepared enzyme. The N_2O reductase from *P. denitrificans* had been shown to have two responses to O_2 (Snyder and Hollocher, 1987). This enzyme lost activity slowly in the presence of O_2 alone ($t_{1/2} = 65$ min.) but in the presence of O_2

and BV^{+} inactivation was rapid ($t_{1/2} = 5$ min.). This rapid inactivation was thought to be as a result of a process resembling turnover inactivation.

Thus in the present work it was necessary to establish that the gas released on reduction of NO_3^- under aerobic conditions was N_2 and not N_2O or some other product. The results presented in section 3.2.1 provide clear evidence that the N_2O reductase of *T. pantotropha* can compete for electrons with the cytochrome oxidases, even when the supply of electrons to these terminal enzymes is limiting (i.e. with physiological substrates). This result is in complete contrast to that observed for N_2O reduction in *P. denitrificans* (Alefounder and Ferguson, 1982). No N_2O reduction was observed in the presence of O_2 in *P. denitrificans*, even when electrons were supplied towards the oxidising end of the respiratory chain at the level of cytochrome *c* (Figure 3.3b). Under such conditions N_2O would only compete with cytochrome *aa₃* for electrons, as the alternative oxidase accepts electrons at the level of ubiquinol. Thus, this evidence strongly agrees with ^{the} hypothesis that O_2 exerts a direct inhibition on the N_2O reductase of *P. denitrificans*, although there is no evidence for any mechanism for this process. An assay for N_2O reductase activity has not yet been developed which will operate in the presence of O_2 in cell free systems. The only known direct electron donors to this enzyme (MV^{+} or BV^{+}) react directly with O_2 and thus it is impossible to determine whether the purified N_2O reductase would be active in the presence of O_2 . The clear evidence that N_2O reduction occurs in *T. pantotropha* implies that either :- (i) the electron transport pathway(s) to O_2 and/or N_2O of *T. pantotropha* in some way differs from those found in organisms which do not denitrify in the presence of O_2 . (ii) The N_2O reductase of this organism is different from that seen in non-aerobic denitrifiers, or (iii) that a

protection mechanism against the deleterious effects of O_2 exists in *T. pantotropha*.

A similar problem is, in principle, faced by aerobic diazotrophs which need to protect their nitrogenase enzymes from the harmful effects of O_2 . *Azotobacter* species have developed a number of protective mechanisms against O_2 including respiratory and conformational protection. It was observed that *Azotobacter vinelandii* reduced O_2 much faster than the energy requirements for the cell if grown at high O_2 concentrations (Phillips and Johnson, 1961). Later the idea that the rate of respiration could be adjusted to respond to the dissolved O_2 concentration, thus maintaining an anaerobic environment was proposed and named as "respiratory protection" (Dalton and Postgate, 1969a,b). The mechanism for this involves use of an alternative oxidase, not linked to proton translocation, and thus bypasses the terminal proton translocating step of oxidative phosphorylation (a site analogous to cytochrome *aa₃*). The proton translocating NADH dehydrogenase is also bypassed and thus respiratory control is minimised so rates of electron transport can increase. However, this response mechanism is not instant, taking many minutes to occur (Lees and Postgate, 1973). In contrast, aerobic N_2O reduction is an immediate response to the availability of both electron acceptors. Branched respiratory chains are observed in many bacteria including *T. pantotropha*. However, the affinities of the oxidases of this bacterium and the conditions under which they are expressed need further investigation before conclusions can be drawn about the existence of a respiratory protection mechanism in *T. pantotropha* analogous to that in *Azotobacter*.

An O_2 stable nitrogenase has been purified from *Azotobacter vinelandii* (Bulen and Le Comte, 1972). This enzyme contained three subunits; the two redox proteins

found in other molybdenum nitrogenases and a third [2Fe-2S] protein. Together with Mg^{2+} , this latter protein partly protects the redox centres of the nitrogenase. It appears the mechanism involves the oxidation of the redox centres followed by the association of the third FeS subunit (Scherings *et al.*, 1977; Lough *et al.*, 1983). A similar mechanism, involving a "protector protein", may be found in *T. pantotropha* for protecting the N_2O reductase from effects of O_2 . This point could be further examined by purification and characterisation of the N_2O reductase from this organism. The characterisation of this enzyme may reveal the reason for any increase in its O_2 stability. In other respects, such as C_2H_2 sensitivity and organisation of the electron transport chain, N_2O reduction in *T. pantotropha* appears similar to that in *P. denitrificans*.

The N_2O reductase of *W. succinogenes*, a non-denitrifying organism, has been reported to be stable in the presence of O_2 (Teraguchi and Hollocher, 1989). However, this reductase, unlike its counterpart in *T. pantotropha*, was insensitive to the presence of C_2H_2 . Thus, it is unlikely that the N_2O reductases of these two distantly related organisms are similar.

Another possibility which needs to be considered, in the context of the observation that N_2O reduction can occur under aerobic conditions in *T. pantotropha*, is that the electron transport chain of this organism may differ in some respect from the respiratory chains found in organisms which do not denitrify aerobically. Robertson and Kuenen (1986; 1990) suggested that the sequence of cytochromes in *T. pantotropha* is essentially similar to that seen in *P. denitrificans* (Stouthamer, 1980; Chapter 1). These workers further suggested that NAD(P)H may accumulate

in cells grown under heterotrophic conditions due to a "bottleneck" in the electron flow between cytochrome *c* and O_2 via cytochrome *aa₃* (Robertson and Kuenen, 1986; 1990). The electrons from NAD(P)H enter the cytochrome chain and are envisaged to lead to an increased level of reduction of the chain to such an extent that electrons can flow to the denitrification pathway even in the presence of O_2 . Electrons have been shown to branch from the "main" cytochrome chain at the level of ubiquinol for NO_3^- reduction and at cytochrome *c* for N_2O , NO_2^- and NO reduction in *T. pantotropha* (section 3.2.5; see also Figure 1.6). If a bottleneck for electrons exists between the cytochrome *aa₃* oxidase and cytochrome *c* an increase in the total flux of electrons within the respiratory chain would be expected if N_2O was added to cells reducing O_2 . No decrease in the rate of O_2 would be expected if this model were to hold true. However, the observations reported in section 3.2.1 showed that the rate of O_2 reduction decreased when N_2O was introduced to cells of this organism. Any bottleneck occurring within the cytochrome chain of this organism must be before the branch point between N_2O and O_2 reduction with such a model. No increase in the total electron flux within the electron transport chain would then be expected with these two terminal electron acceptors present. However, an increase in the total electron flux would be observed if NO_3^- was introduced to cells reducing O_2 since the NO_3^- reductase of this organism accepts electrons before the bottleneck in the chain. The effect of NO_3^- on the electron flux of aerobic cells could be further explored in future work. The model described, however, does not, however, explain why N_2O and O_2 reduction should occur simultaneously in this organism. As discussed above this may be due to a difference in the terminal reductase itself or a respiratory protection mechanism.

NO reductase appears to be active in cells of bacteria such as *P. denitrificans*, which are generally thought to be unable to denitrify aerobically, in the presence of O_2 (Carr and Ferguson, 1990b). This is based on observations that the addition of membrane vesicles, that contain NO reductase, relieves the inhibition of oxidase activity in bovine heart sub-mitochondrial particles seen in the presence of NO (produced in situ using the purified NO_2^- reductase from *P. denitrificans*). Thus the NO reductase of *P. denitrificans* must turnover in the presence of O_2 unlike the other nitrogen-oxide reducing enzymes in this bacterium (Alefounder *et al.*, 1984). Therefore, it could be anticipated that the turnover of the NO reductase of *T. pantotropha* is unlikely to be a step preventing aerobic denitrification. The experiment with the *P. denitrificans* system also provides evidence that the lifetime of NO must be at least sufficient for it to diffuse to the oxidases and bind to give the inhibitory effect. Therefore, NO may ^{be} sufficiently stable to be an intermediate in aerobic denitrification as its lifetime may be sufficient for diffusion from the NO_2^- reducing enzyme(s) which is/(are) located in the periplasm to the active site of the NO reductase which is associated with the membranes in cells of *T. pantotropha*. The subcellular locations of the NO_2^- and NO reductases of *T. pantotropha* were established by the present author but are not reported in this thesis.

This Chapter presents clear evidence that *T. pantotropha* reduces NO_2^- to NO which is reduced further to N_2 via N_2O . The rates of these three reduction processes are comparable on electron basis consistent with NO being an intermediate of denitrification in this bacterium. The trapping of NO with an extracellular cytochrome acts as further evidence that NO can diffuse freely in an O_2 containing

aqueous environment but more importantly confirms NO is a free intermediate of aerobic denitrification. This result may appear to be in conflict with the known high reactivity of NO with O₂ but may be explained on the following basis. The reaction of NO with O₂ has been shown to be a third order reaction in the gas phase (Bodenstein, 1918). Thus the rate of the reaction is dependent on the concentration of O₂ and the square of the concentration of NO. Cells have a high affinity for NO since NO was not detected in cells reducing NO₂⁻ using the NO electrode (section 3.2.2). Thus the steady state concentration of NO must be less than 1 µM. This result agrees with the observation that cells of *P. denitrificans* did not normally release readily detectable levels of NO during denitrification (Garber and Hollocher, 1981; Carr *et al.*, 1989; Gorteski and Hollocher, 1988). A similar result would be expected under aerobic conditions since the NO reductase is apparently not inhibited by O₂. The calculated and observed rate constants for the reaction of NO with O₂ at 300 K in the gas phase are similar (3.3×10^3 and 7.1×10^3 dm³. mol⁻². sec⁻¹ respectively; Laidler, 1978). If the observed rate constant for the reaction of NO with O₂ is similar in the aqueous phase then low concentrations of NO are extremely stable in an aerobic aqueous environment. The low concentration of NO released from denitrifying cells of *T. pantotropha* would explain the apparent lack of inhibition of the oxidase activity which has previously been observed for other organisms in the presence of NO (Carr and Ferguson, 1990b). Preliminary observations from experiments, where NO was added to aerobic cells of *T. pantotropha*, showed O₂ reduction was temporarily inhibited by NO at high NO concentrations (established by present author but not presented in this Thesis). These observations suggest that at high concentrations of NO the oxidases of *T. pantotropha* were reversibly inhibited, because when the NO concentration decreased the rate of

O₂ reduction returned to the rate observed before the introduction of the NO. The aqueous chemistry of NO deserves further investigation as NO has recently been shown to play a role as an endothelium-derived relaxing factor (Palmer *et al.*, 1987).

From the conclusions drawn it is evident that N₂ gas can be produced from NO₃⁻ in the presence of O₂. Thus each step in the denitrification sequence must in some way differ from its counterpart in *P. denitrificans*. In *P. denitrificans* there appear to be different mechanisms switching off three of the reduction steps. In the case of NO₃⁻ reduction the inhibitory effect of O₂ is indirect. It is thought that a redox sensitive component acts to prevent the transport of NO₃⁻ across the cytoplasmic membrane to its site of reduction (Alefounder and Ferguson, 1980; Alefounder *et al.*, 1981). The mechanism by which *T. pantotropha* avoids this transport problem under aerobic conditions is discussed in detail in Chapter 4. Although cells of *P. denitrificans* do not normally reduce NO₂⁻ under aerobic conditions, the enzyme itself can be shown to function under aerobic conditions (for example, in the presence of ascorbate and TMPD as an electron donor, (Alefounder *et al.*, 1983). It has therefore been suggested that NO₂⁻ reduction is prevented under aerobic conditions by a strong competition for electrons from O₂. NO reductase is thought to be functional in the presence of O₂ (discussed above). The final unexplained difference between these two organisms is in the nature of the N₂O reduction step. The electron transport pathways to the reactions of denitrification of these two organisms appear similar from the results presented in section 3.2.5. It appears that the difference most probably lies in the N₂O reductase of *T. pantotropha*.

The observations made in this Chapter agree with much of the previous evidence for absence of aerobic denitrification in *P. denitrificans* (Alefounder *et al.*, 1984; John, 1977). In view of this evidence, it is somewhat surprising that it has recently been reported that *P. denitrificans*, along with several other organisms, denitrifies in the presence of O₂ (Lloyd *et al.*, 1987; Davies *et al.*, 1989). The latter work differs from other investigations of denitrification in that the experimental methodology involved a quadrupole mass spectrophotometer to measure the composition of the gas phase in contact with suspensions of denitrifying bacteria. It is unclear whether the strain of *P. denitrificans*, used by these workers, has gained the ability to denitrify aerobically (see Chapter 7) or whether the results obtained are a consequence of the experimental method. However the results presented here do provide evidence that in at least one bacterium, *T. pantotropha*, all the enzymes of denitrification can function in an aerobic environment.

Chapter 4. Periplasmic and membrane
bound respiratory nitrate reductases
from *Thiosphaera pantotropha*.

4.1 INTRODUCTION.

An evolutionary diverse range of genera of bacteria possess the ability to utilize NO_3^- as an alternative electron acceptor to molecular O_2 . A respiratory membrane bound NO_3^- reductase has been identified and characterised to varying degrees in a number of denitrifying organisms including *P. denitrificans* (Forget, 1971; Craske and Ferguson, 1986). These reductases, as purified vary in molecular mass, tertiary structure and chemical composition although this may be in part due to the different preparative methods employed by different workers (Hochstein and Tomlinson, 1988). All these membrane bound enzymes are iron-sulphur (FeS) proteins and contain molybdenum within a separate cofactor. There appear to be many similarities between the respiratory NO_3^- reductase of *P. denitrificans*, a soil bacterium, and its counterpart in *E. coli* (Stewart, 1988), which is an enteric organism. The enzyme is composed of an integral membrane subunit named γ that contains two *b*-type haems (Ballard and Ferguson, 1988) and which accepts electrons from ubiquinol. The β subunit has been recently proposed to contain the FeS centre, which could be involved in the passage of electrons from the *b*-type cytochromes to the molybdenum centre in the catalytic site in the α subunit (Blasco *et al.*, 1989).

Most species of bacteria that express a respiratory NO_3^- reductase do so only if grown under conditions of O_2 limitation, but in such cells the transfer of electrons to NO_3^- is nevertheless completely blocked by the presence of O_2 (John, 1977; Alefounder *et al.*, 1981). The mechanism by which O_2 acts to inhibit NO_3^- reduction is still not completely resolved. Observations, with *P. denitrificans*, have indicated that this inhibition is exerted indirectly since other electron acceptors, such as

ferricyanide or N_2O , can mimic the inhibitory effect of O_2 and the inhibition can be relieved on addition of antimycin (Alefounder *et al.*, 1981, 1983; Kucera *et al.*, 1981, 1983). It has, therefore, been argued that NO_3^- reduction is controlled by the extent of oxidation of one or more components in the electron transport chain (Alefounder *et al.*, 1983). Irrespective of the identity of such (a) component(s) it appears that O_2 exerts at least part of its control on the transport of the NO_3^- ion across the cytoplasmic membrane to its site of reduction (Alefounder and Ferguson, 1980; Alefounder *et al.*, 1983; Hernandez and Rowe, 1988). This conclusion was based on two experimental observations. First, whereas NO_3^- reduction in whole cells of *P. denitrificans* was completely inhibited by O_2 , inside-out membrane vesicles of this organism could reduce NO_3^- and O_2 simultaneously. NO_3^- reduction was fully inhibited by O_2 in cells of a cytochrome *c*-deficient mutant, so the simultaneous reduction of O_2 and NO_3^- seen in vesicles could not be attributed to the loss of cytochrome c_{550} (Alefounder *et al.*, 1983). Second, inside-out membrane vesicles reduce ClO_3^- , but whole cells can not. Significantly, the same concentration of detergent that allows ClO_3^- to cross the permeability barrier of the cytoplasmic membrane to its site of reduction, also lessens the sensitivity of NO_3^- reduction to O_2 (Alefounder and Ferguson, 1980). More recent work has confirmed that it is NO_3^- uptake that is inhibited under aerobic conditions in whole cells of a number of other bacterial genera (Hernandez and Rowe, 1987; 1988).

A number of bacterial species have been reported to carry out the reduction of NO_3^- in the presence of O_2 as the first step of aerobic denitrification. These include *Aquaspirillum magnetotacticum* (Bazylinski and Blakemore, 1983), *Hyphomicrobium* X (Meiburg *et al.*, 1980) *Alcaligenes* sp. (Krul and Veeningen, 1977) and *T.*

pantotropha (Robertson *et al.*, 1986). This observation raises two questions which will be examined in the present work with *T. pantotropha* as the "model organism". First, is NO_3^- reductase expressed in cells of *T. pantotropha* even when they are grown under aerobic conditions? Second, if the dissimilatory NO_3^- reductase of *T. pantotropha* is structurally similar to the NO_3^- reductase of *P. denitrificans*, how is NO_3^- transported across the cytoplasmic membrane to its site of reduction during aerobic NO_3^- reduction? These questions are central to the theme of this Chapter, which examines the molecular mechanism of the NO_3^- reducing system and the nature of the terminal NO_3^- reductases of *T. pantotropha*.

4.2. RESULTS.

4.2.1. The localization of nitrate reductase activity in *T. pantotropha*.

In order to begin to study the nature of the NO_3^- reduction in *T. pantotropha* the enzyme responsible for the reduction had to be located within the cell. Cells of this organism were grown both anaerobically and aerobically in the presence of NO_3^- and succinate, with NH_4^+ present as a nitrogen source. Washed cells were then tested to check that they possessed NO_3^- reductase activity. The assay procedure used BV^{++} as an electron donor (a detailed protocol is described in section 2.3.7). BV^{++} contains two heterocyclic aromatic rings and thus it is sufficiently hydrophobic to cross the lipid membrane. It can therefore donate electrons to components located in the cytoplasmic compartment of the cell (Jones and Garland, 1977). Cells grown under both anaerobic and aerobic conditions reduced NO_3^- . However, cells grown anaerobically possessed approximately a 4 fold greater $\text{BV}^{++}/\text{NO}_3^-$ oxidoreductase activity than aerobically grown cells (180-270 and 45-65 $\text{nmol. min}^{-1} \text{. mg cell protein}^{-1}$, respectively).

Having established that cells grown aerobically as well as those grown anaerobically contained NO_3^- reductase activity, cells from both growth modes were fractionated into periplasm, cytoplasm and membranes. In order to verify that there was no contamination of the periplasm with cytoplasmic proteins, the preparation was assayed for the presence of malate dehydrogenase (a cytoplasmic marker). Less than 2 % of the total cellular activity of malate dehydrogenase was found in the

Table 4.1. The subcellular location of nitrate reductase activity from anaerobically and aerobically grown *T. pantotropha*.

Fraction	BV ⁺ -NO ₃ ⁻ oxido-reductase activity (% of total)	
	Anaerobically grown cells	Aerobically grown cells
Periplasm	14	97
Membrane	85	3
Cytoplasm	1	n.d.

Cells containing 50 mg protein were fractionated and 100 % activity is equivalent to 200 μ mol BV⁺ reduced. min⁻¹ and 50 μ mol BV⁺reduced. min⁻¹ for the anaerobic and aerobic grown cells, respectively.
All measurements were performed in a reaction medium containing 10 mM Tris/HCl pH 8.0, 2 U. ml⁻¹ glucose oxidase, 25 U. ml⁻¹ catalase, 16 mM D-glucose and 0.1 mM benzylviologen. The reaction was started by the addition of potassium nitrate to a final concentration of 1 mM.

n.d. = not detectable.

periplasmic fraction.

In anaerobically grown cells the $\text{BV}^+/\text{NO}_3^-$ oxidoreductase activity was associated predominantly with the membrane fraction of the cells (Table 4.1). However, a NO_3^- reductase activity was also observed in the periplasmic fraction (Table 4.1). Approximately 1 % of the total activity was also located in the cytoplasm which might have arisen in the following way. The active site of the membrane bound NO_3^- reductase of other organisms is contained within the α subunit of the enzyme. This subunit is water soluble and it retains its activity on dissociation from the membrane (Ballard and Ferguson, 1988). Thus the cytoplasmic activity is most likely to be due to a small degree of dissociation of the membrane bound enzyme during the fractionation procedure. On fractionation of the aerobically grown cells, there was a significant difference in the location of the NO_3^- reductase activity. The $\text{BV}^+/\text{NO}_3^-$ oxidoreductase activity was almost entirely associated with the periplasmic fraction, with only 3 % of the total cellular activity associated with the membranes (Table 4.1). No activity was detectable within the cytoplasmic fraction of aerobically grown cells, which further suggested that the NO_3^- reductase activity located in the cytoplasmic fraction of the anaerobically grown cells is an artefact due to the dissociation of the soluble α subunit of the membrane bound NO_3^- reductase.

The periplasmic and membrane fractions were also assayed for $\text{NADH}/\text{NO}_3^-$ oxidoreductase activity. The membrane, but not the periplasmic, NO_3^- reductase activity could be coupled to NADH oxidation activity since NADH dehydrogenase is a membrane bound enzyme (Table 4.2).

Table 4.2 Distinct catalytic properties of nitrate reductase in periplasm and cytoplasmic membranes of anaerobically grown *T. pantotropha*.

Activity of nitrate reductase ($\mu\text{mol. min}^{-1}$)						
Substrate	BV ⁺			NADH		
Electron Acceptor	NO ₃ ⁻	NO ₃ ⁻ + azide	ClO ₃ ⁻	NO ₃ ⁻	NO ₃ ⁻ + azide	ClO ₃ ⁻
Fraction						
Membrane	230	20	300	100	10	120
Periplasm	40	40	n.d.	n.d.	n.a.	n.d.

Subcellular fractions were prepared from one litre of anaerobically grown cells and activities given represent the total activity found in each fraction.
 All assays were performed in 10 mM Tris/HCl pH 8.0, 2 U. ml⁻¹ glucose oxidase, 25 U. ml⁻¹ catalase and 16 mM D-glucose. Dithionite reduced benzyl-viologen or NADH were present to concentrations of 0.1 mM and 1 mM respectively as indicated. The assay was started by addition of the electron acceptor (NO₃⁻ or ClO₃⁻) to a concentration of 1 mM. The azide concentration when present was 20 μM .
 n.d. = not detectable; n.a. = not applicable

4.2.2. The catalytic properties of the nitrate reductase activities of *T. pantotropha*.

Azide has been shown to act as a competitive inhibitor of NO_3^- reduction with the membrane bound NO_3^- reductase of *P. denitrificans* and *E. coli* (Craske and Ferguson, 1986; Stewart, 1988). This enzyme (in cell free systems) has been shown to catalyse the reduction of ClO_3^- , a substrate analogue of NO_3^- , to ClO_2^- (Craske and Ferguson, 1986). The NO_3^- reductase activities in *T. pantotropha* were tested for their sensitivities to azide and their ability to reduce ClO_3^- .

The NADH- and BV^{++} -dependent NO_3^- reductase activities of anaerobically grown membranes were inhibited by low concentrations of azide (Table 4.2). Typically greater than 90 % of activity was lost in the presence of 20 μM azide. In contrast, the periplasmic $\text{BV}^{++}/\text{NO}_3^-$ oxidoreductase activity was insensitive to this concentration of azide (Table 4.2). Azide did not significantly inhibit the periplasmic NO_3^- reductase activity until its concentration approached 1 mM. The membrane bound NO_3^- reductase activity of *T. pantotropha* further resembled that of *P. denitrificans* since it reduced ClO_3^- , with both NADH and BV^{++} as electron donors. The rate of ClO_3^- reduction was typically slightly greater in magnitude than the rate of NO_3^- reduction (Table 4.2). No detectable $\text{BV}^{++}/\text{ClO}_3^-$ oxidoreductase activity was found in the periplasmic fraction of either aerobically, or anaerobically, grown cells. Therefore, ClO_3^- can not be a substrate for the periplasmic NO_3^- reductase. In principle, the ClO_3^- reductase activity in the membrane fraction could be associated with enzyme other than NO_3^- . The membrane bound DMSO reductase of *E. coli* can utilise ClO_3^- as a substrate (Weiner *et al.*, 1988). The soluble DMSO reductase of *R.*

capsulatus is also thought to possess ClO_3^- reductase activity (McEwan *et al.*, 1987). However no DMSO reductase activity was detected in whole cells, or membranes, of *T. pantotropha* using BV^+ as an electron donor. This lack of DMSO reductase activity suggests that it is the membrane bound NO_3^- reductase that is responsible for the entire rate of ClO_3^- reduction in this organism.

The NO_3^- reductase activity identified within the cytoplasmic fraction in anaerobically grown cells of *T. pantotropha*, reduced ClO_3^- (with a similar specific activity to that of NO_3^- reduction) and was inhibited by 20 μM azide. This similarity in properties with the membrane bound enzyme, further suggests that it is the dissociation of the latter which accounts for the NO_3^- reductase activity in the cytoplasm.

From the evidence presented in this Chapter it appears that there are two NO_3^- reductase activities in *T. pantotropha*, one associated with the membrane and the other with the periplasmic fraction. These two activities arise from distinct enzymes which differ in their catalytic properties. From this preliminary study the membrane bound activity appears to possess the same catalytic properties as the enzyme found in *P. denitrificans* and other denitrifiers. A periplasmic NO_3^- reductase has only previously been identified in one genera of bacteria that of *Rhodobacter* (Sawada and Satoh, 1980; McEwan *et al.*, 1984). With the exception of strain BK5 (Ballard *et al.*, 1990), all strains of *R. capsulatus* that can respire NO_3^- express a soluble NO_3^- reductase which is located in the periplasm. This periplasmic enzyme is structurally and catalytically distinct from the membrane enzyme of *P. denitrificans*. In common with the newly identified periplasmic enzyme in *T. pantotropha*, the periplasmic NO_3^-

reductase from *R. capsulatus* is insensitive to low concentrations of azide and it does not utilise ClO_3^- as a substrate (McEwan *et al.*, 1984).

In conclusion, these results suggest that *T. pantotropha* can express two distinct types of respiratory NO_3^- reductase, each of which being possibly similar to the NO_3^- reductases identified in other organisms. The membrane bound enzyme is expressed only in anaerobically grown cells, whereas the periplasmic enzyme is predominant type of the enzyme synthesised under aerobic conditions although it is also found in anaerobically grown of *T. pantotropha*.

4.2.3. A comparison of nitrate reduction in aerobically and anaerobically grown cells of *T. pantotropha*.

The observation that it is the periplasmic NO_3^- reductase, and not the membrane bound NO_3^- reductase, that is expressed under aerobic conditions, suggests that it is the periplasmic enzyme that confers the ability of intact cells to respire NO_3^- in the presence of O_2 . This possibility was investigated by studying NO_3^- reduction in intact cells of *T. pantotropha* using a physiological substrate. For a complete study cells were grown under both aerobic and anaerobic conditions with succinate as the carbon source. The measurements of NO_3^- reductase activity were performed within the electrode chamber of a NO_3^- electrode maintained under both aerobic and anaerobic conditions as described in Chapter 2. For aerobic experiments, the O_2

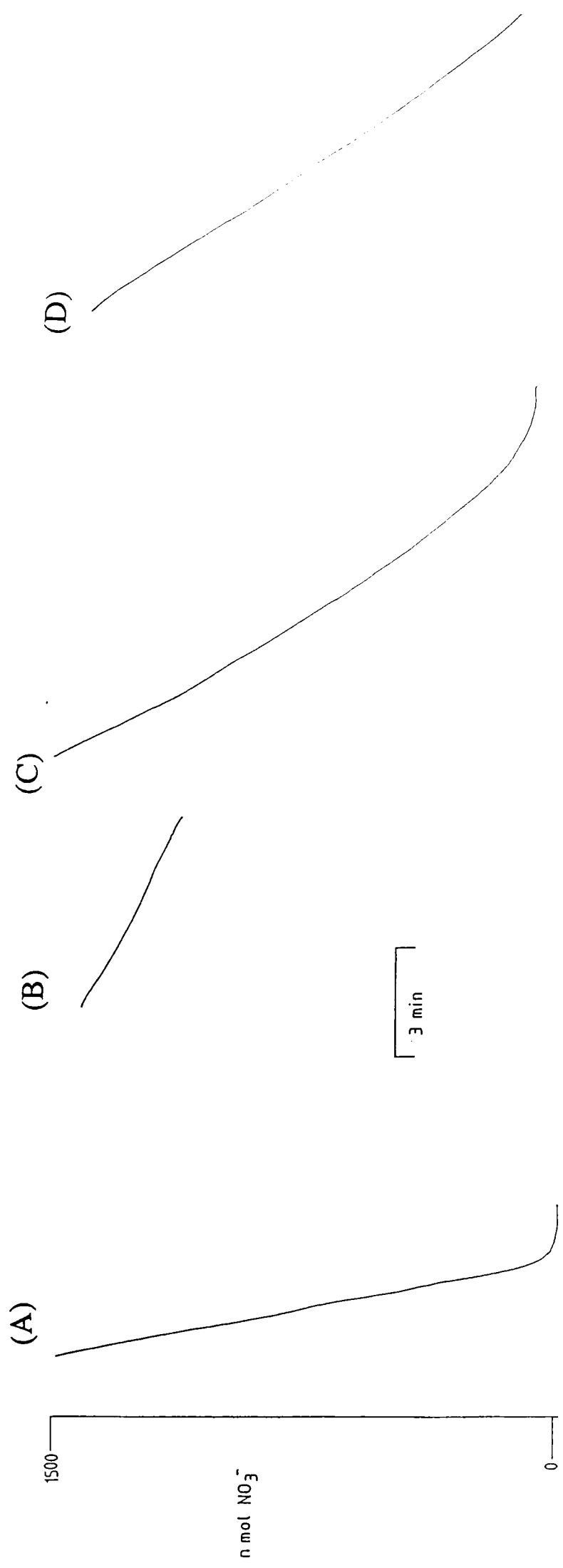


Figure 4.1. The rate of nitrate reduction by intact cells of *T. pantotropha* assayed under anaerobic and aerobic conditions with a nitrate-specific electrode.

Cells (1 mg protein) were resuspended in their succinate growth medium (but this medium lacked nitrate). In aerobic assays the oxygen concentration was 200 μM throughout the experiment. The measurements were started with the addition of nitrate to 50 μM .

Anaerobically grown cells under (A) anaerobic and (B) aerobic assay conditions.
Aerobically grown cells under (C) anaerobic and (D) aerobic assay conditions.

concentration was measured periodically by withdrawing samples from the chamber using a gas tight Hamilton syringe and determining the O_2 concentration in a calibrated O_2 electrode. In this way it was established that the O_2 concentration was stably maintained at $200 \pm 25 \mu M$ throughout the time course of an experiment. The same procedure confirmed that there was no detectable O_2 present in experiments performed under conditions where argon was sparged through the medium.

The rate of NO_3^- reduction was greatest under anaerobic conditions with cells which had been grown anaerobically ($800-950 \text{ nmol } NO_3^- \cdot \text{min}^{-1} \cdot \text{mg cell protein}^{-1}$), and least under aerobic conditions with anaerobically grown cells ($90-100 \text{ nmol } NO_3^- \cdot \text{min}^{-1} \cdot \text{mg cell protein}^{-1}$). Typically, anaerobically grown cells had a five- to eight-fold higher NO_3^- reductase activity than aerobically grown cells, when assayed in the absence of O_2 (Table 4.3 and Figure 4.1). The NO_3^- reductase activity of the anaerobically grown cells was decreased by approximately 90 % in the presence of O_2 (Table 4.3 and Figure 4.1). When anaerobiosis was re-established within the assay medium the rate of NO_3^- reduction was increased to the rate seen before the introduction of O_2 . Low concentrations of azide were shown to inhibit this anaerobic activity by also 90 %, consistent with the cells predominantly expressing the membrane bound enzyme under anaerobic growth conditions (Table 4.3). It should be noted that the remaining activity of these anaerobically grown cells, under aerobic conditions, was not sensitive to azide, suggesting that it was the periplasmic enzyme that was reducing NO_3^- under these conditions (Table 4.3). Aerobically grown cells possessed a rate of NO_3^- reductase activity, with succinate as the electron donor, that was virtually insensitive to the presence of $20 \mu M$ azide (Table 4.3). This acts as further evidence, alongside that obtained in section 4.2.1, that it is the periplasmic

Table 4.3. The sensitivity to azide of aerobic and anaerobic nitrate reduction by cells of *T. pantotropha* grown under aerobic and anaerobic conditions.

Assay conditions	Rate of nitrate reduction ^a	
	Anaerobically grown cells	Aerobically grown cells
Anaerobic	100	100
Anaerobic + azide	8	88
Aerobic	10	75
Aerobic + azide	10	75

The steady state concentration of oxygen was 200 μ M for aerobic experiments and azide was added to 20 μ M where indicated.

^aRates are given as a percentage of the maximum rate of nitrate reduction under anaerobic conditions which was 900 nmol nitrate reduced. min⁻¹. mg protein⁻¹ and 130 nmol nitrate reduced. min⁻¹.mg protein⁻¹ for anaerobically and aerobically grown cells respectively.

NO_3^- that is expressed in aerobically grown cells of *T. pantotropha*. When aerobically grown cells were assayed in the presence of O_2 the rate of NO_3^- reduction was attenuated only by 25 % compared to the rate of reduction measured with the same batch of cells under anaerobic conditions. This aerobic rate never differed significantly when measured in the presence of 20 μM azide. The 25 % inhibition of NO_3^- reductase activity observed can be attributed to a simple competition mechanism for electrons from the respiratory chain between the periplasmic NO_3^- reductase and the cytochrome oxidases, rather than a direct inhibition by O_2 of the NO_3^- reductase enzyme.

These results confirm that (i) the membrane bound NO_3^- reductase is the predominant type of NO_3^- reductase activity expressed in cells of *T. pantotropha* grown under anaerobic denitrifying conditions, and this membrane associated enzyme, in common with the catalytically similar enzyme in *P. denitrificans*, is only operative in the absence of O_2 ; (ii) the periplasmic NO_3^- reductase activity accounts for more than 95 % of NO_3^- reductase activity in cells grown aerobically, in the presence of NO_3^- , and this enzyme can operate both in the presence and absence of O_2 ; (iii) the periplasmic enzyme is present in anaerobically grown cells and explains their capacity for aerobic NO_3^- reduction.

4.2.4. Further properties of the nitrate reductase activities of *T. pantotropha*.

The differential expression of the membrane bound and periplasmic NO_3^- reductases was further indicated by comparative experiments in which NO_3^- reductase activity in intact cells was examined with two non-physiological electron donors MV^{++} and BV^{++} . MV^{++} does not readily traverse the cytoplasmic membrane (Jones and Garland, 1977) and therefore it is a poor electron donor to components located within the cytoplasmic membrane of the cell. BV^{++} is of similar redox potential to MV^{++} but it is more lipid soluble and thus can cross into the cytoplasm of the cell, as discussed in section 4.2.1. Table 4.4 shows data obtained using these two donors with a batch of anaerobically grown cells in which the NO_3^- reductase activity with succinate as an electron donor was seven-fold higher than in the aerobically grown batch of cells. Despite this higher activity when succinate was the electron donor, these anaerobically grown cells had a somewhat lower activity with MV^{++} as the electron donor. This is consistent with MV^{++} being able to donate to the periplasmic, but not to the membrane bound, NO_3^- reductase activity which accounts for 90 % of the activity seen with succinate as the electron donor in anaerobically grown cells. It can also be seen in anaerobically grown cells that the MV^{++} dependent rate was six-fold lower than with BV^{++} . Much of the activity expressed by anaerobically grown cells is clearly not realised with MV^{++} as a substrate. This observation is strong evidence that the site of NO_3^- reduction is at the cytoplasmic face of the cytoplasmic membrane for the membrane bound NO_3^- reductase. A cytoplasmic site of reduction is consistent with the membrane bound enzyme from *T. pantotropha* being similar in organisation to the NO_3^- reductases found in *P. denitrificans* and *E. coli* (Ballard and

Table 4.4. Reduced benzyl-viologen is a substrate for both the periplasmic and membrane bound nitrate reductase in intact cells of *T. pantotropha*, but reduced methyl-viologen is only effective with the periplasmic enzyme.

Growth condition	Activity (nmol. min ⁻¹ . mg cell protein ⁻¹)	
	Anaerobic	Aerobic
Electron donor		
MV ⁺	30	60
BV ⁺	215	54

Cells were resuspended in 100 mM Hepes/NaOH pH 7.3, 2 U. ml⁻¹ glucose oxidase, 25 U. ml⁻¹ catalase and 16 mM D-glucose. The viologen dyes were present at concentration of 0.1 mM. Potassium nitrate (1 mM) was added to start the reaction.

Ferguson, 1988; Stewart, 1988). The activity observed with MV^{+} in the anaerobically grown cells ($30 \text{ nmol. min}^{-1} \text{ mg cell protein}^{-1}$) is presumably in the most part due to the expression of the periplasmic NO_3^- reductase in these cells. In the aerobically grown cells the MV^{+} - and BV^{+} -dependent activities of NO_3^- are comparable (Table 4.4). This is consistent with the expression of a NO_3^- reductase to which both MV^{+} and BV^{+} have free access, and indicates that the site of reduction is located on the periplasmic face of the cytoplasmic membrane. A periplasmic NO_3^- reductase is therefore concluded to be operative.

Bathophenanthroline is a polyimine which can form complexes with metal atoms in a great variety of oxidation states (Hughes and Macero, 1976). The effect of this metal chelator on the NO_3^- reductase activities of *T. pantotropha* was investigated. Bathophenanthroline ($20 \mu\text{M}$) was pre-incubated with cells in the electrode chamber for 15 minutes before the start of each assay. Bathophenanthroline had little significant effect on the rate of NO_3^- reduction in anaerobically grown cells, when assayed under anaerobic conditions (Table 4.5). However, the capacity for aerobic NO_3^- reduction appeared to be lost, or reduced to a level at which the NO_3^- electrode was not sensitive (Table 4.5), in these anaerobically grown cells. In aerobically grown cells bathophenanthroline inhibited the rate of NO_3^- reduction by more than 90 %, both in the presence and absence of O_2 (Table 4.5). These observations can be explained if bathophenanthroline acts to inhibit the periplasmic NO_3^- reductase, but has no significant effect on the membrane bound enzyme in intact cells of *T. pantotropha*. Thus, as observed, the capacity for aerobic NO_3^- reduction by anaerobically grown cells would be lost or significantly impaired by bathophenanthroline, as would any NO_3^- reductase activity expressed by aerobically

TABLE 4.5.

The sensitivity of the nitrate reductase activities of intact cells of *T. pantotropha* to bathophenanthroline.

Cells were resuspended in succinate growth medium lacking nitrate in the chamber of the nitrate electrode. Reactions were started by the addition of potassium nitrate to a final concentration of 50 μ M. The concentration of bathophenanthroline when present was 20 μ M.

Nitrate reductase activity.		
Assay Conditions	Growth conditions.	
	Anaerobic	Aerobic
Anaerobic	100 ^a	100 ^b
Anaerobic + bathophenanthroline	94	8
Aerobic	10	75
Aerobic + bathophenanthroline	< 2	6

Rates are given as a percentage of the maximum rate of nitrate reduction under anaerobic conditions for each batch of cells.

^a = 870 nmol NO₃⁻ reduced. min⁻¹. mg cell protein⁻¹

^b = 125 nmol NO₃⁻ reduced. min⁻¹. mg cell protein⁻¹

grown cells. The basis of this inhibition has not yet been established, so it can not be concluded that the inhibition observed is as the result of the chelation of a metal cofactor associated with the periplasmic NO_3^- reductase. The membrane bound NO_3^- reductase, although not affected by these concentrations of bathophenanthroline in intact cells, was inhibited by as much as 90 % when assayed in a cell free extract with BV^{++} as a reductant. Thus the lack of inhibition of this NO_3^- reductase observed in intact cells is most probably a consequence of the inability of this molecule to traverse the cytoplasmic membrane.

4.2.5. Comparative studies of the capacity for aerobic nitrate reduction in *R. capsulatus* and *P. denitrificans*.

The conclusions from the observations reported so far in this Chapter provide persuasive evidence that it is the periplasmic NO_3^- reductase that allows the simultaneous reduction of NO_3^- and O_2 in *T. pantotropha*. As mentioned previously, a periplasmic NO_3^- reductase with similar catalytic characteristics had been identified in phototrophically grown cells of *R. capsulatus* (McEwan *et al.*, 1984). The activity of this enzyme in cells of this latter organism had been reported to be sensitive to the presence of O_2 (McEwan *et al.*, 1984). However, the latter report was based on an experiment in which O_2 was generated by the endogenous catalase of the cells upon addition of a pulse of H_2O_2 . The rate of NO_3^- reduction was completely, but reversibly, inhibited for several minutes under these conditions (McEwan *et al.*, 1984). However, the time taken to reduce the O_2 produced was not determined, so it

is impossible to calculate the O_2 concentration at which NO_3^- reduction was re-observed. In light of the evidence so presented in this Thesis, the ability of *R. capsulatus* to catalyse aerobic NO_3^- reduction warranted further investigation.

Two strains of *R. capsulatus* were studied, strain N22DNAR⁺ which possesses the periplasmic NO_3^- reductase (McEwan *et al.* 1984) and strain BK5, which unlike other strains of this organism possesses a membrane bound NO_3^- reductase (Ballard *et al.*, 1990). No strain of *R. capsulatus* has ever been reported to contain both a periplasmic and a membrane bound dissimilatory NO_3^- reductase. In addition *P. denitrificans* was studied, as a typical "model" organism, which did not possess the ability for aerobic NO_3^- reduction. Each organism was grown under aerobic and phototrophic (anaerobic for *P. denitrificans*) conditions with NO_3^- present in the growth medium.

Cells of *P. denitrificans* expressed NO_3^- reductase only when grown under anaerobic conditions (Table 4.6). The rate of NO_3^- reduction from this organism was strongly inhibited by the presence of O_2 (Table 4.6), in agreement with the conclusions made by previous workers (John, 1977; Alefounder *et al.*, 1981). *R. capsulatus* strain BK5 behaved as *P. denitrificans*, in that no synthesis of NO_3^- reductase activity was observed when cells were grown aerobically, and the rate of NO_3^- reduction by phototrophically grown cells was completely inhibited under aerobic conditions (Table 4.6). *R. capsulatus* strain N22DNAR⁺ expressed a functional NO_3^- reductase activity when grown under aerobic as well as under phototrophic conditions, although the activity of NO_3^- reductase was typically between two- and four-fold higher in cells grown in the absence of O_2 . This periplasmic

Table 4.6. The sensitivity of the rates of nitrate reduction of aerobically and anaerobically (phototrophically) grown cells of *P. denitrificans* and *R. capsulatus* strains N22DNAR⁺ or BK5 to the introduction of oxygen.

Cells were resuspended in their respective growth medium but lacking nitrate. The reactions were started by the addition of potassium nitrate to 50 µM.

Nitrate reductase activity (nmols. min ⁻¹ . mg prot ⁻¹ .)						
Organism :-	<i>P. denitrificans</i>		<i>R. capsulatus</i> strain BK5		<i>R. capsulatus</i> strainN22DNAR ⁺	
Growth Conditions	Ae	An	Ae	Ph	Ae	Ph
Assay Conditions						
Anaerobic	700	<10	400	<10	300	100
Aerobic	25		<10		240	70

Ae = aerobically grown An = Anaerobically grown

Ph = phototrophically grown

NO_3^- reductase activity was not entirely inhibited by O_2 . A 25-30 % decrease in the rate of NO_3^- reduction was observed in both aerobically, and phototrophically, grown cells of this bacterium when O_2 was present as a second electron acceptor (Table 4.6). This decrease is thought to be the result of competition for electrons between the two acceptors. This observation that simultaneous NO_3^- and O_2 reduction can occur in *R. capsulatus* contrasts with the conclusion made by McEwan *et al.* (1984), but it is consistent with the observation that in *T. pantotropha* the periplasmic NO_3^- reductase is active under aerobic conditions.

4.3. DISCUSSION.

In order for aerobic denitrification to take place the first step, reduction of NO_3^- to NO_2^- , must be possible under aerobic conditions. The results presented in this Chapter verify the findings of Robertson *et al.* (1986) which showed, using O_2 and NO_3^- sensors together, that the simultaneous reduction of NO_3^- and O_2 occur in cells of *T. pantotropha*. The experiment reported by these workers was, however, performed only with cells grown at one O_2 concentration (30 % air saturation), and no evidence was provided for the mechanistic basis for this unusual observation. However, it had been suggested the cytoplasmic membrane of cells of *T. pantotropha* may remain "permeable" to NO_3^- under aerobic conditions (Robertson and Kuenen, 1990). The work presented in this Chapter extends the observation of Robertson and Kuenen, by showing that aerobic NO_3^- reduction can occur in cells grown anaerobically, as well aerobically. The results given here show ^{that} the membrane bound NO_3^- reductase is inhibited immediately, but reversibly, on addition of O_2 to cells of this organism. This clearly argues against any difference in the permeability of the membranes of *T. pantotropha* to NO_3^- compared with those of *P. denitrificans*. The mechanism for the proposed "switch off" of the transport of NO_3^- across the cytoplasmic membrane remains unsolved, although it is expected that the same mechanism will be found to be operative in cells of *T. pantotropha* as in other NO_3^- reducing bacteria. In order to solve this problem it may be first necessary to isolate the protein that is thought to act as a $\text{NO}_3^-/\text{NO}_2^-$ antiport (Alefounder and Ferguson, 1980; Boogerd *et al.*, 1983). This protein remains elusive, although it has recently

been suggested that the *nar* K gene encodes for this protein in *E. coli* (Noji *et al.*, 1989).

The expression of a second NO_3^- reductase in *T. pantotropha*, located in the periplasm, allows NO_3^- to diffuse freely to the active site of this enzyme since the outer cell membrane poses no barrier to small molecules. A periplasmic NO_3^- reductase has also been identified in *R. sphaeroides* f. sp. *denitrificans* (Sawada and Satoh, 1980) and *R. capsulatus* (McEwan *et al.*, 1984). In *R. capsulatus*, also, this periplasmic enzyme has here been demonstrated to be functional in the presence of O_2 . The location of NO_3^- reductase activity in the periplasmic fraction of *T. pantotropha* removes the need under aerobic conditions for the product of its reduction, NO_2^- , to be transported across the cytoplasmic membrane to its site of reduction. All the NO_2^- reductase activity of this organism was located in the periplasmic fraction (see section 4.2.1).

Careful titration of myxothiazol to inhibit the cytochrome bc_1 complex revealed that electron transport for NO_3^- reduction did not involve this complex in *T. pantotropha* (section 3.2.5). Thus, since both NO_3^- reductases would have been expressed in these anaerobically grown cells, it can be concluded that the periplasmic NO_3^- reductase must accept electrons from the electron transport chain at the level of ubiquinol. A similar organisation of the electron transport pathway to NO_3^- reductase in *R. capsulatus* has been reported (McEwan *et al.*, 1985). No more information is available on such pathways but it seems improbable that a quinol confined to the interior of the cytoplasmic membrane could directly reduce a periplasmic protein. Additional membrane bound electron carrier proteins are perhaps involved.

This Chapter has shown that there is expression of a periplasmic NO_3^- reductase, which is functional in the presence of O_2 , in *T. pantotropha*. In such an organism for which the natural habitat undergoes variations in the degree of O_2 saturation this periplasmic NO_3^- reductase may provide the organism with some growth advantages (Robertson, 1988). However, it is perhaps a little surprising that an organism should synthesise a membrane bound NO_3^- reductase when the periplasmic enzyme can catalyse the same reaction. An explanation for this can perhaps be suggested by considering the bioenergetics of the reaction of NO_3^- reduction. Electron transfer from ubiquinol to the membrane bound NO_3^- reductase of *P. denitrificans* is energy conserving (Parsonage and Ferguson, 1983). If the transfer of electrons from ubiquinol to the periplasmic NO_3^- reductase is not linked to the generation of membrane potential, then use of the membrane bound enzyme would be energetically advantageous.

A simple model can be applied to illustrate the above considerations. For the membrane bound enzyme the site of NO_3^- reduction is located on the cytoplasmic face of the membrane. Ubiquinol is oxidised on the same face, thus releases two protons at the periplasmic face. The electrons, however, loop back across the membrane to the cytoplasmic face where they are consumed in the reduction reaction. Thus this process results in a net charge separation (Fig 4.2). For NO_3^- reduction employing the periplasmic NO_3^- reductase there is probably no loop mechanism. The two electrons and protons from the oxidation of ubiquinol are both released into the periplasmic compartment, where they are consumed. Thus this process is electroneutral and does not contribute to energy conservation of the cell.

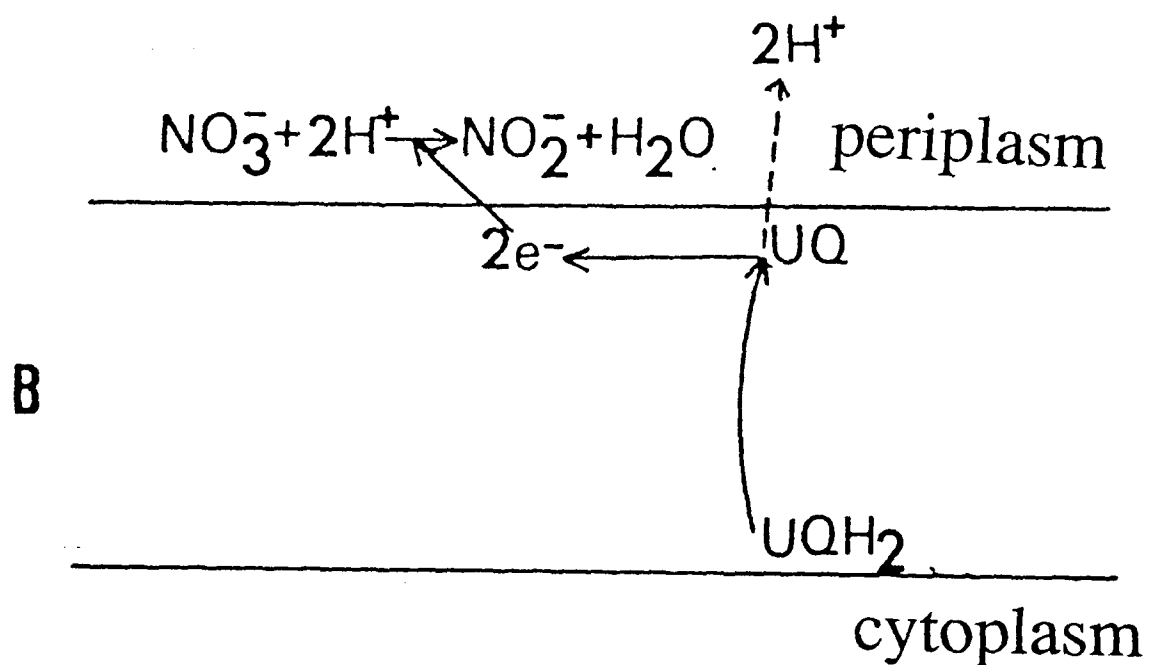
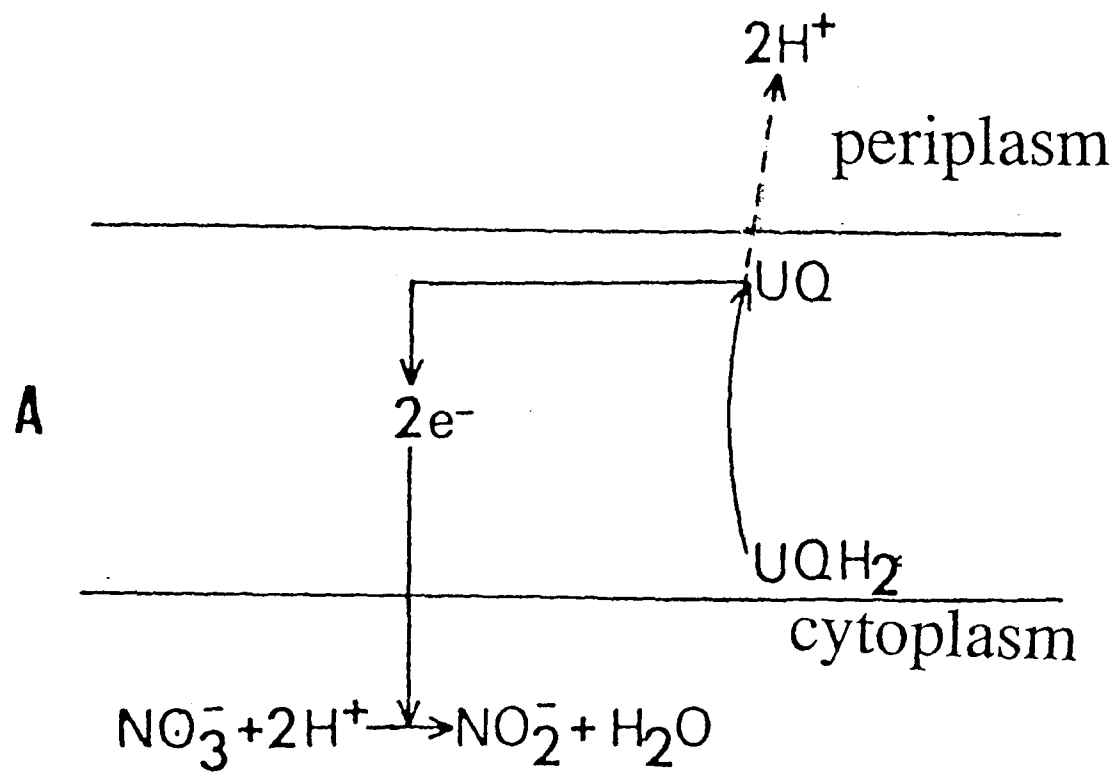


Figure 4.2. Possible mechanisms of charge separation that may accompany quinol oxidation during the reduction of nitrate at either the cytoplasmic face (A), or periplasmic face (B) of the cytoplasmic membrane.

However the situation could in reality be more complex, for example a looping-type mechanism could be operative. The question of energy conservation in these two NO_3^- reductases is addressed in section 5.2.6.

There is a second level of control exerted on NO_3^- reduction in bacteria. This is at the level of controlling the genes for the expression of NO_3^- reductase activity. In *T. pantotropha* the membrane bound NO_3^- reductase is not expressed in cells grown at high levels of aeration. In general the synthesis of the enzymes is repressed by O_2 and induced by the presence of one or more nitrogen-oxides (Kucera *et al.*, 1984). However, anaerobiosis alone may, in some cases, be sufficient to derepress the synthesis of all the enzymes in the pathway of NO_3^- reduction (Kucera *et al.*, 1984). Since denitrifiers are obligate respirers, a sudden shift from aerobic to anaerobic conditions results in the organisms being temporarily unable to generate ATP, and thus a gradual shift in O_2 concentration is better for induction/derepression of the enzymes of denitrification. It seems that for some organisms, e.g. *Pseudomonas sp.*, synthesis of the NO_3^- respiration enzymes occurs if the O_2 concentration is below the biological O_2 demand for the culture (Aida *et al.*, 1986). A chemostat experiment established that NO_3^- reductase of a Klebsiella species was synthesised at O_2 concentrations below 15 mm Hg in the presence of NO_3^- (Dunn *et al.*, 1979). Korner and Zumft (1989) performed an extensive study on the expression of nitrogen-oxide reductases of *Pseudomonas stutzeri*. In conditions of full aerobiosis (7.55 mg of O_2 l^{-1}) cells contained no NO_3^- reductase, the threshold value for the synthesis of this enzyme was found to be 5 mg O_2 l^{-1} , when grown in the presence of NO_3^- . In this organism anaerobiosis in the absence of nitrogen-oxides did not

induce synthesis of this enzyme to any significant degree (Korner and Zumft, 1989).

The data presented in sections 4.2.5 and 4.2.1 show that the periplasmic NO_3^- reductases of *R. capsulatus* and *T. pantotropha* are expressed when these organisms are grown aerobically. The membrane bound NO_3^- reductase was not expressed in aerobically grown cells of *T. pantotropha*, *P. denitrificans* and *R. capsulatus* (strain BK5). This report contrasts to that of Davies *et al.* (1989) which reported the expression of NO_3^- reductase activity in cells of aerobically grown *P. denitrificans*. Although these workers did not demonstrate that this activity was associated with a membrane bound enzyme, a periplasmic NO_3^- reductase has never been identified in *P. denitrificans*.

The mechanism of the control of gene expression of any of the terminal reductases for denitrification is unknown. One hypothesis was that a *b*-type cytochrome acted as a O_2 sensor in the cell to activate the expression of NO_3^- reductase activity. This was supported by evidence that mutants lacking functional cytochromes (*hemA* mutants) had a greatly elevated level of NO_3^- reductase in anaerobically grown *E. coli* K-12 (MacGregor, 1976). However, this theory was discarded when O_2 was shown to repress the synthesis of NO_3^- reductase in these mutants (MacGregor and Bishop, 1977). Most work on the control of the synthesis of NO_3^- reductase has been carried out with the enteric bacteria. There exists strong evidence that under anaerobic conditions induction is brought about by the FNR protein (Lambden and Guest, 1976; Li and Demoss, 1988). Operon fusion studies have established that the FNR protein plays a part in the regulation of the NO_3^- reductase, and other anaerobic respiratory proteins, at the level of transcription (Ruch

et al., 1979; Bonnefoy-Orth *et al.*, 1986; Griffiths and Cole, 1987). The *fnr* gene has been sequenced (Shaw and Guest, 1982). The product of this gene, the FNR protein, shows a great deal of homology with CAP (catabolite activator protein) (Shaw *et al.*, 1983). A cluster of cysteine residues at the amino terminus of this protein resemble a metal binding site (Unden and Guest, 1985). Recent experiments suggest that FNR exists in two forms. The interconversion between the forms appears to be regulated by the availability of O₂ and the binding of metal ions (Trageser and Unden, 1989). The two forms of FNR are proposed to be involved in the regulation of O₂-dependent gene expression (Trageser and Unden, 1989). It is thought that the FNR protein is not directly controlled by molecular O₂, but by the redox potential of the environment of the cells (Unden *et al.*, 1990). This conclusion was drawn from a recent study which showed the extent of expression of fumarate reductase in *E. coli* (an anaerobic respiratory protein) could be related to the Nernst equation (Unden *et al.*, 1990). The situation is complex in *E. coli*. NO₃⁻ is also necessary for the induction of NO₃⁻ reductase activity in this organism, the mechanism of this process involves the product of the *narL* gene, which binds at a promoter upstream of the *narGHJI* operon, and activates the gene expression (Stewart, 1982; Li and Demoss, 1987). To date it remains uncertain whether an FNR-type protein fulfils a similar role in *P. denitrificans*, or any other denitrifier. Zumft *et al.* (1985b, 1988) have isolated many mutants of *P. stutzeri* defective in NO₂⁻ or N₂O respiration. No mutant isolated was reported to be lacking all the enzymes of denitrification. This argues against a common control mechanism, such as an FNR-type system, in these organisms. Clearly the possibility that an FNR-type system may be operative in denitrifying organisms needs further investigation.

**Chapter 5 The characterisation of Tn5-
insertion mutants of *Thiosphaera*
pantotropha deficient in nitrate
reduction.**

5.1. INTRODUCTION.

The studies presented in Chapter 4 established the presence of two NO_3^- reductase enzymes that are involved in the denitrification reactions of *T. pantotropha*. One of these two enzymes was shown to be located within the periplasmic fraction while the second was associated with the membrane fraction of this organism. These two NO_3^- reductase activities had distinct catalytic properties and thus must be the products of different genes. It should, therefore, be possible to isolate mutants which are defective in either the periplasmic or the membrane bound NO_3^- reductase. Molybdenum was required as a component in a cofactor in all the previously identified enzymes, from a diverse range of organisms, which possess the ability to reduce NO_3^- (Ketchum *et al.*, 1970; Nason *et al.*, 1971). The purified periplasmic NO_3^- reductase of *R. capsulatus* strain N22DNAR⁺ has also been shown to contain a similar molybdenum cofactor by complementation analysis of apo- NO_3^- reductase in extracts of *N. crassa nit-1* (McEwan *et al.*, 1987). Thus, it can be assumed with confidence that the two NO_3^- reductases of *T. pantotropha* are also molybdoenzymes. It may, therefore, also be possible to isolate a class of mutants which lack both NO_3^- reductases due to their inability to synthesise some of the enzymes involved in the processing of the molybdenum cofactor. The same doubly deficient phenotype of mutant would be expected if the two NO_3^- reductases possessed other common cofactors or if the electron transport pathways to the NO_3^- reducing enzymes shared a common component.

This Chapter describes experiments designed to produce mutants of *T. pantotropha* defective in some aspect of NO_3^- reduction. If it were feasible to select

for a mutant in which the membrane bound NO_3^- reductase was deleted, it would be possible to investigate the effects of this deletion on anaerobic denitrification. Anaerobic growth, with NO_3^- present as the sole electron acceptor, of such a mutant may be possible but impaired if respiratory NO_3^- reduction could be catalysed by the periplasmic NO_3^- reductase. Such findings could serve as an indication as to whether the transfer of electrons between ubiquinol and the periplasmic NO_3^- reductase is linked to the generation of proton gradient (see section 4.3). Information available from this approach should also, in the long term, be able to give an insight into the organisation of genes for components involved in NO_3^- reduction in this organism.

Mutants of bacteria can be generated in several ways. The oldest established technique involves chemical mutagenesis coupled with a suitable selection procedure. A more recent method involves the insertion of a transposon into the bacterial genome, again linked to a suitable selection procedure. The advantages of the transposon technique include : (i) the mutations generated are more stable than chemically generated point mutations, because the only form of reversion is by deletion of the inserted material; (ii) the locus of the mutation can be identified using a hybridising gene probe for the inserted DNA. It is the inserted DNA of the transposon that causes the disruption of one or more genes, and hence disrupts transcription and/or translation of the gene and generates mutants. A further important point is that such transposons usually only insert once per genome and thus, unlike chemical mutagenesis, there is little chance of mutation in two distant genes on the chromosome.

The methodology of transposon mutagenesis involves the transfer of the genes of a transposable element, in the present work Tn5, into the organism for which the mutants are sought. Transfer can be effected if Tn5 is incorporated into a plasmid that can be maintained in a strain of *E. coli*. Inclusion of a gene encoding for antibiotic resistance, for kanamycin resistance in the case of Tn5, within the transposable element allows the selection of recipient bacteria to which the plasmid has been transferred. A further important feature of the plasmid is that it should not be maintained in the recipient organism.

Simon *et al.* (1983a; 1983b) developed a strategy for the insertion of foreign genes into the genomes of organisms not closely related to *E. coli*. The system required special donor strains (known as mobilizing strains) and derivatives of *E. coli* vector plasmids. The donor strains carry the transfer (*tra*) genes of the broad host range plasmid RP4 integrated into their chromosomal DNA, and these genes facilitate transfer to the recipient strain. The vector plasmid was constructed to contain the *Mob* site (the site for plasmid mobilization) which allows high frequencies of mobilization from the donor strain. The plasmid pBr325 is unable to replicate outside the enteric group of bacteria, and thus is suitable for construction of plasmids which are useful for transposon mutagenesis of a wide variety of Gram negative bacteria. Colonies of the recipient organism resistant to the antibiotic kanamycin will result from insertion of the Tn5 cartridge into their DNA. *E. coli* (from the mating mixture) can be selected against by growth on minimal media which only supports growth of the recipient organism.

5.2. RESULTS.

5.2.1. Transfer frequency of Tn5 to *T. pantotropha*.

A broad host range mobilization system, as described above, was tested for transfer of the Tn5 transposon into *T. pantotropha*. The donor strain *E. coli* S17-1 and the plasmid pSUP2021::Tn5, which had been constructed from pBr325 and a *Mob* site (Simon *et al.*, 1983a), were employed for this test. A pre-requisite for use of the Tn5 mutagenesis in the generation of mutants is that the number of mutants obtained is greater than the rate of spontaneous appearance of mutants resistant to the antibiotic marker. This pre-requisite has not proved attainable in attempts to obtain mutants of some species of bacteria, because the frequency of transfer between the donor and recipient strains may be low and/or the host may possess a restriction mechanism which destroys DNA that is foreign to the host. For example, many attempts to isolate transposon mutants of *P. denitrificans* have failed due to the restriction system of this host. Recently, however, a restriction minus mutant of *P. denitrificans* was constructed, thus allowing the technique of Tn5 mutagenesis to be useful in studies of this organism (de Vries *et al.*, 1989).

Four matings were performed in the present study with *T. pantotropha*. The frequency of transfer ranged from 5×10^{-7} to 5×10^{-6} which generates sufficient numbers of transconjugants to allow selection of the desired mutations caused by Tn5 mutagenesis. No spontaneous kanamycin resistant colonies arose and thus the

frequency of spontaneous mutants must be less than 1×10^{-9} . A plasmid, pSUP5011::Tn5, similar in construction has been used for generation of mutants from a number of organisms, including *T. pantotropha* (Chandra and Friedrich, 1986), for which frequencies of 1.5×10^{-6} transconjugants were previously reported.

5.2.2. Development of a selection procedure for screening mutants defective in activity of the membrane bound nitrate reductase.

The membrane bound NO_3^- reductase of *T. pantotropha* has been shown to utilize ClO_3^- as a substrate (Chapter 4). This ability to reduce ClO_3^- is well known in most other NO_3^- reducing organisms. Such organisms have been shown to be sensitive to ClO_3^- in millimolar concentrations (van Hartingsveldt *et al.*, 1971; Haddock and Jones, 1977). This sensitivity is due to the leakage of ClO_3^- across the cytoplasmic membrane where it is reduced, to ClO_2^- , by the membrane bound NO_3^- reductase. ClO_2^- is highly toxic to cells and so no colony formation is observed in its presence. The periplasmic NO_3^- reductase does not catalyse the reduction of ClO_3^- (Chapter 4), and hence cells of an organism that express only this NO_3^- reductase would not be expected to be sensitive to ClO_3^- , unless there was another ClO_3^- reducing enzyme present. DMSO reductase, an enzyme known to reduce ClO_3^- (Weiner *et al.*, 1988), failed to be identified in *T. pantotropha* (see section 4.2.2). Thus mutants of *T. pantotropha* lacking the membrane bound NO_3^- reductase are expected to be insensitive to ClO_3^- , although they should retain the ability to respire NO_3^- via the periplasmic enzyme.

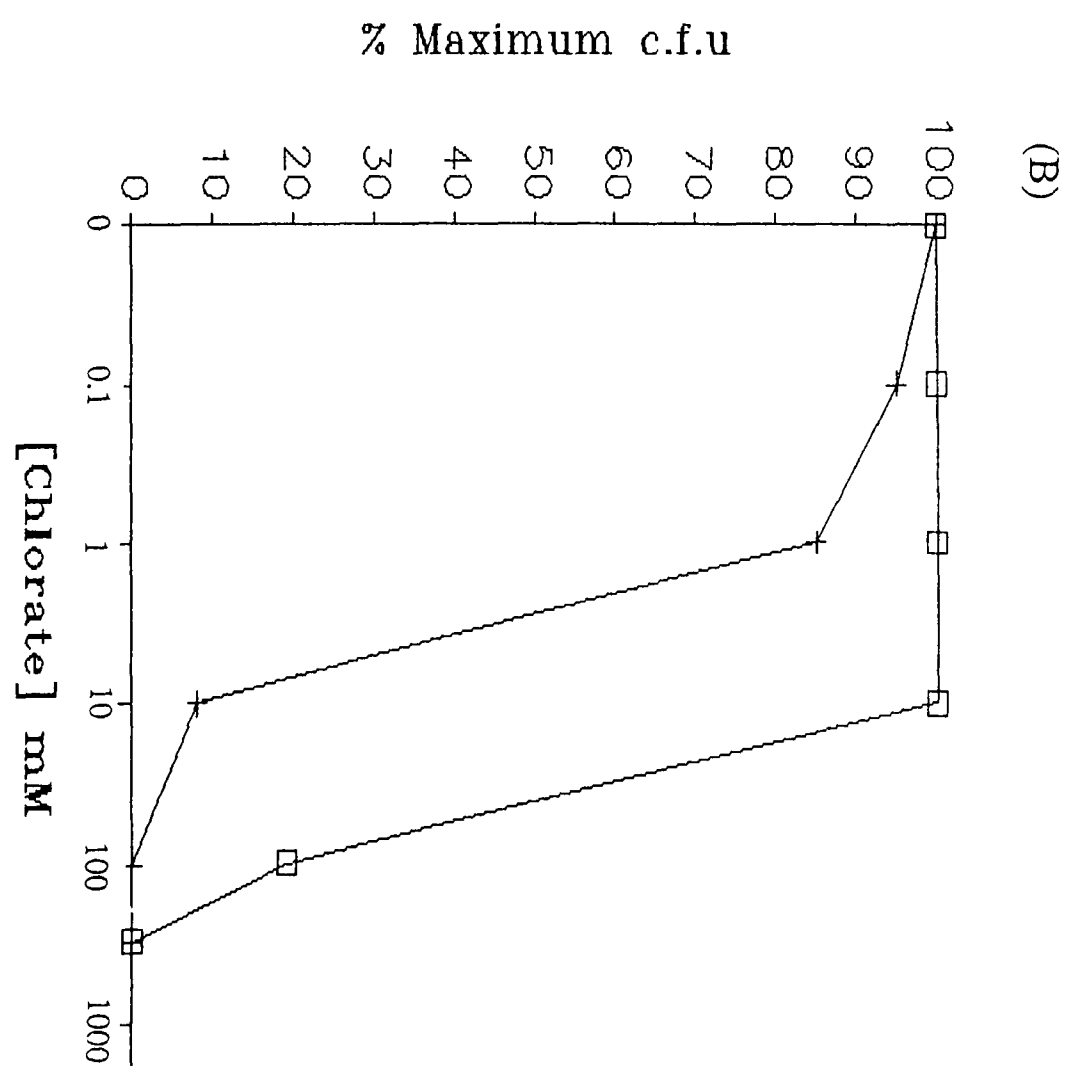
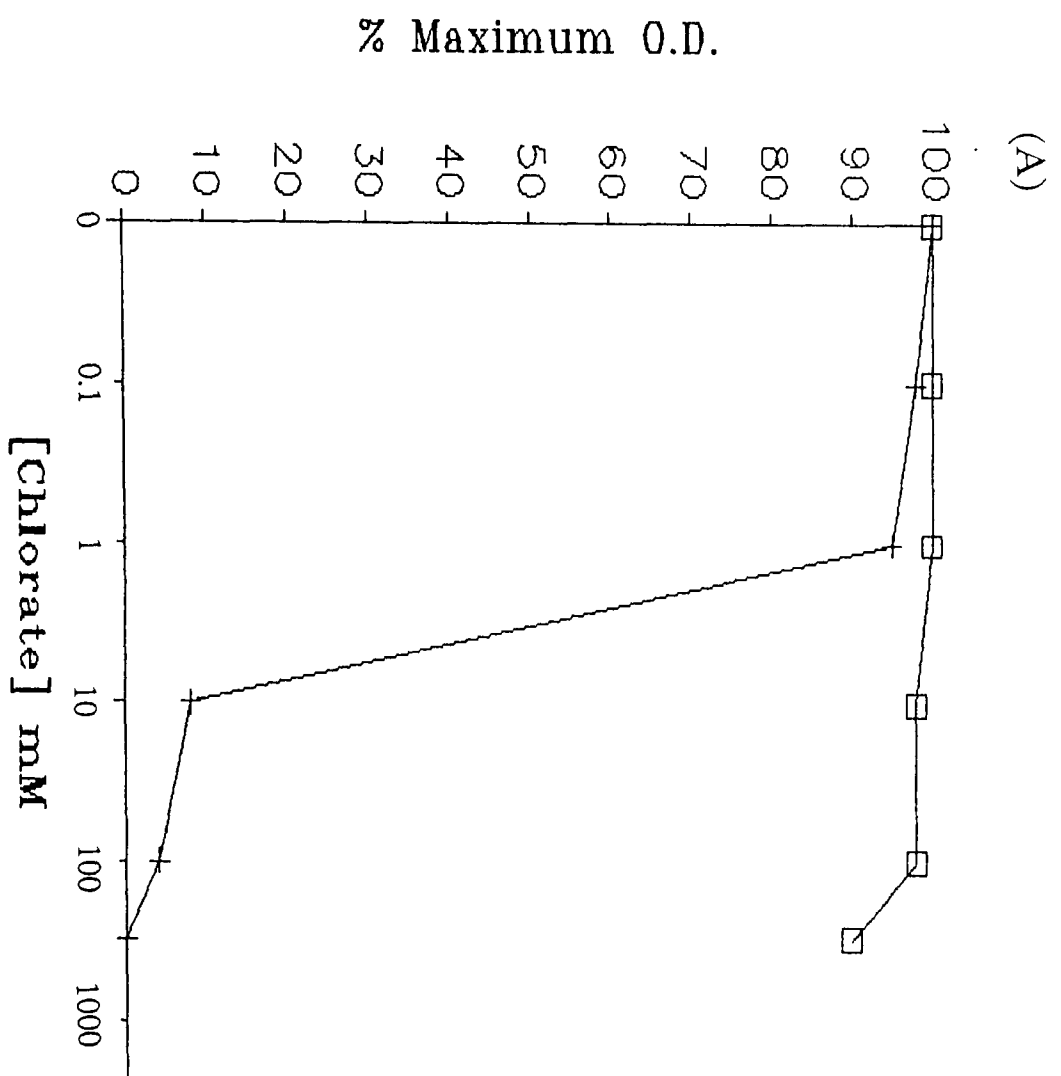


Figure 5.1. Growth of cells of *T. pantotropha* under aerobic and anaerobic denitrifying conditions in the presence of chlorate.

(A) Growth of liquid cultures : The absorbance of stationary phase cultures were recorded at 650 nm. Growth is expressed as the percentage of the maximum optical density (i.e. the optical density recorded in the absence of chlorate) under the specified growth condition.

For aerobic and anaerobic growth the maximum A_{650} =1.0 and 0.8, respectively.

(B) Growth on plates : Growth is expressed as the percentage of the maximum c.f.u. in the absence of chlorate under the specified growth condition.

(□ = aerobic growth, + = anaerobic growth).

c.f.u. = colony forming units.

It was necessary to determine the appropriate concentration of ClO_3^- to select for mutants of *T. pantotropha* defective in the membrane bound NO_3^- reductase. Thus wild type cells of this organism were grown in liquid culture under both anaerobic and aerobic conditions in the presence of NO_3^- . The concentration of ClO_3^- was varied in these cultures and the maximum growth yield of each culture was recorded. Growth under anaerobic conditions was highly sensitive to ClO_3^- concentrations above 10 mM (Figure 5.1A). This is consistent with a high level of expression of the membrane bound NO_3^- reductase under these conditions. Under aerobic conditions, in liquid culture, cells of *T. pantotropha* were insensitive to ClO_3^- at a concentration of 100 mM (Figure 5.1A). A 10% decrease in growth was seen at a concentration of 300 mM (Figure 5.1A), and no growth was observed when 500 mM ClO_3^- was present (not shown). The latter effect appears to be as a result of the increase in the ionic strength of the medium since no growth of this organism was observed when the NO_3^- concentration was increased to 500 mM. A similar pattern of sensitivity to ClO_3^- with cells of *P. denitrificans* was observed in a parallel series of experiments (not shown). The insensitivity of growth of NO_3^- respiring organisms to ClO_3^- under aerobic growth is because most bacteria do not express the membrane bound NO_3^- reductase under this growth condition. Aerobically grown *T. pantotropha* respire NO_3^- , but are insensitive to ClO_3^- , consistent with expression of only the periplasmic NO_3^- reductase (see Chapter 4).

When cells of *T. pantotropha* were grown on solid media the observations were qualitatively similar, reflecting essentially the same phenomenon. Fewer than 10 % of the colonies seen in the absence of ClO_3^- grew anaerobically on plates containing 10 mM ClO_3^- (Figure 5.1B). Under aerobic conditions, comparison of cells grown in

the absence of ClO_3^- with cells grown in the presence of 100 mM and 300 mM ClO_3^- showed that the colony counts were lessened to 20 and 0.1 % respectively (Figure 5.1B). The increase in sensitivity to ClO_3^- observed during aerobic growth on solid medium compared to liquid medium is presumably due to the localised anaerobiosis that accompanies colony growth on solid media. This anaerobiosis leads to the expression of the membrane bound NO_3^- reductase and therefore the ability to reduce ClO_3^- to toxic ClO_2^- .

5.2.3. The selection of Tn5-insertion mutants lacking a functional membrane bound nitrate reductase.

It was expected that mutants of *T. pantotropha* that were resistant to ClO_3^- could result from insertion of the Tn5 into the genes of the membrane bound NO_3^- reductase or into the genes involved in molybdenum cofactor biosynthesis. The latter insertion would result in a phenotype of mutant that was defective in both of the NO_3^- reductase activities found in wild type cells. Thus any selection procedure designed to isolate the doubly defective phenotype must provide an electron acceptor other than NO_3^- to support growth. Two selection procedures were employed in which it was feasible to obtain either the phenotype of mutant lacking the membrane bound NO_3^- reductase or the double mutant phenotype discussed above.

The first selection method employed examined growth on plates containing 300 mM ClO_3^- under "aerobic conditions". It is somewhat surprising in view of the low

levels of expression of the membrane bound NO_3^- reductase under aerobic conditions (Chapter 4), that aerobic growth could provide conditions suitable for the selection of a mutant deficient in the membrane bound NO_3^- reductase. However, the results presented in Figure 5.1 show that this procedure may select for the desired phenotype (see section 5.2.2). Under these conditions the double mutant may grow using the O_2 available as an electron acceptor.

A second selection procedure was employed under anaerobic conditions in which high levels of the membrane bound NO_3^- reductase are expressed in wild type cells of *T. pantotropha*. In this procedure cells were grown in the presence of 50 mM ClO_3^- , 3 mM NO_2^- and 5 mM NO_3^- . NO_2^- was included in this medium as an electron acceptor to enable growth of the double mutant phenotype (which would be competent in NO_2^- reduction). This procedure may fail to select for some types of mutants if there is a defective in a common electron transport component shared between the paths of NO_3^- and NO_2^- reduction.

5.2.4. Classification of the mutants obtained lacking the membrane bound nitrate reductase.

The mutants obtained by the selection procedures described could be divided into two principal classes on the basis of phenotype, classes I and II, that were used for further investigations of NO_3^- reduction in *T. pantotropha*. Two other classes of mutants were isolated (using the second selection procedure), classes III and IV, but no detailed investigation of these mutants was performed in the present work.

Table 5.1.

The sensitivity to azide of rates of aerobic and anaerobic nitrate reduction in cells of wild type *T. pantotropha* and mutant M6, grown under aerobic and anaerobic conditions.

Cells were resuspended in 30 ml of the succinate growth medium which lacked nitrate. The assays were started by the addition of potassium nitrate to a final concentration of 50 μ M. The steady state oxygen concentration was 200 μ M in the aerobic experiments. Rates are given in nmol nitrate reduced. min⁻¹. mg cell protein⁻¹.

Assay conditions	Rate of nitrate reduction.			
	wild type		mutant M6	
	Anaerobic grown	Aerobic grown	Anaerobic grown	Aerobic grown
Anaerobic	900	130	950	135
Anaerobic + 20 μ M azide	70	115	850	130
Aerobic	90	95	750	100
Aerobic + 20 μ M azide	90	95	725	95

5.2.4a. Class I.

Three mutants, M6, M9 and MX1 were isolated by aerobic growth on plates containing 300 mM ClO_3^- (selection procedure 1). These mutants grew anaerobically in liquid culture with NO_3^- as the terminal electron acceptor and were resistant to 300 mM ClO_3^- under this growth condition. The doubling times of growth under anaerobic conditions, using succinate as the electron donor and NO_3^- as the electron acceptor, for cells of the class I mutants and wild type *T. pantotropha* recorded in a typical experiment are shown in Table 5.3. Wild type cells grew with a doubling time of 80 ± 5 min whereas class I mutants grew more slowly with a doubling time of 360 ± 20 min.

Cells of the class I mutants, when grown with NO_3^- as the sole electron acceptor, possessed a total specific rate of NO_3^- reduction under anaerobic conditions which was similar in magnitude to anaerobically grown wild type cells. Table 5.1 shows the results of one experiment with mutant M6; similar observations were made with other batches of cells of the class I mutants. Aerobic growth of these mutants also yielded a specific rate of NO_3^- reduction similar in magnitude to that found in aerobically grown wild type cells when assayed with the NO_3^- electrode (Table 5.1). At first sight, the findings for anaerobically grown cells of these mutants were surprising. If these mutants were defective in the membrane bound NO_3^- reductase activity, as expected on the basis of resistance to ClO_3^- , then a decreased rate of NO_3^- reduction would be predicted (c.f. Table 4.3). However, the NO_3^- reductase activity in the anaerobically grown mutant cells differed from that of wild type in its

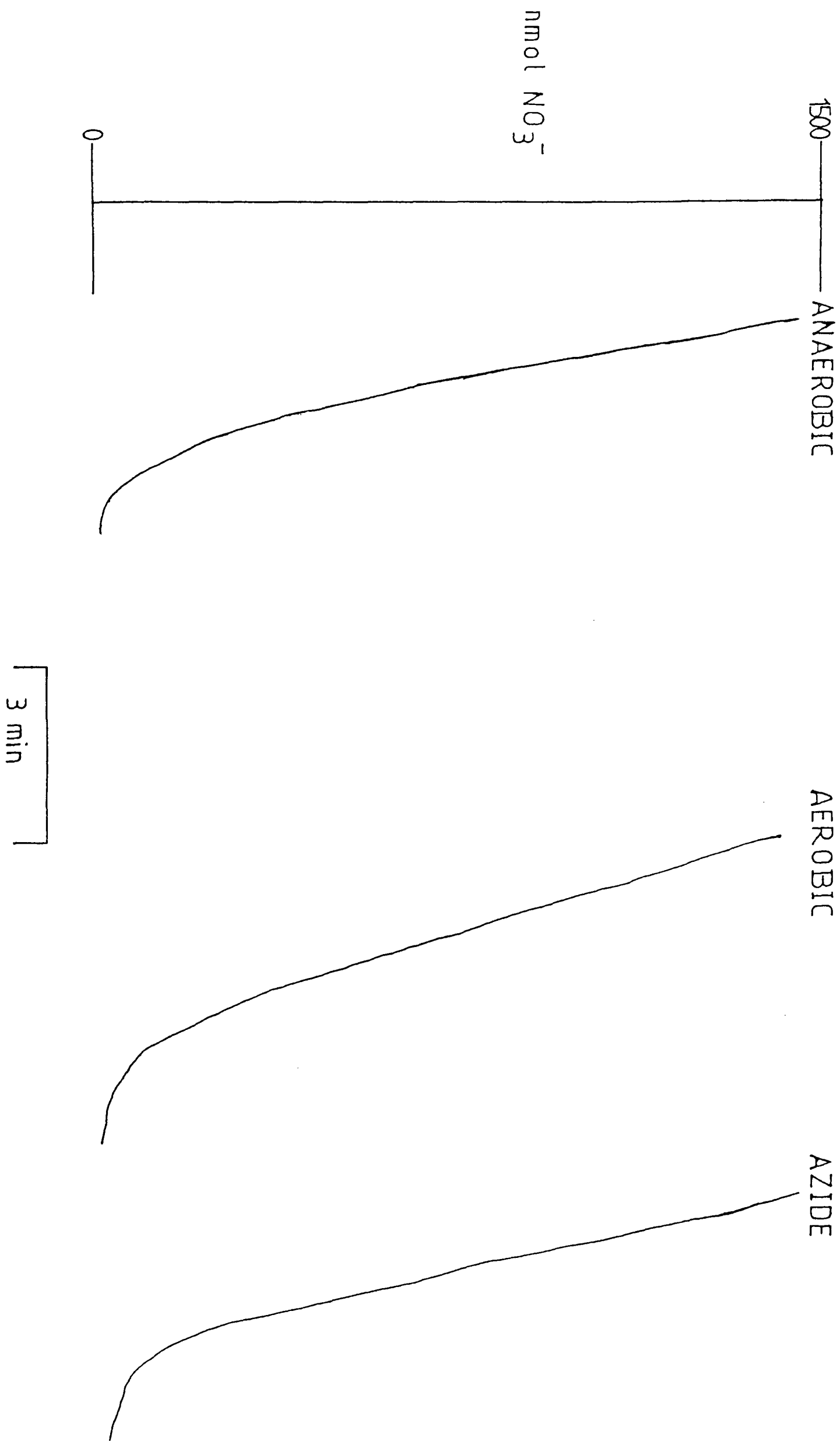


Figure 5.2. The effect of oxygen and azide on reduction of nitrate by anaerobically grown cells of class I mutant, M9, of *T. pantotropha*.

The cells were grown anaerobically on medium containing succinate, nitrate, 50 mM chlorate and 300 $\mu\text{g}.\text{ml}^{-1}$ kanamycin. Cells (1 mg protein) were assayed in a succinate growth medium which lacked chlorate and nitrate. Nitrate (50 μM) was added to the cells at the start of each assay and its disappearance was followed with a nitrate-specific electrode. In the aerobic experiment the oxygen concentration was 200 μM and azide when present (under anaerobic conditions) was 20 μM .

sensitivity to the presence of azide or O_2 (see Figure 5.2 and Table 5.1). As in aerobically grown mutant and wild-type cells, this activity was only attenuated by approximately 20-30 % in the presence of O_2 (at 80-90 % air saturation), presumably due to a competition for electrons within the respiratory chain. Little or no decrease was observed when these assays were performed in the presence of 20 μM azide (Table 5.1, Figure 5.2). This result suggests that these class I mutants lacked a functional membrane bound NO_3^- reductase and "compensated" for this by the expression of elevated levels of the periplasmic NO_3^- reductase which was active under anaerobic conditions.

The BV^{++}/NO_3^- and MV^{++}/NO_3^- oxidoreductase activities of the mutant cells, and cell fractions, were compared to those of wild type cells (Table 5.2). Recall (section 4.2.4) that BV^{++} donates electrons to both the periplasmic and membrane bound NO_3^- reductases of whole cells, whereas MV^{++} donates electrons to the periplasmic, but not to the membrane bound, NO_3^- reductase of intact cells. Anaerobically grown class I mutant cells showed a five- to seven-fold increase in their MV^{++}/NO_3^- oxidoreductase activity compared to the wild type cells but, the BV^{++}/NO_3^- oxidoreductase activity was similar in both cell types. The results of a typical experiment with mutant M6 is shown (Table 5.2). No differences were observed between aerobically grown wild type and class I mutants using this non-physiological donor (Table 5.2). Class I mutants, whether aerobically or anaerobically grown, contained a MV^{++}/NO_3^- and BV^{++}/NO_3^- oxidoreductase in the periplasmic fraction, but a NO_3^- reductase activity associated with either the membrane or cytoplasmic fractions was not found. The cells of class I mutants and their fractions lacked ClO_3^- reductase activity, as expected, indicating that the membrane bound NO_3^- reductase is the only ClO_3^-

Table 5.2. Comparison of the nitrate reductase activities of intact cells of wild type and mutant M6 cells of *T. pantotropha* using methyl- and benzyl-viologen as an electron donor.

Growth conditions	Activity (nmol. min ⁻¹ . mg of cell protein ⁻¹)	
	Electron donor.	
	MV ⁺	BV ⁺
Wild type :-		
Anaerobic	30	215
Aerobic	60	54
Mutant M6 :-		
Anaerobic	235	240
Aerobic	67	64

Cells were resuspended in 100 mM Hepes/NaOH pH 7.3, 2 U. ml⁻¹ glucose oxidase, 25 U. ml⁻¹ catalase and 16 mM D-glucose. The viologen dyes were present at concentration of 0.1 mM. Potassium nitrate (1 mM) was added to start the reaction.

reductase activity in cells of *T. pantotropha*. The MV^{+}/NO_3^- oxidoreductase activity was several fold higher in mutants grown anaerobically rather than aerobically (Table 5.2) which is consistent with the relative specific rates of NO_3^- reductase activity of the cells from the two growth conditions with physiological electron donors (Table 5.1). The lack in the class I mutants of a membrane associated NO_3^- reductase activity, with the non-physiological electron donors, indicates that it is the synthesis of the terminal enzyme or some cofactor of it that is impaired. Thus, these mutants do not result from a deletion in a gene for an electron transport protein to this reductase. The periplasmic NO_3^- reductase of the class I mutants is fully active, suggesting that the genes required for the synthesis or insertion of the molybdenum cofactor have not been inactivated. NO_3^- reductase activity was not found in the cytoplasm, suggesting that a defect existed in the synthesis of some or all of the polypeptides of the normally membrane bound enzyme rather than an inability to assemble the enzyme in the cytoplasmic membrane.

5.2.4b. Class II.

Transconjugants which fell into the phenotype described as class II mutants were also isolated by selection procedure 1. Six mutants designated as M2-1, M2-2, M2-3, M2-4, M2-5 and M2-7 were identified. These mutants were able to grow, albeit slowly (Table 5.3), aerobically in the presence of 300 mM ClO_3^- in liquid culture and on plates. Colonies of this class of mutants appeared small, white and circular. In contrast, the wild type (and class I type mutant) colonies were large, wet-looking and pinkish/brown in colour with slightly darker centres on larger sized colonies. These

class II mutants failed to grow anaerobically either in the presence of NO_3^- on minimal growth media (Table 5.3), or when NO_2^- or N_2O replaced NO_3^- as the electron acceptor in this media. NO_3^- reductase activity was not detectable with succinate as an electron donor in aerobically grown cells of class II mutants. Whole cells, and their fractionated components, also lacked NO_3^- reductase activity with BV^{++} or MV^{++} as electron donors. Aerobic growth by this class of mutant, which was negative according to the Nadi staining test for cytochrome *c* oxidase activity, was slow in comparison with wild type cells and class I mutants which were all Nadi positive (Table 5.3). The inference, from this lack of NO_3^- reductase activity in aerobically grown cells with non-physiological electron donors, is that the synthesis of some component of the periplasmic NO_3^- reductase is impaired. The white colour of these Nadi negative colonies suggests that these mutants may have a general deficiency of cytochromes. This deficiency may, itself, account for the lack of periplasmic NO_3^- reductase activity because in *R. capsulatus* this activity has been shown to be dependent on a specific *c*-type cytochrome, even with non-physiological electron donors (Alef and Klemme, 1979). A *c*-type cytochrome is also known to be associated with the periplasmic NO_3^- reductase of *R. sphaeroides* f. sp. *denitrificans* (Sato, 1981). More recently it has been demonstrated that the periplasmic NO_3^- reductase from *R. capsulatus* co-purifies with cytochrome c_{552} , although the cytochrome is a distinct polypeptide from the catalytic subunit (Richardson *et al.*, 1990). In preparations where the cytochrome has been lost, the enzyme has an activity reported to be more than one hundred-fold less than preparations with the associated cytochrome (Alef and Klemme, 1979; McEwan *et al.*, 1987; Richardson *et al.*, 1990). Thus if the periplasmic NO_3^- reductase of *T. pantotropha* is also associated with a *c*-type cytochrome its stability may be dramatically lessened, or

non-detectable, in the absence of the cytochrome. The phenotype of this class of mutant is consistent with a mutant that possesses a defect in cytochrome synthesis.

The selection procedure for class II mutants would lead one to expect that the membrane bound NO_3^- reductase would be inactive, but this cannot be readily examined because the enzyme is not appreciably expressed under aerobic growth conditions. Anaerobic growth conditions, if they exist, have not been identified for these mutants.

5.2.4c. Class III and class IV.

Three isolates 5001, G1, G2 were identified with phenotype designated as class III. These mutants grew on solid, and liquid medium, aerobically and anaerobically in the presence of NO_2^- , but did not grow anaerobically with NO_3^- as the sole terminal electron acceptor. Aerobically grown colonies were paler than wild type colonies in appearance. These mutants of *T. pantotropha* were resistant to 300 mM ClO_3^- when grown aerobically or anaerobically. Aerobically grown cells reduced NO_3^- , with succinate as an electron donor, under anaerobic conditions at a rate lessened to 20 % of that found in aerobically grown wild type cells. This NO_3^- reductase activity was insensitive to the presence of 20 μM azide, and only partially inhibited by O_2 , suggesting that class III mutants express lower levels of the periplasmic NO_3^- reductase activity found in wild type cells. Cells of class III mutants, using succinate as an electron donor, grown anaerobically in the presence of

NO_2^- , or grown aerobically and then incubated for 4 hours under anaerobic conditions with NO_3^- , expressed 15-25 % of the specific NO_3^- reductase activity found in wild type cells grown under similar conditions. The specific NO_3^- reductase activities of these mutants decreased by 85-95 % in the presence of 20 μM azide or O_2 . This observation indicates that class III mutants must also express the membrane bound NO_3^- found in wild type cells of *T. pantotropha* when grown anaerobically, although at a reduced level of activity. These anaerobically grown cells also contain the periplasmic NO_3^- reductase activity which has approximately 20 % of the activity found in wild type cells grown anaerobically.

The membrane and periplasmic fractions of the anaerobically grown class III mutants reduced NO_3^- , with BV^{++} as an electron donor, at rates which were 15-25 % of the rates found in the corresponding fractions of anaerobically grown wild type cells of *T. pantotropha*. No activity was detected in the cytoplasmic fractions of these cells. The periplasmic fractions of the aerobically grown class III mutants contained 15-30 % of the NO_3^- reductase activities found in the aerobically grown wild type cells. Little, or no, NO_3^- reductase activity was detected in the cytoplasmic fractions of aerobically grown cells of these mutants.

Class III mutants express both the periplasmic NO_3^- reductase and the membrane bound NO_3^- reductases, despite the ClO_3^- resistant properties of these mutants under the selection procedure. This anomaly can be explained as the rate of ClO_3^- reduction, like NO_3^- reduction, in these mutants would be slow. Thus growth would be possible using NO_2^- as an electron acceptor, formed from ClO_3^- by the low level of membrane bound NO_3^- reductase, without ClO_2^- accumulating to a lethal

concentration. As in the wild type *T. pantotropha* cells, the relative expression of these two NO_3^- reductase activities is controlled by the O_2 level present during growth. The decreased levels of activity relative to wild type cells of both enzymes under all growth conditions with a non-physiological electron donor which transfers electrons directly to the NO_3^- reductases, indicates that some common component of these terminal reductases is defective.

One mutant, 5002, isolated was classified as a class IV mutant. This mutant grew aerobically and anaerobically with NO_3^- present as a terminal electron acceptor, unlike the mutants described by class III. The phenotype of mutant 5002 was similar to the class III mutants with levels of terminal periplasmic and membrane bound NO_3^- reductase activities expressed only to 20 % of the levels of those found in wild type cells grown under similar conditions.

5.2.5. Analysis of the growth with formate or thiosulphate as reductant of mutants of *T. pantotropha* defective in nitrate reduction.

The NO_3^- reductase enzymes that have been previously studied have all contained a cofactor that contains a molybdenum centre (Ketchum *et al.*, 1970; Nason *et al.*, 1971). This cofactor is a derivative of pterin (molybdopterin) which is assumed to be identical in all molybdenum containing proteins, with the exception of nitrogenase (Johnson *et al.*, 1980; 1984). Chandra and Friedrich (1986) isolated mutants of *T. pantotropha*, designated Sox⁻ mutants, with an inability to grow by the oxidation of

reduced sulphur compounds. One class of Sox⁻ mutants was also unable to grow with formate or xanthine as an energy source, or with NO₃⁻ as the sole electron acceptor (Chandra and Friedrich, 1986). This class of mutants was assumed to have a deficiency in this molybdenum cofactor biosynthesis.

Two enzymes, formate dehydrogenase and thiosulphate oxidase, which are found in *T. pantotropha* (Chandra and Friedrich, 1986), are known to contain molybdenum in other micro-organisms (Rajagopalan, 1985; Toghrol and Southerland, 1983). Accordingly, cells of the wild type *T. pantotropha* and of the mutant strains generated in the present work were grown autotrophically with thiosulphate as the sole energy source, and heterotrophically with formate as a carbon and energy source; O₂ was the sole oxidant under both these growth conditions. Cells of the wild type and mutants were also grown on medium containing succinate and NO₃⁻ which had been pre-sparged with argon. The latter experimental observations were used to examine any differences in growth rate observed with NO₃⁻ (as an electron acceptor) with those differences seen with formate or thiosulphate (as an electron donor).

Wild type *T. pantotropha* grew with doubling times of 2.5 h. and 4 h. on formate and thiosulphate respectively. The final absorbance reading at 650 nm of cultures grown on formate was 0.3 ± 0.02 and on thiosulphate medium was 0.2 ± 0.02 (results from one such experiment are shown in Table 5.3). Class I mutants grew with similar doubling times and growth yields to the wild type cells (Table 5.3). Class II mutants did not grow aerobically on formate or thiosulphate over a 48 h. period.

Class I mutants grew aerobically as wild type cells on formate and thiosulphate, indicating the activities of formate dehydrogenase and thiosulphate oxidase are not impaired by the insertion of the transposon. This behaviour is consistent with a defect in the structural, or the regulatory genes, for the membrane bound NO_3^- reductase of these mutants. Class II mutants failed to grow on formate and thiosulphate. Thiosulphate oxidase is a periplasmic enzyme in the *Thiobacilli* (Ames, 1986; Lu, 1986), and it has been proposed that electrons are fed into the respiratory chain via *c*-type cytochromes (Kelly, 1988). Thus a mutant deficient in *c*-type cytochromes would be unable to grow using thiosulphate as an energy source. Wild type cells grew aerobically more slowly with formate than with succinate (240 ± 20 min. compared to 55 ± 5 min), whereas class II mutants grew aerobically with succinate, the doubling time being 12 h. Thus formate may fail to support growth of class II mutants, or growth may be very slow with a large lag period such that growth was undetectable in these experiments.

The class III and IV mutants of *T. pantotropha*, which are described in section 5.2.4, contained reduced levels of activity of both periplasmic and the membrane bound NO_3^- reductases. If class III and IV mutants were impaired in the synthesis of the molybdenum cofactor, then these mutants might be expected to contain decreased levels of activity of other molybdoenzymes. These mutants grew on formate to a final optical density similar to wild type cells, but the doubling times of these mutants were increased to 8-10 h and 12-16 h for growth on medium containing formate and thiosulphate respectively. These observations are consistent with the

suggestion that perhaps the Tn5 transposon is inserted into one of the genes involved in the synthesis and processing of the molybdenum cofactor in class III and IV mutants.

5.2.6. Comparison of the anaerobic growth yields of wild type and class I mutants of *T. pantotropha* with nitrate as the terminal electron acceptor.

In section 4.3 the question was raised as to whether the periplasmic and membrane bound NO_3^- reductase activities of *T. pantotropha* might be linked to the conservation of energy by this organism. Since mutants have been identified which possess only a functional periplasmic NO_3^- reductase (class I mutants) the growth yields of these mutants grown under anaerobic conditions can be compared to growth yield of wild type cells grown under similar conditions. Approximately 90 % of the NO_3^- reductase activity expressed in anaerobically grown wild type cells of *T. pantotropha* is associated with the membrane bound NO_3^- reductase (section 4.2.1). Class I mutants possess a specific NO_3^- reductase activity, similar in magnitude to the activity of wild type cells, but associated only with the periplasmic NO_3^- reductase (section 5.2.4a). Therefore, the comparison of the anaerobic growth yields of wild type and class I mutants should reflect the relative degree of energy conservation associated with electron flow to the membrane bound and periplasmic NO_3^- reductases. Table 5.3 indicates that despite similar specific rates of NO_3^- reduction in cells of both the class I mutants and wild type *T. pantotropha*, growth of class I

Table 5.3 Phenotypes and growth characteristics of wild type, class I and II mutants of *T. pantotropha*.

Cells were grown in the specified medium, aliquots were withdrawn at appropriate time intervals and the optical absorbance of the culture at 650 nm was recorded. When nitrate was present as the electron acceptor the medium was sparged with argon before inoculation.

		Doubling times (min).			
		Electron donors/acceptors			
Strain	Phenotype	Succinate/ O ₂	Succinate/ NO ₃ ⁻	Formate/ O ₂	Thiosulphate/ O ₂
wild type	MNR ⁺ PNR ⁺ Nadi ⁺	55	80	160	240
Class I	MNR ⁻ PNR ⁻ Nadi ⁺	55	360	165	250
Class II	MNR ⁻ PNR ⁻ Nadi ⁻	720	N.D.	N.D.	N.D.

MNR = membrane bound nitrate reductase PNR = periplasmic nitrate reductase

N.D. = not detected.

Table 5.4. Comparison of the growth yields of wild type and mutant M6 cells of *T. pantotropha* under anaerobic growth conditions.

Cells were grown in the medium described by Table 2.2a except that (a) the nitrate (the electron acceptor) concentration and (b) the succinate (the electron donor) concentration was varied, values as shown. Cultures were sparged with argon before growth. The optical absorbance at 650 nm of the stationary phase cultures were recorded.

Optical density of culture at 650 nm.		
	wild type	mutant M6
(a) [NO ₃ ⁻] (mM)		
0.5	0.4 ± 0.1	0.1 ± 0.05
2.0	0.6 ± 0.1	0.5 ± 0.1
5.0	0.9 ± 0.2	1.0 ± 0.2
(b) [Succinate] (mM)		
2.0	0.5 ± 0.1	0.2 ± 0.05
5.0	1.0 ± 0.2	1.0 ± 0.2

mutants is approximately four-fold slower than that of wild type cells. One class I mutant, M6, was studied further. Cells of mutant M6 and wild type *T. pantotropha* were grown anaerobically (in medium pre-sparged with argon) with succinate and NO_3^- present, but either the succinate or NO_3^- concentration was varied to become growth limiting. The results from such experiments are shown in Table 5.4. When the concentration of the supplied NO_3^- was 0.5 mM, the final yield of cells of mutant M6 was on average four-fold less than wild type cells (Table 5.4). However, when the concentration of NO_3^- supplied was increased to 2 mM, no significant difference in yield was recorded between the mutant M6 and wild type cells (Table 5.4). Thus 0.5 mM NO_3^- gives limiting electron accepting capacity ($\text{NO}_3^- + \text{NO}_2^- + \text{NO} + \text{N}_2\text{O}$) whereas 2 mM NO_3^- does not.

When 10 mM NO_3^- was included in the growth medium and the succinate concentration was lowered, so that its availability would limit growth, a similar trend to that observed under conditions of NO_3^- limitation was recorded (Table 5.4). At 2 mM succinate a lower growth yield was recorded for mutant M6 than for wild type. However, when the concentration of succinate was raised to 5 mM the wild type and mutant M6 cells grew to a similar final optical density. The results from these experiments with limiting carbon source and oxidant suggest that electron flow to the membrane bound NO_3^- reductase conserves more energy (i.e. has a higher H^+/e translocation) stoichiometry than the periplasmic NO_3^- reductase. This proposal is consistent with the model proposed in section 4.3 for NO_3^- reduction by these two terminal reductases.

5.2.7. Further characterisation of class I and class II nitrate reductase mutants of *T. pantotropha*.

It was postulated (5.2.4a) that mutants falling into the category of "class I" mutants result from the Tn5 insertion into either a structural, or regulatory, gene for the membrane bound NO_3^- reductase. The phenotype of class II mutants may be the result of Tn5 insertion in a gene associated with cytochrome biosynthesis. Two approaches, involving the measurement of cytochrome spectra and probing with antibodies raised against the periplasmic and membrane bound NO_3^- reductase enzymes, were utilised to seek further information on the effect of the transposon insertion in the genomic DNA of *T. pantotropha*.

5.2.7a. Spectral characterisation of the cellular fractions of class I and II mutants of *T. pantotropha*.

Membrane fractions were prepared (as described in section 2.2.1) from class I mutants and wild type *T. pantotropha*, which had in each case been grown anaerobically on medium containing succinate and NO_3^- . These membrane vesicles were resuspended in 10 mM Tris/HCl, pH 8.0, and this suspension was flushed with argon in a sealed glass cuvette. The suspension also contained the glucose oxidase system, described in section 2.3.3, to scavenge for any O_2 leaking into the system. The cytochromes of these membranes were reduced, by careful addition of dithionite,

until a maximum absorbance at 555 nm was obtained. Figures 5.3 (i) and (iii) show the absolute reduced spectra, between 540 and 570 nm, of membrane vesicles of the wild type and mutant M9 cells of *T. pantotropha* respectively. The absorbance at 550 nm is comparable for the wild type cells and the mutant M9, indicating that mutant M9 has a similar *c*-type cytochrome content in its membrane vesicles to the wild type organism. However, there is a significant decrease in the absorption of the membrane fraction of mutant M9 compared to the wild type membranes at 560 nm. Membranes from other class I mutants had spectral properties similar to those of mutant M9. These spectral observations are indicative of decreased levels of *b*-type cytochromes in the class I mutant membranes relative to membranes from wild type cells. The membrane bound NO_3^- reductase of other organisms has been shown to possess *b*-type cytochromes associated with its γ subunit (Ingledew and Poole, 1984; Craske and Ferguson, 1986). The decreased absorbance at 560 nm is consistent with the lack of *b*-type cytochrome associated with the membrane bound NO_3^- reductase.

After incubation of the dithionite reduced membranes with NO_3^- for 2-3 min. the spectra of the membranes were re-recorded. The wild type membranes showed a significant decrease in their absorbance at 560 nm, but not 550 nm, (Figure 5.3 (ii)) suggesting an oxidation of part of the total complement of *b*-type cytochrome, but not the *c*-type cytochrome, content of the membranes. This observation probably reflects the oxidation of the *b*-type cytochromes of the membrane bound NO_3^- reductase by NO_3^- . No changes were detectable even after a 30 min. incubation with NO_3^- when dithionite reduced membrane vesicles of the mutant M9 (Figure 5.3 (iv)), M6 or MX1 (not shown) were studied. This lack of oxidation of cytochromes, along with the decreased *b*-type cytochrome content of these membranes from the mutants,

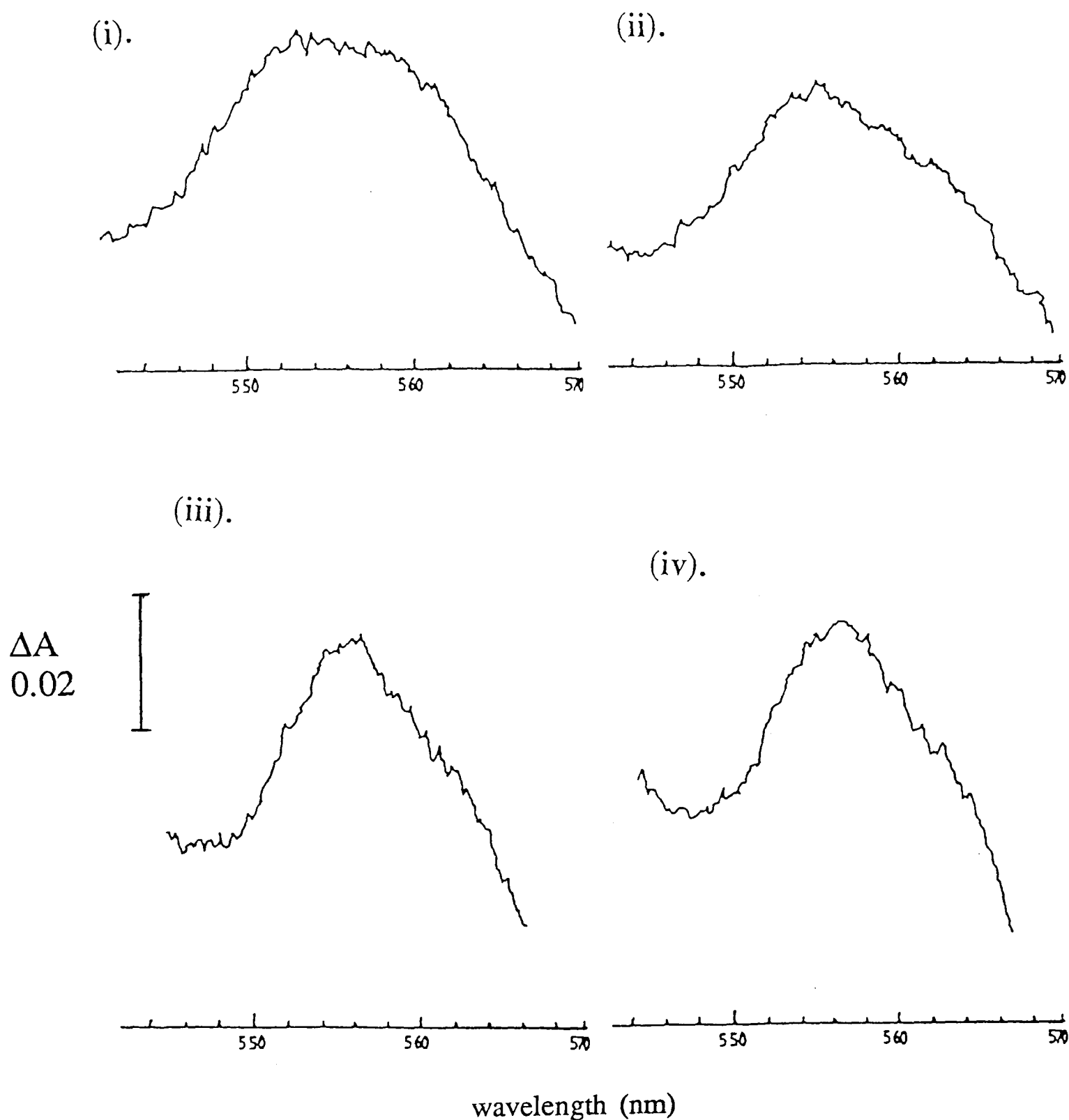


Figure 5.3. The effect of addition of nitrate on the cytochrome spectra from dithionite reduced membrane vesicles of wild type and mutant M6 cells of *T. pantotropha*.

Vesicles (5.0 mg protein) were suspended in 1.5 ml 50 mM Hepes/NaOH pH 7.3, 16 mM Glucose, 50 Units of catalase and 4 Units of glucose oxidase and reduced by the addition of dithionite. Spectra were recorded between 540 nm and 570 nm. [i] Wild type membrane vesicles (reduced) [ii] wild type vesicles (as in [i]) after incubation with 1 mM potassium nitrate for 5 min. [iii] Mutant M9 membrane vesicles (reduced). [iv] Mutant M9 membrane vesicles (as in [iii]) after incubation with 1 mM potassium nitrate for 5 min.

suggests that the γ subunit is lacking from these membranes. These observations are consistent with the lack of a functional membrane bound NO_3^- reductase in the class I NO_3^- reductase mutants of *T. pantotropha*.

Class II mutants were suspected (5.2.4b) of containing a defect in the pathway of cytochrome synthesis. Two such mutants, M2-1 and M2-7, were grown aerobically and fractionated into periplasmic and membrane fractions, which were analyzed for their cytochrome content. No cytochromes were detected in the periplasmic fractions of these mutants. In contrast *c*-type cytochrome absorbances were readily detected in the periplasmic fraction of wild type *T. pantotropha*. The membrane fractions of these mutant membranes contained less than 5 % of the total cytochrome content of the aerobically grown wild type membranes, as judged by the magnitude of absorbance at 410 nm in the preparation which had been reduced with dithionite. The spectral properties of these class II mutants are consistent with a defect in cytochrome biosynthesis in these mutants.

5.2.7b. Immunological studies on the nitrate reductases of wild type, class I and class II mutants of *T. pantotropha*.

The evidence presented in this Chapter has suggested that the Tn5 is inserted into the genomic DNA encoding either the regulatory region, or one of the structural polypeptides, of the membrane bound NO_3^- reductase in the class I mutants of *T. pantotropha*. The NO_3^- reductases of the distantly related *P. denitrificans* and *E. coli*

are immunologically similar (Craske and Ferguson, 1986), such that antibodies raised against the polypeptides of the NO_3^- reductase of *E. coli* cross-react with the polypeptides from the NO_3^- reductase of *P. denitrificans*. Antibodies raised against the polypeptides of the NO_3^- reductase enzyme of *P. denitrificans* were therefore used to elucidate further the consequence of the Tn5 insertion in these mutants.

Wild type cells of anaerobically grown *T. pantotropha* and *P. denitrificans* were fractionated into periplasms, cytoplasms and membranes. These fractions were subjected to SDS-PAGE, blotted onto nitrocellulose membranes (section 2.5.6) and probed with antibodies raised against the NO_3^- reductase of *P. denitrificans*. These antibodies cross-reacted strongly against the α polypeptide (M_r 130 kDa) and the β -polypeptide (M_r 60 kDa) of the NO_3^- reductase in the membranes of *P. denitrificans* (Figure 5.4A). A protein of M_r 117 kDa, in the membrane fraction from *P. denitrificans*, also strongly reacted with these antibodies (Figure 5.4A). This band represents an $\alpha\beta$ complex which does not completely unfold, unless the sample is boiled in the presence of β -mercaptoethanol (Ballard *et al.*, 1990). This $\alpha\beta$ complex, therefore, runs anomalously during PAGE. The α polypeptide and the $\alpha\beta$ complex each stained for NO_3^- reductase activity using the protocol described in section 2.5.5. The antibodies failed to cross-react with the smaller γ subunit (M_r 21,000 kDa) of *P. denitrificans*. The cytoplasmic fraction was also shown to contain the α , β polypeptides and the $\alpha\beta$ polypeptide complex (not shown).

The antibodies raised against the *P. denitrificans* holo- NO_3^- reductase strongly hybridized against polypeptides of M_r 140 kDa, 110 kDa and 65 kDa in the membranes of *T. pantotropha* (Figure 5.4A). These bands were also observed,

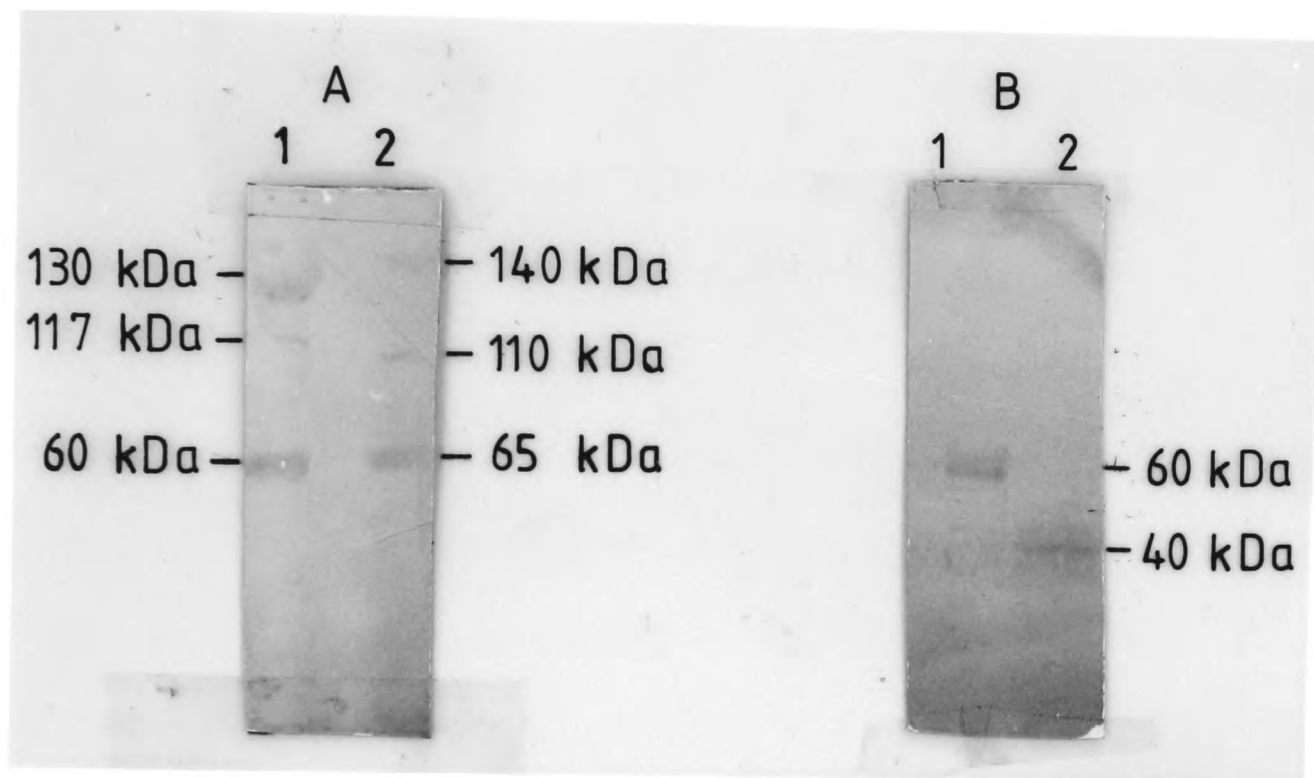


Figure 5.4. Immunoblotting (Western blot) of an SDS gel probed with anti-(*P. denitrificans* nitrate reductase) antibody.

(A) Lane 1, membrane vesicles from *P. denitrificans*. Lane 2, membrane vesicles from *T. pantotropha*. (B) Lanes 1 and 2, membrane vesicles from *T. pantotropha* mutants M6 and M9 respectively.

The samples were not treated with β -mercaptoethanol before loading on the 10 % polyacrylamide gel. Molecular weight markers (not shown) were: β -galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000) and lysozyme (M_r 14,300).

although they were less intense, in the cytoplasmic fraction of this organism. The antibodies raised against the NO_3^- reductase of *P.denitrificans* did not cross-react with any of the polypeptides contained in the periplasmic fraction of *T. pantotropha*. The pattern of cross-reactivity with the polypeptides of *T. pantotropha* indicates that the membrane bound NO_3^- reductase is structurally and immunologically similar to that of *P. denitrificans*, and that the periplasmic NO_3^- reductase is immunologically distinct from this membrane bound NO_3^- reductase.

Following the demonstration that antibodies raised against the NO_3^- reductase of *P. denitrificans* recognised the membrane bound NO_3^- reductase of *T. pantotropha*, the polypeptides of membranes from anaerobically grown class I mutants were also probed with these antibodies. A polypeptide of M_r 60 kDa from mutant M6, and polypeptide of M_r 45 kDa from mutant M9 (Figure 5.4B), but no polypeptide bands from mutant MX1 (not shown), cross-reacted with these antibodies. Similar results were obtained when the cytoplasmic fractions of these mutants were probed with these antibodies. No activity staining bands were detected in the fractions of these class I mutants. Truncated α or β subunits are thus found in the mutants M6 and M9. The antisera recognises both these polypeptides and thus discrimination between them is not possible.

Antibodies raised against the purified 82 kDa polypeptide of the periplasmic NO_3^- reductase from *R. capsulatus* strain N22DNAR⁺ were used to probe the periplasmic, and membrane, fractions of aerobically and anaerobically grown cells of *T. pantotropha* as well as the periplasm from phototrophically grown cells of *R. capsulatus* strain N22DNAR⁺. These antibodies cross reacted with bands of M_r 82

and 50 kDa in periplasmic fractions of *R. capsulatus* strain N22DNAR⁺ (Figure 5.5). The 82 kDa band corresponded to the denatured polypeptide, which contains the site of NO₃⁻ reduction, whereas the 50 kDa band corresponded to the partially folded 82 kDa polypeptide associated with cytochrome *c*₅₅₂ (Richardson *et al.*, 1990). The 50 kDa band stained for haem and for NO₃⁻ reductase activity on similar gels containing the purified NO₃⁻ reductase and crude periplasmic fraction from this organism. The activity stain was short lived, suggesting that this enzyme is labile in the presence of dithionite (Richardson, personal communication). The *c*-type cytochrome, cytochrome *c*₅₅₂, that is associated with purified NO₃⁻ reductase from *R. capsulatus* is a polypeptide of M_r 13 kDa, which can associate with catalytic subunit to give 50 kDa haem staining band seen on SDS-PAGE, as discussed above (Richardson *et al.*, 1990).

The antibodies against the 82 kDa polypeptide of the periplasmic NO₃⁻ reductase of *R. capsulatus* were shown to hybridize against 90 kDa and 40 kDa polypeptides in the periplasmic fractions of both wild type and each of the class I mutants of *T. pantotropha*, all of which had been grown both aerobically (Figure 5.5) and anaerobically. The 40 kDa band also stained for haem and for NO₃⁻ reductase activity (not shown). This observation shows that the periplasmic NO₃⁻ reductase of *T. pantotropha* is immunologically similar to the periplasmic NO₃⁻ reductase of *R. capsulatus*, and in common with the reductase in *R. capsulatus* is associated with a *c*-type cytochrome. Thus the 90 kDa and 40 kDa bands from the periplasmic NO₃⁻ reductase of *T. pantotropha* are analogous to the 82 kDa and 50 kDa bands of the corresponding reductase in *R. capsulatus*. The 50 kDa band corresponds to a partially folded complex of the catalytic and cytochrome polypeptides.

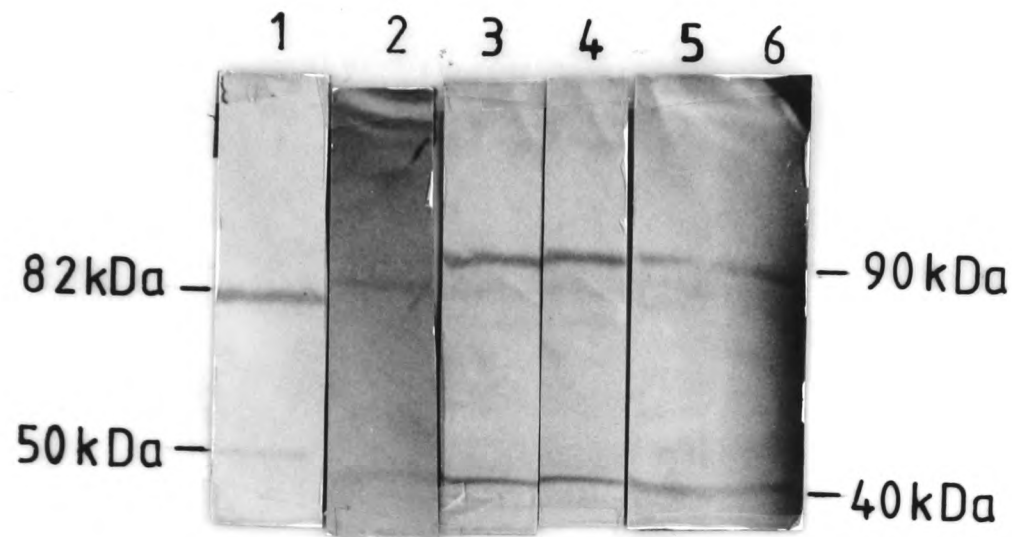


Figure 5.5. Immunoblotting of periplasmic fractions from *T. pantotropha* probed with anti-(*R. capsulatus* strain N22DNAR⁺ nitrate reductase) antibodies.

Lane 1, *R. capsulatus* N22DNAR⁺ periplasm; Lanes 2-6 contain the periplasms from *T. pantotropha* as follows: Lane 2, mutant 2-7; Lane 3, wild type; Lane 4, mutant M6; Lane 5, mutant M9; Lane 6, mutant MX1.

Samples were heated with β -mercaptoethanol before loading onto a 12 % polyacrylamide gel. The approximate molecular weight were estimated from the migration of molecular weight standard on a protein stained portion of the gel: β -galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000) and lysozyme (M_r 14,300).

The antibodies raised against the periplasmic NO_3^- reductase of *R. capsulatus* failed to recognise any of the polypeptides in membrane vesicles from aerobically, or anaerobically, grown cells of *T. pantotropha*, consistent with the membrane bound and periplasmic NO_3^- reductases being immunologically distinct.

The mutants categorised as class II mutants have been shown to lack *c*-type cytochromes in their periplasmic fractions, when grown aerobically on medium containing succinate and NO_3^- . The polypeptides from the periplasmic fraction from one mutant, M2-7, were separated by SDS-PAGE. Polypeptides, with molecular weights in the region of 90 kDa and 40 kDa, were shown to recognise the anti-*R. capsulatus* NO_3^- reductase antibodies, just as was found with material from wild type cells of *T. pantotropha* (Figure 5.5). These polypeptides, however, did not stain for haem and lacked NO_3^- reductase activity. (Indeed, no haem staining bands were detected in the periplasmic fraction of this mutant). On first examination, it is somewhat curious that the 40 kDa band from a mutant, from which *c*-type cytochromes are absent, is electrophoretically similar to the partially folded complex of the same polypeptide with a *c*-type cytochrome from the wild type cells. However, the partially folded polypeptide of the NO_3^- reductase catalytic subunit of *R. capsulatus* retained the same electrophoretic mobility, running as a 50 kDa band on SDS-PAGE, even after it had been dissociated from its complex with cytochrome *c*₅₅₂ (Richardson *et al.*, 1990). It is therefore suggested that the same unusual behaviour applies to the NO_3^- reductase from class II mutants of *T. pantotropha*, in which the cytochrome subunit, at least in its holo-form, is absent. In *R. capsulatus*,

the dissociation of the 90 kDa protein from its associated cytochrome results in a dramatic decrease in its specific NO_3^- reductase activity (Richardson *et al.*, 1990). If the periplasmic NO_3^- reductase from *T. pantotropha* behaves similarly in the latter respect to the enzyme of *R. capsulatus*, this could account for the lack of NO_3^- reductase activity despite the expression of the catalytic subunit of this reductase in the class II phenotype of mutants.

The membranes of class II mutants were not subjected to analysis for recognition by anti-(*P. denitrificans* NO_3^- reductase) antibodies because this class of mutant could not be grown anaerobically and only very low levels of the membrane bound NO_3^- reductase are expressed in aerobically grown wild type cells.

5.2.8. Purification of the periplasmic nitrate reductase of *T. pantotropha*.

Section 5.2.7b has discussed the evidence from which it was deduced that the periplasmic NO_3^- reductase of *T. pantotropha* is associated with a *c*-type cytochrome. Purification would enable further information about this enzyme, and its cytochrome, to be obtained. It has been shown that the levels of expression of the periplasmic NO_3^- reductase are significantly increased in class I mutants grown anaerobically relative to the levels found in anaerobically grown wild type cells (section 5.2.4a). Thus an attempt was made to purify the periplasmic NO_3^- reductase from one such class I mutant, M6, rather than from the wild type cells.

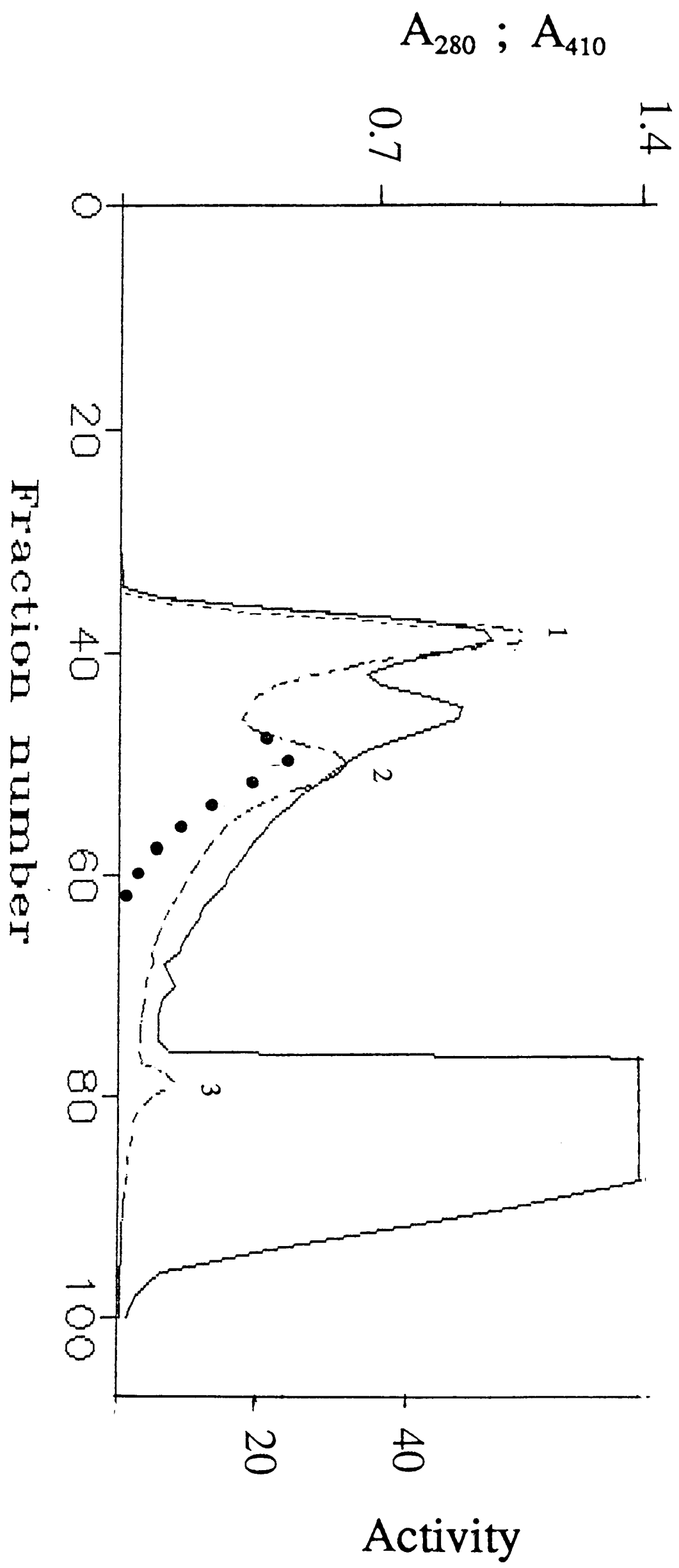


Figure 5.6. Elution profile of the periplasmic nitrate reductase of *T. pantotropha* from Sephacryl S200.

The 20-100 % saturated ammonium sulphate precipitate of the periplasmic fraction of mutant M6 was loaded (in a volume of 15 ml) onto a Sephacryl S200 column (height 100 cm, diameter 2.6 cm) which had been pre-equilibrated in 20 mM Tris-HCl (pH 7.8), 20 % glycerol buffer at 4 °C. The column was eluted with this buffer at a flow rate of 8 ml. hr⁻¹ and 5 ml fraction were collected.

The activity profile is only indicated for fractions 48-60, owing to the presence of nitrite reductase activity in earlier fractions, an accurate measure of nitrate reduction could not be made (see text for further details).

Peaks designated 1, 2 and 3 correspond to cytochromes (see text).

Activity (• • •) is expressed in $\mu\text{mol. min}^{-1}.\text{ml}^{-1}$

A_{280} is represented by (—); A_{410} is represented as (----).

Accordingly mutant M6 was grown on ten litres of medium containing succinate and NO_3^- . A 400 ml volume of periplasm was prepared from these cells using the procedure described in Chapter 1. In wild type cells of *T. pantotropha* approximately 70 % of the periplasmic NO_3^- reductase was shown to precipitate between concentrations of 20 to 50 % $(\text{NH}_4)_2\text{SO}_4$. Less than 30 % of the total periplasmic NO_3^- reductase activity was found in a 20-50 % $(\text{NH}_4)_2\text{SO}_4$ precipitate from mutant M6. The reason for this apparent increase in solubility of the NO_3^- reductase from this mutant is uncertain. In general, the solubility of a particular component is decreased by the presence of other proteins (Scopes, 1987). Thus in wild type periplasm, where the NO_3^- reductase accounts for a substantially lower proportion, relative to mutant M6, of the total periplasmic protein, a decreased solubility of this reductase may perhaps be rationalised. To obtain a maximum yield of the periplasmic NO_3^- reductase from mutant M6, the 0-20 % $(\text{NH}_4)_2\text{SO}_4$ precipitate, which contained only small membrane fragments, was discarded but much of the remaining soluble protein and the NO_3^- reductase was precipitated in a 20-100 % $(\text{NH}_4)_2\text{SO}_4$ cut. This intensely red coloured precipitate was dissolved in 15 ml of 20 mM Tris-HCl (pH 7.8). Spectroscopic analysis of this precipitate revealed the presence of *c*-type cytochromes, all of which were oxidised as prepared. Reduction of this sample with dithionite revealed absorbance bands at 552 nm and 620 nm (broad) which were lost on addition of NO_3^- . SDS-PAGE analysis of this sample showed that approximately 15 polypeptides were present.

The 20-100 % $(\text{NH}_4)_2\text{SO}_4$ cut was loaded onto a Sephacryl S-200 column. The presence of glycerol had previously been shown to increase the stability of the periplasmic NO_3^- reductase of *R. capsulatus* during column chromatography

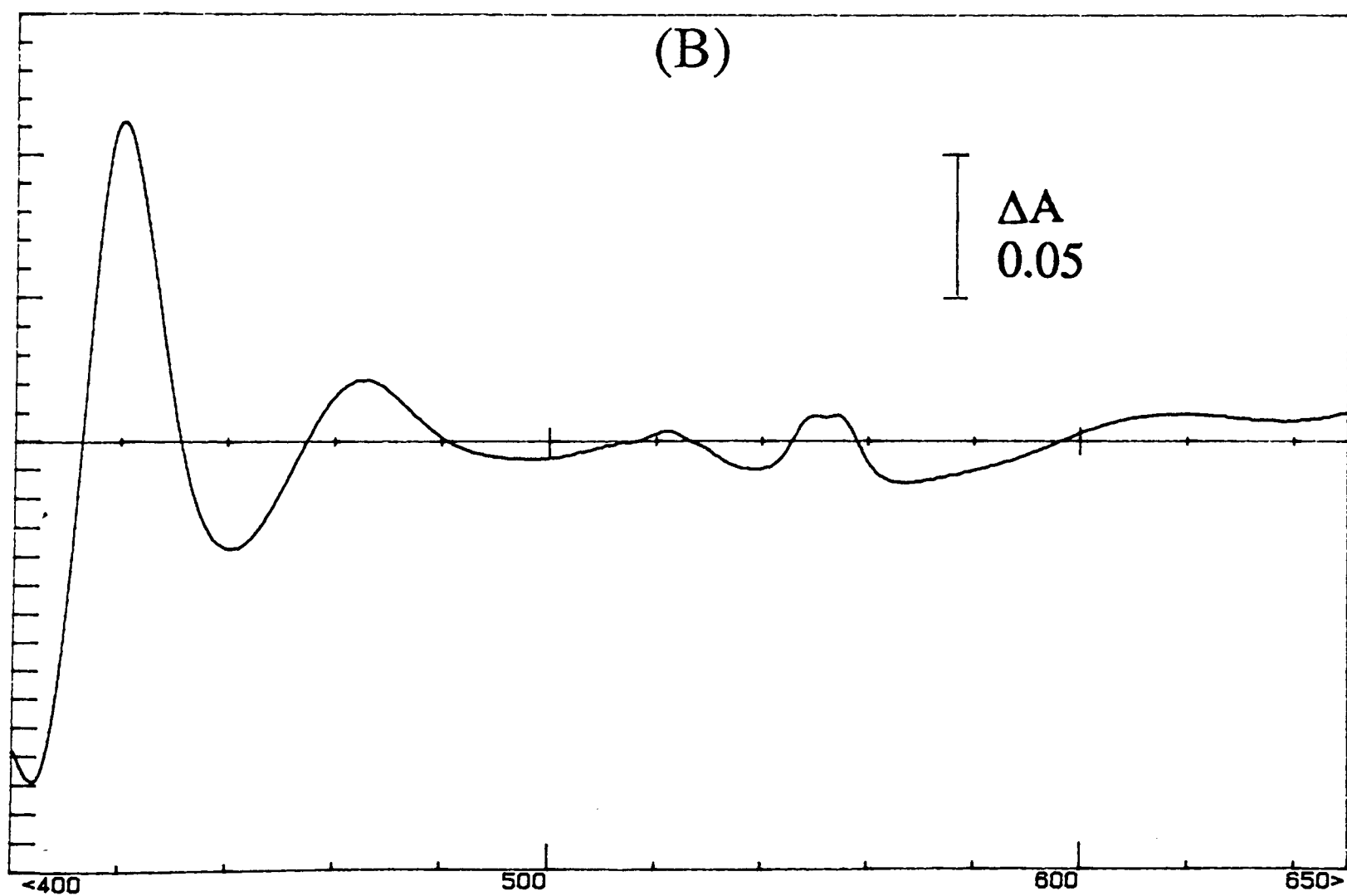
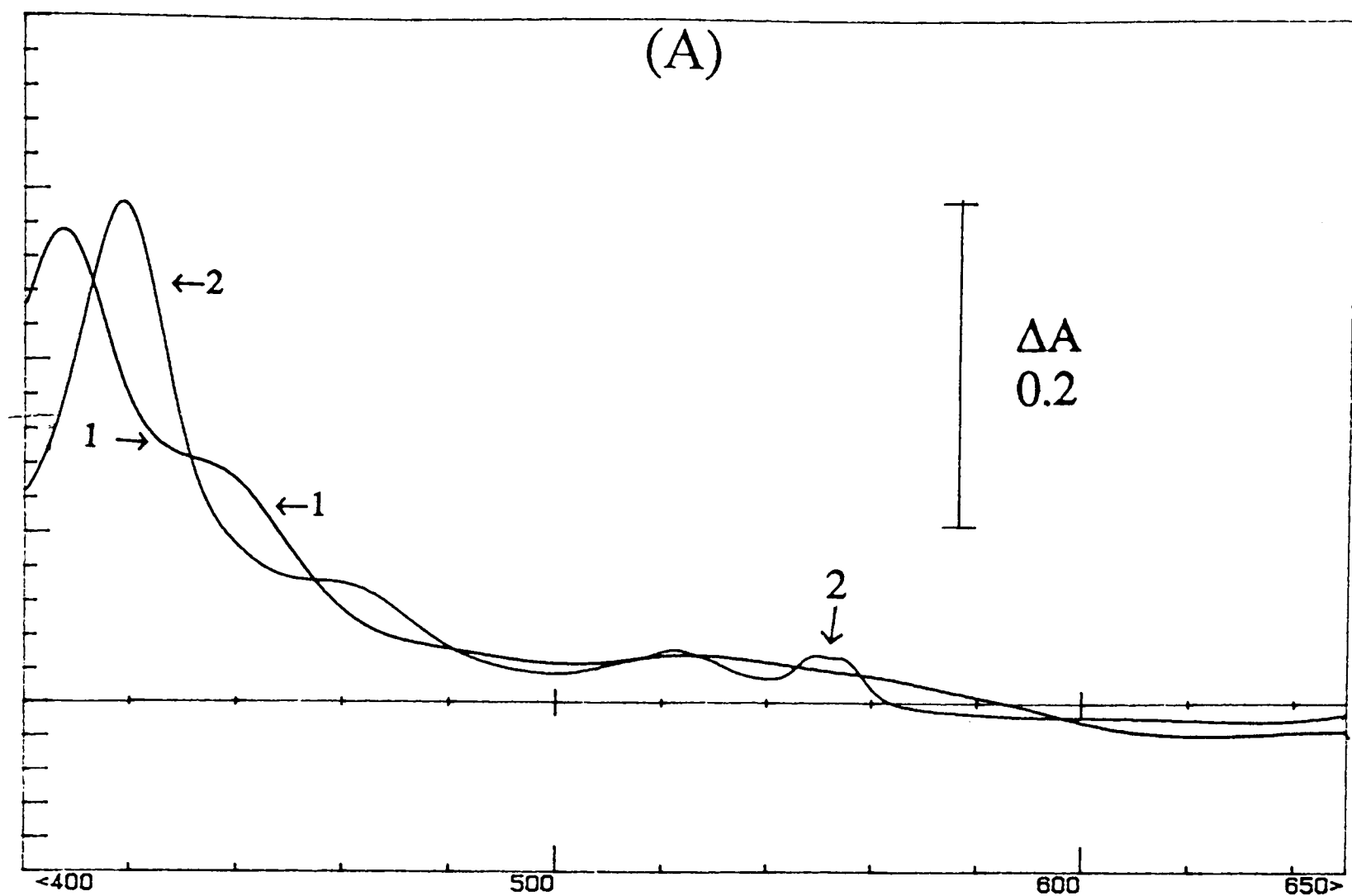
Figure 5.7. The absorption Spectra of cytochrome cd_1 from *T. pantotropha*.

Peak 1 (fractions 36-41) from the Sephacryl S-200 column, in 20 mM Tris/HCl, pH 7.8, 20 % (v/v) glycerol.

(A) 1. the oxidised (as prepared form), 2. the reduced form, obtained by titration of dithionite.

(B). The reduced minus nitrite (1 mM) oxidised difference spectrum.

Scanning was at 50 nm. min⁻¹.



wavelength (nm)

(Richardson *et al.*, 1990), and thus it was included in the buffers during the purification of the NO_3^- reductase from *T. pantotropha*. The absorbance of each of these fractions at 280 nm and 410 nm was monitored to determine, respectively, the protein and cytochrome eluted from the column. The elution profile (Figure 5.6) reveals the presence of three cytochrome peaks labelled as peaks 1, 2 and 3. The MV^{2+} -dependent NO_3^- reductase activity profile corresponded to the position of peak 2 although it overlapped slightly peak 1 (Figure 5.6). Both cytochrome peaks 1 and 2 were oxidised as prepared. The spectral characteristics of the material in peak 1, after reduction with dithionite, resembled cytochrome cd_1 , a NO_2^- reductase, with a split α band at 448 and 552 nm and a broad absorption at 625 nm (see Figure 5.7). This cytochrome was oxidised by NO_2^- . Owing to slight contamination of this peak with NO_3^- reductase activity, addition of NO_3^- to this sample resulted in generation of NO_2^- which in turn acted to oxidise its reductase, cytochrome cd_1 . This phenomenon accounts for the NO_3^- -oxidisable absorption at 625 nm observed in the 20-100 % $(\text{NH}_4)_2\text{SO}_4$ precipitate. Peak 2 contained a NO_3^- -oxidisable *c*-type cytochrome with a α band maxima at 552 nm, designated cytochrome c_{552} , in its reduced form. The third cytochrome peak corresponded to *c*-type cytochrome of M_r 13 kDa.

Peak 2, which contained the periplasmic NO_3^- reductase activity, was further purified using ion-exchange chromatography. Fractions 48-56 from the gel filtration step were combined and further purified using a DEAE-Sephacel column. The elution profile of protein and cytochrome from this ion-exchange is shown in Figure 5.8. The NO_3^- reductase activity co-eluted at 175 mM with first cytochrome peak. The second cytochrome peak eluted at 280 mM KCl and corresponded to cytochrome cd_1 . Peak 1, containing the purified periplasmic NO_3^- reductase of *T. pantotropha*,

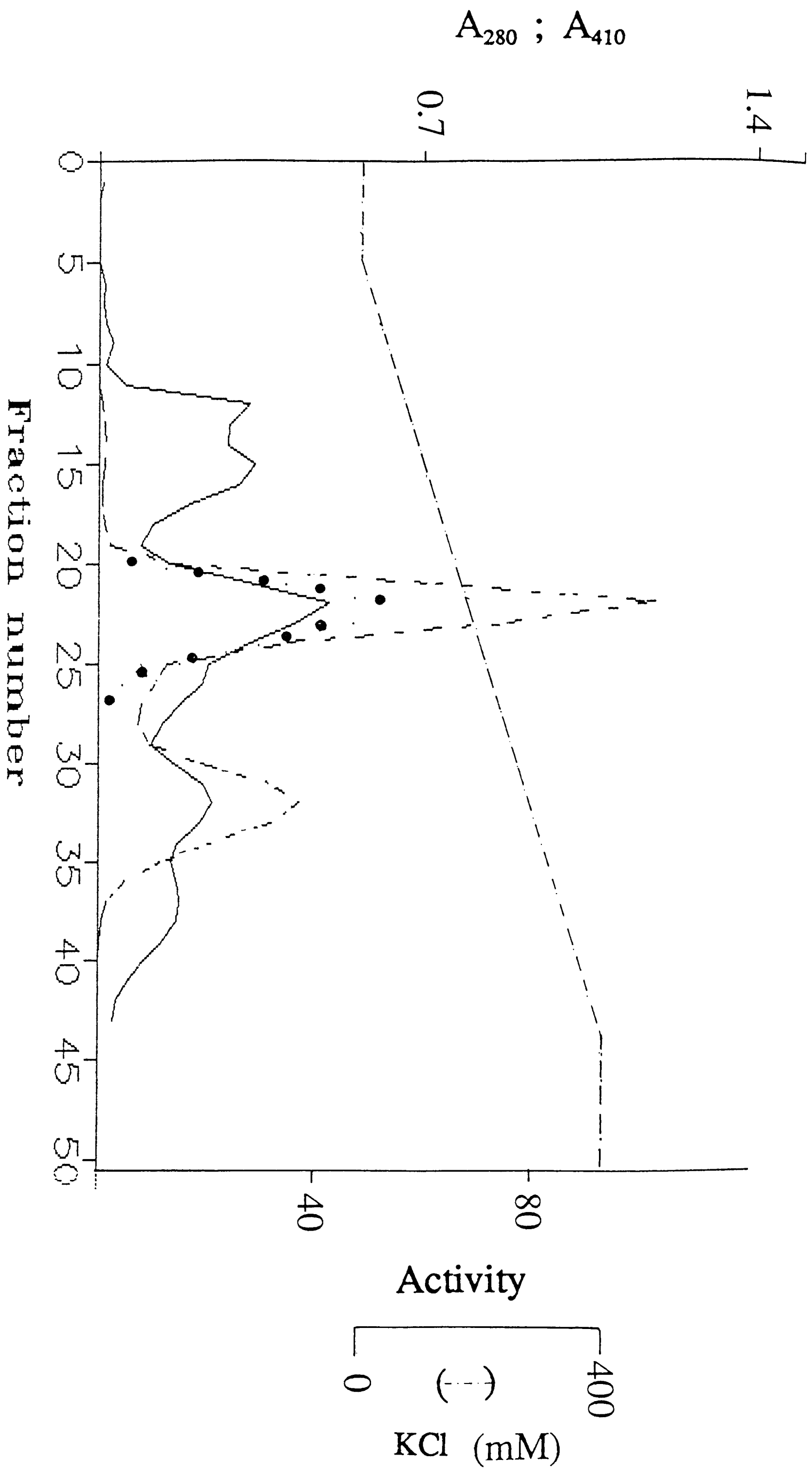


Figure 5.8. Elution profile of the periplasmic nitrate reductase activity of *T. pantotropha* from DEAE-Sephacel.

The combined fractions from the Sephacryl S200 column were loaded onto a DEAE-Sephacel column (height 10 cm, diameter 1 cm) which had been pre-equilibrated with 20 mM potassium phosphate (pH 6.8), 20 % (v/v) glycerol. The column was washed with 25 ml of this buffer to remove unbound protein and then eluted with 85 ml of a 0-400 mM KCl gradient in this buffer. The column was run at 25 ml.h⁻¹ and 2 ml fractions were collected.

Activity (• • •) is expressed in $\mu\text{mol. min}^{-1}.\text{ml}^{-1}$

A₂₈₀ is represented by (—); A₄₁₀ is represented as (----).

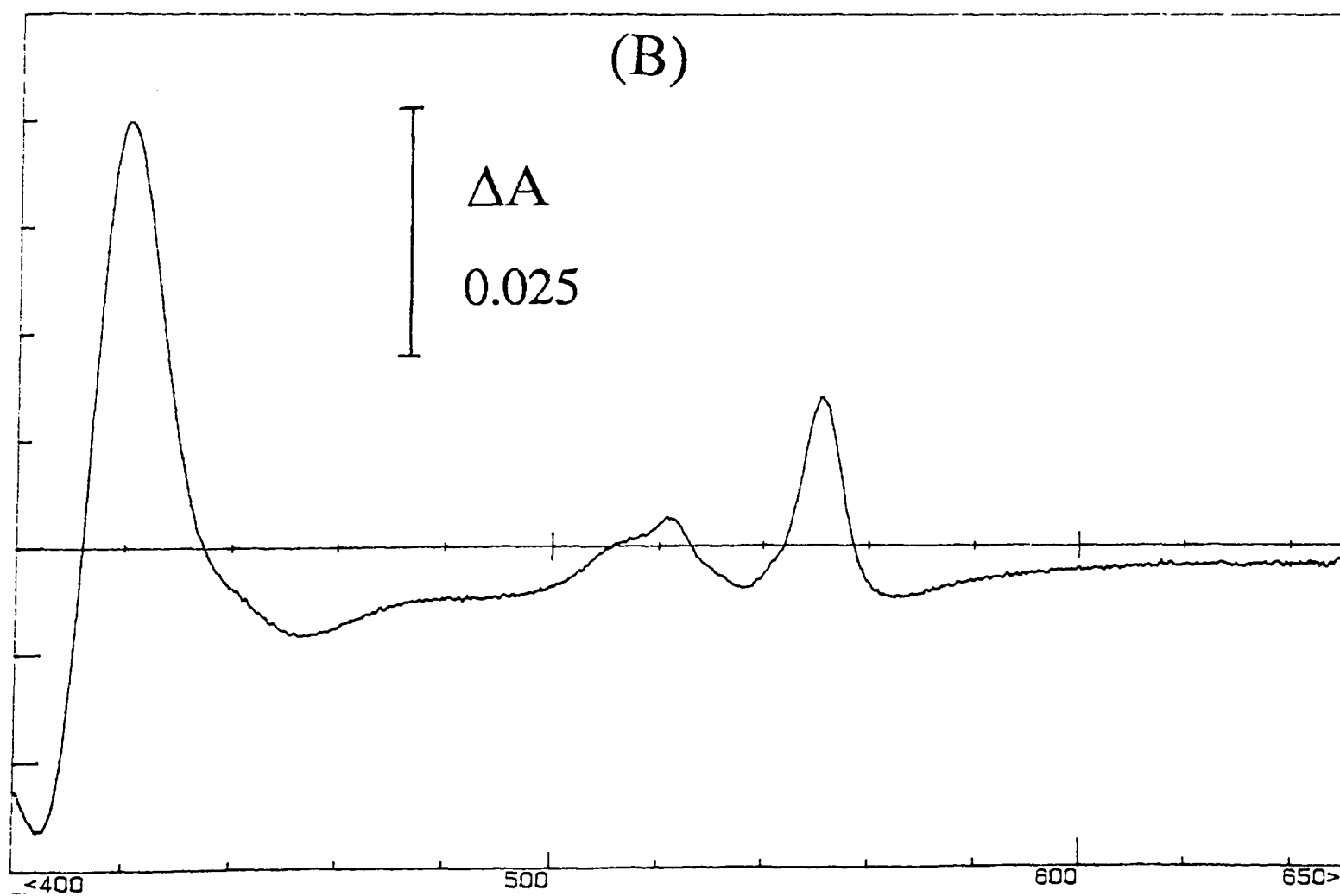
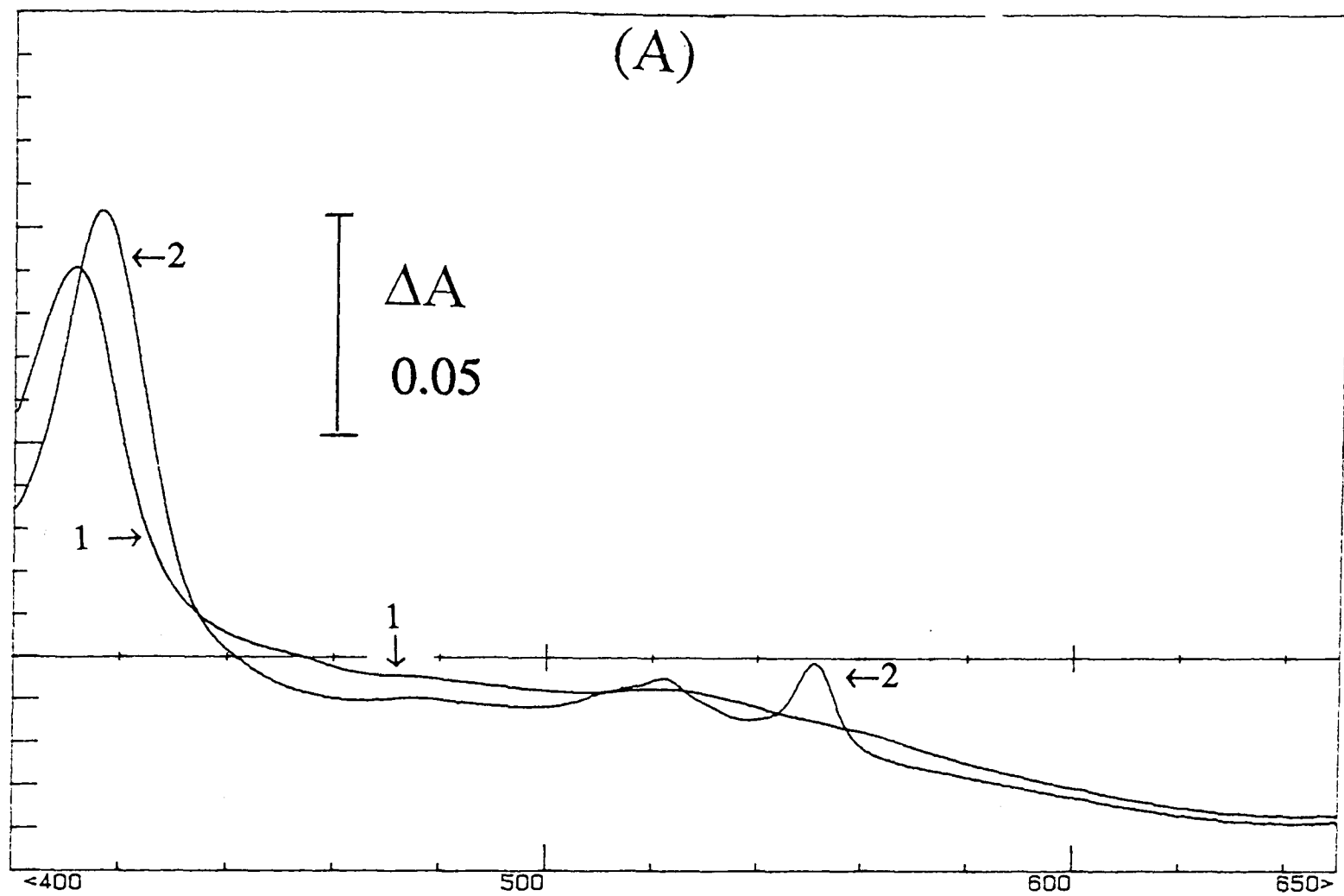
Figure 5.9. The absorption spectra of the purified periplasmic nitrate reductase from *T. pantotropha*.

Fractions 20-24 from the DEAE-Sephacel column contain the purified periplasmic nitrate reductase, in 20 mM potassium phosphate, pH 6.8, 20 % (v/v) glycerol:

(A) 1. The oxidised as prepared form of the enzyme, 2. the dithionite reduced form of the enzyme.

(B) The dithionite reduced minus nitrate oxidised difference spectra. Nitrate was added to 1 mM.

The scan speed was 50 nm. min⁻¹.



wavelengths nm

was oxidised as prepared and had absorbance at 520 nm and 418 nm (Figure 5.9A). In the dithionite reduced form, the absorbance maxima were seen at 552 nm, 528 nm and 408 nm (Figure 5.9A). A spectrum corresponding to the oxidised form was obtained following the addition of NO_3^- . The dithionite reduced minus NO_3^- oxidised spectrum of this cytochrome is shown in Figure 5.9B. These results demonstrate that the association between the NO_3^- reductase activity and a *c*-type cytochrome, designated cytochrome c_{552} , in *T. pantotropha* remains stable at high ionic strengths and therefore indicate that the stabilising forces are not entirely electrostatic in nature.

Fractions 21-24 were combined and analyzed by SDS-PAGE. Coomassie blue staining revealed only one polypeptide, of molecular weight 90 kDa, in the sample (Figure 5.10). This polypeptide did not stain for haem and in view of the cross-reaction with the anti-(*R. capsulatus* NO_3^- reductase) antibody is taken to represent the catalytic subunit of the NO_3^- reductase. No evidence of a second polypeptide corresponding to cytochrome c_{552} was apparent from this gel despite the indication of the presence of a *c*-type cytochrome from the absorption spectra of the sample. Richardson *et al.* (1990) showed that cytochrome c_{552} from *R. capsulatus* was not detected using a Coomassie blue stain on SDS gels.

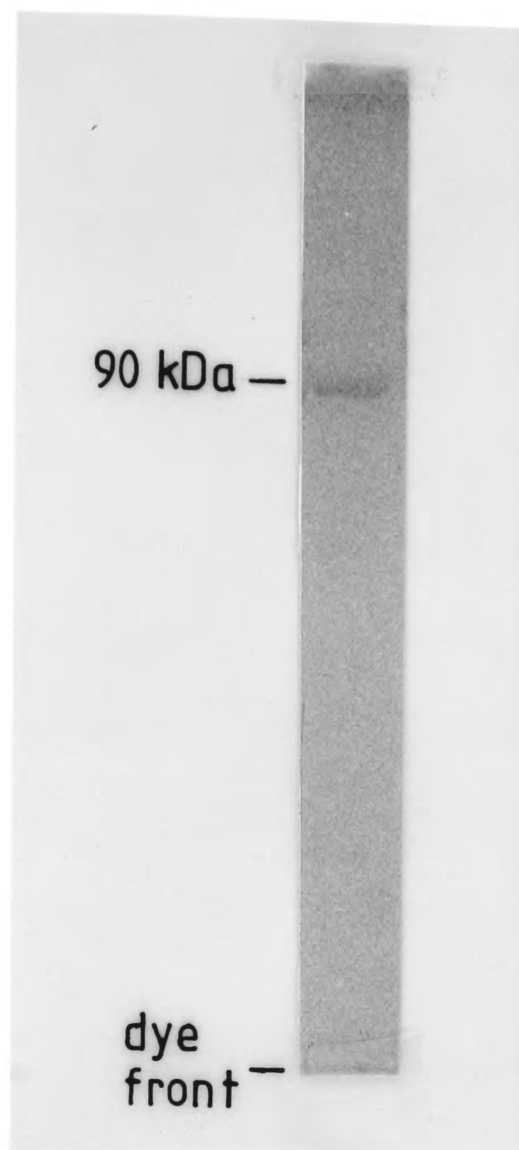


Figure 5.10. SDS-PAGE analysis of the periplasmic nitrate reductase of *T. pantotropha*.

The combined fractions (20-24, 20 μ g protein) from the DEAE-Sephacel column were analyzed on SDS-PAGE gel which was Coomassie stained for protein. The molecular weight of the sample polypeptides were calculated from the migration of molecular weight markers: β -galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000) and lysozyme (M_r 14,300), (not shown).

5.3. DISCUSSION.

The observations established in this Chapter provide genetic and further biochemical evidence for the presence of two distinct NO_3^- reductases in *T. pantotropha* thus complementing the biochemical evidence for two NO_3^- reductases presented in Chapter 4. A mutation which prevents any activity of the membrane bound NO_3^- reductase in this organism does not prevent denitrification from occurring. This is because there is expression of a second NO_3^- reductase, that from the periplasm. Three such mutants, designated as class I mutants, were obtained in the present work. The selection procedure established for these mutants produced the desired phenotype, apparently because this is the only enzyme capable of reducing ClO_3^- to ClO_2^- present in this organism.

However, previous workers screening for ClO_3^- resistance, under anaerobic conditions, to obtain mutants of *E. coli* defective in the membrane bound NO_3^- reductase, obtained mutants that were defective at loci other than in the structural genes of the reductase. ClO_3^- resistant mutants lacked all enzymes that require a functional molybdenum cofactor; such mutations mapped from loci designated as *chlA* through to *chlG*. The *chlA*, *chlB*, *chlD*, *chlE* and *chlG* loci are all implicated in the processing of the molybdenum cofactor of *E. coli*. (The original *chlF* mutation has been lost, so its function has remained unelucidated). The functions of the gene products of the *chl* genes will not be dealt with here (for recent reviews see Cole, 1988; Stewart, 1988; Hinton and Dean, 1990). The screen for ClO_3^- resistance in *E. coli* did not identify mutants deleted in the structural gene of the NO_3^-

reductase because this deletion would not effect other molybdoenzymes which also possess ClO_3^- reductase activity. In *E. coli*, DMSO reductase is an example of one such molybdoenzyme (Weiner *et al.*, 1988). Mutants defective in the structural genes of NO_3^- reductase are ClO_3^- sensitive, and thus were isolated by negative enrichment procedures (Glaser and Demoss, 1972). Early work designated such mutants with deletions in the structural genes as *ChlC* mutants (Demoss, 1978). Subsequent work provided evidence for three structural genes (Bonnefoy-Orth *et al.*, 1981; Stewart and MacGregor, 1982; Edwards *et al.*, 1983). Stewart and MacGregor (1982) proposed the *nar* code to denote individual genes within *chlC* locus, accordingly, the *narG*, *narH* and *narI* genes encode for the α , β and γ subunits, respectively. The properties of transposon mutants indicate that this operon transcribed in the direction *narG* to *narI* (Edwards *et al.*, 1983). Recent work has reported that the *narI* region contains two open reading frames which encode for proteins of M_r 26.5 and 25.5 kDa. The *nar* operon, therefore, encodes for four genes designated and ordered as *narGHJI* (Sodergren and Demoss, 1988). *NarJ* displays no region that is capable of spanning the cytoplasmic membrane and as yet the function of the *NarJ* gene product remains undetermined (Sodergren and Demoss, 1988). The non-translated 5'-region of the *nar* operon contains two functionally distinct domains (Li *et al.*, 1985). One sequence contains the transcriptional start site and a promoter which is under the positive control of the *fnr* gene product (section 4.3). The second domain is involved in the NO_3^- induced expression of the *nar* operon and may contain a site for the binding of the *narL* gene product (Stewart, 1982) (see section 4.3).

In the present study with *T. pantotropha*, in which only the membrane bound NO_3^- reductase reduces ClO_3^- , class I mutants could, in theory, result from insertion

of the Tn5 transposon into either one of the structural genes of the membrane bound NO_3^- reductase or a gene involved in the expression of the enzyme. If organisation of the structural genes of this reductase in *T. pantotropha* is similar to that of *E. coli*, then mutants M6 and M9 could result from an insertion into *narG*. Thus the α subunit would be truncated because Tn5 usually terminates transcription with the consequence that expression of genes distal to the site of insertion is prevented (ie. in this case the β and γ subunits would not be transcribed). Evidence to confirm this proposed gene sequence could be obtained in future work using subunit specific antibodies to the polypeptides of the NO_3^- reductase with many mutants. This approach would verify that it is truncated α subunits that are seen in Figure 5.4B. No polypeptides of the membrane bound NO_3^- reductase were detected in fractions of mutant MX1, thus this mutant may result from an insertion in *narG* or alternatively in a regulatory region upstream of the structural gene.

The selection procedures described in section 5.2.2 could have in theory provided a selection procedure for mutants of several phenotypes/genotypes. Mutations in proteins involved in the electron transport pathway to the membrane bound NO_3^- reductase would be expected to result in a phenotype which is ClO_3^- resistant. No such mutants were isolated in this study or in studies with *E. coli* (Edwards *et al.*, 1983). It should also have been possible in the selection procedure described to isolate mutants with defects in the molybdenum cofactor biosynthesis. Such a mutation would result in the loss of all dissimilatory NO_3^- reductase activity and the inability to grow using thiosulphate or formate as electron donors. This phenotype for a mutant, of *T. pantotropha* has been isolated by previous workers screening for the inability of this organism to use thiosulphate as an electron donor (Chandra and

Friedrich, 1986). Class III and IV mutants, isolated in this study, were impaired in growth on formate and thiosulphate, as well as containing reduced levels of both types of NO_3^- reductase. The phenotype of this mutant suggests a deletion in one of the genes involved in the molybdenum cofactor biosynthesis and assembly or its regulation. The exact nature of this mutation requires further study.

In this study a selection procedure was not devised that successfully produced mutants containing a defect in the periplasmic NO_3^- reductase but retained a functional membrane bound NO_3^- reductase. An attempt made to isolate such mutants, on plates containing a concentration of azide which inhibited the reduction of NO_3^- by the membrane bound, but not the periplasmic NO_3^- reductase, failed. At such concentrations of azide, *P. denitrificans*, an organism which expresses only a membrane bound NO_3^- reductase, failed to grow using NO_3^- as the terminal electron acceptor. However, wild type *T. pantotropha* (and class I mutants) were able to grow under such conditions. Presumably this ability is due to the reduction of NO_3^- by the periplasmic NO_3^- reductase. Thus it should in theory, by replica plating, be possible to identify mutants of *T. pantotropha* that are unable to grow anaerobically on these azide containing plates as result of loss of the periplasmic NO_3^- reductase activity. However, attempts to produce such a mutant failed, perhaps in part because azide appeared to be unstable over the two day period required to grow transconjugants on kanamycin-containing plates. A mutant deficient in the periplasmic NO_3^- reductase is predicted to lose the capacity for aerobic NO_3^- reduction. It would also be interesting to examine what effect, if any, this mutation would have on the aerobic reduction of NO_2^- and N_2O .

A further interesting feature of the studies presented in section 5.2.4a is that deletion of the membrane bound NO_3^- reductase leads to increased expression of the periplasmic NO_3^- reductase under anaerobic but not aerobic growth conditions. The basis for this observation is not understood. It is perhaps possible that some component of the membrane bound nitrate reductase, or a redox effect resulting from expression of the latter enzyme, serves to repress synthesis of the periplasmic NO_3^- reductase activity. The molecular basis for the regulation of expression of both the NO_3^- reductases enzymes requires further investigation.

The mutants with the phenotype designated as class II are thought to result from a lesion in the pathway of cytochrome biosynthesis. These mutants failed to grow anaerobically (on NO_3^- , NO_2^- or N_2O) although they grew aerobically, albeit at rates greatly attenuated when compared to wild type cells (Table 5.3). There appear to be no *c*-type cytochromes and only low levels of *b*-type cytochromes detectable in cells of these mutants. The failure of these mutants to grow anaerobically can be attributed to this lack of cytochromes. Aerobic growth is clearly possible for these mutants with an apparently restricted complement of *b*-type cytochromes. The defect resulting from Tn5 insertion is unclear, but the possibility that the defect exists in haem biosynthesis could be investigated in further work by growth of these cells on complex media containing haem.

The observations in Chapter 4 showed that, like *R. capsulatus*, *T. pantotropha* expresses a periplasmic NO_3^- reductase. These reductases have similar catalytic

properties (i.e. their insensitivity to azide and their inability to reduce ClO_3^-). The evidence presented in the current Chapter shows that there are further similarities between the periplasmic NO_3^- reductases of these two organisms. The reductases from both organisms consist of two polypeptides. One polypeptide, of molecular weight 80-90 kDa, contains the active site of the enzyme and the second polypeptide is a small *c*-type cytochrome. In both organisms this cytochrome has its α -reduced absorbance maxima at 552 nm (section 5.2.8; Richardson *et al.*, 1990).

The periplasmic NO_3^- reductase of *R. capsulatus* strain AD2 has been shown to be encoded on a plasmid (Willison, 1990). *T. pantotropha* has been shown to contain two endogenous plasmids of M_r 450 and 110 kDa (Chandra and Friedrich, 1986). If the genes for the periplasmic NO_3^- reductase of *T. pantotropha* are encoded on one of these plasmids then a strain of *T. pantotropha* which lacks that plasmid would lose the ability for aerobic NO_3^- reduction. Other genes encoding for proteins involved in aerobic denitrification may also be located on the DNA of a plasmid and hence lost if the strain is cured of that plasmid.

Chapter 6 Nitric oxide reduction is ubiquitous in strains of *R. capsulatus*; electron transport pathways to nitric oxide in *R. capsulatus* strain MT1131.

6.1. INTRODUCTION.

Although the observation that many genera of denitrifying bacteria reduce NO had been accepted for many years, the role of NO as a free intermediate in the denitrification process has not been so generally accepted (Chapter 1). However, recent experiments which have involved trapping of NO, formed by cells reducing NO_3^- , (Carr *et al.*, 1989; Goretski and Hollocher, 1988) have provided strong evidence for NO being a freely diffusible intermediate within the overall process of bacterial denitrification. Subsequently, discrete NO reductases that contain *b*- and *c*-type haems have been purified from *P. stutzeri* and *P. denitrificans* (Heiss *et al.*, 1989; Carr and Ferguson, 1990a). The membrane bound NO reductase of *R. sphaeroides* f. sp. *denitrificans* has also been shown to be distinct from the cytochrome *bc₁* complex (Itoh *et al.*, 1989a). Certain strains of the photosynthetic bacterium *Rhodobacter capsulatus* are able to catalyse the reduction of some of the oxy-species of nitrogen that participate in the denitrification process. Thus the possession of a NO_3^- reductase, periplasmic in many strains, e.g. AD2 and N22DNAR⁺ (McEwan *et al.*, 1984), but membrane bound in BK5 (Ballard *et al.*, 1990), and a periplasmic N_2O reductase have been reported (McEwan *et al.*, 1985). Table 2.1 lists the identified nitrogen-oxide oxidoreductase activities in the strains studied in the present work. Recently, it has been shown for *R. capsulatus* strain AD2 that the NO_3^- reductase activity is lost in plasmid-deficient isolates (Willison, 1990). Some strains, e.g. 37b4, do not possess any of these nitrogen-oxide reducing activities (Kelly *et al.*, 1988). Those strains possessing a NO_3^- reductase accumulate NO_2^- when grown phototrophically with NO_3^- present as a terminal electron acceptor. This is because a reductase for the NO_2^- appears to be lacking.

The absence of reports of a NO_2^- or NO reductase activity in *R. capsulatus* correlates with the failure to identify and isolate a strain of this organism which can perform the complete reduction of NO_3^- to N_2 gas. However, the recent identification of a bacterial NO reductase raised the question as to whether, at least, some strains of *R. capsulatus* might possess this enzyme. The possession of the enzymes for reducing some of the oxy-species of nitrogen suggests that *R. capsulatus* encounters such molecules in its natural habitats, and that, therefore, NO reductase may be present. Furthermore, the toxicity of NO, coupled with the evidence of its extensive production in some environments (Remde *et al.*, 1989), suggests that it may be advantageous to possess NO reductase activity.

This Chapter describes strains of *R. capsulatus* that possess a NO reductase and examines the characteristics of this activity. This Chapter also reports the identification of a derivative of *R. capsulatus* strain BK5 that possesses a NO_2^- reductase together with a NO reductase. Therefore, this isolate can be classified as a complete denitrifier. A preliminary attempt to identify the NO_2^- reductase enzyme as either a copper-type, or cytochrome cd_1 , enzyme is also discussed.

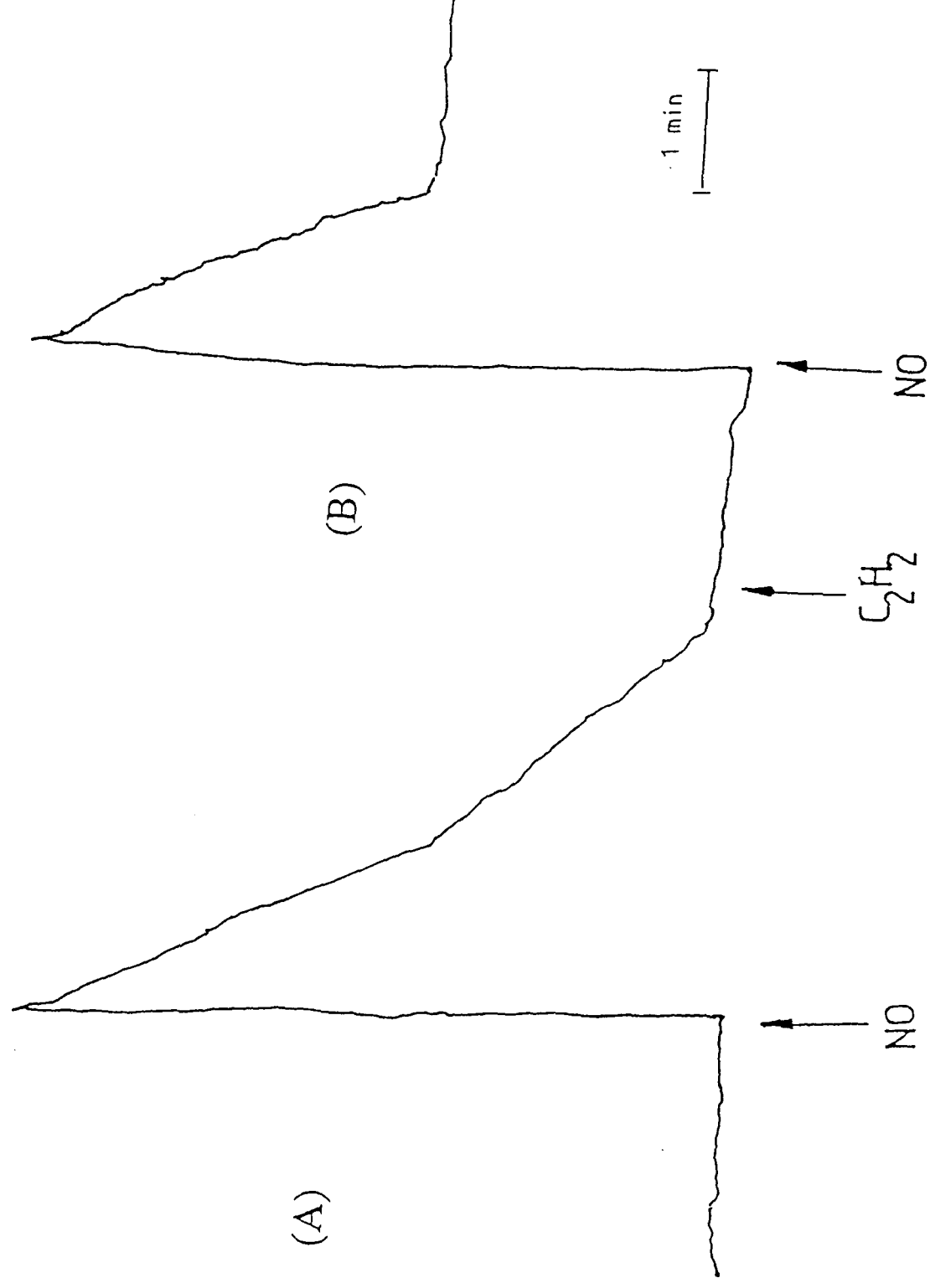
6.2. RESULTS.

6.2.1. The identification of nitric oxide reductase activity in cells of *R. capsulatus* strain MT1131.

As explained previously in Chapter 2, a silver cathode Clark-type electrode responds to both NO and N₂O with the former being detected at slightly higher sensitivity than the latter, whereas the gold cathode NO electrode is many times more sensitive to NO. Figure 6.1 shows the response of the N₂O electrode when NO was introduced to a reaction chamber containing cells of *R. capsulatus* strain MT1131 grown phototrophically on succinate, in the presence of N₂O. NO was clearly reduced to N₂O in this strain. The rate of NO reduction was linearly proportional to the amount of cellular protein present and reduction of NO was not observed in the absence of cells. The first phase of the response in record (A) corresponds to the reduction of NO to N₂O, the second phase to the reduction of N₂O to N₂, as evidenced by the prevention of the second phase by C₂H₂ in record (B). C₂H₂ specifically inhibits N₂O reductase in denitrifying bacteria (Balderson *et al.*, 1976). Comparison of record (A) with (B), Figure 6.1, demonstrates the rate of NO reduction was not attenuated by the presence of C₂H₂. From examination of the amplitudes of the second phases of the electrode response (a measure of the N₂O produced) in records 6.1A and 6.1B, it can be concluded that N₂O respiration does not occur to any significant extent, whilst any trace of NO remains. Once the NO

Figure 6.1. Reduction of nitric oxide by intact cells of *R. capsulatus* strain MT1131 measured with a silver cathode Clark-type electrode.

The reaction chamber contained a total volume of 2 ml: RCV medium; 50 mM sodium succinate, 5.5 mg cell protein, 4 U. glucose oxidase, 50 U. catalase and 16 mM D-glucose. Once the solution was anaerobic, (A) a nitric oxide addition was made to 75 μ M final concentration, (B) an acetylene solution was added to a concentration of 1 mM followed by the addition of 75 μ M nitric oxide. The electrode responds to both nitric and nitrous oxides (see text).



was consumed, the inhibition of N_2O respiration was immediately and completely reversed. This was deduced by comparison with cells that had previously reduced a pulse of added N_2O (not shown). Calibration of the electrode with a known concentration of N_2O demonstrated that NO reduction to N_2O was stoichiometric (i.e. reduction of two moles of NO gave one mole N_2O). The rate of NO reduction ($21 \text{ nmol. min}^{-1} \text{ mg of protein}^{-1}$) was similar to that obtained for N_2O reduction ($8 \text{ nmol. min}^{-1} \text{ mg of protein}^{-1}$). When allowance is made for the number of electrons in the reactions i.e. one for the reduction of NO to N_2O compared to two for the reduction of N_2O to N_2 it appears the rate of electron flow to the two acceptors is similar. Thus the inhibition of N_2O reduction by the presence of NO is unlikely to be due to a competition for electrons between the two acceptors, but rather a consequence of direct inhibition of the N_2O reductase. Cells of *R. capsulatus* MT1131 when grown in the absence of nitrogen-oxides to act as terminal electron acceptors were subsequently found to reduce NO at rates comparable to those found in cells of this strain grown in the presence of N_2O . However, N_2O was reduced at a much attenuated rate by such cells, consistent with previous observations that the N_2O reductase can be induced in this strain (Richardson *et al.*, 1989).

Figure 6.2 shows the electrode response obtained when NO was added to the chamber of a NO gold cathode (NO) Clark-type electrode containing cells of *R. capsulatus* strain MT1131. The record is dominated by the first phase, which corresponds to the reduction of NO, although detailed examination of the experiment reveals a second phase which corresponds to the rate of N_2O reduction, these observations corroborate with the findings in Figure 6.1. Similar rates of NO reduction were measured with both electrodes. The linear rate of NO reduction by

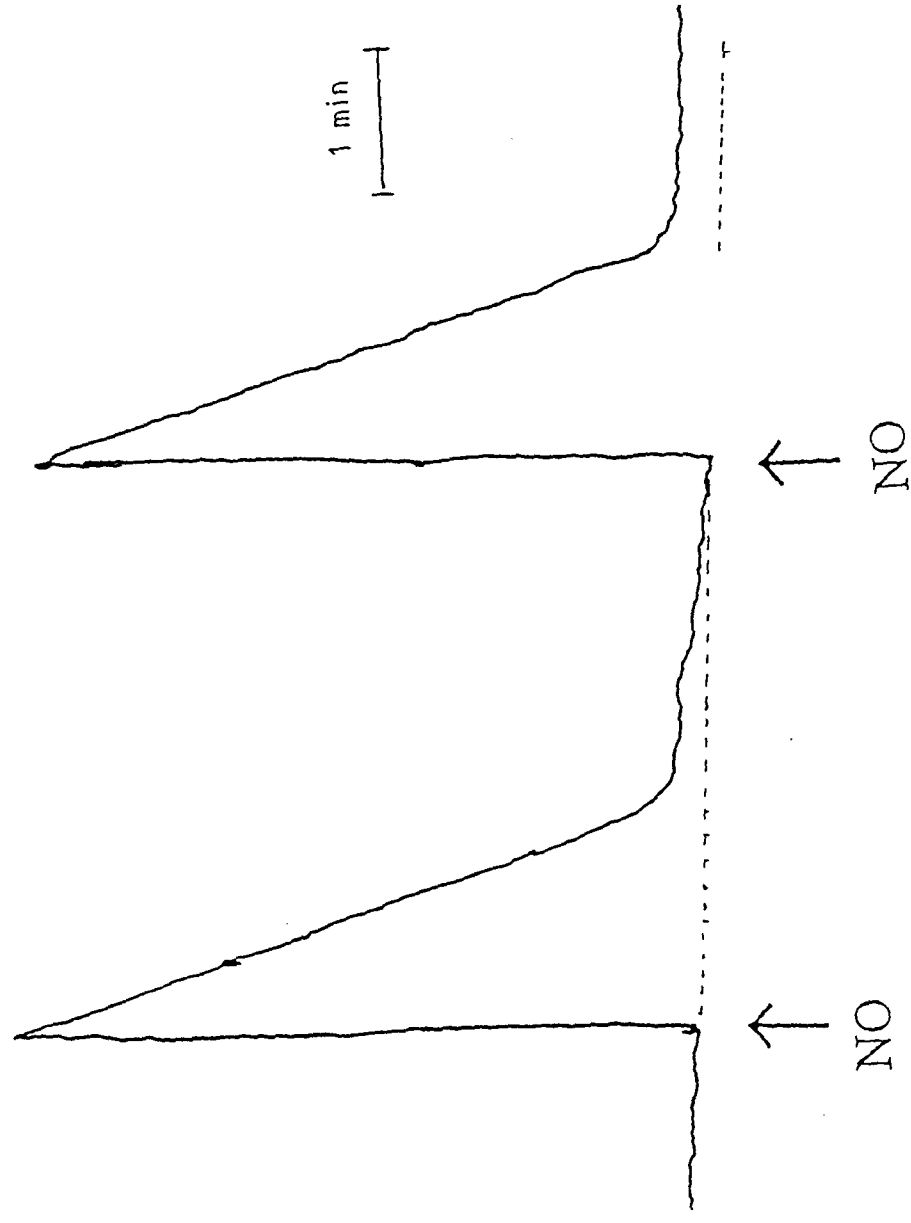


Figure 6.2. Record of the reduction of nitric oxide by cells of *R. capsulatus* strain MT1131 followed by a gold cathode Clark-type electrode.

The reaction chamber contained 2 ml of RCV medium, 50 mM sodium succinate, 5.1 mg cell protein, 4 U. glucose oxidase, 50 U. catalase and 16 mM D-glucose. Once the reaction medium was anaerobic, (A). nitric oxide was added to a final concentration of 75 μ M, (B). (A) was repeated in the presence of acetylene (1 mM).

The broken line represents the baseline of the record. Note the failure to return to this baseline after nitric oxide reduction, in the presence of acetylene, (see text).

cells of *R. capsulatus*, with succinate as a substrate, contrasts with the non-linear rates obtained with an identical concentration of NO and cells of *P. denitrificans* (Carr *et al.*, 1989). The latter non-linear rates have been explained as the direct inhibition of succinate dehydrogenase activity of this organism by NO. The linear rates seen with *R. capsulatus* may be a consequence of the low rate of electron flow to the NO reducing complex, in comparison with the high capacity of the succinate dehydrogenase enzyme and the cytochrome *bc₁* complex. Thus, even substantial inhibition by NO of the succinate dehydrogenase complex may remain undetected in cells of *R. capsulatus*. However, the possibility that the succinate dehydrogenases of these two bacteria may vary sufficiently to account for a difference in the sensitivity to NO cannot be ruled out. The linear rates also indicate that the K_m of cells for NO is less than 10 μ M.

6.2.2. The ubiquity of nitric oxide reductase activity in strains of *R. capsulatus*.

Following the identification of NO reductase activity in *R. capsulatus* strain MT1131 the ability of other strains of this species to reduce NO was investigated. *R. capsulatus* strains N22, N22DNAR⁺, BK5, AD2, 37b4 and Z-1 were grown phototrophically, in the absence of N₂O, as described in Chapter 2. These strains were assayed for NO reductase activity using the NO and N₂O electrodes. All these strains were found to reduce NO, and the specific rates of reduction determined relative to cell protein were similar (10-35 nmol. min⁻¹. mg protein⁻¹) in all strains. The electrode records obtained for a number of strains (N22, N22DNAR⁺, BK5, AD2 and Z-1) were analogous to those obtained for strain MT1131. For strain 37b4, the

response of the electrode was a single phase of reduction of NO to N₂O (as in Figure 6.1B) consistent with the absence of N₂O reductase in this strain. As no strain of *R. capsulatus* tested lacked the ability to reduce NO to N₂O, this activity appears to be ubiquitous in this organism.

All strains of *R. capsulatus* failed to grow phototrophically on RCV growth media which contained NO. This media was pre-sparged with argon before saturation with NO. It can be concluded that this failure of NO to support growth of cells of this organism is due to the toxicity of this gas, since the presence of an inorganic electron acceptor is unnecessary for support of phototrophic growth on a carbon source such as succinate.

6.2.3. Evidence for a discrete nitric oxide reducing enzyme in *R. capsulatus*.

The observation that cells of *R. capsulatus* reduce NO does not provide sufficient evidence to conclude that there is a discrete NO reductase. However, since all tested strains of *R. capsulatus* possessed NO reductase activity, this activity can not be attributed to NO₃⁻ or N₂O reductases, which are only present in some of these strains. Furthermore, in *R. capsulatus* N₂O and NO₃⁻ reductases are periplasmic enzymes (except for NO₃⁻ reductase in strain BK5) whereas the NO reductase activity is associated with the membrane (see section 6.2.5). There is a report of the non-specific reduction of NO by the mitochondrial cytochrome oxidase complex (Brudvig *et al.*, 1980). *R. capsulatus* strain M5 is completely deficient in both the cytochrome *b*₄₁₀ and *b*₂₆₀ oxidase activities. However, it retained a NO reduction rate that was

comparable to that of the parent strain Z-1. Similarly, a mutant MTCBC1 which lacked a functional cytochrome bc_1 complex retained the capacity to reduce NO (see section 6.2.8). In addition, aerobically grown *R. capsulatus* strains which have high oxidase activities, and possess a cytochrome bc_1 complex, did not consume NO. These results clearly rule out the possibility that the disappearance of NO observed in these experiments is associated with either the oxidases of the cell or the cytochrome bc_1 complex. Thus it seems probable that NO reduction is performed by a discrete reductase, which is expressed only under anaerobic conditions.

Cytochrome c' has previously been implicated as playing a physiological role in the binding of NO in cells of denitrifying bacteria, thus protecting the cell from the inhibitory effect of this gas (Yoshimura *et al.*, 1988). Conclusive evidence that the loss of NO from aqueous solutions is not simply a consequence of its binding to cytochrome c' of *R. capsulatus* was sought using the mutant H123, a derivative of strain 37b4, which lacks cytochrome c' . Mutant H123 reduced NO at a similar rate to the wild type strain, thus demonstrating that cytochrome c' is not responsible for the NO reductase activity observed.

Comparison of the rates of reduction of NO with N_2O , as mentioned above, demonstrated that the rates of reduction were similar. This acts as further support for the presence of a discrete NO reducing enzyme with physiological function in cells of *R. capsulatus*.

6.2.4. Evidence that the genes for expression of nitric oxide reductase are located on the chromosomal DNA in *R. capsulatus*.

Recent work (Willison, 1990) has indicated that *R. capsulatus* mutant 10a.2.3. (derived from strain AD2), which has been cured of an endogenous plasmid, no longer has the ability to utilize NO_3^- . Cultures of the plasmid-cured strain had lost both the assimilatory and dissimilatory NO_3^- reductases and failed to revert from this phenotype. An extrachromosomal location for these genes can partially explain the uneven, and sometimes unstable, distribution of these NO_3^- utilising capacities of *R. capsulatus* species (Czichos *et al.*, 1982). This raises the question as to whether other nitrogen-oxide reducing enzymes are also encoded on this, or another, endogenous plasmid. This plasmid-cured isolate was found to reduce both NO and N_2O at rates comparable to *R. capsulatus* strain AD2 which contains only the one endogenous plasmid. This observation indicates that the genes for these activities are located on the chromosomal DNA in strain AD2. Most strains of *R. capsulatus* contain one or more plasmids (Kerber *et al.*, 1981; Hu *et al.*, 1979). However, NO reductase activity was demonstrated in strain 37b4 which had been shown to lack endogenous plasmids (Richardson, Personal communication). Thus it seems probable that NO reductase is chromosomally encoded on all strains of *R. capsulatus*.

6.2.5. Nitric Oxide reductase is a membrane associated enzyme.

Fractionation of cells into periplasm, cytoplasm and membranes revealed that the NO reductase activity was associated with the membrane fraction. Figure 6.3 shows the reduction of NO upon the addition of membrane vesicles to an electrode chamber containing 100 μ M NADH. Addition of rotenone inhibited this NADH/NO oxidoreductase activity by more than 90 % (Figure 6.3). Rotenone inhibits the NADH dehydrogenase of this organism (Baccarini-Melandri *et al.*, 1973). Addition of succinate restored the rate of reduction, but this rate was inhibited by approximately 60 % in the presence of myxothiazol (Figure 6.3). The inhibition observed on addition of myxothiazol will be discussed in detail in section 6.2.7. The rate of NO reduction was fully restored in this experiment when DAD and isoascorbate were added to the vesicles (Figure 6.3). Thus NO reductase activity can be linked to NADH, succinate and the non-physiological electron donating couple DAD/isoascorbate, which can donate electrons to *c*-type cytochromes. The residual signal on the electrode record after NO reduction corresponded to the N₂O produced, since the latter cannot be reduced by membrane vesicles which lack N₂O reductase activity. As in cells, N₂O production from NO had the expected 1:2 stoichiometry.

NO reductase activity was not detected in the soluble fractions with any of the three electron donors that were effective with the membrane fraction. In contrast, over 95 % of the N₂O reductase activity was detected in the periplasm using MV⁺ as an electron donor. This result is in agreement with the established location for the N₂O reductase of *R. capsulatus* (McEwan *et al.*, 1985). MV²⁺ and dithionite

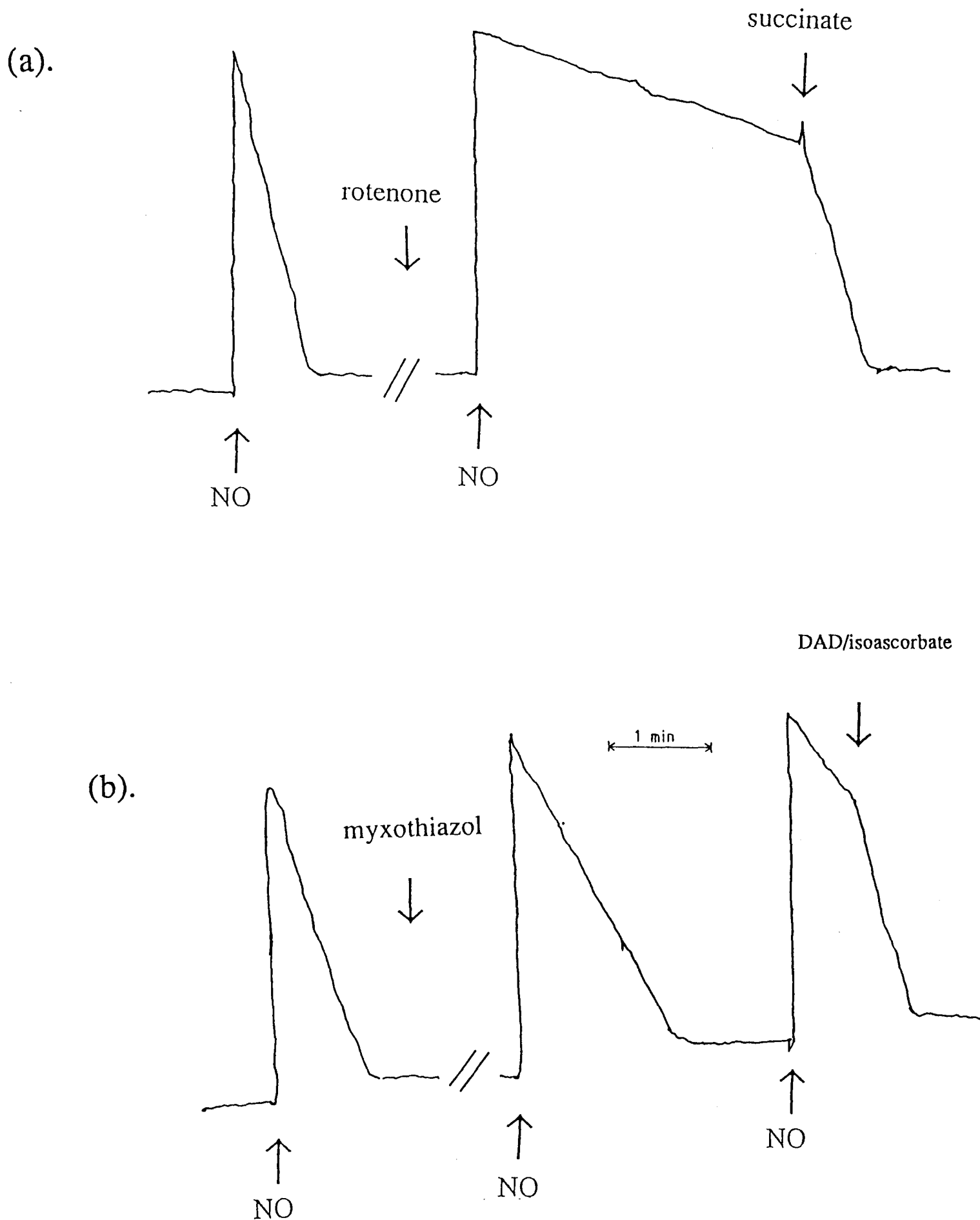


Figure 6.3. The effect of rotenone and myxothiazol on nitric oxide reduction by membrane vesicles of *R. capsulatus* strain MT1131.

(a) Initially 75 μ M nitric oxide was added to 2 ml anaerobic reaction medium (RCV medium without a carbon source, 4 U glucose oxidase, 50 U catalase, 16 mM D-glucose, 250 μ M NADH and 4 mg protein. Following the reduction of the nitric oxide, 25 μ M rotenone was added to the medium. After a 20 min. incubation period nitric oxide was added as before. Succinate was added as indicated to a final concentration of 1 mM.

(b) The contents of the reaction chamber are as in (a) (with succinate as the electron donor). Nitric oxide was added to a concentration of 75 μ M. On reduction of this nitric oxide, 3 μ M myxothiazol was added to the vesicle suspension. After 10 min. another two separate pulses of nitric oxide (75 μ M) were added to the suspension, DAD (20 μ M) and isoascorbate (150 μ M) were added as shown.

together reduce NO non-enzymatically and so were unsuitable for assaying NO reductase activity. Since the specific activity of membrane vesicles (40 nmol. min⁻¹. mg protein⁻¹) is greater than that of cells, and that it has been demonstrated that N₂O reductase, a periplasmic enzyme, is fully released on fractionation of cells this provides persuasive evidence that NO reductase activity is strongly associated with the membrane fraction.

6.2.6. Reduction of nitric oxide produces a proton electrochemical gradient in *R. capsulatus*.

NO-dependent proton translocation had previously been established in cells of several genera of denitrifying bacteria including *P. denitrificans*, *R. sphaeroides* f. sp. *denitrificans*, *Achromobacter cycloclastes* and *Rhizobium japonicum* (Shapleigh *et al.*, 1985 a; Garber and Hollocher, 1981; Garber *et al.*, 1982). *W. succinogenes*, a non-denitrifier, has also been shown to link its NO reducing enzyme to the respiratory chain (Shapleigh and Payne, 1985a).

Connection of NO reduction to energy conservation in *R. capsulatus* was established by the observation of a NO-dependent carotenoid band shift in cells and vesicles. This carotenoid band shift acts as an indicator of cytoplasmic membrane potential (Clark and Jackson, 1981; Ferguson *et al.*, 1987; McEwan *et al.*, 1985). The potential generated after a pulse of NO was smaller than that which was developed upon illumination of both cells and vesicles. Such differences between the

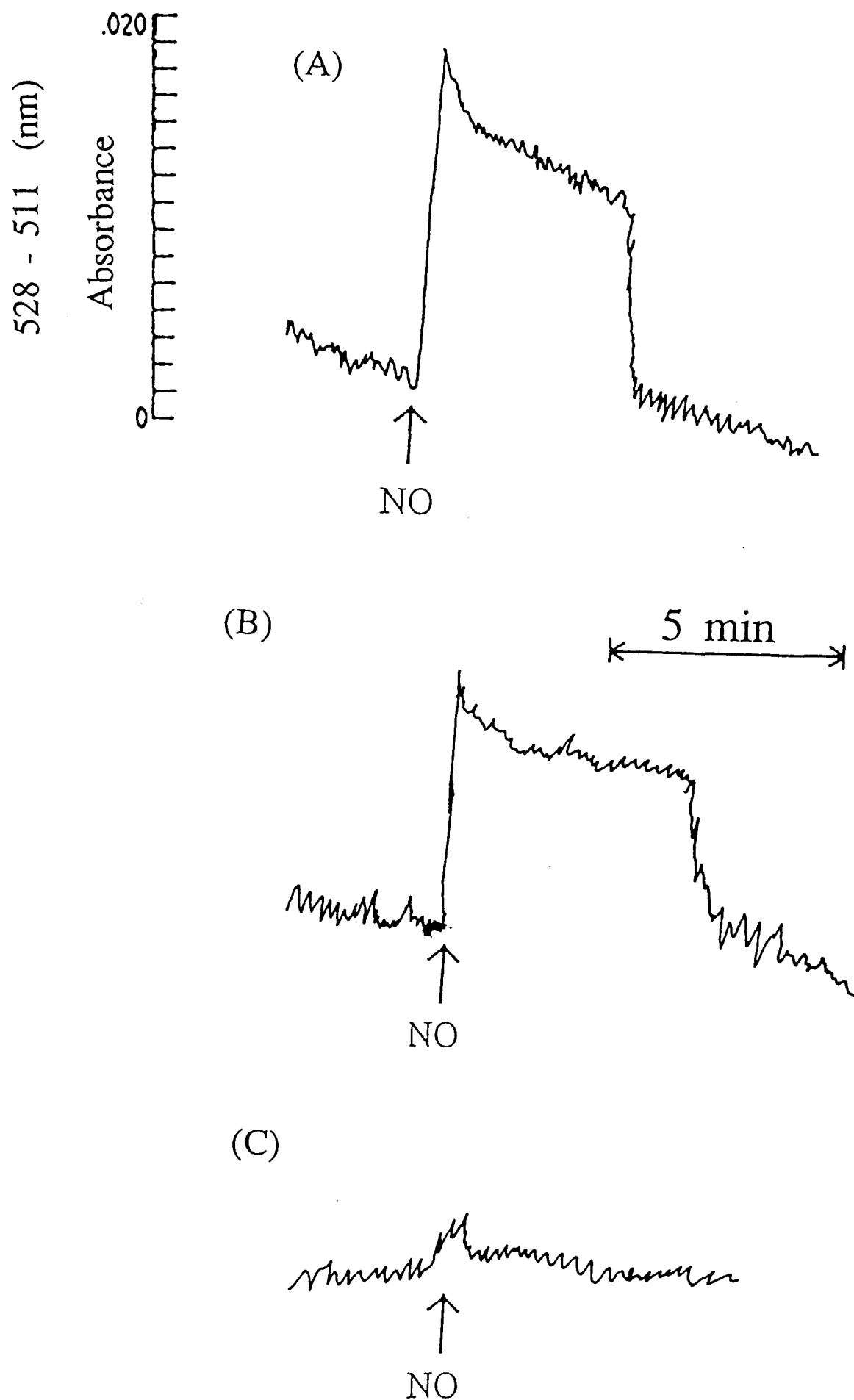


Figure 6.4. Electrochromic absorbance changes of the carotenoid pigments of membrane vesicles of *R. capsulatus* strain MT1131 following the addition of nitric oxide.

Membrane vesicles (0.4 mg protein) were resuspended in 1.5 ml 10 % sucrose, 50 mM KCl, 8 mM MgCl₂, 50 mM Tricine-KOH, pH 7.4, containing 4 U. catalase, 50 U. glucose oxidase and 16 mM D-glucose in a sealed cuvette. The following additions were made: (A) 50 μ M NADH (B) 1 mM succinate (C) DAD (20 μ M) sodium isoascorbate (100 μ M). Once the suspensions had become anaerobic (a steady baseline resulted) nitric oxide was added to a concentration of 10 μ M.

light-dependent and respiration dependent carotenoid shifts have been observed previously (Clark and Jackson, 1979; Ballard *et al.*, 1990).

Inverted membrane vesicles produce a NADH- and succinate-, but not isoascorbate/DAD-dependent membrane potential in response to the addition of NO (Figure 6.4), but not N₂O. All electrochromic changes were completely abolished by the presence of 5 μ M FCCP which collapses both the electrical and chemical components of the transmembrane proton electrochemical gradient.

6.2.7. The involvement of the cytochrome *bc*₁ complex in electron transport pathways to the nitric oxide reductase.

The pathway (or pathways) of electron flow to the NO reductase of *R. capsulatus* was (were) investigated, by first, studying the involvement of the cytochrome *bc*₁ complex in NO reduction. One approach taken was to investigate the effects of myxothiazol and HOQNO, which are known inhibitors the cytochrome *bc*₁ complex, on the reduction of NO.

Work by Myatt *et al.* (1987) demonstrated that the cytochrome *bc*₁ complex inhibitor, myxothiazol, attenuated the steady-state photosynthetic electron transport rate by 97 % in whole cells of *R. capsulatus* when present in a molar ratio of myxothiazol:reaction centre of 2:1. Thus myxothiazol was in 4-fold excess over the cytochrome *bc*₁ complex, since Prince *et al.*, (1986) reported the presence of two reaction centres per cytochrome *bc*₁ complex. The number of moles of cytochrome

bc_1 complex in a given experiment could be calculated by measuring the bacteriochlorophyll concentration, which could be related to the reaction centre content. The ratio of cytochrome bc_1 complex to bacteriochlorophyll of the cells used in the present work was approximately 1:200 (Richardson, 1988). Thus it was possible to calculate the amount of myxothiazol required to saturate the cytochrome bc_1 complex.

The finding that myxothiazol partially inhibited the reduction of NO in cells of strain MT1131 (Table 6.1), indicated that electron flow could proceed via the cytochrome bc_1 complex. NO reduction was restored to the uninhibited rate by the addition of isoascorbate and DAD, which introduces electrons after the Q_z site of myxothiazol inhibition within the cytochrome bc_1 complex. It is notable that, although the rates of NO and N_2O were always fully restored by isoascorbate and DAD to the original rates observed with physiological electron donors, these original rates were never exceeded (Table 6.1). Thus the site of electron donation by DAD, or a site after it, must represent a rate determining step in electron flow to these terminal reductases. It is furthermore noticeable that the inhibition by myxothiazol was never complete. Myxothiazol was similarly found to inhibit NO reduction only partially in the other strains of *R. capsulatus* tested. The extent of this inhibition varied, with the greatest percentage inhibition seen in strains with the greatest specific NO reductase activity. Similar observations have been made for the reduction of N_2O by cells of *R. capsulatus* (Richardson *et al.*, 1989). The observation of partial inhibition suggests that there exists a pathway for electrons that is independent of the myxothiazol-sensitive cytochrome bc_1 complex. The flux through this alternative pathway must be relatively low, and hence unable to support

Table 6.1.The effects of myxothiazol and HOQNO on nitric oxide reduction in *R. capsulatus* strain MT1131 and mutants MTG4/S4, MTCBC1 and MTGS18.

Reactions were performed in the nitric oxide and nitrous oxide electrodes. The cells were added to RCV medium (as described in Table 2.2c containing 30 mM succinate as the carbon source) supplemented with 4 U. glucose oxidase, 50 U. catalase and 16 mM D-glucose. In each case nitric oxide was added to a concentration of 75 μ M. Additions were made as indicated (A). no additions, (B). 3 μ M myxothiazol, (C). 3 μ M myxothiazol, 20 μ M DAD and 250 μ M sodium isoascorbate, (D). as in (B) plus 20 μ M HOQNO, (E) as in (C) plus 20 μ M HOQNO.

Nitric oxide reductase activity (% of maximum activity).					
Strain	Additions.				
	A.	B.	C.	D.	E.
MT1131	100	45	105	17	95
MTG4/S4	75	36	73	N.d.	N.d.
MTCBC1	60 ^a	N.a.	N.a.	5 ^a	55 ^a
MTGS18.	51 ^a	N.a.	N.a.	N.d.	N.d.

N.d. = not determined. N.a. = not applicable.

100 % = 20 nmol. min⁻¹. mg protein⁻¹.
^a100 % = 16 nmol. min⁻¹. mg protein⁻¹ (see text for growth conditions)

the full rate of electron flow diverted from the cytochrome bc_1 complex. Thus an inhibition of rate of NO reduction is seen when flow through the cytochrome bc_1 complex is blocked by the presence of myxothiazol. The lower percentage inhibition of rates seen in some batches of cells may reflect the lower activity of the terminal reductase (or perhaps some other component after cytochrome c_2 on route to the terminal enzyme), because under these circumstances the alternative pathway would cope with a higher fraction of the flux of electrons to the NO reductase.

This alternative pathway also leads to the generation of membrane potential in *R. capsulatus*, as evidenced by the carotenoid band shift observed when a pulse of NO was added to vesicles using succinate as a sole source of electrons in the presence of myxothiazol. The magnitude of this carotenoid shift was approximately 60-80 % of that observed in the absence of this inhibitor, depending on the vesicle preparation.

The effects of HOQNO on electron flux to NO reductase were investigated to elucidate further the pathways of electron transport to NO. The electron flow through the alternative cytochrome bc_1 complex to NO reductase was blocked by HOQNO, demonstrated by the inhibition (greater than 80 %) of NO reduction in cells of *R. capsulatus* strain MT1131 in the presence of both 3 μ M myxothiazol and 20 μ M HOQNO (per 60 μ M BChl) (see Table 6.1). The rate of NO reduction was fully restored by addition of DAD and isoascorbate (Table 6.1), thus demonstrating that HOQNO was not inhibiting the site of NO reduction. NO reduction remained unaffected by 20 μ M HOQNO in the absence of myxothiazol. The cytochrome bc_1 independent pathway of N_2O respiration has also been shown to be blocked by HOQNO (Richardson *et al.*, 1989). Spectroscopic studies suggest that a *b*-type

cytochrome may be involved in this alternative pathway to cytochrome c_2 (Richardson *et al.*, 1989). The effects of HOQNO on the alternative cytochrome bc_1 pathway to NO reductase suggests, but does not conclusively show, that a common component exists in the alternative cytochrome bc_1 electron transport pathways to NO and N_2O reductases.

6.2.8. Nitric oxide reduction in the mutant MTCBC1 of *R.capsulatus*.

Section 6.2.7 has provided evidence that the cytochrome bc_1 complex can be bypassed in the electron transport pathway to the NO reductase in *R. capsulatus*. More support for this conclusion came from studies of a mutant, MTCBC1, that lacked a functional cytochrome bc_1 complex. The MTCBC1 mutant is unable to grow under photosynthetic conditions (Daldal *et al.*, 1987), and thus rates of NO reduction could not be compared directly to photoheterotrophically grown wild type cells. Consequently, precultures of cells of both wild type strain MT1131 and mutant MTCBC1 were grown aerobically in the dark to late exponential phase. Then a 10 % inoculum was transferred to anaerobic RCV medium containing glucose, DMSO and N_2O and incubated in the dark. The doubling times under these conditions were estimated to be 5 h. for strain MT1131 and 10 h. for MTCBC1 and thus the cultures were incubated for 24 h. and 48 h. respectively. The mutant of *R. capsulatus*, MTCBC1, was competent in NO reduction (Table 6.1). The rate of NO reduction in this mutant was only attenuated 40 % relative to the parent strain when grown under these conditions.

Addition of 20 μM HOQNO to cells of *R. capsulatus* strain MTCBC1 led to approximately 80-90 % inhibition of the rate of NO reduction (Table 6.1). This result supports the observations made when HOQNO is added to wild type cells in which the cytochrome bc_1 complex was non-functional due to the presence of myxothiazol (Table 6.1).

6.2.9. The involvement of cytochrome c_2 in both the cytochrome bc_1 -dependent and -independent electron transport pathways to nitric oxide reductase.

The cytochrome bc_1 complex acts as a reductant for cytochrome c_2 in *R. capsulatus* (Crofts *et al.*, 1983). The involvement of cytochrome c_2 in electron transfer from the cytochrome bc_1 complex to the terminal enzyme in NO reduction was therefore anticipated and investigated. Cytochrome c_2 appears to be dispensable for NO reduction because a mutant, MTG4/S4, in which the gene for cytochrome c_2 has been specifically deleted using intersposon mutagenesis, was still able to reduce NO, albeit at a slower rate than the parent strain MT1131 (Table 6.1). In this strain the electrode record of the reduction of NO was monophasic, resembling the parent strain in the presence of C_2H_2 . This similarity arises because cytochrome c_2 has been found to be an obligatory component for N_2O respiration (see Chapters 1 and 7) and, as described earlier, N_2O reduction accounts for the second phase of the electrode response in cells of *R. capsulatus* reducing NO under dark conditions. The cytochrome bc_1 complex was still operative in the cytochrome c_2 -deficient mutant since myxothiazol was observed to give a partial inhibition in the rate of NO reduction (Table 6.1). Thus the cytochrome bc_1 complex can reduce an alternative

component to cytochrome c_2 and this component must also be able to accept electrons from the cytochrome bc_1 complex-independent pathway.

Addition of DAD and isoascorbate to cells of the mutant MTG4/S4 which were reducing NO, with succinate as an electron donor, did not produce a stimulation of rate. However, if this experiment was repeated in the presence of myxothiazol, the rate was restored to that observed in the absence of the inhibitor (Table 6.1). DAD may transfer electrons either to cytochrome c_1 , the redox component which acts as an alternative site to cytochrome c_2 in the reduction of NO, or to some site on the reducing side of this component. However, DAD has been shown not to donate electrons to the purified NO reductase of *P. denitrificans* (Carr and Ferguson 1990a). Electron transport from this unknown component to NO reductase, and not the reduction of this component, must represent the rate limiting step in the reduction of NO in cells which lack cytochrome c_2 since no acceleration of rate is observed on addition of DAD and isoascorbate to these cells. The mutant MTGS18, which lacks both a functional cytochrome bc_1 complex and cytochrome c_2 (Daldal *et al.*, 1987), was grown aerobically as described for mutant MTCBC1. This mutant reduced NO, although the rate was lessened to 51 % (but varied between 40-55 % in different cell batches) of that found in the wild type strain MT1131 grown under similar conditions. This observation of NO reduction in mutant MTGS18 confirmed the conclusion that electron transport to NO reductase could proceed independently of both the cytochrome bc_1 complex and cytochrome c_2 .

As discussed in section 6.2.3 the *R. capsulatus* mutant H123, which lacked cytochrome c' , reduced NO at a rate that was comparable to that of the parent strain

37b4. This provides firm evidence that cytochrome c' is not obligatory for NO reduction in *R. capsulatus*, although it does not conclusively rule out the possibility that cytochrome c' could be one of several c -type cytochromes involved in electron flow to NO reductase.

6.2.10. Isolation of a denitrifying strain of *R. capsulatus*.

The results presented so far in this Chapter have established that strains of *R. capsulatus* can reduce NO. Therefore, some strains of this species have the capacity for NO_3^- , NO and N_2O reduction. This raised the question as to whether there may be strains of *R. capsulatus* that can complete the entire series of denitrification reactions by reducing NO_3^- to N_2 gas. The reduction product of NO_3^- , from the respiratory NO_3^- reductase of this bacterium, is NO_2^- , and so the "missing link" in the ability of *R. capsulatus* to denitrify is in the reduction of NO_2^- . An enzyme which catalyses the reduction of NO_2^- to NO (or to N_2O) needs to ^{be} identified in a strain of this organism for it to be classified as a "complete" denitrifier.

NO_3^- facilitates the maintenance of cellular redox balance when strains of *R. capsulatus* which possess a respiratory NO_3^- reductase are grown phototrophically on a highly reduced carbon source such as butyrate (Richardson *et al.*, 1988). Higher levels of NO_3^- reductase activity have been measured from cells grown on highly reduced carbon sources than from cells grown on succinate or malate (Richardson *et al.*, 1990). The absorbance of the culture at 650 nm was used as a measure of

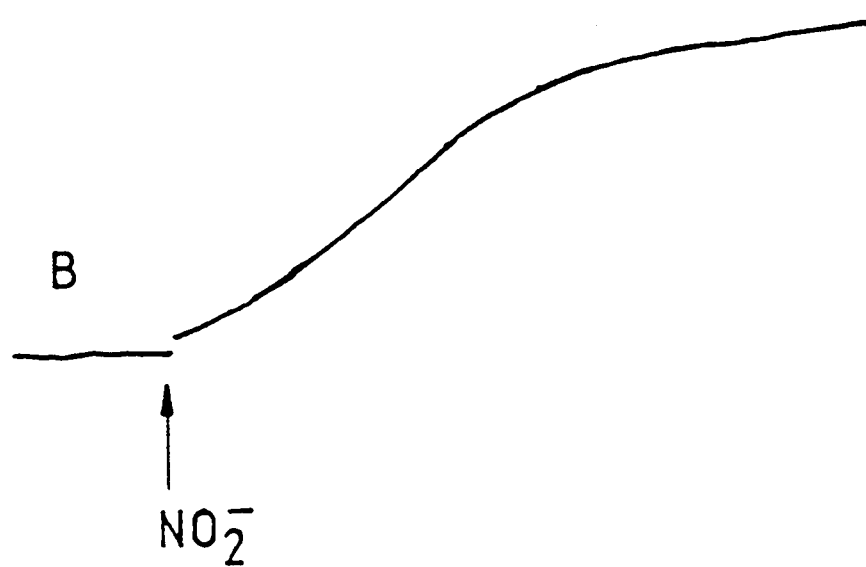
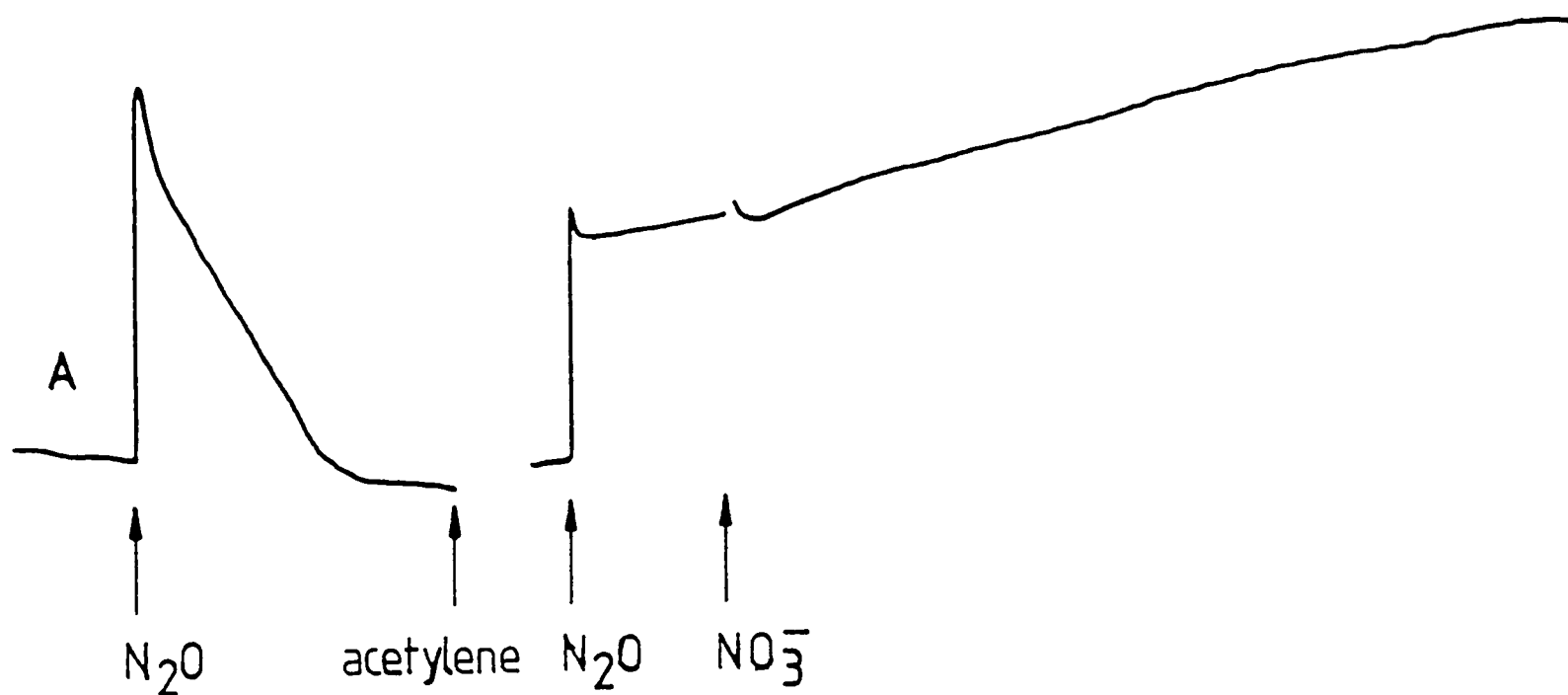
growth yield. When cells of *R. capsulatus* strain BK5 were grown phototrophically in the presence of NO_3^- , with butyrate as the sole carbon source, the absorbance of this culture (at 650 nm) was 0.8. NO_2^- accumulated in this culture until it reached a concentration of 5 mM, (when $\text{O.D.}_{650} = 0.8$), which proved toxic to the cells. When the concentration of NO_3^- in the growth medium was reduced to 1 mM, all of this NO_3^- was reduced to NO_2^- . This culture grew only to an $\text{O.D.}_{650} = 0.2$; thus the growth yield of the culture was limited by the availability of NO_3^- as an electron acceptor. Following repeated subculturing on this medium containing 1 mM NO_3^- , an increase in growth yield was observed (i.e. final $\text{O.D.}_{650} = 0.8$). In addition, extensive release of gas from this culture was apparent and no NO_2^- could be detected in the culture medium. These observations suggested that NO_3^- was being fully reduced to N_2 gas by a denitrifying derivative of strain BK5. Thus instead of only 1 mM NO_3^- being available as an electron sink to retain redox balance, the products of each reduction step (i.e. 1 mM NO_2^- , 1 mM NO and 0.5 mM N_2O) were available. This increase in the availability of oxidant accounts for the increase in growth yield observed with this new isolate in comparison with the parent strain BK5. To obtain a pure culture of this newly detected denitrifier, samples from the gassing culture were serially diluted and plated onto butyrate/ NO_3^- containing agar. These plates were incubated phototrophically until visible colonies appeared. The plates were then overlaid with 3-4 ml butyrate/ NO_3^- top agar and incubated as before. Bubbles, from the release of N_2 gas, were observed over colonies of the denitrifying strain but not the parent strain. This procedure provided a simple means for isolating single colonies of the gas producing isolate. This isolate will be termed *R. capsulatus* BK5DNIT (denitrificans). This isolate, when cultured phototrophically on butyrate/ NO_3^- , or succinate/ NO_3^- , did not accumulate NO_2^- and extensive gas

production was observed. Under similar conditions NO_3^- was reduced only to NO_2^- by the parent BK5 strain. *R. capsulatus* BK5DNIT grew in the dark with the evolution of gas on succinate, or butyrate, under anaerobic conditions with NO_3^- as the sole electron acceptor; NO_2^- did not accumulate during growth of this isolate under these conditions. In contrast, strain BK5 did not possess the capacity for anaerobic growth with NO_3^- as the terminal electron acceptor. The inability of strains of *R. capsulatus* to grow heterotrophically with NO_3^- as the sole oxidant has previously been reported (Klemme, 1979; Klemme *et al.*, 1980).

6.2.11. Measurement of denitrification reactions in *R. capsulatus* strain BK5DNIT.

If [~] bacterial species is to be classified as a "complete" denitrifier it must carry out the reduction of NO_3^- through to N_2 gas. Denitrifying species also possess the capacity to reduce the nitrogen-oxides, which are intermediates of denitrification, to N_2 by the same series of reactions.

Figure 6.5 shows a series of experiments in which respiration of the intermediates of denitrification were followed using the N_2O electrode. In the experiment shown in Figure 6.5A, a pulse of N_2O was added to the cells of *R. capsulatus* strain BK5DNIT. This N_2O was subsequently reduced, thus confirming the presence of N_2O reductase in these cells. Incubation of these cells with C_2H_2 resulted in a complete inhibition in the rate of N_2O reduction. On addition of NO_3^- to this cell suspension, N_2O was produced with the expected stoichiometry. A similar experiment, in the absence of C_2H_2 , N_2O was not detected upon addition of



2 min

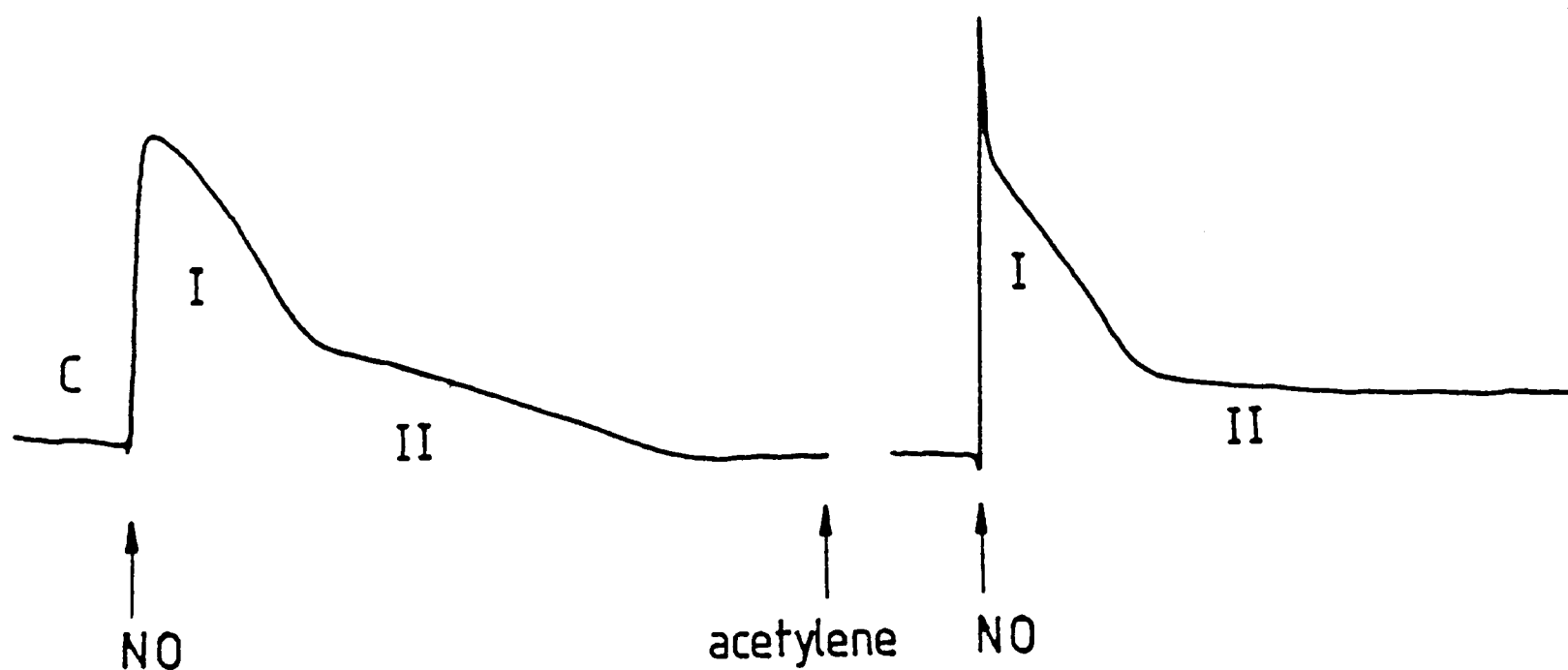


Figure 6.5. *R. capsulatus* strain BK5DNIT catalyses all the reactions of denitrification.

The formation of nitrous oxide was followed using a Clark-type silver cathode electrode : The electrode chamber contained 2 ml RCV medium, 50 mM butyrate, 16 mM glucose, 4 U. glucose oxidase and 50 U. catalase. Once the solution was anaerobic, further additions were made as follows: (A) Cells (5.0 mg protein) followed by a nitrous oxide (125 μ M), acetylene (1 mM) and a second pulse of nitrous oxide (80 μ M) as shown. Subsequently, potassium nitrate was added to 50 μ M. (B) As in (A) but Potassium nitrite (200 μ M) (not potassium nitrate) was added to the chamber. (C) Cells (3.0 mg protein) followed by nitric oxide (75 μ M), acetylene (1 mM) and nitric oxide (75 μ M) were added subsequently as shown.

NO_3^- . These observations provide strong evidence that *R. capsulatus* strain BK5DNIT catalyses the conversion of NO_3^- to N_2 . As predicted, on the basis of absence of a NO_2^- reductase, the production of N_2O from NO_3^- in the presence of C_2H_2 was not observed in strain BK5 (not shown). In a related experiment the addition of NO_2^- to cells of the isolate BK5DNIT, but not to cells of strain BK5, also resulted in the accumulation of N_2O when C_2H_2 was present (Figure 6.5B). The rate of N_2O accumulation was more rapid when NO_2^- , rather than NO_3^- was the oxidant (compare 6.1B with 6.1A). This indicates that NO_3^- reduction represented the rate determining step in the conversion of NO_3^- to N_2O . Figure 6.1C shows an experiment in which NO was added to a dark suspension of cells of BK5DNIT. The electrode record is essentially similar to that obtained for the parent strain BK5 (not shown) demonstrating the ability of both the original strain and the denitrifying strain to reduce NO to N_2O .

6.2.12. Properties of the nitrite reductase of *R. capsulatus* strain BK5DNIT.

A series of preliminary experiments were performed aimed at determining whether cells of BK5DNIT possess a cytochrome cd_1 , or copper-type, NO_2^- reductase. Both types of NO_2^- reductase produce NO as the product of NO_2^- respiration (see Chapter 1).

Work by Matsubara *et al.* (1982) reported the accumulation of N_2O in cultures of cells of *P. stutzeri* grown in copper-deficient medium. This is explained on the basis

that N_2O reductase is a copper-containing protein, and thus in the absence of copper an inactive apoprotein is synthesised (Matsubara *et al.*, 1982). A similar approach was employed to examine whether the lack of copper in the growth medium would impair synthesis of an active NO_2^- reductase, thus suggesting the possession of a copper-type NO_2^- reductase. To prepare a copper-deficient medium the procedure employed by Matsubara *et al.* (1982) was followed. Copper was omitted from the trace element solution for the growth medium, RCV. The RCV medium was prepared as a ten-fold concentrated stock. Contaminating traces of copper were removed by stirring this stock solution with 20 mg of 1,5-diphenylthiocarbazone (DDC) in 3 ml of CHCl_3 per l of medium. This medium was then extracted three times with 100 ml CHCl_3 per l of medium. The medium was then diluted ten-fold and autoclaved before use. As explained in section 6.2.10, NO_2^- is not observed in cultures of BK5DNIT growing phototrophically in the presence of NO_3^- . However, when cells were grown on a copper-deficient medium NO_2^- was detected. When the reductase activities of cells grown on the copper-deficient media were compared with cells grown on the standard media lower rates of NO_2^- and N_2O reduction, but not NO reduction were measured. The N_2O reductase of *R. capsulatus* is known to be a copper-containing enzyme (McEwan *et al.*, 1985), and so the decrease in the rate of N_2O reduction was an indication that the copper content of the medium was sufficiently low to limit its incorporation into cell protein. The decrease in the rate of NO_2^- reduction suggested that the NO_2^- reductase is a copper-containing protein. The lack of attenuation of the rate of NO reduction suggests that NO reductase from *R. capsulatus* is not a copper-containing protein.

Measurement of the expected decrease, in both the rate of N_2O reduction and the rate of accumulation of N_2O from NO_2^- in the presence of C_2H_2 , by the direct addition of DDC to cells within the N_2O electrode chamber could not be performed due to experimental limitations; the concentrations of DDC necessary for chelation of copper from proteins interfered with the sensitivity of the electrode to N_2O . A similar approach was employed^{by} Shapleigh *et al.*, (1985 b) who examined the products of NO_2^- respiration from cytochrome cd_1 , and copper-type, NO_2^- reductases using gas chromatography. They observed that DDC fully inhibited the copper-type NO_2^- reductase whereas the cytochrome cd_1 enzyme remained active. Thus providing a method to differentiate between the two enzymes.

6.2.13. Identification of nitrite reductase activity in fractionated periplasm from *R. capsulatus* strain BK5DNIT.

Cells of *R. capsulatus* strain BK5DNIT from a litre culture, which had been phototrophically grown with butyrate/ NO_3^- RCV medium, were fractionated into periplasm, cytoplasm and membranes as described (section 2.2.1). When these fractions were assayed for the presence of NO_2^- reductase activity, using PES and isoascorbate as an electron donor, the activity was located solely in the periplasmic fraction. The NO_2^- reductase activity was further purified using ion exchange chromatography. The periplasmic fraction was loaded onto DEAE-Sepharose column (height 30, diameter 1 cm) which had been pre-equilibrated with Tris-HCl, pH 8.0. The column was washed with 100 ml of the same buffer then eluted with a linear

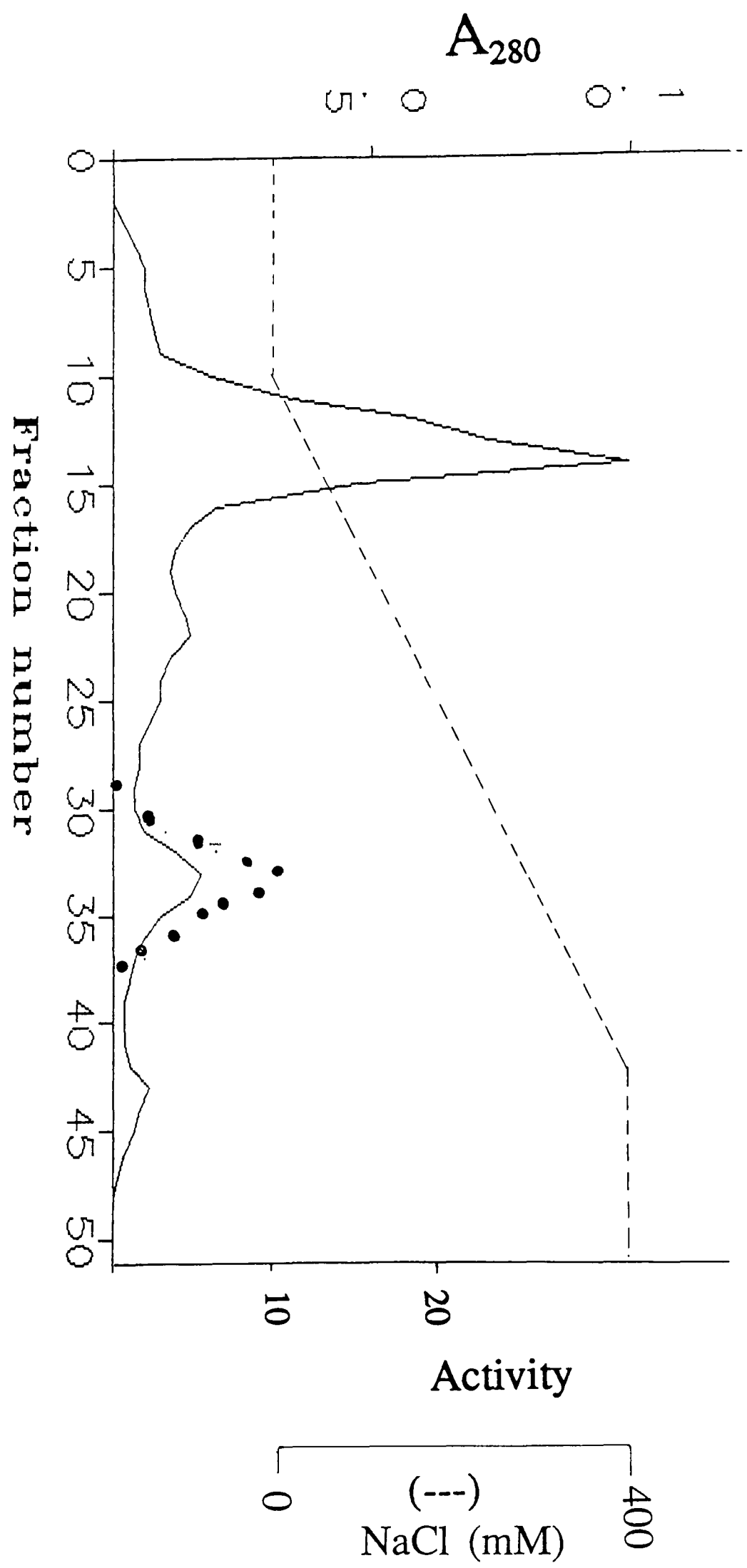


Figure 6.6. Elution profile of the nitrite reductase activity of *R. capsulatus* strain BK5DNIT from DEAE-Sepharose.

The periplasmic from a litre culture of *R. capsulatus* strain BK5DNIT was loaded onto a DEAE-Sepharose column (height 30 cm, diameter 1cm) which had been pre-equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with 100 ml of this buffer to remove unbound protein and then eluted with a 100 ml of a 0-400 mM gradient of NaCl. The column was run at 30 ml. H⁻¹ and 2 ml fractions were collected.

Activity (• • •) is expressed in nmol. min⁻¹.ml⁻¹
A₂₈₀ is represented by (—).

gradient of 0-400 mM NaCl. The fractions corresponding to the maxima of each peak were assayed for NO_2^- reductase activity. Activity was located in fraction 33 which corresponded to 280 mM NaCl (Figure 6.6). The corresponding side fractions were checked for activity so that an activity profile could be constructed (Figure 6.6). Fractions containing activity were pooled and further concentrated.

Absorption spectra of NO_2^- or ferricyanide oxidised minus dithionite reduced material were taken (not shown). These spectra failed to reveal the presence of a cytochrome in concentrated fractions from the ion-exchange column which contained NO_2^- reductase activity. Insufficient sample (0.2 mg) was present to determine the presence of a copper-type absorption. The copper NO_2^- reductase from *R. sphaeroides* f.sp. *denitrificans* has three absorbance bands at 463, 585 and 745 nm, the extinction coefficients of which were 3.7, 4.7 and 0.8 mM. cm^{-1} (Michalski and Nicholas, 1985). If the NO_2^- reductase of *R. capsulatus* BK5DNIT is similar, at least 1 mg of purified protein would be needed to detect the presence of these absorption bands. In contrast, the Soret band of cytochrome cd_1 has an extinction coefficient in the region of 150 mM. cm^{-1} in the oxidised form and 185 mM. cm^{-1} in the reduced form (Henry and Bessieres, 1984) and so should have been detected if present.

Protein staining bands of 40 kDa and 95 kDa (a minor band) were revealed when the fraction containing NO_2^- reductase activity was run on an SDS-PAGE gel in reducing buffer (Figure 6.7). This fraction and the periplasm were loaded into lanes on a gel in non-reducing buffer. One half of the gel was stained for haem and the other half stained with Coomassie-blue for protein. No haem staining band appeared in the partially purified fraction which contained NO_2^- reductase activity. The periplasm

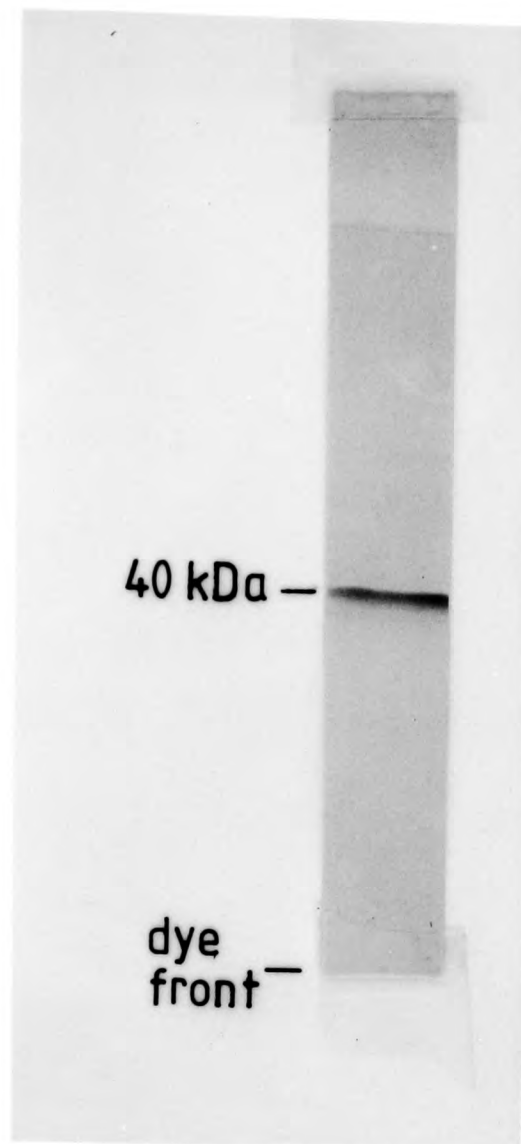


Figure 6.7. SDS-PAGE analysis of the nitrite reductase from *R. capsulatus* strain BK5DNIT.

The combined fractions (32-35, 20 μ g protein) from the DEAE-Sepharose column were analyzed on SDS-PAGE gel which was Coomassie stained for protein. The molecular weight of the sample polypeptides were calculated from the migration of molecular weight markers: β -galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000) and lysozyme (M_r 14,300), (not shown).

contained a haem staining band at approximately 12 kDa which corresponded to cytochrome c_2 , but no band was seen in a position corresponding to the NO_2^- reductase protein, assuming that the 40 and/or 95 kDa polypeptide is (or is part of) the enzyme.

Antibodies raised against the holoenzyme of cytochrome cd_1 from *P. denitrificans* did not cross-react with fractions containing NO_2^- reductase activity of *R. capsulatus* strain BK5DNIT. Samples were run in both reducing and non-reducing buffers. Controls with both periplasm, and the purified cytochrome cd_1 , from *P. denitrificans* when probed with these antibodies revealed a cross reacting band at 70 kDa. This shows that the NO_2^- reductase is immunologically distinct from the cytochrome cd_1 of *P. denitrificans*.

6.3. DISCUSSION.

This is the first report of NO reductase activity in *R. capsulatus*. NO reduction is known not to be restricted to bacteria carrying out all the reactions of denitrification. Its activity has also been reported in *W. succinogenes* (Payne *et al.*, 1982). The presence of a discrete NO reducing enzyme has been identified in several bacteria including the closely related *R. sphaeroides* f. sp. *denitrificans* and *P. denitrificans* (Itoh *et al.*, 1989a; Carr and Ferguson, 1990a). However, the ability, to reduce NO does not appear to ^{be} universal amongst bacteria grown anaerobically, even when NO_3^- is present as the sole electron acceptor. For example, no species of *Campylobacter* possessed the ability to reduce NO despite the possession of NO_3^- and N_2O reductase activities by some strains (Payne *et al.*, 1982). *Campylobacter* species, like related *W. succinogenes*, conserve nitrogen in an assimilable form by reducing NO_3^- , via NO_2^- , to NH_3 and reduce N_2O to N_2 .

The inability of *R. capsulatus* strains to grow in the presence of NO corresponds to results obtained with other bacteria. Only one bacterium, a non-fermenting *Bacillus* species has been reported to grow with NO present as the sole oxidant (Pichinoty *et al.*, 1979). In the case of *R. capsulatus*, no inorganic electron acceptor is necessary in cells growing phototrophically (with succinate or malate). Thus it is not the case that the reduction of NO and its product N_2O fails to supply enough energy to support growth, but that NO directly inhibits one or more cellular functions. This view, that failure to grow is not due to inadequate energy

conservation, is supported further by the ability of N_2O to support growth of *R. capsulatus* under dark conditions (McEwan *et al.*, 1985).

NO reductase activity was found in all strains of *R. capsulatus* tested, which contrasts to the more patchy distribution of the other nitrogen-oxide reducing enzymes. NO_3^- reductase is a periplasmic enzyme in all strains of *R. capsulatus* which possess the capacity for NO_3^- reduction, except strain BK5 (Ballard *et al.*, 1990). The genes for this periplasmic enzyme are located on a plasmid of $\text{Mr } 74 \times 10^6$ in *R. capsulatus* strain AD2 (Willison, 1990). Similarly the genes are expected to be plasmid encoded in other strains of *R. capsulatus*, although this requires further confirmation. In natural habitats endogenous plasmids can be lost or acquired, thus accounting for the ability of only some strains to be able to respire NO_3^- . Whether the genes for N_2O reductase in *R. capsulatus* are located on the bacterial chromosome or on endogenous plasmid remains to be determined. However, in the closely related organism, *P. denitrificans*, the genes for N_2O are located on the chromosomal DNA (unpublished observation of present author). N_2O reductase activity is found in most strains of *R. capsulatus*. Strain 37b4, however lacks this activity although the reason for this remains unclear. However, the occurrence of NO reductase activity in strain 37b4 and mutant 10a.2.3, which both lack plasmids, is strong evidence for a chromosomal location for the genes involved in NO reduction. Further work is necessary to confirm this, but as yet the genes for this enzyme have not been identified in any organism.

The widespread possession of NO reducing ability of *R. capsulatus* should be viewed in the light of the toxicity and abundance of NO. NO is a product of microbial nitrification and denitrification (Payne, 1981; Lipshultz *et al.*, 1981). In addition, it is formed during chemical processes such as the decomposition of NO_2^- in soils and the combustion of fossil fuels (Logan, 1983). NO has been shown to inhibit bacterial respiration; in particular the enzymes cytochrome *cd*₁ (Shapleigh *et al.*, 1987; Frunzke and Zumft, 1986), N_2O reductase (Frunzke and Zumft, 1986; Carr *et al.*, 1989) and bacterial oxidases (Kucera *et al.*, 1987) are inhibited. Section 6.2.1 demonstrates that N_2O reductase activity is also inhibited by NO in *R. capsulatus*. Thus the possession of a NO reductase would be advantageous, as it could act as a detoxification mechanism for the organism.

Cytochrome *c'* has been reported to play a physiological role in the binding of NO (Yoshimura *et al.*, 1988). It is postulated that this binding is part of the protective mechanism of the cell against the toxic effects of NO. It has been demonstrated here, by use of a mutant in which cytochrome *c'* is specifically lost, that the rate of reduction is not attenuated with respect to the rate observed in the parent strain. Thus cytochrome *c'* does not appear to be involved in NO reduction, although it could act as a trap to bind NO, until its reduction via the respiratory chain occurs. This role needs further elucidation. Cytochrome *c'* had been reported to be involved in electron transport to N_2O reductase (McEwan *et al.*, 1985), although more recent work has indicated that this is not the case (see Chapter 1). The result obtained with mutant H123 acts as strong evidence against cytochrome *c'* being involved in electron transport to NO.

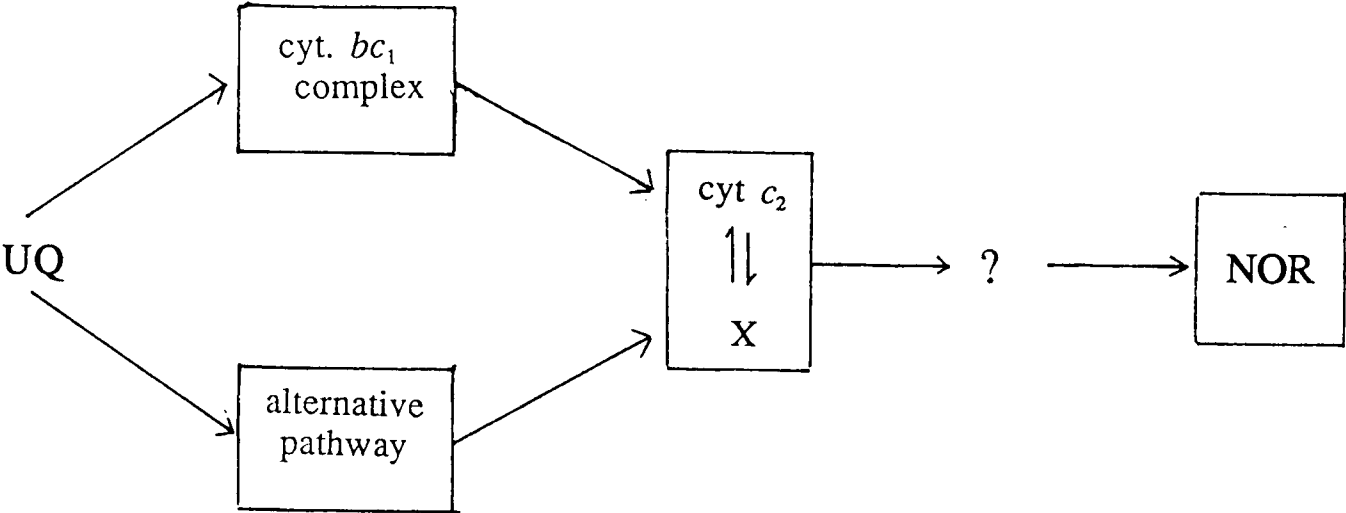
The importance of NO reduction appears to be reflected in the complexity of the electron transport pathways to the terminal reductase. Figure 6.8 summarises the pathways of electron flow to NO and N₂O reductases of *R. capsulatus*. Pathways of electron transport to N₂O, indicated in Figure 6.8B, were elucidated on the basis of studies with inhibitors and the mutants MTG4/S4 and MTCBC1 (Richardson *et al.*, 1989; Richardson *et al.*, 1990). From the results presented in this Chapter it seems probable that electrons can pass via the cytochrome *bc*₁ complex and cytochrome *c*₂ to NO reductase (Figure 6.8A). Involvement of the cytochrome *bc*₁ complex is implicated by the inhibition of NO reduction seen in the presence of myxothiazol. Cytochrome *c*₂ is implicated by the observation that a mutant lacking this cytochrome reduced NO at attenuated rates compared to the parent strain. Direct evidence from visible absorption spectroscopy for NO oxidising redox components of the cell proved impossible to collect owing to the non-specific binding of NO, which causes large changes in the spectrum, to whole cells. Since the inhibition of NO reduction by myxothiazol was never complete the presence of a cytochrome *bc*₁ independent pathway is suggested. This is confirmed by the finding that the mutant MTCBC1 can respire NO. It should be mentioned that there are a number of cytochromes with no identified function in *R. capsulatus*. An increased level of *b*-type cytochromes accompanied by a lower efficiency of coupling is observed in cells of *R. capsulatus* grown in high light intensity. This is thought to reflect the expression of a *b*-type cytochrome which passes electrons to cytochrome *c*₂ independently of the cytochrome *bc*₁ complex (Garcia *et al.*, 1987; Richardson *et al.*, 1989). The involvement of a *b*-type cytochrome in such a pathway in *R. sphaeroides* has also been suggested (Dutton and Prince, 1978).

Figure 6.8. The pathways of electron transport to nitric and nitrous oxide reductases in *R. capsulatus*.

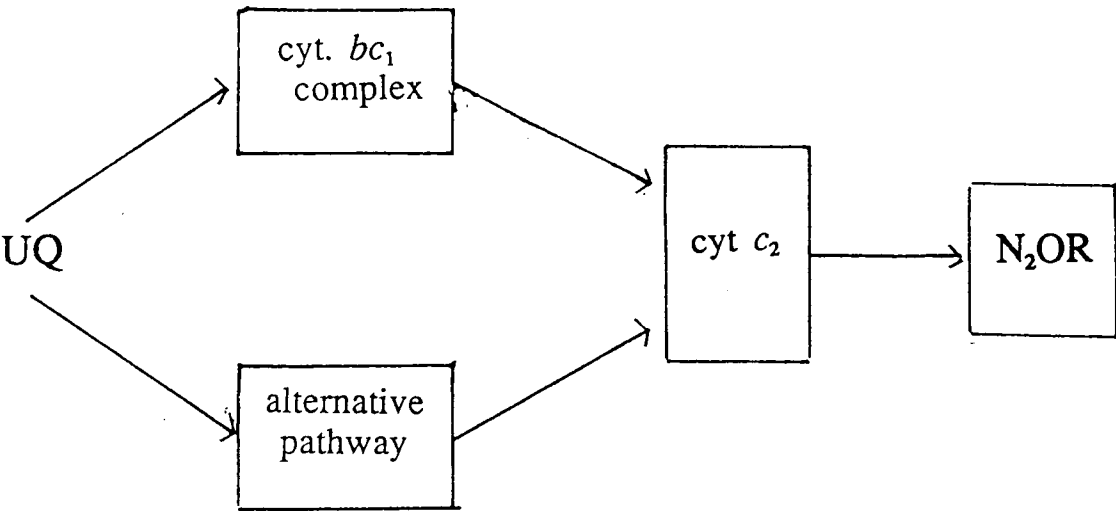
(A) Electron transport to nitric oxide reductase can either use or bypass both the cytochrome bc_1 complex and/or cytochrome c_2 .

(B) Electron transport to nitrous oxide reductase can use or bypass the cytochrome bc_1 complex. Cytochrome c_2 is essential for nitrous oxide reduction.

(A)



(B)



The reduction of NO by the cytochrome c_2 deficient mutant, MTG4/S4, suggests that another component, component X, can accept electrons from the electron transport chain and donate them to ^{the} terminal reductase of NO respiration (Figure 6.8A). Alternatively the properties of this mutant might imply that the cytochrome bc_1 complex can directly donate electrons to NO reductase. This appears less probable because it would require effective collisions between these integral membrane proteins. Whether component X is part of the NO reductase complex, or a physically separate component with perhaps more than one function, is unclear from the present work. Component X accepts electrons from both the cytochrome bc_1 complex and the alternative cytochrome bc_1 independent path (Figure 6.8A). Evidence for these pathways is provided from (a) the observation that NO reduction is partially sensitive to myxothiazol in cells of mutant MTG4/S4 which lacks cytochrome c_2 and (b) mutant MTGS18, which lacks both cytochrome c_2 and the cytochrome bc_1 complex, still reduces NO. Electron flow to the membrane bound NO reductase can proceed independently of cytochrome c_2 , which contrasts to the case observed for the periplasmic N_2O reductase. However, this observation is similar to findings that electron transport between the cytochrome bc_1 complex and the other membrane bound complexes; the reaction centre (Daldal *et al.*, 1986) and the cytochrome b_{410} oxidase (Daldal, 1988), can also proceed independently of this cytochrome. Thus it is postulated that cytochrome c_2 is obligatory only for electron transport to some periplasmic oxidoreductases and not to membrane bound complexes. Recent work has suggested that a previously unidentified membrane bound c -type cytochrome, cytochrome c_x is present in *R. capsulatus* strain MT1131 (and in mutants MTG4/S4 and MTGS18) which can act as an alternative electron donor than cytochrome c_2 to the reaction centre in this strain (Jones *et al.*, 1990). In

P. denitrificans, a membrane bound *c*-type cytochrome (22 kDa) is thought to be able to mediate electron flow between the cytochrome *bc*₁ complex and cytochrome *aa*₃ complex (Berry and Trumpower, 1985). A similar cytochrome has been identified in *R. capsulatus* (Hudig and Drews, 1983), although this component is probably not cytochrome *c*_x, since its redox potential is too low for this function (Jones *et al.*, 1990). These observations add to the evidence that small redox components are necessary for the transfer of electrons between the cytochrome *bc*₁ complex and other large integral membrane complexes.

Strong sequence similarity is observed between the cytochrome *c*₂ from *R. capsulatus* and the corresponding cytochrome *c*₂ and *c*₅₅₀ found in *R. sphaeroides* and *P. denitrificans* respectively (Timkovich and Dickerson, 1976). There are also many similarities in the organisation of the respiratory chains in these organisms (see Chapter 1). However, the obligatory role of cytochrome *c*₂, or cytochrome *c*₅₅₀, appears to vary between these organisms. Daldal *et al.* (1986) reported that phototrophic growth was possible in the absence of cytochrome *c*₂ in *R. capsulatus*. This contrasts to the work of Donohue *et al.* (1988) which provides clear genetic evidence that a *R. sphaeroides* strain devoid of cytochrome *c*₂ is unable to grow phototrophically. It was however possible to isolate a 'pseudorevertant' strain which was photosynthetically competent. These pseudorevertants contained a mutation at a regulatory site which allowed the synthesis of a cytochrome *c*₂-like protein which was not found in wild type cells (Fitch *et al.*, 1989). This cytochrome is able to substitute for cytochrome *c*₂ in photosynthetic electron transport. The cytochrome *c*₂ independent photosynthetic electron transport in *R. capsulatus* has been thought not to require, or accompany the overproduction of, another *c*-type cytochrome (Daldal *et*

al., 1986; Prince *et al.*, 1987). However, a recent report suggests that a membrane bound *c*-type cytochrome acts as an electron mediator between the cytochrome bc_1 complex and the reaction centre of this organism (Jones *et al.*, 1990). The difference in the absolute requirement for cytochrome c_2 cannot be attributed to the inherent properties of the cytochrome bc_1 complexes of *R. capsulatus* and *R. sphaeroides*. Since a plasmid encoding the genes required for a functional cytochrome bc_1 complex, the *pet* (or *fbc*) operon, of *R. sphaeroides* could restore photosynthetic growth in strains of *R. capsulatus* MTCBC1 (cyt bc_1^-) and MTGS18 (cyt bc_1^- , cyt c_2^-) (Davidson *et al.*, 1989). Recent studies with a mutant of *P. denitrificans*, which lacked cytochrome c_{550} , revealed no change in growth yield, maximum specific growth rate or in the duration of the lag phase during anaerobic growth when compared to wild type cells. Furthermore no accumulation of any of the intermediates of denitrification in the growth medium was observed (van Spanning *et al.*, 1990). Thus cytochrome c_{550} can also be discounted as being a obligatory cytochrome in the electron transport to NO in *P. denitrificans*.

Itoh *et al.* (1989a) showed that NO reductase activity in chromatophore membranes of *R. sphaeroides* f.sp. *denitrificans* decreased in the presence of cholate and deoxycholate. This was attributed to the loss of cytochrome c_2 . In this system reconstituted with purified cytochrome c_2 the rate of NO reduction was proportional to the concentration of cytochrome c_2 , and no reduction occurred in the absence of this cytochrome. This does not, however, provide evidence for an obligatory role of cytochrome c_2 in this bacterium, since another periplasmic component not present in the reconstituted system may also be able to transport electrons to NO reductase. This study does, however, provide evidence that direct electron transfer between the

cytochrome bc_1 complex and the site of NO reduction is not possible in this organism, at least. A cytochrome bc_1 independent path in *R. sphaeroides* f. sp. *denitrificans* also seems probable since only partial inhibition (around 60 %) was observed in vesicles of this bacterium in the presence of myxothiazol (Itoh *et al.*, 1989a). These observations further suggest that component X should be in the scheme donating electrons from the cytochrome bc_1 complex, and the alternative cytochrome bc_1 complex, to the NO reductase, as direct donation between these membrane bound complexes seems improbable.

The isolation of a strain of *R. capsulatus* BK5 able to reduce NO_2^- means that all the reactions of denitrification can occur within a single species of this bacterium. Previous work by Dr C.L. George (unpublished) with *R. capsulatus* strain N22DNAR⁺ had also identified the presence of a NO_2^- reducing enzyme as judged by the generation of membrane potential on addition of NO_2^- . However, the observation was not reproducible suggesting that the expression of NO_2^- reductase was not stable. It remains as yet uncertain why other strains of *R. capsulatus* lack this ability. Are the structural and regulatory genes for NO_2^- reduction present, but mutated either on the chromosomal or the plasmid DNA ? A plasmid location for these genes is perhaps likely in view of extra-chromosomal location of the genes for the periplasmic NO_3^- reductase in this organism. The location of these genes on a plasmid may perhaps account for the irreproducibility seen in the possession of NO_2^- reductase activity previously seen. The observation that strains of *R. capsulatus* may gain the ability to reduce NO_3^- is known. The isolation of strain N22DNAR⁺ able to reduce NO_3^- from a non- NO_3^- reducing strain N22 has been reported (McEwan *et al.*, 1982). The mechanism for the gain of this function has not been elucidated. However, the

NO_3^- reductase of strain BK5 is a membrane bound enzyme, and the structural genes are found on a 20 kb EcoR1 fragment of chromosomal DNA (Richardson, unpublished result). Thus this strain bears more similarity to *P. denitrificans* or *R.sphaeroides* f.sp. *denitrificans* (which also express a membrane bound NO_3^- reductase) than to other strains of *R. capsulatus* which express only a periplasmic NO_3^- reducing enzyme. The identification of a strain able to denitrify may be the result of an acquisition new genetic material. Genetic exchange between bacteria populations can occur by a number of mechanisms including transformation, transduction and conjugation (Clarke, 1981). Genes contained on plasmids can recombine with homologous regions of DNA, or the process can occur at random if the DNA is flanked with insertion sequences (Clarke, 1981).

There are a number of advantages in the possession of a NO_2^- reductase by *R. capsulatus* strain BK5 which reduces NO_3^- . These advantages include increased growth yields and the removal of toxic NO_2^- from cultures. On consideration of these advantages, it is somewhat surprising that some strains of *R. capsulatus* lack the ability to reduce NO_2^- , whilst they possess the ability to reduce the other nitrogen-oxides involved in denitrification. These strains of *R. capsulatus* have either lost the gene for NO_2^- reduction or have acquired only some of the genes required for the entire series of denitrification reactions to occur. Perhaps this is a result of evolving as a photosynthetic bacteria and using NO_3^- and N_2O reduction simply as a means of retaining redox poise whereas *P. denitrificans* has evolved as a non-photosynthetic denitrifier.

The distribution of the two types of NO_2^- reductase (copper-type or cytochrome cd_1) does not appear to be generic (see section 1.3.2). The results presented in this Thesis suggest *R. capsulatus* strain BK5DNIT contains the copper-type NO_2^- reductase. This point could be clarified by further large scale purification of this enzyme and spectral characterisation.

This Chapter has clearly established the ability of *R. capsulatus* to reduce NO. Several lines of evidence suggest that this reduction is possible as the result of the expression of a discrete NO reductase. Electron transport to this enzyme involves the cytochrome bc_1 complex and cytochrome c_2 , although neither of these components are obligatory for NO reduction. The isolation of a denitrifying strain has established that NO_2^- reductase activity can be stably expressed in *R. capsulatus*.

Chapter 7 General conclusions

The work presented in this Thesis has clearly established that *T. pantotropha* can carry out the reactions of denitrification under aerobic conditions. It seems probable that the enzymes of denitrification, or the electron transport pathways to these enzymes, must differ in some respect in this organism from those in organisms that do not denitrify aerobically. The first step of this aerobic denitrification process has been shown to be catalysed by a periplasmic NO_3^- reductase. The expression of such a reductase clearly provides a mechanism by which the problem by the inhibition of NO_3^- transport across the cytoplasmic membrane under aerobic conditions can be overcome (see Chapters 1 and 4).

It is not only the reduction of NO_3^- in *T. pantotropha* but also that of NO_2^- , NO and N_2O that needs to be understood. Some progress has been made in the present work. Chapter 5 reports the presence of cytochrome cd_1 in the periplasm of anaerobically grown cells of the *T. pantotropha* mutant, M6. Cytochrome cd_1 was also present in anaerobically grown cells of the wild type organism. These observations contrast to the report of Robertson (1988) in which cytochrome cd_1 was not detected in the soluble fractions of aerobically grown cells of this organism although it possessed a NO_2^- reductase activity. DDC, an inhibitor of the copper-type but not the cytochrome cd_1 , NO_2^- reductase (Shapleigh and Payne, 1985b), was added to whole cells of both *T. pantotropha* and *P. denitrificans* that were reducing NO_3^- to a "gaseous product" (Robertson, 1988). "Gas" production by cells of *P. denitrificans*, which express only a cytochrome cd_1 -type NO_2^- , was unaffected by DDC. However, gas production from cells of *T. pantotropha* was impaired. These observations were taken to suggest that ^{the} latter organism possesses a copper-type NO_2^-

reductase (Robertson, 1988). However, the copper-type enzyme was not identified and to date no organism has been found to possess the cytochrome *cd₁* and the copper-type NO_2^- reductase (Coyne *et al.*, 1989). Future work must determine whether the two types of enzyme are present in *T. pantotropha*, and under what conditions the NO_2^- reductase(s) is (are) expressed. It is tempting to speculate that cytochrome *cd₁* and the copper-type enzyme are expressed under anaerobic and aerobic growth conditions, respectively, and that a common mechanism controlling expression of the "aerobic and anaerobic" NO_2^- and NO_3^- reductases is operative.

It would be surprising from an energetic viewpoint if *T. pantotropha* expressed two NO_2^- reductases. The rationale behind the expression of two NO_3^- reductases is more easily understood, ie. the membrane bound NO_3^- is probably more energy conserving than its periplasmic counterpart, but can not reduce NO_3^- under aerobic conditions for reasons of its cellular location. It is not so clear either why the copper-type NO_2^- reductase would be able to function aerobically or why two reductases should be synthesised. No energetic advantage would be expected if both reductases accept electrons from the same branch point of the respiratory chain. The answers to these questions may be found from a detailed study of the electron transport chain of this organism.

The N_2O reductase of *T. pantotropha* is of considerable interest as there is activity, at least in whole cells, in the presence of O_2 . This contrasts to previous reports of the lability towards O_2 of this enzyme in most other organisms (see Chapters 1 and 3). Ironically, *W. succinogenes*, an obligate anaerobe, has been shown to possess a N_2O reductase that is not O_2 sensitive in cell-free extracts

(Teraguchi and Hollocher, 1988). An associated *c*-type cytochrome replaces one of the copper centres, which is commonly found in the N_2O reductases of other organisms, in purified C_2H_2 -insensitive N_2O reductase of *W. succinogenes*. Purification of the C_2H_2 sensitive N_2O reductase from *T. pantotropha* will reveal whether this enzyme or its metal centres differ from those found in other organisms. Preliminary studies in collaboration with Dr D. J. Richardson indicate that the N_2O reductase gene from *P. denitrificans* hybridises with a 5 kb *Eco*R1 fragment of chromosomal DNA from *T. pantotropha*. This result suggests that there is at least some degree of homology between the enzymes of these organisms. In light of the evidence for the expression of two NO_3^- reductases, and perhaps also two NO_2^- reductases, the possibility that *T. pantotropha* expresses two N_2O reductases must also be considered.

The molecular basis behind the control of expression of the two NO_3^- reductases needs to be investigated. Chapter 5 reports the isolation of a class of mutants (class I) which have a defect in the synthesis of the membrane bound NO_3^- reductase. Curiously, these mutants express elevated levels of the periplasmic NO_3^- reductase under anaerobic conditions. The mechanism of this control is not understood. A possibility is that the synthesis of the periplasmic enzyme is somehow repressed under anaerobic conditions due to the limited availability of the molybdenum cofactor. Thus the inability of mutants to synthesise the membrane bound NO_3^- reductase may alleviate the limitation of cofactor availability. A second possibility is that a component of the membrane bound NO_3^- reductase serves to inhibit the expression of the periplasmic enzyme (see section 5.3). It would be interesting to examine whether the expression of the membrane bound NO_3^- reductase under aerobic

conditions would repress synthesis of the periplasmic enzyme. Further investigation is clearly necessary to investigate these possibilities. It has been established that the structural genes for the membrane bound NO_3^- reductase of *E. coli* hybridizes against the chromosomal DNA from *T. pantotropha* (D. Richardson, personal communication). This probe could, in future work, be used as a probe to clone the structural genes of the membrane bound NO_3^- reductase. These clones could be restriction mapped and the boundaries of the structural genes identified. Antibiotic resistant cartridges could be subcloned into the structural genes and the regions upstream of this DNA. These genes would then be re-introduced to the wild-type organism on a suicide vector. In this way, the effect of an insertion in each gene could be studied with respect to its effect on expression of the two reductases.

Conversely, it would be interesting to see what effect, if any, the failure to express the periplasmic enzyme would have on the membrane bound enzyme. Future work should continue to seek a procedure for the isolation of a transposon mutant of *T. pantotropha* which lacks the periplasmic NO_3^- reductase but retains the membrane bound reductase. If the genes for the periplasmic enzyme are located on a plasmid as suggested in Chapter 5, curing *T. pantotropha* of its plasmids would also create a mutant of the desired phenotype. However, mutants obtained in these ways may also differ in their capacities for NO_2^- , NO and N_2O reduction. *A. eutrophus*, another organism that has been reported to denitrify in the presence of O_2 , has also been reported to express a soluble NO_3^- reductase (Wornecke and Friedrich, 1990). It seems probable in view of the results presented in Chapter 4 of this Thesis, that the latter NO_3^- reductase is also a periplasmic enzyme. The soluble NO_3^- , NO_2^- and N_2O reductases of *A. eutrophus* are plasmid encoded (Wornecke and Friedrich, 1990), and

therefore NO_3^- reductase activity may not be the only enzyme of denitrification lost, if the plasmids are cured from *T. pantotropha*. It would be interesting to examine in future work whether other bacteria which have been reported to be aerobic denitrifiers also express this periplasmic NO_3^- reductase. If the periplasmic NO_3^- reductase is plasmid encoded this raises the possibility that the plasmid could be transferred to organisms such as *P. denitrificans*. This approach could be used to establish whether the capacity for aerobic nitrate reduction (or even aerobic denitrification) could be gained by the simple transfer of an endogenous plasmid.

The fact that NO is an intermediate in the reactions of aerobic denitrification is a novel observation. The reaction of NO with O_2 must be slow at the physiological concentrations encountered during denitrification. It would, however, be of interest to study the aqueous chemistry of NO, because NO has over the recent years been found not only within the denitrification process but also in mammalian cells, e.g. as an endothelial relaxing factor (Palmer *et al.*, 1987). One approach towards solving this problem might be to use an electrode sensitive to NO with a rapid response time.

In view of both the production of NO from nitrification and denitrification reactions and the toxicity of this gas it is perhaps not surprising that *R. capsulatus* should possess the capacity for its removal. Interestingly, it has been established that *R. capsulatus* retains the capacity to reduce NO despite the loss of either, or both, of the cytochrome bc_1 complex and cytochrome c_2 . A complex which is known to function as a ubiquinol cytochrome c oxidoreductase is known to be present alongside the cytochrome bc_1 complex in cells of *R. capsulatus* (Richardson *et al.*,

1989). Recently, Jones *et al.* (1990) have identified a novel *c*-type cytochrome which is associated with the membranes of *R. capsulatus*. This cytochrome is thought to act as substitute for cytochrome c_2 and transfer electrons between ubiquinol and the reaction centre. It is possible that this cytochrome also plays a role in the transfer of electrons to NO in this organism. Except for the case of N_2O reduction in *R. capsulatus*, cytochrome c_2 does not appear to be an obligatory component for other electron transport reactions in this organism. The corresponding cytochrome, cytochrome c_{550} , in the closely related organism, *P. denitrificans*, appears to be non-obligatory in the electron transport pathways of denitrification and electron transport to N_2O can proceed in its absence (van Spanning *et al.*, 1990). The only evidence that cytochrome c_{550} can be involved in the electron transport pathway to N_2O reductase is from spectroscopy. Addition of N_2O to periplasmic fractions of *P. denitrificans* results in the C_2H_2 -sensitive oxidation of cytochrome c_{550} (established by the present author but not presented in this Thesis). Experiments performed in collaboration with Dr A. G. McEwan and Dr D. J. Richardson (not included in this Thesis) have shown that the addition of purified cytochrome c_2 to periplasmic fraction of *R. capsulatus* strain MT1131, results in a N_2O -dependent oxidation of cytochrome c_2 . This reaction is a second order process and thus gives the first evidence that cytochrome c_2 is the direct donor to the N_2O reductase of this organism. Presumably there is an electron transport protein in *P. denitrificans* that can substitute for cytochrome c_{550} .

It can now be seen that some strains of *R. capsulatus* fail to denitrify only because they lack a functional NO_2^- reductase. In the present work an isolate of strain BK5, BK5DNIT, was shown to possess a NO_2^- reductase activity. Thus this

isolate possessed the capacity to denitrify. It is not understood why the genes for the NO_2^- reductase are expressed in this isolate but not in the wild type organism. This observation, however, is not unusual. *R. capsulatus* strain N22DNAR⁺, but not its parent strain N22, expresses a NO_3^- reductase (McEwan *et al.*, 1982). As discussed in Chapter 6, the possibility that these genes are present in the wild type organisms requires investigation. This possibility could be examined by probing the chromosomal DNA of these organisms with the genes for these reductases.

In general terms a great deal of information has been gained in recent years about the enzymology of denitrification. In contrast, studies are only just beginning to investigate the complexities of the metabolic and genetic controls of such systems, some of which have been the focus of the present work.

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