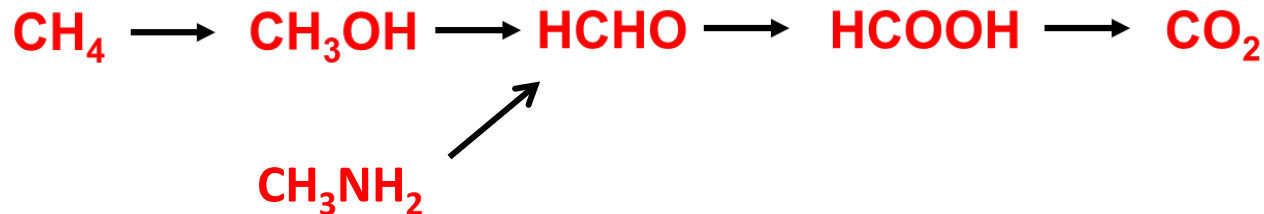


The oxidative metabolism of methylotrophic bacteria

These slides were to support (very) informal seminars at UEA as guest of Professor Colin Murrell.

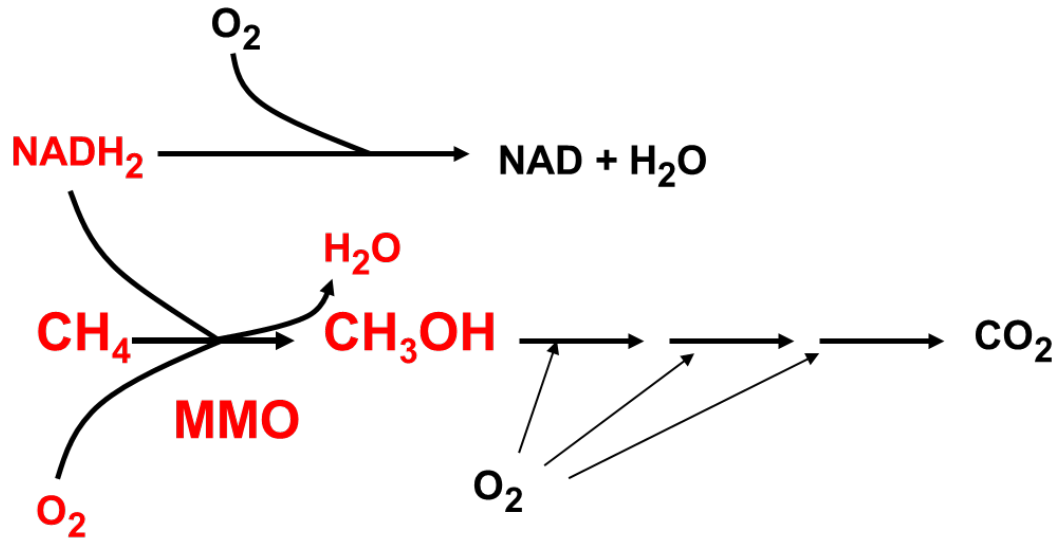
There are separate presentation on cytochromes etc

Almost all the enzymes for the initial oxidation steps to formaldehyde are unique to methylotrophs. These unique enzymes are linked to unusual energy-yielding electron transport chains.



- a) Hydroxylation of methane to methanol [Dalton, Murrell etc]
- b) Oxidation of methanol to formaldehyde [Main topic of this seminar]
- c) Oxidation of methylated amines to formaldehyde [Separate seminar]
- d) Oxidation of formaldehyde to formate [Next slide]
- e) Oxidation of formate to carbon dioxide (typical NAD-linked enzyme)
- f) Electron transport chains for energy production

Hydroxylation of methane to methanol



Catalysed by Methane mono-oxygenase (MMO) (Two types)

Soluble (sMMO)

Membrane bounds (particulate or pMMO)

Mainly solved by Howard Dalton; genetics (etc) by Colin Murrell at University of Warwick

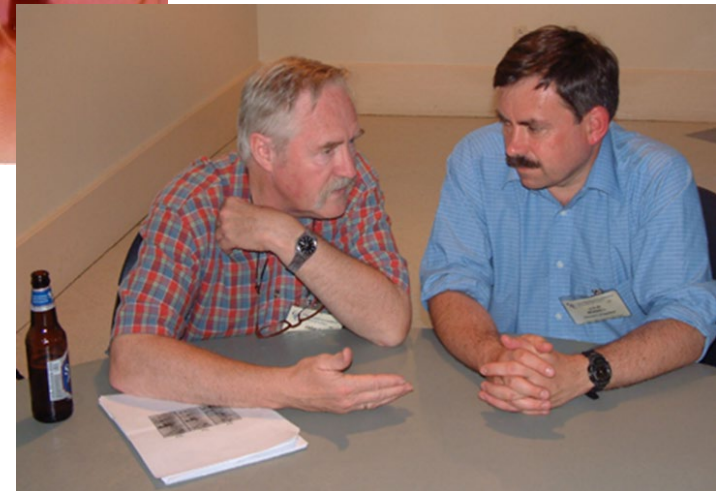
More than half oxygen is used by initial hydroxylation in a reaction that requires input of energy (as NADH)

Important people in study of methane oxidation

The late Sir Howard Dalton FRS. Professor Colin Murrell, Mary Lidstrom, Ed Bellion



My second fiddle (juvenile form of Murrell), when I was his tutor at University of Southampton



Nearly adult form

Oxidation of formaldehyde by methylotrophs

Dye-linked dehydrogenases; so possibly flavoproteins or quinoproteins
NAD-linked dehydrogenases. Absolutely essential during growth on methane with NADH as donor to the hydroxylase
Methanol dehydrogenase. Good substrate but not likely to be relevant.
Complex route involving methanopterin and folates [next slide] (NAD-linked)
Cyclic route in bacteria with RuMP pathway

Note: There must always be routes for removal of toxic formaldehyde. And many aldehyde dehydrogenases are non-specific

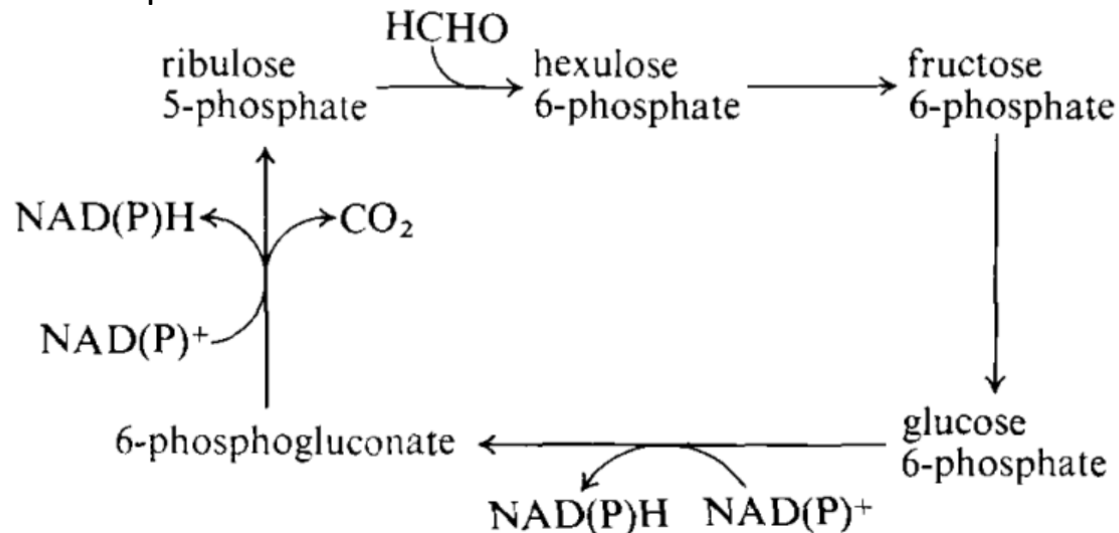
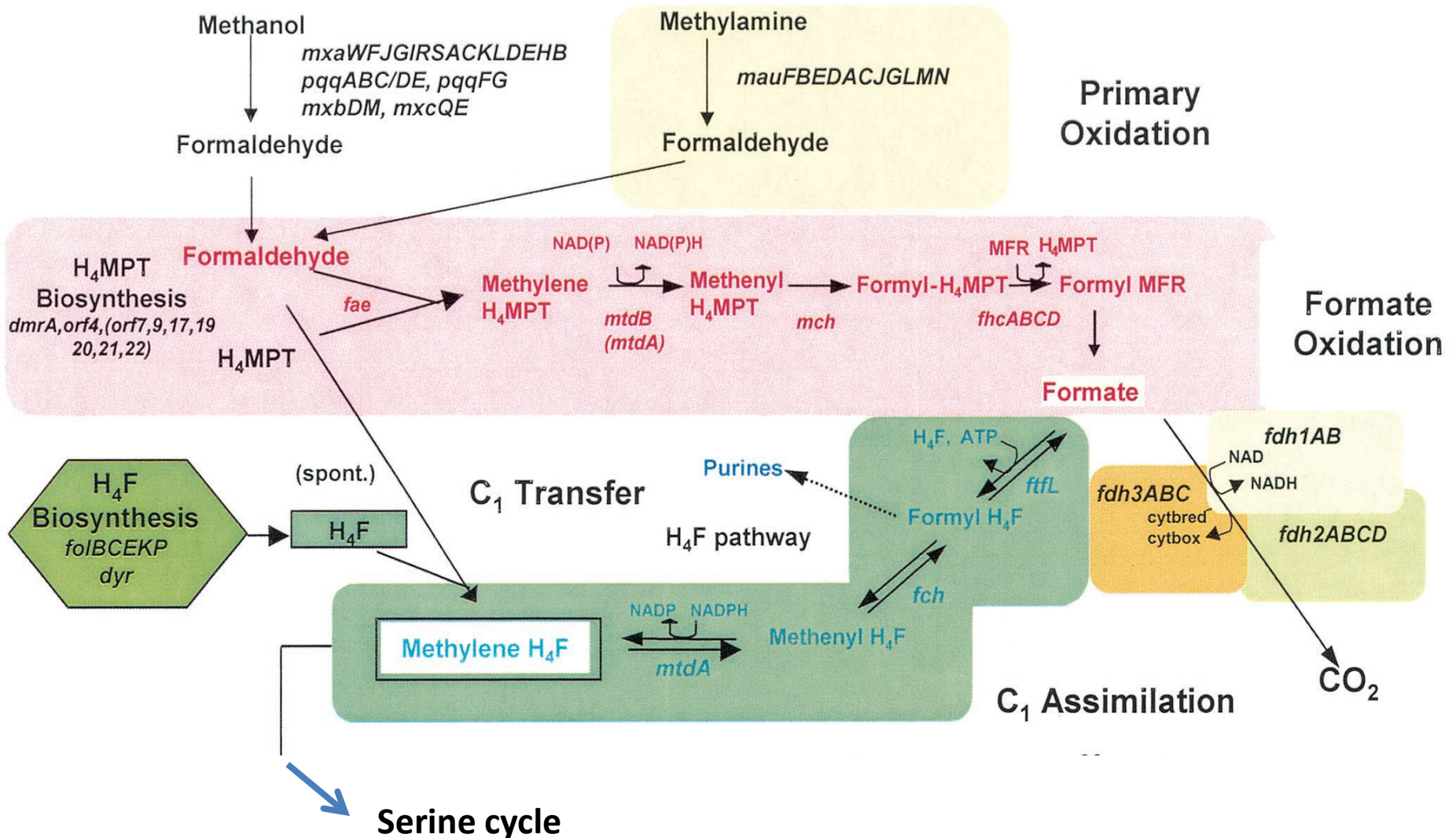


Fig. 25. The cyclic route for oxidation of formaldehyde. This is probably the main route for oxidation of formaldehyde in *Pseudomonas C*, *Methylophilus methylotrophus* and *Pseudomonas oleovorans*. This cyclic pathway is discussed further in Chapter 3.

Formaldehyde oxidation in *Methylobacterium extorquens*

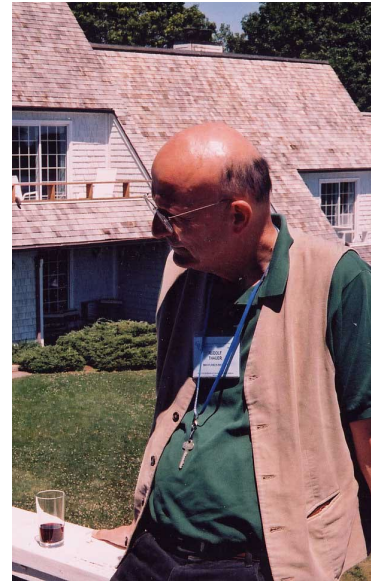
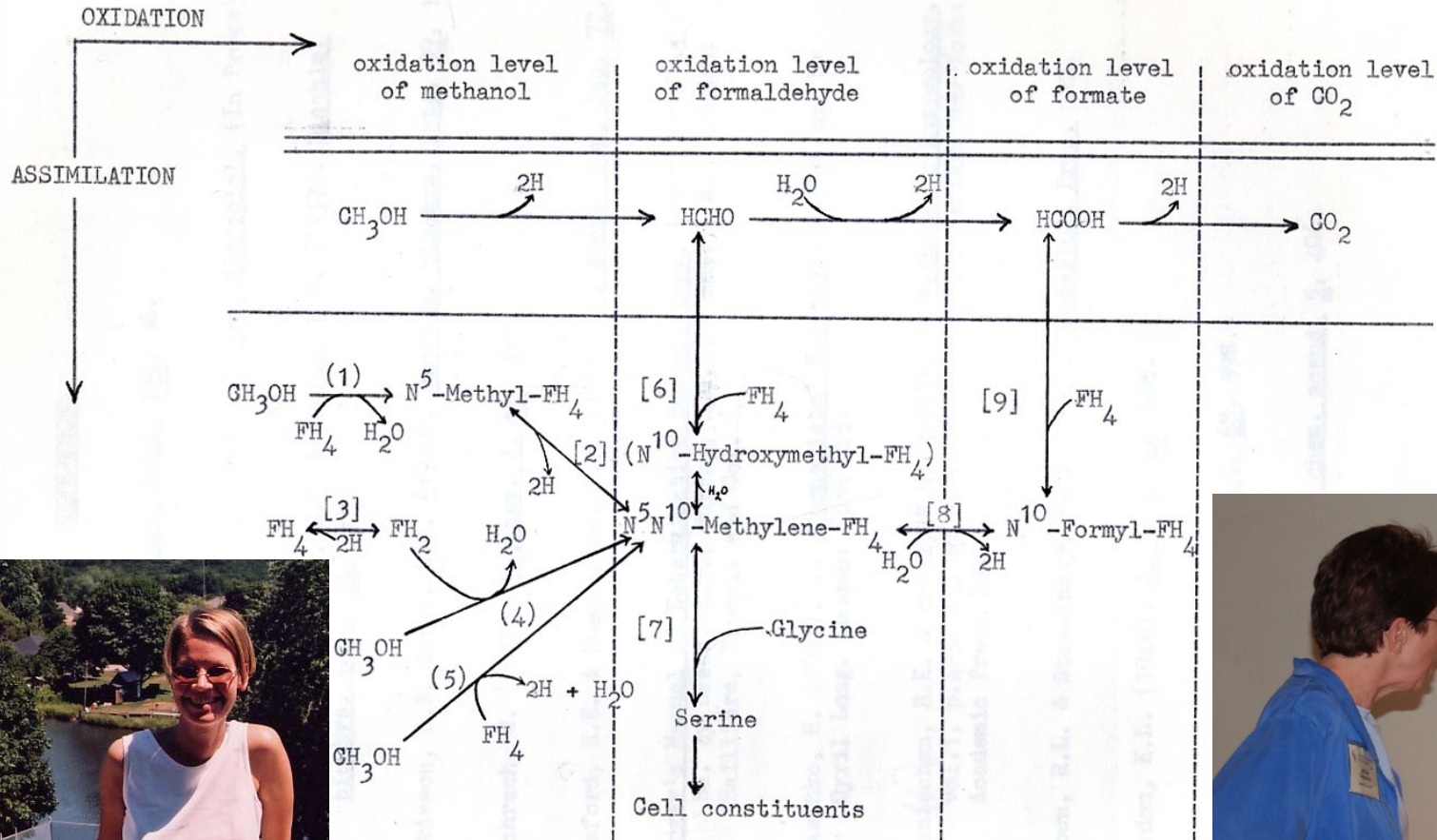
Methanopterin was previously only known as carrier in methanogens. This work is by Lidstrom and colleagues. Much is based on genetic/mutant studies.



Last page of my PhD Thesis (submitted 50 years ago; May 1963) suggesting folate involvement in formaldehyde oxidation

Fig.40.

Hypothetical pathways for the oxidation and assimilation of methanol



The oxidation of methanol in Gram negative methylotrophs

This was my PhD project with Len Zatman (1960-1963). He thought the enzyme for methanol oxidation would be unusual as no NAD-linked alcohol dehydrogenase is able to oxidise methanol.

I started by isolating a soil methylotroph, *Pseudomonas* sp M27. It was the same as Quayle's *Pseudomonas* AM1 (published at end of my first year); now called *Methylobacterium extorquens* AM1

Whole cells oxidised methanol and a very wide range of primary alcohols

Inhibited by p-nitro- phenylhydrazine. (50% inhibition by 1.0 μ M). *This has never been explained or investigated further.*

Inhibited by EDTA. Not due to chelation of metal ions. Unique to alcohol oxidation. Later shown to inhibit competitively the binding MDH to its cytochrome electron acceptor

Extracts did not catalyse reduction by methanol of NAD but it was possible to get reduction of a blue indophenol dye in the presence of an intermediate phenazine methosulphate using whole cells in anaerobic conditions.

We then found that this assay could be used with sonic extracts: this is similar to flavoprotein dehydrogenases like succinate dehydrogenase.

The enzyme is **Methanol dehydrogenase (MDH)**.

Problems with the dye linked system for methanol dehydrogenase

The rate was not proportional to protein concentration.

No dye reduction could be seen with lower concentrations of extract.

When water in the assay was replaced with salts growth medium the rate became proportional to protein concentration.

The active constituent was ammonium chloride NH_4Cl .

The activator species is Ammonia base NH_3 not the ammonium ion NH_4^+

This is unique. In some enzymes ammonium ions replace potassium ions. But with MDH ammonium chloride could only be replaced with methylammonium chloride CH_3NH_3^+

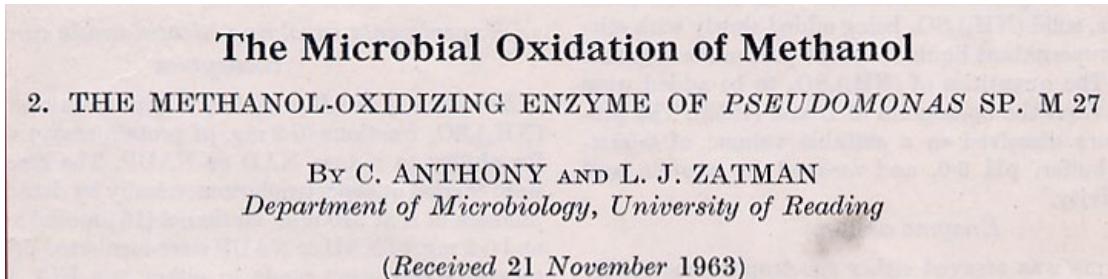
At limiting concentrations of activator 10 times more CH_3NH_3^+ was required than with NH_4^+ . This is because its pK value is 10 times higher than that of ammonium chloride.

AND 10 times more ammonium chloride was required at pH 8, when the proportion of NH_3 was one tenth of the proportion at pH 9.



Methanol dehydrogenase

Responsible for all bacterial methanol oxidation



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

Activator- ammonia (not NH_4^+ ion)

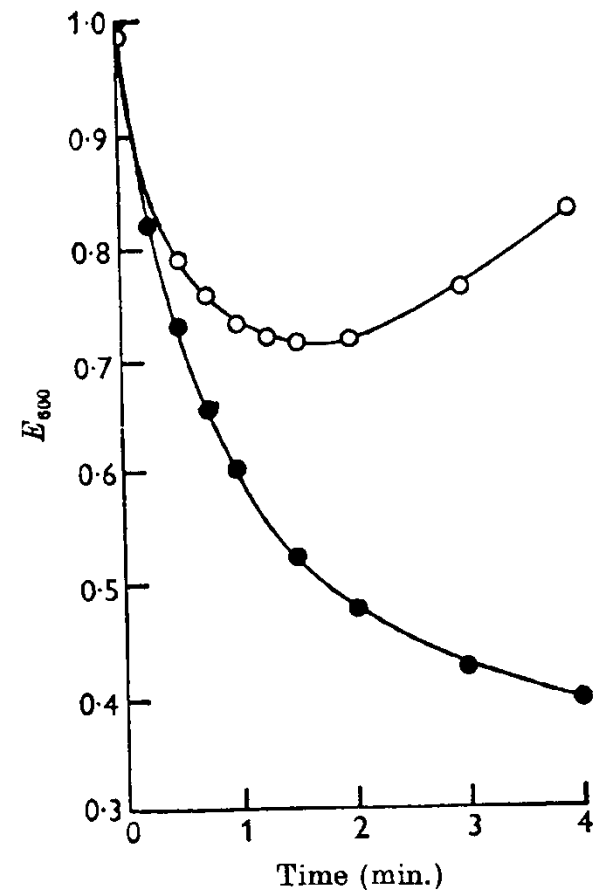
Wide substrate specificity

Unusual absorption spectrum – novel prosthetic group??

High proportion of tryptophans

A homodimer (two 60 kDa subunits); later shown to have two 66kDa subunits and two 8kDa subunits

No metal ion. [Later shown to have Calcium ion]



MDH assay
with PMS/indophenol

A new type of enzyme prosthetic group

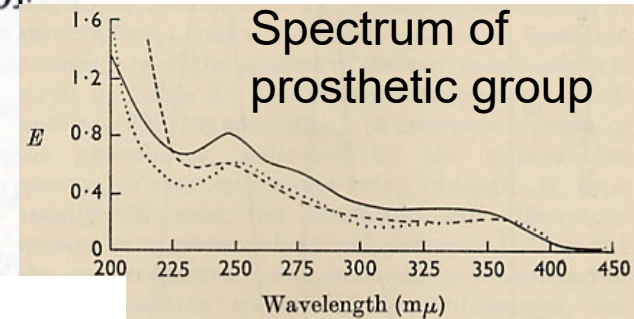
Note: all other dehydrogenases use NAD(P) as hydrogen acceptor or they are membrane flavoproteins that use FMN or FAD as prosthetic group

The Microbial Oxidation of Methanol

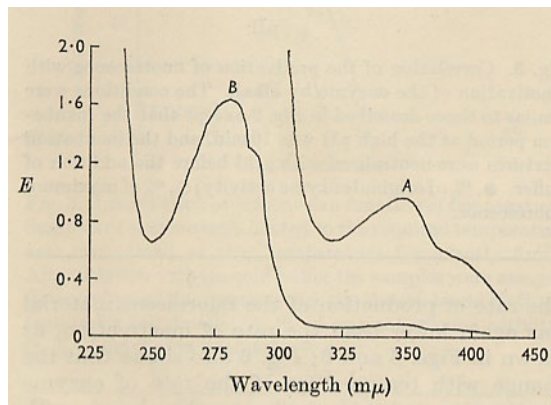
THE PROSTHETIC GROUP OF THE ALCOHOL DEHYDROGENASE OF
PSEUDOMONAS SP. M27:
A NEW OXIDOREDUCTASE PROSTHETIC GROUP

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

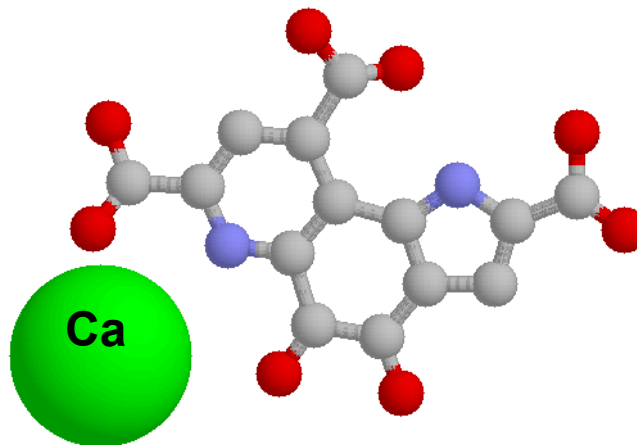
(Received 30 December 1966)



PQQ 1979 Kennard's lab



Spectrum of MDH



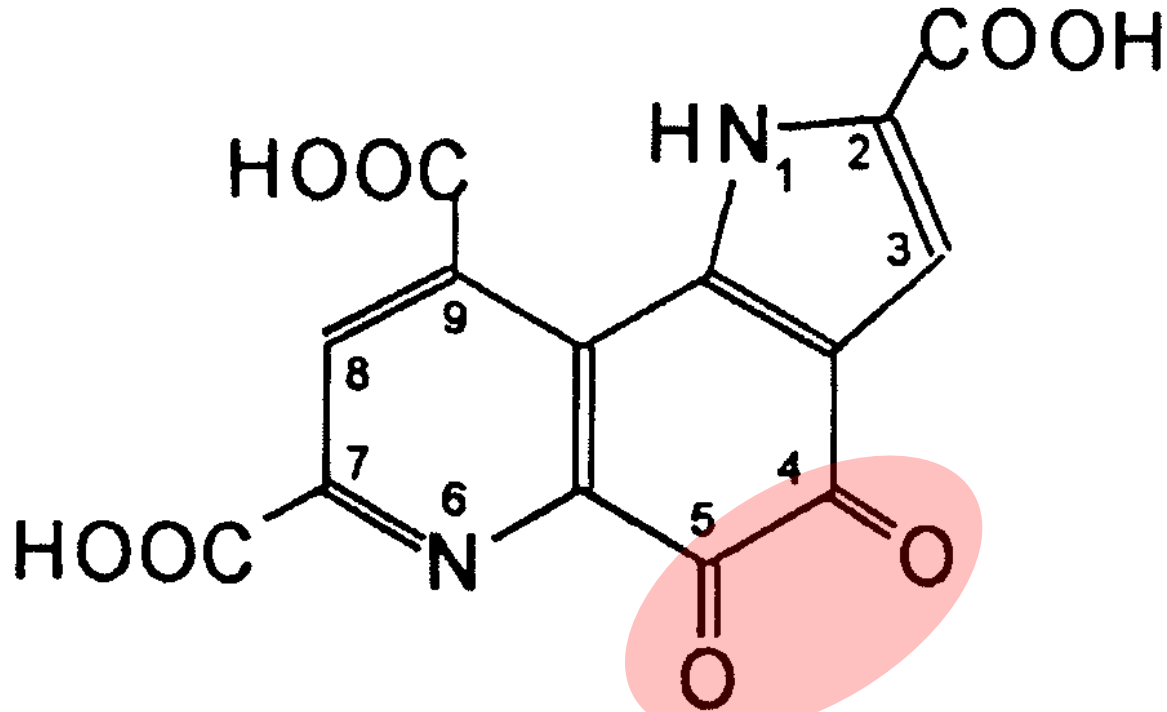
Methanol Dehydrogenase [MDH] 1995

New prosthetic group is PQQ

Pyrrolo-Quinone Quinone

MDH is now known as a Quinoprotein

Pyrroloquinoline Quinone (PQQ)



First isolated and purified
by Anthony & Zatman
in 1967 from bacterial
methanol dehydrogenase

Structure determined by
Kennard's group in 1979

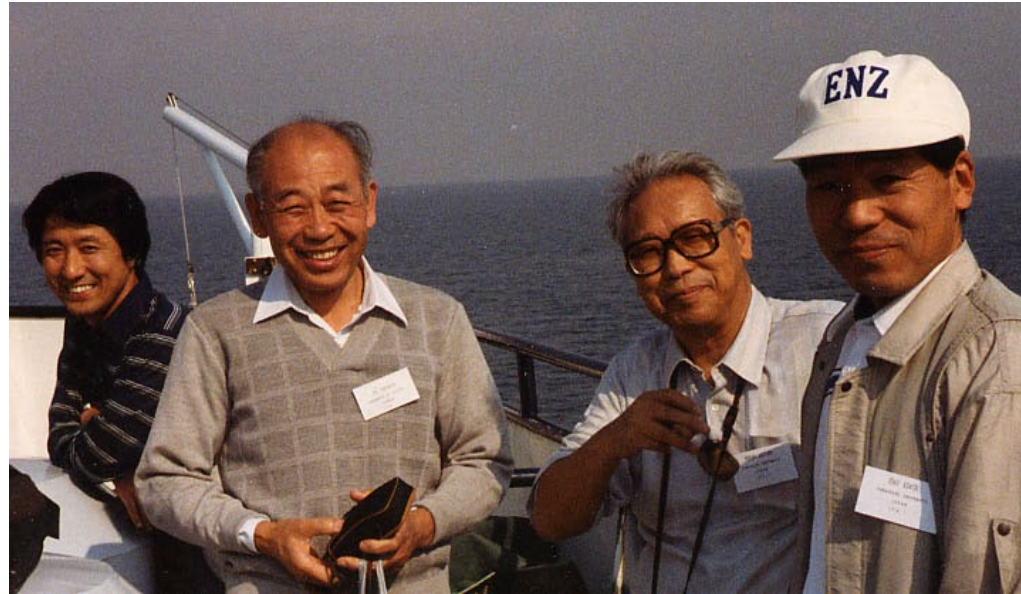
Quinoproteins are enzymes
with an *ortho*-quinone prosthetic
group

They are equivalent to
flavoproteins which has riboflavin
derivatives FMN and FAD as
prosthetic group

PQQ and other quinoproteins The novel prosthetic group of MDH was later in other dehydrogenases and 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



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



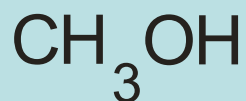
Hans Duine and Hans Frank
'invented' quinoproteins

**Note: No PQQ-containing quinoproteins
have been described in anything other
than bacteria**



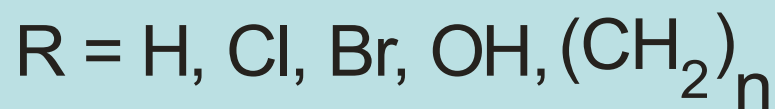
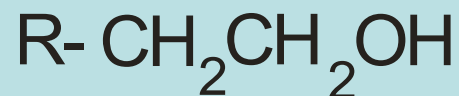
Substrate specificity of methanol dehydrogenase

Substrates oxidised

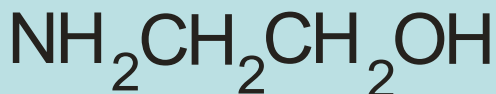


Hydrated formaldehyde

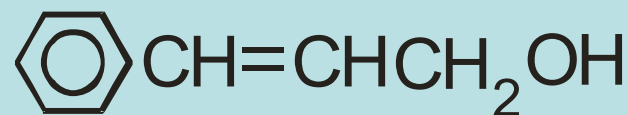
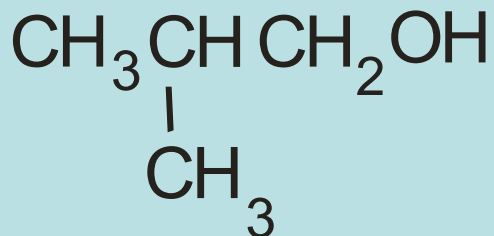
Only primary alcohols



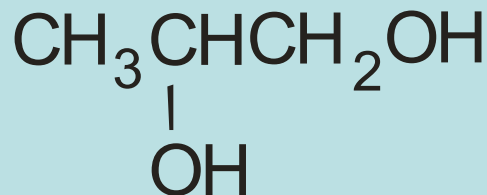
Substrates not oxidised



Alcohols with C2 substituent
are not oxidised



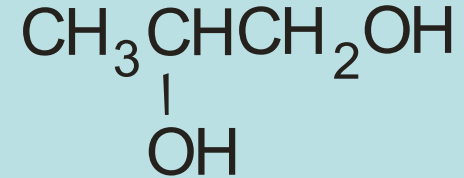
Surprisingly, propane 1,2-diol is oxidised
But only in crude preparations



Modifier protein for methanol dehydrogenase

This was found by accident when investigating growth on propane 1,2-diol

This is oxidised by MDH but **only in crude extracts**;
it does not obey the substrate rules for MDH

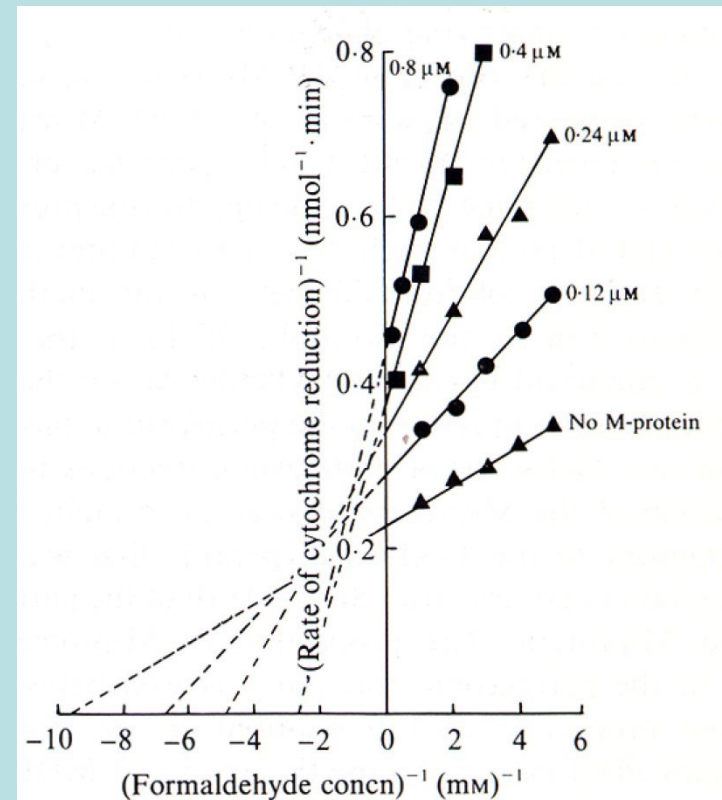


The periplasmic Modifier protein **increases affinity for propanediol**

Surprisingly, it is also present in obligate methylotrophs that do not grow on propanediol

M-protein decreases the $V_{\max}$,
and **decreases affinity, for formaldehyde**

This prevents the wasteful oxidation of methanol
through formaldehyde to formate



Energy production from methanol dehydrogenase

For energy production the MDH must be linked to the electron transport chain

The cytochromes of *M. extorquens* AM1

Membranes have typical b-type cytochromes and a cytochrome aa_3 able to oxidise typical soluble mitochondrial cytochrome c.

The Soluble fraction has 2 cytochromes c; one is small typical high pI (cytochrome c_H) which is oxidised by membrane fractions. And a much larger one with low pI (cytochrome c_L) which is not oxidised by the membrane oxidase.

Cytochrome c_L is a unique cytochrome. It is the electron acceptor for MDH. It is then able to pass the electrons on to cytochrome c_H which is oxidised by the membrane oxidase. [There is a separate presentation on these cytochromes]

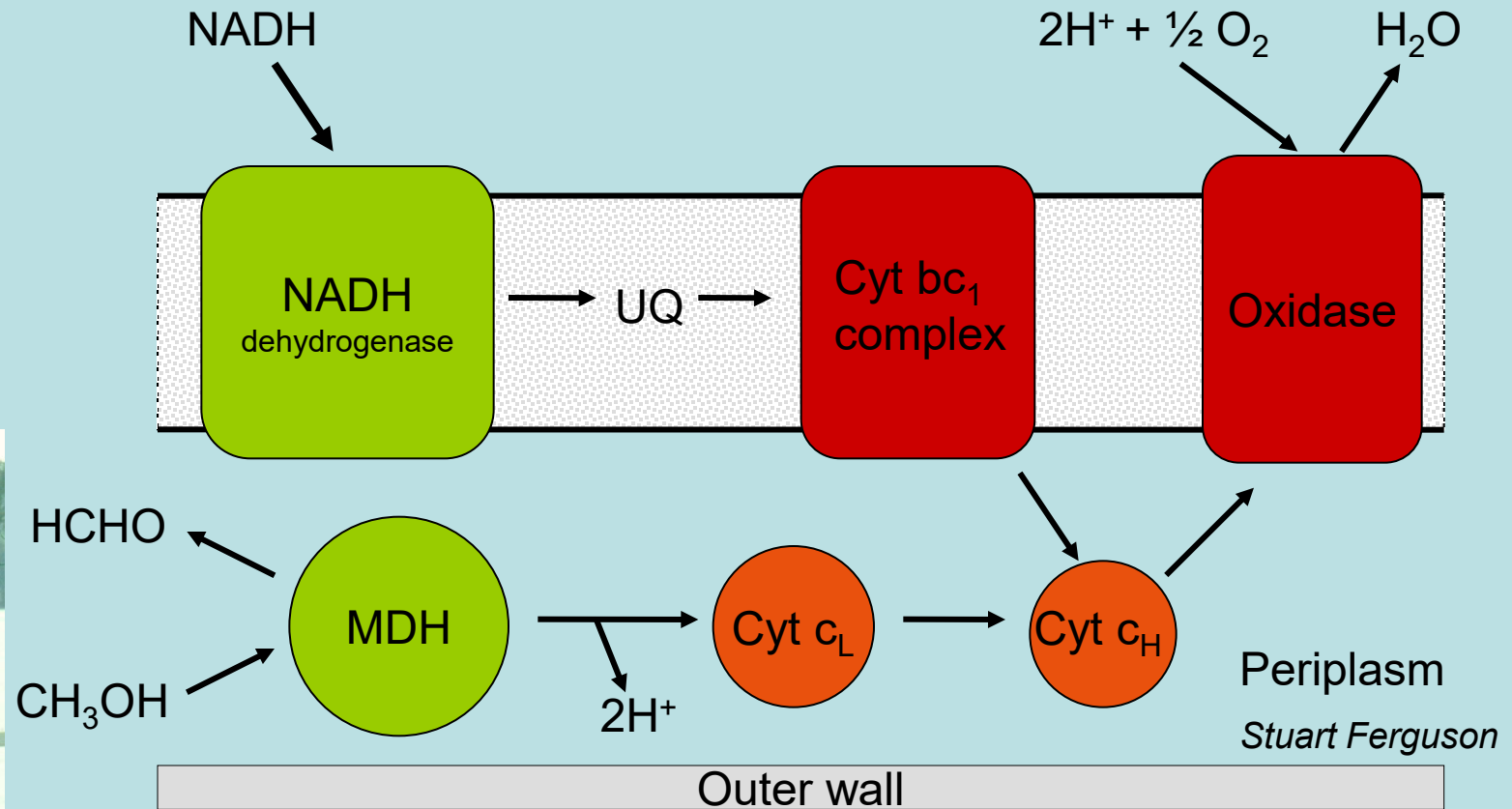
Proton translocation during methanol oxidation

Measured by typical Peter Mitchell method.

The ratio of protons pumped with pyruvate (NADH), succinate and methanol are 3:2:1.

Conclusion: methanol is oxidised by a unique electron transport chain that is similar to those for oxidation of inorganic ions by chemolithotrophs, where ubiquinone and the cytochrome bc_1 complex are not involved.

Electron transport during methanol oxidation



During oxidation of MeOH 2 protons are 'translocated' from inside to outside
 Setting up a protonmotive force supporting production of about 0.6 ATP/MeOH

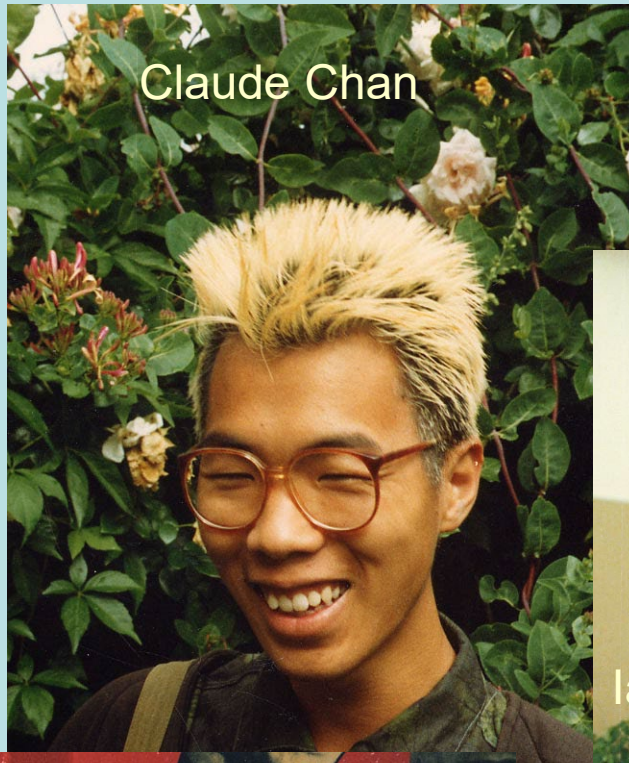
1975 - 1985

David O'Keefe; Sandy Cross; Mathew Beardmore-Gray; Claude Chan;
 Jon Cox; Darren Day; Steve Froud; Andy Elliott; Bill Keevil; Sasha Netrusov; Pat Goodwin

Darren Day



Claude Chan



Research students (Graduated 1990)

Tony Long



Ian Richardson



Darren

Jon Cox



The second subunit of MDH

1965 - 1989 MDH is a homodimer (2 x 60 kDa); 'confirmed' by others for > 20 enzymes

1989 Jim Elliott insisted that his 8.5 kDa 'contaminant' must be a subunit



1986: The key papers starting genetics of the methanol oxidation system

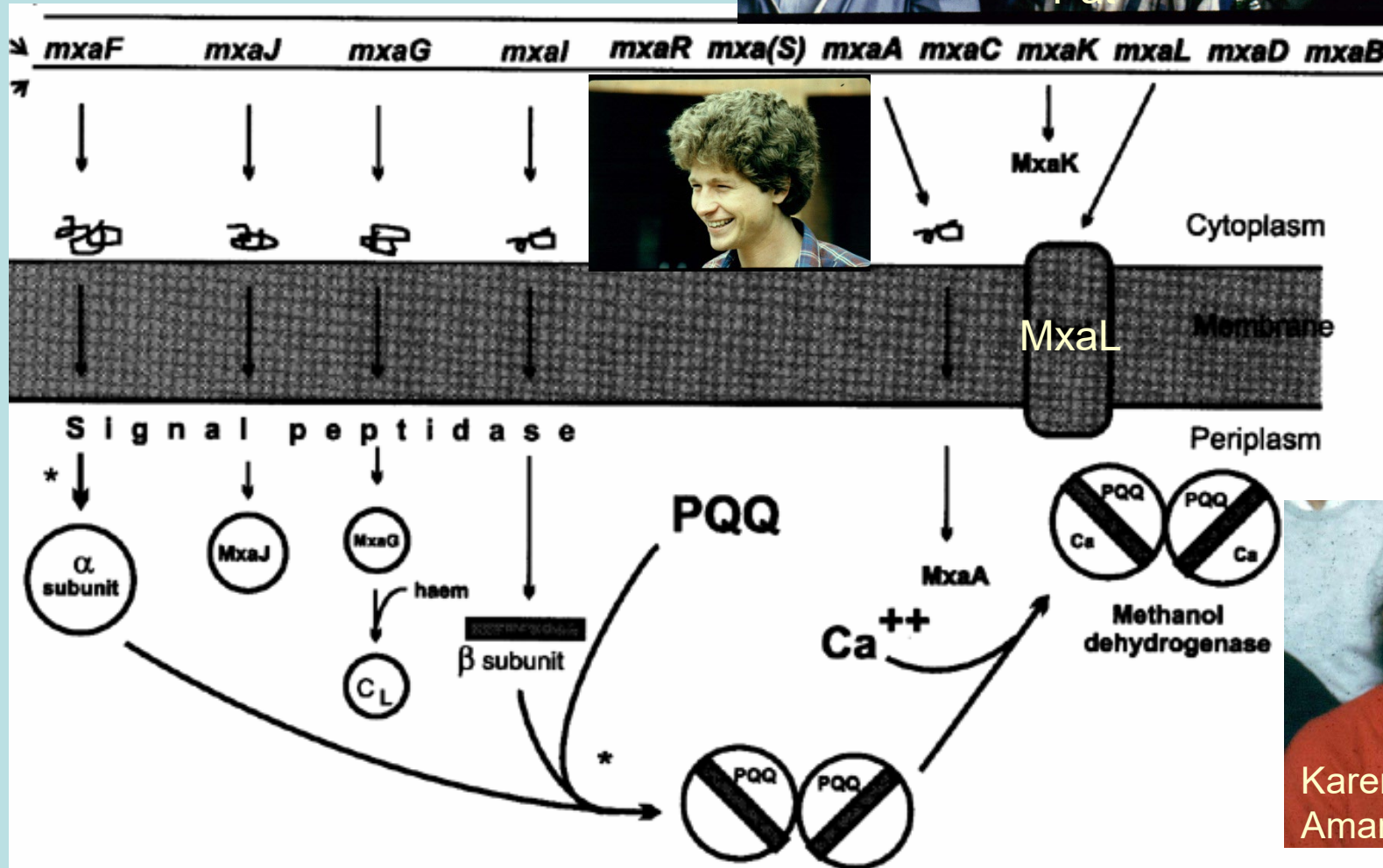
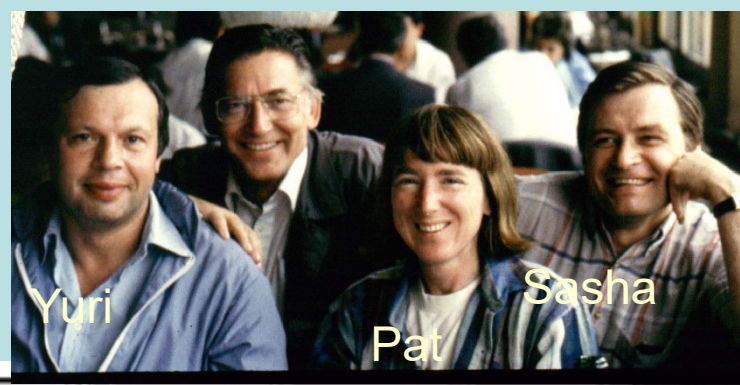
Nunn & Lidstrom (1986a,b) *J. Bacteriol.* **166**

1989 We (David Nunn) sequenced the gene for cytochrome c_L (*mxnG*); and showed that the downstream gene (*mxnI*) in the methanol oxidation operon coded for the 'contaminant' which thus became the 8.5kDa β subunit



1989 Darren Day confirmed that MDH is an $\alpha_2\beta_2$ tetramer

Expression of the mxa operon



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

The calcium ion in MDH

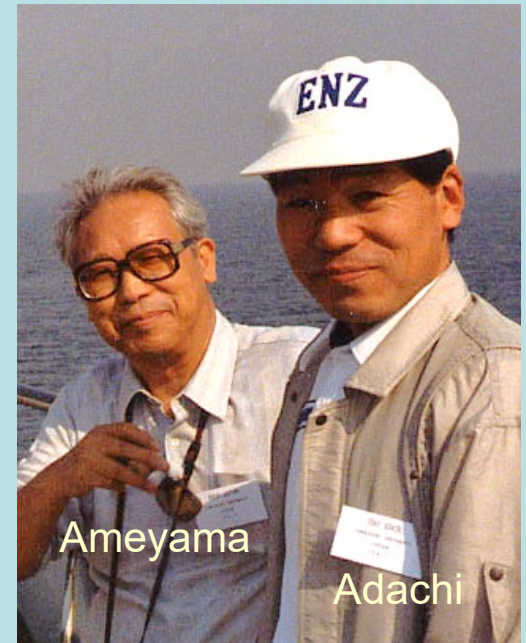
1965-1989 'MDH contains no metal ion' [Anthony & Zatman]

1986 MDH from mutants (*mxmA*, *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 *mxmA*, *K* & *L* lacked Calcium;
incubation with Ca produced active enzyme
& normal spectrum [Ian Richardson]

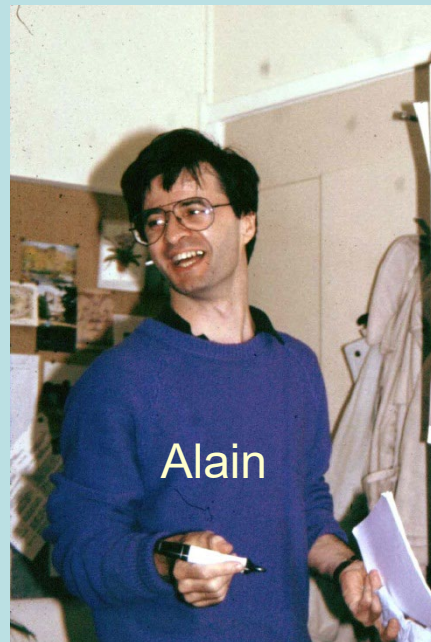
1992 MDH isolated from marine *Methylophaga Marina* is red and inactive;
incubation with Ca produced
normal green active enzyme [Claude Chan]



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]



The Mechanism of MDH

1979: X-ray structure of PQQ; *Salisbury, Forrest, Cruse, Kennard*

1978 – 1991: Main work on the mechanism; *Hans Frank and Hans Duine*

Ping-pong reaction with PQQ quinol produced from quinone

Reaction is catalysed by an active site base;

Addition/elimination reaction with a covalently attached methanol

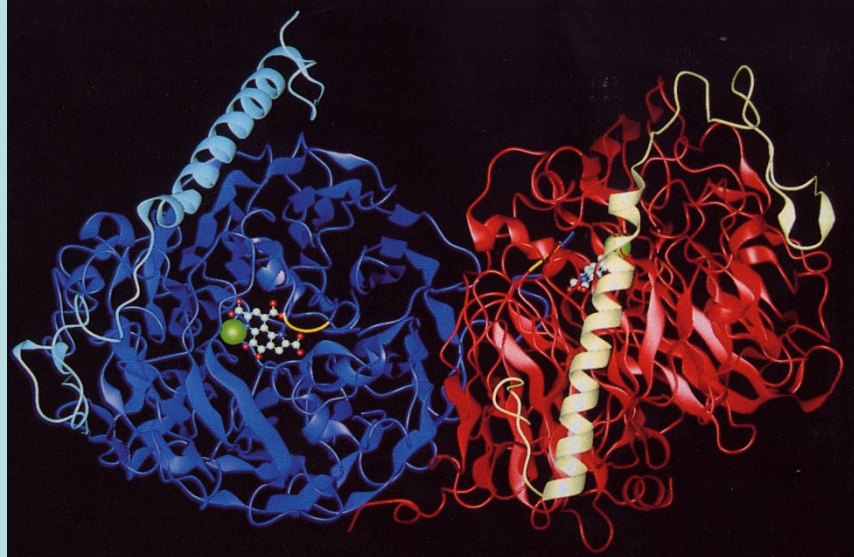


Hans Duine



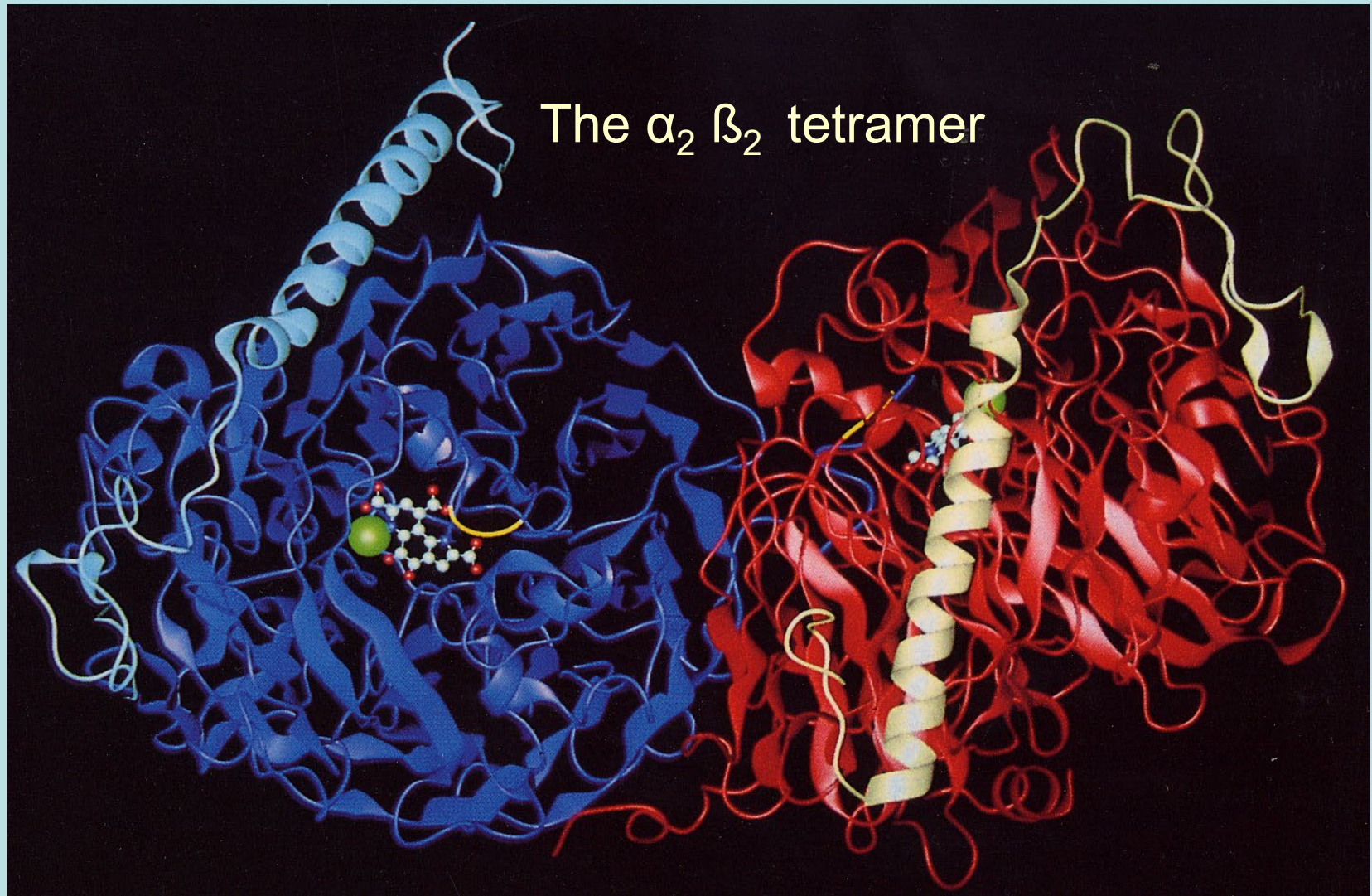
Hans Frank

The X-ray structure of MDH (1992 – 2004)



Meenakshi Ghosh, Chris Anthony; Colin Blake, Alain Avezoux, Matt Goodwin
Scott Mathews, Z-X Xia, Victor Davidson, Scott White et al.

The structure of MDH of *M. extorquens*

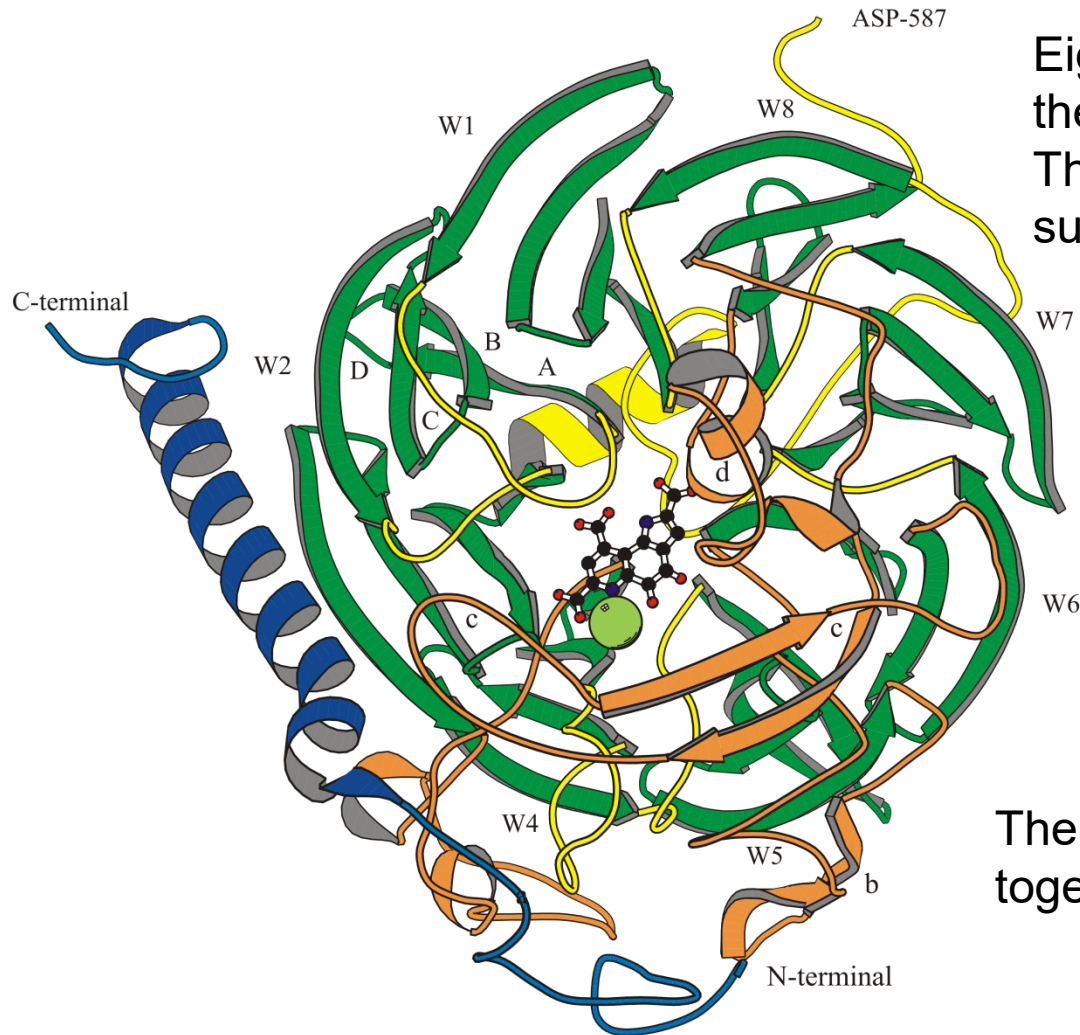


Meenakshi Ghosh, Chris Anthony; Colin Blake, Alain Avezoux, Matt Goodwin

Scott Mathews, Z-X Xia, Victor Davidson, Scott White et al.

1992 - 2003

The β -propeller structure of MDH



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

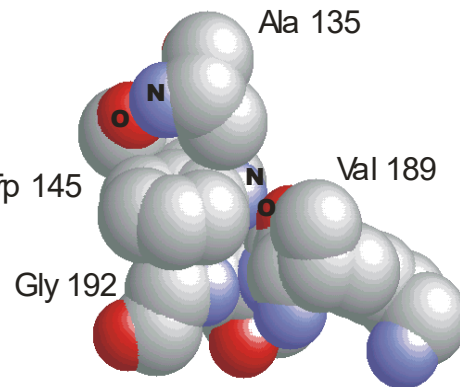
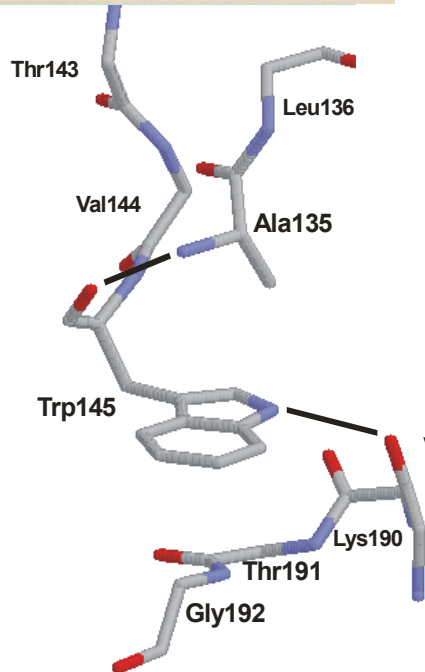
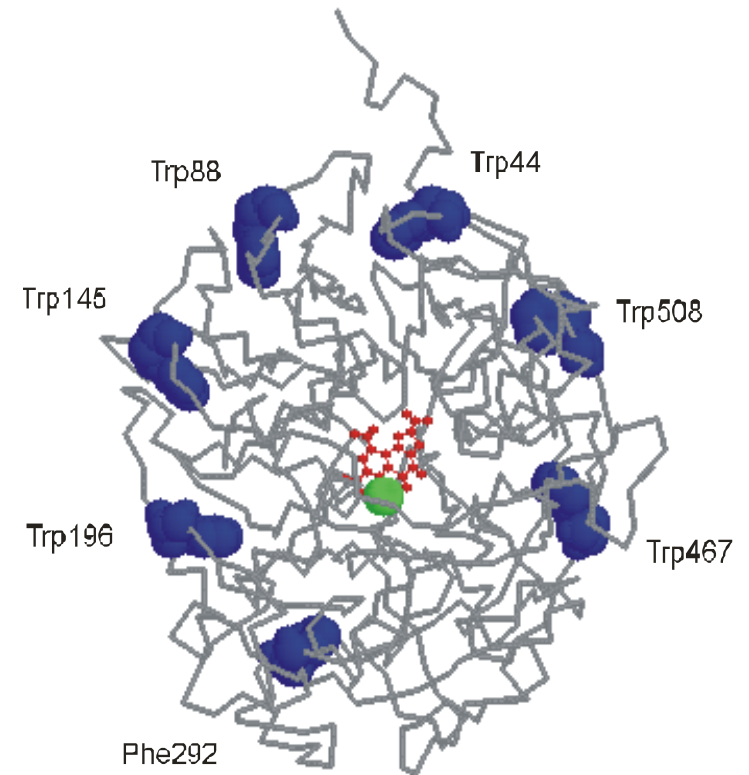
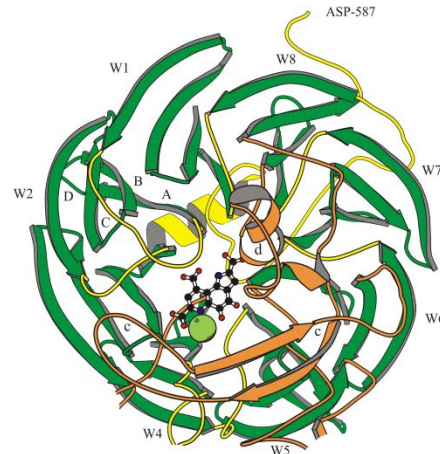
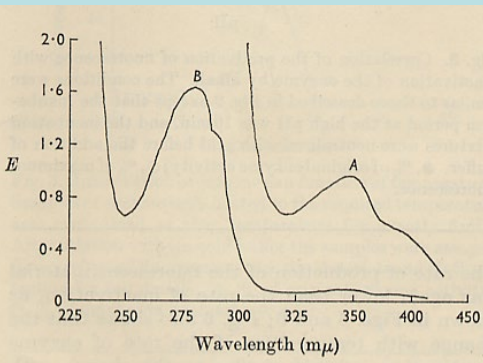
Active site contains PQQ and calcium (green sphere)

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

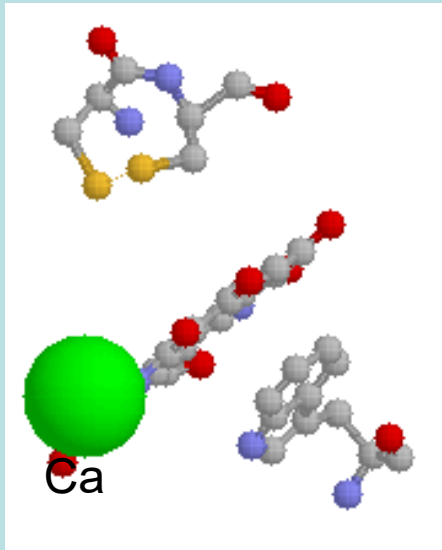
Tryptophan docking system

High proportion of tryptophans;
19 per α subunit

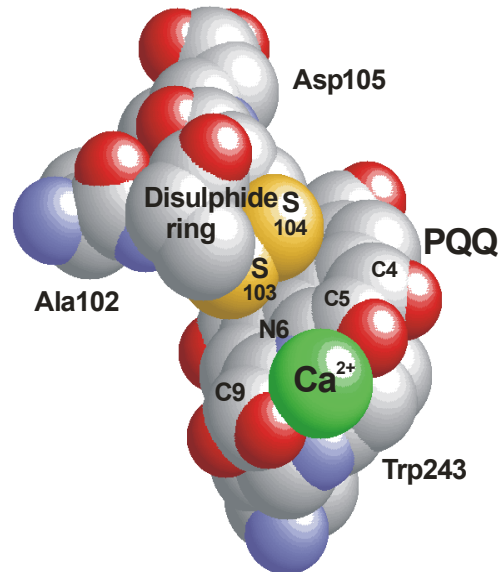
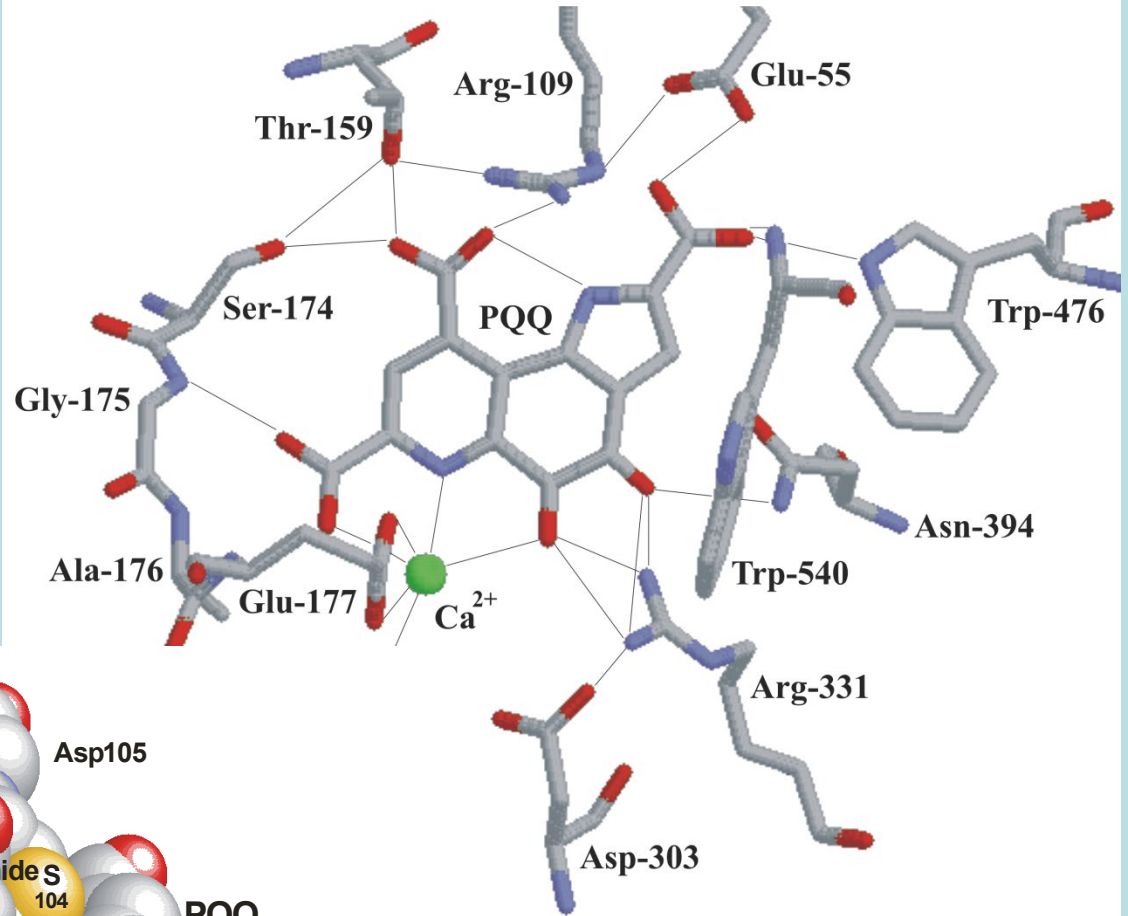
The β -sheet 'propeller blades' are held together by 'tryptophan-docking motifs'



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 (PQQH₂) to 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*.

