Chris Anthony

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Methylotroph stories; dedicated to research students everywhere (May 2024)

This is a power point presentation of a lecture I gave on 20th May to the Microbiology group in Biological Sciences, University of Southampton

The lecture summarised my 40 years of work on methylotroph metabolism in 40 minutes.

I have added a few extra slides to make the stories complete.

VIDEOS can be speeded up by downloading an **accelerator**

HOME

Methylotroph stories

Dedicated to research students everywhere

My CV

Born Watford in 1939, son of a brewery labourer.

1950 – 1957 Watford Grammar School

1957 – 1960 Reading University BSc Microbiology

1960 – 1963 Reading University PhD Thesis: The microbial metabolism of one-carbon compounds

1963 – 1966 Reading University Post Doc: A novel methanol dehydrogenase

1966 - 1967 Sheffield University Post Doc: glutamate fermentation in Clostridia

1967 – 2004 Southampton University: Lecturer etc

2005 – present time: Southampton University: Emeritus Professor

Thank you to my research students (1969 – 2008)

Methanol dehydrogenase	**Cytochromes and electron transport**	**Metabolic pathways**	**3D structures**
Steven Ford	Jim Elliott	Pat Dunstan	Peter James
Dudley Page	Steven Froud	Iain Taylor	Paul Williams
Darren Day	David O'Keefe	John Bolbot	Gyles Cozier
Jonathan Cox	Andrew Cross		Simon Dales
Ian Richardson	Mathew Beardmore-Gray		[John Read]
Alain Avezoux	Anthony Long		[Chenyi]
Karen Amaratunga	Claude Chan		[Leigh Felton]
Tosin Majekodunmi	Helen Ashworth		
Paul Afolabi			
	Also Bill Keevil (Post-doc); Paul Skipp (Technician)		
Amino acid transport	**Electron transport and nitrogen fixation**	**Neomycin biosynthesis**	**Electron transport in methylamine metabolism**
Jeff Robinson	Andrew Smith	Cedric Pearce	Ashley Lawton
Bob Cook	Nick Juty		Kevin Auton

Methylotroph stories

Methylotrophs are bacteria that grow with compounds with a single carbon atom as their sole source of carbon and energy. 'Ordinary bacteria' never grow methylotrophically.

1. The rise of the methylotrophs. Only 2 species in 1960. Many hundreds now – with enormous genetic and metabolic diversity.

2. Methanol dehydrogenase (MDH); the first Quinoprotein.

3. A special electron transport chain for methanol oxidation.

4. A remarkable resurgence of interest in MDH.

My PhD project (1960 – 1963): Isolate a bacterium able to grow on methanol and therefore able to oxidise it. Then find the enzyme responsible.

1. The rise of the methylotrophs.

Always specialised genera; never the usual types of bacteria
Many grow well on 'ordinary' substrates – succinate, lactate etc

Only 2 species in 1960. Only 4 papers in my PhD introduction

Many hundreds of species now – with enormous genetic diversity

A wealth of novel metabolic pathways for conversion of formaldehyde to cell material

Unusual energy transduction

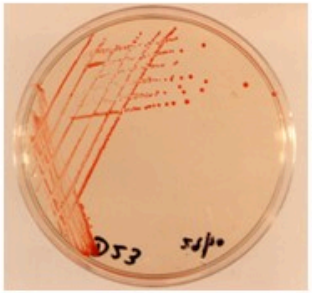
Regular international symposia (from 1970 to present day)

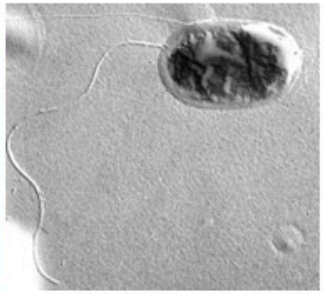
Industrial exploitation. This is because of their metabolic diversity and because their growth substrate, methanol, is inexpensive

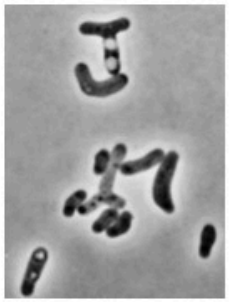
The main 'workhorses' in the study of methylotrophs

- *Pseudomonas* AM1 (Peel & Quayle, 1961)
- *Pseudomonas* sp. M27 (Anthony & Zatman, 1964)

Methylobacterium extorquens. A pink facultative methylotroph

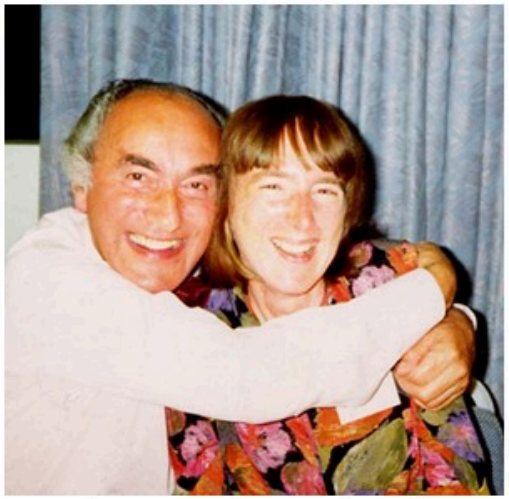






Me in 1963

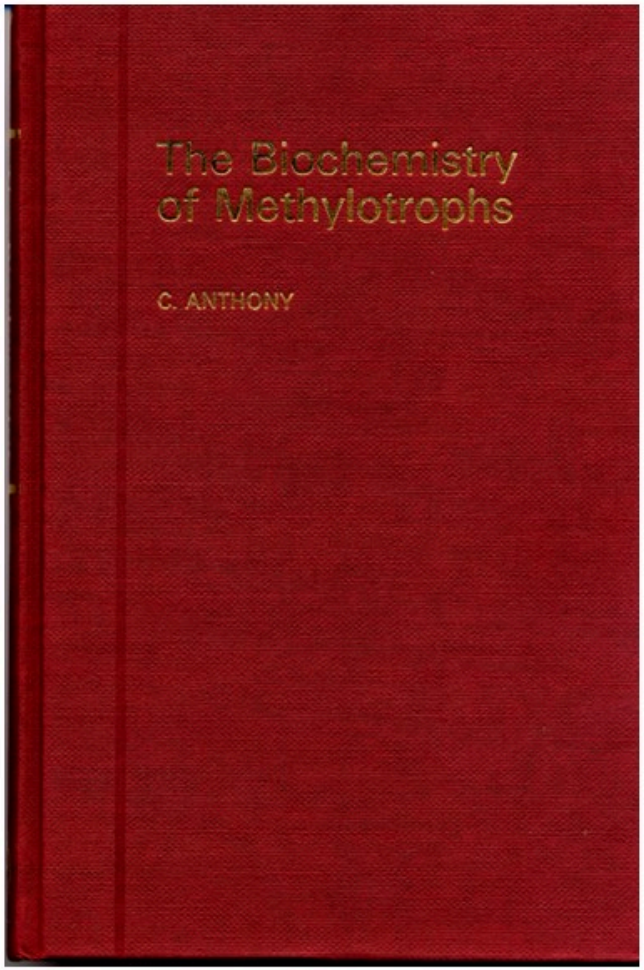
J Rod Quayle



My PhD supervisor and my first research student (Len Zatman and Pat Dunstan, now Goodwin)

Howard Dalton, Rod and me

The Biochemistry of Methylotrophs 1982



The Biochemistry of Methylotrophs

C. ANTHONY

Department of Biochemistry
University of Southampton
England

Chapters 12
Text pages 350
Index 48 pages
Tables 60
Figures 57
References 1300

1982

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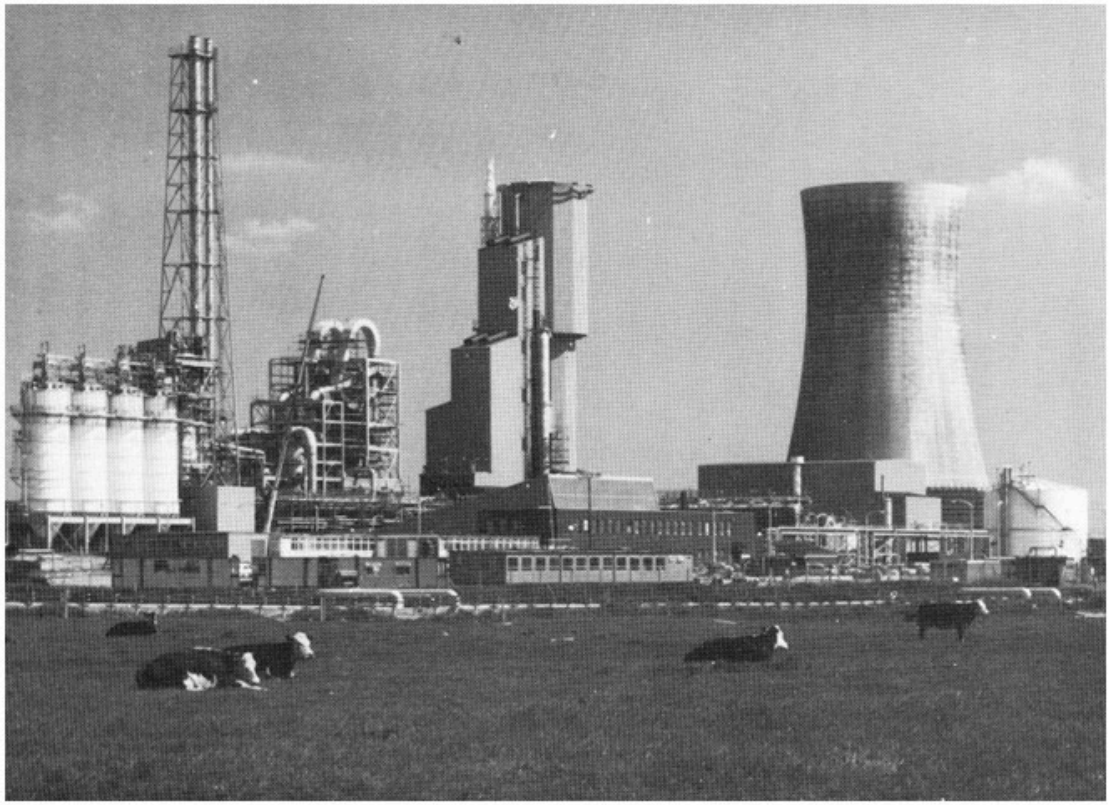
A CD version is available and the complete b
is on my website: chris-anthony.co.uk

The ICI 'Pruteen' plant at Billingham, UK 1980
For production of protein supplement for cattle feed



The centre tower is the 1.5 million litre fermenter

It contains about 100,000,000,000,000,000,000 methylotrophs
10% of their soluble protein is Methanol dehydrogenase

2nd Story: Methanol dehydrogenase

*An enzyme with a remarkable structure and novel type of prosthetic group - **PQQ** (the first Quinoprotein). The false claim that PQQ is a new vitamin*

Oxidising enzymes.

Most common: alcohol + NAD+ → aldehyde + NAD + H+

Alternative oxidising enzymes are membrane bound and have FAD or FMN as a bound prosthetic group: succinate + Enz-FAD → fumarate + Enz-FADH2

Most living things have an NAD-linked alcohol dehydrogenase
BUT none oxidises methanol so my supervisor, Len Zatman, thought there might be an interesting dehydrogenase in bacteria able to oxidise methanol to formaldehyde.

My project: Isolate a bacterium able to grow on methanol and therefore able to oxidise it. Then find the enzyme responsible.
I thought this was a trivial problem but it was not.
My first paper was on a novel methanol dehydrogenase (1964)
My last paper was on the very high (atomic) resolution of this enzyme (2004)

We published 2 papers together. The first described our new methylotroph and the next slide is the second.

Methanol dehydrogenase

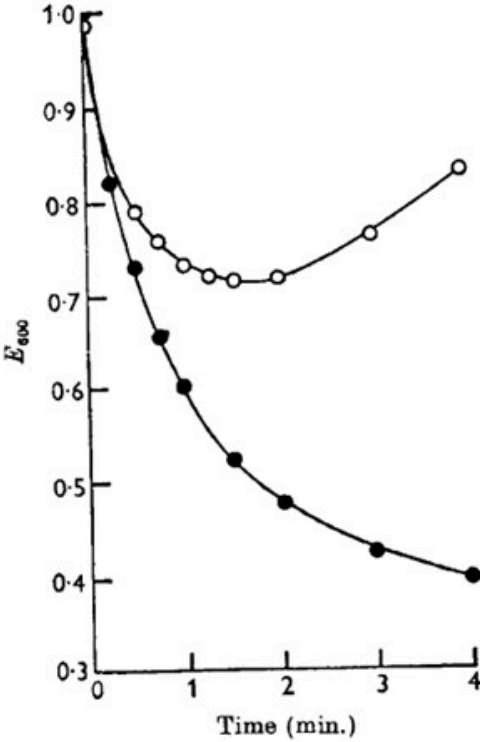
Responsible for all bacterial methanol oxidation

The Microbial Oxidation of Methanol

2. THE METHANOL-OXIDIZING ENZYME OF *PSEUDOMONAS* SP. M 27

By C. ANTHONY and L. J. ZATMAN
Department of Microbiology, University of Reading

(*Received* 21 *November* 1963)



MDH assay
with PMS/indophenol

A soluble enzyme – now known to be in the periplasm

Dye-linked assay (PMS/PIP); pH optimum 9.2; pI 8.2

Methanol + PMS ⟹ Formaldehyde + $PMSH_2$
$PMSH_2$ is oxidised by blue PIP which loses it colour

Activator is ammonia (not NH_4+ ion) (this is unique)

Wide substrate specificity

Unusual absorption spectrum – not FAD or FMN so novel prosthetic group??

High proportion of tryptophans

Isolation of the prosthetic group

The prosthetic group is the part of the enzyme that is not the amino acid chain.

It is FAD or FMN in flavoproteins, or haem in cytochromes

Grow huge amounts of bacteria on methanol. Harvest and break (ultrasound)
Purify the enzyme from the soluble fraction (Ion exchange, Sephadex etc)

Precipitate the protein and release the fluorescent prosthetic group by acid treatment

Purify the prosthetic group (ion exchange etc). After 3 months I had a few red grains.

Characterised by its excitation and emission fluorescence. A beautiful green colour.

The only known compound with a maximum excitation at 360nm with this maximum occurring at low pH. It is tightly bound to the enzyme.

SO a completely new compound. Thirteen years later its structure was determined by X-ray crystallogaphy at Cambridge; then confirmed by us a further 15 years later with the X-ray structure of the whole enzyme.

A new type of enzyme prosthetic group

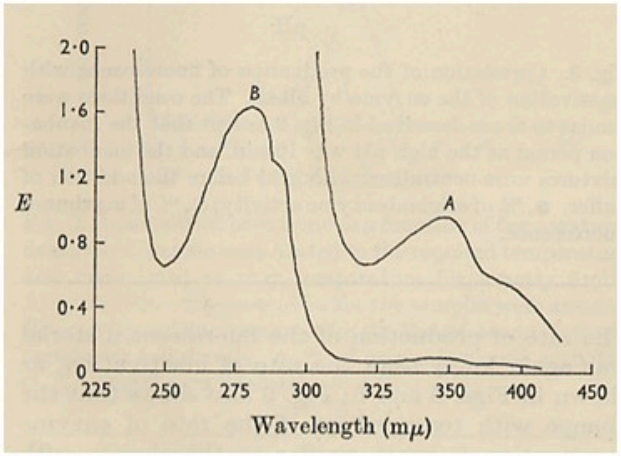
Note: all other dehydrogenases use NAD(P) as hydrogen acceptor or they ar membrane flavoproteins that use FMN or FAD as prosthetic group

The Microbial Oxidation of Methanol

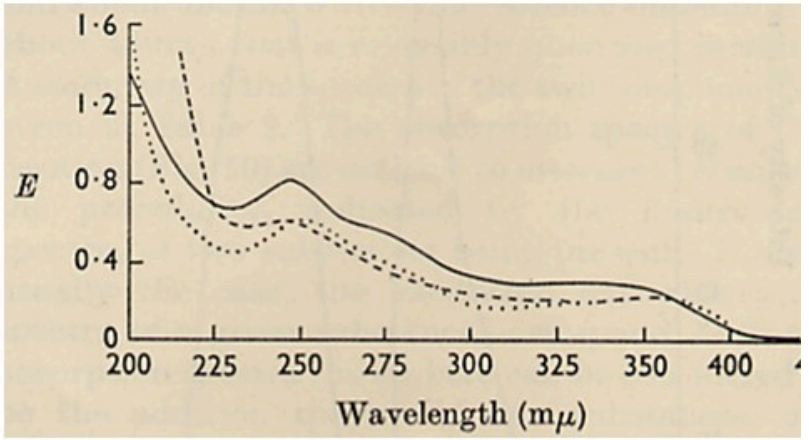
THE PROSTHETIC GROUP OF THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27: A NEW OXIDOREDUCTASE PROSTHETIC GROUP

By C. ANTHONY* AND L. J. ZATMAN
Department of Microbiology, University of Reading

(*Received* 30 *December* 1966)



Spectrum of MDH



Spectrum of prosthetic group

PQQ and other quinoproteins The novel prosthetic group of MDH was later in other dehydrogenases an Hans Duine and Hans Frank called these Quinoproteins. These include the alcohol dehydrogenase of acetic acid bacteria and the typical glucose dehydrogenase in *Pseudomonas*, *E. coli*.

Hauge described GDH and PQQ very early on

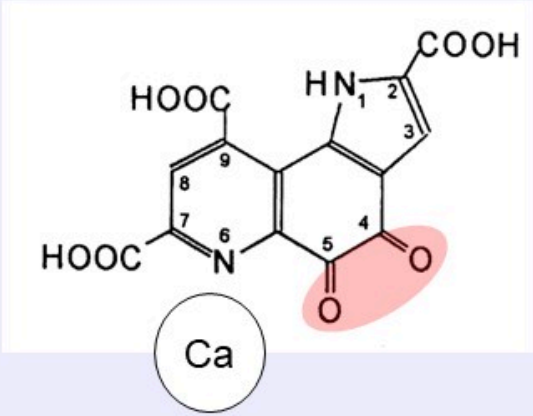
Hans Duine and Hans Frank
'invented' quinoproteins

Friends from Yamaguchi, Japan:
Matsushita, Uragami (?), Ameyama, Adachi.
Found many new quinoproteins especially in
Acetic acid bacteria

Note: No PQQ-containing quinoprote have been described in anything othe than bactera

PQQ and Quinoproteins

Pyrroloquinoline Quinone PQQ)



First isolated and purified by Anthony & Zatman in1967 from bacterial methanol dehydrogenase

Structure determined by Kennard's group in1979

Remains tightly bound throughout reaction cycle

PQQ-containing Quinoproteins

All bacterial dehydrogenases

Usually responsible for 1st step in the oxidation of growth substrate

Mainly alcohols and aldoses

eg ethanol oxidation to acetic acid in acetic acid bacteria

Reaction occurs in the periplasm

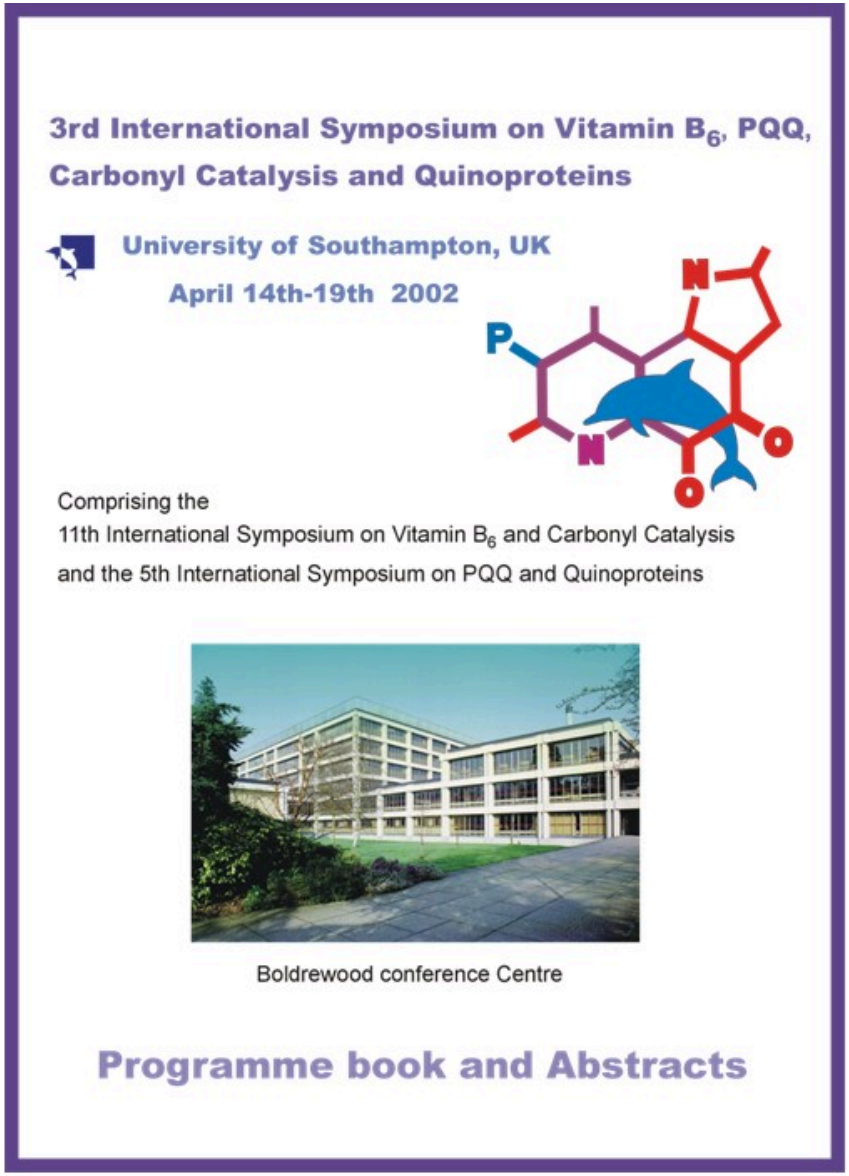
Electron acceptors are cytochromes, blue copper proteins or ubiquinone

May be soluble or membrane-bound

May also have contain haem

None has been described in eukaryotic organisms

From 1988 onwards there have been International Symposia on PQQ and Quinoproteins The 5th was in Southampton in 2002

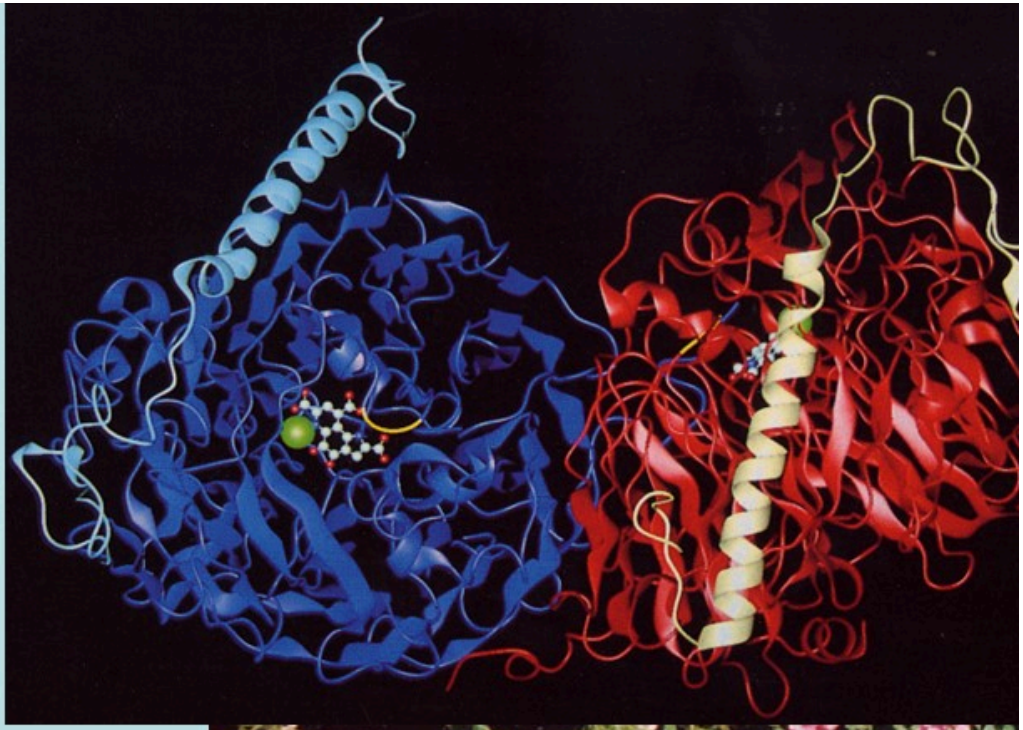


The following slide was included to show what was lost when Boldrewood was demolished. This was my room, complete with coffee machine and comfort. Murali with back showing was a 4th year medical student solv a problem with his project and the other is a research student friend Leigh Felton.
Staff rooms were always availble for students to see members of staf.

My office with Murali and Leigh

The foolowing section is describes our work with MDH which was done some years later in collaboration with Meenakshi Ghosh who came to Oxford from Calcutta to learn how to do X-ray crystallography of proteins. She had previously had experience only with inorganic crystals. She needed a project so i provided it.

The X-ray structure of MDH 1992-2004



Southampton:
Chris Anthony
Alain Avezoux

Oxford:
Meenakshi Ghosh,
Colin Blake

MDH is a dimer
Large subunit is 60k
Small subunit is 9k

A wonderful collaboration; Meenakshi knew no biochemistry and i knew no crystallography and its interpretation

The ß-propeller structure of MDH

Eight 4-stranded ß sheets (W1-8); these are 'propeller blades'. The 'propeller fold' forms a superbarrel with active site inside

Active site contains PQQ and calcium (green sphere)

The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'

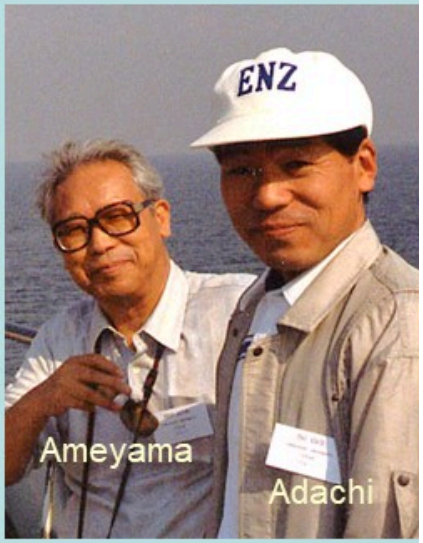
We made the mistake of relying on an 'expert' to asssy our enzyme for metal ion content and he found none. After our Japanes friends showed that their Ethanol dehydrogenase contained calcium we found it in MDH. Our work with a mutant lacking calcium enabled us to reconstitute it with strontium or barium.

The calcium ion in MDH

1965-1989 ' MDH contains no metal ion' [Anthony & Zatman]

1986 MDH from mutants (*mxaA, K & L*) was inactive and had unusual spectrum [Nunn & Lidstrom]

1990 MDH contains Calcium (or strontium) [*Adachi et al.*]



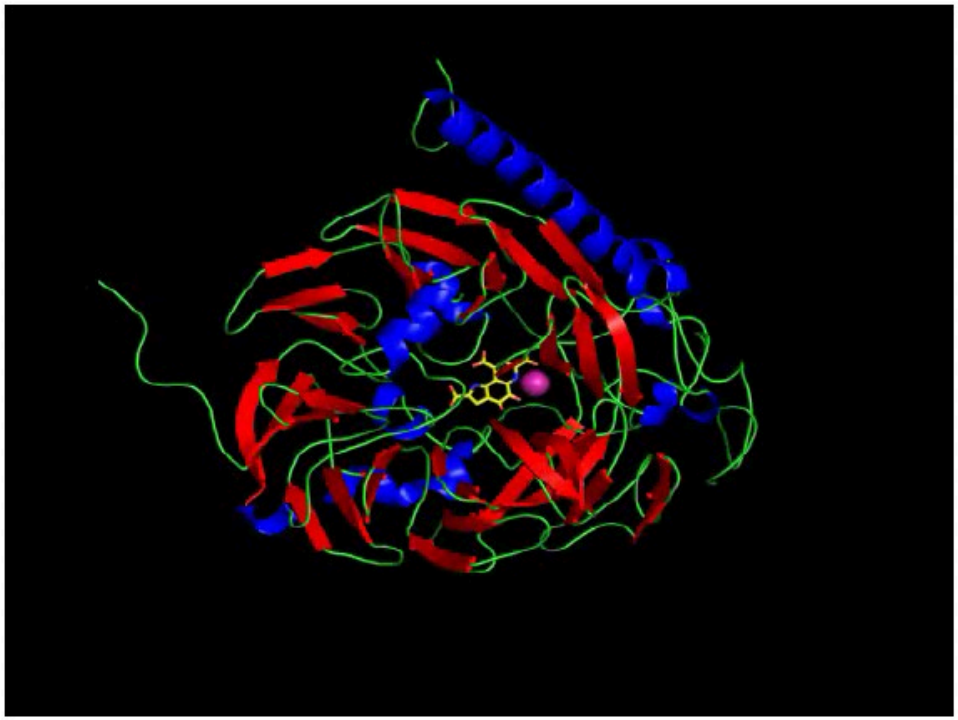
1992 Other MDHs contain calcium.
MDH from *mxaA, K & L* lacked Calcium;
incubation with Ca produced active enzyme & normal spectrum [Ian Richardson]

1992 MDH isolated from marine *Methylophaga Marina is red and inactive;*
incubation with Ca produced
normal green active enzyme [Claude Chan]

The calcium ion in MDH

1994 Kinetics of MDH containing strontium *[Harris & Victor Davidson]*

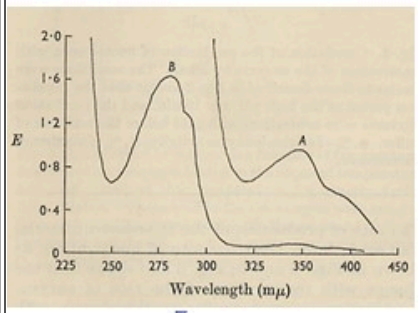
1996 Incubation with Barium produced MDH which was more active than wild-type MDH [Matt Goodwin, Alain Avezoux & Simon Dales]

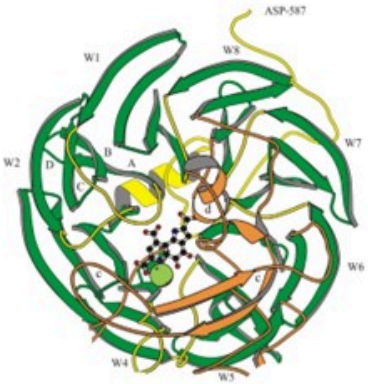


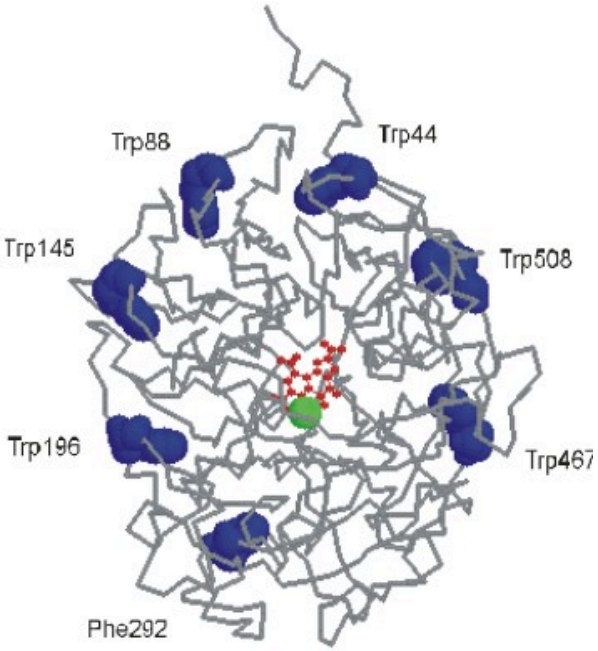
Tryptophan docking system

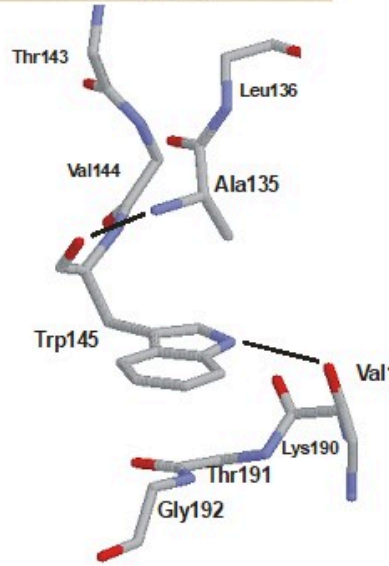
High proportion of tryptophans;
19 per α subunit

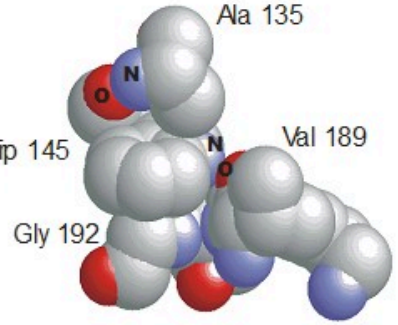
The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'











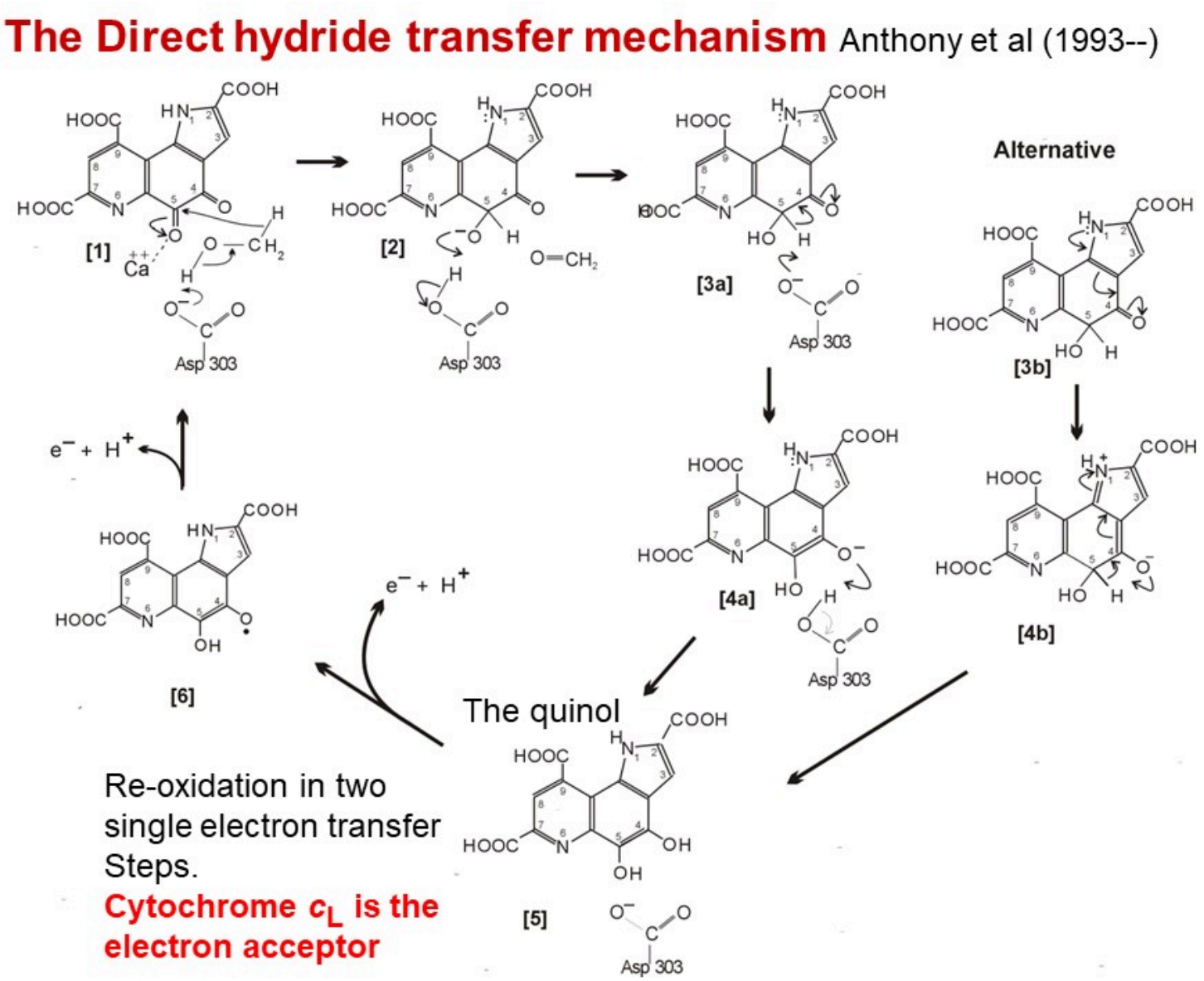
The following slide shows the consensus sequence involved in the docking motifs which demonstrated to Kashahara and Kato that they had a propeller structure which they wrongly interpreted as evidence for a quinopı

The consensus sequence in the tryptophan-docking motifs

Propeller blades W1-W8

Position	1	2	3	4	5	-	6	7	8	9	10	11
Motif	C	C	C				D	D	D	D	D	D
W1	Ala 77	Leu	Gly	Leu	Asp	Asp	Pro	Gly	Thr	Ile	Leu	Trp 88
W2	Ala135	Leu	Asn	Ala	Glu	-	Thr	Gly	Glu	Thr	Val	Trp145
W3	Ala 186	Tyr	Asp	Val	Lys	-	Thr191-Gly192		Glu	Gln	Val	Trp 196
W4	Gly 282	Arg	Asp	Ala	Asp	-	Thr	Gly	Glu	Ala	Lys	Phe 292
W5	Thr 337	Leu	Asp	Arg	Thr	-	Asp	Gly	Ala	Glu	Val	Ser 347
W6	Ala 457	Tyr	Asn	Ala	Ile	-	Thr	Gly	Asp	Tyr	Lys	Trp 467
W7	Ala 489	Arg	Asp	Ser	Asp	-	Thr	Gly	Asp	Leu	Leu	Trp 508
W8	Val 577	Phe	Ser	Leu	Asp 581	-	Gln 39	Leu	Arg	Pro	Ala	Trp 44
MDH Consensus	Ala	X	Asp/ Asn	X	X	-	Thr	Gly	Asp/ Glu	X	X	Trp
GDH/ADH Consensus	Ala	X	Asp/ Asn	X	X	-	Thr	Gly	Lys	X	X	Trp

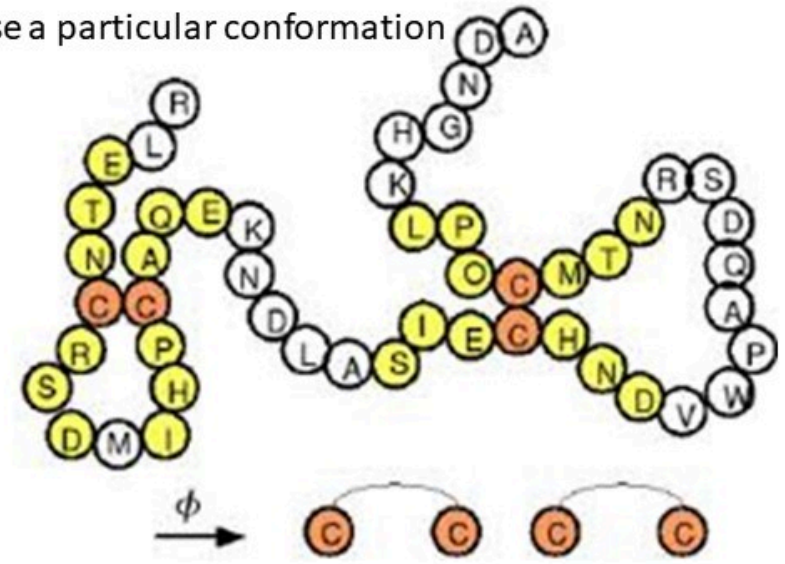
For example: **Trp145** of strand D in W2 is stacked between **Ala135** in strand C in W2 and **Thr-Gly** at position **6-7** in W3



The mechanism involves an initial proton abstraction (by Asp 303) and a hydride transfer from the methyl group of methanol. The quinol then passes its electrons one at a time to the specific cytochrome electron acceptor. After a single electron has been transferred the free radical PQQH is stable, and MDH is usuall isolated with PQQ in this form (Shown by Frank and Duine)

The unique disulphide ring structure in the MDH active site

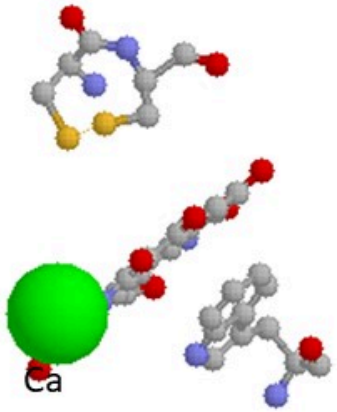
Two distant Cysteine residues in proteins can be oxidised to form a disulphide bridge that is used to stabilise a particular conformation



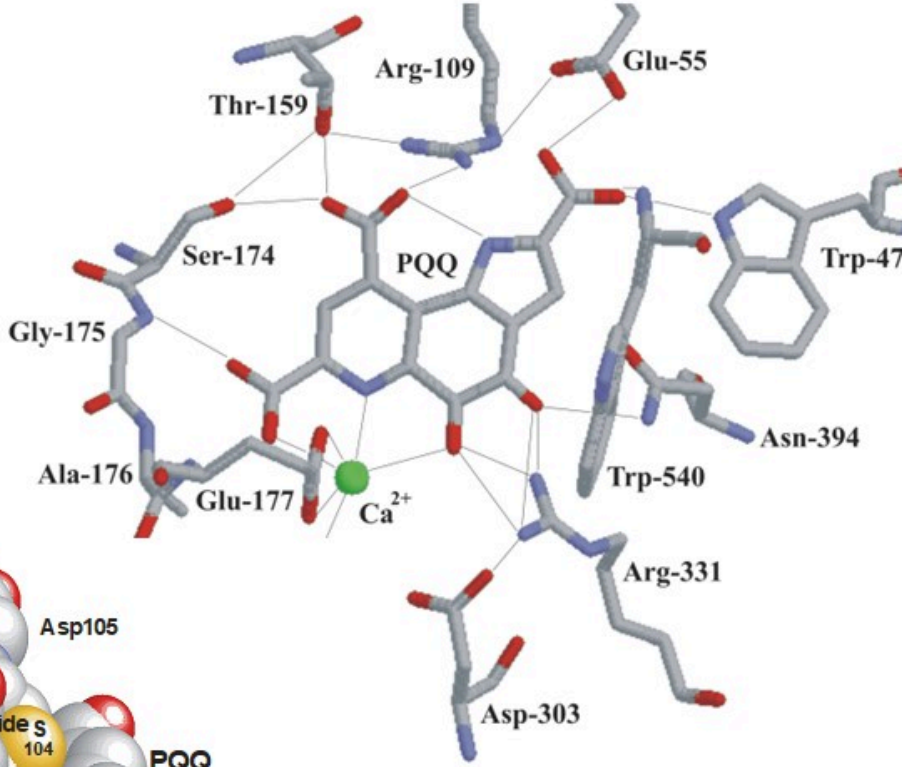
Ramachandran stated that 2 adjacent cysteines would never form a bridge. BUT if they did then they would form an 8-membered ring structure with a 'strained' peptide bond.

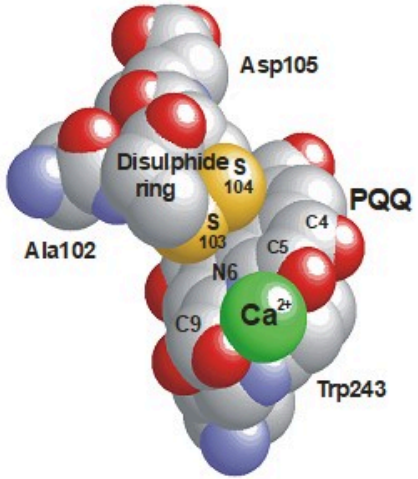
None has ever been seen except in the active site of MDH.
It is very easily reduced, so breaking the bond and inactivating the enzyme.

The active site of MDH



Novel disulphide ring structure from adjacent cysteines



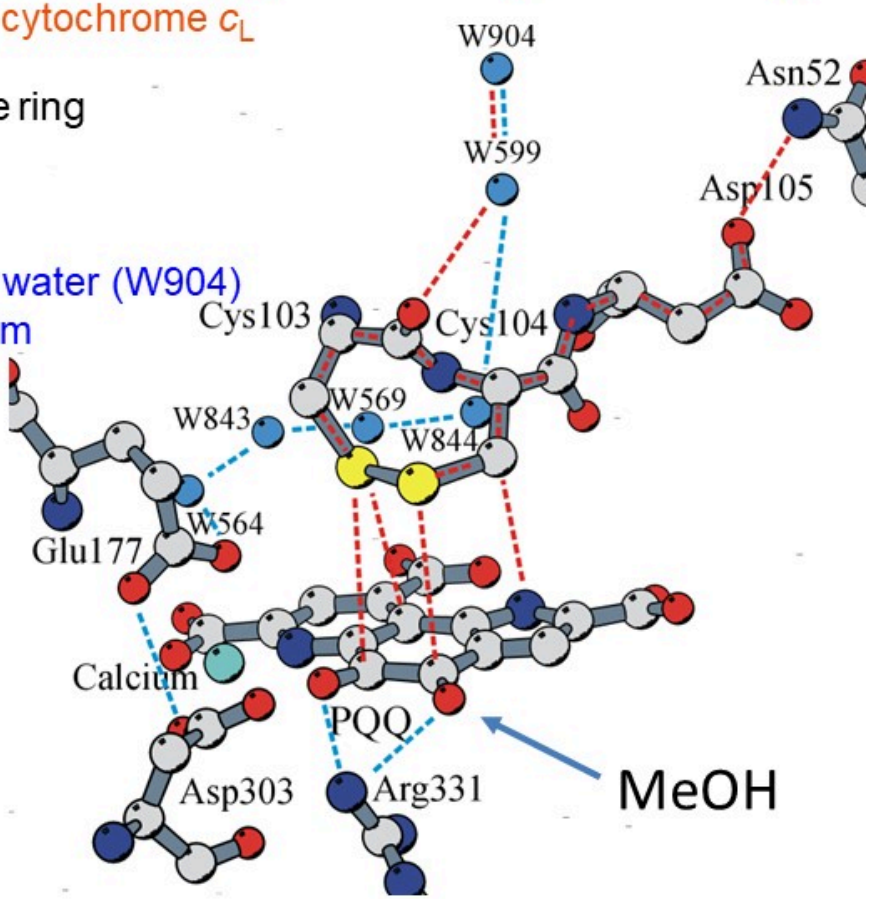


The path of electrons and protons during the oxidative part of the reaction mechanism of MDH

Electrons must pass from reduced PQQ ($PQQH_2$) to a unique type of cytochrome - cytochrome c_L

Note involvement of the disulphide ring

Protons must pass from PQQH2 by way of its hydroxyls, and the hydrogen-bonding network, to the water (W904) which is accessible to the periplasm



The pathways are based on those proposed for the quinohaemoprotein alcohol dehydrogenase by *Oubrie, Dijkstra et al, and Chen, Matsushita, Toyama, Scott Mathews et al.*

False claim that PQQ is vitamin

Most enzyme cofactors cannot be made in humans so have to be provided as their precursors – vitamins. Eg: NAD (Niacin), FAD (riboflavin)

If mammals do have quinoprotein enzymes then PQQ is almost certainly a vitamin.

In 2003 Kashara and Kato published a paper in Nature claiming to have found a gene coding for a quinoprotein and announcing that this meant that PQQ is "The first new vitamin for 55 years".
This was welcomed with a great fanfare

Their argument was so obviously false so I ignored it.

BUT two years later Mitsubishi announced that they were investing 4 million dollars in producing this new vitamin. So I contacted them and published a paper in Nature explaining why it is not. But Mitsubishi went ahead and now it is on the market as a growth nutrient!

Premature announcement that PQQ is a vitamin for mammals

brief communications

Nutritional biochemistry

A new redox-cofactor vitamin for mammals

Takaoki Kasahara, Tadafumi Kato
Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Japan

NATURE | VOL 422 | 24 APRIL 2003 | www.nature.com/nature

Scientists Add New Member to Vitamin B Family
---Sarah Graham, Scientific American, April 24, 2003.

A new page may need to be added to the vitamin B family album. According to a report published today in the journal Nature, researcher have determined that the compound known as pyrroloquinoline quinon (PQQ) should be classified as a vitamin in the same class as vitamins B and B3. Scientists first isolated PQQ from bacteria in 1979.

MITSUBISHI GAS CHEMICAL COMPANY, INC.

Biological Products

September 24, 2003

Mitsubishi Gas Chemical Company Inc. speeds the development of application of coenzyme PQQ-vitamin.

IN THE NEWS

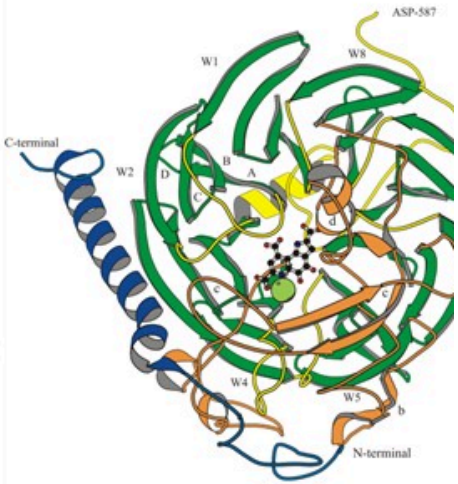
Japanese Discover First New Vitamin in 55 Years

April 24, 2003
By REUTERS

Mind your PQQ - properties of pyrroloquinoline quinine, nutrient - vitamins and supplements in a bodybuilder's di

The argument by Kasahara and Kato

Search engines 'identified' PQQ-binding sites in a protein in mice [predicted from a gene sequence]



Our argument against

We cannot identify PQQ-binding sites in a database

In fact, the search engine found 7 'propeller blades' that typically occur in PQQ-containing quinoproteins

The 'propeller blades' – wrongly identified by the search engine as 'PQQ-binding sites' are not directly involved in binding PQQ; they are purely structural

All trees are green	All PQQ enzymes have propeller structure
My coat is green	My protein has a propeller structure
SO: My coat is a tree	SO: My protein is a PQQ enzyme

Therefore **PQQ is a vitamin**

brief communications

Nutritional biochemistry

A new redox-cofactor vitamin for mammals

Premature announcement that PQQ is a vitamin for mammals

Leigh M. Felton and Chris Anthony:
Nature; Communication Arising

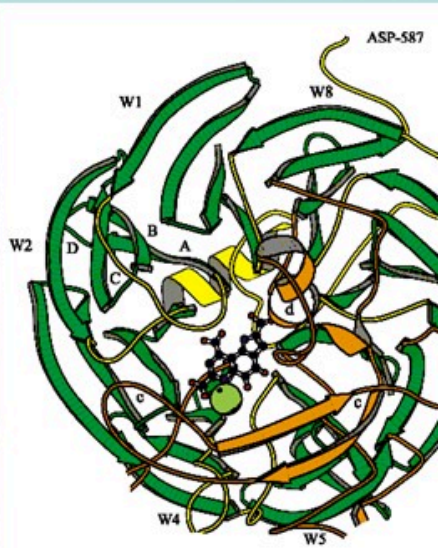
The only evidence that the proposed dehydrogenase is PQQ-dependent is that the Smart and Pfam databases 'identified' '7 PQQ-binding sites' in the C-terminal regio

In fact, the 'sites' are sequences predicted to form a series of ß-sheet 'blades' in a 'propeller fold' as seen in PQQ-dependent dehydrogenases.
This fold has nothing to do with PQQ binding and is found in many other proteins

The proposed enzyme has no sequence similarity to any PQQ-dependent dehydrogenase

There is still no evidence that PQQ
Is a cofactor for a eukaryotic enzyme
Or that it is a vitamin

Thank you,
Goodbye and Good luck



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3rd Story. Before we knew the structure of MDH we started working on how this enzyme with a novel prosthetic group interacts with an electron transport chain so that the oxidation of methanol will be coupled to ATP production.

3rd Story: A special electron transport chain for methanol oxidation involves a novel type of cytochrome c

Methylobacterium has a 'normal' system for NADH and succinate oxidation

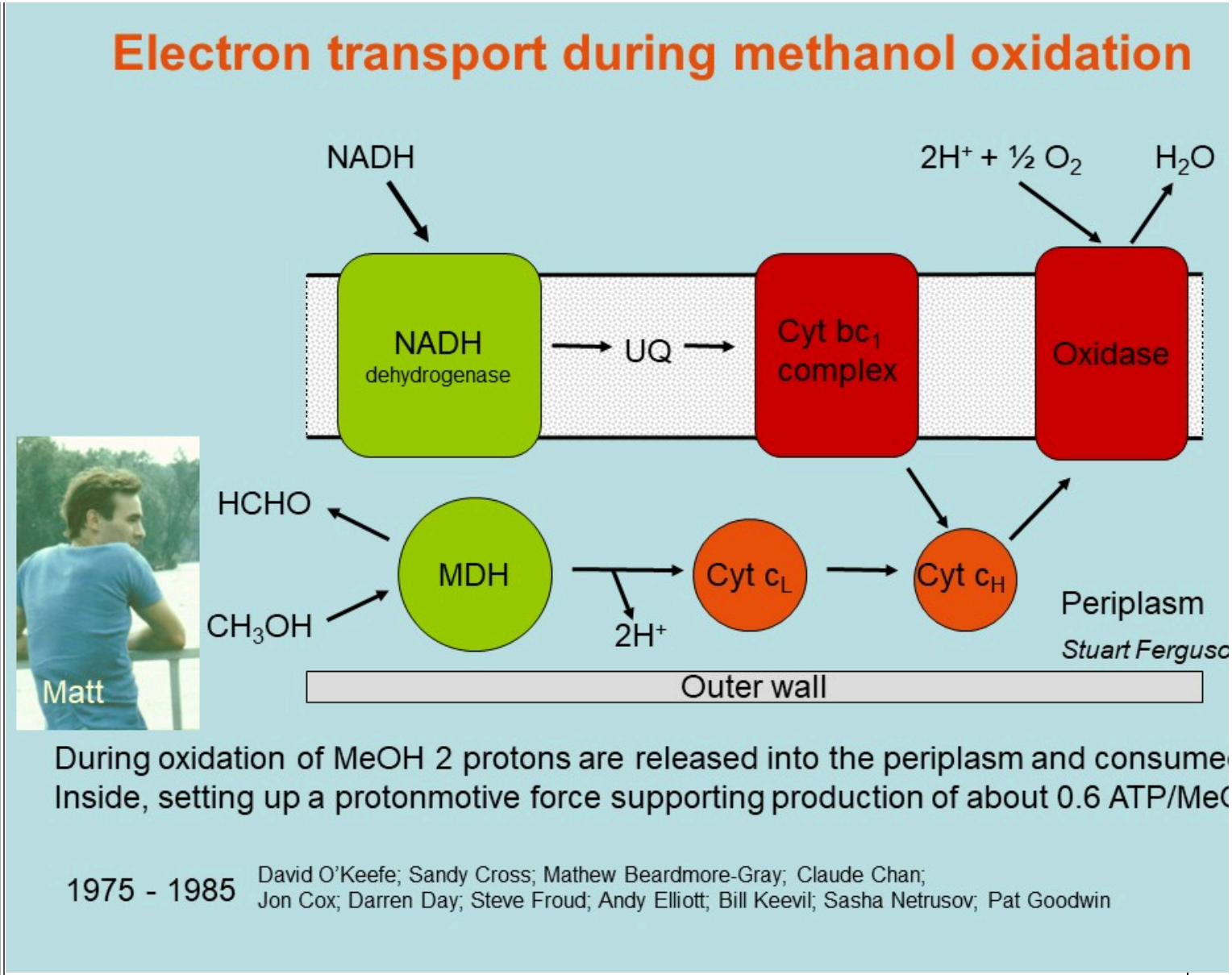
Inhibitors of NADH and succinate oxidation did not inhibit methanol oxidation
Inhibitors of the cytochrome oxidase did inhibit methanol oxidation

Addition of lactate (giving NADH) or succinate reduced cytochromes b, c and oxidase

BUT: Addition of methanol only reduced cytochromes c and oxidase

Methylobacterium and all methylotrophs have an extra cytochrome c called cytochrome c_L This is the specific electron acceptor for MDH. They react in the periplasm.

A student David O'Keefe boldly went against my advice, staying up most of the night and found it after months of my failure.

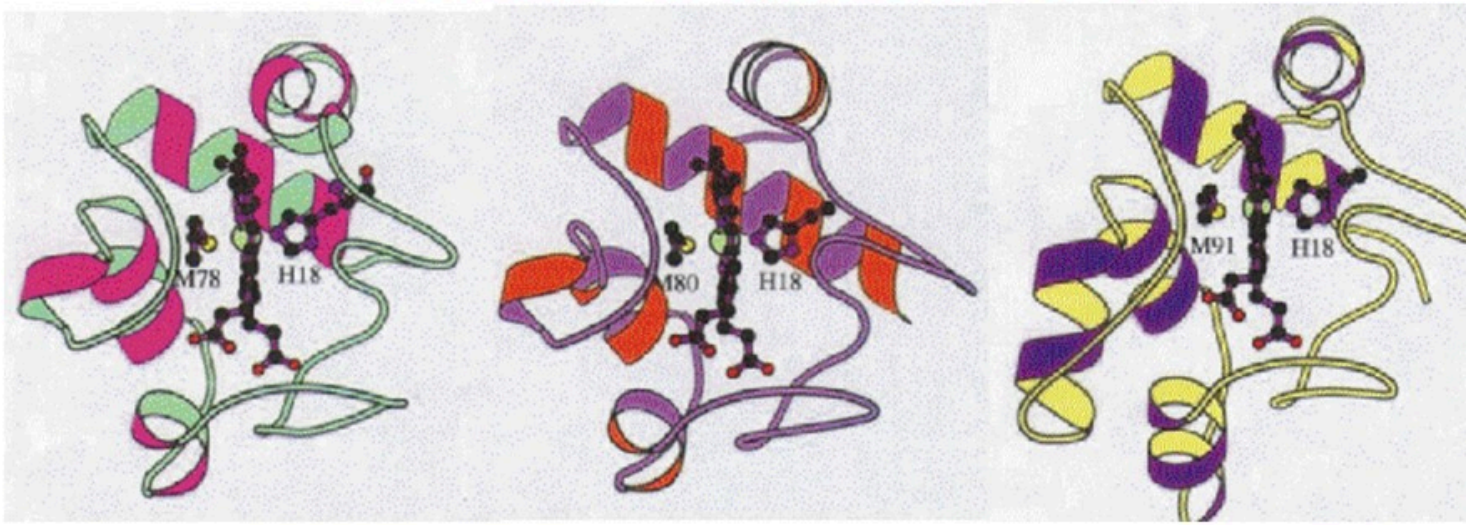


NOTE: The ATP yield from methanol is very low but this does not matter because almost every atom of carbon in these bacteria comes by way of this oxidation process so there is ATP to spare. Cell yields are not limited (as in other bacteria) by ATP but by avaialbility of NADH. Matt Beardmore-Gray calculated that the large amount of cytochrome and MDH in these bacteria is sufficient to cover the surface in a mono-molecular layer.

The solution of the unusual MDH structure helped us get an X-ray crystallogrqphy unit set up in the Department,

with the appointment of John Cooper and Steve Wood, and they helped solve the structure of the two methylotroph cytochromes.

Structure of cytochrome c_H



M. extorquens cytochrome c_H

Mammalian cytochrome *c*

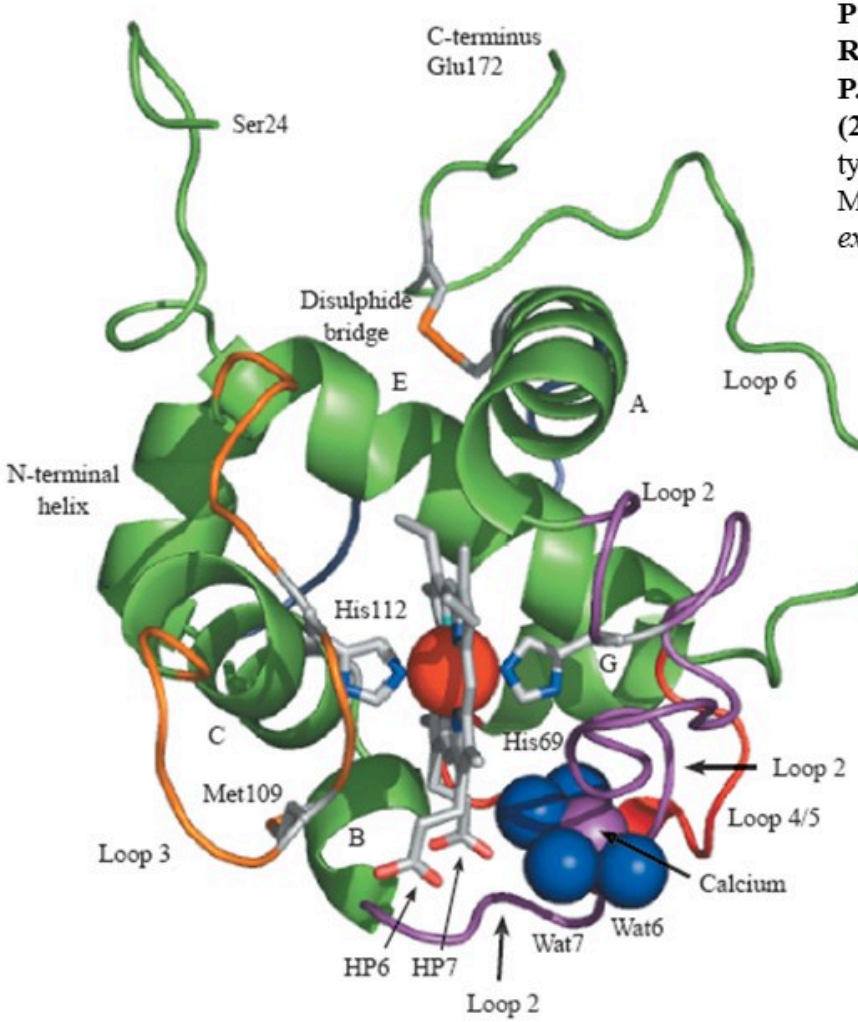
Bacterial cytochrome c_2

Remarkably, cytochrome c_H is unique for a bacterial cytochrome in being similar in structure to mammalian cytochrome c

Its internal structure 'disproves' a key proposal for cytochrome function:
All other cytochromes c have a tyrosine bonded to water in the haem cleft:
Cytochrome cH has tryptophan and no water. So the water has no function.

The X-ray structure of cytochrome c_L, the electron acceptor from methanol dehydrogenase

Paul Williams and Southampton X-ray structure group



Paul Williams, Leighton Coates, Fiyaz Mohamm Raj Gill Peter Erskine, Dominique Bourgeois, St P. Wood, Chris Anthony and Jonathan B. Coope (2006). The 1.6 A° X-ray Structure of the Unusual c type Cytochrome, Cytochrome cL, from the Methylotrophic Bacterium *Methylobacterium extorquens*. *J. Mol. Biol*. 357, 151–162

The amino acid sequence is unique for cytochromes

The core structure is exactly as see in other cytochromes c

Two cytochromes c have evolved ir the same organism, functioning in t same electron transport chain, havi similar core structure but no sequer identity

NOTE: the unique calcium ion and the exposed haem propionates

4. A resurgence of interest in MDH

In 1986 Mary Lidstrom and David Nunn published the methanol operon.

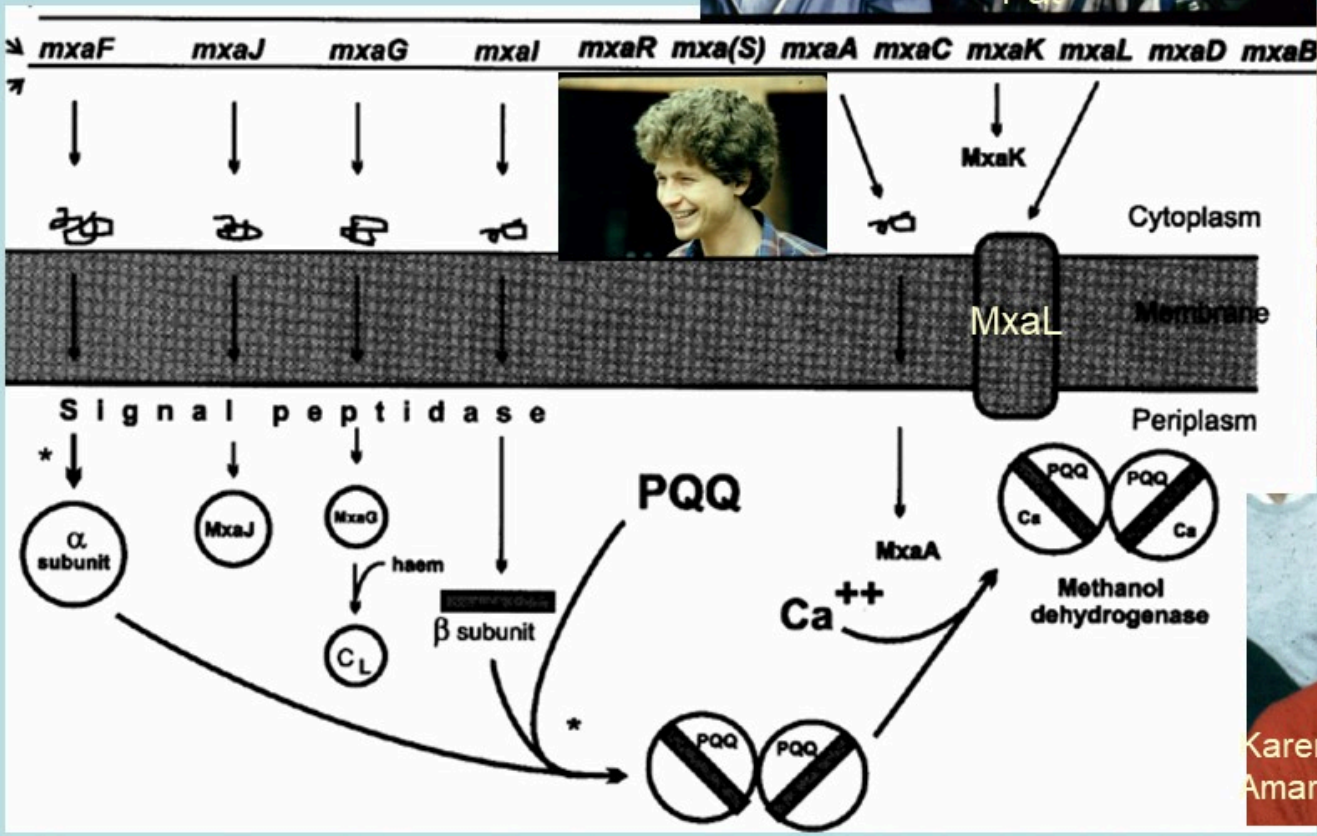
David came to work in my lab and he sequenced the genes for MDH and cytochrome c_L

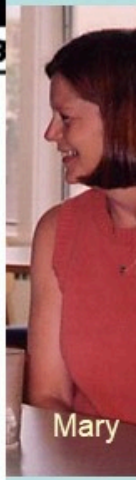
A very remarkable observation

Mary Lidstrom showed that all methylotrophs have two methanol oxidation operons.

The 2nd (Mox2) is never expressed. Mutations in Mox2 do not affect growth o methanol.

Expression of the mxa operon





The genes: Nunn, Lidstrom, Amaratunga, Anderson, Anthony, Goodwin, Morris, O'Connor

NOTE: the immense amount of difficult work on the genetics of our *Methylobacterium* by Karen Amaratunga and Simon Dales led them to provide important evidence for all out ideas about structure, mechanism and electron transport by a programme of site-directed mutagenesis.

A novel methanotroph (Methanotrophs grow on methane which is oxidised to methanol which is then oxidised by MDH)

In 2017 a novel methanotroph (*Methylacidiphilum fumariolicu*) was isolated from acidic volcanic mud. It grows at high temperature and low pH.
It requires a rare earth element for growth (eg Lanthanum, Cerium, Neodymium).

It only uses the 2nd methanol oxidation operon to produce MDH which uses one of the lanthanides instead of calcium at the active site. As a result during the last few years I have weekly reports on ResearchGate of my 1964 paper being cited!

A final strange thing

In 2018 Colin Murrell's group at UEA described a novel methylotroph isolated from seawater using a methanol medium containing lanthanum salts.

"We propose the name *Marinibacterium anthonyii* (in honour of the British microbiologis Professor Christopher Anthony)". Note: I was Colin Murrell's tutor here and he played second violin in my string quartet.

Marinibacterium anthonyii is the only methylotroph that does not have 'my' MDH); it only has the 2nd methanol oxidation operon.

Our last publication on MDH 40 years after my first

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The atomic resolution structure of methanol dehydrogenase from *Methylobacterium extorquens*

P. A. Williams, L. Coates, F. Mohammed, R. Gill, P. T. Erskine, A. Coker, S. P. Wood, C. Anthony and J. B. Cooper*

School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, England

The crystal structure of methanol dehydrogenase (MDH) from *Methylobacterium extorquens* has been refined without stereochemical restraints at a resolution of 1.2 Å. The high-resolution data have defined the conformation of the tricyclic pyrroloquinoline quinone (PQQ) cofactor ring as entirely planar. The detailed definition of the active-site geometry has

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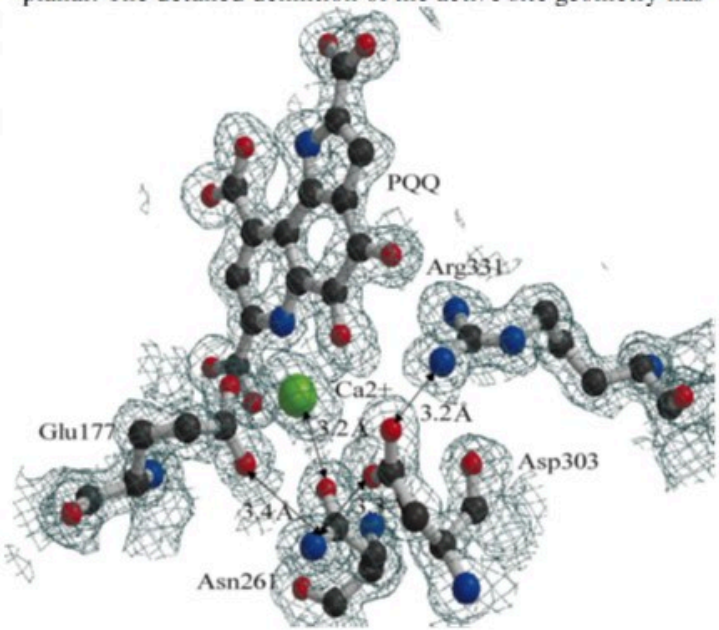
PDB Reference: methanol dehydrogenase, 1w6s, r1w6ssf.



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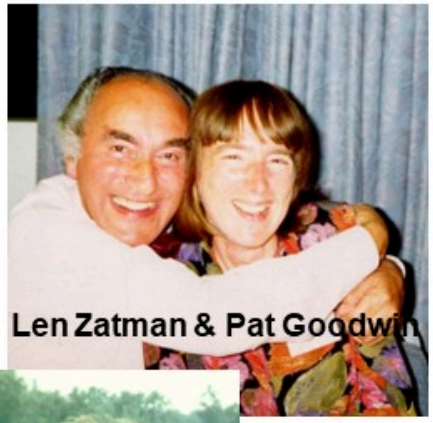

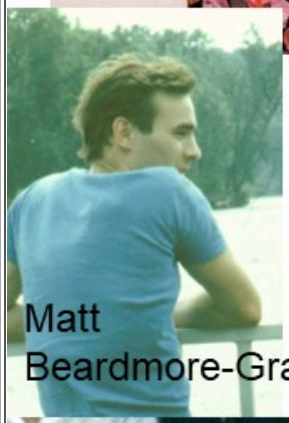

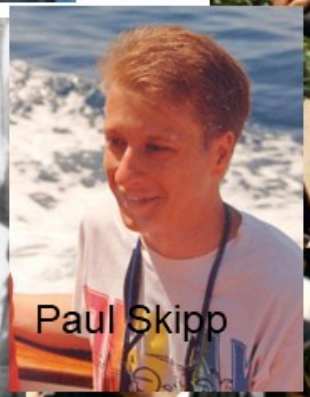

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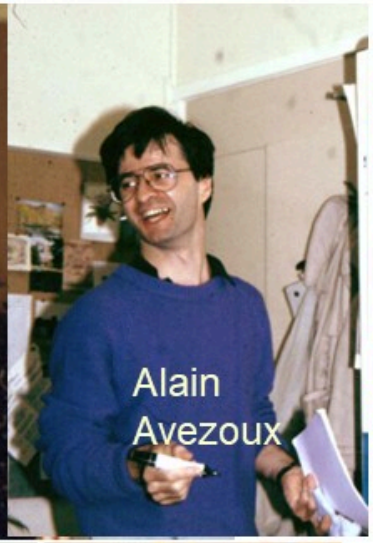
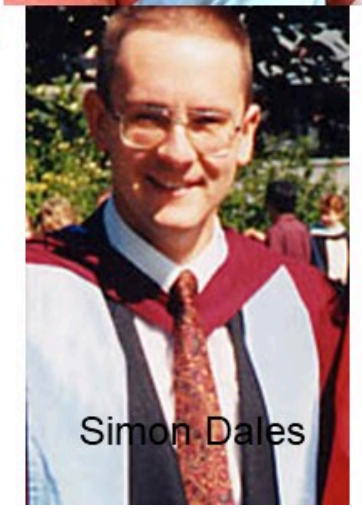


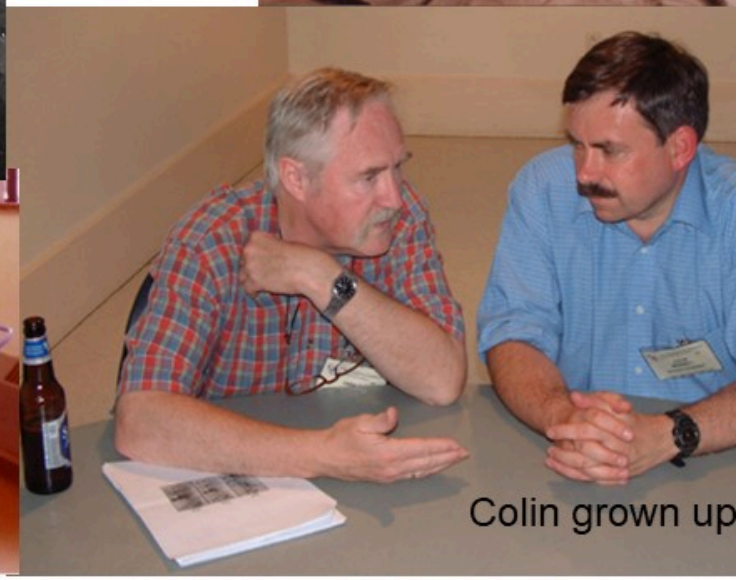


A few more pictures of methylotroph friends


Chris
1959


A BioCellist


Colin Murrell
2nd fiddle
1977


Colin grown up

More friends

HOME **CONTACT ME**: Email **THANKS** for help to Hugh Anthony and Carl Gore