The scientific legacy of Stuart Ferguson

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1. Introduction

Stuart Ferguson was an exceptionally insightful and inspiring research scientist. to set the scene for the topics that follow in subsequent Chapters of this volume. We briefly summarise his career and highlight some of the most significant contributions he made to bioenergetics, biochemistry and microbial physiology, In particular, we draw attention to his work on bacterial electron transport chains and the key role of the periplasm in electron transfer reactions, denitrification and cytochrome *c* biogenesis. Stuart's influence on the field of bioenergetics was not confined to his own research area, however. He co-wrote (with David Nicholls) the textbook *Bioenergetics*, now in its 4th edition, that has become respected worldwide as one of the most authoritative texts in this field.

2. The early years at Oxford: ATP synthase and *Paracoccus*

Stuart began his university career in the 1970s as an undergraduate chemistry student at Merton College, Oxford. He stayed in Oxford to do his DPhil, under the supervision of George Radda FRS, working on the mitochondrial ATPase. This was a time when studies on the molecular mechanism of ATP synthesis were of growing interest. Using chemical modification experiments, Stuart was one of the first to identify the ATP/ADP-binding site subunit co-operativity of ATP synthase that is key to understanding its operation (Ferguson et al., 1975a, 1975b, 1976). Indeed, this discovery underpins the binding-change mechanism later proposed by Paul Boyer, who won the 1997 Nobel Prize. Other work going on at Oxford at this time was to have a profound influence on the direction of Stuart's research. In 1975 Philip John and Bob Whatley first highlighted the similarities between the mitochondrial respiratory chain and that of the Gram-negative bacterium *Paracoccus denitrificans* and they proposed a plausible evolutionary origin of the mitochondrion from a *Paracoccus*-like ancestor (John and Whatley, 1975). This work inspired Stuart to turn his attention to microbial biochemistry and to the bioenergetics of *P. denitrificans* which he continued to study throughout the rest of his career. As a postdoctoral fellow at Oxford, he produced a series of papers continuing his work on the ATP synthase and, working with Douglas Kell

and Philip John, investigated basic bioenergetic principles, measuring membrane potential and ΔpH in submitochondrial particles and *Paracoccus* membrane vesicles (Kell et al., 1978; Sorgato et al., 1978).

3. To Birmingham: initial work on denitrification and the role of the periplasm

In 1978 Stuart left Oxford for a lectureship in the Department of Biochemistry at the University of Birmingham. Here he was able to start building his own research group and he naturally wanted to expand his interests in bioenergetics. He decided to use *Paracoccus* as his model system as it was clear that this bacterium was not just a “free-living mitochondrion” but was able to use a wide range of additional electron acceptors as well as molecular oxygen. Employing electron transport measurements with different donors and acceptors in intact cells, along with careful localisation and purification of the enzymes involved, he and his group started to investigate the composition and functions of the branched electron transport chains of this versatile bacterium. For example, Stuart showed that the methanol dehydrogenase of *P. denitrificans* was located in the periplasm and he was able to elucidate how electrons from the oxidation of methanol in this compartment entered the respiratory chain and allowed respiration (Alefounder and Ferguson, 1981); see the Chapter by Stephen Spiro in this volume). Work on another electron donor system, the NADH dehydrogenase was more challenging and was undertaken by Tina George (George and Ferguson, 1984), who Stuart later married. But it was at Birmingham that Stuart started research that was to become a major focus of his work in subsequent years - the process of denitrification.

In dentification, nitrate is reduced in a series of energy-conserving respiratory first to nitrite (via nitrate reductase), then to nitric oxide (via nitrite reductase), then nitrous oxide (via nitric oxide reductase) and finally to nitrogen gas (via nitrous oxide reductase). This process is a key part of the global nitrogen cycle and a greater understanding of it is of more than academic interest; it is essential for both maximising agricultural yields and to mitigate the production of the greenhouse gas nitrous oxide. Stuart’s group initiated studies on the reductases needed for each step in the pathway and showed that some of the enzymes

involved in denitrification are located in the periplasm. Stuart realised that the periplasm was a metabolically active sub-cellular compartment with a key role in electron transport at a time when its full role in microbial physiology was still unclear, and he rejected the widely used term ‘periplasmic space’. As he put it in a particularly prescient letter to *Trends in Biochemical Sciences* entitled “periplasm underestimated” he opined “…the periplasm cannot be considered as a 'space'; it appears to be bursting at the seams with many different types of protein as well as the murein layer” (Ferguson, 1990).

During his time at Birmingham, Stuart collaborated and shared a research laboratory with the late J. Baz Jackson. Their interests were very complementary. Baz was using purple photosynthetic bacteria as models to study fundamental aspects of bioenergetics and the generation of the proton-motive force during photosynthesis, taking advantage of the “carotenoid bandshift” as an intrinsic reporter of membrane potential. Using this and other techniques, and with Alastair McEwan (a contributor to this volume) as their PhD student, they pioneered investigations into the role of anaerobic electron transport in *Rhodobacter.* They showed that electron flow to alternative acceptors such as dimethylsulphoxide, trimethylamine-N-oxide and nitrous oxide could generate a membrane potential in this organism (McEwan et al., 1983, 1985). This work led to Research Council funding, which gave the opportunity for two of us (Richardson and Kelly) to join Stuart and Baz in 1985 as doctoral student and post-doc respectively, building on the work of Al McEwan in establishing a new role for anaerobic respiration in redox poising of the photosynthetic electron transport chain (Richardson et al., 1988).

4. Return to Oxford: Unravelling denitrification and cytochrome c biogenesis

Stuart had always wanted to return to Oxford and did so in 1985, to take up a Tutorial Fellowship at St. Edmund Hall. In 1994 he was appointed to a Readership and then Professor of Biochemistry three years later. After finishing his PhD in 1988, David Richardson (see his Chapter in this volume) worked with Stuart in Oxford as post-doctoral researcher, joined by Ben Berks and James Moir (see Chapter X) as Doctoral Students. This

marked the beginning of a long period of highly productive research in which Stuart and his group made many significant contributions to bacterial bioenergetics and deepened our understanding of the biochemistry of denitrification. For example, initial characterisation (with David Richardson) of a novel type of periplasmic nitrate reductase (now known as the Nap enzyme) in the aerobic denitrifier *Thiosphaera pantotropha*, (Bell et al., 1990) showed it was distinct from the membrane bound nitrate reductase (Nar) that had been well studied in a range of bacteria. Further studies by David Richardson and later collaboration with Jeff Cole (another contributor to this volume) revealed that Nap has physiological roles independent of denitrification and is widespread in many groups of bacteria including *E. coli* and a range of pathogens.

Another important discovery was that nitric oxide (NO), which is also both a potent toxin and a signalling molecule in mammalian cells),was a free intermediate in denitrification. Stuart's group was amongst the first to identify the reductase that reduces and detoxifies NO (Carr et al., 1989; Carr and Ferguson, 1990). In retrospect this work was not only pivotal in the denitrification field but also highlighted a key physiological role for nitric oxide, well before NO became famous as "molecule of the year" in *Science* in 1992! The enzyme that produces nitric oxide is the cytochrome cd_1 nitrite reductase and a major achievement was its crystallisation and structural characterisation, with James Moir and Vilmos Fülöp (Fülöp et al., 1995). This work led on to novel mechanistic insights, for example the crystal structure of the oxidized enzyme showed that the d_1 haem iron of the active site is ligated by His/Tyr side chains, and the *c* haem iron is His/His ligated, but during catalysis both haems undergo re-ligation; upon reduction, the tyrosine ligand of the d_1 haem is released to allow substrate binding and, concomitantly, a refolding of the cytochrome *c* domain takes place, resulting in an unexpected change of the *c* haem iron coordination from His/His to Met/His (Allen et al., 2000; Williams et al., 1997). Nitrous oxide is also a free intermediate in denitrification. It is now clear that denitrifying bacteria are a major agricultural source of this environmentally damaging greenhouse gas; again, Stuart's group was one of the first to identify and

characterise the copper containing nitrous oxide reductase enzyme that can destroy nitrous oxide by converting it to the more inert dinitrogen gas (Berks et al., 1993; McEwan et al., 1985; Richardson et al., 1989).

Many of the electron transport enzymes involved in denitrification are *c*-type cytochromes located in the periplasm. It is now known that the apoproteins are translocated there via the Sec system and that the heme is attached covalently and postranslationally to a CXXCH motif via the complex multiprotein cytochrome *c* maturation (Ccm) system (Stevens et al., 2011). Stuart had long been fascinated by the question of *why* the hemes need to be covalently bound in *c*-type cytochromes and discussed this in several publications e.g. Barker and Ferguson (1999). He also wanted to know *how* this process was achieved. In the 1990s, Stuart's group started investigating this problem, using *Paracoccus* and *E. coli* as model systems and applying molecular genetics and mutagenesis approaches to complement their biochemical work. This work continued for the rest of Stuart's career at Oxford. With Dudley Page, Yoshihiro Sambongi and others Stuart first started to probe the roles of the different *ccm* genes and the importance of disulfide bond reduction in cytochrome *c* assembly e.g. (Page and Ferguson, 1997; Page et al., 1997; Sambongi and Ferguson, 1996). In 2000, Oliver Daltrop in Stuart's group showed - "contrary to opinion of 30 years standing" - that a *c*-type cytochrome can in fact be assembled from heme and apoprotein *in vitro* under mild conditions and in the absence of any biosynthesis machinery, provided formation of a disulfide bond within the CXXCH motif is avoided (Daltrop et al., 2002). With James Allen, the essentiality of the CXXCH heme attachment motif was investigated further and a novel biogenesis system in trypanosomes discovered that attached heme to an unprecedented XXXCH motif in apocytochromes (Allen et al., 2004). Collaboration with Julie Stevens and Christina Redfield at Oxford over many years resulted in deeper insights into the mechanism of the Ccm proteins, especially CcmE (Harvat et al., 2005, 2009; Stevens et al., 2003). Because of Stuart's interest in the cd_1 nitrite reductase, the biogenesis of the *d* heme became a focus, in collaboration with Martin Warren (a

contributor to this volume). They showed that in denitrifying and sulfate-reducing bacteria as well as in Archaea, a novel branch of the standard tetrapyrrole pathway exists, which involves the “hijacking” of siroheme, the prosthetic group of sulfite and nitrite reductase, and its processing into both heme and d_1 heme (Bali et al., 2011). This surprising finding uncovered a previously unknown additional role for siroheme in biology.

The periplasm is an oxidising environment and cytochrome c biogenesis must ensure that the cysteine residues in the CXXCH motif remain reduced to allow the formation of thioether bonds to the heme. This occurs by channelling of electrons via DsbD, a membrane bound component of the Dsb (disulfide bond) system with a thioredoxin fold. With Despoina Mavridou (see Chapter X in this volume), Julie Stevens and Christina Redfield, the interplay between the Dsb and Ccm systems was investigated and the mechanism of DsbD was studied in detail using a multi-disciplinary approach. It became clear that an extended active-site motif controls the reactivity of the thioredoxin fold, with implications for the mechanism of the whole family of such proteins e.g. Mavridou et al. (2014). This is a yet another example of where Stuart’s work on what may appear at first to be a specialised area of microbial biochemistry turned out to have general implications.

Dedication and perspective

We dedicate this article to the memory of our mentor, colleague and friend Stuart Ferguson, whose science was rigorous and humorous stories legendary (if prone to outrageous exaggeration!). Our very brief and selective summary cannot do justice to the breadth of Stuart’s research, which is contained in over 250 publications. His achievements were recognised by the award of the Keilin Medal by the UK Biochemical Society in 2001; highly appropriate given the discovery of cytochromes by David Keilin in1925. There is no doubt that Stuart has had an important influence on the field of bacterial bioenergetics and his approach and thinking is reflected in the following Chapters in this special volume of *Advances in Microbial Physiology*.

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