fiery fast bowler and very useful left-handed batsman. He was a lifelong Spurs supporter and a highly competitive member of the Biological Sciences football team, aptly names 'Biohazard'. In the 1970s Howard performed with distinction in the 'Biohazard' team that played a friendly match with the Saudi Arabia national team, thereby adding to his illustrious international career. A great passion of Howard's was real tennis and he was a member of Leamington Real Tennis Club where his competitive spirit, guile and ability won him many tournaments. It was here, while playing in a friendly doubles tournament, that he tragically collapsed and died on 12 January 2008. He had just returned from a month in The Gambia assisting his wife Kira in her extensive humanitarian work, setting up new schools and medical centres there. This work will now be assisted through generous contributions made to the African Oyster Trust in his memory. He was also excited by the prospect of advising the Gambian government on a number of important environmental issues.

Howard was a down-to-earth, self-effacing man, outgoing and witty and in the 1980s was a 'leading light' at gatherings of the staff of Biological Sciences at Warwick in weekly socials at local pubs (code-named 'Choir-Practice'!). He also enjoyed the occasional 'poker-night' with selected colleagues who invariably relieved him of his hard-earned cash. Howard's penetrating questions and insightful comments at national and international scientific meetings always made for a lively and stimulating debate and discussing science with him was always immensely rewarding. He was extremely generous of his time with well over 100 PhD students and postdoctoral researchers, and it was a real privilege for me to work with him as a PhD student and then as a colleague for nearly 30 years. Above all else, he made science fun and was an inspirational mentor, a much-loved colleague and a dear friend. He will be very sorely missed. Howard is survived by his wife Kira and children (Christopher, Eric, Jeremy and Amber).

Colin Murrell, University of Warwick

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A tribute to Howard Dalton and methane monooxygenase

CHRISTOPHER ANTHONY

ABSTRACT

This is a brief account of the scientific contribution of Professor Sir Howard Dalton FRS, who died in January 2008. It starts with his important study of the physiology of nitrogen-fixing bacteria, but concentrates on his work on methane oxidation. This started with the development of reliable assays for the methane monooxygenase, leading to the discovery of two types of enzyme, soluble and membrane-bound and their regulation by copper levels in the growth medium. His contribution to our understanding of the structure and function of these enzymes is then discussed and the review closes with a brief summary of our present knowledge of these important enzymes.

Keywords: *Dalton, methane, methane oxidation, methylotrophs, methanotrophs*

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Introduction

Howard Dalton was one of the most distinguished and influential microbiologists of his generation. This review is based on my contribution to *A Tribute to Celebrate the Life of Professor Sir Howard Dalton FRS*, held at the University of Warwick in May 2008.

His research career started when he undertook a DPhil with Professor John Postgate FRS at the ARC Unit of Nitrogen Fixation, Sussex University, where he worked on nitrogen fixation in the soil bacterium *Azotobacter*. He then worked for two years with Professor Len Mortensen at Purdue University, Indiana, on the biochemistry of nitrogenase in the anaerobic bacterium *Clostridium*. Recognising that physico-chemical spectroscopy techniques were going to be of great importance in studying metal-containing enzymes, he returned to Sussex University in 1970 to work with Professor Bob Bray on molybdenum enzymes. Professor Roger Whittenbury persuaded Dalton to take up a lectureship in Microbiology at the Department of Biological Sciences in the University of Warwick in 1973 where was awarded a Personal Chair in 1983. He was elected as a Fellow of the Royal Society in 1993, was appointed President of the Society for General Microbiology, 1997–2000, and awarded the Leeuwenhoek Medal in 2000. In 2002, Dalton was seconded to become Chief Scientific Adviser to Defra. He was Knighted in the New Year's Honours list in 2007.

This review starts with a brief description of Dalton's contribution to the physiology of bacterial nitrogen fixation, but most of it describes his work on methane oxidation. The earlier 'historical' part concentrates on his direct contributions, and the later part provides a brief summary of our present knowledge about the methane monooxygenases, much of which was directly contributed, or was inspired, by Dalton's group at Warwick.

Nitrogen fixation

During bacterial nitrogen fixation, atmospheric nitrogen gas is reduced to ammonia in a reaction catalysed by the nitrogenase complex, consisting of two proteins containing the metals iron and molybdenum. This enzyme is famously extremely sensitive to oxygen but many nitrogen fixing bacteria require oxygen for growth, and sometimes the efficiency of fixation is higher when oxygen is present. This raises the question that formed the basis of Dalton's PhD work: How does the oxygen-sensitive nitrogenase function in bacteria in a highly aerobic environment? The chosen microbe for study was *Azotobacter*, which fixes nitrogen in aerobic conditions, during which the rate of oxygen consumption is extremely high, while its nitrogenase remains very sensitive to molecular oxygen. His approach provides an excellent example of the use of continuous culture to sort out a puzzling problem of bacterial physiology[1,2]. Dalton's extensive imaginative study showed convincingly that this is due to a process of *Respiratory Protection*: ''Nitrogenase is inactivated by oxygen but the organism's respiration normally scavenges oxygen and keeps it away from the enzyme.''

Appreciating that a better understanding of nitrogenase would be based on a physico-chemical approach, Dalton spent the next five years with Mortenson in Purdue and with Bray in Sussex studying nitrogenase and related metal-containing enzymes. These studies extended his expertise in protein purification, spectrophotometric analysis and complex EPR spectroscopy of metal enzymes in complex multi-protein systems[3–5], all of which supported his subsequent work on methane oxidation.

Methane oxidation

Methylotrophs are microbes able to grow on reduced carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds; such compounds include methane, methanol, methylamine and trimethylamine[6]. The end product of all anaerobic microbial degradation of organic material is methane and some of this reaches the atmosphere where it is a powerful greenhouse gas. The methanotrophs are a major group of methylotrophs, able to use methane (and usually methanol) as sole source of carbon and energy; the vast majority of these are obligate and so are unable to grow on multicarbon compounds[6–8]. The methanotrophs clearly play an important role in the carbon cycle, diminishing the amount of methane liberated into the atmosphere[9], and they have become of considerable importance as they can be exploited in biotransformation and bioremediation processes[10].

In 1973 Dalton joined the new Biological Sciences Department in the University of Warwick as a lecturer in Roger Whittenbury's group and set out to solve the problem of the bacterial oxidation of methane. In this he was highly successful and the Warwick group continues to be internationally known for its work on the biochemistry, physiology, molecular biology and ecology of methanotrophs.

Previously, Whittenbury and colleagues at Edinburgh University had laid the foundation for all later work on methanotrophs by their magnificent work in isolating and characterizing a wide range of new methanotrophs [100 new isolates][12]. These methanotrophs can be placed in two groups. Type I methanotrophs (such as *Methylococcus*) have internal membranes arranged as bundles of vesicular discs and assimilate methane by way of the Ribulose monophosphate pathway; Type II methanotrophs (such as *Methylosinus*) have membranes arranged around the cell periphery, and assimilate methane by the Serine pathway[6]. In much of the early work on methane oxidation it was thought possible that the systems for methane oxidation might be different in these fundamentally different types of bacteria.

All of the energy used for growth of methanotrophs is obtained by oxidation of methane to carbon dioxide:

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

In 1973, the first step in this process was very poorly understood, and Dalton set out to solve it using the expertise in multicomponent metal-containing enzyme systems that he had acquired during his work on nitrogenase and related enzymes. Work from many laboratories using a variety of methanotrophs had led to the general conclusion that the first step in methane oxidation is catalysed by a mixed function monooxygenase system[12–14]. This is now called methane monooxygenase (MMO), which hydroxylates methane to methanol using molecular oxygen and a reductant (AH_2):

$$CH_4 + AH_2 + O_2 \longrightarrow CH_3OH + H_2O + A$$

The reductant was usually assumed to be NAD(P)H but there was considerable confusion and disagreement about results in the earlier studies which was often due to the use of different bacteria, or different membrane preparations, different assays *etc*. The obvious assay systems would involve measurement of the product methanol, or spectrophotometric measurement of NADH disappearance, or methane- and NADH-dependent consumption of oxygen. However most cell-free preparations used membrane fractions containing NADH oxidase which also consumes NADH and oxygen, and the product methanol may also be further metabolized (Figure 1).

An essential first step in Dalton's solution of the problem was the development of reliable, unambiguous assay systems, that are still in use today. These were based on the use of alternative subtrates whose oxidation by MMO depended on its wide substrate specifi-

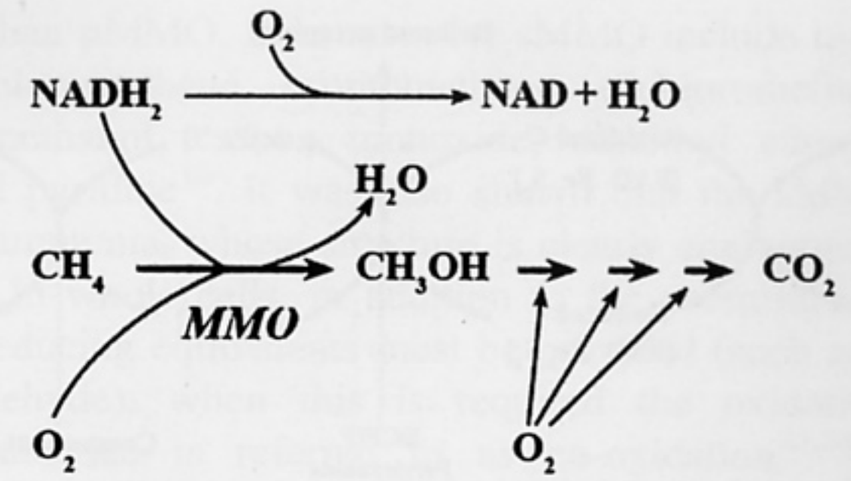

Fig. 1. *Methane oxidation by methane monooxygenase in methanotrophs. Assay of MMOs is made difficult by alternative routes for oxidation of NADH, consumption of oxygen, and metabolism of the product methanol.*

city. These methods included the oxidation of bromomethane, whose disappearance was measured by gas–liquid chromatography (GLC)[15] and the oxidation of ethylene or propylene, the epoxy products also being measured by GLC[16].

Discovery of methane monooxygenases

Using these assay methods the most definitive description of MMO was achieved, using the enzyme purified from soluble extracts of the Type I methanotroph *Methylococus capsulatus*[17,18]. This soluble MMO (sMMO) has subsequently been shown to be present in all methanotrophs. It catalyses the hydroxylation of methane to methanol using NAD(P)H as reductant. It is made up of three components and, as with nitrogenase, sMMO contains metal ions. Of course, resolution of this enzyme was a considerable achievement. Only one of the components could be assayed independently of the other two. This was component C, a flavoprotein containing FAD and an iron sulfide centre as found in spinach ferredoxin and putidaredoxin. Component A is the hydroxylase and contains non-haem iron. Component B is a small colourless protein. Component C transfers electrons from the donor NADH to the hydroxylase (Figure 2).

About this time a 3-component MMO was partially purified by John Higgins and colleagues from membranes from a Type II methanotroph, *Methylosinus trichosporium*; the electron donor was NADH in crude extracts but it was necessary to use ascorbate or cytochrome c in purified preparations[19]. The enzyme was relatively unstable and some results were not always easy to

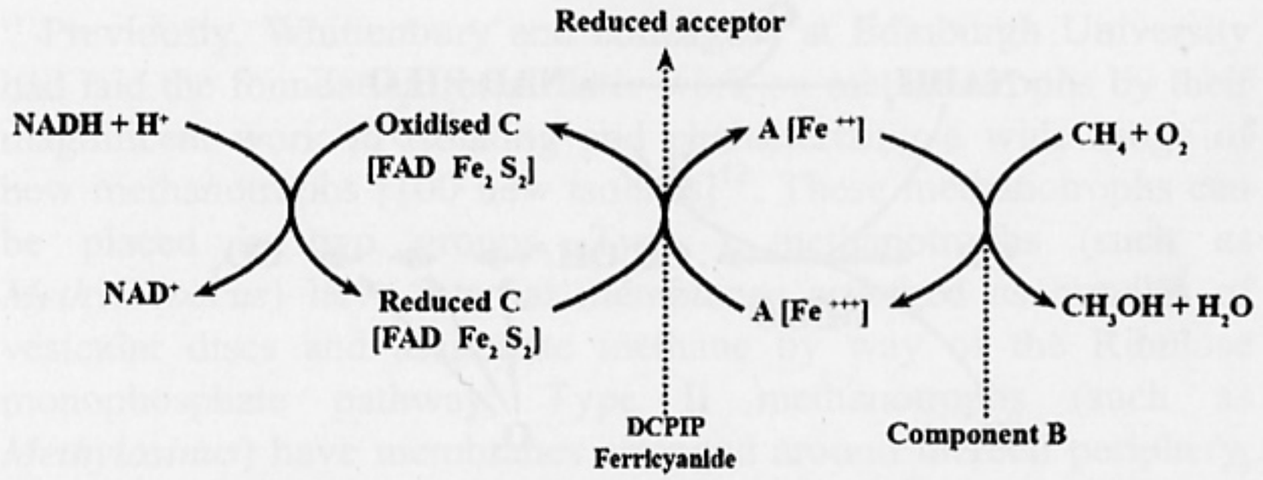

Fig. 2. The pathway of electron transfer between the components of sMMO during oxidation of methane to methanol as proposed by Dalton in 1981.

reproduce. It therefore appeared that there might be two different kinds of MMO or that there might be a single membrane-bound MMO in both types of methanotroph, but that it may be more readily released from its normal association with membranes to produce the sMMO.

This was eventually resolved by Dalton's group in an elegant study using continuous culture, reminiscent of his work on respiratory protection of nitrogenase. It was shown that there are two completely different enzymes in *Methylococcus capsulatus*, the soluble sMMO and also a membrane (or particulate) mMMO. Which enzyme is produced depends on the copper availability; pMMO is produced when the copper/biomass ratio is high whereas sMMO is produced when the copper/biomass ratio is low[20]. In batch culture both MMOs may be produced as the copper/biomass ratio cannot be as well-controlled or defined. Dalton's group subsequently developed reproducible solubilisation and purification methods and showed that the pMMO also has 3 components and that the two types of MMO are present in other methanotrophs, including the type II *Methylosinus trichosporium*[21]. Some methanotrophs synthesise only a single type of MMO and in that case it is the mMMO that is produced. The two families of MMO share no detectable similarity in amino acid sequence or three-dimensional structure.

The substrate specificity of MMOs

A remarkable feature of the MMOs, possibly related to their normal small, unfunctionalised methane substrate is their extraordinary wide substrate specificity, the sMMO having a wider range of

substrates than pMMO. Substrates for sMMO include n-alkanes, n-alkenes, chloromethane, bromomethane, trichloromethane, nitromethane, methanol, carbon monoxide, dimethyl ether, benzene, styrene and pyridine[16]. It was also shown that the sMMO is able to oxidize ammonia, whose structure is clearly analogous to that of methane[22]. In whole cells, in addition to the potential substrate, a source of reducing equivalents must be provided (such as methanol or formaldehyde); when this is required the oxidation of the potential substrate is referred to as co-oxidation[23,24]. Because methanotrophs can co-oxidise a range of hydrocarbons and chlorinated pollutants they have a biotechnological interest that extends far beyond their ability to oxidize methane to methanol. Important examples include the industrial production of methanol from methane, the co-oxidation of propene to epoxypropane, bioremediation of chlorinated hydrocarbons and production of valuable recombinant proteins using methane as the starting material[10].

The structure and function of methane monooxygenases

The structure and function of sMMO

As originally proposed, it is now known that the three components of sMMO are a hydroxylase (originally, component A) with an $(\alpha\beta\gamma)_2$ structure, a reductase [NAD(P)H-dependent] (originally, component C) and the small 16 kDa protein B which acts as a coupling/gating protein. The hydroxylase contains a μ-(hydr)oxo-bridged binuclear iron site which is the site of oxygen activation; it is located in a hydrophobic pocket in the α subunit which is also likely to be the substrate binding site. The reductase component which passes electrons to the hydroxylase from NAD(P)H has FAD and Fe_2S_2 prosthetic groups. Protein B has no metal or prosthetic groups and has been implicated in several roles, including the coupling of electron transfer between the reductase and the hydroxylase, and affecting the rate and regioselectivity of substrate oxidation. It has been shown that it shifts the midpoint redox potential of the hydroxylase, suggesting that it facilitates oxygen binding.

The three dimensional structures of the hydroxylase, the reductase and protein B have been published[25–27] and these are shown in Figures 3–6.

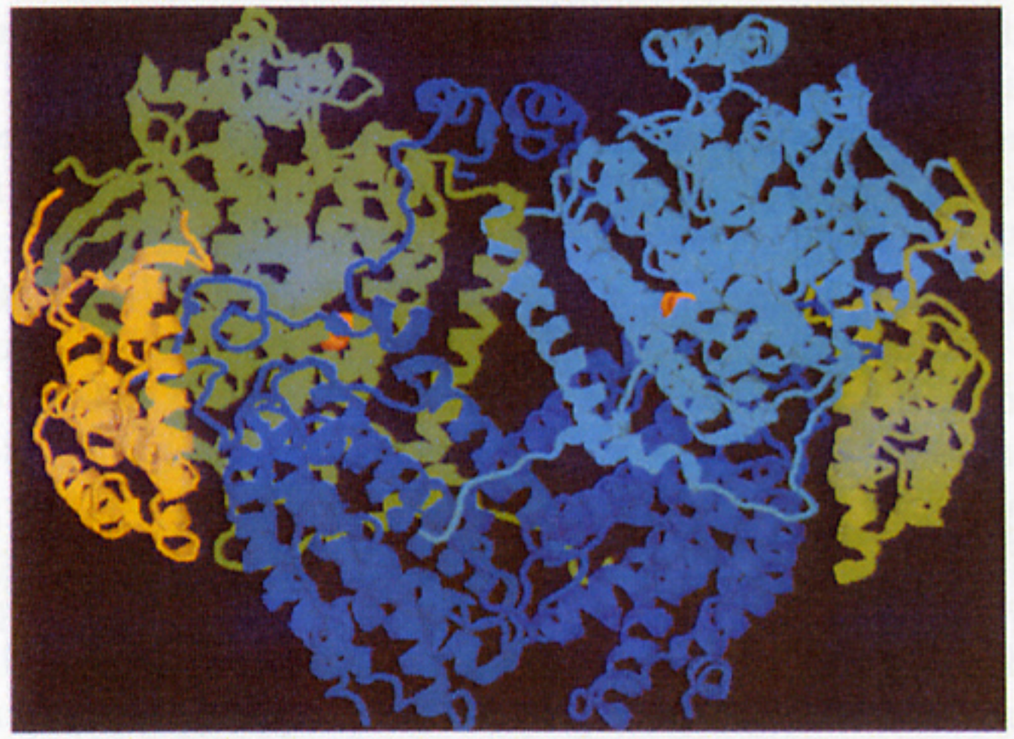

Fig. 3. *Structure of the sMMO hydroxylase from* Methylococcus capsulatus *(Bath) at 2.2 A° resolution. The subunits are coloured as follows: α are pale blue and green; β are royal blue and mid-blue and γ are yellow–green and yellow. The binuclear iron centres are represented by two orange spheres on the α subunits. This Figure is from Dalton's Leeuwenhoek Lecture 2000[8], based on the structure of Rosenzweig* et al.*[25]. Original: Figure 7 from Dalton 2005.* [Dalton, H. *Phil. Trans. R. Soc. B.* **360**, 1207–1222].

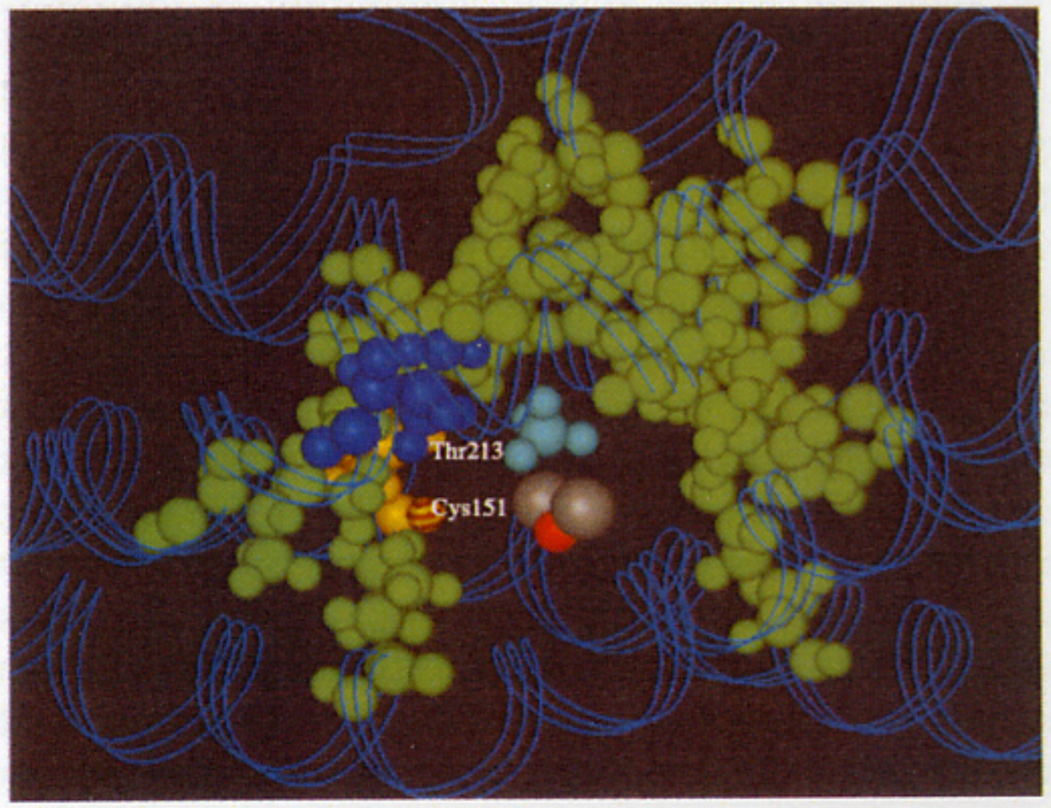

Fig. 4. *The possible binding site for methane in the hydroxylase of sMMO. The hydrophobic residues forming the 'horseshoe-shaped' pocket are shown in green. The binuclear iron centre is in silver/grey, and the bound methane molecule is shown in blue. This Figure is from Dalton's Leeuwenhoek Lecture 2000[8], based on the structure of George* et al.*[26]. Original: Figure 5 from Dalton 2005* [Dalton, H. *Phil. Trans. R. Soc. B,* **360**, 1207–1222]

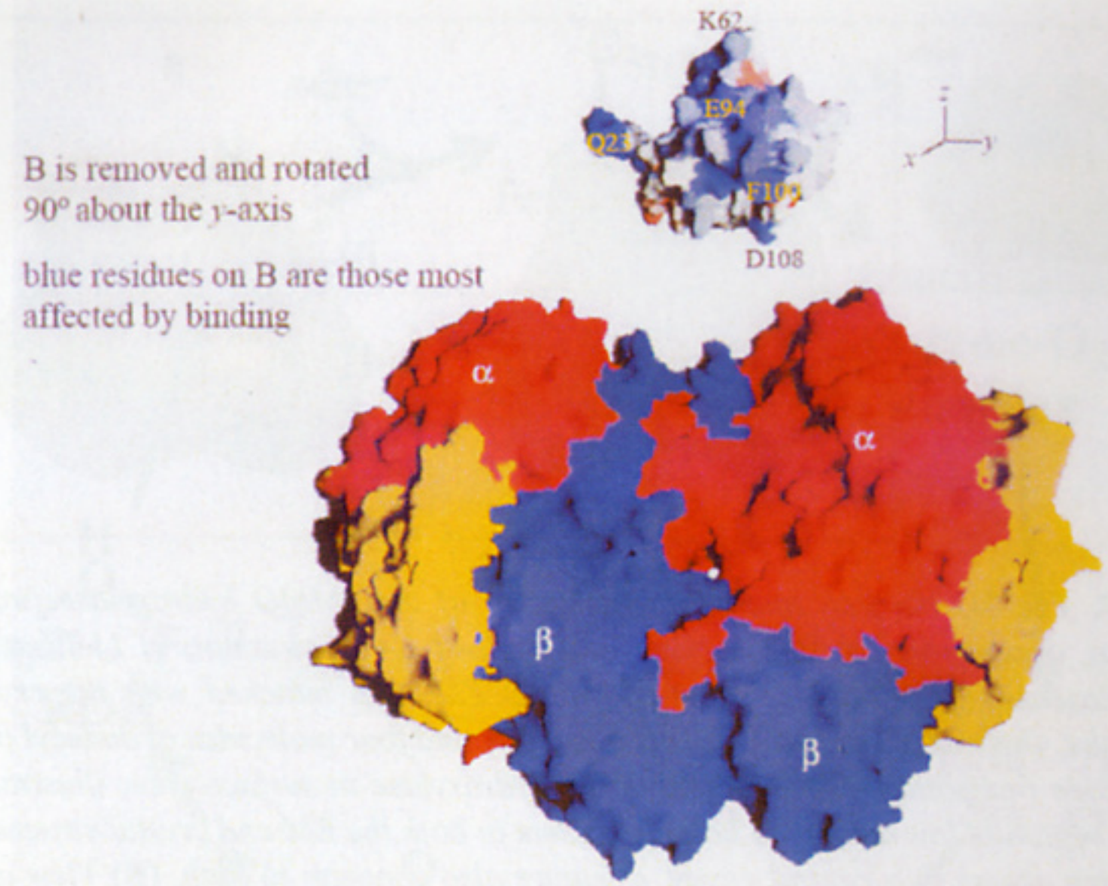

Fig. 5. *Surface diagram model showing the docking of protein B into the canyon on the sMMO hydroxylase). The α subunits of the hydroxylase are shown in red, β in blue and γ in yellow. Protein B has been translated away from its proposed docking site on the surface of the hydroxylase; it has been rotated 90° clockwise about the y-axis to expose the residues most involved in binding. The residues coloured blue are the most affected by binding. This Figure is from Dalton's Leeuwenhoek Lecture 2000[8], based on the structure of Walters et al.[27]. Original: Figure 8 from Dalton 2005* [Dalton, H. *Phil. Trans. R. Soc. B,* **360**, 1207–1222]

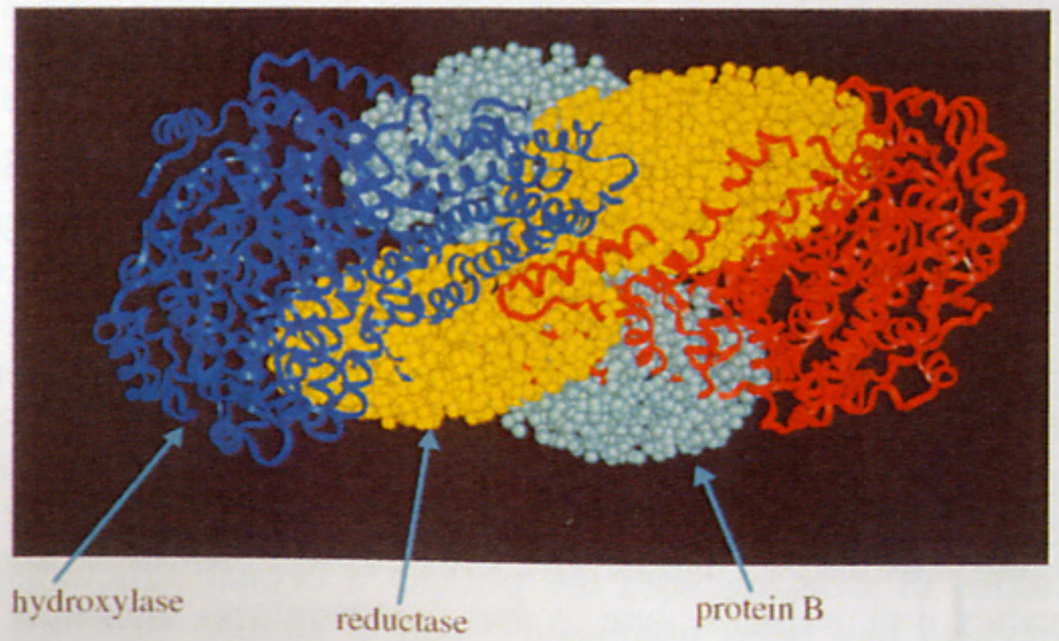

Fig. 6. *Interaction between sMMO components. This Figure is from Dalton's Leeuwenhoek Lecture 2000[8]. Original: Figure 9 from Dalton 2005.* [Dalton, H. *Phil. Trans. R. Soc. B,* **360**, 1207–1222].

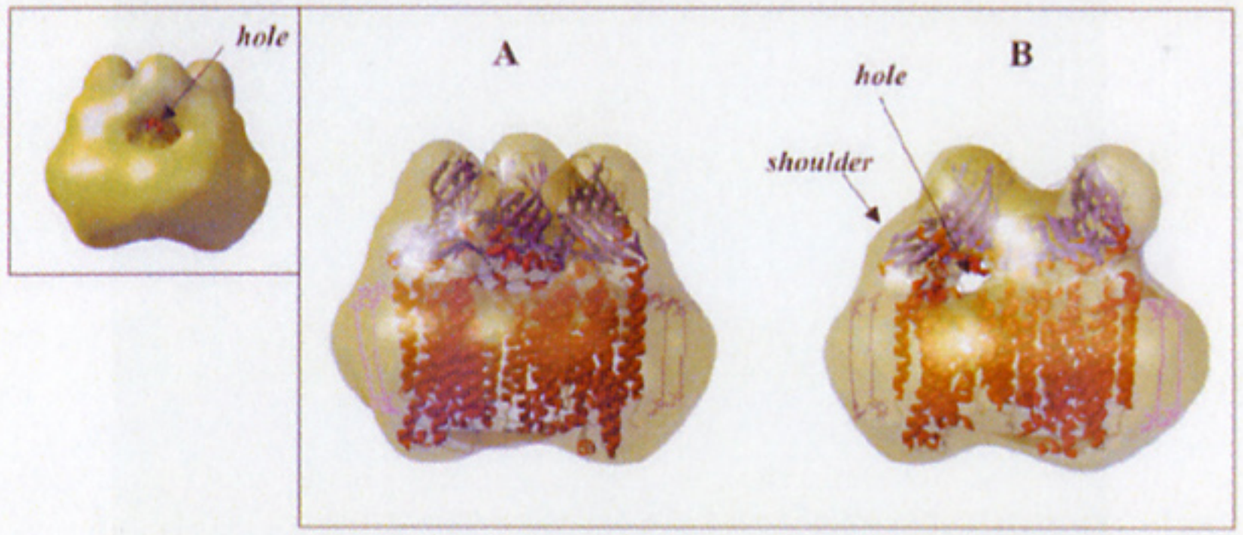

Fig. 7. *The 3D electron microscope structure of the pMMO hydroxylase (transparent), accommodating the previously described X-ray structure of Liebermann and Rosenzweig[20]. **(A)** Side view of the 3D EM map matched with the crystal structure, (helices in red and β-sheets in purple) and four molecules of dodecyl β-D-maltoside (magenta). The inset shows the hydroxylase in surface view, illustrating that a region of low density (a hole) is present in both the EM and crystal structures, allowing access to a central cavity, a feature also common to both.* **(B)** *View as in panel A rotated around the vertical axis illustrating a good match between the orientation of the neck domains and fitting of a shoulder domain with alignment of holes in both structures. This Figure is taken from one of Daltons last publications on MMO[31]. Original: Figure 7 from Kitmitto, Myronova, Basu and Dalton (2005) Biochemistry,* **44**, 10954–10965.

The structure and function of pMMO

Because of the difficulty of working with this membrane MMO relatively little is known of the details of its structure or mechanism. It was shown to contain a three-subunit hydroxylase and a two-subunit reductase but there was considerable confusion about the amounts and nature of the iron and copper in the pMMO[21,28]. It appears most likely that the electron donor to the hydroxylase is quinol from the membrane pool. In the extracted enzyme, this can be provided by duroquinol, as used in Dalton's earlier work on this enzyme[21]. *In vivo*, this may be produced by reverse electron transport from formaldehyde or from methanol by way of methanol dehydrogenase and cytochromes *c*. It was earlier shown by Dalton, using acetylene as a suicide substrate, that the α-subunit of the hydroxylase contains the active site of the enzyme[29]. In spite of the difficulties of working with the pMMO a crystal structure of the hydroxylase has been published[30] and a similar structure using electron microscopy and single particle analysis (SPA), this being one of the last of Howard Dalton's published research papers[31]; Figure 7 is taken from that paper.

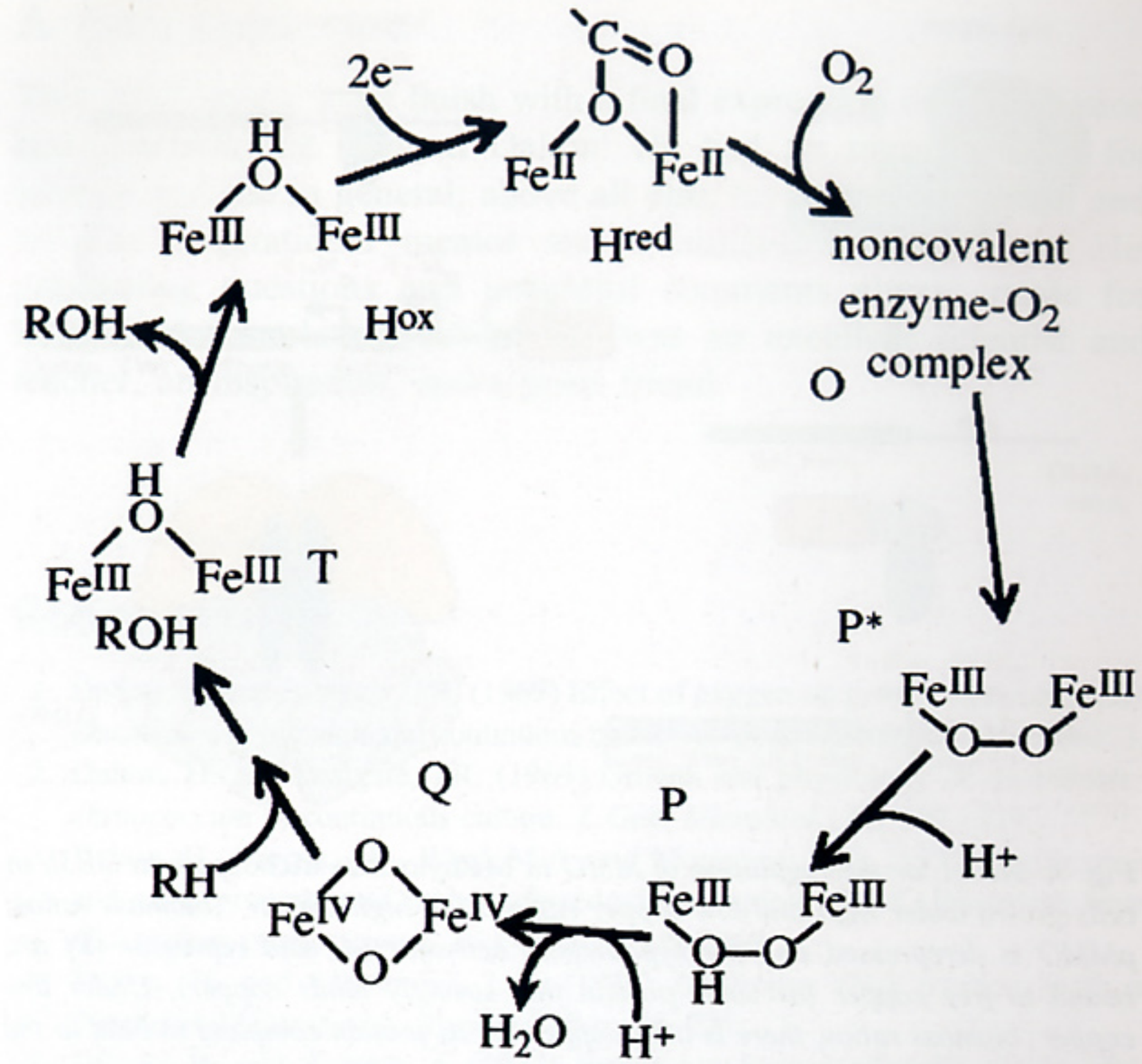

Fig. 8. *Principal intermediates during the sMMO catalytic cycle. This Figure is based mainly on the work of the groups of Dalton, Lippard and Lipscomb; it is taken from Dalton's Leeuwenhoek Lecture 2000[8] in which the significance of the intermediates is fully discussed. Original: Figure 6 from Dalton 2005.* [Dalton, H. *Phil. Trans. R. Soc. B,* **360**, 1207–1222].

The catalytic cycle of sMMO

Figure 8 shows the catalytic cycle of sMMO. In the resting state the binuclear iron centre is in the diferric oxidation state and this must be reduced to the diferrous form to allow oxygen to bind. The two electrons for this reduction are provided by the reductase component which feeds the two electrons from NAD(P)H one at a time into the binuclear centre[32–34]. The actual mechanism for **C—H** bond cleavage is uncertain[8] but it may involve free radicals as Dalton had originally suggested[8,35]; full references to his work and to the important work on the catalytic cycle by the groups of Lipscomb and Lippard are given in the 2005 review by Dalton[8].

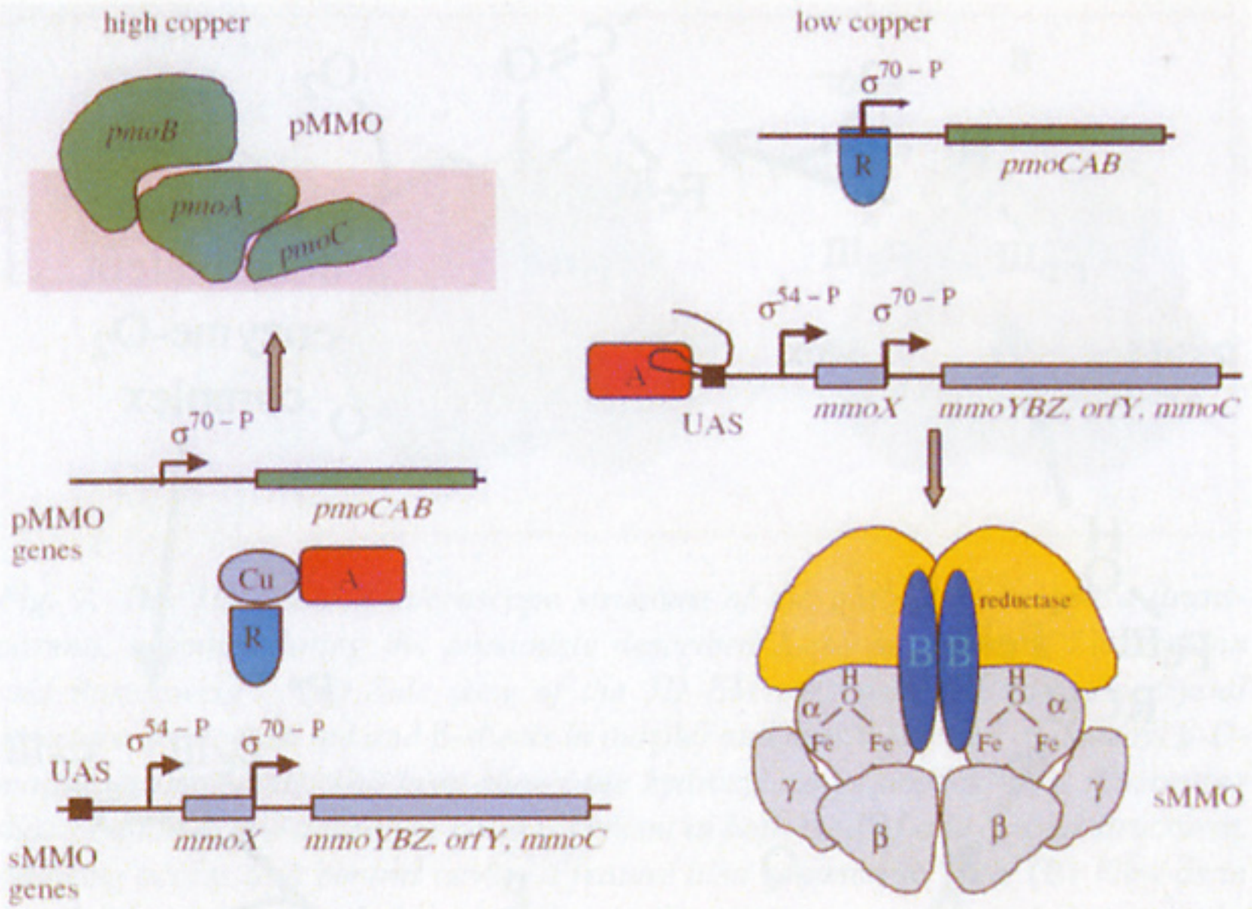

Fig. 9. *Model for the regulation of MMO in* Methylosinus trichosporium *OB3b in cells grown under high and low copper regimes. At high copper : biomass ratios, pMMO is derepressed and the hypothetical activator (A) and repressor (R) are bound to free copper (or some protein that strongly binds copper). Under low copper : biomass ratios, there is little copper (or its protein complex) to bind to the repressor, so R binds to repress pMMO transcription. The free activator can now bind to the upstream activating sequence (UAS) of* mmoX *to permit transcription of the sMMO-encoding genes This Figure is from Dalton's Leeuwenhoek Lecture 2000[8], based on the review by Murrell* et al.*[36]. Original: Figure 4 from Dalton 2005.* [Dalton, H. *Phil. Trans. R. Soc. B,* **360**, 1207–1222].

The molecular biology of methane oxidation systems

Work on the molecular biology of MMO was initiated by Colin Murrell in Dalton's group at Warwick, first showing that sMMO is encoded by a six-gene operon; the genes for pMMO are encoded in a similar way and expression of the two operons is regulated by the copper in the growth medium[36] as indicated in Figure 9. Summaries of developments in the analysis of genetics of methanotrophs and their exploitation in the understanding of methanotroph physiology and biochemistry, and production of heterologous proteins is described more extensively elsewhere[10].

A final thank you

This brief review must finish with a final expression of appreciation and gratitude for Howard Dalton. He had an immense zest for science and life in general; above all else, he made science fun and was an inspirational mentor and a much-loved colleague. His penetrating questions and insightful comments always made for lively and stimulating debate. He was an excellent scientist and teacher, an inspiration, and a great friend.

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