Paracoccus denitrificans oxidative phosphorylation, retentions, gains, losses, and lessons *en route* to mitochondria.

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Abstract

There are many similarities between the oxidative phosphorylation apparatus of mitochondria and those found in the cytoplasmic membranes of alpha-proteobacteria, exemplified by *Paracocus denitrificans.*These similarities are reviewed here alongside consideration of the differences between mitochondrial and bacterial counterparts, as well as the loss from the modern mitochondria of many of the bacterial respiratory proteins. The assembly of *c*-type cytochromes is of particular evolutionary interest as the post-translational apparatus used in the alpha-proteobacteria is found in plants**,** and for example in eukyarotic species including algae of various kinds together with jacobids, but has been superseded by different systems in mitochondria of metazoans and trypanosomatids. All mitochondrial cytochromes *c* have the N-terminal sequence feature that is recognized by the metazoan system whereas the bacterial counterparts do not, suggesting that the loss of the bacterial system from eukaryotes occurred in the context of an already present recognition sequence in the eukaryotic cytochromes. Interestingly, in the case of cytochromes c_1 the putative recognition features for the metazoans appear to be substantially present in the bacterial proteins. The ability to prepare from *P. denitrificans* inverted membrane vesicles with classic respiratory control presents a

valuable system from which to draw lessons concerning the long debated topic of what controls the rates of respiration and ATP synthesis in mitochondria.

Introduction

It was the John and Whatley (1975) paper in Nature that really put *Paracoccus denitrificans* on the map, by bringing together the then accumulating information on the aerobic respiratory chain and membrane phospholipid composition. On this basis they argued *P. denitrificans* had in this respect the most features in common with the modern-day mammalian mitochondrion of any then characterised bacterium. An expanded version of their initial paper was subsequently published (John and Whatley 1977). We now appreciate that most, if not all, of these features are shared with other members of the alpha-proteobacteria, and that DNA sequence information has provided more points of comparison, revealing both further confirmation of the similarities but also things that are different.

A variety of lesser known eukaryotes are discussed herein. Their phylogenetic relationships, particularly in respect of c-type cytochrome biogenesis, are provided by Allen et al (2008). The relationship between mitochondrial DNA genomes and those of alpha proteobacteria are summarised by Roger at al (2017), their Fig 2 in particular**.**

The aerobic respiratory chain

It was the core respiratory chain that attracted the attention of John and Whatley (1975) and we now know that Complexes I (NADH-ubiquinone oxidoreductase, III (ubiquinol- cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase) contain essentially the same redox groups in *P. denitrificans* and mitochondria, while the

striking difference is that the bacterial counterparts have fewer subunits, for unknown reasons. Often called the supernumerary subunits, both *P. denitrificans and Rhodobacter sphaeroides /R.capsulata* have three such proteins associated with complex I, subunits that are absent from many other bacteria, e.g. *Escherichia coli* (Yip et al (2011). Strikingly, no doubt originating with the publicity given to *P. denitrificans* by John and Whatley (1975), *P. denitrificans* cytochrome c oxidase (cytochrome aa_3 oxidase) was only the second type of integral membrane protein to have its high resolution structure determined, remarkably published the same week as that for the bovine heart mitochondrial enzyme – a dead heat (Iwata et al 1995; Tsukihara et al, 1995).

Other mitochondrial-type components seen in *P. denitrificans* are the electron transfer flavoprotein-ubiquinone (ETF-UQ) oxidoreductase and the periplasmic sulphite oxidase. The former is a frequently overlooked monotopic membrane protein that catalyses the oxidation of ETF and the reduction of ubiquinone, the reduced ETF being generated as it reoxidises bound $FADH_2$ formed at the active sites of certain enzymes, e.g. the those catalysing the dehydrogenation of acyl-CoA species in the oxidation of fatty acids. This role of mobile ETF thereby explains why the modern 'concept' of mobile FAD/$FADH_2$, analogous to NAD^+/ NADH, and the redesignation of succinate dehydrogenase as $FADH_2$ dehydrogenase, are figments of the imagination. The sulfite oxidase is a molybdenum-containing enzyme that has its molybdenum cofactor assembled into the folded protein in the cytoplasm and is then exported, presumably, but not certainly, with its heme, to the periplasm via the Tat pathway of bacterial folded protein transport. The counterpart mode of assembly of the mitochondrial protein in the intermembrane space of poses an interesting problem;

it appears to involve the separate transport of the Mo cofactor, contrary to what is found in bacteria, to the intermembrane space where it binds to, and induces folding of, the apo sulphite oxidase that has been targeted to that location by a standard mitochondrial import process, the polypeptide being coded for in the nucleus (Klein and Schwartz 2012). Thus the Tat transport system is not required and indeed is absent from most non-photosynthetic eukaryotes. However, Tat components are encoded by the unusually large mitochondrial genome of the jacobid, *Recliomonas americana NZ* (Burger et al 2013), but what role they play therein is unclear (Hewtt et al 2014). A candidate function is assembly of the Rieske FeS centre of the cytochrome bc_1 (Complex III), analogous to the mode of assembly of this protein in bacteria. The Tat system is a well known component of the thylakoid membranes of chloroplasts where it is often known as the ΔpH transport system. This is probably responsible for the delivery of Riekse-type protein of the cytochrome b_6f complex as well as components of Photosytem II. A recent development is the evidence that the Tat system is functional in plant mitochondria (Carrie et al 2016) which,perhaps coincidentally, use a 'bacterial' system for synthesis of cytochromes c (see below).

Cytochrome c assembly

It has been known since the 1960s that a protein first termed cytochrome *c* heme lyase, but now more appropriately known as holo cytochrome *c* synthase (HCCS) is responsible in some organisms for the post-translational attachment of heme to a polypeptide to give a *c*-type cytochrome. The distinguishing feature of a *c*-type cytochrome is that heme is attached to the polypeptide chain by two thioether bonds , the formation of which involves the saturation of the two vinyl groups in heme, the

species found in non-ovalently bound in *b*-type cytochromes (Fig.1). The early studies on *Sacchromyces cerevisae* and *Neurospora crassa* implicated a second protein, cytochrome c_1 heme lyase (but now called holo cytochrome c_1 synthase HCC_1S), with specificity for the maturation of cytochrome c_1. However, analysis of a variety of genomes indcates that in most, if not all multicellular eukaryotic organisms (excluding plants eukaryotes a single HCCS protein appears to suffice (Allen et al 2008). These proteins are always coded for by the nuclear genome; there is no evidence for a bacterial origin for HCCS (Allen et al 2008). Interestingly, *c*-type cytochromes are relatively uncommon in the archaea but their biosynthesis appears to share components with the bacterial pathway that is introduced below.

The natural expectation was that an analogous system to HCCS would be identified in bacteria, although given the diversity of *c*-type cytochromes in the latter it was uncertain whether there would be many variants. In the event it transpired that many proteobacteria, including the alpha-proteobacteria, but also *E coli* and other organisms of the γ group, use a multicomponent system known as the Cytochrome *c* maturation proteins (Ccm) (also sometimes known as System I), the functional parts of which are localised on the periplasmic side of the bacterial membrane, where the post-translational modifications involved in the the production of c-type cytochromes occur. It subsequently became apparent that the Ccm system is possessed by plant mitochondria and other non-metazoan mitochondria (Allen at al 2008). There is another cytochrome *c* biogenesis system (known as Ccs or System II) found in bacteria, in particular Gram-positive organisms and in proteobacteria other than the α and γ groups. This Ccs system is never found in mitochondria, but functions in

thylakoid membranes to produce the *c*-type cytochrome, known as cytochrome *f*, that is needed for the electron transport pathway from Photosystem II to Photosystem I.

Whereas it was once thought that CP heme binding motifs were required for the action of HCCS, it is now known that these proteins have conserved histidines that are critical for binding heme and directing its attachment to the apo cytochrome *c* (Babbit et al 2015). It is also clear that the supposed CP heme-binding motifs are not essential for catalytic activity (Moore et al 2010). HCCS recognises very specific features of the N-terminus of the apo cytochrome, especially a phenylalanine residue that occurs separated by three residues from the N-terminal side of the characteristic CXXCH motif (Kleingardner and Bren, 2011; Stevens et al 2011) . It is the absence of this particular feature, coupled with some others, that prevents bacterial *c*-type cytochromes from being handled by the HCCS. However, a fusion of the N-terminal sequence of a yeast cytochrome *c* to the sequence of the *P. denitrificans* protein cytochrome c_{550}, including the CXXCH of the bacterial protein, does result in covalent attachment and production of a *bona fide* c-type cytochrome (Stevens *et al*. 2011). It is noteworthy that plant mitochondrial cytochromes c possess the critical F residue and other features identified as being important for maturation, yet in plants the Ccm, and not the HCCS, system functions. Strikingly, a plant mitochondrial cytochrome *c*, expressed in *E.coli*, has been shown to be matured by co-produced yeast HCCS (Moreno-Beltran et al 2014). It appears that HCCS evolved to recognise a pre-exisiting sequence motif at the N-terminal side of the CXXCH motif.

The Ccm system will attach heme to almost any periplasmic protein (see Mavridou et al 2008 for exceptions) that has a CXXCH motif. As mentioned earlier, in some

organisms, e.g. humans, there is one HCCS that is believed to attach heme to both cytochrome *c* and cytochrome c_1 in contrast to other eukaryotes, e.g *S cerevisea and N crassa*, where there is the second type of HCCS enzyme. In organisms that have only one HCCS the essential N-terminus of cytochrome *c* required for maturation is not obviously present in cytochromes c_1 that must be matured by this single HCCS. However, there is a tyrosine residue in exactly the same sequence position relative to the CXXCH as the F in cytochrome c. Yeast HCCS and human HCCS have been shown to process an F to Y variant of mitochondrial cytochrome *c* almost as well as, or as well as the wild-type protein (San Francisco et al 2013; Zhang et al 2014). Furthermore, *C. elegans* cytochrome *c* (and the corresponding proteins for very closely related organisms) have Y rather than F at this position, thus adding to the view that a consensus residue rather than a specific residue is recognised at this sequence position by HCCS enzymes. An important experiment showed that the essential F residue was needed only for HCCS recognition. F to A variants could be produced as folded heme proteins if the cytochrome was directed to the bacterial periplasm and processed there by the Ccm system (Kleingardner and Bren 2011; Stevens et al. 2011).

The sequence recognized in mitochondrial cytochromes *c* and c_1 by HCCS appears to be at a minimum those shown below:

K/R G X X I/L F x x K/R (CXXCH)… cyt c

K/R G X X V Y x x V (CXXCH) cyt c_1

where the F is Y in some (but few) mitochondrial cyts *c* and the Y is F in some (but few_ cyts c_1.

It should be stressed that it is not clear that c_1 is recognised in this way – obvious experiments, e.g. with a chimera of a cytochrome c_1 N-terminal sequence fused to a cytochrome *c* sequence from the CXXCH onwards need now to be done to clarify this issue.

What is striking is comparison of the sequence immediately to the N-terminal side of CXXCH in *P. denitrificans* cytochrome c_1 with that in the human protein

R G L Q V Y T E V **C** S A **C** H *P. denitrificans*

R G F Q V Y K Q V**C** A S **C** H human

A comparable sequence is found in cytochromes c_1 from other alpha-proteobacteria. This similarity is in marked contrast to the corresponding distinct sequences in mitochondrial cytochromes *c* and similar proteins, e.g., cytochrome c_{550}, in *Paracoccus denitrificans* and related proteins, e.g.*Rhodobacter* cytochromes c_2 , in other bacteria. If this motif is the N-terminal recognition sequence for HCCS (in organisms where there is no HCC_1S) and HCC_1S (where, as in yeasts, both proteins are present) it looks as though it was already substantially present in the cytochromes c_1 of alpha-proteobacteria. A similar sequence is found in many plant mitochondrial cytochromes c_1 and thus as is the case for cytochrome *c* it seems that HCC_1 S evolved to recognise a pre-exisiting sequence motif.

The N-terminal recognition sequences are present in trypanosomes (and the Euglenozoa in general) which do not have HCCS systems for either of their mitochondrial cytochromes c; these proteins are notable for the attachment to heme via a single thioether in either an AXXCH (cytc) Or FXXCH (cytc1) sequence. The evolutionary advantage, if any, of the unusual single thioether mode of attachment in

the heme is unknown, but the most closely related phyla to the Euglenozoa are Heterolobosea and Jakobida both of which possess the Ccm system, as does, by inference, *Naegleria gruberi* since its cytochrome *c* has a CXXCH motif (Allen et al 2008). Thus it is likely that loss of the Ccm system, which shows only very weak activity towards a ZxxCH motif (where Z is an amino acid residue other than C but in practice to date only A or F in trypanosomes) in substitution for CXXCH was accompanied by the appearance of a novel, as yet unknown, cytochrome c biogenesis system and the single cysteine heme attachment motifs, whereas the HCCS evolved separately to handle the CXXCH motif. It has been speculated that this might have arisen to avoid conflict with formation of a disulphide bond in the inner mitochondrial membrane space within the CXXCH motif. However, Ginger et al (2012) showed that replacement in *Trypanosoma brucei* of the native cytochrome *c* by a variant possessing a standard CXXCH motif enabled the cells to maintain mitochondrial respiration, albeit without complete complementation. Production of the CXXCH cytochrome in another trypanosomatid, *Crithidia fasciclata,* showed that although heme was attached to both cysteines it was not with the same stereochemistry that is universally observed in the c-type cytochromes. Thus it is still a puzzle. If there is an advantage to the formation of single cysteine cytochromes in euglenoids it is nevertheless surprising that it is not catalyzed by an enzyme easily identifiable as an HCCS, as the human and yeast enzymes have been shown to be reasonably effective at attaching heme to ZxxCH motifs. (Babbit et al 2015; Zhang et al 2016)). Furthermore, Jasion and Poulos (2012) readily produced recombinant *Leishmania major* (a trypanosomatid) cytochrome *c* by coexpression of its gene with yeast HCCS in *Escherichia coli*, thus demonstrating that the N-terminal recognition sequence required for HCCS to act is not only discernible but also functional, despite an

atypical N-terminal extension and the rare AXXCH heme attachment motif. This again indicates that the N-terminal recognition sequence was present in mitochondrial proteins that were (ands are) processed by the Ccm system and then the sequence was retained as both the HCCS system, and the as yet unidentified trypanosomatid system, evolved

Loss of some components of the aerobic respiratory chain

When John and Whatley wrote their 1975 paper it was not appreciated that *P. denitrificans* had more than one cytochrome oxidase, but later work has shown that it possesses at least two additional oxidases, cytochrome cbb_3 and cytochrome ba_3. Why have neither of these been retained in the modern-day mitochondrion? The ba_3 oxidase is an example of an electron transport pathway with, at first sight, a suboptimal proton translocation stoichiometry, this oxidase receiving electrons directly from ubiquinol such that the proton-translocating Complex III is bypassed. In contrast to animal mitochondria, it seems that under some circustances bacteria are not seeking to maximise the P/O ratio, probably because they are growing on carbon sources that are more reduced than the average cell biomass and thus disposal of excess reductant is as important as ATP yield. Plant and other mitochondria, notably those of trypanosomes, have an alternative heme-independent oxidase that can serve the same function and analogues of this alternative oxidase (AOX) are found in some bacteria. Thus the disappearance of ba_3 (or bo_3 in some organisms e.g. *E. coli*) from

mitochondria, can be accounted for, but the *cbb*$_3$ oxidase achieves essentially the same protonmotive function as *aa*$_3$ and yet with a much higher affinity for oxygen. Furthermore, it does not need the biosynthetic effort involved in converting *b*- to *a*-type heme. The *cbb*3 oxidase has a lower kcat than the *aa*$_3$ oxidase which is presumably reflects a tradeoff for the higher substrate affinity. In this context, there nevertheless still seems to be no rationale for the adoption of the specialized *a*-type heme in cytochrome oxidase.

Independent compexes or solid state 'wire' model of the P. denitrificans electron transport chain?

For many years the widely accepted model of the mitochondrial respiratory chain was one in which independent Complexes I, II (succinate-ubiquinone oxidoreductase) , III, and IV moved by slow lateral diffusion and were connected by the much more mobile ubiquinone/ubiquinol and cytochrome *c*, which are respectively located within the membrane bilayer and on the intermembrane space side of the inner membrane. Thus ubiquinone/ubiquinol was envisaged as a pool and thus able to collect reducing equivalents from any of complexes I and II (and, but rarely mentioned, also from the monotopic ETF ubiquinone oxidoreductase whose motion has not been studied) and delivered to Complex III. Some of the most persuasive evidence for this view was provided by Kröger and Klingenberg (1973) who showed that the pseudo-first order rate constant for a pool of ubiquione reduction and ubiquinol oxidation was consistent with the respiratory rate, whereas of course the half time for oxidation of ubiquinol, being in excess, was slower, leading to a mistaken view that there was not a pool of the ubiquinone. The analysis also showed why titration of the respiration by

antimycin could follow a quasi sigmoidal profile; this is not a reflection of cooperativity but rather of an excess, under some conditions, in the capacity of Complex III which is the site of action of antimycin. Thus a pool role for ubiquinol allowed oxidation to continue almost unabated as part of the Complex III activity was inhibited by titration with antimycin.

Cytochrome *c* has not often been seen as a pool but in some mitochondria it has a role beyond shuttling electrons from Complex III to Complex IV, for example in mediating electron transfer from sulphite oxidase in liver mitochondria.

In recent years the notion of independent protein complexes has been challenged by observations of super complexes in the mitochondrial respiratory chain. The structural evidence for these is strong but the kinetic properties of the respiratory chain, including those studied by Kröger, and Klingenberg (1973), do not readily support the sandwiching, for example, of ubiquinone between a complex I and a complex III (see eg Letts and Sazanov, 2017; Blaza et al 2014; Mikenovich et al., 2017). Such 'sandwich' models frequently overlook the need for electron input from ETF-ubiquinone oxidoreductase and old observations, and now fully confirmed (Blaza et al. 2014) that rates of NADH and succinate oxidation in submitochondrial particles (inverted inner mitochondrial membranes) are not additive, contrary to the predictions of a simple 'sandwich' model.

Some of the earliest evidence for supercomplexes came from analysis of *P. denitrificans* respiratory chain components (Berry & Trumpower 1985; Stroh et al 2004), but as with mitochondria the functional significance remains unclear. There is much debate about supercomplexes in bacterial membranes (see eg. Lorente-Garcia I. et al (2014); Magalon & Alberge (2016**)).** As with mitochondria, they may have a

structural rather than a catalytic and functional purpose. A role is often envisaged as facilitating in the direct channelling of products and substrates between individual complexes.

The composition of the respiratory chain system of *P denitrificans* depends on how the cells are grown, and under most, if not all, conditions is more complicated that that of the mammalian mitochondrion. Thus when cells are grown anaerobically on succinate plus nitrate the cytoplasmic membranes contain a number of additional components that are not present in mitochondria and that have therefore been lost in the transition from alpha-proteobacterium to mitochondrion. These components include the ba_3 ubiquinol oxidase, the membrane-bound ubiquinol/nitrate oxido reductase (Nar), the cbb_3 oxidase, and the nitrate, nitric oxide and nitrous oxide reductases, with the latter four enzymes receiving electrons via the cytochrome bc_1 complex. Thus in *P. denitrificans* inhibition of oxygen reduction by antimycin, with NADH as substrate, follows a non-hyperbolic (pseudo-sigmoidal) profile as a consequence of the combination of the availability of the ba_3 pathway and an excess capacity of the bc_1 complex. This view is reinforced by the observation that a titer of antimycin that gave 50% inhibition of respiration in the presence of an uncoupler had no inhibitory effect on the controlled rate (so called state 4) in the presence of a sizeable proton motive force (Ferguson, 1975).

A notable observation is that the inhibition by antimycin in intact cells of anaerobic nitrite respiration follows a standard hyperbolic pattern, suggestive of a sole route to nitrte reductase via the *bc*1 complex and no great excess of capacity at the *bc*1 level (Alefounder et al 1981). It should be borne in mind that nitrite reduction will be followed contemporaneously by nitric oxide and nitrous oxide reduction (Alefounder

at al, 1983; John, 1977) which also depend on bc_1 a for electron supply. In contrast, the appearance of nitrate reductase activity under aerobic conditions as electron flow to oxygen is blocked by titration with antimycin exhibits a insensitive to the inhibitor phase which indicates a excess of capacity for electron flow to oxygen; thus only gradually does inhibition of the nitrate reduction by oxygen attenuate (Alefounder et al 1981), consistent with a pool function for ubiquinone/ubiquinol. The basis of the essentially complete inhibition of nitrate reduction until oxygen is exhausted is still not properly understood but it is hard to understand in the context of a solid state supercomplex model which woud imply that nitrate and oxygen respiration should occur simultaneously. A broad current interpretation is that the pool of ubiquinol is kinetically blocked from supplying nitrate reductase when the electron flow rate to oxygen is sufficiently high, but when that rate attenuates nitrate reduction commences. The switch from oxygen to nitrate and vice-versa occurs on a timescale that is probably within the response time of the electrodes used for measurement, and thus on a timescale of less than 10 seconds, and most probably faster (John, 1977); switching is not likely to be accompanied by rearrangement of supercomplexes in the membrane.

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ATP synthase

The FoF_1 ATP synthase from *P. denitrificans* is the first complete ATP synthase structure to be determined by X-ray diffraction, thus remarkably following in the footsteps of the cytochrome oxidase from the same organism (Morales-Rios et al 2015). The core structures of the mitochondrial and bacterial ATP synthases are very

similar but there are notable differences. One of the most unexpected has been the realization that the number of c subunits in the F_0 part of the enzyme is variable. Amongst the mitochondrial enzymes either 8 (animals) or 10 (other mitochondria) (Ferguson, 2015) copies have so far been reported whereas in bacteria and plants values up to 15 have been reported. The *P. denitrificans* structure shows that it has 12. There is a clear expectation that the c subunit stoichiometry relates to the H+/ATP ratio; thus whereas passage of 8 protons through F_0 of the animal mitochondrial enzyme is predicted to drive synthesis of 3 ATP by causing a 360^o rotation of the central stalk and hence the synthese of 3 ATP by the 3 beta subunits, for the *P. denitrificans* enzyme 12 protons are required for the same outcome. The implication of this difference is that the maximum [ATP]/[ADP][Pi] ratio that can be achieved (more strictly $\Delta G_{\text{ATP SYNTHESIS}}$) by the mitochondrial enzyme would be lower than that of the *Paracoccus* enzyme if equal magnitudes of the proton electrochemical gradient were present. Interestingly, and perhaps coincidentally, old experiments of this kind did suggest that the H+/ATP ratio must be bigger, by approximately a ratio of 2:3 (Kell et al 1978a; Sorgato et al 1978, similar to the ratio, predicted on structural grounds, of 2.66 (8/3):4 (12/3). The higher H+/ATP ratio in the bacterial system may reflect the absence of a contribution to the energetics of ATP synthesis provided by the adenine nucleotide and phosphate transporters in mitochondria (Ferguson, 2010).

It was recognized over 40 years ago that oxidative phosphorylation in *P. denitrificans* is not reversible, in the sense that addition of ATP to inside-out membrane vesicles does not result in its hydrolysis and generation of a protonmotive force. Even when respiring vesicles were incubated with an estimated equilbrium mixture of ATP, ADP

and P_i in the presence of a respiration-dependent proton motive force exchange of ^{32}P from [$\gamma\,^{32}P$]ATP into phosphate was not detected, contrary to expectations (Ferguson et al. 1976). Many years later a subunit of the enzyme, known as zeta and possessing some inhibitory activity, was identified; this protein is distinct both structurally and in behaviour from the mitochondrial ATP synthase inhibitor protein (IF_1). Recently there have been two reports of studies in which a gene knock out of the zeta subunit has been made. Varghese et al. (2018) could find no effect on growth or *in vitro* ATPase activity whereas Mendoza-Hoffman et al. (2018) did report impaired growth of cells. Clearly the role of the zeta subunit, also found in other alpha-proteobacteria, requires more attention

There is no reason why the F_oF_1 ATP synthase of *P. denitrificans* should ever reverse, as there is no evidence for non-respiratory growth (i.e. fermentation) which might require ATP-dependent protonmotive force generation such as is seen in some organisms, for example in mitochondria from trypansosomses in the blood stream stage of the life cycle when oxidative phosphorylation is absent amd glycolytically generated ATP allows the ATPase activity of F_oF_1 to maintain the still essential membrane potential (Schnaufer et al 2005).

Lessons – what controls the rate of ATP synthesis?

John and Whatley (1975) showed that it is possible to prepare inside-out membrane vesicles from *P. denitrificans* that exhibit respiratory control. No such counterparts (submitochondrial particles) have ever been prepared from mitochondrial membranes or from another bacterial species. Thus these vesicle preparations provide a singular

opportunity to investigate some aspects of the problems of 'what controls the respiratory rate?' and 'what controls the rate of ATP synthesis?' The effect of progressively adding rotenone to attenuate the respiratory rate provides some insight into these issues. It was found that addition of rotenone to progressively inhibit the NADH-ubiquinone oxidoreductase (Complex 1) activity has little effect on the ADP/O ratio for *P. denitrificans* vesicles (Ferguson et al 1976). At first sight this may seem a trivial observation since in general the stoichiometry of a process is not influenced by the rate at which it occurs. However, in the case of oxidative phosphorylation this is not a trivial finding as the indirect nature of the coupling between respiration and ATP synthesis means that the stoichiometry could change, for example as a result in variation of the rate of proton leakage back across the membrane relative to the rate of proton flow through the ATP synthase as the electron transport rate slows. Indeed the effects of the inhibitor malonate on the succinate-dependent mitochondrial P/O ratio gave some marginal suggestion of such an effect (Ernster and Nordenbrand, 1974). It seems that as the rate of electron transport is, say, halved the rate of ATP synthesis must also halved. At this point it could be argued that if each *P denitrificans* vesicle had just one Complex I then this result would be entirely trivial. However, this is extremely unlikely and is contradicted by the finding (Kell et al 1978b) that the extent of stimulation by an uncoupler of the electron-transport rate declines with increasing titre of rotenone. Had the titration resulted in populations of completely inhibited and completely uninhibited vesicles (i.e. if just one Complex I per vesicle) then the % increase in electron transport rate should surely have stayed the same.

More recently Zharova and Vinogradov (2014) have done related experiments with *P. denitrificans* vesicles and found that modulating the rate of respiration through

lowering the steady state NADH concentration similarly barely affects ATP/ADP ratio achieved. Further observations were that the $\Delta G_{\text{ATP SYNTHESIS}}$ (i.e. proportional to [ATP]/[ADP][Pi]) generated by these vesicles was essentially unchanged whatever the rate of respiration achieved by rotenone titration of NADH oxidation (Kell et al 1978b)). Again in general terms it is not unexpected that the thermodynamics of the output should be independent of the rate at which the output is achieved, but in chemiosmotic terms this observation is not necessarily automatically predicted. An additional observation was that the protonmotive force was not detectably attenuated as the respiratory rate declined to approximately 20% of the uninhibited value. This was the case for both respiration during ATP synthesis (state 3) and in its absence (state 4) rates (Kell et al 1978b), although the protonmotive force was about 15 mV lower in the former condition than in the latter.

An explanation of these observations in chemisomotic terms is as follows. It can be assumed that in these vesicles the protonmotive force is large enough that one or more proton-translocating steps in the respiratory chain are close to equilibrium with it but the electron transport rate is not zero, as it should be at true equilibrium, owing to proton leakage across the membrane. As rotenone is added there is a tiny attenuation in the size of the protonmotive force such that the stimulation by an uncoupler is progressively lost. This description requires that Ohms's law does not apply to the proton leakage rate across the membrane. When ATP synthesis is in progress the protonmotive force drops as would be expected by analogy with an electrical circuit and is just as has been seen before in studies with mitochondria. One must surmise that the protonmotive force slightly declines as rotenone is added but this decline escapes the relatively imprecise method of measuring protonmotive force. This means

that the rate of ATP synthesis by the synthase has very strong dependence on the size of the protonmotive force such that the rate of ATP synthesis reflects the rate of electron transport. It is known from a variety of other studies that the rate of ATP synthesis is sharply dependent on protonmotve force. Similarly when ATP synthesis is complete, the proton motive force is scarcely affected by the attenuation of respiration such that small declines are not reflected in a measured $\Delta G_{\text{ATP SYNTHESIS}}$.

In vivo both mitochondria and bacteria such as *P. denitrificans* will experience fluctuating respiration rates which will need to be accompanied by changes in the rate of ATP synthesis. It follows that for *P. denitirficans* vesicles, at least, the rate of ATP synthesis should be directly determind by the protonmotive force which in turn is a delicate function of the respiration rate (or in other words the proton translocation rate). The rate of respiration must be independent of the [ATP/[ADP][Pi] ratio and thus the rate of ATP synthesis cannot be directly determined by the concentration of ATP or ADP as has often been discussed for mitochondria where analysis is made more complex by the contribution of adenine nucleotide exchange process across the inner membrane. The lack of demonstrated reversibility (Ferguson et al., 1976) of the ATP synthase correlates with this conclusion, as do the more recent observations of Zharova and Vinogradov (2012) who could find no effect of considerable variation in the phosphate concentration on $\Delta G_{\text{ATP SYNTHESIS,}}$ contrary to what would be expected from a model in which the respiratory chain is effectively in equilibirium with the ATP synthase reaction. This does require that the $\Delta G_{\text{ATP SYNTHESIS}}$ never reaches its maximum theoretical value if true equilibrium were to be reached; the kinetic properties (lack of reversibility and relative affinities for ATP and ADP) of the ATP synthase are consistent with failure to reach equilibrium. It has been more difficult to

address this issue with mitochondria and so *P. denitrificans* as a model system may have a lesson to teach here in terms of understanding the factors that generally determine the rate of ATP synthesis in oxidative phosphorylation.

Conclusion

The molecular and structural work on the oxidative phosphorylation apparatus subsequent to 1975 have added flesh to the proposal made in 1975 that *P. denitrificans* (and as it turns out other alphaproteobacteria (see Fig 2 in Roger et al (2017), some of which may be closer relatives but have less well characterised oxidative phosphoryaltion systems) is a convincing mitochondrial ancestor. The bioenergetic changes, for example for example the change in number of c subunits in the ATP synthase and *c*-type cytochrome biogenesis system we now recognise, are themselves of evolutionary and bioenergetics interest, as is the indication that fundamental questions about what controls the rate of ATP synthesis may be best illuminated through studies with *P. denitrificans.*

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Figure legend

Fig. 1. The structure of the heme found (A) in *b*-type cytochromes, as well as in hemoglobin and cytochrome P450 amongst other hemoproteins, and (B) in c-type

cytochromes.The heme in cytochrome c_1 is identical to that found in cytochrome *c* as shown in (B). The designation c_1 (and in bacterial examples c_2 c_3 c_{550} etc) refers, in an unsystematic way for historical reasons, to the type of protein and not the type of heme.