The modifier protein of formaldehyde dehydrogenase from *Methylococcus capsulatus* (Bath).

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Abstract.

Stirling & Dalton (1978) isolated a formaldehyde dehydrogenase (mFDH) from *Methylococcus capsulatus* (Bath) which required a small heat stable component for the catalysis of formaldehyde oxidation to occur. The mFDH was purified and shown to be a dimer of 57kDa subunits. The heat stable component was purified and shown to modify the function of FDH and was therefore termed a modifier protein (protein F) (Millet *et al*., (unpublished)). In this study purification procedures for protein F and mFDH are described. The N-terminal sequence of purified mFDH and protein F are described and data are presented which indicates that mFDH is a tetramer of 63 kDa subunits.

It was known that protein F altered the function of mFDH from a specific FDH to a general aldehyde dehydrogenase. The data presented in this study demonstrate that only formaldehyde oxidation is catalysed in the presence of protein F and in its absence formaldehyde oxidation can not be detected. The data also show that the oxidation of a range of aldehydes and alcohols are catalysed by mFDH in the absence of protein F. The data demonstrate that formaldehyde association to mFDH is cooperative resulting in a sigmoidal plot for rate vs. formaldehyde concentration while acetaldehyde oxidation follows Michaelis-Menten kinetics. Data are also presented which demonstrate that protein F may induce a conformational change in mFDH which allows formaldehyde oxidation catalysis to occur and inhibits the oxidation of acetaldehyde.

The data also show that protein F has limited effects on other dehydrogenase enzymes. An enzyme whose activity is altered by protein F was identified in a commercial sample of calf liver glucose dehydrogenase (GDH) and this was purified and shown not to be GDH. Also described is the characterisation of a second NAD^+-linked FDH (nFDH) isolated from *Methylococcus capsulatus* (Bath) which does not require a modifier protein.

From the data presented it is proposed that the function of mFDH may be to aid in reducing toxic formaldehyde concentrations in the organism and the role of the nFDH could be the generation of NADH for methane and carbon assimilation.

Abbreviations.

6PG	6-phosphogluconate
CV	Column volume
dFDH	Dye linked formaldehyde dehydrogenase
E4P	Erythrose-4-phosphate
EPR	Electron paramagnetic resonance
ESI-MS	Electrospray ionisation mass spectrometry
F6P	Fructose-6-phosphate
FD-FDH	Factor - dependent formaldehyde dehydrogenase
FDH	Formaldehyde dehydrogenase
FPLC	Fast protein liquid chromatography
FoDH	Formate dehydrogenase
G6P	Glucose-6-phosphate
GAP	Glyceraldehyde-3-phosphate
GDH	Glucose dehydrogenase
gFDH	Glutathione-dependent formaldehyde dehydrogenase
GSH	Glutathione
H6P	Hexulose-6-phosphate
HTSE	Heat - treated soluble extract
KDGP	2-keto-3-deoxy-6-phosphogluconate
MDH	Methanol dehydrogenase
mFDH	Modifier protein linked formaldehyde dehydrogenase
MMO	Methane monooxygenase
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide
NAD(P)	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced form)
nFDH	NAD^+-, factor independent formaldehyde dehydrogenase
mFDH	NAD^+-, modifier protein dependent formaldehyde dehydrogenase
pMMO	Particulate methane monooxygenase
protein F	Modifier protein
PQQ	Pyrrolo-quinoline quinone
Ri5P	Ribose-5-phosphate
Ru5P	Ribulose-5-phosphate
RuBP	Ribulose bisposphate pathway
RuMP	Ribulose monophosphate pathway

S7P	Sedoheptulose-7-phosphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sMMO	Soluble methane monooxygenase
UV	Ultra violet
X5P	Xylulose-5-phosphate
XuMP	Xylulose monophosphate pathway

1. Introduction

The oxidation of formaldehyde to formate by a NAD^+ linked formaldehyde dehydrogenase (FDH) in *Methylococcus capsulatus* (Bath) was first described in 1978 (Stirling & Dalton, 1978). These workers isolated the FDH component and demonstrated that this enzyme required the presence of a small heat stable protein for formaldehyde oxidation. The characterisation of the heat stable component was not performed at that time. *M. capsulatus* (Bath) is a methylotrophic organism i.e. an organism which can utilise carbon compounds containing one or more carbon atoms (although these compounds cannot contain carbon-carbon bonds) as the sole carbon and energy source for growth and assimilation as formaldehyde (Colby & Zatman, 1975). This definition of methylotrophs has been a matter of discussion (Anthony, 1982; Colby *et al.*, 1979; Zatman, 1981) which is outside the scope of this document. Unlike *M. capsulatus* (Bath) not all methylotrophs can utilise methane as their sole source of carbon and energy, those which can are now termed methanotrophs (Higgins *et al.*, 1981).

One central feature of all methylotrophic organisms is their ability to assimilate carbon in the form of single carbon compounds and synthesise an intermediate metabolite containing a three carbon (C_3) skeleton such as pyruvate. The pathways for assimilation use either formaldehyde or carbon dioxide as their initiation point. Extensive research over the past 40 years has led to the discovery of four principal pathways for the synthesis of C_3 compounds (Anthony, 1982). Figure 1.1 demonstrates the relationship of the XuMP, RuMP, SERINE pathway and RuBP or Calvin cycle, for carbon assimilation in methylotrophic organisms (Dijkhuizen *et al.*, 1992).

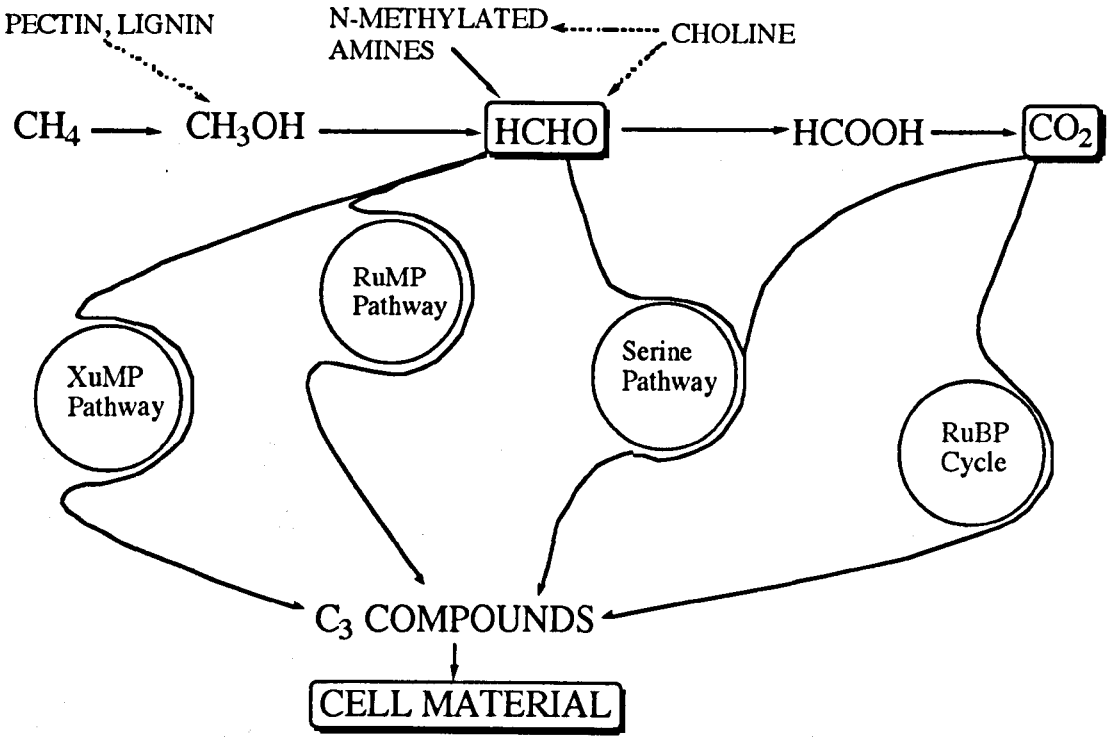


Figure 1.1 Interaction of the four principal pathways for carbon assimilation in methylotrophic organisms.

For a discussion of assimilatory pathways other than the RuMP pathway which will be mentioned below the reader is directed to (de Vries *et al.*, 1990; Anthony, 1982). Figure 1.1 demonstrates the central position of formaldehyde to these metabolic pathways, with the exception of the RuBP cycle.

Formaldehyde is synthesised in *M. capsulatus* (Bath) from the oxidation of growth substrates, such as methane and methanol. These direct sources of formaldehyde rely upon the oxidation of reduced substrate to produce formaldehyde. Of particular interest to this study is the oxidation of methane via methanol as this is the growth substrate used throughout this study. The following section discusses initially the production of formaldehyde from methane and then its subsequent removal either by assimilation using the RuMP pathway, oxidation by the RuMP pathway or direct oxidation via formate. After discussing how formaldehyde is formed and removed the control of the metabolic processes by protein interactions will be discussed.

1.1 Formation of formaldehyde from methane via methanol.

Figure 1.2 shows the oxidation of methane to methanol by methane monooxygenase (MMO) and methanol dehydrogenase (MDH).

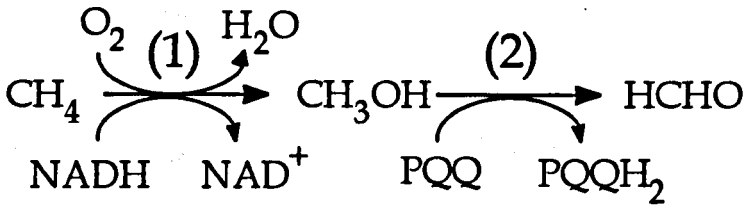


Figure 1.2 Oxidation of methane to formaldehyde by (1) MMO and (2) MDH.

The first stage of methane oxidation in methanotrophic organisms is catalysed by MMO, this enzyme exists in two forms: a particulate (membrane associated) MMO (pMMO) (Ribbons & Michalover, 1970) and a soluble form (sMMO) (Colby & Dalton, 1976). Methanotrophic organisms in general do not express both pMMO and sMMO although *M. capsulatus* (Bath) is able to express both forms, unlike the organism *Methylomonas albus* BG8 where only the particulate form has been identified (Stanley *et al.*, 1983). Expression of the different forms of MMO in *M. capsulatus* (Bath) has been linked to environmental conditions, in particular the level of copper in the growth medium (Stanley *et al.*, 1983; Prior & Dalton, 1985). The pMMO enzyme to date has not been purified from *M. capsulatus* (Bath) although partial purification has been reported (Smith & Dalton, 1989) showing it to consist of three peptides of MW 49, 23 and 22 kDa. Studies have demonstrated that pMMO is a copper - containing enzyme which can oxidise a limited number of alkanes (Chan *et al.*, 1993). Recently the pMMO from *M. capsulatus* (Bath) has been purified (Zahn & Dispirito, 1996). After

purification it was shown to be a trimer of 47, 27 and 25 kDa subunits and bound 14.5 copper ions per 99 kDa.

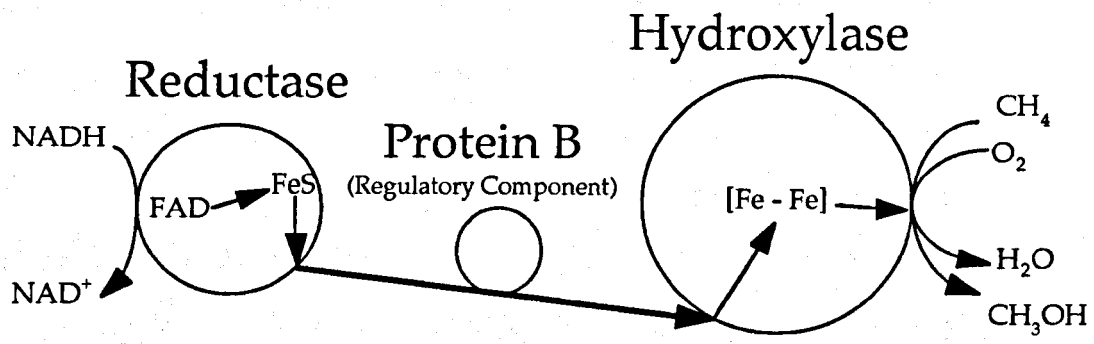


Figure 1.3 Schematic representation of the sMMO Components.

sMMO has been more extensively studied than pMMO because the sMMO complex has been purified from *M. capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. sMMO is a three component enzyme consisting of a reductase, a hydroxylase and a regulatory component (Green and Dalton, 1985). sMMO is schematically represented by Figure 1.3 which demonstrates the flow of electrons from NADH to an active site located in the hydroxylase component.

The mechanism of each component is well understood with the exception of protein B which is the small regulatory component. The proposed role of protein B will be discussed later. The electron transfer from NADH to the reductase and the resulting oxidation of methane have recently been reviewed (Dalton, 1992; Dalton *et al.*, 1993; Froland *et al.*, 1993) and the crystal structure of sMMO hydroxylase has been determined (Rosenzweig *et al.*, 1993). Consequently the complete mechanism of methane oxidation by sMMO at the structural level can be determined while the crystallisation of the whole sMMO complex may also be completed in the near future allowing an understanding of the overall control of this biologically important reaction.

The second reaction step in Figure 1.2 is the conversion of methanol to formaldehyde by MDH. The principal enzyme responsible for this reaction in Gram

negative methylotrophs is a periplasmic pyridine - nucleotide independent alcohol dehydrogenase. These enzymes contain the co-factor pyrroloquinoline-quinone (PQQ), as do many other dehydrogenase enzymes (reviewed in Duine *et al.*, 1986). MDH catalyses the oxidation of methanol to formaldehyde, although it can also catalyse the oxidation of formaldehyde. The role of MDH in the oxidation of formaldehyde is not believed to be of physiological significance as mutant strains which lack MDH are not affected in their ability to convert formaldehyde to formate (Heptinstall & Quayle, 1970). The MDH isolated from *Methylobacterium extorquens* AM1 is composed of two sub-units and the native complex is in the configuration of $\alpha_2\beta_2$. The α sub-unit is a 62 kDa protein which contains the PQQ and calcium binding sites. The β sub-unit is 8.5 kDa in size and contains a large number of lysine residues (Nunn *et al.*, 1989) although the function of this component is unknown. The α and β subunits of PQQ -dependent MDH have been crystallised and the three dimensional structure deduced, shown in Figure 1.4 (Xia *et al.*, 1992; and reviewed in Anthony *et al.*, 1994).

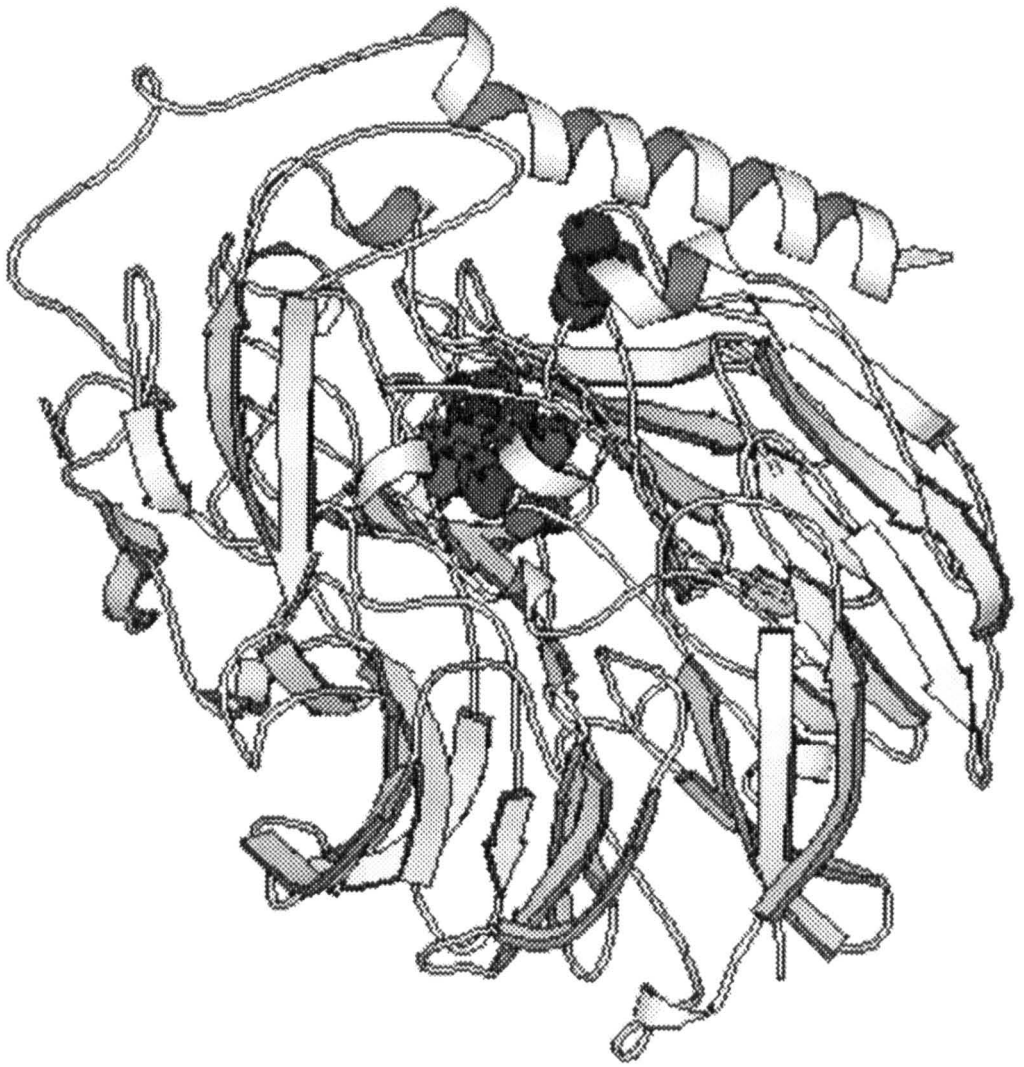


Figure 1.4 Schematic representation of the 3 dimensional structure of MDH (Brookhaven code, 3aah).

The β subunit is orientated at the top of the molecule and comprises of a single α-helix and a loop region. The α subunit comprises the bulk of the structure containing the PQQ and Ca^{2+} which are shown in CPK format in the centre.

Catalytic activity of PQQ - dependent MDH enzymes in soluble extract requires the addition of artificial electron acceptors, such as phenazine methosulphate. The interaction between MDH and its proposed *in vivo* specific electron acceptor, cytochrome C_L has also been characterised (reviewed in Anthony *et al.*, 1993).The current evidence indicates that cytochrome C_L associates through hydrophobic interactions with residues present in the α sub-unit of MDH (Cox *et al.*, 1992; Chan & Anthony, 1991). The cytochrome C_L interacts with a typical Class 1 C-type cytochrome (Cytochrome C_H) which is the substrate for the membrane oxidase cytochrome aa_3 or cytochrome co (Anthony *et al.*, 1993).

The MDH isolated from *Bacillus* strain C1 (Vonck *et al.*, 1991; Arfman, 1991), differs from the PQQ - dependent MDH enzyme described above. This MDH is a NAD-dependent MDH which oxidises C1-C4 primary alcohols. The MDH has been shown to consist of ten identical subunits of 43 kDa and each subunit contains 1 mole of zinc atoms and 1 mole of magnesium atoms. Interestingly these enzymes also contain 1 mole of NADH which is tightly bound to the enzyme and activity requires the addition of exogenous NAD^+.

There is also evidence that MDH enzymes isolated from *Hyphomicrobium* X and *Bacillus* strain C1 have a regulatory component which can effect the enzyme activity (Arfman, 1991). Moreover there are reports of a modifier protein (Page & Anthony, 1986) which can decrease the affinity of formaldehyde for the MDH enzyme isolated from *M. extorquens* AM1.

The formation of formaldehyde is therefore central to the metabolism of methanotrophs. When grown on methane, *M. capsulatus* (Bath) produces 1 mole of formaldehyde per mole of methane oxidised. Such formaldehyde formation could generate problems if there were no method available for its removal as formaldehyde is toxic to cells at concentrations > 15 %. Methanotrophic organisms have developed three principal systems for the metabolism of formaldehyde. In *M. capsulatus* (Bath) these are:

1) assimilation into the biomass by RuMP pathway,

2) oxidation of formaldehyde via the RuMP pathway and

3) direct oxidation by aldehyde dehydrogenase enzymes.

Each of these formaldehyde removal systems will now be discussed.

1.2 Metabolism of formaldehyde.

1.2.1 Assimilation of formaldehyde.

The principal method of formaldehyde fixation in *M. capsulatus* (Bath) is the RuMP pathway which can be conveniently divided into three stages: (1) fixation of formaldehyde into a six carbon compound (C_6), 2) cleavage of the C_6 compound to generate two C_3 compounds and (3) rearrangement of the products from fixation and cleavage stages to form a C_5 compound. These are highlighted in Figure 1.5 which is the proposed RuMP pathway of *M. capsulatus* (Bath) (Strom *et al.*, 1974).

The fixation of formaldehyde is common to all methylotrophs where three formaldehyde molecules are assimilated by combination with three, ribulose-5-phosphate (Ru5P) molecules, by the enzyme hexulose-6-phosphate synthetase. The products hexulose-6-phosphate (H6P) are then isomerized by hexulose-6-phosphate isomerase to produce three, fructose-6-phosphate (F6P) molecules. One of these F6P molecules is then cleaved into two C_3 while the other two F6P molecules are retained for the final stage.

The cleavage of F6P is achieved by two possible routes: either conversion of F6P to fructose-1,6-bisphosphate (F16BP) by phosphofructokinase and subsequent cleavage by fructose-1,6-bisphosphate aldolase (FBPA), known as the FBPAase

variation. Alternatively F6P is oxidised by enzymes of Enter-Douderoff pathway to 2-keto-3-deoxy-6-phosphogluconate (KDPG) and ultimately cleaved by KDPG aldolase (known as the KDPGA variation). The former pathway requires the use of an ATP molecule for the formation of F16BP, while the latter produces NAD(P)H from the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate (6PG). In *M. capsulatus* (Bath), F6P is converted to C_3 molecules by the KDPGA variation involving the enzymes described above. Utilising this pathway one molecule of pyruvate is produced from the assimilation of three formaldehyde molecules (Strom *et al.*, 1974).

Ru5P is regenerated by a series of sugar rearrangement reactions. This third stage of the cycle has also been identified in methylotrophic organisms in two forms. The two sugar phosphate interconversions involve transketolase enzymes, the TA variation present in *M. capsulatus* (Bath) utilises the enzymes ribose-5-phosphate isomerase and ribulose-5-phosphate epimerase while the second variation (SBPase variation) found in the facultative methylotrophic organisms, such as *Bacillus* PM6 (Colby and Zatman, 1975), uses sedoheptulose-1,7-bisphosphate aldolase and sedoheptulose-1,7-bisphosphatase for the conversion. In both cases the second C_3 molecule generated from the cleavage stage above is combined with the two remaining F6P molecules which, after the rearrangement reactions produces, three Ru5P molecules.

This pathway is the principal system for formaldehyde fixation in *M. capsulatus* (Bath). This allows pyruvate to be constructed which can then be used for the construction of other metabolites. In *M. capsulatus* (Bath) one cycle of the pathway generates 1 mole of pyruvate from 3 moles of formaldehyde and also produces 1 mole of NAD(P)H.

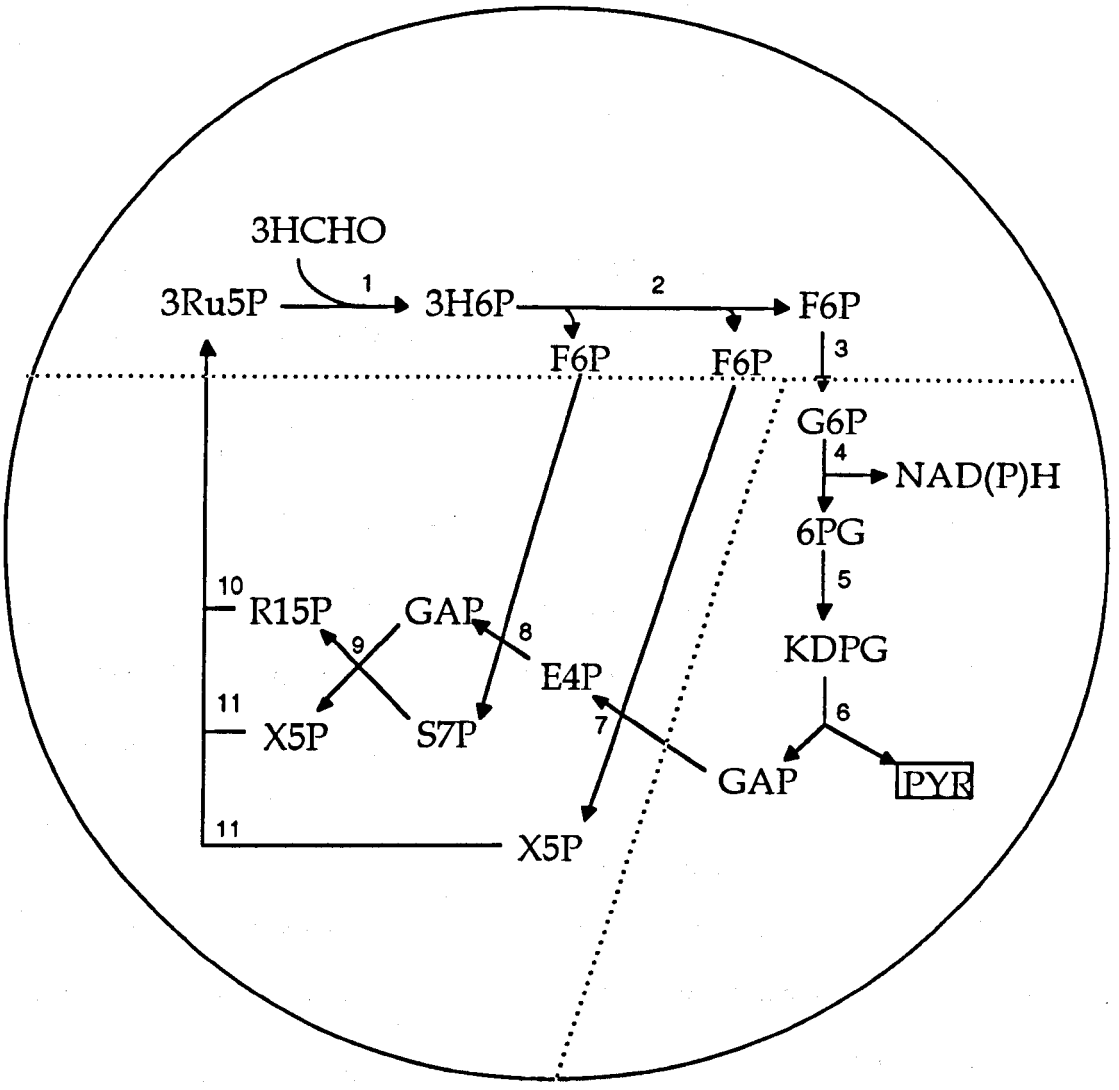


Figure 1.5 The RuMP pathway for formaldehyde fixation in *M. capsulatus* (Bath).

The various reactions are catalysed by the enzymes marked: 1. Hexulose phosphate synthetase; 2. Hexulose phosphate isomerase; 3. phosphoglucose isomerase; 4. glucose-6-phosphate dehydrogenase; 5. 6-phosphogluconate dehydrogenase; 6. 2-keto-3-deoxy-6-phosphogluconate aldolase; 7. transketolase; 8. transaldolase; 9. ribulose-5-phosphate 3 epimerase.

Abbreviations. 6PG. 6-phosphogluconate; E4P. erythrose-4-phosphate; F6P. fructose-6-phosphate; G6P. glucose-6-phosphate; GAP. glyceraldehyde-3-phosphate; H6P. hexulose-6-phosphate; KDPG. 2-keto-3-deoxy-6-phosphogluconate; Ri5P. ribose-5-phosphate; Ru5P. ribulose-5-phosphate; S7P. sedoheptulose-7-phosphate; X5P. Xylulose-5-phosphate.

1.2.2 Oxidation of formaldehyde in methanotrophs.

Methylotrophic organisms oxidise formaldehyde to carbon dioxide via formate, utilising formaldehyde dehydrogenase (FDH) and formate dehydrogenase (FoDH) enzymes. The oxidation of formaldehyde generates NADH which can be used in general metabolism for the synthesis of cellular components, formation of ATP or the oxidation of methane by MMO. A wide range of FDH enzymes have been isolated and/or characterised from methanotrophs and other methylotrophs (Table 1.1).

The FDH enzymes isolated from methylotrophs thus far can be conveniently divided into 2 broad groups depending upon their electron acceptor: the NAD^+ - dependent FDH (nFDH) enzymes and dye-linked FDH (dFDH) enzymes. Activity of dFDH enzymes can only be determined in cell free extract by the addition of artificial electron acceptors, such as phenazine methosulphate and/or cytochromes. *In vivo* it is probable that dFDH enzymes directly associate with a cytochrome, leading to direct ATP synthesis via the electron chain, although as yet this has not been characterised. A dFDH has been described in *M. capsulatus* (Bath) which was found to be present in soluble extracts at low levels (Hay, 1990) .

Table 1.1 Comparison of FDH enzymes from methylotrophic and non-methylotrophic organisms.

Organism	FDH		FoDH	Oxidative RuMP	Reference
	NAD^+	DCPIP	NAD^+		
Methanotrophs					
M. capsulatus (Bath)	+*	+	+		1,2
M. capsulatus (Texas)	-	-	+	+	3
Methylomonas methanica	$+^{\psi}$	+	+	+	3
Methylomonas GB3	+		+		4
Other methylotrophs and non methylotrophic organisms					
Methylophilus methylotrophus	$+^{\psi}$	-	+	+	4
Amycolatopsis methanolica	$+^{\psi}$				4
Rhodococcus erythropolis	$+^{\psi}$				4
Paracoccus denitrificans	$+^{\psi}$				4
Thiobacillus versutus	$+^{\psi}$				4
Methylophaga marina	$+^{\psi}$				4
Hansenula polymorpha	$+^{\psi}$				4
Pseudomonas olevorans	$+^{\psi}$		+	+	4
Acetobacter methanolicus MB58	$+^{\psi}$	+	+	+	4

FDH enzymes labelled ψ are GSH-factor dependent formaldehyde dehydrogenase enzymes and * modifier protein factor dependent. 1. Stirling & Dalton, 1978, 2. Hay, 1990, 3. Strom *et al.*, 1974, 4. From Dijkhuizen *et al.*, 1992

nFDH enzymes can be further classified depending upon their requirement for additional compounds for formaldehyde oxidation. nFDH enzymes which do not require additional compounds for the catalysis of formaldehyde oxidation have been isolated from *Methylomonas* GB3 although the best example is the enzyme isolated from *Pseudomonas putida* C83 (Ando *et al.*, 1979). This enzyme has a narrow substrate specificity, only oxidising aliphatic aldehydes with less than four carbon atoms. FDH enzymes which require extra cofactors for formaldehyde oxidation can again be divided into two groups, those that require a thiol cofactor or those that require a protein. In recent years thiol - dependent FDH enzymes have been isolated from many methylotrophs and non-methylotrophs. FDH activity of these enzymes can only be determined after a thiol compound has been added to the reaction mixture. In most cases the thiol compound used is glutathione (GSH) and these are termed GSH - dependent FDH (gFDH) enzymes. gFDH enzymes have been isolated/identified in many methylotrophs and non-methylotrophs (Table 1.1). The enzyme isolated from *Amycolatopsis methanolica* is a trimeric zinc - containing dehydrogenase which appears to be similar to the enzyme isolated from *Rhodococcus erythropolis* (van Ophem *et al.*, 1992; van Ophem *et al.*, 1994). The gFDH enzymes isolated from *R. erythropolis* and *A. methanolica* are similar at the primary sequence level, and the gFDH enzymes have been identified as Type III alcohol dehydrogenase enzymes isolated from liver tissue (Holmquist & Vallee, 1991). A FDH enzyme isolated in *Hyphomicrobium* X is also a factor dependent enzyme. Studies into the nature of the factor have proved unsuccessful and it is believed that this enzyme is identical to gFDH enzymes as FDH activity is stimulated in the presence of GSH (Poels & Duine, 1989). The FDH enzyme isolated from *M. capsulatus* (Bath) differs from gFDH enzymes in that it requires the presence of a small heat stable protein for FDH activity (Stirling & Dalton, 1978). The enzyme activity is not stimulated by the presence of thiol compounds and constitutes the final group of FDH enzymes that have been identified. It has been demonstrated that the absence of this small protein cofactor allows FDH to oxidise a range of aliphatic aldehydes except formaldehyde and only in the presence of this protein cofactor is

formaldehyde oxidised (Millet *et al* ., unpublished) . This FDH is therefore termed a modifier protein dependent FDH (mFDH).

The formate that is generated by the direct oxidation of formaldehyde is further oxidised by FoDH enzymes. These enzymes are generally soluble NAD^+ - linked and are uniform in methanotrophs which utilise the linear formaldehyde oxidation pathway (Zatman, 1981). For a review of the structure and function of these enzymes see Anthony, 1982 and Popov & Laminz, 1994.

1.2.3 Oxidation of formaldehyde by the RuMP pathway.

The RuMP pathway described above not only allows formaldehyde assimilation, but can be involved in the oxidation of formaldehyde to CO_2. Enzymes involved in dissimilatory RuMP pathway were first discovered in 1974 (Strom *et al.*, 1974). The cycle is outlined in Figure 1.6 and demonstrates that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are key enzymes in the pathway.

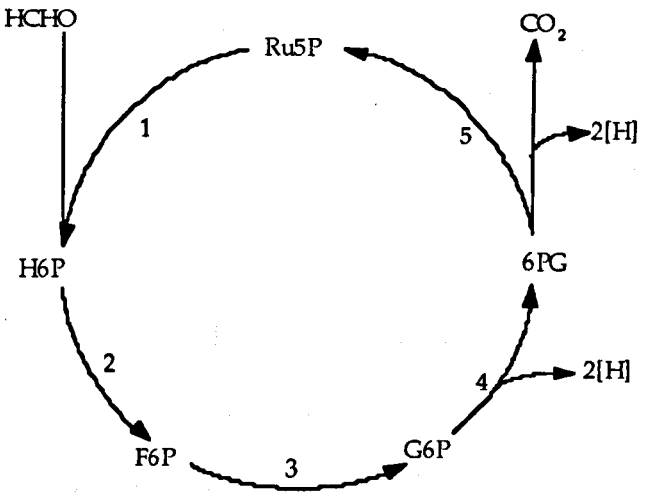


Figure 1.6 Oxidation of formaldehyde by enzymes involved in the RuMP pathway.

1. Hexulose phosphate synthetase, 2. Hexulose phosphate isomerase, 3. Phosphoglucose isomerase, 4. Glucose-6-phosphate dehydrogenase, 5. 6-Phosphogluconate dehydrogenase. Abbreviation of compounds as in Figure 1.5

In organisms where high FDH activities have been reported the role of the RuMP pathway in the oxidation of formaldehyde is questioned as only low activities of the key enzymes involved have been determined. Studies on the organism *Psuedomonas* C have shown that linear oxidation is used for the oxidation of formaldehyde, even in the presence of RuMP enzymes (Basset *et al.*, 1980). The validity of these studies has been questioned and from the same data it can be determined that >90% of formaldehyde oxidation may occur via the RuMP pathway (Anthony, 1982). The role of the RuMP pathway in formaldehyde oxidation in *M. capsulatus* (Bath) is unknown, although it is an important formaldehyde oxidation pathway in some methylotrophs.

As demonstrated above *M. capsulatus* (Bath) has evolved three systems for the removal of formaldehyde. These systems must work not only together, but also in harmony with those for the formation of formaldehyde and the assimilation pathway generating C_3 compounds. In *M. capsulatus* (Bath) the key enzyme in maintaining this metabolic balance is FDH. This point in the metabolism is a committal step as oxidation

by FDH enzymes is generally irreversible and results in a reduction of formaldehyde concentration. Therefore *M. capsulatus* (Bath) has evolved regulatory components for enzymes involved in the formation of formaldehyde and its subsequent oxidation.

1.3 Control of metabolic pathways by protein interactions.

Most metabolic pathways are controlled by protein interactions though some enzymes have specific regulatory proteins which modify their functions. Those proteins which effect only the substrate specificity of a target enzyme are termed specifier proteins, while those which alter the substrate specificity and the kinetics of a target enzyme are termed modifier proteins.

The following is an account of specifier and modifier proteins which have been characterised. The proteins which will be discussed are Protein B, the regulatory component of sMMO; M-protein, the regulatory component of MDH; α-lactalbumin, the regulatory component of lactose synthetase. Others proteins have also been identified and these are given in Table 1.2. Only limited studies have been made into these proteins and consequently very few data available.

Table 1.2 Proteins which modify metabolic processes.

Source	Name	Effector Protein	Effect	Reference
Bacillus subtilis	Cwb A product	autolysin and *spoIID*	stimulate enzyme activity.	1
Rat liver	unknown	glucokinase	association allows glucokinase to be antagonistically regulated by fructose-6-phosphate and fructose-1-phosphate	2
Chicken liver	unknown	fructose-1,6-bisphosphotase	stimulation of enzyme activity.	3
Rate kidney epithelial cells	glyceraldehyde-3-phosphate dehydrogenase activator protein	glyceraldehyde-3-phosphate dehydrogenase	stimulates enzyme activity	4

1. Kurodua *et al.*, 1992; 3. van Schaftingen, 1989; 4. Han *et al.*, 1992; 5. Aithal *et al.*, 1994.

The above list is not complete as the vast number of DNA - binding, cell cycle regulatory proteins e.g. G-protein. can also be described as modifier or specifier proteins. The DNA - binding proteins and cell cycle regulatory components are generally specifier proteins in that they alter the substrate specificity of an enzyme e.g. sigma factors alter the substrate specificity of RNA polymerase.

1.3.1 Regulation of formaldehyde formation by protein interactions.

As described above the oxidation of methane to formaldehyde utilises MMO and MDH. sMMO is a three component enzyme which has a small regulatory component while MDH, isolated from *M. extorquens* AM1, is a two component enzyme one of which is a regulatory component (Page & Anthony, 1982). The MDH enzyme isolated from Bacillus sp. has an activator protein (Arfman, 1991) which stimulates the oxidation of methanol.

Early studies on protein B confirmed its ability to regulate sMMO activity (Green & Dalton, 1985). The endogenous rate of NADH oxidation, catalysed by the hydroxylase and reductase components, was unaffected by the presence of substrate but greatly reduced in the presence of protein B. The presence of protein B was required for substrate oxidation and in its absence no product formation could be detected, as demonstrated in Table 1.3.

In addition to reducing the rate of NADH oxidation, protein B was shown to reduce the rate of electron transfer from the reductase to the hydroxylase (Table 1.4) in the absence of substrate. The presence of an oxidisable substrate stimulated oxygen uptake while in the absence of the substrate protein B reduced the rate of NADH consumption/oxygen uptake. From this data it was proposed that the presence of protein B uncoupled the electron transfer from the reductase to hydroxylase thus inhibiting the oxidation of substrate.

Table 1.3. The effect of protein B on NADH oxidation by sMMO (Green & Dalton, 1985).

sMMO Assay Components	Rate of NADH oxidised ($nmol/min^{-1}$)	Rate of propylene oxide formation ($nmol/min^{-1}$)
hydroxylase, reductase, protein B and propylene	150.9	161.8
hydroxylase, reductase and protein B	3.3	0
hydroxylase, reductase and propylene	53.4	0
hydroxylase and reductase	59.4	0
hydroxylase	0	ND
protein B	0	ND
reductase	4.5	ND

ND - not determined

Table 1.4 Coupled and uncoupled activities of the sMMO complex.

Assay Components	Oxygen consumed	Cyanomethane oxidised	NADH consumed
hydroxylase, reductase, protein B and CH_3CN	102.3	108	114
hydroxylase, reductase and protein B	16.7	ND	32.1
hydroxylase, reductase and CH_3CN	53.4	0	116.2
hydroxylase and reductase	54.6	ND	111.8

ND - not determined.

The specific binding site for each component in the sMMO complex has been mapped using cross linking studies. EPR spectroscopy provided evidence that protein

B association results in an alteration in the active site environment of the hydroxylase. The recent x-ray structure of the hydroxylase revealed a possible binding site for protein B. A wide canyon is identified on the hydroxylase (Figure 1.7) which contains the exposed iron - binding domains (Rosenzweig *et al.*, 1993). It is proposed that both protein B and the reductase bind in this region.

Figure 1.7 Schematic representation of the 3D structure of the hydroxylase component of sMMO (Brookhaven code 1mmo).

The molecule is displayed with its symmetry axis from top to bottom. The centre of the molecule is identified as the possible binding region for protein B and the reductase.

From these studies a hypothesis of the regulation of methane oxidation by protein B has been put forward (Figure 1.8) (reviewed in Lipscomb, 1994). It is believed that protein B is associated with the hydroxylase *in vivo* as they are present in approximately equal quantities and their association constant is high. The reductase is present in much lower concentrations, although it has a large association constant. Therefore it is likely that it is also present in the complex *in vivo*. It is believed that the catalytic cycle is initiated by the reduction of the hydroxylase by the reductase. This reduction decreases the association constant of protein B which then leaves the complex, although evidence does indicate that this only occurs after the reductase has dissociated. This allows a two electron transfer from the reductase to the hydroxylase before oxygen activation commences. The dissociation of protein B does not result in a rapid relaxation of the hydroxylase structure and this allows the oxygenation reaction to occur. This idea is not supported by evidence from other workers and studies of the *M. capuslatus* (Bath) sMMO show that the hydroxylase structure relaxes after dissocation of protein B (Patricia Wilkins, Personal communication)

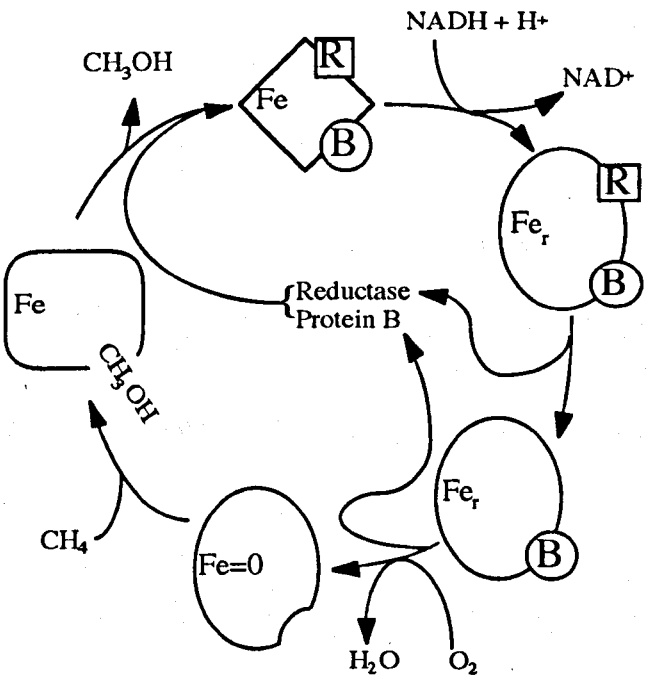


Figure 1.8 Proposed mechanism of protein B regulation of sMMO activity (Lipscomb, 1994). Fe - Diiron active site of hydroxylase, Fe_r - reduced diiron active site, R - Reductase, B - Protein B.

It would therefore appear that protein B controls the action of sMMO by altering its conformation and slowing electron transfer from the reductase to the hydroxylase. The role and function of protein B described above has been deduced from studies of proteins isolated from *M. capsulatus* (Bath) and *M. trichosporium* OB3b. These proteins differ slightly both in size and amino acid composition and it may be that the physiological function of the two proteins is different. In *M. capsulatus* (Bath) the presence of protein B is essential for substrate oxidation activity unlike protein B from *M. trichosporium* OB3b which stimulates sMMO activity 10 fold though is not essential for activity. Studies of sMMO from the organism *Methylobacterium* SP. CRL26 show that it does not require any regulatory component for sMMO activity (Patel & Savas, 1987). It has been proposed that in this case the regulatory component may be tightly bound to the hydroxylase.

The catalytic activity of MDH isolated from *M. extoquens* AM1 and Bacillus sp. are also regulated by protein interactions. There have been two regulatory components identified: a modifier protein which alters the substrate specificity of *M. extoquens* AM1 MDH (Page & Anthony, 1982) and an activator protein which stimulates Bacillus sp. MDH activity (Arfman *et al.*, 1991).

The *M. extorquens* AM1 MDH modifier protein has been isolated from periplasmic extracts, which is the location of MDH in methylotrophic organisms (Long & Anthony, 1986). The purified modifier protein was shown to be a 45 kDa protein which effected the substrate specificity of MDH and is termed M-protein. This protein is believed to associate with MDH and subsequently increase the affinity of methanol for MDH (Table 1.5)

Table 1.5 Rates of substrate oxidation by MDH in the presence and absence of MDH modifier protein (Page & Anthony, 1986).

Substrate	Rate of cytochrome reduction in the presence of the modifier protein. (nmol/min^{-1})	Rate of cytochrome reduction in the absence of the modifier protein. (nmol/min^{-1})
Methanol	3.0	5.4
Formaldehyde	3.3	1.4

M-protein has been isolated from two other methylotrophic organisms, *M. methylotrophus* and *P. denitrificans* (Long & Anthony, 1991). The effect of the modifier protein on cytochrome linked methanol oxidation activity in these organisms differed. In *M. methylotrophus* M-protein activated MDH by up to 50 %, while in *P. denitrificans* M-protein decreased the activity by up to 50 %, despite increasing the affinity for this substrate. In both cases the effect of the modifier protein on the rate of formaldehyde oxidation was identical. Both the V_{max} and K_m for formaldehyde were reduced and the maximum effect was observed in a 5:1 molar ratio of MDH:modifier protein (Long & Anthony, 1991). The role of the MDH modifier protein is unknown as its concentration is believed to be insufficient to maintain the 5:1 molar ratio mentioned above.

The second form of MDH regulatory protein identified is able to activate the NAD(H) containing MDH isolated from the thermotolerant *Bacillus* strains (Arfman *et*

al., 1991). The activator protein is a dimeric protein of 27 kDa subunits, which lacks any chromophore. The purified activator protein is able to bind 1 mole of NAD(H) per mole of protein and forms a loose complex with the MDH. Formation of the loose complex results in a 40 fold increase in the methanol turnover rate, at physiological concentrations of methanol. The activator protein facilitates the reoxidation of the MDH bound NAD(H) and appears to be a more favourable electron acceptor than free NAD^+. The physiological significance of this activator protein is unknown and its mechanism appears completely different from the M-protein.

1.3.2 Regulation of lactose synthesis in mammary glands.

The regulatory proteins involved in controlling the activity of sMMO and MDH, are specifier proteins. Protein B controls electron transfer between the reductase and the hydroxylase component, thus altering the activity of sMMO, while the modifier protein specifies methanol oxidation by MDH. The only documented example of a modifier protein is α-lactalbumin (Hill & Brew, 1975). This small 16 kDa protein is released in mammary glands in response to hormonal control and alters the specificity of the enzyme γ-galactosyl transferase. Both α-lactalbumin and γ-galactosyl transferase are part of the lactose synthetase complex. γ-galactosyl transferase catalyses reaction (B) in Figure 1.9 and is primarily involved in the biosynthesis of oligosaccharide prosthetic groups of certain glycoproteins. It is also able to catalyse the formation of N-acetyllactosamine by the reaction with free N-acetylglucosamine (Figure 1.9, A). *In vitro* γ-galactosyl transferase is also able to catalyse the formation of lactose (Figure 1.9, C) although the K_m value for glucose for this reaction is ~ 2.5 M. It is therefore believed that at physiological glucose concentrations the formation of lactose by γ-galactosyl transferase is unlikely to occur. γ-galactosyl transferase has been isolated from most cellular tissues where its function is to catalyse reactions A and B in Figure

1.9. α-lactalbumin has only been isolated from mammary gland tissue and was initially identified in milk. As it is only found in these tissues its effect is very specific. α-lactalbumin was shown to alter the substrate specificity of γ-galactosyl transferase by reducing the K_m for glucose.

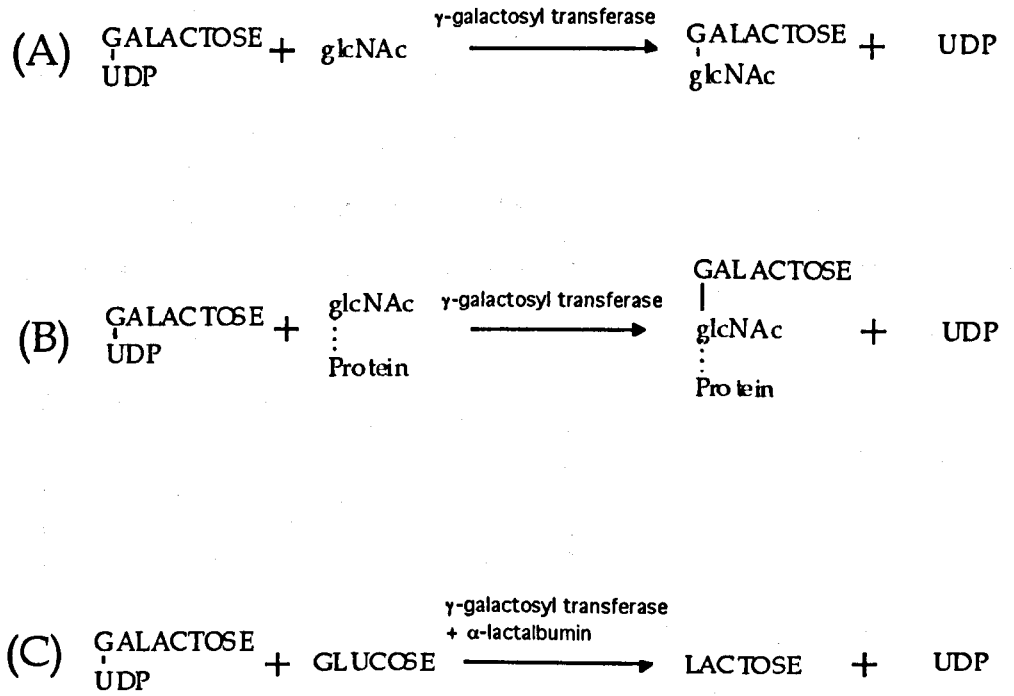


Figure 1.9 Reactions catalysed by γ-galactosyl transferase.

(A) Formation of N-acetyllactosamine (galactose-glcNAc) from UDP-galactose and N-acetylglucosamine (glcNAc).

(B) Formation of oligosaccharide prosthetic groups.

(C) Formation of lactose in the presence of α-lactalbumin.

It was determined that α-lactalbumin specified the reaction catalysed by γ-galactosyl transferase and was designated a 'specifier protein' by virtue of its unique ability to alter γ-galactosyl transferase activity. It was determined that in the absence of α-lactalbumin, γ-galactosyl transferase shows an ordered kinetic mechanism with respect to substrate binding and product dissociation (Morrison & Ebner, 1971). While it is proposed that in the presence of α-lactalbumin dead-end inhibitory complexes are formed between UDP-galactose and γ-galactosyl transferase which stop the N-acetyllactosamine synthesis reactions (Morrison & Ebner, 1971). The formation of dead-end complexes in the presence of α-lactalbumin and subsequent 10,00 fold decrease in the K_m value for glucose allows lactose synthesis. As α-lactalbumin alters both the substrate specificity and kinetics of γ-galactosyl transferase it should therefore be termed a modifier protein.

1.4 Historical information, aims and outline of this study.

Prior to this study very little information was available about the FDH isolated from *M. capsulatus* (Bath) in 1978 (Stirling & Dalton, 1978) . It was known that for the oxidation of formaldehyde a small heat stable protein was required. This heat stable component was characterised and shown to be a small peptide which modified the function of FDH and was therefore described as a modifier protein (Millet *et al.*, unpublished) . It was shown that FDH catalysed the oxidation of formaldehyde and some long chain aldehydes although formaldehyde oxidation could only be detected in the presence of the modifier protein. In the absence of the modifier protein the oxidation of higher aldehydes was demonstrated by FDH.

The oxidation of formaldehyde in the presence of the modifier protein was shown to follow sigmoidal kinetics, while in the absence of the modifier protein the oxidation of acetaldehyde was shown to follow saturated kinetics (Millet *et al.*,

(unpublished) . Thess data contradicted that presented in 1978 when the oxidation of formaldehyde by FDH was presumed to follow saturated Michaelis Menten kinetics (Stirling & Dalton, 1978). The reason why FDH has a modifier protein was unknown. The role of this FDH enzyme in the metabolism of C_1 compounds in *M. capsulatus* (Bath) was presumed to be for the regeneration of NADH for the assimilation of methane (Stirling & Dalton, 1978). In light of the information previously known the aims of this study were to:

1. Develop methods for the purification of both FDH and the modifier protein.
2. Study the substrate specificity of FDH in both the presence and the absence of the modifier protein.
3. Investigate the effect of the modifier protein on the kinetics of FDH.
4. Study how the modifier protein affects FDH.
5. Investigate whether the modifier protein was active with other dehydrogenase enzymes.

This thesis describes the results of studies into the biochemistry of the modifier protein and FDH isolated from *M. capsulatus* (Bath). The development of purification methods for both components and structural characterisation of FDH and the modifier protein are described in Chapter 3. Investigation of the kinetics of the modifier protein and FDH describing the effect that the modifier protein has on FDH is given in Chapter 4. Also described in Chapter 4 is a possible mechanism of modifier protein action. In Chapter 5 data are presented which shows that the modifier protein can alter the activity of other dehydrogenase enzymes. The purification and characterisation of an enzyme from a commercial preparation of calf liver glucose dehydrogenase which has its function altered by the modifier protein is also reported in Chapter 5. In Chapter 6 evidence is presented for a second NAD^+ - linked FDH enzyme present in soluble

extracts of *M. capsulatus* (Bath). This enzyme was partially purified and characterised and shown to be different from the modifier protein dependent FDH enzyme.

2. Materials and Methods.

2.1 Materials.

All chemicals were purchased from Sigma chemical company (UK) and were of the highest grade available, unless otherwise individually stated. All water used in this study was double distilled before use.

FPLC equipment, media and columns were purchased from Pharmacia Biotech Limited (St Albans, UK). FPLC columns were used on either a Phamacia FPLC system, comprising two Pharmacia P500 pumps and a Pharmacia LCC 500 programmable pump controller, or a Phamacia Biopilot system, comprising three Pharmacia P6000 pumps. The Biopilot was programmed by the Pharmacia Unicorn Control system. Eluted protein in all FPLC methods was monitored by absorbance at 280 nm using a Pharmacia UV-M detector system, unless otherwise stated in the text. For all purification methods fractions were collected using a Pharmacia FRAC 100.

Isocratic Gel Permeation HPLC was performed using a single LKB 2152 pump and a LKB 2512 UV detector (Pharmacia - LKB, UK) set to 280 nm. Separation was performed using a TSK G3000 SW Gel Permeation column, TosoHaas (USA). A Beckman System Gold HPLC (Beckman instruments, Buckinghamshire, UK) with a programmable UV-visible detector was used for analytical HPLC methods.

Gel electrophoresis was performed using a BIO - RAD mini PROTEAN II and a BIO - RAD PowerPac 300 power supply (BIO-RAD, Hemel Hempstead, UK). UV/visible spectrophotometric measurements were performed using a Hewlett Packard diode array spectrophotometer (HP8452A) (Hewlett Packard, Paolo Alto, California, USA). A temperature controlled stage (HP98090A) was also employed to maintain a

constant temperature. Fluorescence experiments were performed using a Perkin Elmer LS-5 fluorimeter (Perkin Elmer, Warrington, UK).

Stopped flow kinetic experiments were performed using a HI-TECH SF80 instrument, using a xenon source and fluorescence detector. The data from the stopped flow experiments were collected using OLIS SF (On-line instruments systems Inc. Boggart. USA) software run on a Compaq 386/25 computer.

Electrospray mass spectrometry experiments were carried out using a Quattro II triple quadrupole mass spectrometer (VG Biotech, Altringham, UK) equipped with an atmospheric pressure ionisation chamber.

Centrifuges used in this study included a Beckman J2 - 20 (Beckman, UK) with JA 10, JA 20 and JA 21 rotors (Beckman, UK). A Microcentaur (MSE, Fisons, Crawley, UK) and a MSE 2000 (MSE, UK) bench centrifuges were also used. Cell cultures were centrifuged using a Westfalia continuous centrifuge (Westfalia Separator Ltd. Milton Keynes, UK).

Methylococcus capsulatus (Bath) cells were grown in a LH 210 or a LH 2000 fermenter (LH fermentation, Berkshire, UK).

2.2 Preparation of standard formaldehyde solution.

Formaldehyde was produced by the hydrolysis of the more stable polymer paraformaldehyde. Paraformaldehyde was suspended in water to a final concentration of 0.1g (w/v) and sealed in a glass ampoule. The sealed ampoules were then autoclaved at 121 °C for 3 hours until complete hydrolysis had occurred resulting in a 1 M formaldehyde solution. The concentration of the resulting solution was assayed using the Nash method described below.

2.3 Analytical determinations.

2.3.1 Formaldehyde concentration determination.

The acetylacetone method for formaldehyde concentration determination (Nash, 1953) was used. Assay reagents were prepared as described by Nash (1953). In summary, the reaction mixture (2 ml) and formaldehyde solution (0.1 ml) were incubated at 60 °C for 5 minutes and then diluted to a total volume of 10 ml with water. The absorbance of the diluted reaction mixture was determined spectrophotometrically at 412 nm. A standard curve was prepared using a standard solution of 1 M formaldehyde and unknown formaldehyde concentrations were determined from this curve.

2.3.2 Determination of formate and acetate.

The rates of formate and acetate produced in enzyme assays was determined by HPLC analysis. A 50 µl sample was taken from an enzyme reaction mixture at 0, 5 and

10 minute time intervals and diluted with 50 µl of concentrated phosphoric acid. This sample was centrifuged at 16,500 x g for 5 minutes prior to a 25 µl aliquot being loaded onto a HiChrom Licrosphere HPLC column equilibrated with an aqueous solution of 0.01 M octylamine, adjusted to pH 6.6 with phosphoric acid. The sample was eluted at a flow rate of 1.0 ml/min in the same buffer. Formate and acetate were determined by comparing their absorbance at 210 nm with the absorbancies of known standard concentrations.

2.3.3 Continuous culture of *Methylococcus capsulatus* (Bath).

Methylococcus capsulatus (Bath) was grown in continuous culture under oxygen - limiting conditions on nitrate mineral salt medium, buffered with HCl at pH 7.2. The cells were grown at 45 °C at a growth rate of 0.024 hr^{-1}. Methane (25 % (v/v) in air) was supplied to a 2 L vessel as the carbon source.

2.3.4 Preparation of cell free soluble extract.

Cells were harvested from the 20 L fermenter overflow vessel from the chemostat by passing the cell suspension through a Westfalia continuous centrifuge at 4,000 x g. The resulting cell paste was washed by resuspending it in 100 ml of 20 mM phosphate buffer pH 7.2. The resuspended cells were centrifuged at 10,000 xg for 10 minutes and the supernatant removed. The cell paste was resuspended in 20 ml of the same buffer and the cells were stored drop frozen in liquid nitrogen at -80 °C.

When required, a small amount of stored frozen cells was removed and resuspended in an equal volume of 20 mM phosphate buffer, pH 7.2. To this approximately 5 mg of DNAase III was added and the cells were passed 3 times

through a Constant systems (LH fermentation, UK.) cell disrupter with a breakage pressure of 24,000 p.s.i. The fractured cell suspension was clarified by centrifuged at 4 °C using a relative centrifugal force of 58,000 xg for 1.5 hours. After centrifugation the supernatant was removed and stored drop frozen in liquid nitrogen at -80 °C.

Alternatively, when small volumes of cells were being fractured (<50 ml) a French pressure cell was used. The cell suspension was passed through 3 times at a breakage pressure of 14,000 p.s.i. After fracturing, the cell suspension was centrifuged as above.

2.3.5 Preparation of heat - treated soluble extract (HTSE).

To prepare crude modifier protein, soluble extract was heated to 70 °C for 10 minutes. The precipitated protein material was removed by centrifugation at 4000 xg for 15 minutes. The supernatant was retained and is termed the heat - treated soluble extract (HTSE).

2.3.6 Definition of an enzyme unit.

One unit of enzyme activity used in this study is defined as the amount of protein required to produce 1 μmol of product, or remove 1 μmol of substrate, per minute.

2.3.7 Determination of formaldehyde dehydrogenase activity.

The rate of formaldehyde oxidation catalysed by formaldehyde dehydrogenase (FDH) in crude soluble extract was determined at 45 °C by measuring the formation of

NADH from NAD^+ spectrophotometrically at 340 nm. A typical reaction mixture contained 4 mg protein, 1 mg heat - treated soluble extract (Section 2.3.5), 3 mM KCN, 2 mM NAD^+, 2 mM formaldehyde and 25 mM phosphate buffer, pH 7.2, made to a final volume of 1 ml with water. All reagents, except formaldehyde, were added to a 1.5 ml semi-micro quartz cuvette. The solution was pre-incubated at 45 °C for 3 minutes, after which time the reaction was started by the addition of formaldehyde. When the activity of purified enzyme was being assessed the 3 mM KCN was omitted from the assay mixture.

Enzyme activity was also determined by following formaldehyde disappearance using the Nash assay as described above. The production of formate was also determined in some cases to assess the rate of formaldehyde oxidation (Section 2.3.2).

2.3.8 Determination of modifier protein activity.

Activity of the modifier protein was determined by its ability to restore formaldehyde oxidation activity to partially purified FDH. A typical reaction mixture contained 4 μM FDH, 4 mg of protein sample containing modifier protein, 3 mM KCN, 2 mM NAD^+ and 25 mM phosphate buffer pH 7.2 made to a final volume of 1 ml with water. All reagents, except formaldehyde, were added to a 1.5 ml semi-micro quartz cuvette and the solution preincubated at 45 °C for 3 minutes. The reaction was initiated by the addition of 2 mM formaldehyde. The rate of NADH formation was measured at 340 nm over a 5 minute period.

2.3.9 Determination of soluble methane monooxygenase activity.

Methane monooxygenase activity was determined by gas chromatography at 45 °C with propylene as substrate, as described in Green & Dalton, 1985.

2.3.10 Determination of alcohol dehydrogenase (E.C 1.1.1.1) activity.

Alcohol dehydrogenase catalytic activity was determined at 37 °C by measuring the rate of NADH formation at 340 nm. The reaction mixture contained 1 unit of alcohol dehydrogenase (from Bakers Yeast, Sigma A3263), 2 mM NAD^+, 1 mM ethanol and 25 mM phosphate buffer, pH 7.5, made to a final volume of 1 ml with water. All reagents, except ethanol, were added to a semi-micro quartz cuvette and the reaction mixture was preincubated for 1 minute at 37 °C. The reaction was started by the addition of ethanol to a final concentration of 1 mM. The formation of NADH was measured at 340 nm for 3 minutes.

2.3.11 Determination of glucose dehydrogenase (E.C. 1.1.1.47) activity.

The catalytic activities of glucose dehydrogenase from *Bacillus megaterium* (Sigma G7653) and calf liver (Sigma G5625) were determined spectrophotometrically at 340 nm and 37 °C by the appearance of NADH. The assay solution contained 2.5 units of glucose dehydrogenase, 2 mM glucose, 2 mM NAD^+ and 25 mM phosphate buffer pH 8.8 made to a final volume of 1 ml with water. All reagents, except glucose, were added to a semi-micro quartz cuvette and the reaction mixture was preincubated for 1 minute at 37 °C. The reaction was started by the addition of 1 mM glucose and NADH formation was followed for 1 minute.

2.3.12 Determination of glyceraldehyde-3-phosphate dehydrogenase activity (EC. 1.2.1.12).

The catalytic activity of glyceraldehyde-3-phosphate dehydrogenase was determined spectrophotometrically at 25 °C, by following the formation of NADH at 340 nm. A typical reaction mixture contained 1 unit of glyceraldehyde-3-phosphate dehydrogenase, 2 mM glyceraldehyde-3-phosphate, 2 mM NAD^+ and 25 mM phosphate buffer pH 7.6, made to a final volume of 1 ml with water. All reagents, except glyceraldehyde-3-phosphate, were added to a 1.5 ml semi-micro quartz curette and the reaction initiated by the addition of 2 mM glyceraldehyde-3-phosphate. NADH formation was followed over a 5 minute period.

2.3.13 Determination of protein concentrations.

Total protein concentrations of FDH containing solutions were determined using the BIORAD protein determination kit (BIORAD, Hemel Hempsted UK.). The reagent was used according to the manufacturers instructions. In summary, 20 µl of protein sample was added to 980 µl of dye reagent. The mixture was incubated at room temperature for 5 minutes prior to the absorbance being determined at 595 nm. Bovine serum albumin was used as a protein standard for calibration of the reagent. Protein solutions which gave an absorbance greater than 1.0 were diluted and the protein concentration predetermined.

The concentration of purified modifier protein was determined spectrophotometrically at 205 nm as described in Stoscheck (1987). Briefly modifier protein solutions were diluted until their concentration was approximately 1 - 10 µg/ml. The absorbance at 205 nm was determined and the protein concentration then estimated.

2.3.14 Stopped flow kinetics experiments.

Pre-steady state kinetics were analysed using a stopped flow spectrophotometer Figure 2.1.

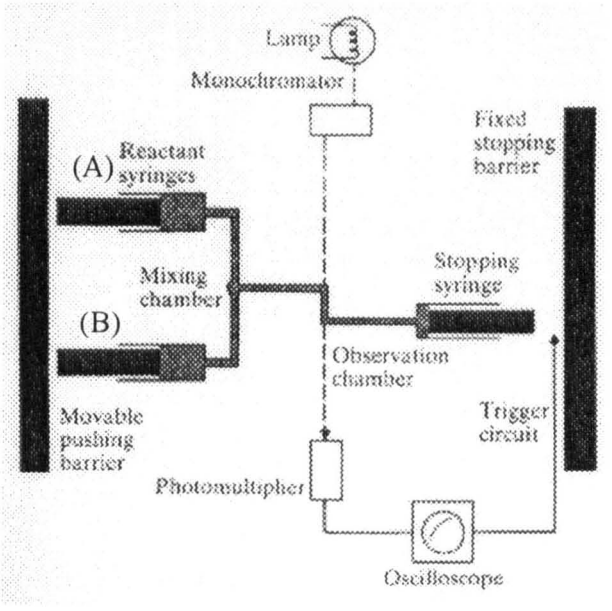


Figure 2.1 Diagram of a standard stopped flow instrument.

Figure 2.1 shows a schematic diagram of a standard stopped flow apparatus. The reactant syringes contained different reagents which, when mixed in equal amounts initiate the reaction. A solution containing all reaction reagents, but one, was placed in syringe B and the analyte solution under study was in syringe A. The contents of the syringes (A and B) and reaction mixtures are described in the results chapter. A rapid shot of compressed air (100 μsec) against the movable barrier forces equivalent volumes of solution from syringes A and B simultaneously into the mixing chamber. This rapidly mixes the reactants before the solution moves into the observation chamber. The stop syringe arrests the flow abruptly and at a fixed time after mixing

initiates the data collection system. This allows measurements to begin a few milliseconds after reagent mixing.

In these experiments, the instrument stop syringe was set at 170 μl, and the reactant syringes each contained 5 ml of solution. The reactants were maintained at 45 °C, prior to mixing. NADH formation was determined by fluorescence detection using an excitation wavelength of 340 nm and emission at 435 nm. In each experiment 100 data points were collected over a 5 second period (in studying the modifier protein the reaction was complete in less than 1 second so 100 data points were collected over a 1 second range). The data from 10 individual experiments were averaged and used to calculate the first order rate constant (k_{obs}) using OLIS stopped flow software.

To standardise the instrument the interaction of 4-methylumbelliferyl α-D-mannopyranoside with concanavalin A was used (Harrington & Wilkins, 1978).

2.3.15 Protein UV-visible absorption spectra.

UV - visible spectra of protein samples were measured using a Hewlett Packard diode array spectrophotometer. All proteins were diluted with 25 mM phosphate buffer prior to measuring the absorbance over a wavelength range of 220 - 800 nm.

2.3.16 Determination of fluorescence emission spectra.

Fluorescence emission spectra of protein samples and reagents were performed with a Perkin Elmer LS-5 fluorimeter using an excitation wavelength of 340 nm. Samples for analysis were diluted with 25 mM phosphate buffer, pH 7.2, and the emission spectra determined over the wavelength range 300 - 700 nm.

2.3.17 Determination of protein molecular mass.

The molecular weight of FDH was estimated by SDS - PAGE using a 15 % (w/v) gel system. The relative mobilities of FDH and molecular weight standards were compared and the molecular weight determined graphically. On all SDS - PAGE gels, unless otherwise stated, Pharmacia low molecular weight protein markers were used (Pharmacia 17-0446-01). The native molecular weight of the enzyme was determined by elution from a Pharmacia Superdex 200 gel filtration column equilibrated with 50 mM Tris - HCl pH 7.2 buffer containing 100 mM KCl. A Pharmacia Superose 12 HR 10/30 gel filtration column equilibrated with the same buffer, was also used for determination of FDH native molecular weight. The relative elution time of FDH was compared to those proteins of known molecular weight. For calibration of the gel filtration columns, Pharmacia HMW markers (Pharmacia 17-0445-01) were used.

The native molecular weights of proteins were also determined by native PAGE using a 10 % (w/v) gel system and Pharmacia high molecular weight protein standards were used for calibration of the gels (Pharmacia 17-0445-01).

Accurate molecular weight measurements of FDH and modifier protein were made using electrospray ionisation mass spectrometry (ESI-MS). ESI-MS experiments were carried out in the nebuliser - assisted electrospray mode. The potential of the electrospray needle was set at 3.5 kV and the extraction cone voltage was set at 50V. FDH was diluted to a concentration of 40 *p*mol/ml in Tris-HCl buffer, pH 7.2, containing 5 % acetic acid. 10 µl aliquots were introduced into the source at a flow rate of 5 µl/min. Mass spectra were acquired over the range m/z 600 - 1700 during a 10 second scan and by operating the data system in the multichannel (MCA) mode several scans were summed to produce the final spectrum. Calibration was carried out using a solution of horse heart myoglobin.

2.3.18 Determination of NAD^+ bound to FDH.

The amount of NAD^+ bound to FDH was determined by unfolding the protein and removing the cofactor. The protein sample was denatured by the addition of 6 M urea and boiling for 2 minutes. The precipitated material was removed by centrifugation at 16,500 x g for 5 minutes. The supernatant was loaded onto a Pharmacia Mono Q (HR 5/5) ion exchange column equilibrated with 10 mM Tris-HCl, pH 8.8, containing 6 M urea. The sample was eluted with a linear gradient of 0 - 1.0 M KCl in the same buffer and eluted material was monitored by absorbance at 254 nm.

2.4 Purification methods.

2.4.1 General information.

The following is a list of the different media and column sizes used in the purification of the protein components. Specific information about the separation methods and elution conditions can be found in the relevant sections of the text.

2.4.2 Gel filtration.

Superdex gel filtration columns.

The gel filtration media used in this study were Pharmacia Superdex 200 preparative grade (pg) and Superdex 75 pg. These media were used in both the modifier and dehydrogenase component purifications, described later. The media were packed into various columns from the Phamacia HR and XK series. The sample (equal

to 10% column volume (CV)) was loaded at 1.5 CV/hr and eluted at a flow rate of 3.0 CV/hr.

Sample desalting.

Samples which required desalting were passed through a Pharmacia Fast Desalt column or a Pharmacia HR 10/10 column packed with Sephadex G25, fine grade. A 1 ml sample was loaded onto an equilibrated desalting column and eluted at a linear flow rate of 15 CV/hr.

HPLC TSK G3000 SW gel permeation chromatography.

A TSK G3000 SW gel permeation HPLC column was used for the purification of protein components. Two ml samples were loaded onto the column at a maximum flow rate of 0.6 CV/hr. The protein material was eluted at a flow rate of 3.0 CV/hr.

The specific conditions used for each gel filtration column and media are given in Chapter 3.0, Appendix 1, 2 and 3.

2.4.3 Ion exchange chromatography.

Anion exchange chromatography.

Ion exchange chromatography was performed using Q-Sepharose, Mono Q and Resource Q media (Pharmacia, UK). For all ion exchange separations the protein sample was loaded onto the column in a buffer containing no KCl. Protein was eluted from the column using a gradient of 0 - 1.0 M KCl.

DEAE - cellulose batch separation.

Whatman DEAE - Cellulose was used in a batch separation method. The medium was prepared as instructed by the manufacturer and stored in 20 % ethanol. The medium was washed and then equilibrated with sample buffer (described later, Appendix 1). The DEAE - Cellulose was slurried into the protein extract in 2 g quantities and the mixture was allowed to stand on ice for 30 minutes. This suspension was checked for FDH activity, as described above (Section 2.3.7) and the addition of DEAE - Cellulose repeated until FDH activity began to decrease. The resulting suspension was centrifuged at 4000 xg to remove the DEAE - cellulose and the supernatant retained for further purification.

2.4.4 Affinity chromatography methods.

Phenyl Superose hydrophobic interaction.

Ammonium sulphate (1.7 M) was added to the protein sample and centrifuged at 16,500 xg prior to loading onto a prepacked Pharmacia HR 5/5 Phenyl Superose column. The sample was eluted from the column using a linear gradient of 1.7 - 0 M ammonium sulphate buffer at a flow rate of 15 CV/hr. Protein eluted from the column was detected and collected as described above.

Affinity dye medium.

A Sigma dye agarose affinity development kit comprising nine 2.5 ml columns prepacked with blue 3GA, blue 4, blue 72, brown 10, green 5, green 19, red 120, yellow 3 and yellow 86 media was used as directed by the manufacturer. Each column was equilibrated with 25 mM phosphate, pH 7.2, buffer and 1 mg of protein solution was loaded. After loading the columns were washed with 3 CV of equilibration buffer

and all effluent was collected. The sample was eluted from the column with 3 CV of buffer with 1 M KCl. Alternatively sample was eluted with 3 CV of buffer with 1 M NAD^+.

Immobilised metal affinity chromatography.

Immobilised metal affinity chromatography (IMAC) was investigated in an attempt to purify the FDH component, (Belew *et al* (1987)). The IMAC column was prepared by absorbing 1 CV of 0.2 M $CuSO_4$ in equilibration buffer. Unbound Cu^{2+} was removed by washing the column with 10 CV of 25 mM phosphate buffer, pH 7.2. Sample was eluted at a flow rate of 0.2 CV/hr with a linear gradient of 0 - 1.0 M imidazole.

2.5 Gel electrophoresis of protein samples.

12.5 % (w/v) monomer concentration SDS-PAGE and native PAGE were prepared as described in the instructions for the BIORAD Mini protean II system and used to resolve FDH from other proteins. All gels were run at a constant voltage of 200 V for 30 minutes. The proteins separated by PAGE were visualised by staining with Coomassie brilliant blue.

2.6 Western blot analysis and preparation of samples for N - terminal sequencing.

Protein samples for either N-terminal sequencing or western blot analysis were run on duplicate SDS gels, as described above. Protein in one of the gels was stained

with Coomassie blue and the other was used for electroblotting the protein onto a nitro-cellulose membrane, or PVDF membrane.

Western blot analysis of FDH protein against soluble methane monooxygenase hydroxylase component antibodies was performed as described in West *et al.*, (1992). The method described in Packman, (1993), using cyclohexylamino-propanesulphonic acid buffer, was used for preparation of protein samples for N-terminal sequence analysis.

2.7 N-terminal sequence analysis.

Protein bands visualised on PVDF membranes (above) were excised and sent for N-terminal sequence analysis. N-terminal sequencing was performed at University of Southampton, Protein Sequencing Unit, Department of Biochemistry, School of Biological Sciences, Southampton, SO16 7PX.

3. Purification and characterisation of formaldehyde dehydrogenase enzyme components.

3.1 Introduction.

Stirling & Dalton (1978) identified a formaldehyde dehydrogenase (FDH) in *Methylococcus capsulatus (Bath)* which required a small heat stable cofactor for catalytic activity. FDH activity could only be determined by the addition of heat-treated soluble extract (HTSE) to the FDH reaction mixture (Stirling & Dalton, 1978). The FDH was purified in a four step procedure; Step 1: ammonium sulphate precipitation, Step 2: Sephadex 75 gel filtration chromatography, Step 3: Sephadex 200 gel filtration chromatography and Step 4: DEAE - Cellulose treatment (Stirling & Dalton, 1978). The purified FDH was shown to have a MW of 110 kDa and a subunit MW of 57 kDa (Stirling & Dalton, 1978).

Isolation of the active component of HTSE was not performed at that time, although it was determined by trypsin digestion that the active component was a protein, with a MW of less than 16 kDa (Stirling & Dalton, 1978). The active component of HTSE was isolated in a three step procedure and shown to be a 10 kDa protein which modified the function of FDH. It was therefore called a modifier protein (Millet *et al.*, unpublished).

Subsequent attempts to purify the FDH components by the above methods have proved unsuccessful, though small quantities of impure protein have been isolated and used in characterisation studies (Millet *et al.*, unpublished). The data presented here demonstrates methods for the purification of both FDH and the active component from HTSE suitable for the production of large quantities of pure protein. Data is also

presented demonstrating that the FDH enzyme purified in this study is different from other FDH enzymes purified and characterised from methylotrophic bacteria.

3.2 Experimental.

3.2.1 Growth of *M. capsulatus* (Bath) and preparation of soluble extract.

M. capsulatus (Bath) was cultured in an oxygen limiting chemostat, as described in the Materials and Methods (Chapter 2, Section 2.3.15). Cell free soluble extract was prepared as described in Chapter 2, Section 2.3.16.

3.2.2 Purification of formaldehyde dehydrogenase.

In an attempt to purify FDH four methods using a variety of different chromatographic procedures were evaluated. The first two methods were unsuitable for further use in this study as they produced insufficient protein and were difficult to scale up. These are described in Appendices 1 and 2. Method three co-purified FDH with the hydroxylase component of soluble methane monooxygenase (sMMO) and the results of the copurification are discussed in Appendix 3. The following method was used for the purification of FDH throughout this study:

Step 1: Ammonium sulphate precipitation.

Crude extract was saturated to 30 % (w/v) with solid ammonium sulphate and allowed to stand on ice for 30 minutes. Precipitated material was removed by centrifugation at 48,500 xg. The supernatant was then saturated to 55 % (w/v) with ammonium sulphate. Precipitated material was removed by centrifugation as above.

The supernatant was discarded and the pellet redissolved in a minimum volume of 25 mM Tris - HCl, pH 7.2, containing 50 mM KCl. The resulting solution was assayed for formaldehyde oxidation activity as described in the Materials and Methods (Chapter 2, Section 2.3.4).

Step 2: Gel filtration chromatography.

The FDH active solution from Step 1 was loaded onto a Pharmacia Superdex 200 pg (HR 26/60) gel filtration column which had been equilibrated with 2 column volumes (CV) of 25 mM Tris - HCl, pH 7.2, containing 50 mM KCl and 1 mM benzamidine. The protein was eluted at a flow rate of 0.75 CV/hr in the same buffer and 4 ml fractions were collected. Eluted protein was monitored by absorbance at 280 nm and the fractions collected were assayed for formaldehyde oxidation activity. Those fractions which showed activity were pooled and concentrated in an Amicon ultrafiltration unit over a 30 kDa membrane at 4 °C. After concentrating the protein solution, FDH activity was again determined, as in Step 1.

Step 3: Ion exchange chromatography.

The concentrated active protein from the gel filtration column was loaded onto a Q - Sepharose ion exchange column equilibrated with 25 mM Tris - HCl, pH 7.2. After sample loading, the column was washed with equilibration buffer for 1 CV and then the concentration of KCl was increased to 0.3 M in the same buffer over 0.25 CV. The FDH protein was eluted in a linear gradient of 0.3 - 0.5 M KCl in the same buffer, over 3 CV. The column was regenerated by raising the KCl concentration to 1.0 M for 2 CV, then re-equilibrated in equilibration buffer. Eluted protein was monitored as in step

1 and 8 ml fractions were collected. Each fraction was tested for formaldehyde oxidation activity; active fractions were pooled and concentrated over a 30 kDa Amicon ultrafiltration membrane.

Step 4: Gel filtration chromatography.

To remove low molecular weight proteins, the FDH solution was loaded onto a Pharmacia Superdex 75 (HR 10/30) column equilibrated with 25 mM Tris - HCl buffer, pH 7.2, containing 100 mM KCl. Protein was eluted at a flow rate of 0.75 CV/hr and 1 ml fractions were collected with eluted protein monitored as in Step 1. Formaldehyde oxidation activity in each fraction was assessed and those which demonstrated FDH activity were pooled, drop frozen in liquid nitrogen and stored at -80 °C.

3.2.3 Purification of the modifier protein.

Step 1: Heat treatment.

Crude extract was heated to 70 °C for 12 minutes. The precipitated material was removed by centrifugation at 10,000 xg for 20 minutes. The supernatant was used as heat-treated soluble extract (HTSE).

Step 2: Ion exchange chromatography.

The HTSE solution was loaded onto a Pharmacia Q - Sepharose (HR 26/10) ion exchange column equilibrated with 20 mM Hepes buffer, pH 7.2, containing 1 mM benzamidine. After sample loading the column was washed for 2 CV with equilibration buffer at a flow rate of 6 CV/hr. The sample was eluted from the column using a linear gradient of 0 - 1.0 M KCl in 5 CV of equilibration buffer. Eight ml fractions were collected and those showing modifier protein activity were pooled and concentrated in an Amicon ultrafiltration unit over a 3,000 Da membrane until the volume had been reduced to less than 5 ml.

Step 3: Gel filtration chromatography.

The concentrated protein from Step 2 was loaded onto a Pharmacia Superdex 75 pg (HR 26/60) column. The fractions were eluted with 20 mM Hepes buffer, pH 7.2, containing 1 mM benzamidine at a flow rate of 0.75 CV/hr. Eluted protein was monitored by absorbance at 280 nm and 4 ml fractions were collected and assayed for modifier protein activity. Those exhibiting activity were pooled and concentrated over a 3,000 Da ultrafiltration membrane until the volume was 10 % of the starting volume. This material was sterile filtered through a 0.2 μm Whatman PVDF filter and stored at room temperature.

3.3 Results.

3.3.1 Purification of FDH.

The method described in Section 3.2.2 consistently produced a sufficient yield of FDH to perform enzyme characterisation and kinetic experiments. The method is identical to the third method evaluated (described in Appendix 3) except that a Tris - HCl buffer was used in Step 2 to facilitate the separation of FDH from the hydroxylase of sMMO. This purification is summarised in Table 3.1.

Table 3.1 Purification of formaldehyde dehydrogenase.

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity [mU. (mg protein)$^{-1}$]	Yield (%)	Purification Factor
Crude Extract	190	9,500	36,100	4	100	1
Ammonium Sulphate Precipitation	60	3,720	33,500	9.3	92	2.3
Gel Filtration, Superdex 200 pg	40	134	4,600	34	12.7	8.5
Ion Exchange, Q - Sepharose	24	16.8	3,900	232	10.8	58
Gel Filtration, Superdex 75 pg	10	16.4	3,850	234	10.7	58.5

Two peaks of formaldehyde oxidation activity were identified in the fractions collected from the Q - Sepharose FPLC column, one required the presence of modifier protein for FDH catalytic activity while the other did not (Figure 3.1).

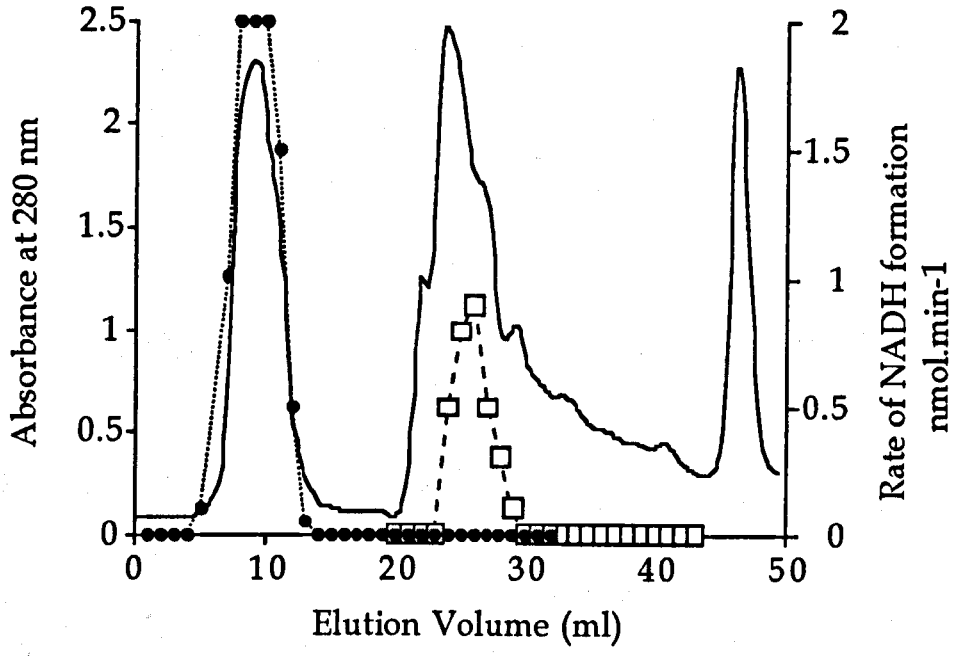


Figure 3.1 Chromatograph of FDH activities eluting from the Q-Sepharose ion exchange column.

● - FDH activity requiring the presence of the modifier protein,
□ - FDH activity not requiring the presence of the modifier protein.

The first peak eluted in the void volume and SDS - PAGE analysis of this protein showed two bands when stained with Coomasie Blue (Figure 3.2, lanes D and E). The second peak with FDH activity did not require the addition of modifier protein for formaldehyde oxidation. Further more the second peak showed four bands on a SDS - PAGE, three had the same molecular weights as the hydroxylase component of soluble methane monooxygenase (sMMO) and the fourth had a molecular weight of

approximately 30 kDa. This protein was further purified and found to be an NAD^+ dependent, secondary cofactor independent FDH (Chapter 6.0).

From the SDS-PAGE and ESI-MS data it is estimated that the FDH protein isolated in this study was > 95 % pure.

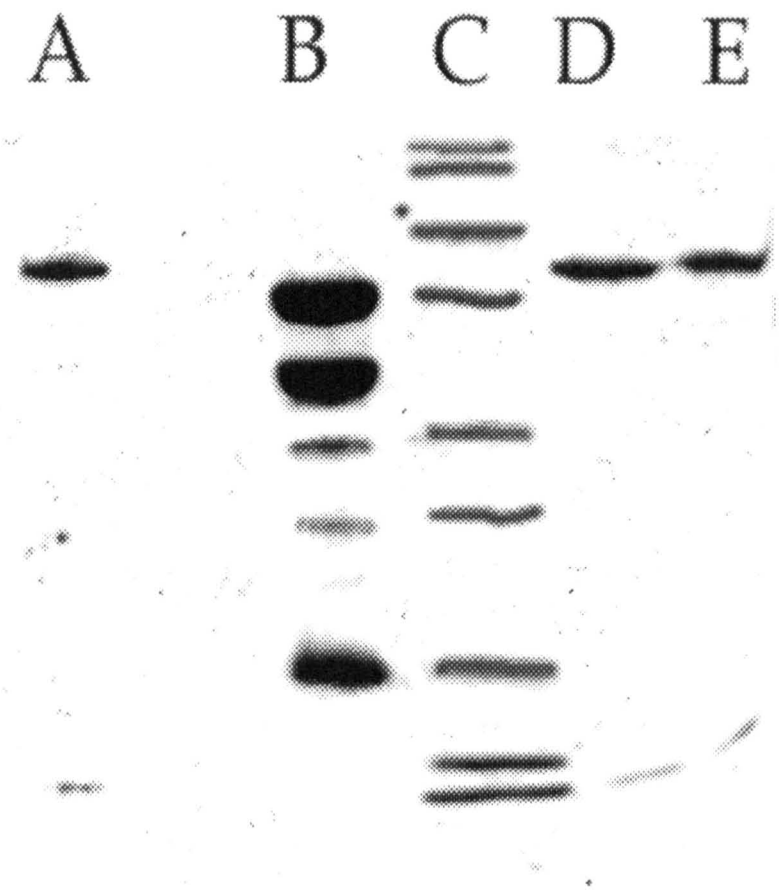


Figure 3.2 15% SDS PAGE of purified formaldehyde dehydrogenase.

Lane A, 10 μg purified FDH;
Lane B, 50 μg purified hydroxylase of sMMO;
Lane C, Novex UK wide range protein standards comprising: 116.3 kDa β-galactosidase, 97.4 kDa phosphorylase B, 66.3 kDa bovine serum albumin, 55.4 kDa glutamate dehydrogenase, 36.5 kDa lactate dehydrogenase, 31 kDa carbonic anhydrase, 21.5 kDa trypsin inhibitor (soybean), 14.4 kDa lysozyme and 6.5 kDa aprotinin;
Lane D and E, 10 μg and 20 μg purified FDH demonstrating the presence of a small molecular weight contaminant.

3.3.2 Isolation of the modifier protein.

The activity of the modifier protein during the purification procedure was calculated from its ability to restore formaldehyde oxidation to purified FDH. Modifier protein activity could not be determined in crude extract due to the presence of both FDH and modifier protein. The purification procedure for the modifier protein is shown in Table 3.2.

Table 3.2 Purification of modifier protein.

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity* [mU. (mg protein)-1]	Yield (%)	Purification Factor
Heat treatment	75	2250	49.5	22	100	1
Ion exchange, Q-Sepharose	220	2046	94.6	46	190	2.0
Gel filtration Superdex 75	60	24	72.6	3,025	145	138

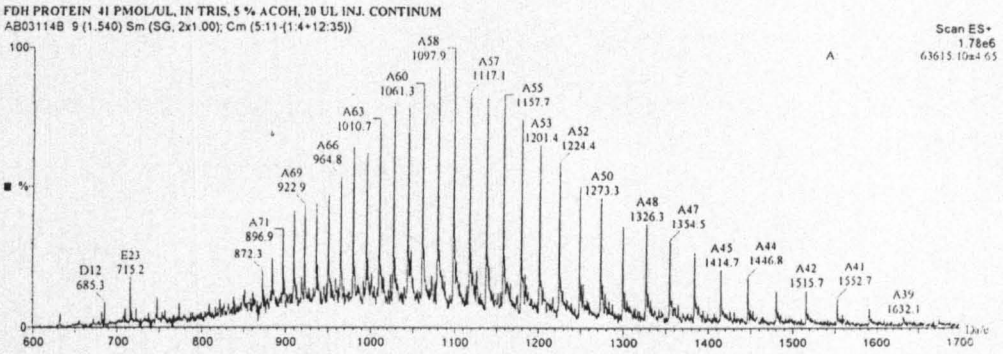
*Specific activity of the modifier protein was based on the restoration of formaldehyde oxidation activity to FDH. FDH was added to the assay mixture in a ratio of 2 : 1 (w/w) FDH:modifier protein solution.

When crude extract was heated to 70 °C approximately 70 % of the protein material was precipitated. Attempts to purify the modifier protein without heat treatment were unsuccessful, possibly due to the low levels of protein present *in vivo*.

Analysis of the amount of modifier protein recovered during the purification procedure showed that the standard BIORAD protein estimation method was unsuitable for this small protein. Estimation of the amount of modifier protein from its absorption at 205 nm was found to be more accurate (Chapter 2, Section 2.3.13).

Analysis of the purified modifier protein by ESI-MS showed that there was a single species in the sample. As with the FDH preparation, the ESI-MS data were used to estimate the purity of the modifier protein preparation and indicated that it was > 90% pure.

(A)



(B)

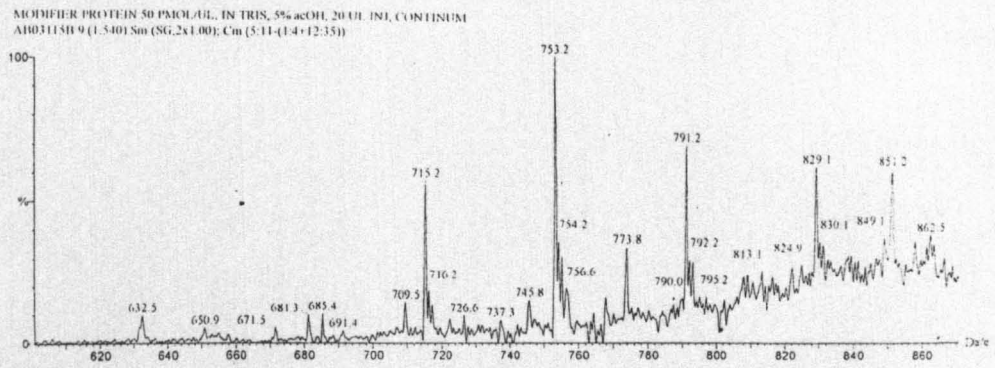


Figure 3.3 ESI-MS spectrum of (A) purified formaldehdye dehydrogenase and (B) modifier protein. ESI-MS experiments were performed as described in the Materials and methods (2.3.17)

3.4 Characterisation of formaldehyde dehydrogenase and modifier protein.

3.4.1 Molecular weight determination of formaldehyde dehydrogenase and modifier protein.

The subunit MW of FDH was determined by ESI-MS to be 63620 ± 63 Da (Figure 3.3). The MW of native FDH was determined by gel filtration chromatography with the active protein eluting from a Superdex 200 FPLC column in the region of proteins with a MW of 250 kDa (Figure 3.4).

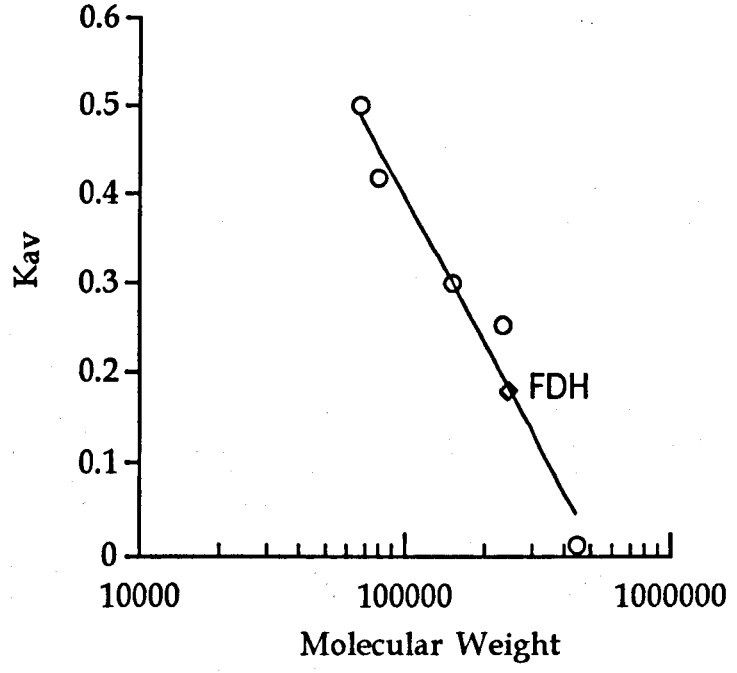


Figure 3.4 Determination of the molecular weight of native FDH by gel filtration.

Molecular weight standards used were, bovine serum albumin (66 kDa), alkaline phosphatase (80 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa) and ferritin (440 kDa).

The MW of the modifier protein was determined by ESI-MS as 8,211 Da. The molecular weight of the native modifier protein was determined by gel filtration chromatography and two peaks of activity were eluted from a Superdex 75 FPLC column. The regions at which these peaks eluted corresponded to proteins of MW 10 and 20 kDa.

3.4.2 UV- visible spectra of formaldehyde dehydrogenase and modifier protein.

Purified FDH and modifier protein exhibit a single UV/visible absorption peak. λ_{max} of FDH was 278nm and the modifier protein was 280nm (Figure 3.5).

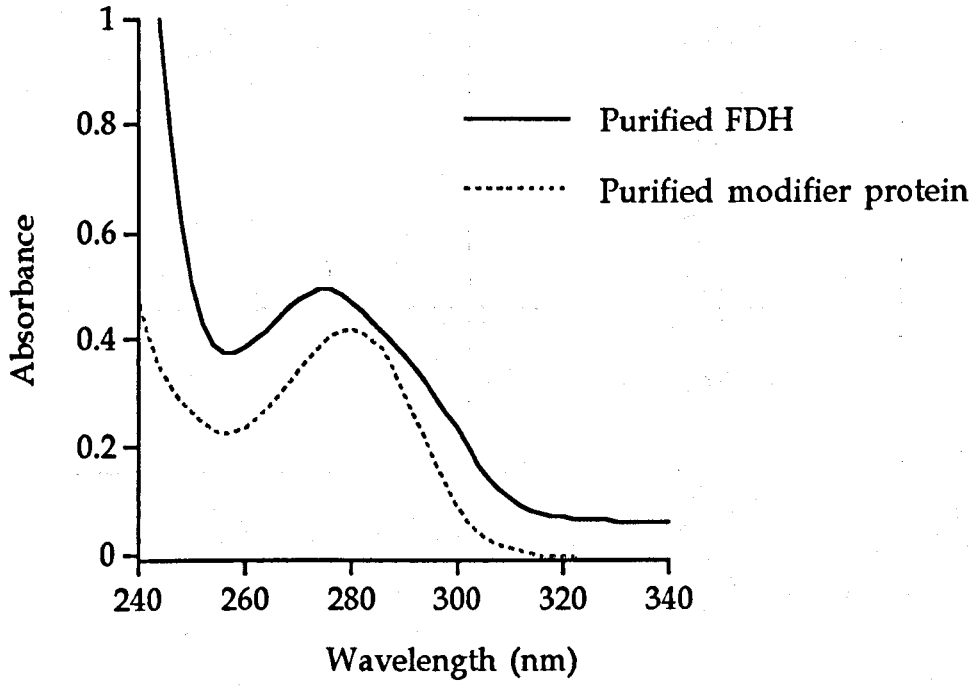


Figure 3.5 UV-visible absorption spectra of purified formaldehyde dehydrogenase and modifier protein.

3.4.3 N - terminal sequence analyses of formaldehyde dehydrogenase and modifier protein.

Purified samples of FDH and modifier protein were sent for N - terminal sequence determination as described in Materials and Methods (Chapter 2, section 2.7) and the results are shown in Table 3.3. The resulting sequences (Table 3.3) were used to search the current SWISS - PROT and EMBL protein sequence databases for proteins which have similar N -terminal sequences (the date of the search was May 1995). In both cases no proteins with similar N - terminal sequences were found.

Table 3.3 N - terminal sequence data for purified formaldehyde dehydrogenase and modifier protein.

Protein Component	N - terminal Sequence
FDH	NSELDRLXKFXRNVVML
Modifier protein	SDGGHXVAPXN

Amino acids are described by their single letter amino acid code and X signifies a residue which could not be identified.

3.5 Discussion.

3.5.1 Purification of protein components.

The initial aim of this study was to develop a purification procedure for both FDH and the modifier protein which would generate sufficient quantities of both for the completion of the work. In developing the FDH purification method described above (Section 3.2.2) four different procedures were attempted (Section 3.2.2 and Appendices 1,2 and 3). The first two methods attempted produced protein which was chromatographically pure, but were difficult to scale up. The use of ammonium sulphate precipitation, gel filtration chromatography, ion exchange chromatography and a final gel filtration step (Section 3.2.2 and Appendix 3), allowed large volumes of soluble extract to be processed rapidly and with relative ease.

Initially the purification procedure generated FDH which was heavily contaminated with the hydroxylase component of soluble methane monooxygenase (sMMO) which comprises approximately 30 % of the soluble cell protein. The hydroxylase was identified by N - terminal sequence analysis and the implications of this co-purification are discussed in Appendix 3. Changing the elution buffer from non ionic Hepes buffer to Tris - HCl proved important in the separation of FDH from the hydroxylase of sMMO. The reason for the separation of FDH from the hydroxylase in Tris - HCl is unknown, although it is possible that the increase in the ionic strength of the elution buffer was responsible.

FDH activity was observed to decrease during the purification procedure, principally during the first gel filtration step. However, the stability of the purified FDH did not appear to be a problem when stored at -80 °C. In an attempt to stabilise FDH during the gel filtration step various compounds (e.g. dithiothreitol, benzamidine,

mannitol) were added to the elution buffer. All proved to be unsuccessful in the stabilisation of FDH. The decrease in activity was limited when steps 1 and 2 were performed as quickly as possible.

In an attempt to combine the FDH and modifier protein purification procedures modifier protein activity was determined at various stages of the ammonium sulphate precipitation of FDH. In all cases no modifier protein activity was detected. Attempts were also made to purify the modifier protein without the heat treatment step and these were also unsuccessful. It appeared that the modifier protein was activated by the heat treatment.

During purification of the modifier protein, the yield in activity units after the ion exchange step, increased to 146.8 %. This suggests that this step may remove an FDH inhibitor or a component which interferes with the FDH assay. Stirling & Dalton (1978) report an increase in the activity units of the FDH component in their purification. They attribute the increase in protein activity to the chromatographic separation of FDH from a cyanide resistant NADH oxidase which interferes in the FDH assay. It is also likely that this is also what is happening in this purification of the modifier protein.

The BIORAD method was found to be unreliable for the determination of modifier protein concentration. The BIORAD method involves the determination of an absorbance shift from 495 nm to 595 nm in the dye reagent after stabilisation with protein. The dye interacts with the protein by hydrophobic and ionic interactions. Protein - dye interactions can also occur with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. A similar problem also occurred when staining the protein on SDS-PAGE using Coomassie brilliant blue. A possible explanation for the unreliability of this method is that the modifier protein lacks residues which are reactive to the BIORAD dye. As the modifier protein was unreactive to Coomassie stain and the

BIORAD reagent the concentration was estimated by absorption measurements at 205 nm. The peptide bond absorbs light strongly below 210 nm and although some absorption by tyrosine and tryptophan occur in this region there is less variability in absorbance between proteins at 205 nm than measurement at 280 nm. This assumes that the modifier protein does not have a significant number of aromatic residues which would interfere with these measurements.

3.5.2 Comparison with other FDH enzymes.

The ESI-MS data gave a MW of 63,620 Da for the FDH subunit, while gel filtration demonstrated that native FDH had a molecular weight of 250 kDa, therefore indicating that the enzyme is a tetramer and not a dimer as previously thought (Stirling & Dalton, 1978). FDH enzymes which have previously been purified from methylotrophic bacteria are usually dimers or trimers (van Ophem *et al.*, 1992; Eggling & Sahm, 1985) and this is the first report of a tetrameric FDH isolated from a methylotrophic organism. The FDH isolated in this study also has a MW greater than those of enzymes characterised from other methylotrophic bacteria (Table 3.6).

All the FDH enzymes included in Table 3.6, except those isolated from *M. capsulatus* (Bath) and *Pseudomonas putida* C-83, require the addition of glutathione (GSH) for FDH activity and are called GSH-dependent FDH (gFDH) enzymes. It has been demonstrated that the amino acid sequence of these FDH enzymes is homologous with the sequence of Type III alcohol dehydrogenases which can also catalyse the oxidation of formaldehyde in the presence of glutathione (Holmquist & Vallee, 1991).

Table 3.4 Comparison of molecular weights of FDH enzymes isolated from methylotrophic bacteria.

Organism	MW (Native)	MW (Subunit)
[1] *M. capsulatus (Bath)*	250,000	63,600
Arthobacter P1	115,000	56,000
P. putida C-83	150,000	75,000
Amycolatopsis methanolica	120,000	40,000
Rhodococcus erythropolis	130,000	44,000
[2] *M. capsulatus (Bath)*	100, 000	30,000

[1]*M. capsulatus (Bath)* - modifier protein dependent FDH,
Arthobacter P1 - Attwood *et al.*, 1992,
P. putida C-83 - Ando *et al.*, 1979,
A. methanolic - van Ophem *et al.*, 1992,
R. erythropolis - Eggling & Sahm, 1985,
[2]*M. capsulatus (Bath)*,- Chapter 6 this study.

The FDH isolated in this chapter differs from these enzymes in that it requires the presence of a small protein for the oxidation of formaldehyde and is unaffected by GSH (Chapter 4). The oxidation of methane and methanol in *M. capsulatus (Bath)* is regulated by control proteins. The regulation of formaldehyde oxidation by a control protein, such as the modifier protein, is therefore unsurprising. FDH is possibly the most important enzyme in the oxidation pathway as it is involved in the initial NADH formation reaction. As methylotrophic organisms assimilate carbon via formaldehyde, FDH must be closely regulated to maintain both the level of formaldehyde and also the level of NADH formed by its oxidation. The modifier protein is ideally suited to this function as dissociation from FDH stops formaldehyde oxidation. Therefore control of the modifier protein expression would control formaldehyde oxidation. As the modifier protein regulates FDH it will now be referred to as protein F.

3.5.3 N- terminal sequence analysis.

Analysis of the current SWISS - PROT and EMBL databases for proteins with similar N - terminal sequences indicates that FDH and protein F are both unique. The N - terminal sequences for FDH enzymes present in the databases are all different from that of the FDH from *M. capsulatus (Bath)*, as demonstrated in Figure 3.6.

Figure 3.6 N-terminal sequence comparison of FDH enzymes.

```
M-FDH                          N S E L D R L X K F X R N V V
FD-FDH          P Q T V R G V I A R S K G A P V E L T D F I R H P P G G H
NMDA-FDHM             K T K A A V L H S A G K P F E I E E L E L D G P R E H E V
GD-FALDH    M Y V I V P T G R A D Q T K L S S R S Y E A G R L A T R
PD-FALDH            M R T R A A V A L E A G K P L E V M E V N
HORSE FDH  S T A G K V I K C K A A V L W E
S.CER-FDH  A T A G K V I K C R K A V T W E
E.COLI-FDH          M K S R A A V A F A P G K P L E
```

Amino acid residues are displayed by their single letter code, X represents an unknown residue. Sequences were aligned using MACAW software for the Macintosh.

M-FDH, protein F dependent FDH from *M. capsulatus* (Bath),
FD-FDH, factor dependent FDH from *A. methanolica* (van Ophem *et al.*, 1992),
NMDA-FDH, 4-nitroso-N,N-dimethylaniline dependent FDH from *R. rhodoccocus* (van Ophem *et al.*, 1992),
GD-FALDH, Glutathione dependent FDH from *M. marinus* (Ras *et al.*, 1995),
PD-FALDH, Glutathione dependent FDH from *Paracoccus denitrificans* (Ras *et al.*, 1995),
HORSE FDH, Glutathione dependent FDH from horse tissue (Jornvall *et al.*, 1987),
CER-FDH, Glutathione dependent FDH from *S. Cerevisiae* (Jornvall *et al.*, 1987),
E.COLI-FDH, Glutathione dependent FDH from *E.coli* (Gutheil *et al.*, 1992).

Figure 3.6 shows the available N-terminal sequence data for both factor dependent FDH enzymes, such as gFDH, and non-factor dependent FDH (nFDH) enzymes such as those purified from *P. putida* C-83. The data in Figure 3.6 shows that there are no similarities between the FDH isolated in this study and those reported in the literature. It is therefore concluded that FDH is a novel protein.

In conclusion the procedures described in this chapter provide reproducible purification methods for both protein F and FDH. The data demonstrate that FDH is a tetramer and not a dimer as previously reported (Stirling & Dalton, 1978). The data also suggest that both FDH and protein F may be unique proteins.

4. Steady state kinetic analysis of formaldehyde dehydrogenase and its modifier protein (protein F).

4.1 Introduction.

Stirling & Dalton, 1978 demonstrated that heat-treated soluble extract (HTSE) was necessary for the catalysis of formaldehyde oxidation by the NAD^+-linked formaldehyde dehydrogenase (mFDH) enzyme in *Methylococcus capsulatus* (Bath). The oxidation of other aldehydes such as glycoaldehyde, glyoxal and DL-glyceraldehyde by mFDH in the presence of HTSE was also reported (Stirling & Dalton, 1978). However the substrate specificity of mFDH in the presence and absence of HTSE was not fully investigated. In this study the active component of HTSE has been purified as a modifier protein (protein F) (Chapter 3) and the oxidation of formaldehyde and other potential substrates by mFDH has been further characterised in the presence and absence of protein F.

The mFDH purified in this study (Chapter 3) is similar to FDH enzymes isolated and characterised from *Amycolatopsis methanolica* (van Ophem *et al.*, 1992) and *Rhodococcus erythropolis* (Eggeling & Sahm, 1984; Eggeling & Sahm, 1985) in that they also require a small cofactor for formaldehyde oxidation. However the cofactor required for these enzymes is glutathione (GSH) or an uncharacterised GSH type molecule and therefore this group of enzymes is called NAD^+ linked GSH - dependent FDH (gFDH). The enzyme isolated from *M. capsulatus* (Bath) reported here which requires a protein cofactor.

The K_m value for formaldehyde and the limited range of aldehydes oxidised by mFDH, in the presence of protein F, was calculated (Stirling & Dalton, 1978). These

data indicated that the enzyme purified from *M. capsulatus* (Bath) was a FDH with a narrow substrate specificity. The substrate specificity of mFDH in the absence of protein F was not determined. Previous work had shown that mFDH was able to catalyse the oxidation of a range of simple aliphatic aldehydes in the absence of protein F (Millet *et al*., unpublished).

4.2 Steady state kinetic analysis of mFDH.

4.2.1 Determination of formaldehyde dehydrogenase substrate specificity in the presence and absence of protein F.

The substrate specificity of purified mFDH was determined in the presence and absence of purified protein F. All protein components were purified as previously described (Chapter 3). The rates of oxidation of substrates, other than formaldehyde, were determined as described in Materials and Methods (Chapter 2, Section 2.3.7) except that after the three minute preincubation at 45 °C, 2 mM aldehyde or alcohol substrate was added to initiate the reaction. The oxidation rate for each aldehyde or alcohol was determined in the presence and absence of protein F and was expressed as a percentage of the rate of 2 mM formaldehyde oxidation in the presence of protein F.

The data from these experiments (Table 4.1) show that a range of aliphatic substrates can be oxidised by mFDH in the absence of protein F. However in its presence only the catalysis of formaldehyde oxidation was observed. In order to determine whether formaldehyde oxidation was catalysed by mFDH in the absence of protein F any decrease in formaldehyde concentration over a two hour period was followed. There was no reduction in the concentration of formaldehyde over this time

period and it is therefore concluded that mFDH has an absolute requirement for protein F for the catalysis of formaldehyde oxidation.

Table 4.1 Substrate specificity of FDH in the presence and absence of protein F.

Substrate	% Activity + Protein F	% Activity - Protein F
Formaldehyde	100	0
Acetaldehyde	0	57
Propanal	0	37
Butanal	0	8
Pentanal	0	very low
Glyoxal	20	20
Methanol	0	18
Ethanol	0	50
Propanol	0	21
Butanol	0	10
Benzaldehyde	0	0
Glyceraldehyde	0	0

100% activity is equivalent to 240 nmol NADH formed.min^{-1}.mg FDH^{-1} in the presence of 20 μM protein F. The rate of oxidation of each substrate was determined using a substrate concentration of 2mM and 0.4mg FDH.

The rate of pentanal oxidation catalysed by mFDH was almost indistinguishable from the baseline. Oxidation of aliphatic aldehydes with more than four carbon atoms by mFDH in the absence of protein F could not be detected by the available method. It is therefore concluded that mFDH does not catalyse the oxidation of these substrates. The oxidation of glyceraldehyde was not observed in the presence or absence of protein F as previously reported (Stirling & Dalton, 1978). Glyoxal oxidation occurred in the presence and absence of protein F and may be due to break down products of glyoxal being present in the solution.

The reverse reaction, the formation of formaldehyde from formate, was also investigated by the addition of 2 mM formate and 2 mM NADH to mFDH in both the presence and absence of protein F. However, no formaldehyde production was observed in either reaction using the Nash method (Nash, 1953).

4.2.2 Stoichiometry of formaldehyde and acetaldehyde oxidation catalysed by mFDH in the presence and absence of protein F.

The stoichiometry of formaldehyde loss, NAD^+ consumed, formate formed, and NADH produced, catalysed by mFDH was previously reported to be 1:1:1:1 in the presence of HTSE (Stirling & Dalton, 1978). This experiment was repeated using purified protein F and the stoichiometry for the catalysis of acetaldehyde oxidation in the absence of protein F was also investigated. A known amount of formaldehyde (100 nmol) was added to the mFDH reaction mixture (Chapter 2, Section 2.3.7) and the concentrations of formate and NADH formed were determined after 30 minutes. This experiment was repeated using 100 nmol of acetaldehyde as substrate and the amounts of acetate and NADH formed was determined after 30 minutes. The oxidation of 100 nmol of formaldehyde produced 94 nmol of formate and 116 nmol of NADH while the oxidation of 100 nmol acetaldehyde, in the absence of protein F,

produced 107 nmol of acetate and 119 nmol of NADH. The data show that the amount of product formed is in the same molar ratio as the amount of substrate added to the reaction mixture.

4.2.3 Effect of protein F concentration on formaldehyde and acetaldehyde oxidation catalysed by mFDH.

The data in Table 4.1 demonstrate that in the presence of protein F mFDH is specific for formaldehyde, whilst in its absence oxidation of higher aldehydes by mFDH is observed. Figure 4.1 shows the effect of increasing concentrations of protein F on the rates of formaldehyde and acetaldehyde oxidation catalysed by mFDH.

Table 4.1 and Figure 4.1 demonstrate a switch in the function of mFDH from a specific formaldehyde oxidising enzyme to a non-specific aldehyde dehydrogenase which is controlled by the presence or absence of protein F. The data also demonstrate that for maximum catalysis of formaldehyde oxidation the molar ratio of protein F:mFDH must be 4 : 1.

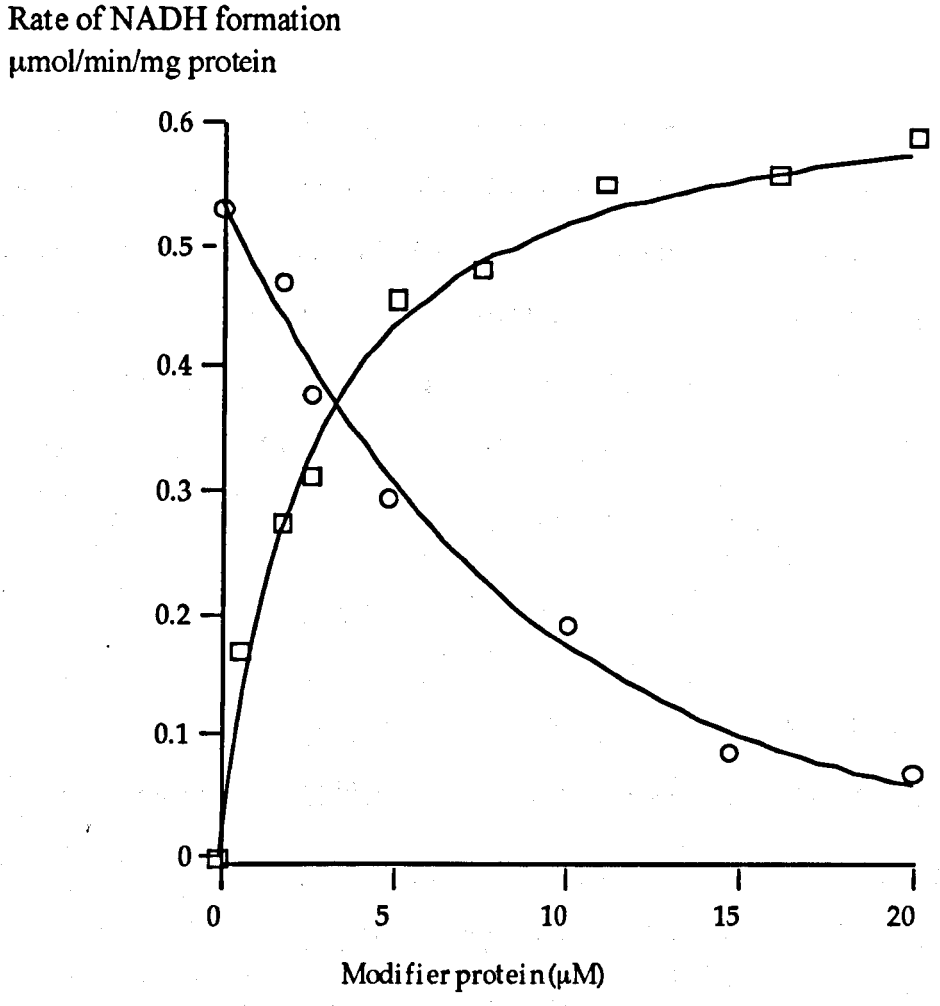


Figure 4.1 Effect of increasing concentrations of protein F on the rates of formaldehyde and acetaldehyde oxidation.

O - oxidation of acetaldehyde,
□ - oxidation of formaldehyde.

All reactions were performed as described in the Material and Methods with 4.7 μM FDH and variable amounts of protein F.

4.2.4 Effect of glutathione and methanol on the catalytic activity of mFDH in the absence of protein F.

Investigation of the substrate specificity of mFDH demonstrated that mFDH can catalyse the oxidation of alcohols in the absence of protein F (Table 4.1). Similar activity has been observed for FDH enzymes purified from *A. methanolica* and *Hyphomicrobium X* (van Ophem *et al.*, 1992; Poels & Duine, 1989). These enzymes are able to catalyse the oxidation of alcohols and aldehydes but require the presence of reduced glutathione for the catalysis of formaldehyde oxidation. The effect of reduced glutathione on the rate of formaldehyde oxidation by mFDH, isolated from *M. capsulatus* (Bath), was therefore investigated. Glutathione concentrations up to 2 M were added to the mFDH reaction mixture in the absence of protein F. At all glutathione concentrations no formaldehyde oxidation was detected. It was therefore concluded that glutathione is unable to replace the function of protein F.

Van Ophem *et al.*, (1992) demonstrated that high methanol concentrations have a similar effect to glutathione in FDH enzymes isolated from *A. methanolica*. To test this effect on mFDH methanol was added to the reaction mixture up to a final concentration of 2 M in the absence of protein F and formate production measured. As was observed with glutathione all concentrations of methanol failed to restore formaldehyde oxidation activity to mFDH.

4.2.5 Calculation of kinetic constants (K_m, V_{max}) for the catalysis of formaldehyde and acetaldehyde oxidation by mFDH.

Previously reported K_m values for formaldehyde, glyoxal and DL-glyceraldehyde with mFDH, in the presence of HTSE, were 0.68 mM, 0.075 mM, 2.0 mM respectively (Stirling & Dalton, 1978). Determination of the K_m and V_{max} values for formaldehyde and acetaldehyde with mFDH, in the presence and absence of purified protein F was attempted. This was done by monitoring the rate of NADH formation at various concentrations of formaldehyde or acetaldehyde, as shown in Figure 4.2

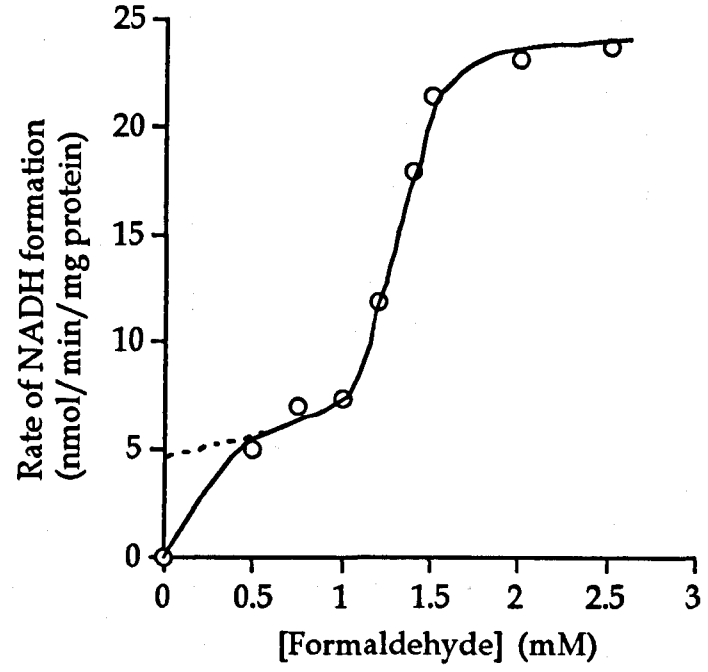
These data demonstrate that formaldehyde oxidation by mFDH does not follow a Michaelis Menten kinetic relationship. Acetaldehyde oxidation appears after a certain concentration to follow Michaelis Menten kinetics. It is concluded from this data that the sigmoidal kinetics for formaldehyde oxidation, with respect to formaldehyde concentration, demonstrate that formaldehyde binding to mFDH is cooperative, unlike the binding of acetaldehyde which does not show cooperativity.

To verify the data in Figure 4.2 these experiments were repeated while monitoring the rates of formate and acetate formation (Figure 4.3). The data in Figure 4.3 demonstrate that the relationship between the rate of product (acetate and formate) formation and substrate concentration is very similar to the relationship between the rate of NADH formation and substrate concentration. As shown in Figure 4.2 and Figure 4.3 the data demonstrate that there is a sigmoidal dependence of the rate of formaldehyde oxidation on formaldehyde concentration. This demonstrates that the binding of formaldehyde to mFDH is cooperative

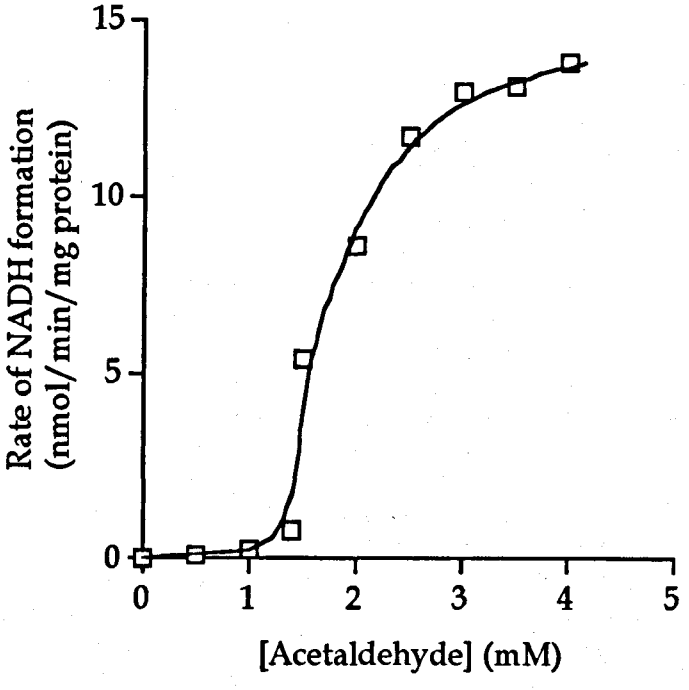
The data in Figure 4.2 and Figure 4.3 show that there was a need for a certain concentration of acetaldehyde to be added before catalysis was observed. This

(formaldehyde or another substrate) was bound to the active site of the purified enzyme. The data demonstrate that it was necessary to displace this bound molecule by high concentrations of acetaldehyde before oxidation could occur. This bound substrate has been partially characterised as described in the next section.

The affinity of NAD^+/$NADP^+$ was not determined in this study. Previous work has shown that NAD^+ and $NADP^+$ are equivalent substrates (Millet *et al.*, unpublished)



(A)

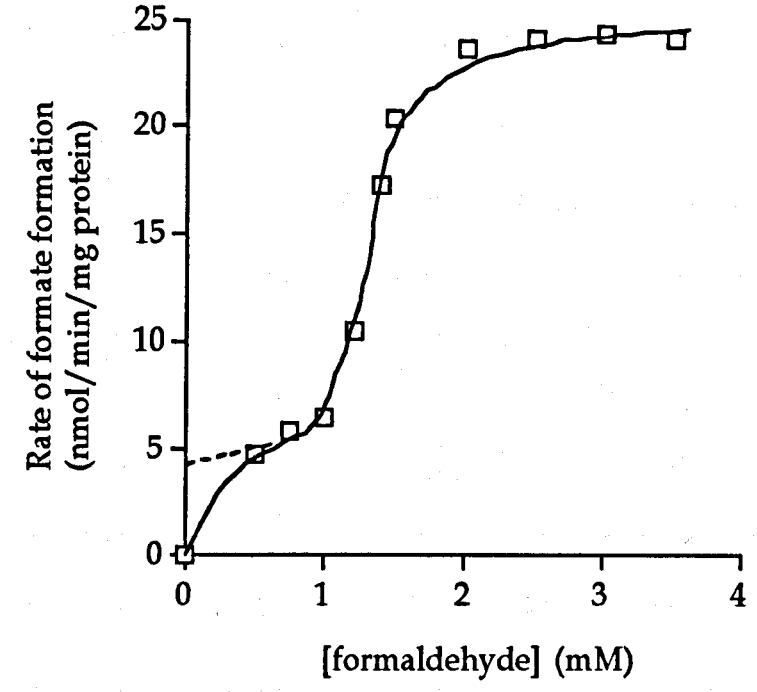


(B)

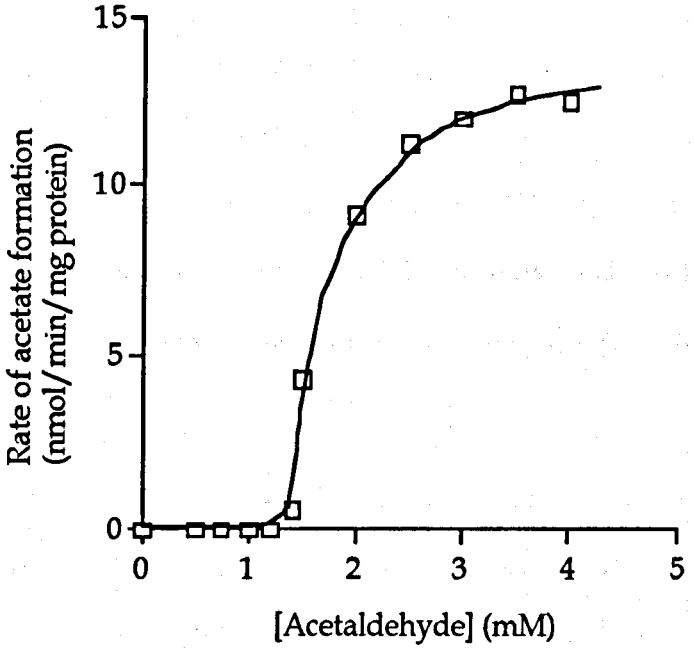
Figure 4.2 Effect of protein F on the catalysis of formaldehyde and acetaldehyde oxidation by mFDH.

(A) Formaldehyde oxidation catalysed by 4.7 μM mFDH in the presence of 20 μM protein F. Solid line = line of best fit, dotted line shows that at 0 formaldehyde concentration a rate of NAD^+ reduction is predicted. This was not experimentally determined here.

(B) Catalysis of acetaldehyde oxidation by 4.7 μM mFDH in the absence of protein F.



(A)



(B)

Figure 4.3 Rates of formate and acetate formation in the presence and absence of protein F.

(A) Rate of formate production from formaldehyde catalysed by 4.7 μM FDH with 20 μM protein F. Solid line = line of best fit, dotted line shows that at 0 formaldehyde concentration a rate of NAD^+ reduction is predicted. This was not experimentally determined here.

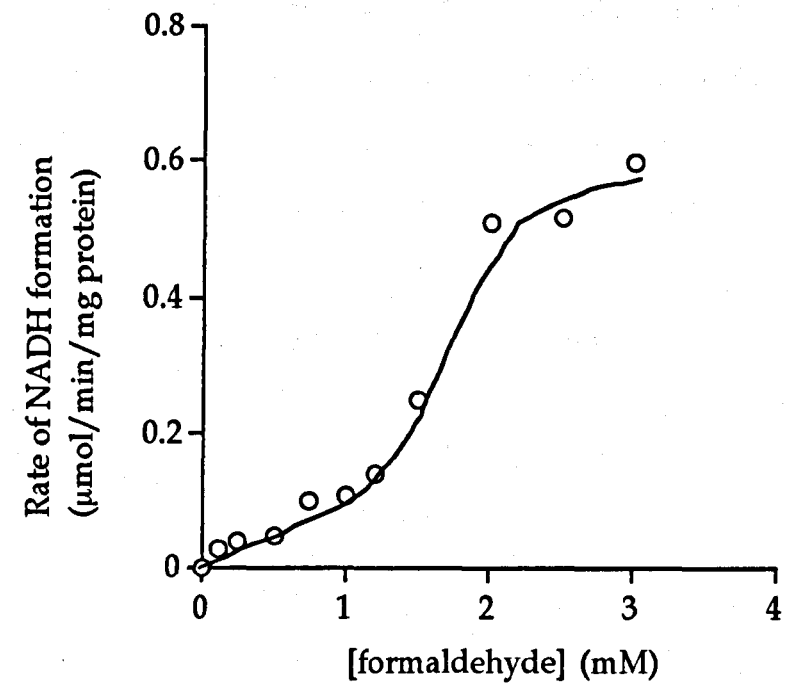
(B) Rate of acetate formation from acetaldehyde catalysed by 4.7 μM FDH in the absence of protein F.

4.2.6 Characterisation of substrate bound to mFDH.

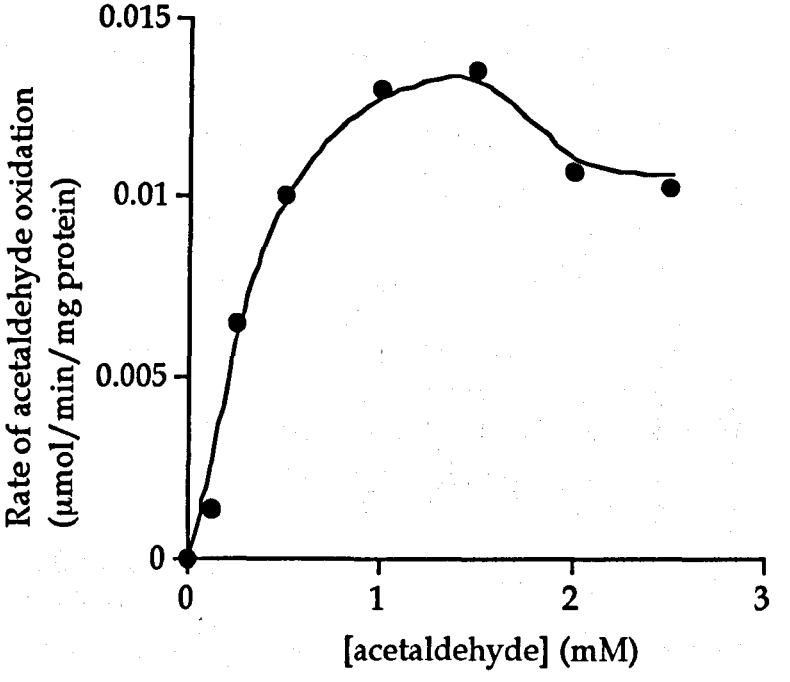
The data in Figure 4.2 indicate that NADH formation may occur in the presence of protein F and absence of formaldehyde which may not be detectable by the method above. In addition, no oxidation of acetaldehyde was observed at low substrate concentrations (< 1.5 mM under these experimental conditions). These data indicate that there is an oxidisable substrate which is bound to purified mFDH. This molecule inhibits acetaldehyde oxidation at low substrate concentrations and allows NAD^+ reduction in the presence of protein F and absence of formaldehyde.

To test this hypothesis, mFDH was allowed to turn over in the presence of excess NAD^+ (2 mM) and protein F. In the experiment 2.11 μmol of NADH were produced when 2.00 μmol of mFDH were used in the reaction mixture. These data show that the amount of NADH produced was equal to the amount of mFDH present in the reaction mixture. The formation of NADH only occurred in the presence of protein F and the data from Section 4.1.5 demonstrated that some formate production did occur in the absence of added formaldehyde. These observations indicate that the substrate bound to mFDH was formaldehyde.

The turned over mFDH from above was repurified as described in the Materials and Methods (Chapter 2, Section 2.3.18). The repurified mFDH was then used to determine the rates of formaldehyde and acetaldehyde oxidation in the presence and absence of protein F respectively. The data from these experiments are shown in Figure 4.4.



(A)



(B)

Figure 4.4 Effect of removing bound substrate on the catalysis of formaldehyde and acetaldehyde oxidation by mFDH.

(A) Rate of formaldehyde oxidation by 4.7 μM mFDH in the presence of 20 μM protein F.

(B) Rate of acetaldehyde oxidation by 4.7 μM mFDH in the absence of protein F.

The data (Figure 4.4 (A)) demonstrate that in the absence of formaldehyde no NAD^+ reduction occurs, indicating that the bound substrate had been removed from mFDH. Figure 4.4(B) clearly demonstrates that after removal of the bound compound mFDH catalyses acetaldehyde oxidation, even at low substrate concentrations. From these data the K_m value calculated for acetaldehyde was 0.45 mM and V_{max} was 0.184 $mM.min^{-1}$ (Figure 4.5). Figure 4.4 (A) also demonstrates that the rate of formaldehyde oxidation, after mFDH has been turned over, still shows cooperativity towards formaldehyde binding.

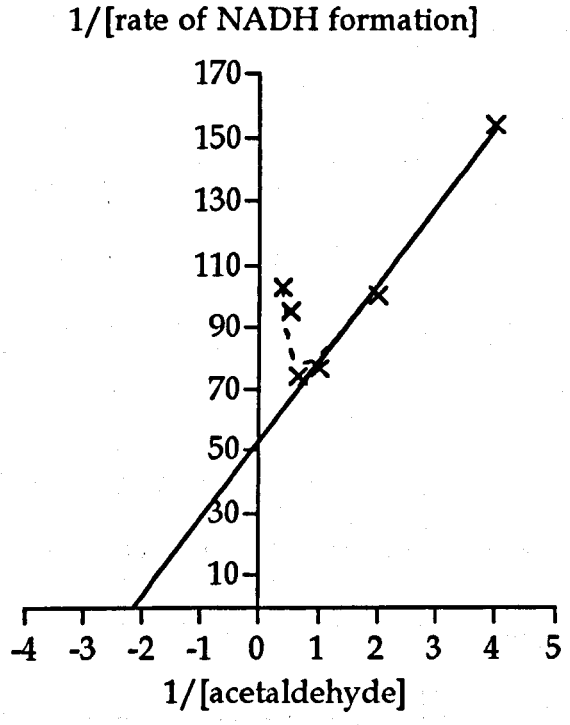


Figure 4.5 Determination of K_m, and V_{max} for acetaldehyde oxidation by mFDH from a double reciprocal plot.

Figure 4.5 also shows that acetaldehyde demonstrates negative cooperativity. This could be caused by a number of reasons, such as substrate inhibition, product inhibition, or a compound present in the acetaldehyde solution which at high concentrations inhibits oxidation. Any of these could be correct and without further study the reason can not really be commented on.

4.2.7 NAD^+ bound to mFDH is responsible for a change in the protein spectrum after mFDH has been turned over.

Reacting mFDH with NAD^+ in the presence of protein F removed the bound substrate and resulted in a marked change in the spectrum of the turned over enzyme. Purified mFDH exhibited a normal 278 nm absorption peak and no other spectral features (Figure 4.6). After reacting the enzyme with excess NAD^+ in the absence of added substrate, the absorbance peak shifted from 278 nm to 260 nm in the spectrum of repurified mFDH (Figure 4.6).

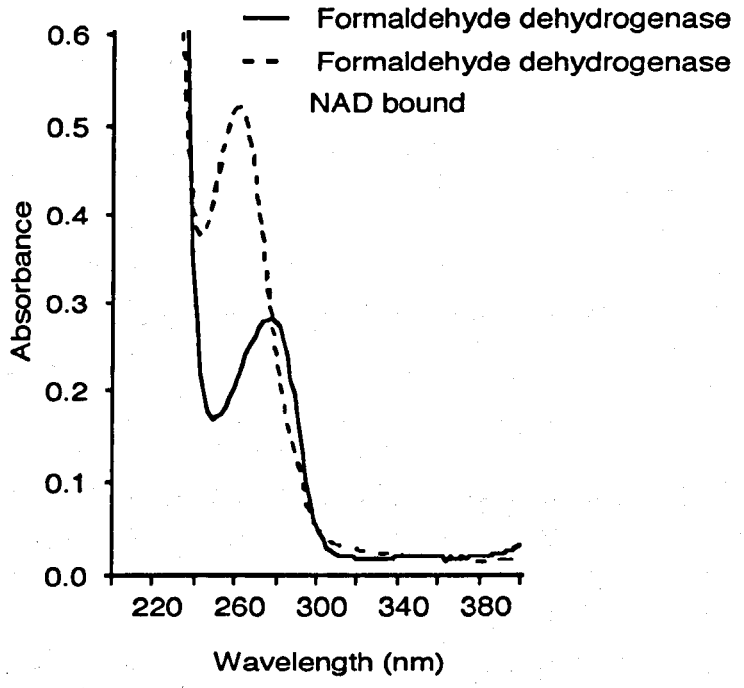


Figure 4.6 Alteration of protein spectrum of mFDH after binding of NAD^+.

This shift in the absorbance maximum from 280 nm to 260 nm was attributed to the association of NAD^+ with mFDH. The presence of bound NAD^+ was confirmed by its removal from the mFDH by unfolding the protein in the presence of 6M urea and then separating the NAD^+ from the mFDH on a Mono Q ion exchange column (as

described in Materials and Methods, Chapter 2, Section 2.3.18). The material eluted from the Mono Q column at the same elution volume as authentic NAD^+. The concentration of NAD^+ was calculated from the absorbance of the mFDH sample at 254 nm, after correcting for the contribution of mFDH to this absorbance. At a concentration of 2 μM mFDH the corrected absorbance value at 254 nm accounted for 8 μM NAD^+. Thus the amount of NAD^+ bound is in a molar ratio of 4:1 (NAD^+:mFDH) indicating the presence of four high affinity binding sites for the cofactor on the enzyme. The retention of bound NAD^+ after repurification of mFDH by gel filtration indicated that NAD^+ was not non-specifically bound and that the binding constant for NAD^+ is very large. ESI-MS experiments indicated that the NAD^+ was not present in the protein sample after electrospray ionisation and therefore the mass of mFDH with bound NAD^+ could not be determined in this way.

4.3 Determination of a kinetic model for formaldehyde oxidation catalysed by mFDH.

4.3.1 Evidence for four binding sites for NAD^+, formaldehyde and protein F on mFDH.

It is assumed that Figure 4.2(A), Figure 4.3(A) and Figure 4.4(A) demonstrate that formaldehyde binding to mFDH is cooperative. Replotting the data in Figure 4.4(A) using the Hill equation gives a Hill constant of 3.8, suggesting four (or multiples of four) binding sites for formaldehyde on mFDH (Figure 4.7). The data in Figure 4.2(A) were also replotted using the Hill equation but due to NADH formation in the absence of formaldehyde the results are unreliable.

For maximum catalysis of formaldehyde oxidation to occur there must be a molar ratio of 4:1, protein F:mFDH, demonstrating that there are probably also four protein F association sites (Chapter 4, Section 4.2.3). In addition it was shown (Chapter 4, Section 4.2.7) that there are four high affinity association sites for NAD^+ on mFDH. It is therefore concluded that there are four binding sites for formaldehyde or acetaldehyde, NAD^+ and protein F on mFDH.

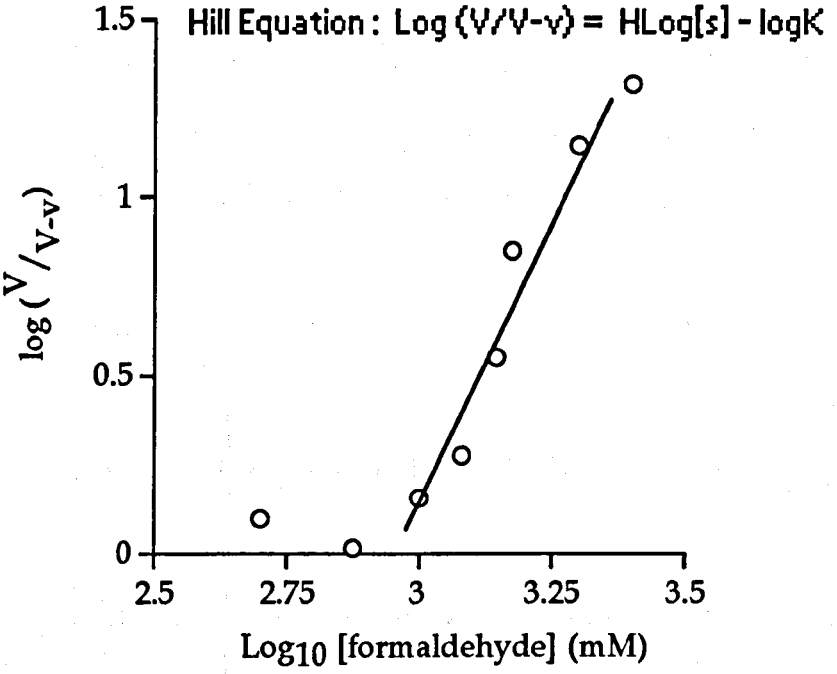


Figure 4.7 Hill plot for formaldehyde oxidation catalysed by mFDH in the presence of protein F.

Data from Figure 4.4(A) rearranged using Hill equation and plotted as above. For this plot the gradient of the regression line was calculated and this is the Hill constant. The Hill constant (H) was calculated from a plot of log (V/V-v) vs. Log [s]. Regression was used to determine the line of best fit through the linear region of the data and the gradient

4.3.2 The binding of formaldehyde and acetaldehyde to mFDH are mutually exclusive.

The effect of protein F on mFDH is very dramatic, as demonstrated in Table 4.1 and Figure 4.1. An important question about the mechanism of catalysis of formaldehyde oxidation by mFDH is whether there is a single or multiple binding sites for aldehyde substrates on the enzyme. Competition for aldehyde binding sites between formaldehyde and acetaldehyde in the presence and the absence of protein F was investigated by the addition of increasing concentrations of acetaldehyde to formaldehyde oxidation reaction mixtures and *vice versa.*

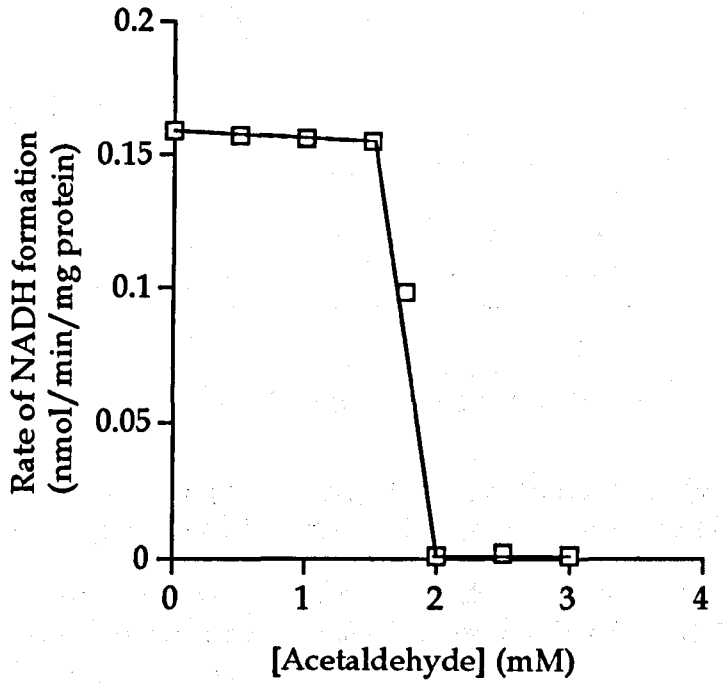


Figure 4.8 Effect of increasing acetaldehyde concentrations on the rate of formaldehyde oxidation catalysed by mFDH.

The reaction mixture contained 4.7 μM FDH, 20 μM protein F. Acetaldehyde was added to the reaction mixture at various concentrations before the 3 minute incubation at 45 °C and the reaction initiated by the addition of formaldehyde.

Figure 4.8 demonstrates that formaldehyde oxidation catalysed by mFDH is inhibited in the presence of acetaldehyde. Complete inhibition of formaldehyde oxidation occurred when the molar ratio of formaldehyde : acetaldehyde was 1 : 1. A similar relationship was observed when increasing concentrations of formaldehyde were added to an analogous acetaldehyde oxidation reaction mixture in the absence of protein F (Figure 4.9).

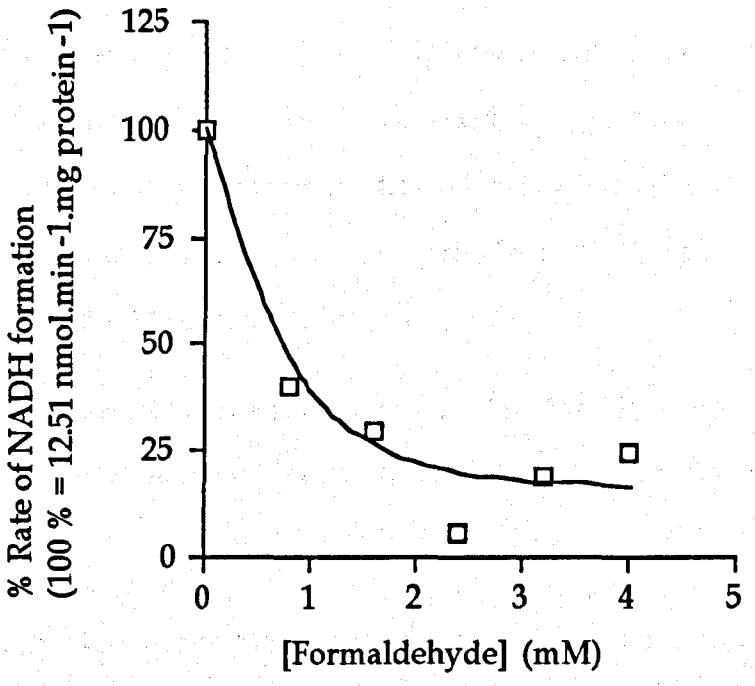


Figure 4.9 Effect of increasing formaldehyde concentrations on the rate of acetaldehyde oxidation catalysed by mFDH in the absence of protein F.

The reaction mixture contained 4.7 μM formaldehyde dehydrogenase. Formaldehyde was added to the reaction mixture before the 3 minute incubation at 45 °C and the reaction initiated by the addition of acetaldehyde.

Inhibition of acetaldehyde oxidation in the absence of protein F appears to exhibit competitive inhibition kinetics (Figure 4.9). If we assume from the data (Chapter 4, Section 4.2.6) that formaldehyde forms a tight complex with mFDH, then

the apparent inhibition constant, (K^*_i) for formaldehyde can be calculated using equation (1) (Cha, 1975, Williams & Morrison, 1979).

$$V_s/V_o = \{((([E_o]-[I_o]-K_s)+(I_o+K_s-E_o)^2)+4K_sE_o)^{0.5}\}/2E_o \qquad (1)$$

In Equation (1) V_o is the uninhibited initial rate of acetaldehyde oxidation, V_s is the initial rate of acetaldehyde oxidation in the presence of varying formaldehyde concentrations, E_o is the initial mFDH concentration, I_o is the concentration of formaldehyde at the start of the reaction and K_s is the inhibition constant K^*_i . Using non-linear regression the K^*_i value can be calculated from a plot of V_s/V_o against $[I_o]$. When this analysis was carried out on the data in Figure 4.9 the K^*_i calculated for formaldehyde was 0.41 mM. This value is very similar to the K_m value for acetaldehyde of 0.45 mM (Chapter 4, Section 4.2.6).

It must be noted that the scatter in the data shown in Figure 4.9 is sufficiently large to question the reliability of this data and in future experiments this should be repeated.

4.3.3 Presteady state kinetic analysis of formaldehyde and acetaldehyde oxidation catalysed by mFDH.

The steady state analysis above (Chapter 4, Section 4.1.3 and 4.1.5) showed that the rate of substrate oxidation was sigmoidal in relation to formaldehyde concentration. This supports the theory that formaldehyde association with mFDH is cooperative. Pre-steady state kinetics were undertaken to investigate the effect of the reaction components (mFDH, aldehyde, protein F and the electron acceptor NAD^+)

on the rate determining step of aldehyde oxidation and to determine if the mechanism of aldehyde oxidation is similar to published aldehyde dehydrogenase mechanisms.

Prior to undertaking the stopped flow pre-steady state experiments, the fluorescence emission spectra of all the reagents were measured with excitation at 340 nm and scanning emission wavelengths between 300 and 700 nm. The emission spectra of the different components used indicated that none of them interfered with the determination of NADH by this method (Figure 4.10).

The stopped flow experiments were performed as described in Materials and Methods (Chapter 2, Section 2.3.14). The analyte under study in each experiment was placed into one syringe (syringe A) and the other reagents placed in the second syringe (syringe B). The contents of each syringe in each specific experiment is given at the bottom of each figure legend (Figure 4.11).

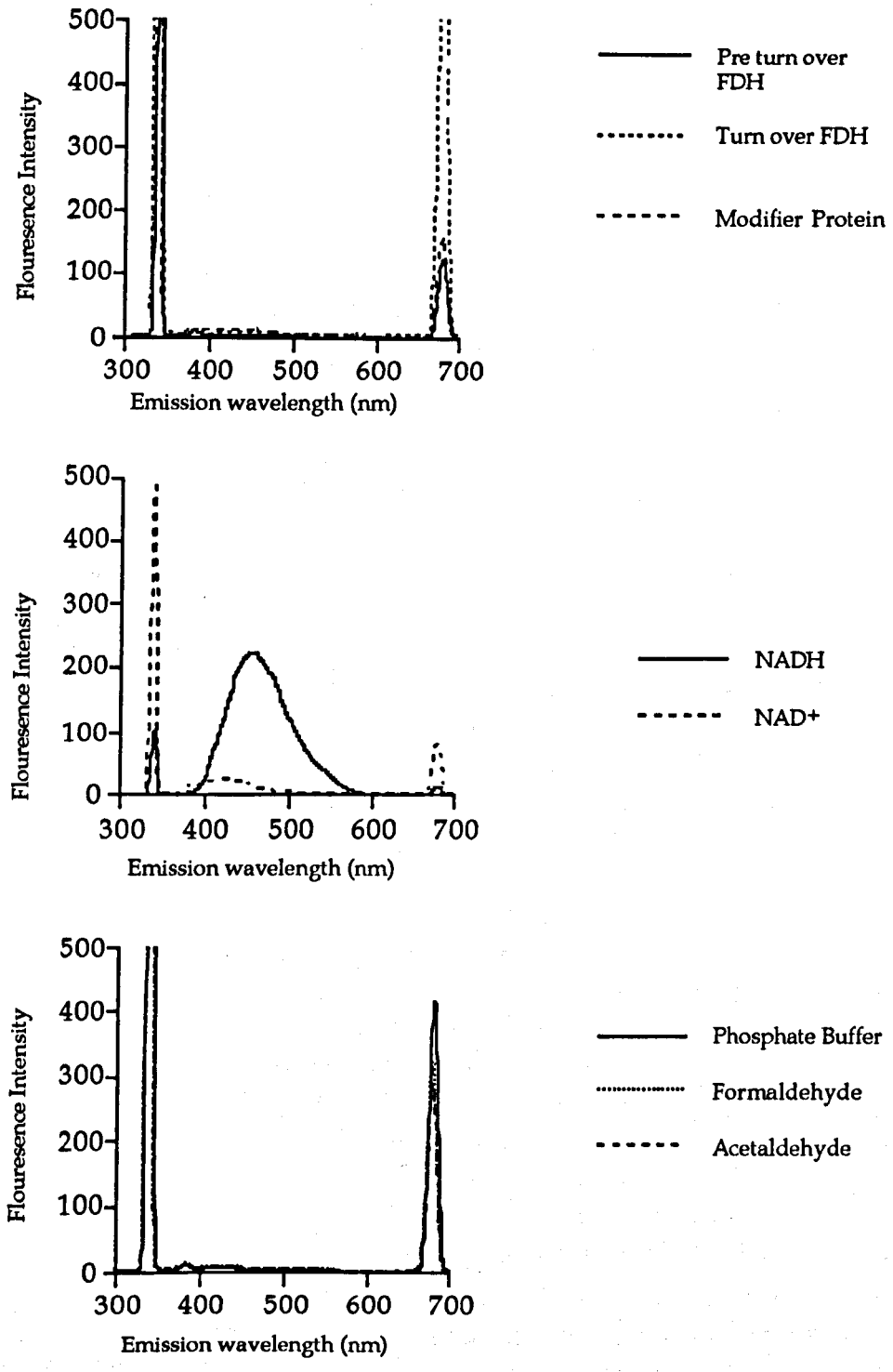
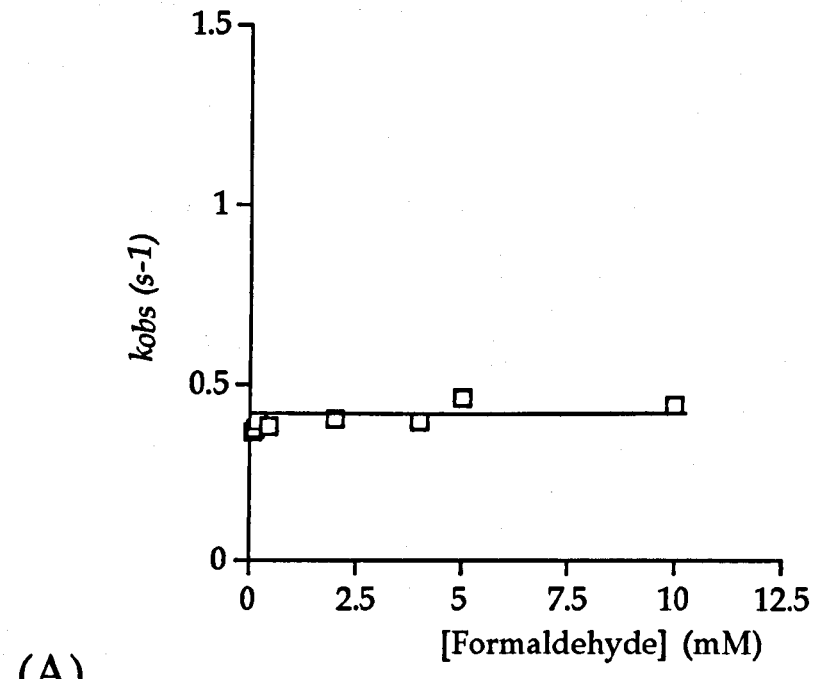
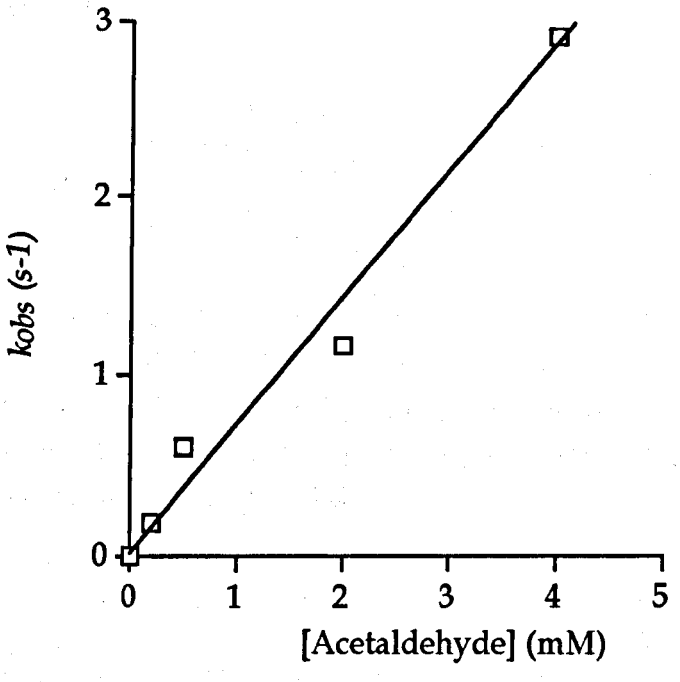


Figure 4.10 Fluorescence spectra of mFDH protein F and all reagents used in the determination of k_{obs} by stopped flow spectrophotometry.

Fluorescence emission spectra for each analyte were determined over the wavelength range 300 - 700 nm with excitation at 340 nm. The concentration of each analyte was identical to the concentration used in the stopped flow experiments.



(A)



(B)

Figure 4.11 Effects of formaldehyde and acetaldehyde concentrations on k_{obs} for their oxidation.

(A) Various concentrations of formaldehyde (0 - 20 mM) were placed in syringe A, with 0.4 μM mFDH, 4 mM NAD^+ and 4 μM protein F in syringe B.

(B) Syringe A contained various concentrations of acetaldehyde (0-8 mM) and syringe B contained 0.4 μM mFDH and 4 mM NAD^+.

In both experiments syringe A and B contained 25 mM phosphate buffer pH 7.2.

Figure 4.11 shows that formaldehyde binding to mFDH was not involved in the rate limiting step of its own oxidation since k_{obs} was independent of formaldehyde concentration. This contrasts with acetaldehyde where there is a linear relationship between k_{obs} and acetaldehyde concentrations, demonstrating that acetaldehyde is involved in the rate determining step of its oxidation by mFDH.

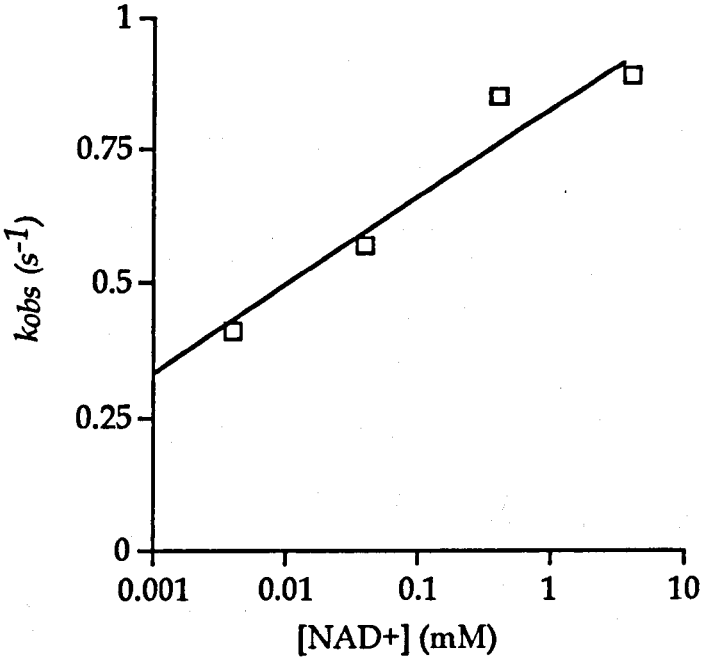
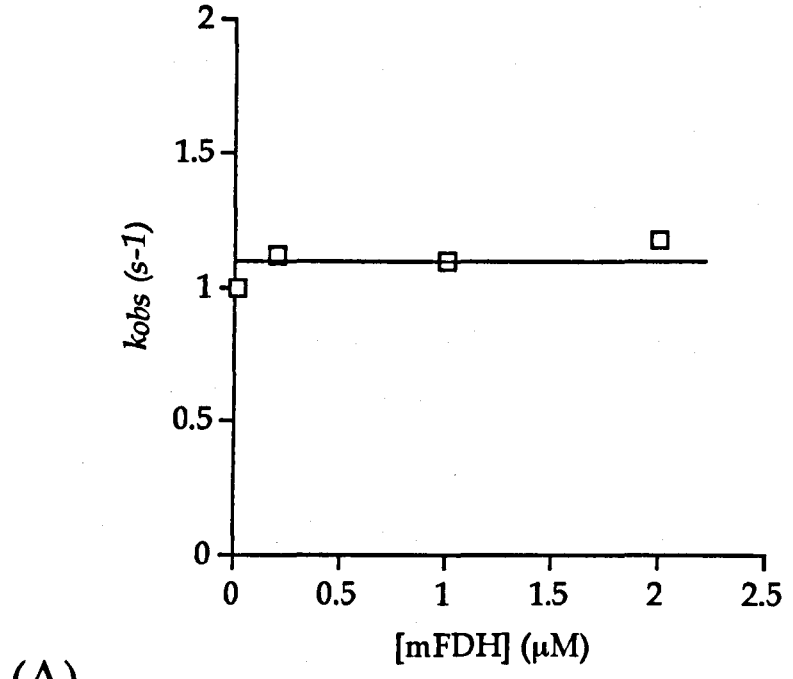


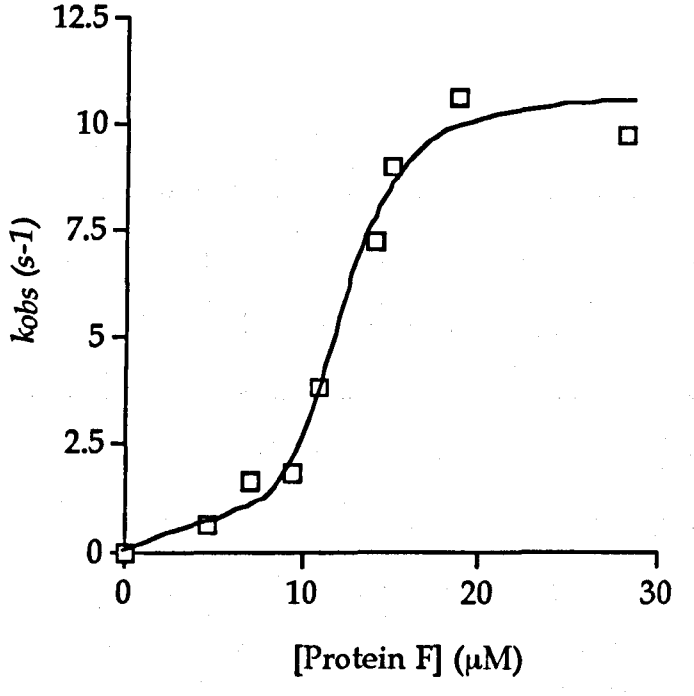
Figure 4.12 Effect of NAD^+ concentration on the observed rate constant (k_{obs}) of formaldehyde oxidation.

NAD^+ was placed into syringe A up to a final concentration of 8 mM while syringe B contained 0.4 μM mFDH, 8 μM protein F and 4 mM formaldehyde. Both syringe A and B contained 25 mM phosphate buffer pH 7.2.

Figure 4.12 demonstrates that NAD^+ association is not involved in the rate determining step of formaldehyde oxidation. There is a decrease in the k_{obs} value with decreasing concentrations of NAD^+, but the k_{obs} only decreases by 0.5 s^{-1} over a 10^5 range of NAD^+ concentrations, therefore the decrease is insignificant.



(A)



(B)

Figure 4.13 Effect of mFDH and protein F concentrations k_{obs} for formaldehyde oxidation.

(A) various concentrations of mFDH were placed in syringe A (0 - 4 μM), with 4 mM NAD^+ and 4 μM protein F in syringe B.

(B) syringe A contained various concentrations of protein F (0-52 μM) and syringe B contained 0.4 μM mFDH, 4 mM NAD^+ and 4 mM formaldehyde.

In both (A) and (B) all reagents were in 25 mM phosphate buffer, pH 7.2.

Figure 4.13 (A) shows that the rate determining step in formaldehyde oxidation is independent of the concentration of mFDH. However, Figure 4.13 (B) indicates that the rate determining step of formaldehyde oxidation does depend on the concentration of protein F. The sigmoidal dependence of k_{obs} on protein F concentration is characteristic of a third or higher order reaction, for formaldehyde oxidation. A third order reaction implies that there are 3 species involved in the rate equation i.e. Rate = k[A][B][C], or Rate = k[A][B]^2 etc. where k is the rate constant. If a multiple substrate reaction was involved then other reactants would have had an effect on the k_{obs} for formaldehyde oxidation.

4.3.4 Construction of a kinetic model for formaldehyde oxidation by mFDH.

The experimental data show that the rate determining step of formaldehyde oxidation is dependent upon protein F concentration. It is possible to deduce a model for the association of protein F with mFDH that is consistent with the sigmoidal shape of Figure 4.13 (B). When designing a kinetic model for this relationship it was assumed that the reactions are completely reversible and that the rate of the association and dissociation of mFDH with protein F was diffusion controlled, i.e. approximately $10^9\ M^{-1}.s^{-1}$, (Samuel, C., Personal communication).

Considering:

$$\text{mFDH} + \ n\ \text{protein F} \underset{}{\overset{\text{FAST}}{\rightleftharpoons}} \ n\text{protein F.mFDH complex}$$

where multiple protein F molecules (*n*protein F) form a complex with a single mFDH (*nprotein F:mFDH*). This complex undergoes a rate determining conformational change after which products are formed in a rapid reaction (Figure 4.14). Using steady state kinetic experiments it was demonstrated that mFDH can bind both NAD^+

and formaldehyde in the absence of protein F (Section 4.2.6 and 4.2.7). It must be assumed that the reactions of formaldehyde and NAD^+ with mFDH are independent of the presence of protein F. The rates at which these substrates associate with mFDH is assumed to be faster than the rate determining step. These assumptions are incorporated in Figure 4.14.

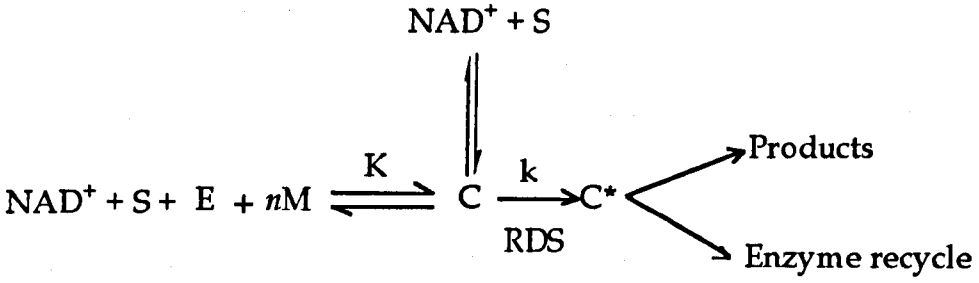


Figure 4.14 Proposed enzyme protein F interactions resulting in a catalytically active complex.

S represents substrate, E represents mFDH, M is protein F, C the *nprotein F:mFDH* complex, C^* the complex after undergoing the rate determining conformational change, K is the overall equilibrium constant for the formation of the nmodifier.enzyme complex and k is the rate constant for the conformational change. The figure also shows that NAD^+ and S may associate at two points.

The rate the *n*protein F molecules associate with mFDH to form the *n*protein F: mFDH complex (C) is determined by the overall equilibrium constant K. This is composed of several equilibrium constants for the individual reaction steps in the formation of the *nprotein F:mFDH* complex. For the equation in Figure 4.14 to describe a sigmoidal curve more than one protein F molecule must be involved and this has been demonstrated from the steady state experiments described earlier in this chapter (Section 4.2.3). Using the equation in Figure 4.14 a kinetic model can be constructed.

The concentration of the *protein F:mFDH* complex, [C], at any given time in the reaction is defined by equation 2.

$$[C] = K[E][M]^n \qquad (2)$$

Likewise the concentration of mFDH, [E], at any given time in the reaction is defined by equation 3, where E_o is the initial enzyme concentration.

$$[E] = E_o - [C] - [P]$$

$$\therefore [C] = E_o - [E] - [P] \qquad (3)$$

Solving equations 2 and 3 simultaneously for [E] gives equation 4.

$$[E] = (E_o - [P]) \cdot \frac{1}{(1 + K[M]^n)} \qquad (4)$$

From equations 2 and 3 we know that E_o - $[P]$ is equal to the overall equilibrium constant K times the concentration of protein F, equation 5.

$$K[M]^n = [E_o] - [P] \qquad (5)$$

Rearrangement of equations 4 and 5 leads to equation 6 which defines [E] in relation to the overall equilibrium constant K and the concentration of protein F.

$$[E] = \frac{K[M]^n}{1 + K[M]^n} \qquad (6)$$

Combining equation 3 and 6 gives:

$$[C] = ([E_o] - [P]) \cdot \frac{K[M]^n}{1 + K[M]^n} \qquad (7)$$

Equation 7 describes a sigmoidal relationship between [C], $[M]^n$ and K. From the equation in Figure 4.14, the rate of product formation is related to [C] and the rate constant k, as shown in equation 8.

$$\frac{\delta[P]}{\delta t} = k[C] \qquad (8)$$

Combing equation 7 and 8 leads to equation 9.

$$\frac{\delta[P]}{\delta t} = k.(E_o - [P]) \cdot \frac{K[M]^n}{1 + K[M]^n} \qquad (9)$$

The rate of product formation can also be expressed in terms of the observed rate constant, equation 10.

$$\frac{\delta[P]}{\delta t} = k_{obs}(E_O - [P]) \qquad (10)$$

From equations 9 and 10 the observed rate constant is seen to be dependent on both the concentration of protein F the rate constant k and the equilibrium constant K, equation 11.

$$k_{obs} = k.\frac{K[M]^n}{1+K[M]^n} \qquad (11)$$

Equation 11 describes a sigmoidal relationship between [M] and k_{obs} when n is greater than two.

Equation 11 was used in non-linear regression analyses to determine K and k from the experimental data. Microsoft Excel 5.0 (Microsoft Corporation) and Mac Curve Fit (Shareware, Kevin Raner) software were used for the non-linear regression analyses. Initial values for the overall equilibrium constant, K, of 10^{20} and of 15 for k_{obs} were used for regression analysis and both of these parameters were varied to produce the best fit to the experimental data. Figure 4.15 shows the result of the non-linear regression analysis. The number of moles of protein F required for maximum catalysis of formaldehyde oxidation was previously determined to be four from the steady state kinetic data (Chapter 4, Section 4.2.3) and this was confirmed by the regression analysis. Altering the value of n from 2 to 6 gave poor correlation with the experimental data while n=4, gave the best fit.

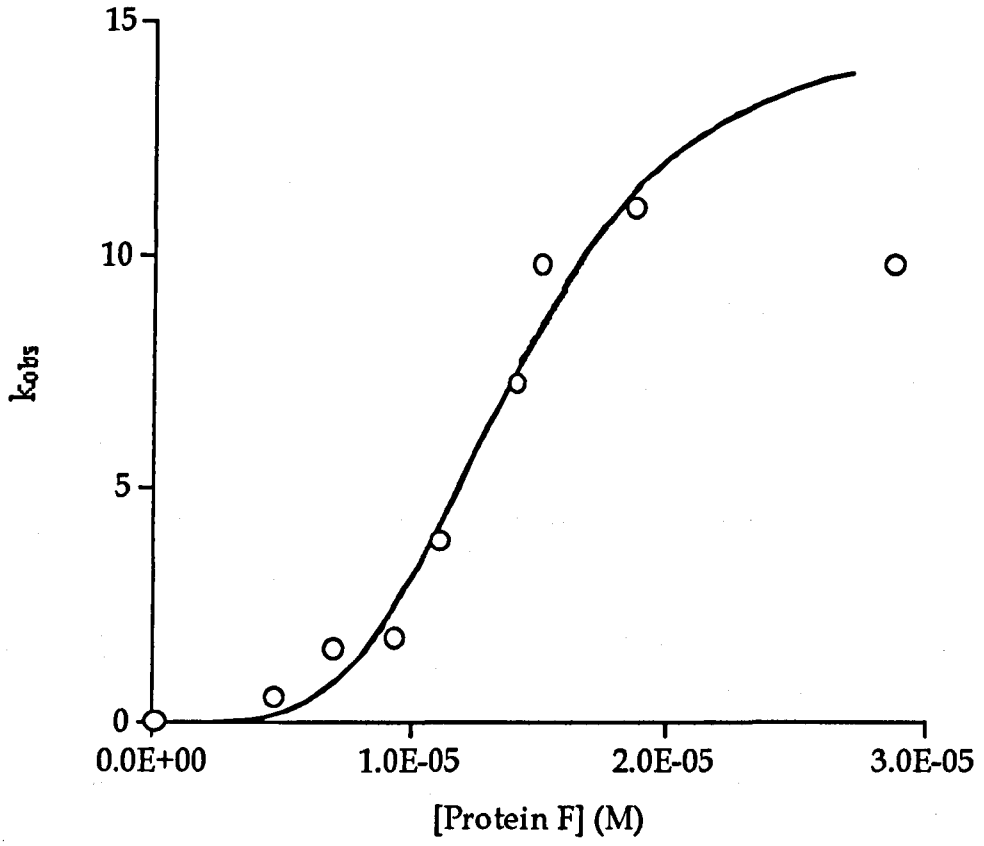


Figure 4.15 Line of best fit from non-linear regression analysis of stopped flow experimental data to equation 11.

—— Line of best fit.

O - Experimental data.

As is clearly demonstrated in Figure 4.15 the model is consistent with the experimental data. The equilibrium constant for the formation of the *nprotein F:mFDH* complex was determined as 2.6×10^{20} M^{-4} and k, the rate constant of the proposed conformational change, was calculated as 15 s^{-1}.

4.4 Discussion.

The alteration of enzyme specificity/function by regulatory proteins is not unique to mFDH. The two initial enzymes in the linear methane oxidation pathway, soluble methane monooxygenase (sMMO) and methanol dehydrogenase (MDH), both have regulatory protein components. sMMO is a three component enzyme that is regulated by protein B, a small 16 kDa peptide (Green & Dalton, 1985). Protein B may regulate the transfer of electrons from the reductase to the hydroxylase which contains the di-iron active site (Green & Dalton, 1985). MDH, a two component enzyme, is regulated by a 45 kDa peptide called M protein (Page & Anthony, 1986; Long & Anthony, 1991). The function of M protein is somewhat different from that of protein B as MDH is able to oxidise both methanol and formaldehyde and M protein specifies which is oxidised (Long & Anthony, 1991). In the presence of M protein, MDH catalyses the oxidation of methanol preferentially while in the absence of M protein, MDH will catalyse the oxidation of both methanol and formaldehyde (Page & Anthony, 1986). Protein M performs this function by altering the K_m for each substrate (Page & Anthony, 1986). The functions of protein B in sMMO and protein M in MDH are compared to protein F of mFDH in Table 4.2.

Table 4.2 Comparison of the modifier proteins involved in the methane oxidation pathway.

Property	Protein F	M protein	Protein B
Molecular Weight	9,000	45,000	16,000
Prosthetic Groups	None detected	None detected	None detected
Mode of action	Alteration of the substrate specificity and the type of kinetic model of aldehyde oxidation	Alters the Km values for formaldehyde and methanol.	Changes the type of reaction catalysed by sMMO, converting it from an oxidase to a monooxygenase.

The control of sMMO and MDH by proteins B and M respectively is not as striking as the control of mFDH by protein F. In the absence of protein B, methane oxidation can be detected in some sMMO enzymes, such as that isolated from *Methylosinus trichosporium* OB3b (Froland *et al.*, 1992; Fox *et al.*, 1991) and likewise in the absence of M protein MDH can still catalyse the oxidation of either methanol or formaldehyde *in vitro* (Long & Anthony, 1991). However mFDH is not able to catalyse the oxidation of formaldehyde in the absence of protein F and in the presence of protein F the oxidation of higher aldehydes is not observed. It is known that formaldehyde has a pivotal role in the metabolism of methylotrophic organisms at the point of carbon assimilation into the cellular biomass (Anthony, 1982) . Protein F may enable close regulation of *in vivo* concentrations of formaldehyde, maintaining a sufficient rate of formate formation, producing NADH for carbon assimilation and creating a 'carbon' pool in the form of formaldehyde available for assimilation.

4.4.1 Substrate specificity of formaldehyde dehydrogenase.

In an attempt to gain a clearer understanding of the role which mFDH plays in the metabolism of single carbon compounds in *M. capsulatus* (Bath) the substrate specificity in the presence and absence of protein F was determined for a range of simple aldehydes and alcohols. The data showed that formaldehyde oxidation, catalysed by mFDH, only occurred in the presence of protein F whereas the oxidation of higher aldehydes by mFDH was only detected in the absence of protein F. The alteration in substrate specificity from a general aldehyde dehydrogenase to a specific FDH demonstrates that protein F functions as a very powerful regulatory protein which can switch on and off two different enzyme functions.

The oxidation of formaldehyde by Type III alcohol dehydrogenases in the presence of glutathione (GSH) has been documented for methylotrophic organisms (van Ophem *et al.*, 1992) and these enzymes have been termed GSH dependentformaldehyde dehydrogenases, gFDH (van Ophem *et al.*, 1992; Eggeling & Sahm, 1984; Eggeling & Sahm, 1985). The oxidation of alcohols catalysed by mFDH suggested that it could be a gFDH but attempts to replace protein F with glutathione were unsuccessful. The N - terminal sequence of mFDH presented earlier (Chapter 3, Section 3.5.3) is distinctly different from those reported for gFDH enzymes (van Ophem *et al.*, 1992). Therefore the mFDH purified from *M. capsulatus* (Bath) and kinetically characterised in this study is not a gFDH enzyme.

It was previously reported that mFDH catalysed the oxidation of glyceraldehyde in the presence of HTSE, although the observed oxidation of this substrate may have been due to the presence of formaldehyde in the glyceraldehyde solution used (Stirling & Dalton, 1978). The glyceraldehyde used in the present study was purchased from Sigma and no formaldehyde was detected when the sample was analysed by the Nash method (Nash, 1953).

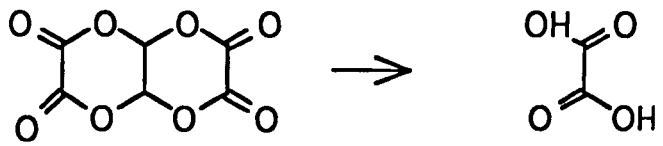


Figure 4.16 Structure and proposed oxidation product of glyoxal oxidation.

Glyoxal was the only substrate investigated which was oxidised in the presence and absence of the modifier protein. Figure 4.16 shows the structure of glyoxal and its proposed oxidation product. Glyoxal exists as a hydrated diol in solution and it is unclear whether or not the hydration of glyoxal contributes to its oxidation by mFDH both in the presence and absence of protein F.

The effect of the extent of aldehyde hydration on the catalysis of their oxidation by dehydrogenases is unknown. It is known that the rate of formaldehyde hydration and the equilibrium constant for the reaction is relatively large (i.e. k = 10 $M^{-1}s^{-1}$, K= 2000 M^{-1}, Bell & Evans, 1965). This means that the majority of formaldehyde (approximately 99.9%) in solution is in the hydrated form (methane diol). This percentage is reduced for acetaldehyde where only 60% is hydrated in solution and the amount of aldehyde hydration decreases further as the number of carbons in the molecule increases (Bell & Evans, 1965). The data presented in this study suggest that those substrates with a low percentage of diol in solution have a lower rate of oxidation. However if mFDH was catalysing the oxidation of the diol form of aldehydes then oxidation of more alcohols might be expected.

As formaldehyde is predominately methane diol in solution, protein F may reduce the equilibrium constant for methane diol formation thus stabilising formaldehyde in solution. Such a function of protein F would be similar to that of GSH, where gFDH enzymes oxidise the more stable S-(hydroxymethyl)-glutathione,

which is formed spontaneously in solution from formaldehyde and GSH (Holmquist & Vallee, 1991).

4.4.2 Stoichiometry of formaldehyde and acetaldehyde oxidation.

The calculated stoichiometry of formaldehyde and acetaldehyde oxidation is shown in Figure 4.17.

$$HCHO + NAD^+ + H_2O \longrightarrow HCOOH + NADH + H^+$$

$$CH_3CHO + NAD^+ + H_2O \longrightarrow CH_3COOH + NADH + H^+$$

Figure 4.17 Stoichiometry of formaldehyde and acetaldehyde oxidation by mFDH.

The data (Chapter 4, Section 4.2.2) show that one mole of NADH is produced for every mole of formaldehyde or acetaldehyde oxidised. As the data showed that one mole of formate was produced from the oxidation of one mole of formaldehyde dismutase activity was not investigated. If mFDH was catalysing a dismutase reaction the amount of formate produced would be equal to half the amount of formaldehyde reactant, due to the formation of ethanol as the other product (Kato *et al.*, 1988; Bystrykh *et al.*, 1993).

Therefore the function of mFDH in the presence of protein F is to oxidise formaldehyde to formate and produce NADH, thus supplying reducing energy for catabolic reactions and carbon assimilation by the RuMP pathway. In Appendix 3 it was shown that mFDH and the hydroxylase of soluble methane monooxygenase copurified suggesting a strong affinity between the two enzymes and a possible

synergistic relationship. The substrate specificity studies of mFDH showed that methanol oxidation can also be catalysed by mFDH in the absence of the modifier protein. If we assume that the stoichiometry of methanol oxidation is the same as for formaldehyde oxidation then for every two moles of methane oxidised, one mole can be assimilated via formaldehyde and one mole is oxidised to produce two moles of NADH for methane oxidation, or carbon assimilation (Figure 4.18). From the data available there is little relability in this model. As this may be an important route of the formation of NADH further work should be performed to investgate this further.

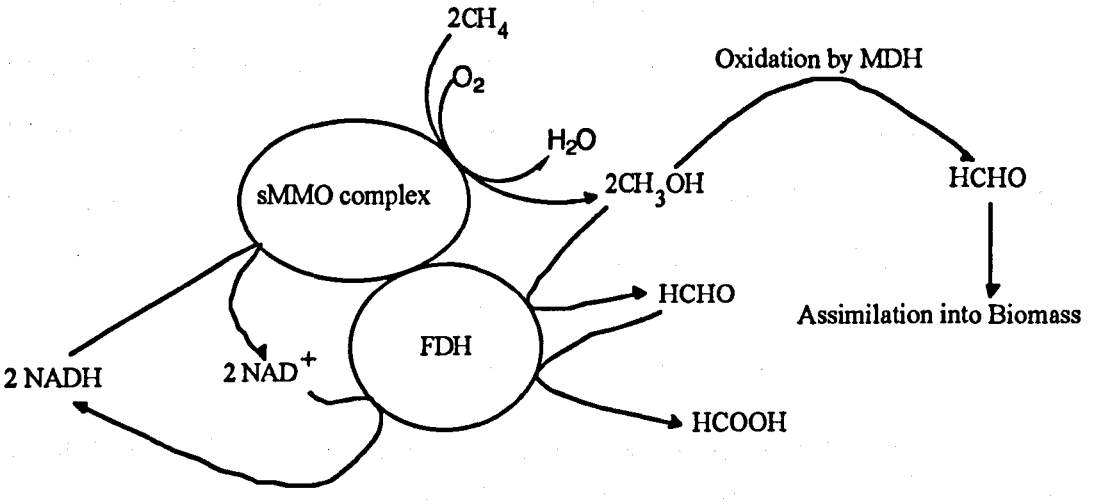


Figure 4.18 A possible role for mFDH in the metabolism of single carbon compounds in *M. capsulatus* (Bath).

sMMO - soluble methane monooxygenase, mFDH - formaldehyde dehydrogenase, MDH - methanol dehydrogenase.

4.4.3 The binding of formaldehyde and acetaldehyde occurs at the same site on mFDH.

In an attempt to understand the regulatory mechanism of protein F the competition between formaldehyde and acetaldehyde binding was investigated. The difference in shape of the inhibition profiles for formaldehyde and acetaldehyde may

be related to different binding constants for each substrate. It could, however, indicate that binding of formaldehyde to one of the aldehyde binding sites on mFDH does not inhibit acetaldehyde oxidation at the other sites and only when all sites are occupied is oxidation of acetaldehyde completely inhibited. The ability of formaldehyde to inhibit acetaldehyde oxidation and *vice versa* suggests that these substrates bind at the same sites on mFDH. It also demonstrates that protein F does not stop the binding of acetaldehyde to mFDH.

Purification of mFDH with either formaldehyde or NAD^+ bound to the enzyme shows that protein F is not required for the binding of these molecules to mFDH. Thus a mFDH.SUBSTRATE.NAD^+ complex can be formed in the absence of protein F and when the substrate is a higher aldehyde this complex produces product. When the substrate is formaldehyde, association of protein F is required for the formation of products. The purification of mFDH containing bound formaldehyde or NAD^+ indicates that the association constants for these molecules is large.

4.4.4 Formaldehyde demonstrates cooperative binding to mFDH.

Previous in this study the molecular weight of mFDH isolated from *M. capsulatus* (Bath) was determined to be 255,000 Da, with a subunit molecular weight of 63,620 Da indicating that it is a tetramer (Chapter 3, Section 3.5.2). The tetrameric structure of the enzyme is also suggested from the kinetic experiments from which the number of binding sites for formaldehyde, NAD^+ and modifier protein were calculated. Earlier work indicated the presence of four or multiples of four binding sites for protein F on mFDH (Millet *et al.*, (unpublished)) and this was confirmed in this study. Evidence has been presented here which indicates that there are also four binding sites on mFDH for formaldehyde and NAD^+. There appears to be cooperativity in the binding of the four formaldehyde molecules but not in protein F

binding. The enzyme purified and characterised in this study is different from other FDH's purified from methylotrophic organisms in that formaldehyde binding is cooperative (Ando *et al.*, 1979; Attwood *et al.*, 1992; van Ophem *et al.*, 1992; Eggeling & Sahm, 1985).

The observation of cooperative formaldehyde binding suggests that the substrate is able to induce a conformational change which increases the affinity of mFDH for each successive formaldehyde bound. Formaldehyde binding in this respect is similar to oxygen binding to haemoglobin (Pertuz, 1989). Formaldehyde may be capable of inducing conformational changes in other subunits or active sites of mFDH, therefore enhancing the binding of subsequent formaldehyde molecules to other binding sites, which would result in the cooperativity observed experimentally. The cooperativity is probably a function of the association of protein F. Upon binding of protein F a conformational change in mFDH results in cooperative binding of formaldehyde. This would explain why acetaldehyde oxidation by mFDH in the absence of protein F does not show a sigmoidal relationship while formaldehyde oxidation in the presence of formaldehyde does.

Purified mFDH was shown to have one formaldehyde molecule tightly bound to the enzyme. This could be an artefact of the growth conditions or may be important for stabilisation of mFDH *in vivo*. This tight binding of one formaldehyde molecule to mFDH and the cooperativity of formaldehyde binding suggests that one role of mFDH could be to rapidly prevent formaldehyde from accumulating within the cell, which would be toxic. Such a 'safety valve' system is extremely important in methylotrophic bacteria since the formation of formaldehyde is an integral step in the growth of these organisms (Chapter 1, Section 1.1). Without a system to remove toxic concentrations of formaldehyde from the cell, death would result. If the function of this mFDH is solely to serve as a 'safety valve' it would mean that formaldehyde oxidation must be catalysed by other enzymes. It has been proposed that there are

three principal enzymes responsible for formaldehyde oxidation, MDH (Long & Anthony, 1991), mFDH (Stirling & Dalton, 1978) and a cytochrome - dependent FDH (Hay, 1990). This would mean that the yet unpurified and uncharacterised cytochrome - dependent FDH could be the principal system for formaldehyde oxidation in *M. capsulatus* (Bath). Chapter 6 identifies a second NAD+-linked FDH which may be responsible for the oxidation of formaldehyde for the formation of NADH.

4.4.5 Effect of protein F on mFDH.

mFDH isolated from *M. capsulatus* (Bath) is not unusual in requiring a cofactor for formaldehyde oxidation. What is unusual is that the cofactor is a protein and not a small molecule such as glutathione. Protein F does not covalently modify mFDH since it can be removed from crude soluble extract by dialysis. This indicates that protein F must have an allosteric effect or cause a conformational change in mFDH which allows formaldehyde oxidation to proceed. In this respect Protein F is similar to α-lactalbumin causing a switching of substrate specificity, as described in Chapter 1 (Section 1.3.2).

The association of protein F with mFDH is not cooperative and titration of protein F against mFDH showed that the molar ratio of mFDH: protein F required for maximum activity was 1 : 4. As increasing concentrations of protein F did not inhibit formaldehyde oxidation this could indicate that the binding site(s) for protein F on mFDH are distinct from the formaldehyde binding site(s). If aldehyde and protein F shared a common binding site then increasing concentrations of protein F would inhibit the oxidation of formaldehyde.

4.4.6 A conformational change in mFDH induced by the association of protein F, allows formaldehyde oxidation.

The stopped-flow experiments allow a better understanding of the role of protein F and determination of possible rate determining steps in the oxidations of acetaldehyde and formaldehyde. It appears from the experimental data that the rate determining step in acetaldehyde oxidation is associated with the binding of acetaldehyde to mFDH. These data cannot distinguish the rates of substrate binding or of product dissociation. Previous studies of aldehyde dehydrogenase enzymes have demonstrated that the rate determining step occurs after a hydride transfer reaction Figure 4.19 (Dickinson, 1988).

The rate determining step in the mechanism of aldehyde dehydrogenase enzymes is the dissociation of NADH caused by the association of NAD^+ (Figure 4.19). This appears to be different from the rate determining step of acetaldehyde oxidation by mFDH. In the case of acetaldehyde oxidation by mFDH if the dissociation of NADH was the rate determining step then there would not be a dependence of k_{obs} on the acetaldehyde concentration. This therefore suggests that the rate determining step in acetaldehyde oxidation catalysed by mFDH is probably the association of substrate or dissociation of acetate.

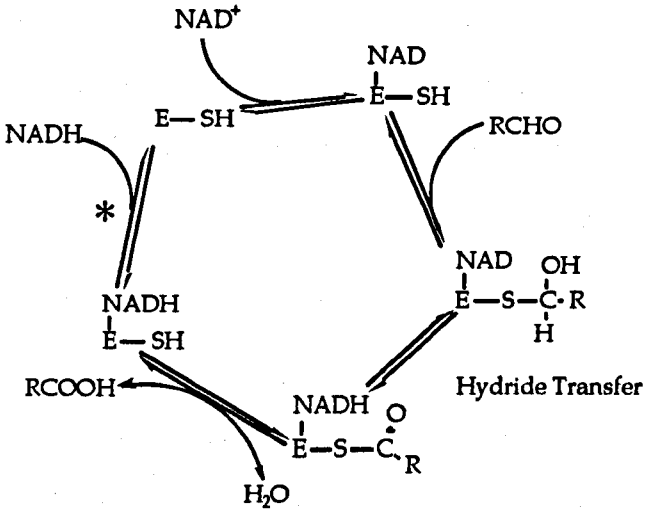


Figure 4.19 The proposed mechanism for aldehyde dehydrogenase showing the hydride transfer step which precedes the rate determining step* (Dickinson, 1988).

E-SH - Aldehyde dehydrogenase enzyme with free sulphydryl group, RCHO - aldehyde substrate, RCOOH - carboxylate product.

The stopped-flow kinetic analysis of formaldehyde oxidation demonstrated that only protein F was involved in the rate determining step. The relationship between k_{obs} and protein F concentration indicated a complex interaction between the modifier and mFDH. The unique relationship between protein F and mFDH does not allow a direct comparison to be made with other aldehyde dehydrogenases which have been characterised.

The proposed model indicates that the rate determining step in formaldehyde oxidation is a protein F induced conformational change in mFDH which allows product formation. From the proposed mathematical model and the experimental evidence from the steady state kinetic analysis it is proposed that the association of protein F with mFDH induces a conformational change which then enables formaldehyde to bind to the other three proposed sites on mFDH. When protein F concentration is in excess of other reagents the cooperative formaldehyde binding is demonstrated as in section 4.2.5. If formaldehyde was able to cause cooperativity

then purified mFDH would have four moles of substrate bound and not just one mole of substrate bound per mole of mFDH. It is therefore concluded that the conformational change induced by the binding of protein F to mFDH causes formaldehyde binding to become cooperative and allows the formation of products.

The experimental evidence also indicates that although the occupation of all protein F binding sites is not essential for formaldehyde oxidation to occur there must be four protein F molecules bound to mFDH for maximum activity (Section 4.1.3).

In conclusion, all of the data from both the steady state and presteady state kinetic experiments support the hypothesis that the association of formaldehyde and NAD^+ to mFDH are independent of protein F and that formaldehyde oxidation, catalysed by mFDH, only occurs after protein F has induced a conformational change. The sigmoidal dependence of oxidation rate on formaldehyde concentration occurs because of the conformational change induced by the association of protein F. In contrast the rate of catalysis of acetaldehyde oxidation is dependent on the concentration of substrate, and exhibits normal michaelis mentenkinetics. The function of mFDH in the biochemistry of *M. capsulatus* (Bath) is still uncertain. It would appear that there are two possibilities:

(1) formation of one mole of NADH from one mole of methane oxidised which can be used in carbon assimilatory pathways and methane oxidation.

(2) A safety system for the removal of toxic formaldehyde which produces NADH for cell repair, which may be caused by high *in vivo* concentrations of formaldehyde.

As formaldehyde oxidation by mFDH in the presence of protein F is sigmoidal its function is more likely to be (2) above. It may be that protein F is expressed in response to cellular damage or high intracellular formaldehyde concentrations where higher amounts of NADH could be required.

5. The effect of protein F on other dehydrogenases.

5.1 Introduction.

The effect of the modifier protein (protein F) on the activity of formaldehyde dehydrogenase (mFDH) from *Methylococcus capsulatus* (Bath), is an important finding in metabolic control and prompted the search for similar control proteins in other organisms which are, as yet, undescribed. The only known example of a protein which alters the substrate specificity and kinetics of an enzyme is α-lactalbumin, isolated from mammalian cells, which controls the activity of γ-galactosyl transferase (Chapter 1, Section 1.3.2).

Several commercially available dehydrogenases were tested to determine whether protein F had any effect on their activities. Alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and glucose dehydrogenase (GDH) were chosen. ADH is relatively ubiquitous, having been isolated from liver tissue and most other mammalian tissues and from the seeds and leaves of higher plants. It has also been isolated from many micro-organisms (Conn *et al.*, 1987). ADH is the last enzyme in the alcoholic fermentation pathway and is responsible for the conversion of acetaldehyde to ethanol, as shown in Figure 5.1.

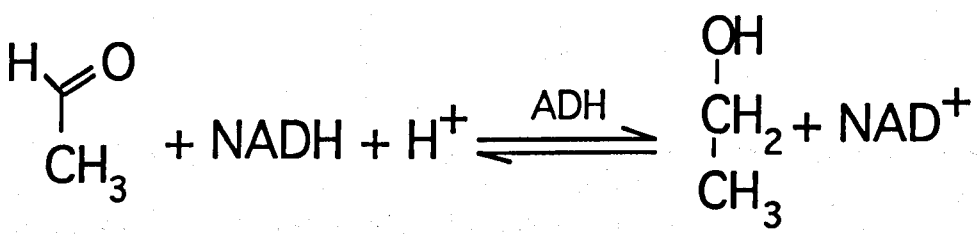


Figure 5.1 Physiological reaction catalysed by alcohol dehydrogenase.

The metabolic role of ADH is well understood and documented in most biochemistry text books (Stryer, 1988; Conn *et al.*, 1987). The crystal structure of horse liver alcohol dehydrogenase (HLADH) was solved in 1976 (Eklund *et al.*, 1976) and subsequently the crystal structures of many ADH mutant enzymes have been determined (Brookhaven Protein Databank). These have helped in the understanding of the mechanism of action by ADH. HLADH and other ADH enzymes are used extensively in bio-transformation reactions for the production of fine chemical products with high optical purity (Matos, 1985).

G3PDH is also ubiquitous, being found in all cells which metabolise glucose and holds a pivotal position in the glycolysis pathway. G3PDH catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, as shown in Figure 5.2.

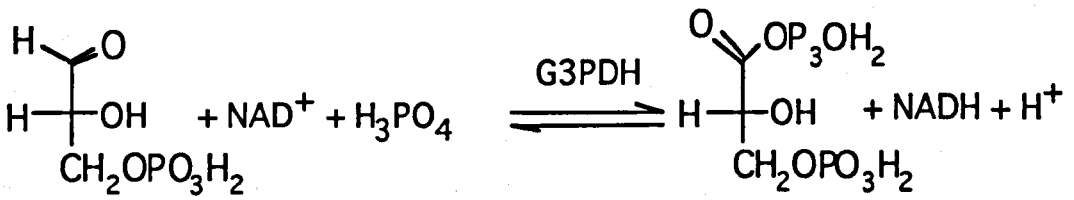


Figure 5.2 Conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate catalysed by G3PDH.

This is the first reaction which produces a high energy phosphate compound in the glycolysis pathway (Conn *et al.*, 1987). As with ADH, the mechanism of the reaction is very well documented (Liu & Huskey, 1992) and the crystal structure of G3PDH has been solved (Kim *et al.*, 1995; Vellieux *et al.*, 1993). It has been reported that an activator protein for G3PDH has been purified from kidney epithelial cells (Aithal *et al.*, 1994). This is a 60 kDa cytosolic protein which is expressed in both rat

and monkey tissues in response to a potassium deficient extracellular environment and stimulates the activity of G3PDH (Aithal *et al.*, 1994).

The final enzyme chosen, glucose dehydrogenase (GDH), catalyses the reaction shown in Figure 5.3. There is very little information about GDH in the literature.

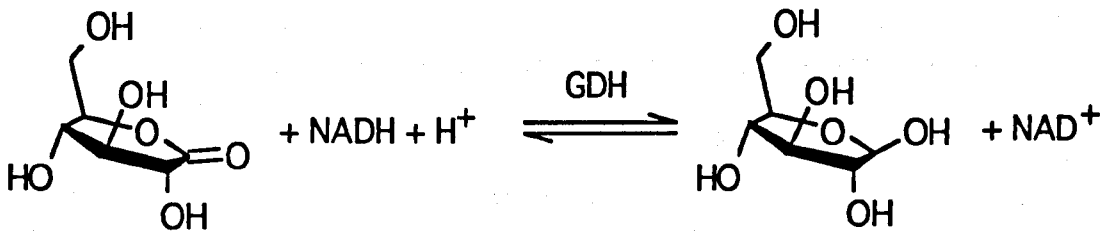


Figure 5.3 Conversion of gluconolactone to glucose by GDH.

5.2 Results.

5.2.1 Effect of Protein F on commercially available dehydrogenase enzymes.

Each of the dehydrogenase enzymes studied were assayed for native activity as described earlier (Chapter 2, Section 2.3.6 - 2.3.8). The rate of NADH formation calculated from these assays is termed *the enzyme 100% activity.* After determination of the unmodified activities of each dehydrogenase, the activities were redetermined in the presence of 4.7μM protein F.

The activity of each dehydrogenase enzyme was then determined, using formaldehyde as the substrate, in the absence and presence of protein F. The formaldehyde oxidation activity of the different enzymes was expressed as a percentage of *the enzyme 100% activity.*

The effect of protein F on the activity of commercially available dehydrogenase enzymes is shown in Table 5.1. These data demonstrate that FDH catalytic activity was only detected in GDH samples and only when protein F was added to the reaction mixture. Table 5.1 shows that GDH samples isolated from two sources, calf liver and *Bacillus megaterium*, each exhibited some FDH catalytic activity in the presence of protein F. The calf liver GDH was a crude liver homogenate (Sigma G5625) and the GDH from *B. megaterium* was purchased as a chromatographically purified enzyme (Sigma G7653). Analysis of these samples on SDS-PAGE showed that there was a single species of 60 kDa present in both preparations.

Table 5.1 also demonstrates that protein F was able to enhance the activity of ADH by 27%. Investigations into the effect of the molar ratio of protein F : ADH on

ADH activity were not carried out. However, investigations of the formaldehyde oxidising species in the GDH sample from Calf liver were pursued.

Table 5.1 Effect of protein F on commercially available dehydrogenases.

Enzyme and Source.	Native Dehydrogenase Activity (%)		Formaldehyde Dehydrogenase Activity (%)	
	- Modifier	+ Modifier	- Modifier	+ Modifier
Alcohol Dehydrogenase from Bakers Yeast. (ADH, Sigma A3263)	100	127 (124-130)	0	0
Glyceraldehyde - 3 - Phosphate Dehydrogenase from *Bacillus stearothermophilus.* (G3P, Sigma G5892)	100	100	0	0
Glucose Dehydrogenase from Calf liver (GDH, Sigma G5625)	100	103 (94-109)	0	7.4 (7.2-7.65)
Glucose Dehydrogenase from *B. megaterium.* (GDH1, Sigma G7653)	100	100	0	0.2 (0.1-0.3)

100% activity of each dehydrogenase is the rate of oxidation of the native substrate of each enzyme. Each result is the average of 3 experiments.

5.2.2 Purification of a protein F dependent FDH from commercially available calf liver GDH.

It was clear from the data presented in Table 5.1 that the calf liver GDH preparation was able to catalyse the oxidation of formaldehyde in the presence of protein F. To determine whether calf liver GDH was responsible for this oxidation activity, purification of the catalytically active protein was attempted. The formaldehyde oxidising species present in the GDH preparation was purified in three chromatographic steps: gel filtration, ion exchange and gel filtration chromatography.

Step 1: Gel filtration,

A 1 ml sample of 67 mg/ml calf liver glucose dehydrogenase (GDH) solution (Sigma G5625) was loaded onto a Pharmacia Superdex 200 pg gel filtration column (Pharmacia HR 16/20) equilibrated with 20 mM Tris - HCl, pH 7.5. The sample was eluted at a flow rate of 4 ml/min in the same buffer and 4 ml fractions were collected. Eluted protein was monitored by absorbance at 280 nm. FDH activity in the presence of protein F and GDH activity were determined for all fractions. Those which showed FDH activity were retained for further purification and stored at -20 °C.

Step 2: Ion exchange.

Protein which demonstrated FDH catalytic activity from Step 1 was loaded onto a Pharmacia Mono Q column (HR 5/5) equilibrated with 20 mM Tris - HCl pH 7.5 at a flow rate of 0.5 ml/min. Protein was eluted with a linear gradient of 0 - 1.0 M KCl over 10 column volumes in the same buffer. Eluted protein was monitored at 280 nm and 4 ml fractions were collected. FDH and GDH enzyme activities in all fractions were

determined and those fractions which had FDH activity were pooled and stored at -20 °C for further purification.

Step 3: Gel filtration.

Material which demonstrated FDH activity isolated from Step 2 was concentrated to a final volume of 1 ml with an Amicon ultrafiltration unit using a PM30 membrane. The concentrated solution was loaded in 125 µl aliquots at a flow rate of 0.25 ml/min onto a Pharmacia Superose 12 column (HR 10/30), which had been equilibrated with 20 mM Tris - HCl, pH 7.5 buffer containing 50 mM KCl. Protein was eluted from the column at a flow rate of 0.5 ml/min in the same buffer and 2 ml fractions were collected. Eluted protein from the column was monitored by absorbance and the enzyme activities of each fraction determined as previously described.

Those fractions which had FDH activity were pooled and concentrated to a final volume of 1 ml using an Amicon ultrafiltration unit over a PM30 membrane. The concentrated material was stored at -20 °C

During the purification procedures both GDH and FDH activities were measured in all the fractions collected. Table 5.2(A) and Table 5.2(B) show the activities of GDH and FDH respectively at different stages of purification. Table 5.2(A) demonstrates that purification of the formaldehyde-oxidising species from calf liver GDH completely removes GDH activity. The specific activity for the FDH species increased throughout the purification (Table 5.2(B)) indicating that these are distinct enzymes. Figure 5.4 shows an SDS-PAGE of the proteins which were purified. The FDH species clearly has a different electrophoretic mobility from GDH and must be masked in the commercial preparation by the relative quantity of GDH in the sample.

Table 5.2 Purification of a FDH from calf liver GDH (Sigma G5625).

(A) *GDH activity.*

Purification Step	Total Volume (ml)	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity for GDH (nmol/min/mg protein)	Protein Recovery (%)	Fold Purification
Crude Extract	1	67	1300	19	100	1
Gel Filtration	16	19.2	1260	66	97	3.5
Ion Exchange	8	3.3	224	68	17	3.6
Gel Filtration	3	0.6	0	0	0	0

(B) *FDH activity.*

Purification Step	Total Volume (ml)	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity for FDH (nmol/min/mg protein)	Protein Recovery (%)	Fold Purification
Crude Extract	1	67	95	1.4	100	1
Gel Filtration	16	19.2	290	15	305	11
Ion Exchange	8	3.3	88	27	93	19.3
Gel Filtration	3	0.6	75	125	79	89

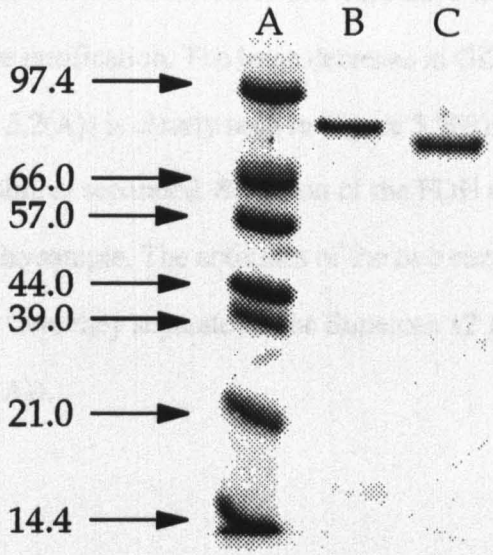


Figure 5.4 12.5 % SDS-PAGE of protein material with protein F dependent FDH activity purified from calf liver GDH.

Lane A. sigma low molecular weight markers, in kDa.

Lane B. protein material with protein F dependent FDH activity after the purification procedure above.

Lane C. protein material which retained GDH activity after the purification procedure.

Figure 5.5 shows chromatograms of the first two steps of the purification procedure with FDH and GDH activities overlaid. The chromatograms clearly demonstrate that proteins with GDH and FDH activities have different chromatographic mobilities throughout the purification. The large decrease in GDH activity through the Mono Q column (Table 5.2(A)) is clearly seen in Figure 5.5(B) where the activities of the GDH and FDH are almost separated. Selection of the FDH active protein removes most of the GDH from the sample. The activities of the two enzymes coincide until the final purification stage where they separate on the Superose 12 gel filtration column (Figure 5.6) (Table 5.2(A)).

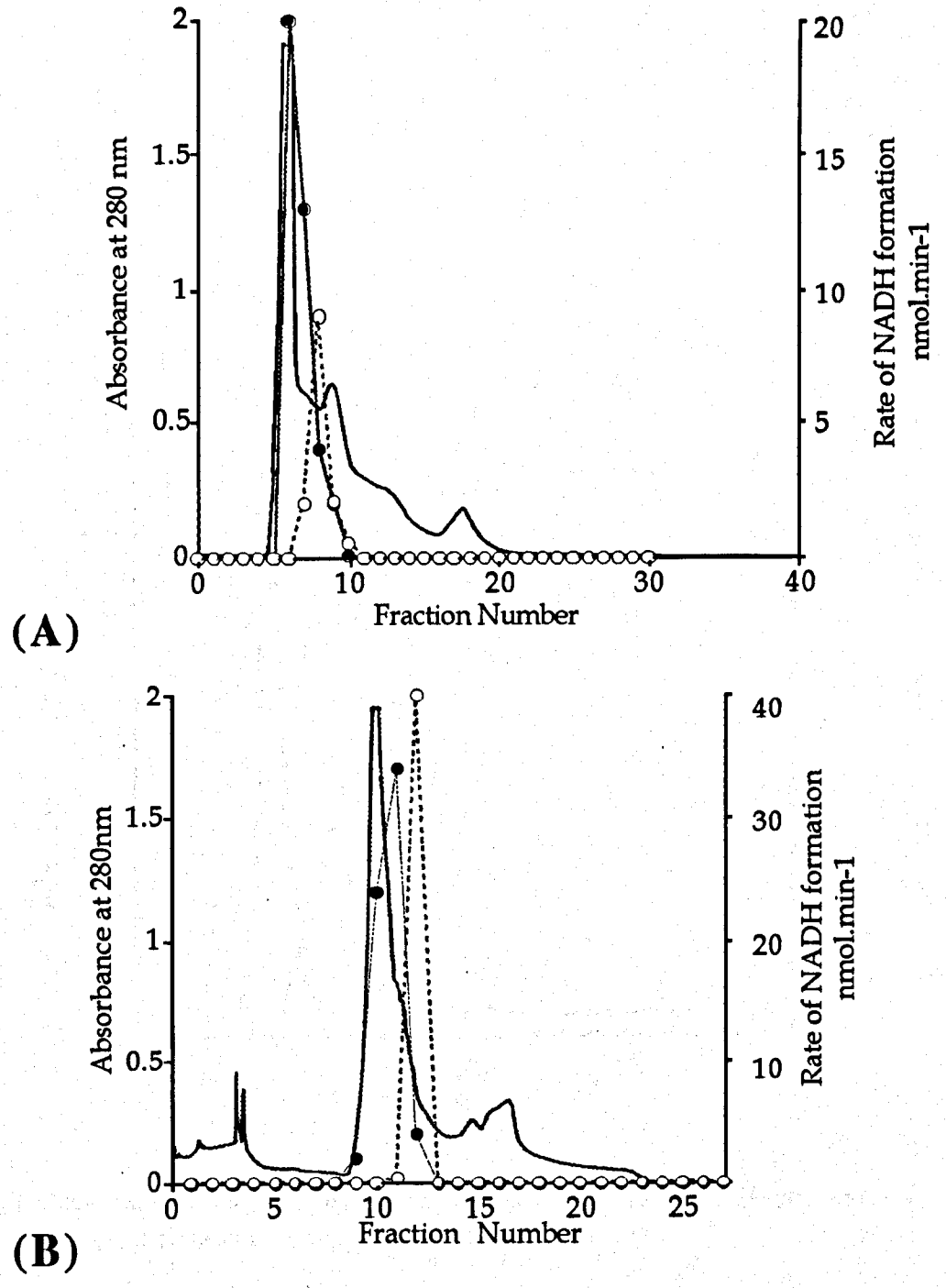


Figure 5.5 Chromatograms from the first 2 purification steps of protein F dependent FDH from calf liver GDH.

○ - FDH activity, ● - GDH activity, ----- Absorbance at 280 nm.

(A) Superdex 200 pg gel filtration chromatography.

(B) Mono Q ion exchange chromatography.

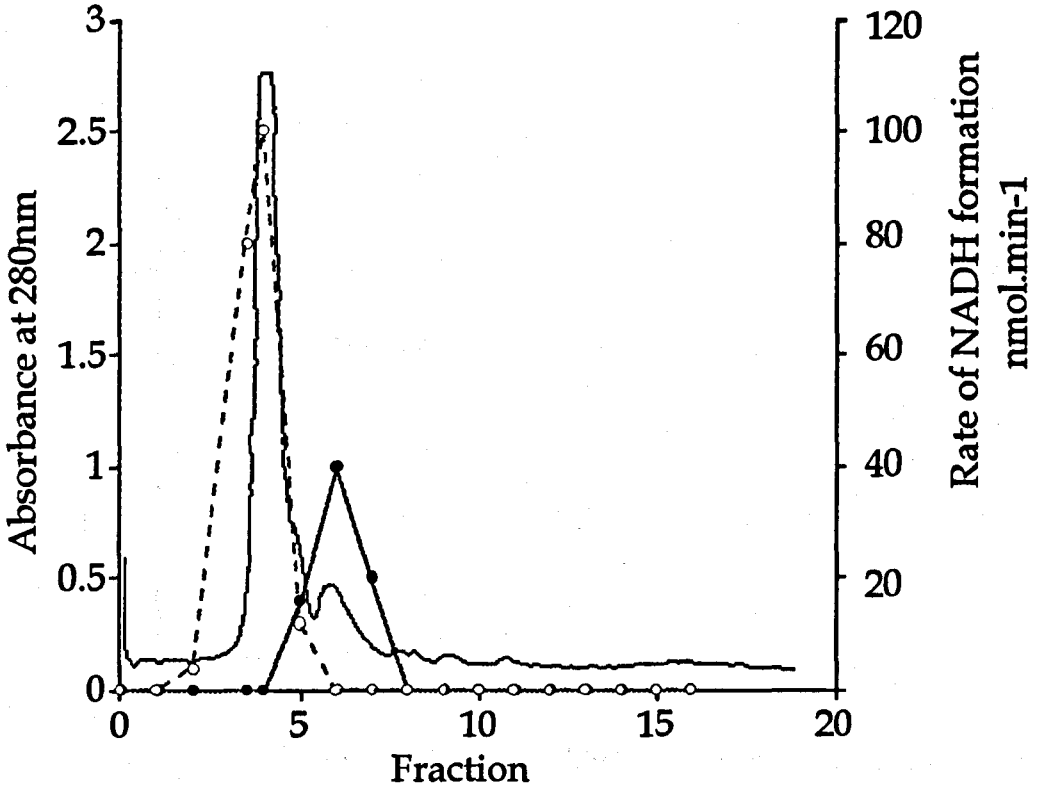


Figure 5.6 Chromatogram of the protein F dependent FDH separated from GDH on a Superose 12 gel filtration column.

O - FDH activity, ● - GDH activity, ----- Absorbance at 280 nm.

The molecular weight of the FDH enzyme isolated from the GDH preparation was determined to be 75 kDa by SDS-PAGE (Figure 5.4) and 80 kDa ± 15 kDa by gel filtration (Figure 5.7). This indicates that this formaldehyde dehydrogenase is a single component enzyme with a molecular weight of approximately 80 kDa.

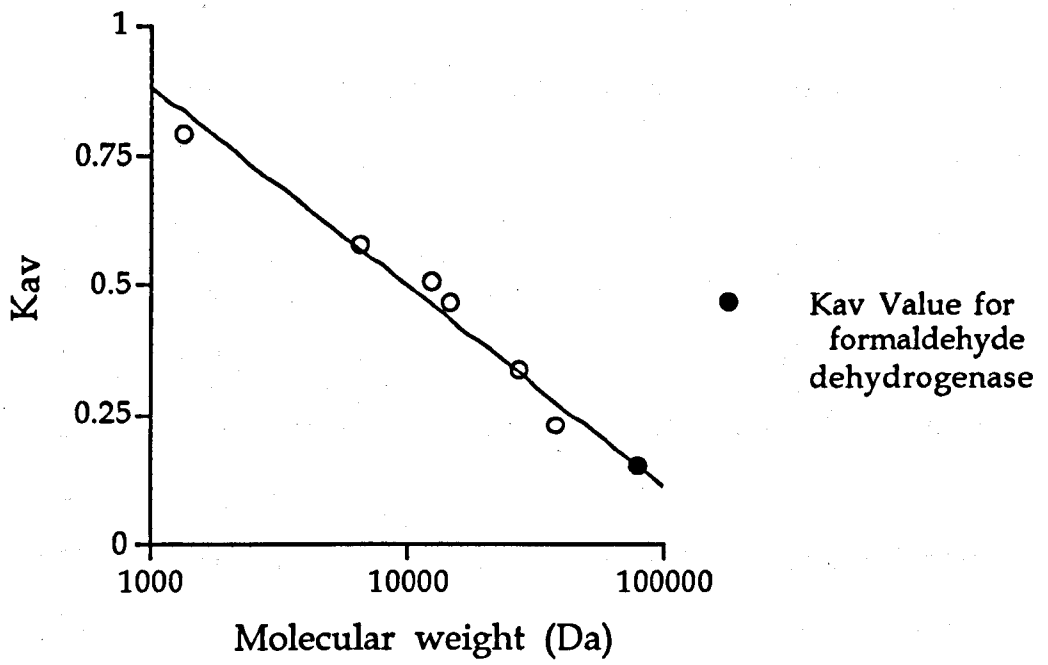


Figure 5.7 Molecular weight estimation of the FDH purified from calf liver GDH.

Molecular weight markers used included, vitamin B12 (1.5 kDa), aprotinin (6.5 kDa), cytochrome C (14 kDa), Soya bean trypsin inhibitor (27 kDa), carbonic anhydrase (36.8 kDa) and bovine serum albumin (63 kDa). V_o (Exclusion volume) was calculated by blue dextran, and V_t (Total elution volume) was calculated by acetone, K_{av} was determined as $K_{av} = \frac{V_e - V_o}{V_t - V_o}$ where V_e is the elution volume of each standard and the unknown.

5.2.3 Substrate specificity of FDH purified from a GDH preparation.

The substrate specificity of the FDH purified from commercial GDH was determined in both the presence and absence of protein F using a range of aliphatic aldehydes (C_1-C_4) and alcohols (C_1 - C_3) (Table 5.3). As shown in Table 5.3 the function of this enzyme is modulated by protein F and formaldehyde oxidation occurs only in the presence of protein F. This enzyme is clearly unable to catalyse the oxidation of alcohols, or higher aldehydes, other than acetaldehyde.

Replacement of NAD^+ with 2,6-dichlorophenolindol/phenazine methosulphate was attempted, but no oxidation could be detected when these were included in the reaction mixture, with either formaldehyde or acetaldehyde as substrate. Glutathione was added to the reaction mixture up to a concentration of 2 M to see whether formaldehyde oxidation could be stimulated in the absence of protein F. This was unsuccessful and no formaldehyde oxidation was detected. The stoichiometry of formaldehyde oxidation was also determined. The oxidation of 200 μmol of formaldehyde in the presence of 2 mM of NAD^+ and 4.7 μM of protein F, produced 180 μmol of formate and 165 μmol of NADH. The reverse reaction, which would result in the formation of NAD^+, did not occur when up to 20 mM sodium acetate or sodium formate was added to the reaction mixture with NADH as the electron donor.

Table 5.3 Substrate specificity of protein F dependent FDH purified from commercial calf liver GDH.

Substrate	% Activity	
	- Protein F	+ Protein F
Formaldehyde	1.8	100
Acetaldehyde	178	55
Propanal	0	0
Butanal	0	0
Methanol	0	0
Ethanol	0	0
Propan-1-ol	0	0
β-D-Glucose	0	0

All reactions were performed as described in Section 5.2.1 except that reactions were initiated by the addition of the substrate being studied to a final concentration of 2 mM. 100% activity is the rate of formaldehyde oxidation by 7.5 mU of purified enzyme and the absence of a rate of glucose oxidation was used as a control

5.3 Discussion.

The results demonstrate that protein F affects both the substrate specificity and relative activities of some commercially available dehydrogenases. Of the four enzymes examined only G3PDH showed no effect when protein F was added to the reaction mixture (Table 5.1). The lack an effect on G3PDH is probably due to a difference in the size and nature of the substrate of FDH and G3PDH enzymes. It is also likely that the mechanism of G3PDH (Segal & Boyer, 1953, Harris & Waters, 1976) is very different from mFDH.

The enhancement of alcohol dehydrogenase (ADH) activity by adding protein F (Table 5.1), is interesting mechanistically. Since protein F probably alters the substrate specificity of mFDH by causing a conformational change (Chapter 4.0), it is possible that protein F brings about a conformational change in ADH, which results in the increase in the activity demonstrated. It is known that alteration of either the NAD^+ pyridinium C3 atom substituent (Figure 5.8) or its location in the cofactor binding cleft of ADH is important in the reaction mechanism (Samana *et al.*, 1986).

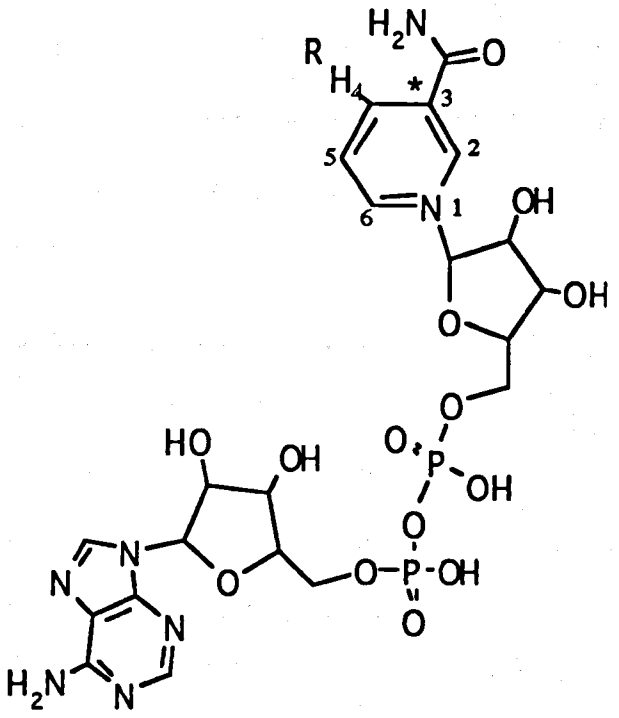


Figure 5.8 Structure of NAD^+.

The pyridinium ring is at the top of the structure and the C3 amide group (*) and reactive centre (R) are labelled.

The replacement of the C3 amide in NAD^+ with a hydrophobic group, e.g. benzoyl, enhances the activity of horse liver ADH by both increasing the hydrophobicity of the pyridinium ring and by moving NAD^+ closer to the substrate (Samana *et al.*, 1986). Using 3-acetylpyridine adenine dinucleotide as electron acceptor and ethanol as substrate, the first order rate constant for the reaction increased to 300 s^{-1}, compared to 130 s^{-1} for NAD^+ and ethanol (Shore *et al.*, 1974). It is therefore proposed that the association of protein F with ADH alters the structure of the enzyme sufficiently to reposition the C3 pyridinium atom into a more hydrophobic region of the active site. This would result in a more hydrophobic environment for the C3 amide group, which could increase the rate of alcohol oxidation catalysed by ADH.

Both samples of GDH investigated show FDH activity in the presence of protein F (Section 5.2.1). The calf liver material showed a higher level of activity than

the *B. megaterium* and this could to be due to the relative quantities of contaminating material in each sample. Only after purification of the formaldehyde oxidising enzyme from the calf liver sample was the catalyst found not to be GDH itself. It is therefore possible that a mFDH is also present in *B. megaterium.*

The identification of a mFDH type enzyme in a sample of calf liver GDH and its subsequent purification, has potentially important implications for metabolic control in the liver. Many compounds are inactivated or removed from the body via the liver. Some are excreted in bile, while in other cases the metabolised compound passes via the blood to the kidneys for excretion. This detoxification role of the liver invariably involves enzyme catalysed chemical change in the toxic compound, which renders it inert, stable and water soluble (for full reviews see Moffat & Mottram, 1978 and Tortora & Anagnostakos, 1987). One important function of the liver is the processing of alcohol and its metabolites. There is much interest in the control and expression of aldehyde dehydrogenase and ADH enzymes in various human tissues in an attempt to determine whether there is a link between ADH expression level and susceptibility towards alcoholism (Ohmori *et al.*, 1986; Thomasson *et al.*, 1993). The presence of an enzyme which requires a modifier protein for the oxidation of aldehydes to occur would allow a high degree of control over the metabolic process since aldehyde dehydrogenase is a key step in the metabolism of alcohol in the liver.

Aldehyde dehydrogenases have been purified from both mitochondrial and cytosolic liver cell preparations (Hart & Dickinson, 1978; Allanson & Dickinson, 1984). The native molecular weight (80 kDa) of the enzyme purified in this study is very different from those found in liver tissue (Allanson & Dickinson, 1984; Dickenson, 1988). The kinetic behaviour of the enzyme purified from calf liver is similar to the cytosolic form of aldehyde dehydrogenase (Allanson & Dickinson, 1984). Aldehyde dehydrogenase enzymes isolated from liver cell mitochondria show an enzyme concentration dependent lag phase (Hart & Dickinson, 1978; Allanson &

Dickinson, 1984). This lag phase can be eliminated by increasing the ionic strength of the mitochondrial aldehyde dehydrogenase enzyme reaction mixture (Takahashi & Weiner, 1980; Vallar & Pietruszko, 1984). The ionic strength of the buffer used in the enzyme reaction mixtures in this study was similar to those used by Hart & Dickinson (1978) and therefore the enzyme concentration lag phase should have been evident. Since no such lag phase was observed during this study it is proposed that this is a cytosolic aldehyde dehydrogenase and not a mitochondrial isoform.

The FDH enzyme purified from calf liver has a very narrow substrate specificity and in the absence of protein F only acetaldehyde oxidation was observed. However in the presence of protein F, acetaldehyde oxidation was not completely inhibited, as was observed with the mFDH isolated from *M. capsulatus* (Bath) (Chapter 4). The product of the formaldehyde oxidation reaction was identified as formate and the stoichiometery indicated that the enzyme was a dehydrogenase and not a dismutase.

The inability of this FDH to oxidise 1° alcohols indicates that it is different from the type III ADH or glutathione (GSH) dependent formaldehyde dehydrogenases (gFDH) previously isolated from liver. The latter of these dehydrogenases can only catalyse formaldehyde oxidation in the presence of GSH which when mixed with formaldehyde spontaneously forms S-(hydroxymethyl)-glutathione (Holmquist & Vallee, 1991). Recently, gFDH enzymes isolated from methylotrophic bacteria, have been shown to be similar to type III ADH enzymes from mammalian tissue (Guthiel *et al.*, 1992; Speer *et al.*, 1994). Since one important function of the liver in mammals is to remove toxic compounds utilising GSH, the use of GSH in aldehyde and specifically in formaldehyde oxidation would remove GSH which is present in concentrations of 0.1 - 10 mM in most mammalian cells (Meister, 1995).

The use of GSH as an antioxidant and for the removal of free radicals for subsequent excretion has been reviewed recently, as has its roles in cellular metabolism

(Meister, 1995). The presence of a modifier protein controlled enzyme for aldehyde/alcohol detoxification could play an important role in reducing the use of GSH thus maintaining levels of this molecule for other detoxification reactions. The presence of this type of control protein in eukaryotes cells, except α-lactalbumin (discussed in Chapter 1) has not been reported. The isolation of the FDH protein from a sample of calf liver suggests that there may be additional control proteins which help to regulate metabolic processes in mammalian cells.

6. Purification of a second NAD^+-linked formaldehyde dehydrogenase from *Methylococcus capsulatus* (Bath).

6.1 Introduction.

The formaldehyde dehydrogenase (FDH) enzymes isolated from methylotrophs thus far can be conveniently divided into 2 broad groups depending upon their electron acceptor: the dye-linked FDH (dFDH) enzymes and NAD^+ -dependent FDH enzymes. The dFDH enzymes are so called because FDH activity can be determined in cell free extract by the addition of artificial electron acceptors, such as 2,6-dichlorophenolindophenol (DCPIP) and or cytochromes. It is assumed that these directly reduce with a cytochrome *in vivo*, leading to direct ATP synthesis via the electron chain. dFDH's are not generally specific to formaldehyde and have a broad substrate specificity (Marrison & Attwood, 1980). A dFDH has been described in *Methylococcus capsulatus* (Bath) which was found to be present in soluble extracts at low levels, using an enzyme assay in the absence of ammonium ions (Hay, 1990).

The NAD+-linked enzymes can also be divided into two groups depending on their requirement for an extra cofactor for formaldehyde oxidation. NAD^+-linked FDH (nFDH) enzymes which do not require an extra cofactor have been reported from methylotrophs and non -methylotrophs (Attwood *et al.*, 1992; Poels & Duine, 1989; Ando *et al.*, 1978). FDH enzymes which require extract cofactors are either NAD^+-linked glutathione dependent formaldehyde dehydrogenases (gFDH) (van Ophem *et al.*, 1992; Eggeling & Sahm, 1984) or modifier protein dependent formaldehyde dehydrogenases (mFDH) (Stirling & Dalton, 1978 and Chapters 3 and 4 of this study).

Three enzymes have been assumed to be responsible for formaldehyde oxidation in *M. capsulatus* (Bath); the modifier protein dependent FDH (mFDH),

methanol dehydrogenase and a dFDH. It has been postulated that enzymes important in the oxidative RuMP pathway are also present in *M. capsulatus*(Bath) as previously described (Chapter 1, Section 1.2.2). The respective role of each enzyme and the RuMP pathway in the general oxidation of formaldehyde, to generate a reduced compound in *M. capsulatus*(Bath) is unknown.

The characterisation of two distinct FDH's isolated from a single organism under the same growth conditions has not been reported. The results presented in this chapter characterise a protein F independent formaldehyde dehydrogenase (identified in Chapter 3). This nFDH enzyme was identified in soluble extracts of *M. capsulatus* (Bath) but dismissed and the activity assumed to be that of mFDH which had protein F associated. The activity of this nFDH was initially masked during the purification of mFDH as both co-purified with the hydroxylase component of soluble methane monooxygenase (sMMO) (Chapter 3, Section 3.3.1). After isolation of mFDH the activity of nFDH became apparent and the enzyme responsible was partially purified and characterised. The enzyme was investigated as it may help in the understanding of the role of mFDH in the metabolism of formaldehyde in *M. capsulatus* (Bath).

6.2 Experimental.

6.2.1 Determination of formaldehyde oxidation activity.

The rate of formaldehyde oxidation catalysed by the purified nFDH was determined as described in Chapter 2 (Section 2.3.7) except that protein F was omitted from the reaction mixture. Each reaction mixture contained 2 mM formaldehyde, 2 mM NAD^+, 0.5 mg protein and 25 mM phosphate buffer pH 7.2 made to a final volume of 1 ml with water.

6.2.2 Partial purification of NAD^+ dependent FDH.

The nFDH enzyme was partially purified in a three step procedure.

Step 1: Ammonium sulphate precipitation.

Crude extract was saturated to 30 % (w/v) with solid ammonium sulphate and allowed to stand on ice for 30 minutes. Precipitated material was removed by centrifugation at 50,000 xg for 20 minutes. The supernatant was then saturated to 55 % (w/v) solid ammonium sulphate and the pellet redissolved in a minimum volume of 20 mM Tris - HCl, pH 7.2. Formaldehyde oxidation activity was determined in the redissolved pellet before proceeding to step 2.

Step 2: Gel filtration chromatography.

The redissolved pellet was loaded onto a Superdex 200 pg gel filtration column equilibrated with 20 mM Tris - HCl , pH 7.2 containing 50 mM KCl. The protein was eluted at a flow rate of 4 ml/min in the same buffer and 4 ml fractions were collected.

The fractions were assayed for FDH activity and those that showed activity were pooled and concentrated in an Amicon ultrafiltration unit over a 30 kDa membrane.

Step 3: Ion exchange chromatography.

The concentrated active protein from the gel filtration column was loaded onto a Q - Sepharose ion exchange column equilibrated with 20 mM Tris - HCl, pH 7.2. After sample loading the column was washed for 2 column volumes (CV) with equilibration buffer. The concentration of KCl was then increased to 0.3 M in 0.25 CV before a linear gradient of 0.3 - 0.5 M, over 3 CV, was used to separate the nFDH protein. After the gradient elution the column was regenerated to remove bound protein by increasing the concentration of KCl to 1 M for 0.5 CV. Fractions of 8 ml were collected and tested for FDH activity. Those which showed activity were pooled and concentrated over a 30 kDa Amicon ultrafiltration membrane and stored at -80 °C.

6.3 Results.

6.3.1 Purification and molecular weight estimation.

A summary of the purification of nFDH is shown in Table 6.1.

Table 6.1 Purification of nFDH.

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity [mU. (mg protein)$^{-1}$]	Yield (%)	Purification Factor
Crude Extract	325	13,650	53.6	3.9	100	1
Ammonium Sulphate Precipitation	80	7500	22.8	3.0	43	0.77
Gel Filtration, Superdex 200 pg	70	748	19.6	26.2	36	6.7
Ion Exchange, Q - Sepharose.	25	105	11.5	109	22	27.9

The data above indicate that the ammonium sulphate precipitation step was inefficient in this purification method as nFDH activity decreased significantly.

Table 6.1 also shows a purification factor of 27.9, though analysis of the sample by SDS-PAGE showed that more than one protein remained in the purified preparation (Figure 6.1). The major species in the sample was a protein of approximately 30 kDa

with contaminating bands at 60, 40 and 20 kDa. The contaminating protein was presumed to be the hydroxylase component of soluble methane monooxygenase (sMMO) and this was confirmed by the standard sMMO assay. Attempts were made to remove the hydroxylase from the nFDH sample by further chromatography but these were unsuccessful.

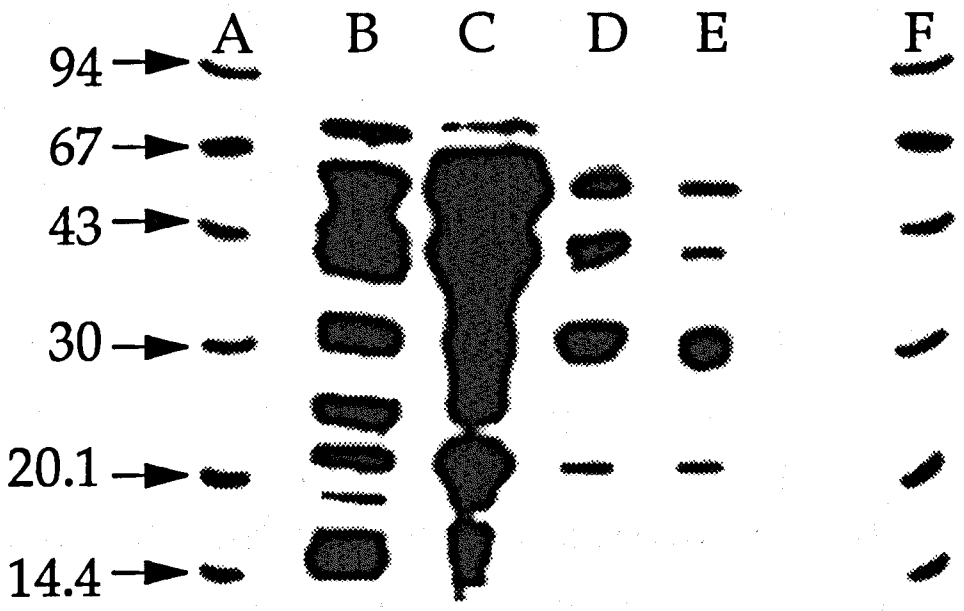


Figure 6.1 12.5% SDS - PAGE of nFDH.

Lanes A and F. Pharmacia low molecular weight markers;

Lane B. Soluble extract;

Lane C. Ammonium sulphate precipitated material;

Lane D. Superdex 200 pg purified nFDH;

Lane E. Q-Sepharose ion exchange purified nFDH.

The subunit molecular weight of nFDH was estimated as 30 ± 10 kDa by SDS - PAGE by comparing the relative mobility of nFDH with known molecular weight

markers (Figure 6.1). The native molecular weight of nFDH was determined by PAGE as 100 ± 20 kDa. This indicates that this enzyme is probably a trimer similar to FDH enzymes isolated from *Rhodococcus erythropolis* (van Ophem *et al.*, 1992). The molecular weight of nFDH was also determined by gel filtration but this method gave a value of 250 kDa. This high native molecular weight estimate may be the result of the association of nFDH with the sMMO hydroxylase. The molecular weight data therefore indicate that nFDH and mFDH are different species.

6.3.2 Substrate specificity of nFDH.

The oxidation rates by nFDH of a range of simple aldehydes, including formaldehyde, acetaldehyde, propanal, butanal, pentanal, two higher aldehydes and two alcohols were determined (Table 6.2). All enzyme assays were performed as described in the experimental section (Section 6.2.1). As the results in Table 6.2 show, nFDH does not catalyse the oxidation of aliphatic aldehydes which have more than four carbon atoms.

The data in Table 6.2 and Table 4.1 demonstrate that nFDH differs from mFDH in that the former catalyses the oxidation of both glyceraldehyde and benzaldehyde. In addition the rate for formaldehyde oxidation is a minimum of 20 % lower than the rate of oxidation for other aliphatic aldehydes, with propanal having the highest oxidation rate catalysed by nFDH.

The products of formaldehyde and acetaldehyde oxidation were identified as formate and acetate respectively using the HPLC assay described above (Chapter 2, Section 2.3.2). The stoichiometry of formaldehyde and acetaldehyde oxidation was determined by the addition of either 2 mM formaldehyde or 2 mM acetaldehyde to the enzyme reaction mixture and the amount of products formed determined. 1.4 mM

formate and 1.7 mM acetate were recovered from the reaction mixtures after 20 minutes demonstrating the stoichiometery of formaldehyde and acetaldehyde oxidation by nFDH is probably the same as that of mFDH (Chapter 4.0).

The requirement for NAD^+ as the nFDH electron acceptor was assessed by attempting to replace it with DCPIP or phenazine methosulphate (PMS) or a mixture of the two. The reduction of cytochrome C by nFDH was not attempted as neither of above compounds were reduced by nFDH. $NADP^+$ was not tested and this should be repeated using $NADP^+$ as the electron acceptor.

Table 6.2 Substrate specificity of nFDH.

Substrate	% Activity
Formaldehyde	100
Acetaldehyde	124
Propanal	421
Butanal	0
Pentanal	0
Benzaldehyde	21
Glyceraldehyde	122
Methanol	0
Ethanol	0

100 % activity is equal to 190 nmol NADH formed.min^{-1}.mg^{-1} nFDH with 2 mM formaldehyde as substrate. All substrates were tested at a concentration of 2 mM.

6.3.3 Determination of kinetic parameters (K_m, V_{max} and k_{cat}) for substrates oxidised by nFDH.

In an attempt to determine the possible role of nFDH in the metabolism of *M. capsulatus* (Bath) it was important to calculate K_m, V_{max} and k_{cat} values for each substrate whose oxidation was catalysed by nFDH (Table 6.3). The effect of substrate concentration on the rate of NADH formation was determined for each substrate (Figure 6.2). As shown in Figure 6.2 all the substrates exhibited michaelis menten kinetics. The data from the plots in Figure 6.2 were used to calculate K_m and V_{max} using double reciprocal plots and both k_{cat} and the specificity constant were calculated from this data. The results are shown in Table 6.3.

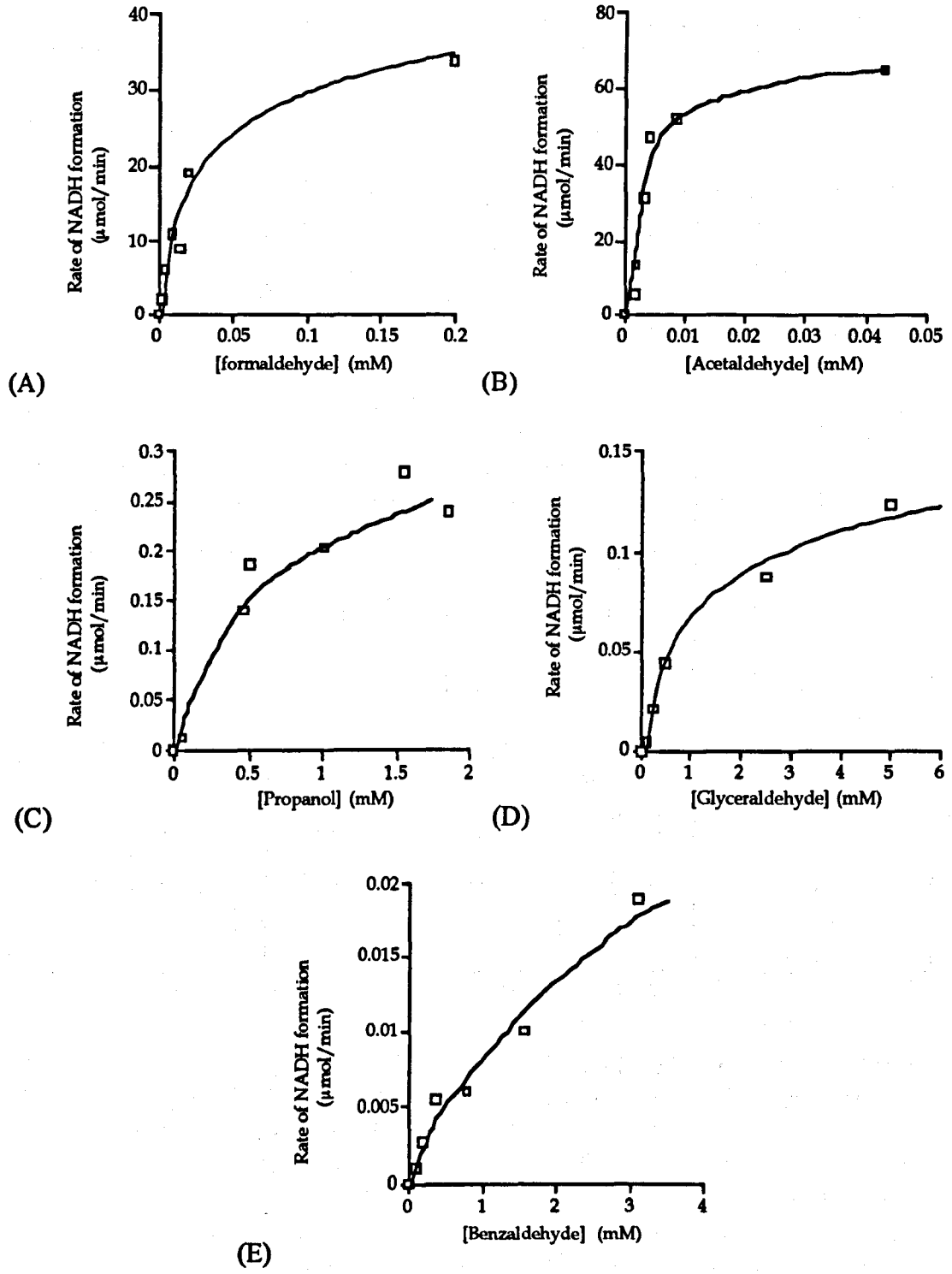


Figure 6.2 Rate (v) vs. [S] plots for substrates oxidised by nFDH. Each graph shows the line of best fit using the Michaelis Menten equation to the reactions data.

(A) oxidation of formaldehyde by nFDH,
(B) oxidation of acetaldehyde by nFDH,
(C) oxidation of propanal by nFDH,
(D) oxidation of glyceraldehyde by nFDH and
(E) oxidation of benzaldehyde by nFDH.

Table 6.3 Kinetic constants for substrates whose oxidation is catalysed by nFDH.

Substrate	K_m (mM)	V_{max} (μmol/min)	k_{cat} (min^{-1})	Specificity Constant ($min^{-1}.mM^{-1}$)
Formaldehyde	0.002	0.021	10.5	5250
Acetaldehyde	0.001	0.040	40.0	40000
Propanal	0.524	0.328	0.626	1.2
Benzaldehyde	2.36	0.029	0.012	.005
Glyceraldehyde	1.74	0.174	0.10	.06

All reactions were performed with 0.4 mg/ml nFDH and the kinetic constants were calculated using the Lineweaver-Burk plots. K_{cat} is defined as V_{max}/K_m and the specificity constant is defined as k_{cat}/K_m.

From the kinetic constants shown in Table 6.3 acetaldehyde would appear to be the 'preferred' substrate as it has the highest specificity constant. The data in Table 6.2 showed that propanal was oxidised four times faster than formaldehyde, yet Table 6.3 shows that the specificity constant for propanal is 4000 fold lower than for formaldehyde.

6.3.4 Effect of compounds known to stimulate formaldehyde oxidation.

The activity of nFDH in the presence and absence of compounds which are known to stimulate other FDH's was investigated. Purified protein F (up to 9.4 μM) was added to the reaction mixture to determine its effect on the rates of formaldehyde and acetaldehyde oxidation by nFDH. At all concentrations of protein F, no change in the rates of formaldehyde or acetaldehyde oxidation, catalysed by nFDH, was observed.

The effects of glutathione and methanol (0.1 - 2.0 mM) on the rate of formaldehyde oxidation was also determined by their addition to nFDH reaction mixtures. As with protein F no increase in the rate of formaldehyde oxidation was observed.

If a loosely bound cofactor were associated with nFDH, the effect of exogenous stimulants might be negated. To remove any such cofactor nFDH was dialysed against 20 mM phosphate buffer , pH 7.2 overnight. The rate of formaldehyde oxidation by this nFDH was similar to that prior to dialysis (50 nmol NADH formed.min^{-1} *vs.* 43 nmol NADH formed.min^{-1}).

The absorption spectrum of nFDH exhibited a single absorbance maximum at 280 nm with no indication of the presence of a bound chromophore. The fact that dialysis did not alter nFDH activity and the absence of an additional chromophore in the

UV-visible spectrum indicate that there was no non-protein cofactor bound to the enzyme. A protein cofactor may have been visible on SDS-PAGE (Figure 6.2), but due to the presence of more than one protein in the preparation a protein cofactor can not be discounted.

6.4 Discussion.

FDH's are vital to the metabolism of single carbon compounds in methylotrophs as all their primary growth compounds (methane, methanol, methylamines etc.) use formaldehyde as an intermediate in their oxidation (Anthony, 1982). FDH enzymes have been isolated from a range of methylotrophic organisms and can be broadly grouped by the nature of their electron acceptors, either dyes or NAD^+.

dFDH enzymes are assumed to have cytochrome electron acceptors *in vivo* (Marrison & Attwood, 1980). These enzymes are identified by their ability to reduce DCPIP/PMS in a reaction mixture when formaldehyde is added as substrate. dFDH enzymes do not catalyse oxidation of formaldehyde in the presence of NAD^+ (Marrison & Attwood, 1980). Such enzymes have been identified in *Hyphomicrobium zavarzinii*, (Klein *et al.*, 1994) *Hyphomicrobium X, Pesudomonas* AM1 and *Hyphomicrobium vulgae 3* (Marrison & Attwood, 1980) and *M. capsulatus* (Bath) (Hay, 1990).

The NAD+-linked FDH enzymes isolated from microorganisms can be further divided by the requirement of additional factors for formaldehyde oxidation. Enzymes which have been shown to require additional small cofactors for the oxidation of formaldehyde include mFDH, isolated from *M. capsulatus* (Bath) (Stirling & Dalton, 1978, Chapter 3 this study) and gFDH enzymes, isolated from *Amycolatopsis methanolic* (van Ophem *et al.*, 1992) and *Rhodococcus erythropolis* (Eggeling & Sahm, 1984; Eggeling & Sahm, 1985). The gFDH enzymes require glutathione (GSH)

for formaldehyde oxidation to occur. These gFDH enzymes are similar to Type III alcohol dehydrogenase (ADH) enzymes isolated from mammalian tissues (Speer *et al.*, 1994).

NAD+-linked FDH (nFDH) enzymes which do not require an additional cofactor have been isolated from the methylotrophic bacteria *Arthobacter* P1 (Attwood *et al.*, 1992), *Hyphomicrobium* X (Poels & Duine, 1989), *Methylobacter marinus* A45 (Speer *et al.*, 1994). An enzyme isolated from *Pseudomonas putida* C83 (Ando *et al.*, 1979) is commercially available from Sigma (see Table 6.4).

The nFDH isolated here from *M. capsulatus* (Bath) is quite different from the mFDH enzyme described in Chapter 3.0. nFDH is an aldehyde dehydrogenase with a narrow substrate specificity and does not catalyse the oxidation of alcohols. nFDH appears to be similar to the FDH enzyme purified from *Pseudomonas putida* C-83 (Ando *et al.*, 1979) (Table 6.4) and not FDH enzymes which require a second cofactor for the catalysis of formaldehyde oxidation.

Table 6.4 Comparison of the properties of some NAD^+-linked FDH enzymes.

Source.					
Property	*Arthobacter* P1	*P. putida* C - 83	*nFDH*	*mFDH*	*A. methanolica*
Molecular Weight					
Mr (native)	115,000	150,000	100,000	250,000	120,000
Mr (subunit)	56,000	75,000	30,000	63,600	40,000
K_m *(mM)*					
Formaldehyde	0.1	0.067	0.002	ND	9.6
NAD^+	0.8	0.056	12.7	ND	ND
V_{max} *(µmol/min/mg protein)*					
Formaldehyde	0.70	10	0.021	ND	0.030
NAD^+	0.42	ND	0.36	ND	ND
General Data					
Substrate specificity	Formaldehyde	Formaldehyde	Formaldehyde and a narrow range of aldehydes	Formaldehyde and a narrow range of aldehydes and alcohols	Formaldehyde only and a range of alcohols
Addition of reducing reagents	stimulates activity	no effect	no effect	no effect	Required for FDH activity.

Enzymes from *Arthobacter* P1 (Attwood *et al.*, 1992), *P. putida* C - 83 (Ando *et al.*, 1979) and nFDH (this study) are NAD+-linked factor-independent FDH enzymes.

The enzyme from *A. methanolica* (van Ophem *et al.*, 1992) and mFDH (Chapters 3 and 4) are both NAD^+-linked factor dependent FDH enzymes (ND = not determined)

In organisms from which a factor dependent FDH has been isolated, a second NAD^+ -dependent enzyme has not been found although there are believed to be two NAD^+-linked FDH's in *Hyphomicrobium X*, although it is not known whether either is a factor dependent FDH (Poels & Duine, 1989). The results in this study show that in *M. capsulatus* (Bath) there is strong evidence for two distinct NAD^+-linked FDH's namely mFDH and nFDH.

The data in Table 6.4 show that nFDH has a molecular weight similar to the NAD^+-linked, GSH - dependent FDH isolated from *A. methanolica* (van Ophem *et al.*, 1992). Its molecular weight is not similar to the mFDH isolated from *M. capsulatus* (Bath) which has a native molecular mass of 250,000 kDa and a subunit mass of 63,600 kDa (Chapter 3, Section 3.4.1). The data indicate that nFDH is a factor independent FDH since attempts to stimulate its activity with protein F, methanol or glutathione were unsuccessful and dialysis of the nFDH preparation failed to remove any loosely bound cofactor which may have been present after purification. Therefore, nFDH is dissimilar to the FDH enzymes isolated from *A. methanolica*, *R. erythropolis* and mFDH from *M. capsulatus* (Bath) in which dialysis of crude preparations of these enzymes causes loss of formaldehyde oxidation activity (van Ophem *et al.*, 1992; Eggeling & Sahm, 1984; Eggeling & Sahm, 1985; Stirling & Dalton, 1978).

The oxidation of formaldehyde catalysed by nFDH followed michaelis menten kinetics, unlike mFDH for which sigmoidal kinetics were observed (Chapter 4, Section 4.2.5). This could indicate separate roles for mFDH and nFDH in the metabolism of single carbon compounds in *M. capsulatus* (Bath). It was shown that nFDH has a high affinity for both formaldehyde and acetaldehyde and the K_m values measured here are an order of magnitude lower than other values reported in literature for nFDH enzymes (Attwood *et al.*, 1992; Ando *et al.*, 1979). The k_{cat} values for the oxidised substrates indicate that nFDH is a non-specific aldehyde dehydrogenase which is capable of catalysing the oxidation of aliphatic aldehydes with fewer than four carbon atoms and

also of aldehydes such as benzaldehdye and glyceraldehyde. In comparison to mFDH, nFDH appears to oxidise acetaldehyde better than formaldehyde although it is not possible to calculate the specificity constant of formaldehyde for mFDH from the data available (Chapter 4) so a comparison with the specificity constant for nFDH can not be made.

In conclusion the physiological significance of the presence of two NAD^+-linked FDH in *M. capsulatus* (Bath), which appear to be simultaneously expressed, is unknown. Although nFDH catalyses the oxidation of more complex aldehydes such as benzaldehdye and glyceraldehyde, it must catalyse formaldehyde oxidation *in vivo* because of its high specificity constant in comparison to the values for other substrates. It was proposed in Chapter 4 that the role of mFDH was possibly two fold:

(1) A 'safety system' to remove toxic levels of formaldehyde *in vivo.*

(2) A system to generate NADH for methane and carbon assimilation into the cell biomass (Chapter 4, Section 4.4).

As nFDH exhibits Michaelis Menten kinetics and has no requirement for an additional cofactor for formaldehyde oxidation it is likely that its role *in vivo* is to generate NADH for the oxidation of methane and/or assimilation of formaldehyde into the biomass.

7. General discussion.

The initial aims of this study were to isolate and characterise the two component FDH from *Methylococcus capsulatus* (Bath). The purification of this two component mFDH initially proved difficult although the method finally chosen (Chapter 3, Section 3.2.2) allowed the purification of large quantities of mFDH and protein F. However, the inability to purify protein F from soluble extract without heat treatment gives rise to some concern. The heat treatment step in protein F purification questions the validity of protein F suggesting it may be an artefact of heating. It has been demonstrated that dialysis of crude extract removes mFDH activity. This activity can only be restored by the addition of protein F thus indicating that protein F is present in soluble extract (Hay, 1990; Stirling & Dalton, 1978). It could therefore be concluded that protein F exists in an inactive form *in vivo* and that heat treatment of soluble extract is required to activate protein F. The precise nature of the inactive molecule is unknown and the lack of any other chromophoric group present in purified protein F could indicate that heat treatment removes an unknown cofactor which activates protein F. Alternatively the inactivity could be due to the blocking or modification of an amino acid which is reversed by the heat treatment.

Analysis of the deduced N-terminal sequence for both protein F and mFDH did not identify any similar proteins in the SWISS-PROT and EMBL databases. This was unsurprising due to the unique properties of this mFDH enzyme (Chapter 3 Section 3.4.3). Identification and isolation of the genes encoding mFDH and protein F were not attempted in this study though would prove useful in future for 3 reasons:

1) Sequencing of the genes responsible would allow complete determination of the primary structure of these proteins. This structure could then be used to initiate a

better study of the protein and DNA databases to identify other proteins similar to protein F and mFDH.

2) Construction of a clone for each protein would allow over expression and the increased quantity of the protein would ease the purification of the protein components. Protein engineering could also be performed to add a specific affinity tag to each protein which can aid in single step purification.

3) The active region of protein F and mFDH could be mapped using site directed mutagenesis. This would allow the mechanism of protein F interaction with mFDH to be characterised fully.

Whilst purifying the mFDH a second NAD^+ -linked FDH (nFDH) enzyme was identified (Chapter 3, Section 3.3.1). The data presented on this enzyme demonstrate that it is a factor independent FDH which is very sensitive to formaldehyde (Chapter 6, Section 6.3.3). Purification of nFDH was hindered by the co-purification of the hydroxylase component of sMMO (Chapter 6, Section 6.3.1). A role for sMMO in formaldehyde metabolism in *M. capsulatus* (Bath) was investigated because of the copurification of the enzyme activities. Investigation of NAD^+ -linked FDH catalytic activity by sMMO in the presence and the absence of protein F demonstrated that sMMO could not oxidise formaldehyde. Likewise the epoxidation of rate of sMMO was not affected by the addition of mFDH or HTSE (Appendix 3).

It is therefore concluded that even though the 2 enzyme activities co-purify, the mixture contains separate enzymes responsible for FDH and sMMO catalytic activity. The idea of a synergistic relationship between sMMO and FDH, with FDH supplying NADH for methane assimilation following its oxidation of formaldehyde, could be important in determining the role of both mFDH and nFDH. Further work should be undertaken to investigate the localisation of both sMMO and FDH enzymes in an

attempt to determine whether they associate *in vivo* thus supporting the synergistic relationship theory.

Kinetically mFDH is a unique enzyme, the control of mFDH catalytic activities displayed by protein F is very dramatic (Chapter 4, Section 4.2.4). The ability of mFDH to have both substrate specificity and kinetics altered by protein F demonstrate an important role for mFDH in formaldehyde metabolism. In the absence of protein F it is concluded that mFDH is a general aldehyde/alcohol dehydrogenase enzyme which obeys Michaelis Menten kinetics (Chapter 4, Section 4.2.4). It is also concluded that the association of protein F with mFDH induces a conformational change which inhibits the oxidation of substrates other than formaldehyde. It was demonstrated that the presence of protein F was not required for formaldehyde association and its presence did not inhibit the association of acetaldehyde with mFDH (Chapter 4, Section 4.3.2). It can therefore be concluded that the conformational change induced by protein F alters the substrate binding site allowing formaldehyde to orientate correctly within the active site so that oxidation can occur. In the presence of protein F the association of higher aldehydes, such as acetaldehyde, are incorrectly orientated within the active site of mFDH and inhibit their oxidation.

The role of FDH enzymes in single carbon metabolism is fundamental to the maintenance of correct NADH levels for further methane oxidation or assimilation of formaldehyde molecules to form metabolites (Anthony, 1982). The role of mFDH in formaldehyde oxidation in *M. capsulatus* (Bath) is difficult to hypothesise especially when two NAD^+ -linked FDH enzymes have now been demonstrated (Chapter 3, Section 3.3.1). The role of mFDH in the metabolism of formaldehyde was proposed to be for the generation of NADH for methane oxidation and carbon assimilation (Stirling & Dalton, 1978). The experimental evidence from this study demonstrates that mFDH with its unique protein control system, is more likely to play a role in cell stress conditions either by reducing the level of formaldehyde or by providing a higher amount

of NADH for metabolic pathways involved in cell repair. Therefore protein F is similar to those proteins in *E. coli* whose expression has been linked to heat shock (Ingraham *et al.*, 1983). The function of these heat shock response proteins is to aid in and control the repair of cellular damage. In the case of protein F it can be hypothesised that protein expression is in response to high formaldehyde concentrations which are toxic to the cell. By interacting with mFDH, protein F could remove the formaldehyde and produce NADH for cellular repair enzymes. Such a system would account for the sigmoidal kinetics of formaldehyde oxidation by mFDH as when the formaldehyde concentration falls below the toxic threshold the enzyme function would stop and waste of formaldehyde would be limited. A similar detoxification role has been proposed for Type III alcohol dehydrogenase enzymes isolated from animal liver tissue which require glutathione (GSH) to oxidise formaldehyde (NAD^+-linked, GSH - dependent FDH , gFDH) (Gutheil *et al.*, 1992; Fernandez *et al.*, 1995). The gFDH enzymes which have been isolated from methylotrophic bacteria are also believed to have a formaldehyde detoxification role *in vivo* (Gutheil *et al.*, 1992; van Ophem *et al.*, 1992). It is unknown whether GSH is present in *M. capsulatus* (Bath) so it is therefore likely that mFDH plays an equivalent role to the gFDH enzymes isolated from both animals and other methylotrophic bacteria.

As the function of mFDH would appear to be in detoxification of formaldehyde the role of nFDH is therefore proposed to be for the regeneration of NADH for methane oxidation and carbon assimilation. The NADH from the oxidation of formaldehyde could subsequently be used by sMMO for further methane assimilation or for the generation of cellular components. From the data nFDH would be ideally suited to this function with its low rate of formaldehyde oxidation and the high specificity constant formaldehyde (Chapter 6, Section 6.3.3). This means that formaldehyde would be oxidised at the same rate even at very low concentrations, unlike the oxidation of formaldehyde by mFDH where there is a greatly reduced rate of formaldehyde oxidation

at low formaldehyde concentrations. It is also possible that nFDH is associated with sMMO *in vivo* and could explain why they co-purify so tightly (Chapter 6). The association of nFDH with sMMO would allow a direct control between the two enzymes and therefore close regulating the rate of methane and formaldehyde oxidation by the level of NAD^+/NADH.

Identification of a mFDH type enzyme in calf liver glucose dehydrogenase preparation has important implications in metabolic control. There are no reported cases where a protein modulates enzyme function, except α-lactalbumin, and methods for metabolic control are based around covalent modification, allosteric interactions, product inhibition or substrate activation (Stryer, 1988, Conn *et al,* 1987). It has been demonstrated that protein F does not covalently modify mFDH as crude preparations which have been incubated with HTSE still loose mFDH catalytic activity after dialysis of the extract (Hay, 1990). Experimental evidence (Chapter 5) indicates the possibility of more proteins that are controlled by protein/protein interactions such as protein F : mFDH and α-lactalbumin : γ-galactosyl transferase. It could therefore be hypothesised that there are a range of modifier proteins which alter the function of metabolic enzymes thus controlling a range of cellular processes. The only reported example to date of a modifier protein in animals is α-lactalbumin. This small 16 kDa peptide is released in mammary glands of pregnant females. The peptide associates with γ - galactosyl transferase and switches the function of this enzyme to lactose synthesis (Hill & Brew, 1975).

Further extensive studies should be undertaken to investigate the real physiological role of protein F and mFDH. Now that N-terminal sequence data is available for both protein F and mFDH, construction of clones from the M. capsulatus (Bath) DNA will aid in both the biochemical and microbiological studies. DNA probes to mFDH and protein F would also allow other methylotrophic bacteria to be investigated to determine if they also contain a mFDH type of enzyme. The probes

could would also allow the investigation of animal tissues for protein F type molecules in an attempt to determine if modifier proteins are wide spread in nature or isolated to protein F and α-lactalbumin.

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Appendix 1.

Purification method 1.

Step 1: DEAE - cellulose.

DEAE - cellulose, pre-equilibrated with 20 mM phosphate buffer, pH 7.2, was slurried into crude extract in 2 g batches. The mixture was stirred and left to stand on ice for 10 minutes. A 1 ml fraction of the supernatant was analysed for FDH activity. This slurring with DEAE - cellulose was repeated until enzyme activity started to decrease.

Step 2: Gel permeation chromatography.

The supernatant was applied to a TSK G3000 SW gel permeation column in 2 ml aliquots and eluted at 4 ml/min, with 20 mM phosphate buffer, pH 7.2. Fractions were collected (4 ml) and formaldehyde oxidation activity assessed. Fractions that showed formaldehyde oxidation activity were pooled and concentrated on an Amicon 30 kDa membrane. The concentrated material was stored at -80 °C for further purification.

Step 3: Affinity chromatography.

Active material collected from the gel permeation column was applied to a 1 ml Cibacron blue F3G - A 2 - Sepharose CL - 6B column and eluted using a gradient of 0 - 10 mM NAD^+ in the same buffer. Fractions were collected and analysed for catalysis of formaldehyde oxidation activity.

Table A1.1 Purification of mFDH by method 1.

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity [mU. (mg protein)$^{-1}$]	Yield	Purification Factor
Crude Extract	50	375	31.125	83	100	1
DEAE - Cellulose	50	285	35.625	125	114	1.5
TSK G3000 SW	40	47.6	3.28	68	10	0.82
Reactive Blue Sepharose	40	0.3	0.72	2,400	2	28.9

Appendix 2

Purification method 2.

Step 1: DEAE - cellulose, as in method 1 (Appendix 1).

Step 2: Gel filtration chromatography.

The FDH active material was loaded on to a Pharmacia Superdex 200 pg column equilibrated with 20 mM phosphate buffer, pH 7.2, containing 10 % glycerol and eluted at 0.75 CV/hr in the same buffer. Four ml fractions were collected and catalysis of formaldehyde oxidation activity assessed; those which showed activity were pooled and concentrated using an Amicon ultrafiltration unit and a 30 kDa membrane.

Step 3: Hydrophobic interaction chromatography.

Ammonium sulphate, 1.7 M, was added to the FDH active material. This solution was then loaded on to a Pharmacia Phenyl Superose (HR 5/5) hydrophobic interaction column and eluted with a linear gradient of 1.7 - 0 M ammonium sulphate in 20 mM phosphate buffer, pH 7.2, containing 10 % glycerol. One ml fractions were collected and assayed for catalysis of formaldehyde oxidation activity. Fractions that showed activity were pooled and concentrated using an Amicon ultrafiltration unit and a 30 kDa membrane.

Step 4: Immobilised metal affinity chromatography.

Concentrated FDH active material was loaded on to an immobilised metal ion affinity column which had Cu^{II} ions bound to the column matrix. The sample was eluted with a linear gradient of 0 - 1.0 M imidazole in 20 mM phosphate buffer, pH 7.2, containing 10 % glycerol, at a flow rate of 0.1 CV/hr. Fractions of 1 ml were collected and analysed for FDH activity.

Table A2.1 Purification method 2.

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity [mU. (mg protein)-1]	Yield	Purification Factor
Crude Extract	10.0	240	1.350	5.6	100	1
DEAE - Cellulose	10.0	92	0.36	4.9	26.6	0.98
Superdex 200 pg	17.5	10.1	0.184	18.2	13.6	3.3
Phenyl Superose	12.0	3.6	0.168	46.6	12.4	8.3
IMAC - Cu^{2+}	11.0	0.16	0.110	687.5	8.1	123

Appendix 3

A3.1 Purification method 3.

Step 1: Ammonium sulphate precipitation.

Crude extract was saturated with 30 % (w/v) solid ammonium sulphate and allowed to stand on ice for 30 minutes. Precipitated material was removed by centrifugation at 48,500x g for 20 minutes. The supernatant was then saturated to 55 % (w/v) ammonium sulphate and the pellet resuspended in a minimum volume of 20 mM Hepes buffer, pH 7.2, containing 50 mM KCl and 1 mM benzamidine. Catalysis of formaldehyde oxidation was assayed in the solution resulting from resuspending the pellet.

Step 2: Gel filtration chromatography.

The resuspended pellet solution was loaded on to a Superdex 200 pg gel filtration column which had been equilibrated with 20 mM Hepes buffer, pH 7.2, containing 50 mM KCl and 1 mM benzamidine. The protein was eluted at a flow rate of 0.75 CV/hr in the same buffer and 4 ml fractions were collected. The collected fractions were assayed for FDH activity and those that showed activity were pooled and concentrated in an Amicon ultrafiltration unit over a 30 kDa membrane.

Step 3: Ion exchange chromatography.

The concentrated active protein from the gel filtration column was loaded onto a Q - Sepharose ion exchange column equilibrated with 25 mM Hepes, pH 7.2. After sample loading the column was washed with equilibration buffer for 1 CV, after which the

concentration of KCl was increased to 0.3M in the same buffer over 0.25 CV. The FDH protein was eluted in a linear gradient of 0.3 - 0.5 M KCl in the same buffer over 3 CV. The column was regenerated by raising the KCl concentration to 1.0 M for 2 CV then equilibrating in equilibration buffer. Eluted protein was monitored at 280 nm and 8 ml fractions were collected. Each fraction was tested for formaldehyde oxidation activity; active fractions were pooled and concentrated over a 30 kDa Amicon ultrafiltration membrane.

Table A3.1 Purification method 3.

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity [mU. (mg protein)$^{-1}$]	Yield	Purification Factor
Crude Extract	100	5200	5.900	1.1	100	1
Ammonium Sulphate Precipitation	35	1015	2.415	2.3	36	2.1
Superdex 200 Gel Filtration	25	197	0.650	3.3	11	3
Mono Q Ion Exchange	30	105	0.840	8.0	14	7.3

A3.2 Results.

It was noted that mFDH co-purified with the hydroxylase component of sMMO when the non ionic buffer Hepes was used for elution of the enzyme. The method described in Table A3.1 shows a very low purification factor and this is due to the presence of the hydroxylase protein further investigations were carried out. Due to the problem of co-purification it was thought that the sMMO hydroxylase might be the mFDH enzyme and therefore this possibility was investigated.

A3.2.1 Co-purification of mFDH with three other proteins and their identification as the hydroxylase components of sMMO.

The elution profile from the ion exchange column using the above method suggested that mFDH was chromatographically pure when using Hepes buffer. When the purified protein sample was loaded onto a 12.5 % SDS gel there were four Coomasie Blue stained bands visible (Figure A3.1).

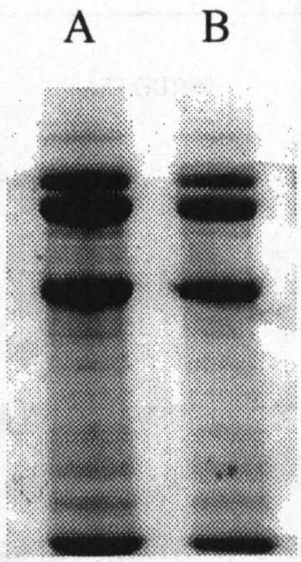

Figure A3.1 12.5% SDS PAGE of mFDH co-purified with three other proteins.

Lane A, pooled active FDH fractions from Superdex 200;
Lane B, purified sMMO hydroxylase.

The active mFDH eluted from the Superdex 200 gel filtration column was western blotted against antibodies for the hydroxylase of sMMO and the bands at 60, 38 and 16 kDa, corresponding to the hydroxylase, were stained. Confirmation that the protein material was the hydroxylase of sMMO was obtained by N - terminal sequencing of the 38 kDa and 16 kDa proteins, as described in Chapter 2, Section 2.7. The results of the N- terminal sequencing are shown in Table A3.2.

Table A3.2 N - terminal sequence analysis of proteins co-purified with FDH.

Component	N- terminal sequence of FDH active protein.	N - terminal sequence of sMMO components.
38 kDa Band (β subunit)	SMLGERR	SMLGERR
16 kDa Band (γ subunit)	AKLGIHSN	AKLGIHSN

In attempting to separate the hydroxylase component from mFDH, different chromatographic methods, such as gel filtration with high salt concentrations in the elution buffers, affinity methods and various ion exchange systems, were used. All methods failed to separate mFDH from the sMMO hydroxylase.

A3.2.2 Formaldehyde dehydrogenase activity of sMMO.

To investigate whether sMMO has a role in formaldehyde oxidation in *Methylococcus capsulatus* (Bath), purified hydroxylase was assessed for formaldehyde oxidation catalytic activity, in the presence and absence of protein F. A maximum formaldehyde oxidation activity of 5 mU.mg protein^{-1} was observed with purified hydroxylase. SDS - PAGE analysis of the hydroxylase sample showed a protein band with the same electrophoretic mobility as mFDH (Figure A3.1). It is therefore likely that the mFDH impurity was responsible for the observed formaldehyde oxidation activity and not the hydroxylase.

The effect of the modifier protein on the catalysis of propylene epoxidation by sMMO was also investigated by adding HTSE to a standard sMMO assay solution in place of protein B, as described in Materials and. The results are shown in Table A3.3. As the data demonstrate, there was an increase in the rate of propylene oxide formation in the presence of HTSE but the observed activity was only 10% of that when pure protein B is used in the normal assay (activity of protein B was 1400 mU.mg protein^{-1}).

Table A3.3 Effect of the HTSE on propylene epoxidation activity of sMMO.

Protein	**Rate of propylene epoxidation. (mU/mg protein)**	
	+ HTSE	- HTSE
sMMO Hydroxylase + Reductase	142	71
HTSE	0	0
sMMO Hydroxylase	0	0
sMMO Reductase	0	0

Propylene epoxidation activity was assayed as described in Materials and Methods. HTSE was added to the propylene oxide assay in place of protein B.

A3.3 Discussion.

The difficulty in separating the hydroxylase of sMMO from mFDH led to the suggestion that the former might be responsible for modifier protein dependent catalysis of formaldehyde oxidation. It is known that the oxidation of methanol and to a very limited extent formaldehyde, (Howard, T. personal communication) are catalysed by sMMO. Thus there could be modifier protein dependent formaldehyde oxidation activity catalysed by sMMO. The FDH catalytic activity that was observed however, could not be attributed to sMMO as the hydroxylase samples contained a small amount of protein with a molecular weight similar to that of mFDH. Therefore it must be assumed that the observed activity was due to the mFDH present in the hydroxylase preparations.

It is known that the association of proteins to form complexes allows close regulation of metabolic pathways (Stryer, 1988). The association of the sMMO hydroxylase with mFDH could play a role in controlling the oxidations of methane and formaldehyde in *Methylococcus capsulatus* (Bath). The advantage to the organism from such a symbiotic relationship would allow the efficient use of carbon resources and the generation of NADH as required for further methane assimilation. The results in Table A3.3 suggest that a small amount of protein B was present in the HTSE sample and may have caused the observed increase in the rate of propylene epoxidation.