MECHANISTIC STUDIES ON

FLAVOCYTOCHROME b_2

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ABSTRACT

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase E.C. 1.1.2.3) from the yeast *Saccharomyces cerevisiae* is a bifunctional enzyme located in the mitochondrial inter-membrane space where it catalyses the oxidation of L-lactate to pyruvate and the reduction of cytochrome *c*. The X-ray crystal structure has recently been determined to 2.4Å. The enzyme is a tetramer of identical subunits with M_r = 57.5kDa. Each subunit consists of two functionally distinct domains, one containg flavin mononucleotide (FMN), the other a protoheme IX group.

Wild-type and seven point mutant forms of flavocytochrome b_2 have been expressed in the bacterium *Escherichia coli*. The enzyme expressed in E.coli contains both prosthetic groups, has an electronic absorption spectrum identical with that of the yeast protein and has an identical M_r value of 57.5kDa as estimated by SDS-polyacrylamide gel electrophoresis. N-terminal amino-acid sequence data indicate that the flavocytochrome b_2 isolated from *E. coli* begins at position 6 (Met) when compared with mature flavocytochrome b_2 from yeast. The absence of the first five amino acids appears to have no effect on the catalytic properties of the enzyme. The high-level expression of flavocytochrome b_2 (500-1000 fold more than from a similar wet weight of yeast) has enabled a fuller characterisation of the wild-type and mutant enzymes.

Structural and mechanistic studies have led to the proposal of a mechanism for lactate oxidation. Site-directed mutagenesis has been used to probe the role of seven amino-acid residues thought to be involved in the mechanism of action of flavocytochrome b_2. Typical saturation kinetics were observed for five of the mutant enzymes. Data were analysed to give values of k_{cat} and K_M. Two of the mutant enzymes had no observable activity. A change in the rate-limiting step in the mechanism of flavocytochrome b_2 is thought to have occurred for one of the mutant enzymes indicating that this amino-acid residue has a key role in the

turnover mechanism. The implication of these results are discussed in relation to the mechanism of L-lactate oxidation.

A flavocytochrome b_2 mutant has been constructed in which the C-terminal tail (23 amino-acid residues) has been deleted. This tail appears to form many intersubunit contacts in the tetrameric wild-type protein, and it was expected that its removal might lead to the formation of monomeric flavocytochrome b_2. However, the isolated tail-deleted mutant enzyme (TD-b_2) was found to be tetrameric. TD-b_2 exhibited loss of activity during turnover in a biphasic process. The rate of the faster of the two phases was dependent on L-lactate concentration. The slower phase, however was independent of L-lactate concentration and was found to correlate with dissociation of FMN. Thus the C-terminal tail of flavocytochrome b_2 appears to be required for the structural integrity of the enzyme around the flavin active site.

Cytochrome *c*, the physiological partner for flavocytochrome b_2, has been used as the electron acceptor in steady-state kinetic studies of the wild-type and mutant enzymes. With wild-type and the point mutant enzymes, typical saturation kinetics were observed. The data were analysed to yield k_{cat} and K_M values. K_M for cytochrome *c* was found to vary directly with k_{cat} indicating that the K_M value is not a true reflection of the dissociation constant. The reduction of cytochrome *c* by flavocytochrome b_2 has been significantly affected by the tail deletion. This has been interpreted as the involvement of the C-terminal tail in the interaction of flavocytochrome b_2 with cytochrome *c*.

First-order rate constants have the units s^{-1}, second-order rate constants have the units $M^{-1}s^{-1}$, and molar absorbance coefficients have the units $M^{-1}cm^{-1}$. Other abbreviations of units are of a standard form, eg:-

grams	g
millilitre(s)	ml
dalton units	Da

The following nomenclature has been adopted in referring to flavocytochrome b_2 and related enzymes:-

S.c.b_2	flavocytochrome b_2 as isolated from the yeast *Saccharomyces cerevisiae*
H.a.b_2	flavocytochrome b_2 as isolated from the yeast *Hansenula anomala*
E.c.b_2	flavocytochrome b_2 as expressed and isolated from the bacterium *Escherechia coli*
TD-b_2	tail-deleted flavocytochrome b_2

The single-letter code for amino-acid residues has been used, eg:-

R376 denotes an arginine at position 376 in the amino-acid sequence

Y143F denotes a mutant enzyme in which the residue at position 143 in the amino-acid sequence has been changed from a tyrosine to a phenylalanine.

The following abbreviations have been used:-

FMN	flavin mononucleotide
heme	Fe protoporphyrin IX
K_M	Michaelis-Menten constant
k_{cat}	rate of catalysis at a saturating concentration of substrate
SGO	spinach glycolate oxidase
LDH	lactate dehydrogenase

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INTRODUCTION

1.1. Historical Perspective

Harden and Norris observed in 1915, that dried yeast mixed with lactic acid readily reduced methylene blue and that pyruvic acid was formed [1]. Four years later Meyerhof demonstrated that this process occurred in the absence of any added coenzyme [2]. The first partial purification of flavocytochrome b_2 was reported by Bernheim who extracted a turbid solution from acetone-dried Delft yeast which had lactate dehydrogenase activity [3]. Bach and co-workers found evidence to suggest that the lactate dehydrogenase activity was associated with a *b*-type cytochrome and named the enzyme cytochrome b_2 [4]. In 1954, the enzyme was crystallised facilitating the isolation of pure preparations and the identification of flavin mononucleotide (FMN) as a second prosthetic group [5].

Since then, significant advances have been made in the development of structural models, electron-transfer schemes and lactate oxidation mechanisms, particularly by Appleby and Morton, and the groups of Labeyrie and Lederer. However, it was not until recently that the first accurate structural model of flavocytochrome b_2 was made by Xia *et al* [6]. It is now possible to identify residues which may be important in structural and/or functional aspects of the enzyme, and with the availability of the DNA sequence, these residues can be specifically altered to generate mutant flavocytochromes b_2 which may give an insight into the role of particular amino acids.

1.2. Biological Background

Flavocytochrome b_2 is synthesised in the cytoplasm with an 80 amino acid N-terminal extension which directs the enzyme to the mitochondria. The pre-protein is imported into mitochondria and subsequently processed in two steps resulting in soluble mature flavocytochrome b_2, located in the intermembrane space [7-9], where it transfers electrons from L-lactate to cytochrome *c* [10,11].

Pajot and Claisse demonstrated that yeast cells could respire on L-lactate in the presence of Antimycin A [11], a Streptomyces antibiotic which inhibits respiratory electron transfer by preventing oxidation of cytochrome *b*. However, the antimycin resistant respiration was prevented by cyanide, a known inhibitor of cytochrome *c* oxidase. A short electron-transfer chain was proposed involving flavocytochrome b_2, cytochrome *c* and cytochrome *c* oxidase as shown in figures 1.1 and 1.2. Oxygen and lactate uptake measurements in the presence and absence of Antimycin A supported the proposed scheme. Path A produces 1 mole of adenosine triphosphate (ATP) per mole of lactate and path B produces 11 moles of ATP per mole of lactate. Thus, the role of flavocytochrome b_2 in yeast mitochondria may be to provide an alternative electron-transfer pathway providing an extra mole of ATP for every mole of lactate oxidised* and, in the event of the oxidative phosphorylation pathway being blocked, it will provide enough energy for cell growth to continue.

1.3. Isolation and Purification

The first isolation of flavocytochrome b_2 was carried out by Bernheim in 1928, who extracted a turbid solution from acetone-dried Delft yeast which had dehydrogenase activity [3]. Since then, there have been many reports of new improved purification methods.

The first major advance in the purification method was devised by Morton and Appleby who used crystallisation of the enzyme as the major step in the procedure [5]. The purification involved air drying and grinding of yeast, extraction of lipid material with n-butanol, fractionation of a lactate extract with acetone at low temperatures, and finally crystallisation at low ionic strength. The flavocytochrome b_2 crystals were later found to contain DNA [12], which was removed by dialysis [13] or chromatography [14], to give type II DNA free flavocytochrome b_2 crystals. However, flavocytochrome b_2 purified

* Note however that the conventional NAD-linked lactate dehydrogenase will produce cytoplasmic NADH which may be available to mitochondrial oxidation by means of a shuttle mechanism.

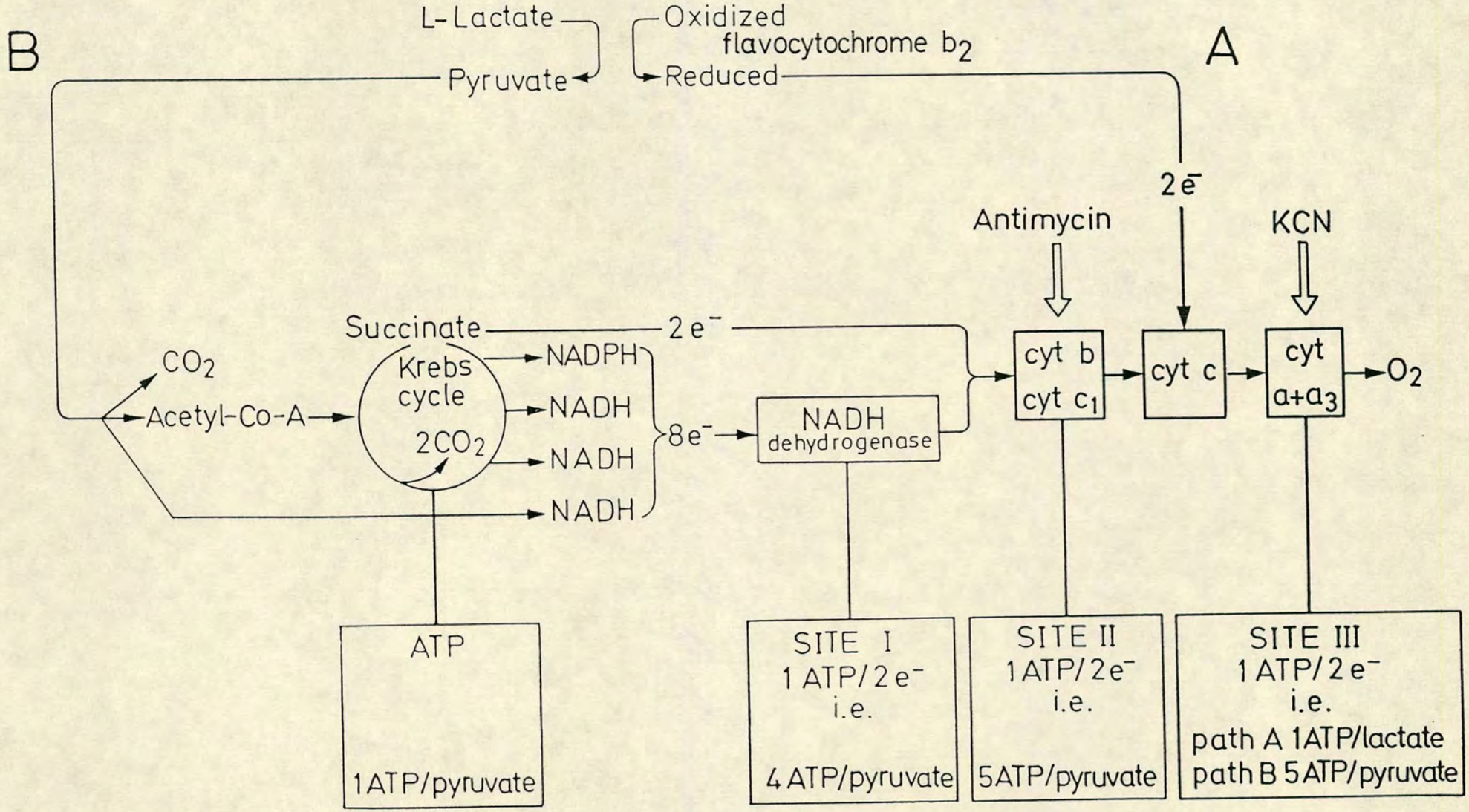


Figure 1.1 The repiratory chain of yeast mitochondria. Path A is the direct electron transfer from flavocytochrome b_2 to cytochrome *c* and produces 1 mole of ATP per mole of L-lactate. Path B produces 11 moles of ATP per mole of L-lactate.

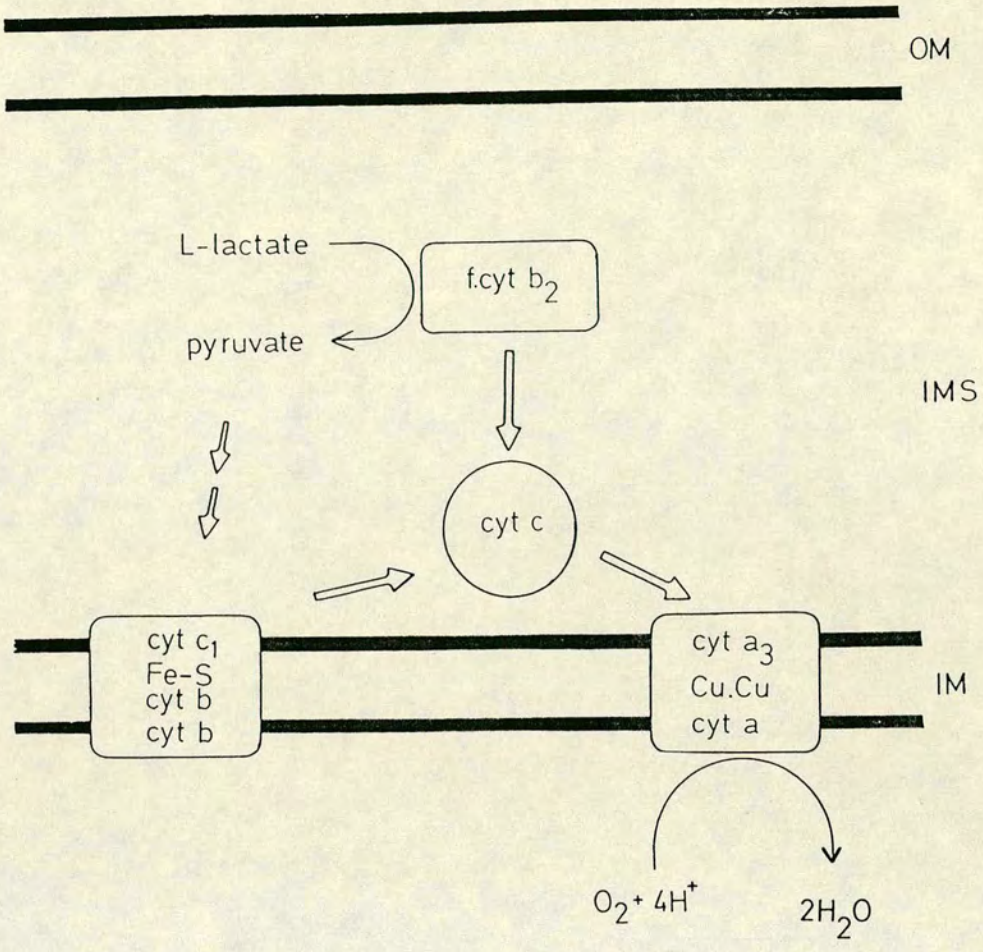


Figure 1.2 The position of flavocytochrome b_2 in relation to the intermembrane space of the mitochondria, and the other electron-transport enzymes. OM = outer membrane, IMS = intermembrane space, IM = inner membrane.

⇨ ⇨ represents path B in figure 1.1 (p4).

by crystallisation was found to be unstable and the enzyme activity as measured by different groups was found to be inconsistent [12–20].

Nicholls *et al* observed changes in the properties of the enzyme during crystallisation, which were a prerequisite to crystal formation [21]. The K_M for lactate binding and the electrophoretic mobility of flavocytochrome b_2 were observed to increase. The cause of these changes was not determined, but it was suggested that they may have been due to the action of a proteolytic enzyme or by protein contamination. At the same time, Somlo and Slonimski noted that a modification within the enzyme had to take place before it would crystallise [22]. The "physiological" enzyme (the form present in crude extracts) had different kinetic properties to that of the crystalline type. Analysis of the crystalline type enzyme by polyacrylamide gel electrophoresis showed that it consisted of two polypeptide chains of unequal length (36 and 21kDa) [23]. The heavier chain was shown to be the heme-binding fragment and the lighter chain was part of the flavin-binding fragment [23,24].

Jacq and Lederer purified flavocytochrome b_2 extracts in the presence of phenylmethylsulphonyl fluoride (PMSF), a known inhibitor of proteases [25], and reported that crystallisation did not take place [27]. On analysis, this flavocytochrome b_2 was more stable, and it was shown by polyacrylamide gel electrophoresis that the enzyme consisted of a single polypeptide chain of molecular weight 57kDa (similar to *H. anomala* flavocytochrome b_2 [26]) and with kinetic properties identical to the "physiological" flavocytochrome b_2. Therefore, it was concluded that in the absence of PMSF a peptide bond was cleaved by an indigenous protease prior to crystallisation. New purification procedures were developed in which all steps were carried out in the presence of PMSF (1mM) [27–29]. The crystalline and non-crystalline enzymes were termed "cleaved" and "intact" flavocytochromes b_2 respectively.

A comparison between the purification procedures of these enzymes, and

flavocytochrome b_2 from *H. anomala* has been reported by Labeyrie et al [29]. Analysis by gel electrophoresis showed a single band at ≈57kDa, and two bands at approximately 21 and 36kDa for intact and cleaved flavocytochromes b_2 respectively. The cleaved enzyme can be kept for up to one month and the intact form for four to five months, without any significant loss in activity, if stored at 4°C under an atmosphere of nitrogen. The best preparations from *S. cerevisiae* gave around 50mg of flavocytochrome b_2 per kilogram of dried yeast.

1.4. Structural Studies

Flavocytochrome b_2 is a tetramer of molecular weight 230kDa [30]. Each subunit contains two prosthetic groups, protoheme IX and flavin mononucleotide (FMN) (figure 1.3) [5], and is folded into two functionally distinct domains, a heme-binding domain and a flavin-binding domain [31]. The DNA and amino-acid sequences have been determined [34,35]. The single polypeptide chain consists of 511 amino acids. Figure 1.16 (section 1.7) shows the amino-acid sequence of flavocytochrome b_2 from *S. cerevisiae*. (The recently deduced flavocytochrome b_2 sequence from *H. anomala* [36] and the amino-acid sequence of spinach glycolate oxidase are also shown in this figure.)

Before the crystal structure was obtained there was some argument as to the quaternary structure of the enzyme. There was evidence of two peptide chains of molecular weight 36 and 21kDa [23], which arise due to a cleavage site in a protease sensitive region of the polypeptide chain [27]. The position of this sensitive site was found to be in the flavin-binding domain [28] and led to the suggestion of a biglobular or even a polyglobular flavin domain structure [31-33]. Powder X-ray diffraction and electron-microscopy studies have shown that cleaved flavocytochrome b_2 is a true tetramer with molecular symmetry 222 [30,37]. The crystals were shown to contain large cylinders of

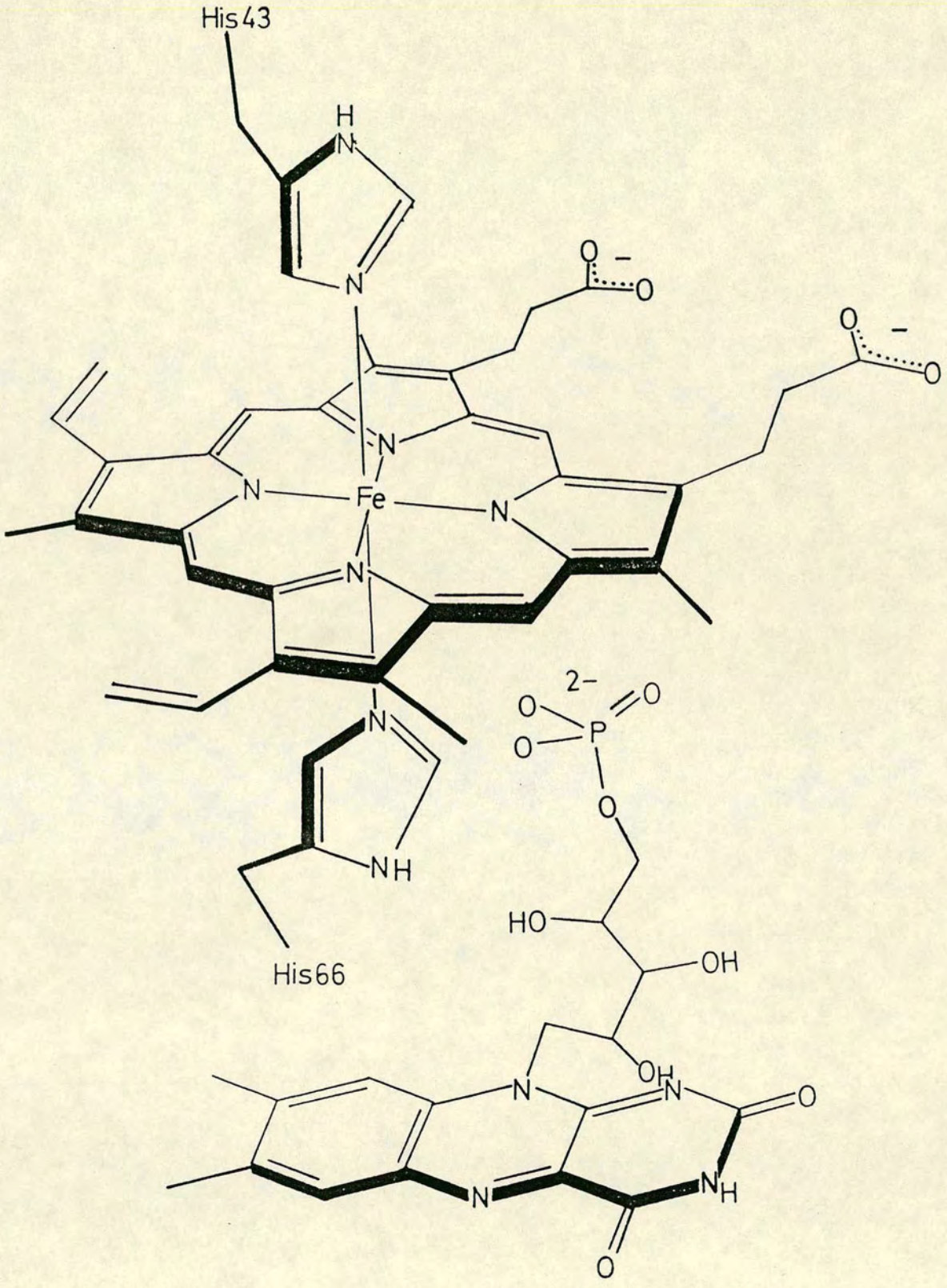


Figure 1.3 Structure of the flavocytochrome b_2 prosthetic groups, flavin mononucleotide and heme.

water running parallel to the crystal c axis. These cylinders account for the 37% water content and facilitate diffusion of lactate and cytochrome *c* through the crystal (section 1.6).

Mathews *et al* have recently solved the three dimensional structure of the intact flavocytochrome b_2. The original 3.0Å structure [6] has since been refined to 2.4Å [38]. The protein is a tetramer of identical subunits with a four-fold axis of rotation. The subunits are packed so that the four flavin domains form an oblate ellipsoid approximately 100Å in diameter and 60Å thick with the cytochrome domains protruding from the edge of the ellipsoid above the mid plane. Residues 1-100 form the heme-binding domain which is very homologous to liver microsomal cytochrome b_5 (section 1.7.2). The heme domain can be isolated by proteolysis to yield the so-called cytochrome b_2 core which is resistant to further proteolysis [39]. Residues 101 to 486 comprise the flavin-binding domain containing a $\beta_8\alpha_8$ motif similar to glycolate oxidase (section 1.7.3). The remaining residues, 487-511, form an extended C-terminal tail, which forms many intersubunit contacts with the other three subunits. Mild treatment of flavocytochrome b_2 with proteases can cleave the peptide chain at the domain-domain connection. This results in a flavodehydrogenase protein (a tetramer of flavin domains) and cytochrome b_2 core, with no affinity between the two [33,40].

The heme group, contained within a hydrophobic crevice, is situated close to the domain-domain interface. The two propionates extend towards the flavin, each hydrogen bonded to a tyrosine, one to Y97 of the heme domain and the other to Y143 of the flavin domain. The heme is bound noncovalently with the iron chelated at the fifth and sixth positions by two histidines (H43 and H66). The flavin is also noncovalently bound, situated towards the carboxy end of the β barrel. The heme and flavin groups are almost coplanar (17° twist) and the distance from the iron to the centre of the flavin isoalloxazine

ring is ≈16Å. The shortest distance from the edge of the porphyrin ring to the N5 of the flavin is ≈11Å. In two of the four subunits, an isolated piece of electron density was located at the flavin binding site. This electron density has been associated with a molecule of pyruvate bound close to the flavin. In these subunits, the cytochrome domains are disordered and Y143 is hydrogen bonded to the carboxylate group of pyruvate instead of the heme propionate. Figure 1.4 shows the structure around the active site. There is also a region of disorder, residues 300–315, which forms a surface-exposed loop. The C_α chain is shown in figure 1.5. The secondary and tertiary structures of the polypeptide chain are shown schematically in figures 1.6 and 1.17.

Labeyrie *et al* have shown by nmr linewidth studies on flavocytochrome b_2 from *H. anomala*, that the cytochrome domain appears to be mobile relative to the central flavodehydrogenase [41]. Under certain conditions, resonance linewidths are proportional to molecular weight for rigid molecules and are narrowed by motion within the molecule. ^1H nmr spectra were recorded for holoflavocytochrome b_2 and the isolated cytochrome b_2 core at a frequency of 400 MHz. The molecular weight ratio of flavocytochrome b_2 to the cytochrome domain is about 18, but the ratio of linewidths was only about 4. Thus there appears to be mobility in the cytochrome domain which may be important in the function of the enzyme, for example in controlling electron transfer, allowing easy entrance/exit of substrate to and from the active site, or to allow formation of a complex between flavocytochrome b_2 and cytochrome *c*.

1.5. Solution Studies

1.5.1. Spectral Properties

Flavocytochrome b_2 electronic absorption spectra were first recorded in 1942 [42]. Since then visible absorption has been useful for measuring the

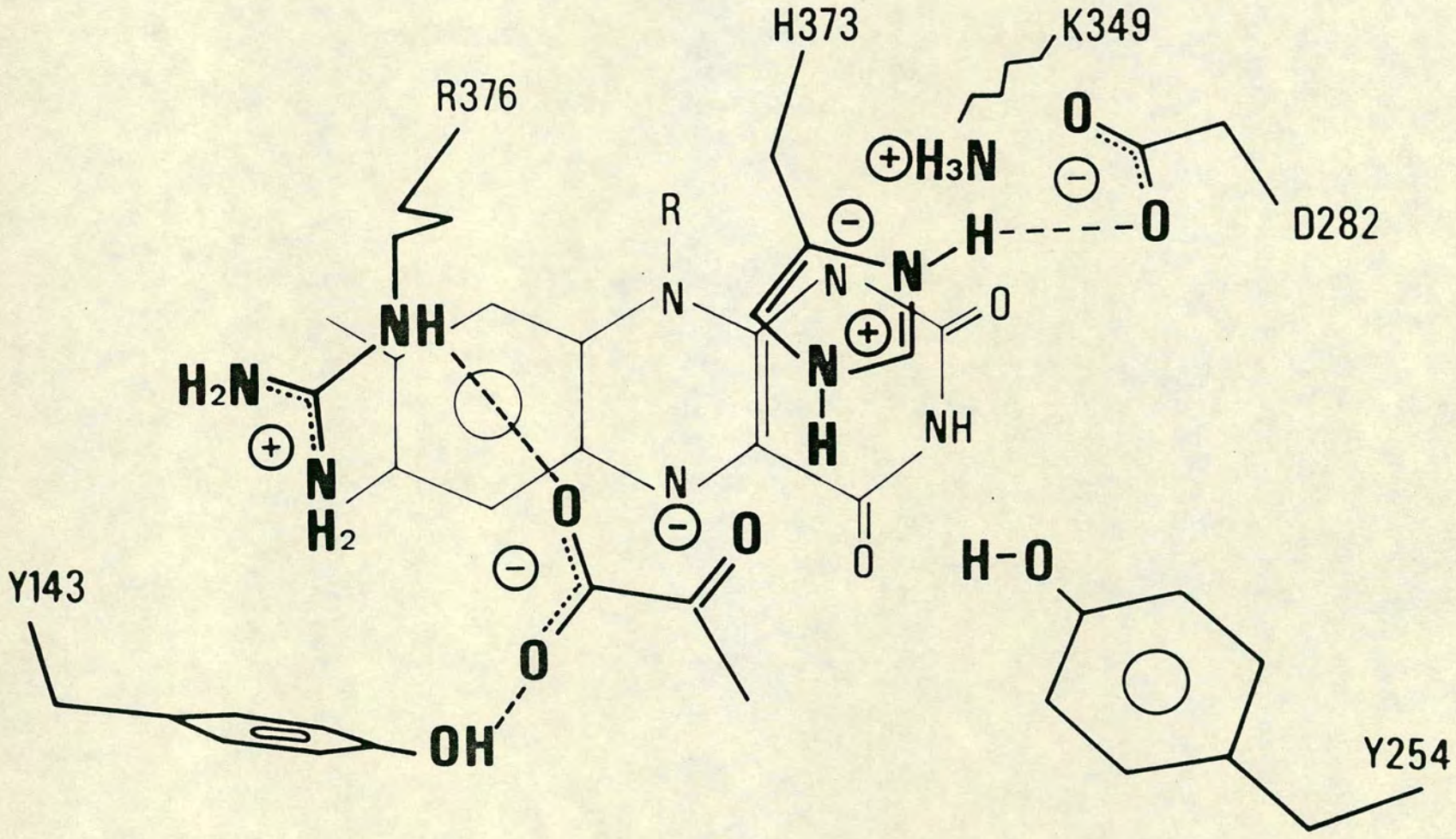


Figure 1.4 Structure of the flavin active site in subunit 2 containing the bound pyruvate molecule.

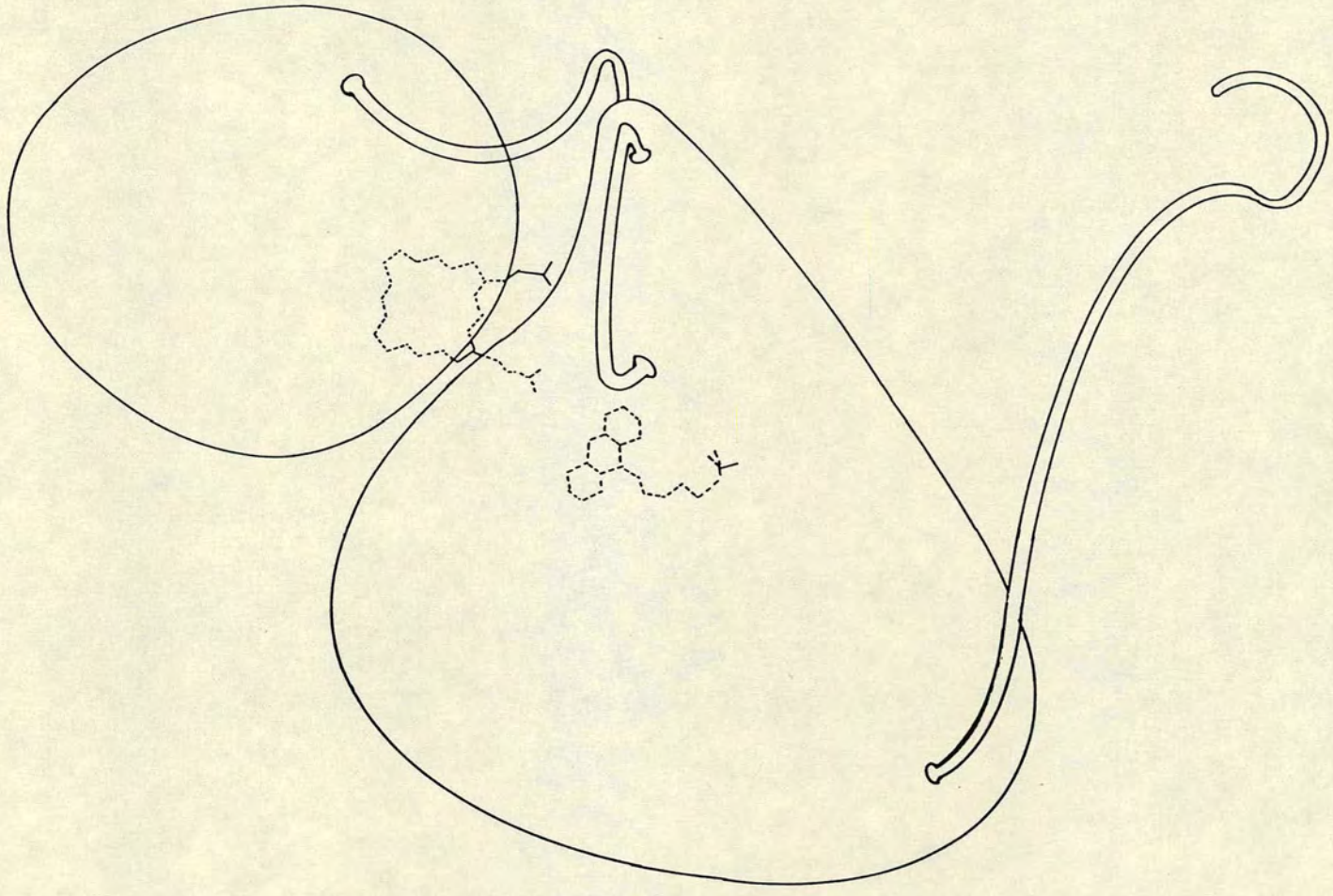


Figure 1.6 Schematic diagram of the flavocytochrome b_2 monomer showing the C-terminal tail, the disordered loop and the hinge region.

enzyme concentration in the calculation of enzymatic activity. Figure 1.7 shows the UV–vis spectrum of flavocytochrome b_2 which is typical of a *b*-type cytochrome with sharp peaks (in the reduced state) at 557nm and 528nm and a large sharp peak due to the soret absorbance at 423nm (reduced) and 413nm (oxidised). The absorbances at 360nm and 265nm are due to the flavin and the aromatic residues of the polypeptide chain, respectively. The ratio of the UV and soret absorbance gives a useful indication of enzyme purity.

The extinction coefficients generally accepted as most accurate were determined by Pajot and Groudinsky (table 1.1) [43]. There are no significant differences between the forms of flavocytochrome b_2. Cytochrome b_2 core has a slightly smaller absorbance at 413nm in the oxidised state ($121 mM^{-1}cm^{-1}$) due to the absence of the flavin contribution at this wavelength ($\approx 8 mM^{-1}cm^{-1}$). The core also has a smaller UV absorbance due to its smaller molecular weight.

1.5.2. Catalysis and Substrate Specificity

There have been many kinetic studies on flavocytochrome b_2 since the enzyme was first discovered. Early work was carried out using methylene blue as an electron acceptor, monitoring the rate of turnover by the Thunberg tube method [44]. Cytochrome *c* was first used as an electron acceptor in 1935 [45], but it was not until 1974 that cytochrome *c* was shown to be the physiological partner (section 1.2). Ferricyanide was first suggested as a useful electron acceptor by Morton and Appleby in 1954 [5], and it has been used in most subsequent kinetic studies.

As mentioned above (section 1.3), the cleaved form of the enzyme is less stable and activity values vary according to preparation procedure. Many of the kinetic studies on flavocytochrome b_2 have been carried out using this cleaved form. Pompon *et al* suggested that proteolysis affects the steps involved in flavin reduction and not the internal electron transfers [46,47] so that although

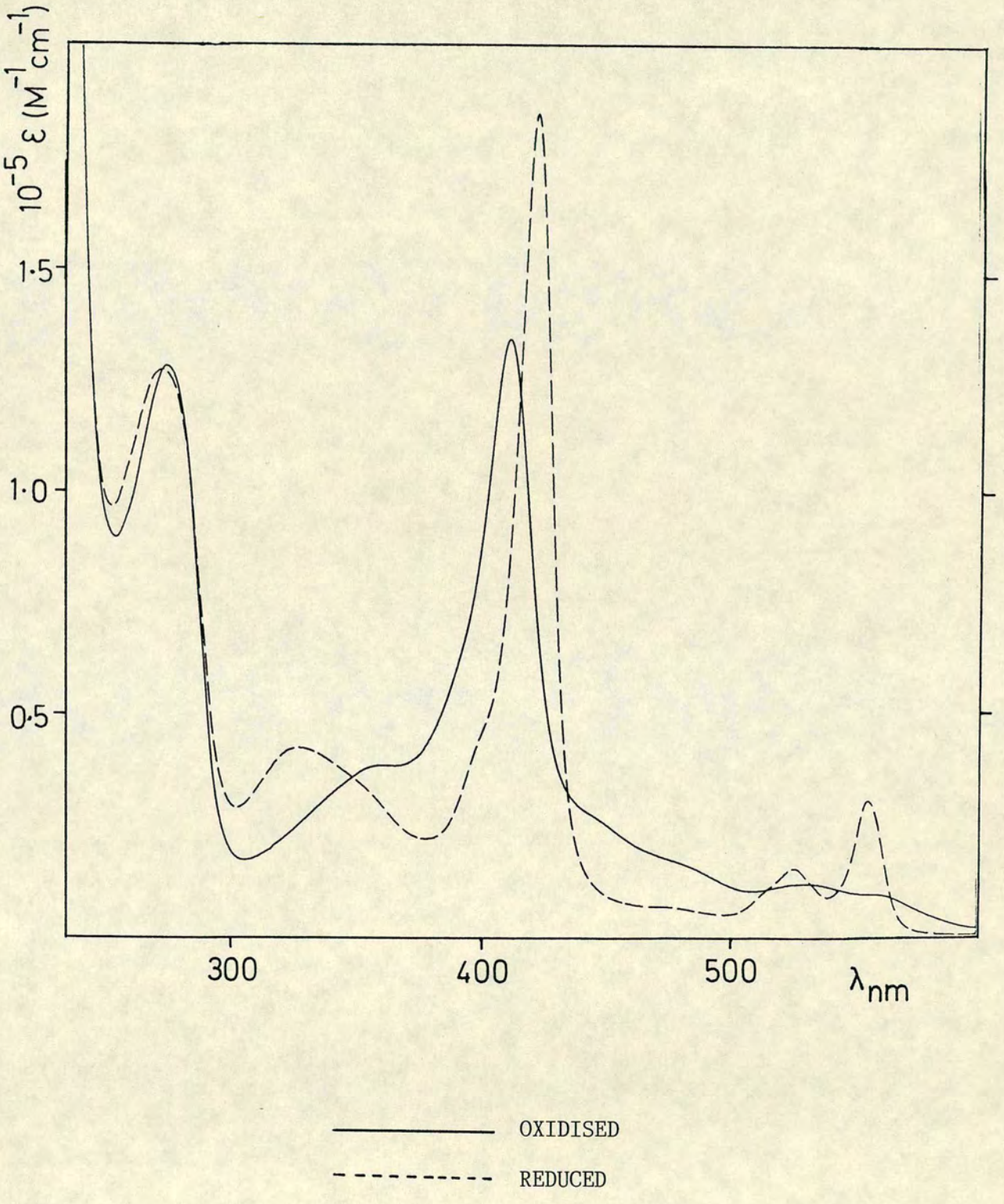


Figure 1.7 The electronic absorption spectrum of flavocytochrome b_2. The extinction coefficients are listed in table 1.1.

Band	Oxidised form		Reduced form			
	λ max nm	ε $mM^{-1}cm^{-1}$	λ max nm	ε $mM^{-1}cm^{-1}$	λ nm	red-ox $mM^{-1}cm^{-1}$
α	560	9.2	557	30.9	557	21.5
β	530	11.3	528	15.6	438.3	-7.9
γ	413	129.5	423	183		
δ	362	34.4	328	39		
UV	275	89	269	88		

[$b_2$core (oxidised) λ_{max} = 413 nm ε = 121.5 $mM^{-1}cm^{-1}$]

Table 1.1 Extinction coefficients of flavocytochrome b_2 [43]. The contribution of flavin at 413nm is $\approx 8 mM^{-1}cm^{-1}$.

the two forms of the enzyme are structurally different, some of the findings from these studies are still relevant. The "best" estimates of k_{cat} and K_M according to Labeyrie *et al* are shown in table 1.2 [29].

Flavocytochrome b_2 has been shown to have a very broad substrate specificity [48-50]. Some substrates and inhibitors are shown in tables 1.3 and 1.4. Table 1.5 lists some substances which have no interaction with the enzyme. The highest turnover rate is obtained with L-lactate which suggests that this is the true substrate. It can be seen from tables 1.3 and 1.4 that all substrates and inhibitors apart from p-toluenesulphonate are carboxylates and that the minimum structural requirements for a substrate are a carboxylate with an α-hydrogen and an α-hydroxyl group. From the data in table 1.4.A, it can be seen that as the number of CH_2 units increases, the inhibitory effect increases. From this dependence upon the length of the aliphatic inhibitor, the free energy of interaction of one carboxylate with the positive charge on the enzyme can be calculated. Knowing this energy and selecting from the possible amino acids, Dikstein suggested that the carboxylates of the substrates and inhibitors were interacting with the guanidinium group of an arginine side-chain [49].* Hart *et al* have shown that a strong carboxylate-arginine interaction is important with lactate dehydrogenase from *Bacillus stearothermophilus* [51].

Most of the substances which are neither substrates nor inhibitors are neutral, but it is surprising that alanine and serine show no interaction, considering the similarity of their structures with lactate and remembering that amino-acid oxidases oxidise their substrates in a similar manner to flavocytochrome b_2 (sections 1.5.5 and 1.7).

* The situation is more complex than Dikstein suggests since either the interaction between the carboxylate and the positive charge or the association energy of the aliphatic chain or a combination of the two could be the variable parameter.

Enzyme	k_{cat} (s^{-1})	K_M (mM)	Ref
Cleaved (S.c.)	210	1.6	[69]
Intact (S.c.)	550	0.4	[28]
Intact (H.a.)	1000	1.3	[70]

Table 1.2 k_{cat} and K_M values for various forms of flavocytochrome b_2.

Substrate	Formula	Relative Rate
L-lactate	$^{-}O_2C.\underset{OH}{CH}.CH_3$	1.00
	$^{-}O_2C.\underset{OH}{CH}.CH_2X$	
DL-chlorolactate	X = Cl	0.35
DL-fluorolactate	X = F	0.10
DL-bromolactate	X = Br	0.91
L-glycerate	X = OH	0.50
DL-3-phosphoglycerate	X = OPO_2OH	0.25
	$^{-}O_2C.\underset{OH}{CH}.(CH_2)_nH$	
Glycollate	n = 0	0.04
DL- -hydroxybutyrate	n = 2	0.30
DL- -hydroxy-n-caproate	n = 4	0.18
DL- -hydroxycaproate	n = 8	0.30
DL- -hydroxyisocaproate	$^{-}O_2C\underset{OH}{CH}.CH_2\underset{CH_3}{CH}CH_3$	0.17
	$^{-}O_2C\underset{OH}{CH}\text{-}y\text{-}CO_2^{-}$	
DL-isocitrate	y = $\underset{-CH.CH_2-}{\overset{CO_2^{-}}{\vert}}$	0.01
L-malate	y = $-CH_2-$	0.01
L-tartate	y = $\underset{-CH-}{\overset{OH}{\vert}}$	0.05

Table 1.3 List of substrates for flavocytochrome b_2.

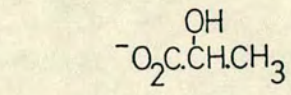
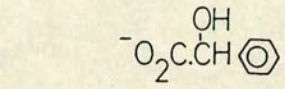
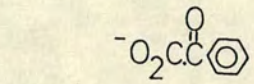
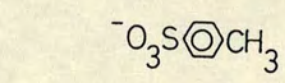
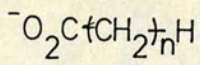
Inhibitor	Formula	K_I
D-Lactate		150 mM
DL-Mandelate		2 mM
DL-Phenylpyruvate		10 mM
p-Toluenesulphonate		100 mM
		
Formate	n = 0	6 M
Acetate	n = 1	2-3 M
Propionate	n = 2	800 mM
Butyrate	n = 3	300 mM
Valerate	n = 4	400 mM
Caprylate	n = 7	10 mM

Table 1.4 List of inhibitors for flavocytochrome b_2.

Inhibitor	Formula	Relative Inhibition (%)	Inhibitor Concentration (mM)
	$^{-}O_2C\overset{OH}{C}H\text{-}R$		
DL-glycolate	R = H	21	100
DL-glycerate	R = CH	40	100
DL-mandelate	R = 	72	100
DL- -hydroxybutyrate	R = CH_2CH_3	45	100
DL- -hydroxyisobutyrate	R = $(CH_3)_2$	32	100
DL- -hydroxy-n-caproate	R = $(CH_2)_3CH_3$	65	100
DL- -hydroxyisocaproate	R = $CH_2CH(CH_3)_2$	51	100
DL- -hydroxyisovalerate	R = $CH_2CH_2CH(CH_3)_2$	76	100
	$^{-}O_2C\overset{OH}{C}H(CH_2)_nCO_2^{-}$		
DL-malate	n = 1	95	10
D-malate	n = 1	86	100
L-malate	n = 1	53	50
DL-hydroxymalonate	n = 0	8	100
DL- -hydroxyglutarate	n = 2	7	50

Table 1.4 List of inhibitors for flavocytochrome b_2.

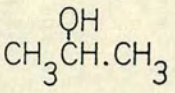
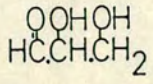
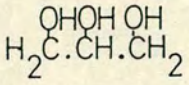
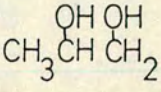
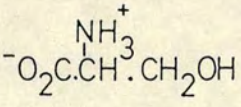
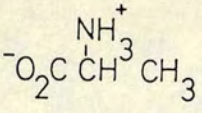
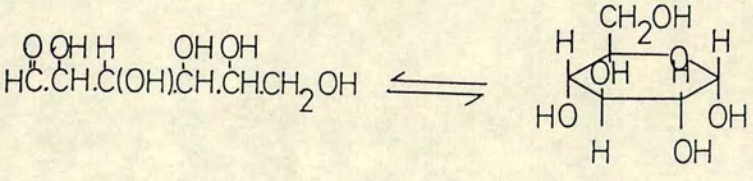
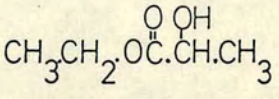
isopropanol	
DL-glyceraldehyde	
glycerol	
propyleneglycol	
serine	
alanine	
glucose	
ethyllactate	

Table 1.5 List of chemicals which are neither substrates or inhibitors for flavocytochrome b_2.

1.5.3. pH Dependence Studies

From studies of the pH dependence of enzyme catalysis, information can be gained about the active site. The results of several pH dependence studies are summarised in table 1.6. It is clear from this table that the pH optimum is between 7 and 8 and that the free enzyme has pK_a values around 6.5 and 9, which are shifted upon substrate binding to around 5.5 and 10. Lactic acid has a pK_a of 3.86 [52], therefore groups at or near to the active site, must be giving rise to these pK_a values. The values quoted would be consistent with histidine and/or tyrosine or even the flavin group itself. As shown in figure 1.4, there are two tyrosines (143 and 254), and a histidine (373) present at the active site.

In a detailed pH dependence study by Hinkson and Mahler [53], the apparant pK_a of a group or groups affecting lactate binding was measured as 6.5 to 7.0. There was no corresponding pK_a around this region for the binding of D-lactate. Since D-lactate is identical to L-lactate except for the orientation of the groups attached to the α-carbon, it was suggested that the residue on the free enzyme responsible for the pK_a around 6.5 to 7.0 interacts with one of the groups attached to the α-carbon of L-lactate, and most probably the hydroxyl group. D-lactate and caprylate are both competitive inhibitors of flavocytochrome b_2, with similar dissociation constants (table II of ref. 53). This strongly suggests that it is mainly the carboxylate group which is responsisble for the binding of these inhibitors. It was also suggested that L-lactate binds more strongly through the carboxylate group than by any R-OH-enzyme interaction, since the decrease in dissociation constant ascribable to this second effect is only one order of magnitude, (cf 7.2×10^{-4} M for L-lactate, 4.2×10^{-3} M for caprylate) ie approximately 4.2kJ mol^{-1} for R-OH binding and 17.6kJ mol^{-1} for the binding of R-OH and $R\text{-}COO^-$.

From the pH dependence of pyruvate inhibition, pK_a values of

Enzyme	pH (opt)	pK_a	Temp (^oC)	Ref
E	7.2	6.0, 8.8	25	[57]
E-S		5.3, 9.7		
E-S	7.5	5.65	20	[58]
E-S	7.3		25	[59]
E-S	8.0			[5]
E	8.0	6.5-7.0	20	[53]
E		6.3		
E-S		5.68		

E = free enzyme

E-S = enzyme-substrate complex

Table 1.6 Results of various pH studies.

approximately 7.3 and 8.0 can be calculated [53]. It was suggested that this pK_a (7.3) is predominantly that of the same group measured with the free enzyme (6.5 to 7.0). From this it was inferred that this residue is involved in the binding of substrate (L-lactate) and product (pyruvate), and so is capable of interacting with either an α-hydroxy or an α-keto group.

1.5.4. Kinetic Rates and Schemes

The pathway of electron transfer within flavocytochrome b_2 is from bound lactate to flavin, then flavin to heme, (figure 1.8) [54,55]. The first step, oxidation of lactate and electron transfer to flavin, is the slowest step in the enzyme turnover [55]. A kinetic isotope effect of 5 was obtained by measuring the oxidation of DL-lactate deuterated at the C_α position [56], suggesting that the rate limitation of the first step was due to the cleavage of C_α-H bond. Flavin and heme reduction by 2-^{2}H-lactate measured by means of a stopped-flow spectrophotometer, resulted in kinetic isotope effects of 8 and 6 for flavin and heme reduction respectively, indicating that the C_α-H bond cleavage is not totally rate limiting [46].

As flavin is the first acceptor in the electron-transfer pathway, it is obviously essential for activity [65]. Is the heme also essential? Forestier and Baudras found a linear relationship between heme content and activity [66]. Extrapolation to a zero heme content indicated zero cytochrome *c* reductase activity and a lowered ferricyanide reductase activity. In 1977 Iwatsubo *et al* demonstrated with dehemoflavocytochrome b_2 [67], (heme-free preparations of flavocytochrome b_2) that the flavin hydroquinone is a poor electron donor to ferricyanide. The subsequent flavin semiquinone is a very good electron donor to ferricyanide (showing a 200-fold greater second-order rate constant than that seen with flavin hydroquinone) suggesting that the role of the heme moiety of flavocytochrome b_2 in ferricyanide reductase activity is to accept the first electron of flavin hydroquinone to produce two very good one-electron

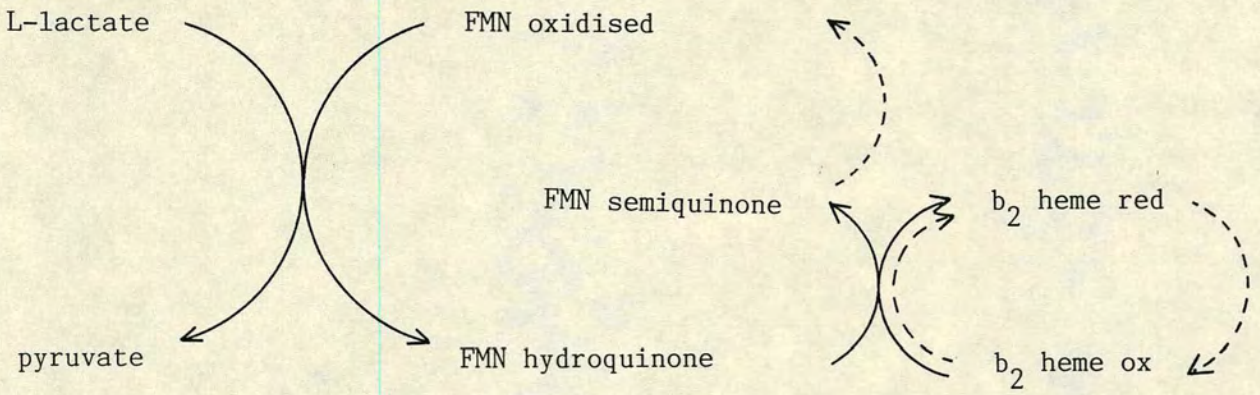


Figure 1.8 The electron-transfer scheme for flavocytochrome b_2.

donors, which are then oxidised by ferricyanide. Moreover, when the dehemoflavocytochrome b_2 is mixed with lactate and cytochrome *c* there is no cytochrome *c* reductase activity, implying that the heme is essential for cytochrome *c* reduction. This is supported by the fact that a flavodehydrogenase form of flavocytochrome b_2 (tetramer of flavin-binding domains with no cytochrome b_2 cores) from *H. anomala* also has no cytochrome *c* reductase activity [40]. The mid-point oxidation-reduction potentials of the various redox groups have been measured (table 1.7) and are consistent with the proposed electron-transfer pathway discussed above.

From the redox potentials (table 1.7) electron transfer from bound substrate (E_{m7} = -190mV) to flavin (E_{m7} = -55mV) will proceed far to the right while there is little difference in the redox potentials of the flavin (-55mV) and the heme (0mV).

In 1975, Capeillere-Blandin *et* al carried out combined stopped-flow absorbance and rapid-freezing experiments on cleaved flavocytochrome b_2 [62]. Using simulation studies, they tried to derive an electron-transfer scheme which would correlate with all the data [68]. Fully oxidised flavocytochrome b_2 was reduced by lactate in the absence of acceptor. The change in absorbance due to reduction was measured at two wavelengths (to monitor heme and flavin reduction separately) by means of a stopped-flow spectrophotometer. The time courses of flavin and heme reduction, which were biphasic in nature, were superimposable (no lag phase detectable). The duration of Phase I was ≈30-35ms and accounted for approximately 85% of the total absorbance change. Phase II accounted for the remaining 15% of the total absorbance change, the rate constant of phase II being 20 fold smaller than in phase I. EPR spectroscopy was used to follow the levels of oxidised heme and flavin

Enzyme	$E_{m7}(H_o/H_r)$	$E_{m7}(F_o/F_{sq})$	$E_{m7}(F_{sq}/F_r)$	$E_{m7}(F_o/F_r)$	Ref
S_X	120				[60]
S_X	0 ± 3				[61]
$b_2$core (S_X)	-28				[39]
S_X	0				[39]
deflavocyt b_2 (S_X)	0				[39]
S_X	6 ± 2			-52 ± 7	[62]
S_X	6 ± 2	-44 ± 8	-57 ± 9		[63]
H_i	-19 ± 5	-23 ± 10	-45 ± 12	-34 ± 10	[63]/*
$b_2$core (H_i)	-10 ± 5				[64]
deflavocyt b_2 (H_i)	-5 ± 5				[64]

E_{m7} (lactate/pyruvate) = -190 mV (Ref 71)

E_{m7} (free flavin) = -220 mV (Ref 72/73)

E_{m7} (ferri/ferro-cytochrome c) = 273 mV (Ref 74)

Table 1.7 Redox potentials of various forms of flavocytochrome b_2. All values are reported in mV. * = Capeillere-Blandin, unpublished results.

S_x = Saccharomyces cleaved form flavocytochrome b_2, H_i = Hansenula intact form flavocytochrome b_2, H_o = oxidised heme, H_r = reduced heme, F_o = oxidised flavin, F_{sq} = semiquinone flavin, F_r = reduced flavin.

semiquinone. At the end of phase I, up to 80% of the heme was reduced, up to 50% of the flavin was semiquinone and 25–35% was hydroquinone. The electron distribution was equivalent to 2 electrons per protomer. Phase II corresponded to the entry of a third electron. The model scheme includes reversibility of electron transfer between flavin and heme, and an interprotomer electron transfer between two flavin semiquinones, so that entry of a third electron pair in each dimer can be achieved to give full reduction of flavocytochrome b_2 (figure 1.9). The rate constants were generated by simulation analysis. The dismutation step was very slow and would therefore not be involved in catalytic turnover. The rate-determining step of the slow phase was considered to be related to a conformational change.

The high isotope effect obtained by reduction of flavocytochrome b_2 with 2-^{2}H-Lactate allowed Pompon *et al* to study flavin and heme reduction at a very low rate of electron entry [46]. The data obtained for cleaved flavocytochrome b_2 were analysed by computer simulation [47]. In addition to the one-electron interprotomer transfer between flavin semiquinones, the model according to Pompon allowed for interprotomer electron transfer between heme pairs and between flavin and heme. Since there was no evidence of cooperativity between protomers within a dimer, the initial entries of lactate were not assumed to be synchronised in the first step. (A simplification introduced in the model of Capeillere-Blandin [68]). Pompon devised three variations of his model, the interprotomer electron transfer being different in each: a) flavin to flavin; b) flavin to heme; and c) heme to heme. These models allow transient species to exist, for example one bound lactate or one reduced flavin per pair of active sites. For the detailed schemes of the three variants, see figure 1 of ref [47]. The two simulation models yield similar kinetic rates for the first steps: $k_{1\ max} = 120s^{-1}$, k_{+2} , k_{-2} estimated to be 600 and $120s^{-1}$ respectively, 24°C (Capeillere-Blandin [25]). $k_1 = 0.6–30s^{-1}$

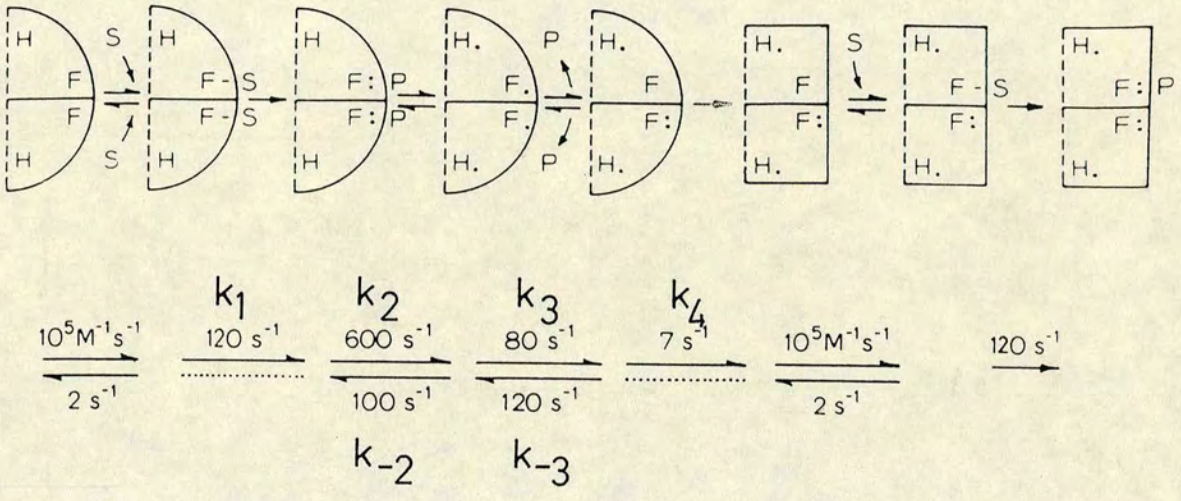


Figure 1.9 A kinetic model of flavocytochrome b_2 reduction. Each semicircle represents a flavocytochrome b_2 dimer during phase I, the entry of the first two electron pairs per dimer. After the flavin to flavin interprotomer electron transfer to give one flavin hydroquinone and one flavin semiquinone, the dimer undergoes a change in conformation (rectangles) to allow the entry of the third electron pair (phase II) to give fully reduced flavocytochrome b_2 [62,68].

(deuterated lactate), k_{+2} , k_{-2} = 500 and 120s^{-1} respectively, 5°C (Pompon [47]).

There is disagreement on the rates of the further steps leading to complete reduction of flavocytochrome b_2. Capeillere-Blandin proposed estimates of 80 and 120s^{-1} for k_{+3} and k_{-3} , 20°C, for the interprotomer dismutation reaction and k_4 = 7s^{-1} for the limiting irreversible step [68]. Pompon's model produces best estimates of 8 and 12s^{-1}, 4°C for the various interprotomer electron transfers [47].

To summarise, the two models agree on: 1) similar kinetic rate constants for the first two steps of electron transfer; 2) that irreversible flavin reduction by lactate is followed by a reversible electron transfer from flavin hydroquinone to an oxidised heme; 3) flavocytochrome b_2 is a three electron acceptor, but acts as a two-electron transferase in turnover. The main differences are: 1) The initial flavin reduction is synchronised for any given dimer in Capeillere-Blandin's model, whereas Pompon assumes this step is random; 2) There is an order of magnitude difference in interprotomer electron-transfer rates. Pompon also includes interprotomer transfer between heme pairs and flavin and heme; 3) The slow phase limiting step is an electron transfer between protomers in Pompon's model, whereas Capeillere-Blandin interprets this as a conformational change allowing a third lactate molecule to be oxidised per dimer.

Thus, the models describe the steps involved in enzyme turnover, but differ concerning the electron-transfer steps which facilitate full reduction of flavocytochrome b_2 in the absence of any acceptor. Full reduction would be unlikely to occur in this way *in vivo* since cytochrome *c* would always be present. It seems unlikely in view of the 3-D structural determination (section 1.4), that certain electron-transfer steps are possible (interprotomer heme → heme and flavin → heme), due to the distances involved. The two simulation models fail to take into account any mobility of the cytochrome domain,

relative to the flavin domains, which may occur during turnover. Any change in conformation may increase or decrease the probability of electron transfer between the prosthetic groups. Such a movement has been proposed (section 1.4).

1.5.5. Lactate Oxidation Mechanisms

Although the previous section details the kinetic rates of the overall electron-transfer steps, it does not describe the chemistry which actually takes place at the active site. In other words, how lactate is oxidised to pyruvate and how the resultant electrons are transferred to the oxidised flavin. Work carried out on homologous enzymes has led to theories as to how this might occur in flavocytochrome b_2. Walsh *et al* found that pyruvate was obtained as a by-product of the oxidation of β-chloroalanine to chloropyruvate by the flavoenzyme D-amino acid oxidase [75]. The product ratio was dependent on oxygen concentration, resulting exclusively in pyruvate when the reaction was carried out under nitrogen. Deuterium kinetic isotope measurements showed that C_α-H abstraction was partially rate limiting ($k_H/k_D \approx 2$). This was interpreted as evidence for a carbanion type mechanism, in which the initial step was C_α-H abstraction to form a carbanion or an adduct derived from a substrate carbanion and the flavin coenzyme. This intermediate would then be oxidised to form chloropyruvate, or undergo halogen elimination to form an enamine with subsequent ketonisation to yield pyruvate (figure 1.10). The analogous reaction of β-chlorolactate oxidation by the flavoenzyme lactate oxidase gave similar results [76] and Walsh proposed that these flavoenzymes catalysed the reactions by a common mechanism. Further evidence consistent with these proposals was obtained by inactivation studies of flavin oxidases with acetylenic substrates, where the carbanion intermediate can lead to a stable covalent adduct [77].

The suggestion of a carbanion mechanism prompted work with many

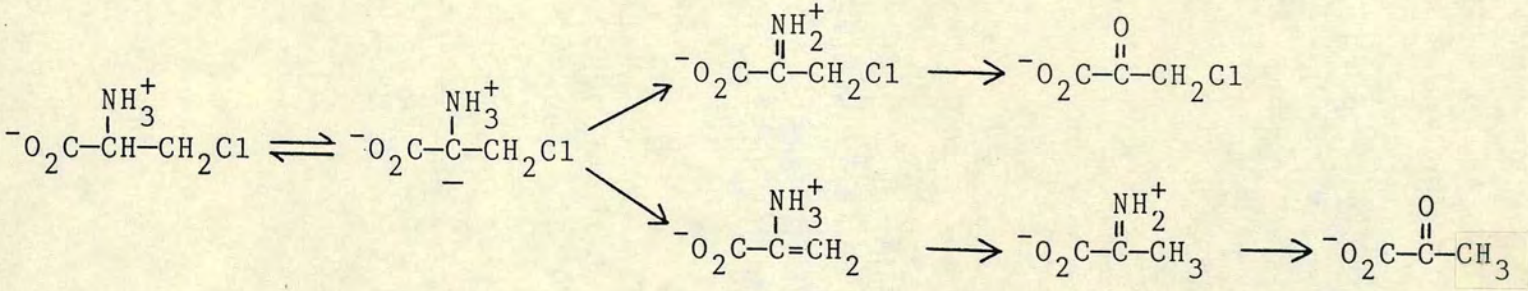


Figure 1.10 The oxidation of chloroalanine to chloropyruvate by D-amino acid oxidase. Halide elimination occurs from the carbanion intermediate to give pyruvate as a by-product [75].

enzymes and model analogues. Arguments for this mechanism, or the alternative radical and hydride transfer mechanisms, (where the α-hydrogen is abstracted as an H· radical leaving a substrate radical, or there is direct hydride transfer from the substrate to the flavin) have been extensively reviewed [78-83]. The evidence tends to support the carbanion mechanism, but does not provide conclusive proof.

A series of reactions similar to those previously mentioned were carried out to try to elucidate the mechanism involved with flavocytochrome b_2. Urban *et al* demonstrated that bromopyruvate could be reduced to bromolactate when it was incubated with flavocytochrome b_2 in the presence of excess lactate [84]. The heme group remained reduced throughout the reaction, indicating that the enzyme was acting as a simple transhydrogenase. With transhydrogenation of β-halogenosubstrates, with the exception of fluoride, halide elimination was found to be a competitive reaction [85]. The halide elimination was easier to detect in the reverse mode (α-keto substrates → α-hydroxy substrates) than the forward. A partition ratio of 500 was found between oxidation and halide elimination during the forward reaction (1 mole of pyruvate formed for every 500 moles of halopyruvate) compared to a value of 2 for the reverse reaction. Scheme I (figure 1.11) was proposed in which the substrate reduction pathway is the reverse mechanism of the normal oxidation. This scheme allows for a carbanion intermediate which could lead to halide elimination [85].

If for α-hydroxyacid oxidation, the C_α-H bond cleavage is considered to be rate limiting, then according to the principle of microreversibility, formation of a C_α-H bond in the reduction of α-ketoacids should be rate limiting. Deuterium kinetic isotope effects were measured for the reduction of α-keto-β-halogeno acids by flavocytochrome b_2, which had previosly been reduced with 2-^{2}H-lactate. A kinetic isotope effect of 4.4 was obtained in

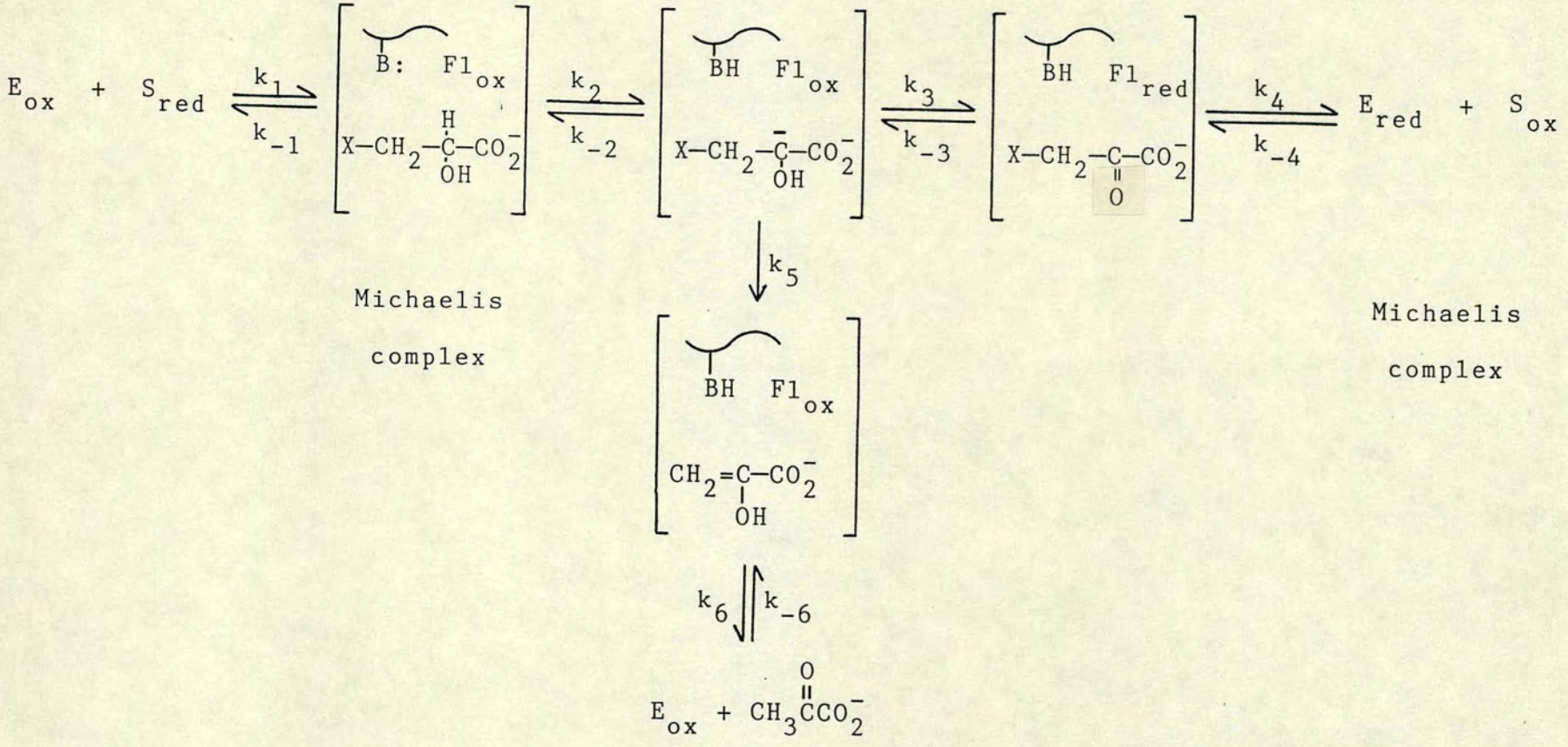


Figure 1.11 Scheme I, the proposed mechanism of halosubstrate oxidation by flavocytochrome b_2 via a carbanion intermediate.

agreement with a rate-limiting step as proposed above. Furthermore, on analysis of the rates of dehydrohalogenation with bromopyruvate, an inverse isotope effect was apparent [86]. This can easily be explained by scheme I. If the protonation of the carbanion intermediate, k_{-2} , is rate limiting, then with flavocytochrome b_2 reduced with 2-^{2}H-lactate, there will be more intermediate present for a longer period of time and the competitive dehydrohalogenation will become more favourable. This accounts for the partition ratios reported above.

When transhydrogenation reactions were carried out with 2-^{3}H-lactate and halogenopyruvate, tritium was found in water and on the halogenolactate at the C2 position [86], and when the halogenopyruvate underwent halide elimination, tritium was found in the C3 position. This could be explained if the tritium transiently resides on a monoprotic active site base after it has been abstracted to form the carbanion. Hydrogen exchange between this active site group and solvent was found to correlate with the level of free reduced enzyme.

Studies with the suicide inhibitor 2-hydroxy-3-butynoate presented further support for the involvement of a carbanion mechanism [56,87]. 2-hydroxy-3-butynoate can modify flavocytochrome b_2 in both the forward and reverse reaction. Partition ratios were measured as 3200 and 5 for the forward and reverse modes respectively. Scheme II (figure 1.12) analagous to the one for halogen elimination, was proposed, in which the intermediate carbanion can resonate to an allenic carbanion, which can form a covalent adduct. This adduct is highly reactive and cyclises to form a modified flavin group [79,87].

There is a lot of evidence then to support a carbanion mechanism, but how do the electrons transfer to the oxidised flavin? Three possibilities are: a) The substrate carbanion could attack the N5 position of the flavin in a

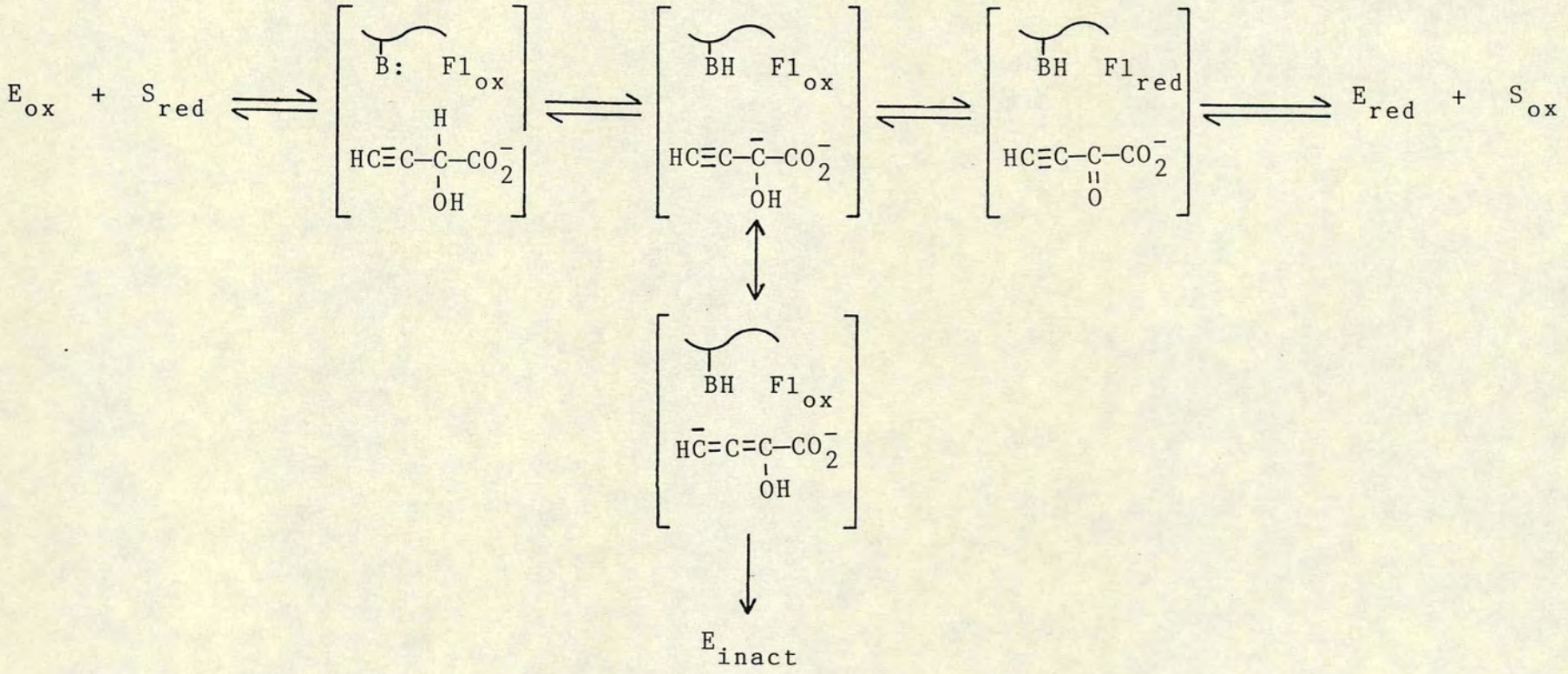


Figure 1.12 Scheme II, the oxidation of the suicide inhibitor 2-hydroxybutynoate. Inhibition occurs by nucleophilic attack of the flavin by the highly reactive allenic anion.

nucleophilic manner to form a covalent adduct, with subsequent cleavage reducing the flavin; or b) One-electron transfer followed by collapse of the radical pair to form a covalent adduct; or c) by a second one-electron transfer to give oxidised substrate and reduced flavin (figure 1.13) [78-80,88]. Differentiation between direct nucleophilic attack and a radical mechanism to form a covalent intermediate could be difficult if the collapse of the radical pair was fast [88].

By considering the solution studies mentioned above, and the refined 3-D structure of the flavocytochrome b_2 active site, Lederer and Mathews proposed a scheme for the reverse reaction, the reduction of pyruvate [89] (see figure 1.4). Electrons are transferred from the N5 position of the dianion form of reduced flavin to the pyruvate carbonyl function. The authors were unable to decide whether the reduced flavin existed as the dianionic form in the free reduced enzyme, or whether it was protonated and the proton was expelled on pyruvate binding. Also they did not discuss how the transfer of electrons would take place, except to say that the structure did not rule out the possibility of a covalent intermediate. On electron transfer to the C2 position, the substrate becomes tetrahedral. The carbonyl oxygen would be protonated by Y254 and the final (rate limiting) step would be protonation of the carbanion by H373. Recently, Urban and Lederer have shown by fluoropyruvate inactivation and radioactive labelling studies, that H373 is indeed involved in the α-hydrogen abstraction/protonation step [90]. Ghisla and Massey disagreed with Lederer and Mathews' interpretation [88]. They considered the anionic N5 to be too close to the pyruvate carbonyl (3.7Å) without the formation of a covalent adduct taking place. Covalent intermediates have been observed between substrates and flavin for lactate oxidase [82,91] and D-amino acid oxidase [92], but to date there has been no evidence for a covalent link to substrate with flavocytochrome b_2. Ghisla and Massey proposed a mechanism

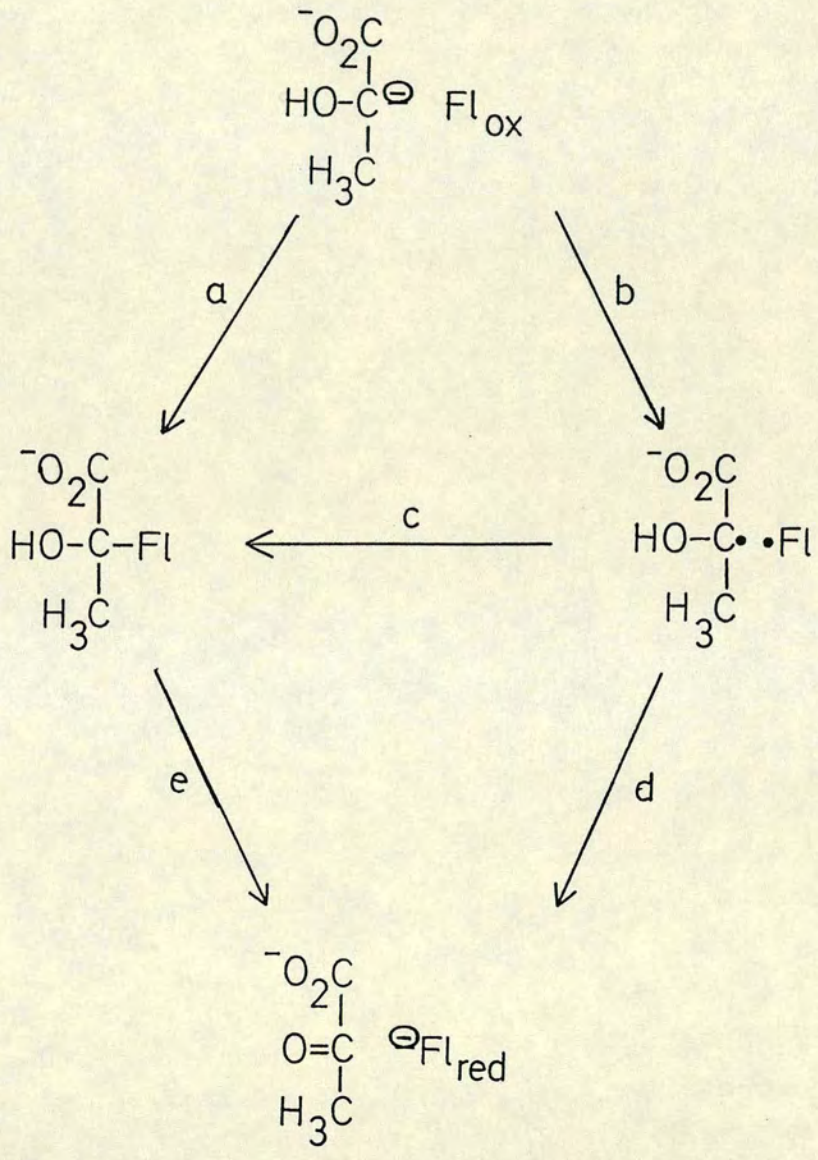


Figure 1.13 The alternate routes of electron transfer from the substrate carbanion to the oxidised flavin. Oxidation of substrate can occur by a two-electron transfer via a covalent intermediate (a+e) or by formation of a radical pair (b) followed by a second one-electron transfer to give the covalent intermediate (c) or directly to give the reduced flavin (d).

in which a transient covalent intermediate, analogous to those observed with lactate oxidase and D-amino acid oxidase, is formed.

In contrast to the above results, there are a set of experiments which are inconsistent with a carbanion mechanism. The deazaflavins are a series of synthetic flavin analogues in which one of the nitrogens has been replaced by a methyne bridge (CH) [93,72,73,79,94]. It was hoped that 5-deaza-FMN would act as a probe for the mechanism of action of flavocytochrome b_2 [95]. Studies using flavocytochrome b_2 incorporated with 5-deaza-FMN would help to distinguish between hydride and carbanion mechanisms: if a covalent adduct was formed along the reaction pathway, the substrate-flavin bond with 5-deaza-FMN would not be prone to cleavage to yield products; any incorporation of the C_α-H onto the flavin would support a hydride mechanism rather than a carbanion one (figure 1.14). However, although flavocytochrome b_2 reconstituted with 5-deaza-FMN was reducible by lactate (very slowly) and oxidisable by pyruvate, electrons could not be transferred to the heme or to ferricyanide, ie the 5-deaza-FMN could not stabilise the semiquinone radical. 5-deaza-flavocytochrome b_2 is expected to have poor transhydrogenase activity and not to stabilise the semiquinone since the redox potentials for oxidised → hydroquinone and oxidised → semiquinone are -233mV (enzyme-bound) [95], and -770mV (enzyme-free) [96] compared to -52mV [62] and -44mV [63] (both enzyme bound). More importantly, there was no evidence for a covalent adduct between substrate and 5-deaza-FMN even for 2-hydroxy-3-butynoate, the suicide inhibitor. Furthermore, labelling studies with tritium show that substrate α-hydrogen is incorporated onto the flavin analogue. Similarly there was no halide elimination or adduct formation with 5-deazaflavin lactate oxidase [97].

Hemmerich has suggested that deazaflavins should be regarded as analogues of flavin-shaped pyridine nucleotides and not actual flavins [94,98].

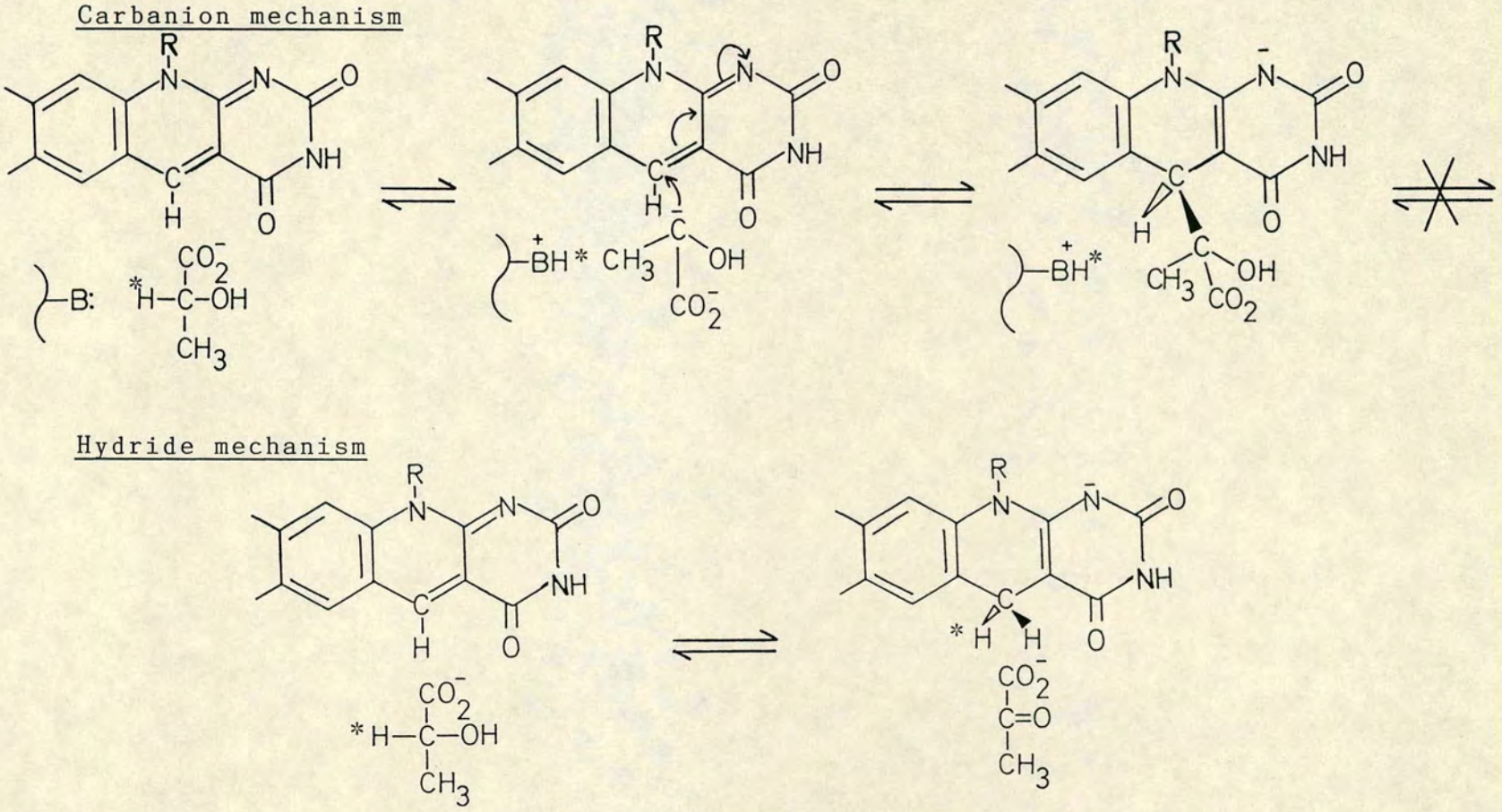


Figure 1.14 The carbanion mechanism versus the hydride mechanism for 5-deaza-FMN. Substrate oxidation by the carbanion mechanism would result in a trapped covalent intermediate, whereas oxidation by the hydride mechanism would result in a transfer of tritium from the substrate to the flavin C5 position.

Lederer and Mathews argue that the protein may switch from a carbanion to a hydride mechanism when flavin is replaced by deazaflavin [89]. Ghisla and Massey consider that an enzyme active centre switching to the opposite mechanism on changing to the flavin analogue is "a rather unlikely possibility" [99]. Thus the deazaflavins pose a "mechanistic dilemma" for the flavoproteins which are proposed to operate via a carbanion mechanism.

In conclusion, from all the solution studies carried out and the information available from the highly refined 3-D structure of the active site, a mechanism of flavin reduction/substrate oxidation can be proposed (figure 1.15). The ketosubstrate reduction is simply the reverse of this mechanism, and in the case of halogenosubstrates, the halide elimination occurs from B, and the rate-limiting step in either direction is k_2/k_{-2} (figure 1.15).

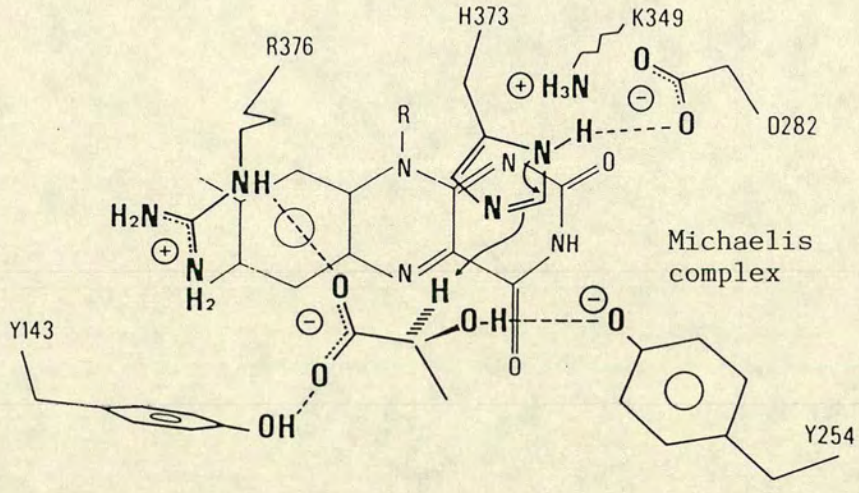
1.6. Flavocytochrome b_2 Interaction With Cytochrome *c*

As mentioned in section 1.5, dehemoflavocytochrome b_2 has no cytochrome *c* reductase activity, [67] but retains some of its ferricyanide reductase activity, implying that the heme is essential for cytochrome *c* reduction and that the two acceptors are reduced by a different mechanism.

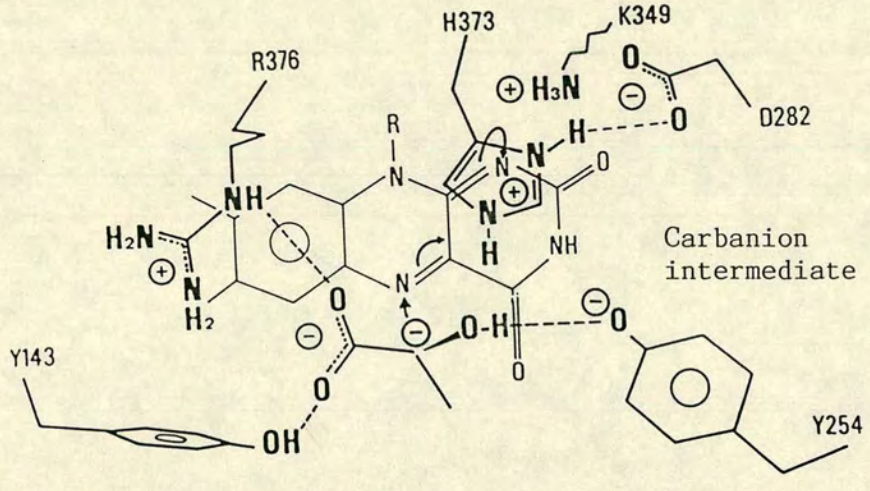
Cytochrome *c* is known to form a stable complex with flavocytochrome b_2 in solution and in the crystalline state [100-104], with $K_d = 10^{-8}M$ for cleaved flavocytochrome b_2. The complex stability is strongly dependent upon ionic strength and pH (the ratio of cytochrome *c* : flavocytochrome b_2 increases as pH is lowered or ionic strength decreased) suggesting that the interaction is largely due to electrostatic effects. Baudras *et al* obtained a flavocytochrome b_2:cytochrome *c* heme ratio of 4 [101,102] (one cytochrome *c* per flavocytochrome b_2 tetramer) using cleaved enzyme when studying the protein complex in both solution and crystalline states. Complex formation in solution was monitored using ultracentrifugation studies in sucrose gradients. Yoshimura *et al* also obtained a heme ratio of 4 using flavocytochrome b_2

$E_{ox} + S_{red}$

$k_1 \rightleftharpoons k_{-1}$



$k_2 \rightleftharpoons k_{-2}$ Rate Determining Step



$k_3 \rightleftharpoons k_{-3}$

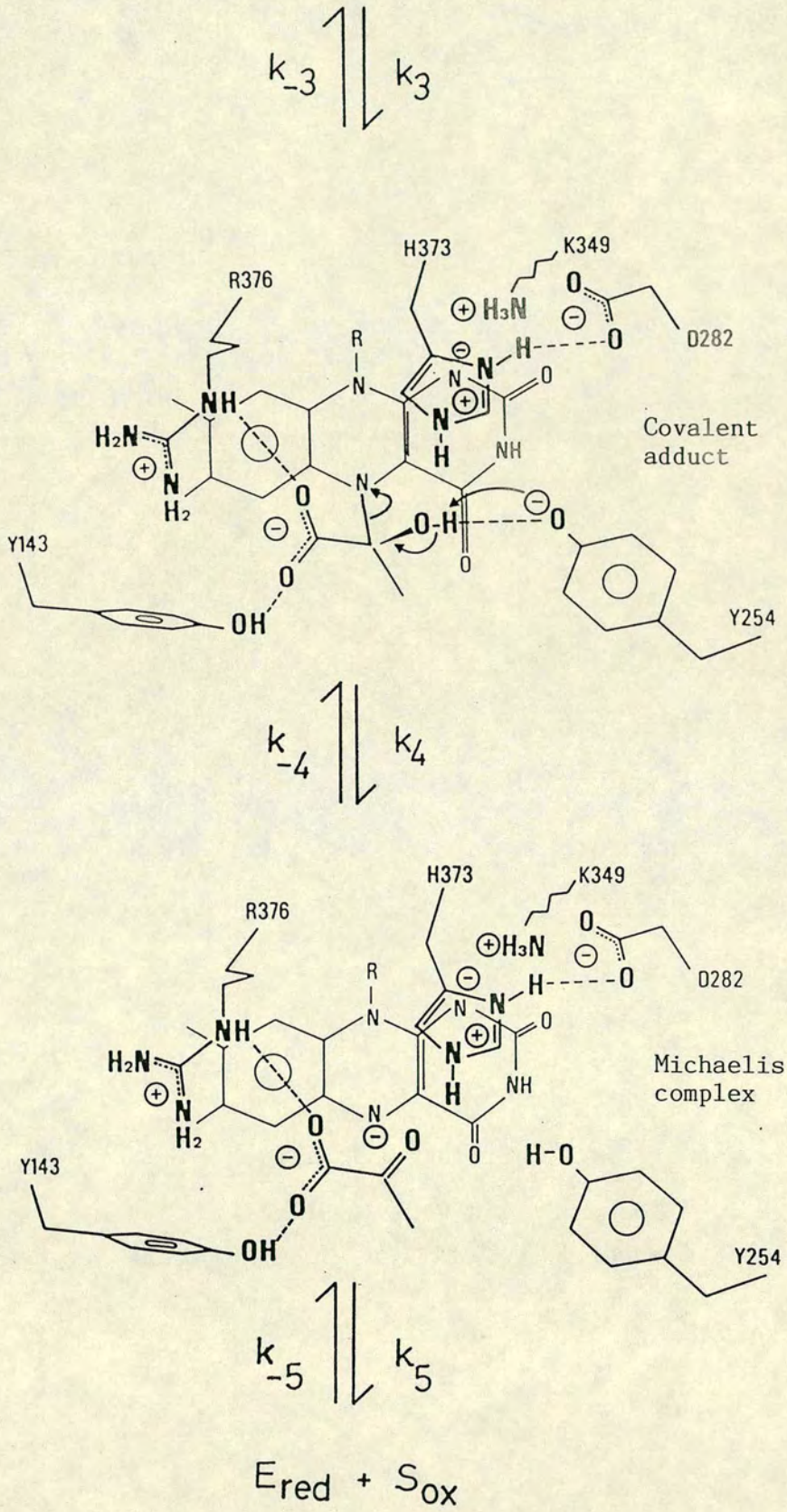


Figure 1.15 The proposed mechanism of lactate oxidation by flavocytochrome b_2.

purified by Hasegawa's method (thought to be cleaved type enzyme since it forms the same trigonal crystals as type II, DNA-free enzyme) but observed that the ratio decreased to 2 in the presence of free cytochrome *c*. These ratios were measured using ultracentrifugal and gel chromatographical analyses. Tegoni *et al* diffused cytochrome *c* through crystals of intact flavocytochrome b_2 and obtained a heme ratio of 1.0 [100].

Crystals of a flavocytochrome b_2/cytochrome *c* complex have been made either by diffusing cytochrome *c* into flavocytochrome b_2 crystals [100], or by co-crystallising flavocytochrome b_2 and cytochrome *c* [101-103]. However, no flavocytochrome b_2/cytochrome *c* crystals were formed in the earlier purification procedures by Morton and Appleby [5], when crystallisation was used as a purification technique to separate flavocytochrome b_2 from cytochrome *c* and other proteins. In the former case, the crystals of intact flavocytochrome b_2 were washed, and then soaked in a solution of cytochrome *c* at low ionic strength. The cytochrome *c* diffused through the large solvent channels (100Å diameter) to form a complex of one molecule of cytochrome *c* to every subunit of flavocytochrome b_2. If the crystals were then placed in a buffer of high ionic strength, cytochrome *c* would diffuse out of the crystals into the buffer solution. Both flavocytochrome b_2 and cytochrome *c* could be oxidised by ferricyanide and when washed and soaked in a lactate solution, both enzymes became reduced, giving direct evidence of a productive interaction between the two enzymes within the crystalline state, since lactate cannot reduce cytochrome *c* directly. The enzyme system could turnover without cracking or disrupting the crystals. However, these crystals are not good enough for X-ray diffraction, probably due to cytochrome *c* being located in a variety of positions [105].

Cytochrome *c* has been the subject of many studies to determine how it recognises and binds to other proteins [106-111]. Models of protein-protein

interactions between cytochrome *c* and a variety of other proteins have been proposed. In all these studies, it has been concluded that the three or four lysine residues close to the exposed heme edge are important. This is also the case for the flavocytochrome b_2/cytochrome *c* interaction. Rates of electron transfer between flavocytochrome b_2 and a series of singly modified cytochromes *c* have been measured, indicating that the lysines located near to the exposed heme edge play a role in the protein–protein interface [112].

Finding the region on flavocytochrome b_2 where cytochrome *c* binds has not yet been accomplished. There are no patches of negative charge similar to those on cytochrome *c* oxidase or cytochrome b_5 (see section 1.7.2). The "front" face of the flavocytochrome b_2 cytochrome domain is largely covered by the flavin domain, so a complex of the type seen for cytochrome b_5 and cytochrome *c* as predicted by Salemme [107], is difficult to envisage unless there is a very large conformational change between the cytochrome and flavin domains. This seems unlikely in view of the large amounts of energy that would be required. In a fluorescence study by Vanderkooi *et al*, it was shown that the metalloporphyrin fluorescence in Zn(II) and Sn(IV) cytochromes *c* was affected by the flavocytochrome b_2 heme [113], and a distance of about 18Å between the heme centres was proposed. This distance can be compared with distances proposed for other cytochrome *c*–protein complexes (table 2. of Ref.113)

1.7. Comparison With Other Proteins

1.7.1. *H. anomala* Flavocytochrome b_2

Flavocytochrome b_2 from *Hansenula anomala* (*H.a.*b_2) was first discovered by F.Pichinoty when he observed that crude extracts from the yeast had very high lactate dehydrogenase activity. By 1967 Baudras had developed a procedure for obtaining pure enzyme [114]. *H.a.*b_2 is much more stable to

indigenous proteases, so the enzyme has always been isolated as an intact form [114], although Gervais *et al* have shown that a cleaved form of $H.a.b_2$ can be generated by the action of clostripain and trypsin [115]. As expected, $H.a.b_2$ shows many similarities to flavocytochrome b_2 from *S. cerevisiae* ($S.c.b_2$). The recently deduced amino-acid sequence shows an overall identity of 60% with many areas well conserved (figure 1.16) [36]. All of the amino acids identified as potentially important in functionality by Lederer and Mathews [89] are identical. However, it is the differences between the two enzymes which are most interesting. There are two regions of the enzyme where the sequence differs greatly from $S.c.b_2$. The first is the crystallographically disordered loop (residues 300-310 in $S.c.b_2$) where the charge is -6 compared to +4 for $S.c.b_2$ and the second is the short loop which forms a hinge joining the cytochrome domain to the flavin-binding domain, where the charge is -6 compared to -1. As already mentioned, the enzyme is more stable to proteolysis and is also expressed in higher quantities in *H. anomala* (500-1000mg Λ per kg of cells compared to 30-50mg from *S. cerevisiae* [29]) making purification easier. The specific activity is higher, $1000s^{-1}$ Λ mol^{-1} compared to $550s^{-1}$ Λ mol^{-1} at 30°C [29]. The differences in kinetic properties may be due to the change of charge in the two surface loops.

A kinetic analysis by Capeillere-Blandin *et al* has shown that there is a detectable lag phase (1.3 $\pm$ 0.5ms) between flavin and heme reduction, indicating that lactate oxidation or in particular α-hydrogen abstraction from lactate is no longer rate limiting and that intramolecular electron transfer from flavin to heme is partly rate limiting [63]. Kinetic isotope studies with 2-^{2}H-lactate should verify this. With flavin reduction now faster than intramolecular electron transfer, a more accurate evaluation of electron-transfer rate constants can be calculated from simulation studies. Preliminary values calculated at 5°C from an incomplete simulation (the model

Figure 1.16

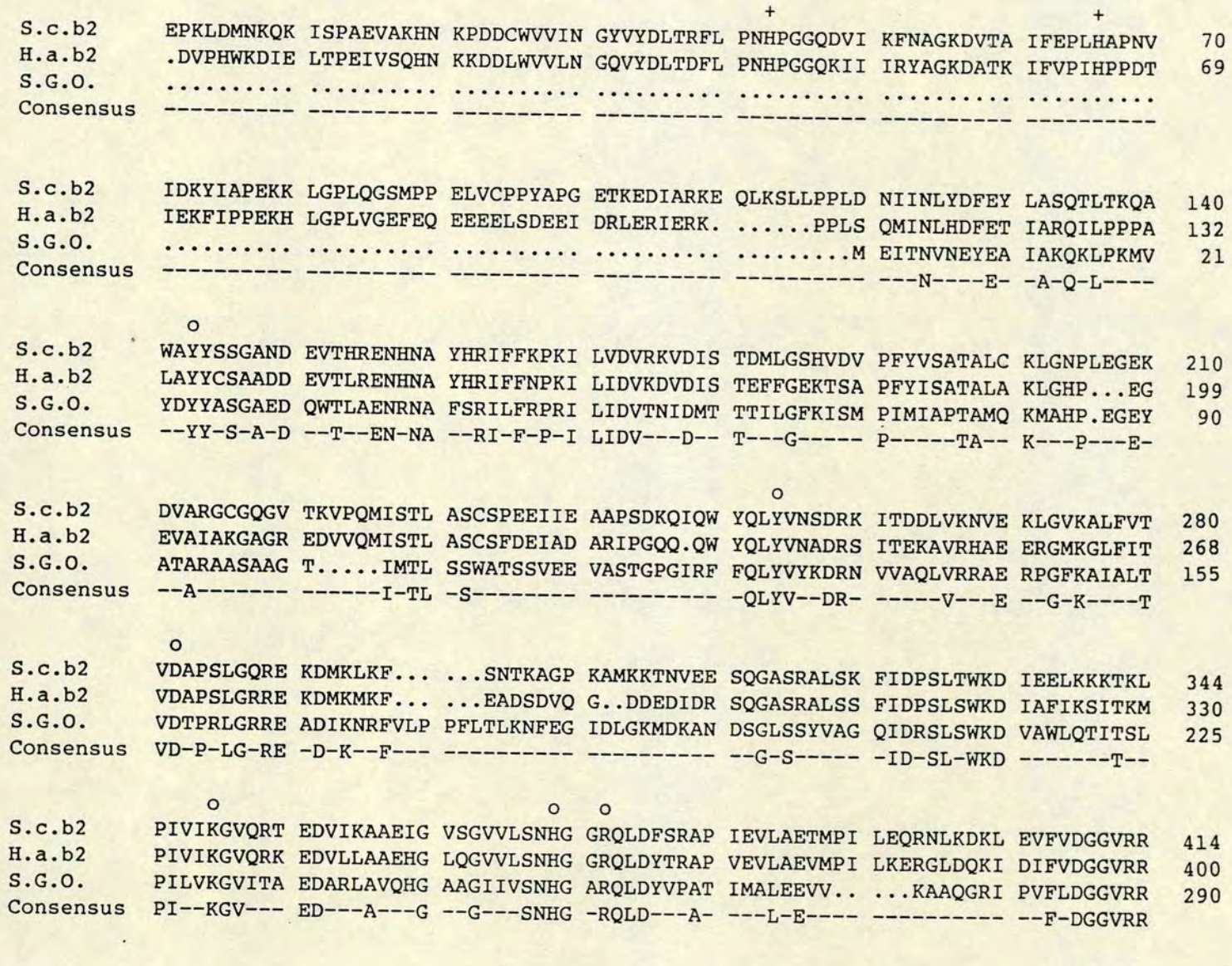

S.c.b2 EPKLDMNKQK ISPAEVAKHN KPDDCWVVIN GYVYDLTRFL PNHPGGQDVI KFNAGKDVTA IFEPLHAPNV 70
H.a.b2 .DVPHWKDIE LTPEIVSQHN KKDDLWVVLN GQVYDLTDFL PNHPGGQKII IRYAGKDATK IFVPIHPPDT 69
S.G.O. ....
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ----------
S.c.b2 IDKYIAPEKK LGPLQGSMPP ELVCPPYAPG ETKEDIARKE QLKSLLPPLD NIINLYDFEY LASQTLTKQA 140
H.a.b2 IEKFIPPEKH LGPLVGEFEQ EEEELSDEEI DRLERIERK. ......PPLS QMINLHDFET IARQILPPPA 132
S.G.O. ....M EITNVNEYEA IAKQKLPKMV 21
Consensus ---------- ---------- ---------- ---------- ---N----E- -A-Q-L----
S.c.b2 WAYYSSGAND EVTHRENHNA YHRIFFKPKI LVDVRKVDIS TDMLGSHVDV PFYVSATALC KLGNPLEGEK 210
H.a.b2 LAYYCSAADD EVTLRENHNA YHRIFFNPKI LIDVKDVDIS TEFFGEKTSA PFYISATALA KLGHP...EG 199
S.G.O. YDYYASGAED QWTLAENRNA FSRILFRPRI LIDVTNIDMT TTILGFKISM PIMIAPTAMQ KMAHP.EGEY 90
Consensus --YY-S-A-D --T--EN-NA --RI-F-P-I LIDV---D-- T---G----- P-----TA-- K---P---E-
S.c.b2 DVARGCGQGV TKVPQMISTL ASCSPEEIIE AAPSDKQIQW YQLYVNSDRK ITDDLVKNVE KLGVKALFVT 280
H.a.b2 EVAIAKGAGR EDVVQMISTL ASCSFDEIAD ARIPGQQ.QW YQLYVNADRS ITEKAVRHAE ERGMKGLFIT 268
S.G.O. ATARAASAAG T.....IMTL SSWATSSVEE VASTGPGIRF FQLYVYKDRN VVAQLVRRAE RPGFKAIALT 155
Consensus --A------- ------I-TL -S-------- ---------- -QLYV--DR- -----V---E --G-K----T
S.c.b2 VDAPSLGQRE KDMKLKF... ...SNTKAGP KAMKKTNVEE SQGASRALSK FIDPSLTWKD IEELKKKTKL 344
H.a.b2 VDAPSLGRRE KDMKMKF... ...EADSDVQ G..DDEDIDR SQGASRALSS FIDPSLSWKD IAFIKSITKM 330
S.G.O. VDTPRLGRRE ADIKNRFVLP PFLTLKNFEG IDLGKMDKAN DSGLSSYVAG QIDRSLSWKD VAWLQTITSL 225
Consensus VD-P-LG-RE -D-K--F--- ---------- ---------- --G-S----- -ID-SL-WKD -------T--
S.c.b2 PIVIKGVQRT EDVIKAAEIG VSGVVLSNHG GRQLDFSRAP IEVLAETMPI LEQRNLKDKL EVFVDGGVRR 414
H.a.b2 PIVIKGVQRK EDVLLAAEHG LQGVVLSNHG GRQLDYTRAP VEVLAEVMPI LKERGLDQKI DIFVDGGVRR 400
S.G.O. PILVKGVITA EDARLAVQHG AAGIIVSNHG ARQLDYVPAT IMALEEVV.. ...KAAQGRI PVFLDGGVRR 290
Consensus PI--KGV--- ED---A---G --G---SNHG -RQLD---A- ---L-E---- ---------- --F-DGGVRR

Figure 1.16 contd

```
S.c.b2      GTDVLKALCL GAKGVGLGRP FLYANSCYGR NGVEKAIEIL RDEIEMSMRL LGVTSIAELK PDLLDLSTLK  484
H.a.b2      GTDVLKALCL GAKGVGLGRP FLYAMSSYGD KGVTKAIQLL KDEIEMNMRL LGVNKIEELT PELLDTRSIH  470
S.G.O.      GTDVFKALAL GAAGVFIGRP VVFSLAAEAE AGVKKVLQMM RDEFELTMAL SGCRSLKEIS RSHIAADWDG  360
Consensus   GTDV-KAL-L GA-GV--GRP ---------- -GV-K----- -DE-E--M-L -G-----E-- ----------

S.c.b2      ARTVGVPNDV LYNEVYEGPT LTEFEDA*       511
H.a.b2      NRAVPVAKDY LYEQNYQRMS GAEFRPGIED *   500
S.G.O.      PSSRAVARL*                           369
Consensus   -----V---- ---------- ----------
```

Figure 1.16 Amino-acid sequence alignment between flavocytochromes b_2 from *S. cerevisiae* (*S.c.*b_2), *H. anomala* (*H.a.*b_2) and spinach glycolate oxidase (SGO) [34,36,145]. + = 5th and 6th position ligands to the heme iron in *S.c.*b_2 and *H.a.*b_2. o = amino-acid residues which are thought to be important in the mechanism of substrate oxidation.

only simulates for phase I, the entry of one electron pair) are listed in table 1.8. It was suggested that the corresponding values for *S.c.*b_2 would be the same. The one-electron transfer from fully reduced *H.a.*b_2 to cytochrome *c* has been studied by Capeillere-Blandin using stopped-flow spectrophotometry [116]. Using further simulation studies, the rate of electron transfer at 5°C was estimated to be $380s^{-1}$. Using temperature-jump techniques, Tegoni *et al* studied the electron transfer from flavin semiquinone to oxidised heme at 16°C (pH 7.0) [117]. The rate calculated was $160s^{-1}$, but in view of the large errors involved in the calculation, it was considered that the rate corresponded to the value of k_{cat} at 16°C ie $225s^{-1}$.

The whole electron-transfer scheme for *H.a.*b_2 from lactate through to cytochrome *c* at 16°C was summarised by Tegoni *et al* as follows [117]: Step 1) An irreversible two-electron transfer from bound lactate to flavin ($k_1 \approx 2000s^{-1}$ [118]). Step 2) A reversible one-electron transfer from flavin hydroquinone to oxidised heme ($k_2 \approx 600s^{-1}$ and $k_{-2} \approx 160\text{-}200s^{-1}$ [119]. Step 3) A one-electron oxidation of heme by cytochrome *c* ($k_3 \approx 550s^{-1}$ [116]) which is never rate limiting in turnover under normal assay conditions. The products of the first three steps are flavin semiquinone, oxidised heme and a molecule of reduced cytochrome *c*. Step 4) A reversible electron transfer from flavin semiquinone to oxidised heme (k_4 unknown). Step 5) The oxidation of the reduced heme by a second molecue of cytochrome *c*. Its rate is assumed to be the same as that of step 3, ie $550s^{-1}$. The value of k_{cat} at 16°C is $225s^{-1}$ [118]. k_4 should be at least equal to $225s^{-1}$ or even higher if the rate-limiting step in turnover was not step 4 but for example pyruvate dissociation.

Tegoni *et al* [120,121] observed that pyruvate, the product of lactate oxidation, stabilises the semiquinone form of the flavin. Dissociation constants of pyruvate were estimated to be 10 and 20mM for oxidised and reduced flavins respectively and 0.2mM for semiquinone [122]. The redox potentials of

Enzyme	Flavin		Heme	
	Rate constant (s^{-1})		Rate constant (s^{-1})	
	Phase I	Phase II	Phase I	Phase II
H.a.b_2	360 ± 40	30 ± 7	170 ± 15*	15 ± 6
S.c.b_2	42 ± 4	2 ± 1.5	35 ± 4	1.5 ± 0.3

Table 1.8 Rates of electron transfer for *S.c.*b_2 and *H.a.*b_2. Phase I is the fast rate of electron transfer corresponding to the entry of the first two electron pairs per flavocytochrome b_2 dimer. Phase II corresponds to the entry of the third electron pair giving fully reduced flavocytochrome b_2. A lag of 1.3 ± 0.5ms was observed for *H.a.*b_2 heme reduction indicating that the rate-limiting step has changed from lactate oxidation to flavin to heme electron transfer.

the two one-electron flavin reductions (oxidised → semiquinone and semiquinone → hydroquinone) change from −16mV and −60mV to +74mV and −133mV in the presence of 10mM pyruvate. The two-electron reduction of flavin (oxidised → hydroquinone) changes slightly from −54mV to −31mV while the heme (oxidised → reduced) potential remains constant within experimental error at ≈ −17mV. It is clear from these data that a bound molecule of pyruvate would block the electron transfer from semiquinone to heme. One interpretation of this is that product inhibition may be a form of metabolic regulation of lactate oxidation. This work has not been repeated with *S.c.*b_2, but it is thought that a similar result would be obtained.

S.c.b_2 has been shown to form a complex with cytochrome *c* with a heme ratio varying between 1 and 4. However, *H.a.*b_2 forms a more stable complex with the ratio always being 1.0 (at low ionic strength) [123].

see p43

1.7.2. The Cytochrome Domain and Related Proteins

As mentioned above, cytochrome b_2 core shows a great deal of homology to cytochrome b_5. The two proteins have molecular weights of around 11kDa and give very similar absorbance spectra [124]. Both proteins are members of a family of proteins which all share a common fold, the so-called "cytochrome b_5 fold" [125]. This family of proteins include: cytochrome b_5 from a variety of species including *S. cerevisiae* [126−130]; cytochrome b_2 core from *S. cerevisiae* and *H. anomala* ; sulphite oxidase from chicken, rat and bovine liver [131−133]; and assimilatory nitrate reductase from tobacco, *Neurospora crassa* and *H. anomala* [134−136]. Tables of amino-acid sequence alignment [125,134] show a high level of sequence homology. There are 13 residues which are invariant in the proteins. These include the two iron ligands histidines 43 and 66 (using the flavocytochrome b_2 numbering scheme), W26 whose indole ring stacks parallel to the imidazole ring of H19 (H19 conserved in all members of the b_5 family except in rat mitochondrial outer membrane

cytochrome b_5 where it is replaced by arginine [127]) quenching the tryptophan fluorescence [137], and a three amino acid segment (P44, G45, G46) which forms a bend in the Cα backbone, reversing the direction of the chain. Table 1.9 summarises some of the sequence information available for some of the enzymes. When the three dimensional structure of cytochrome b_5 (2.0Å) [138] is superimposed with the heme-binding domain of flavocytochrome b_2, the two proteins show a close alignment [38]. An X-ray diffraction study of the isolated cytochrome b_2 core is presently being carried out [139]. In cytochrome b_5 the heme is rotated by about 30° compared to the heme orientation of flavocytochrome b_2, pointing one of the heme propionates back towards the protein and partially shielding it from solvent [6]. It is clear from the sequences that these proteins are evolutionary products of a common ancestor.

In Salemme's model of the cytochrome b_5/cytochrome *c* complex [107], three negatively charged amino acids from b_5 (E44, E48 and D60) were thought to interact electrostatically with positively charged lysines around the cytochrome exposed heme edge. Of these three amino acids, only one (E44) is conserved in any of the other proteins. The rest have been changed non-conservatively. This makes complexes analogous to Salemme's b_5/c model difficult to envisage with the other members of the protein family, unless the heme propionates become involved as the remaining two negative charges.

1.7.3. The Flavodehydrogenase Domain and Related Proteins

There are three FMN-binding enzymes of known structure which have been shown by structural comparisons to be homologous to each other. The three are flavocytochrome b_2, glycolate oxidase from spinach [140] and trimethylamine dehydrogenase from the methylotrophic bacterium W_3A_1 [141]. All three structures are based on a $\beta_8\alpha_8$ barrel motif with loop and helical extensions. Trimethylamine dehydrogenase covalently binds FMN (via position

Enzyme	$b_2$core (S.c.)	$b_2$core (H.a.)	b_5 mc (beef)	b_5 om (rat)	b_5 mc (rat)	NR (tobacco)	SO (chicken)
$b_2$core (S.c)	-	53.3	30.6	36.5	31.8	33.3	32.1
$b_2$core (H.a)	49/92	-	32.9	31.8	32.9	28.2	28.6
b_5 mc (beef)	26/85	28/85	-	60.5	88.5	37.6	32.9
b_5 om (rat)	31/85	27/85	52/86	-	59.8	37.2	31.0
b_5 mc (rat)	27/85	28/85	77/87	52/87	-	37.2	34.5
NR (tobacco)	29/87	24/85	32/85	32/86	32/86	-	31.8
SO (chicken)	27/84	24/84	28/85	27/87	30/87	27/85	-

Table 1.9 Sequence identities between the b_5 family. The top half of the table lists the percentage homologies. The bottom half of the table lists the homologies in the form 'x/y', where 'x' = the number of identities in 'y' comparable positions.

6 and a cysteine side-chain [142]), whereas in the other two enzymes the FMN is non-covalently bound. Figure 1.17 shows a comparison of the structures. It can be seen that flavocytochrome b_2 is more closely related to glycolate oxidase than to trimethylamine dehydrogenase. Glycolate oxidase shows 39% and 40% sequence identities with the flavin-binding domains of *S.c.*b_2 and *H.a.*b_2 (figure 1.16). The amino-acid sequence of trimethylamine dehydrogenase has not yet been determined. The $\beta_8\alpha_8$ structural motif has been found in a variety of enzymes usually of different function and very different amino-acid sequences. The active sites of these enzymes are all located at the carboxy end of the β barrel. For reviews of the αβ barrel enzymes see refs 143 and 144.

The recently determined sequence of glycolate oxidase [145] has been fitted to the previously determined electron density map [146]. The refined model gives an active site description which is very similar to the one in flavocytochrome b_2. There are no high resolution data available for substrate bound enzyme, but a model of the active site with a molecule of glycolate in an assumed position shows several protein-substrate interactions already described for flavocytochrome b_2 (figure 1.18). In the model, the glycolate carboxylate group forms an ionic bond with the guanidinium group of R257; one of the carboxylate oxygens hydrogen bonds to Y24; the hydroxyl goup attached to C2 position of the glycolate molecule hydrogen bonds to Y129, and the C2 position is close in space to H254 (which is hydrogen bonded to D157). There is very little mechanistic information available for glycolate oxidase, but presumably the glycolate molecule is oxidised in a similar manner to that of lactate by flavocytochrome b_2 (section 1.5.5). As with flavocytochrome b_2, the N1-O2 region of the flavin interacts with a lysine, K230, presumably to stabilise the anionic form of the reduced flavin.

L-2-hydroxy acid oxidase from rat kidney is also an FMN-binding protein,

The dotted line between helices E and F represents the disordered loop.

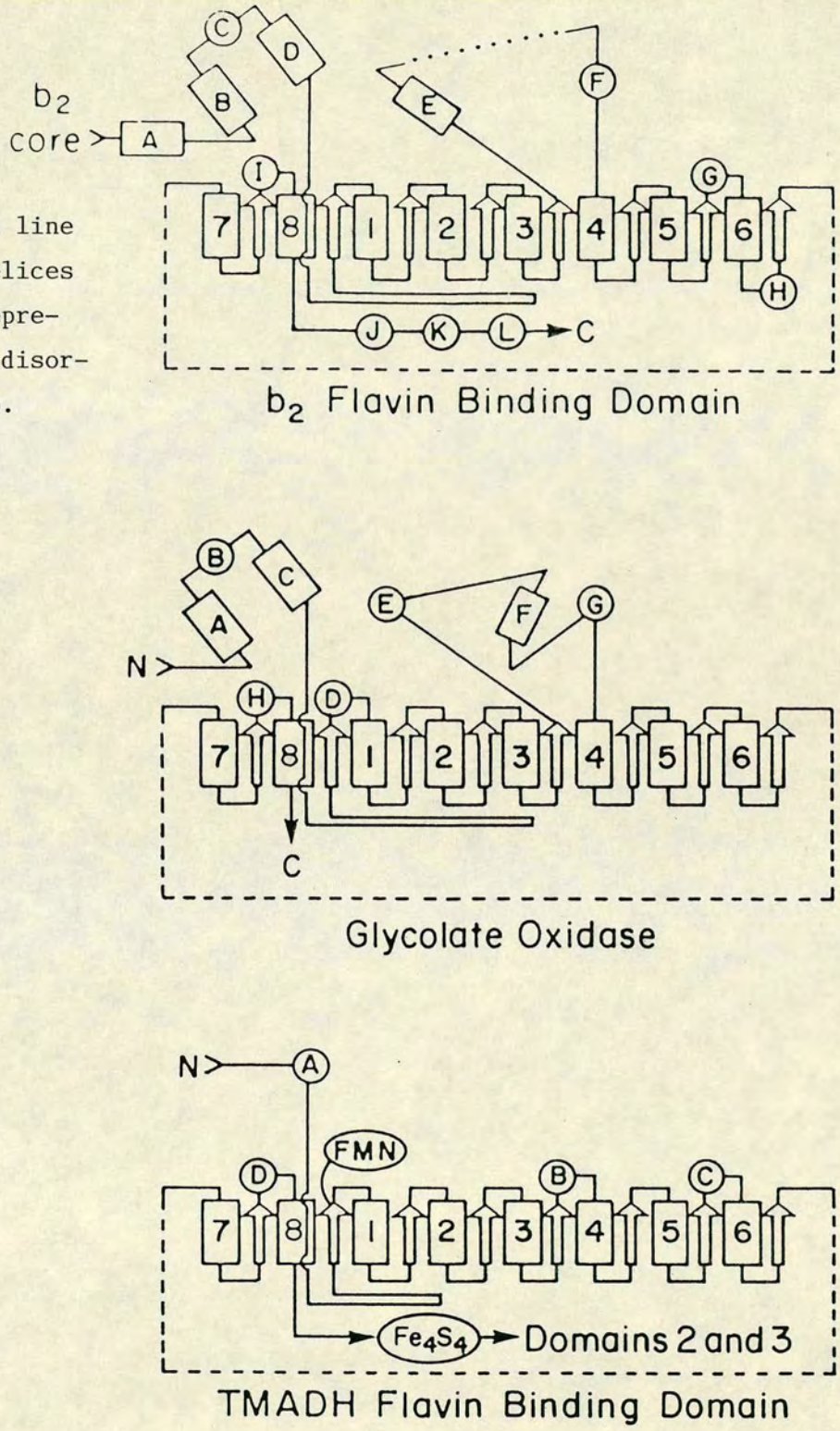
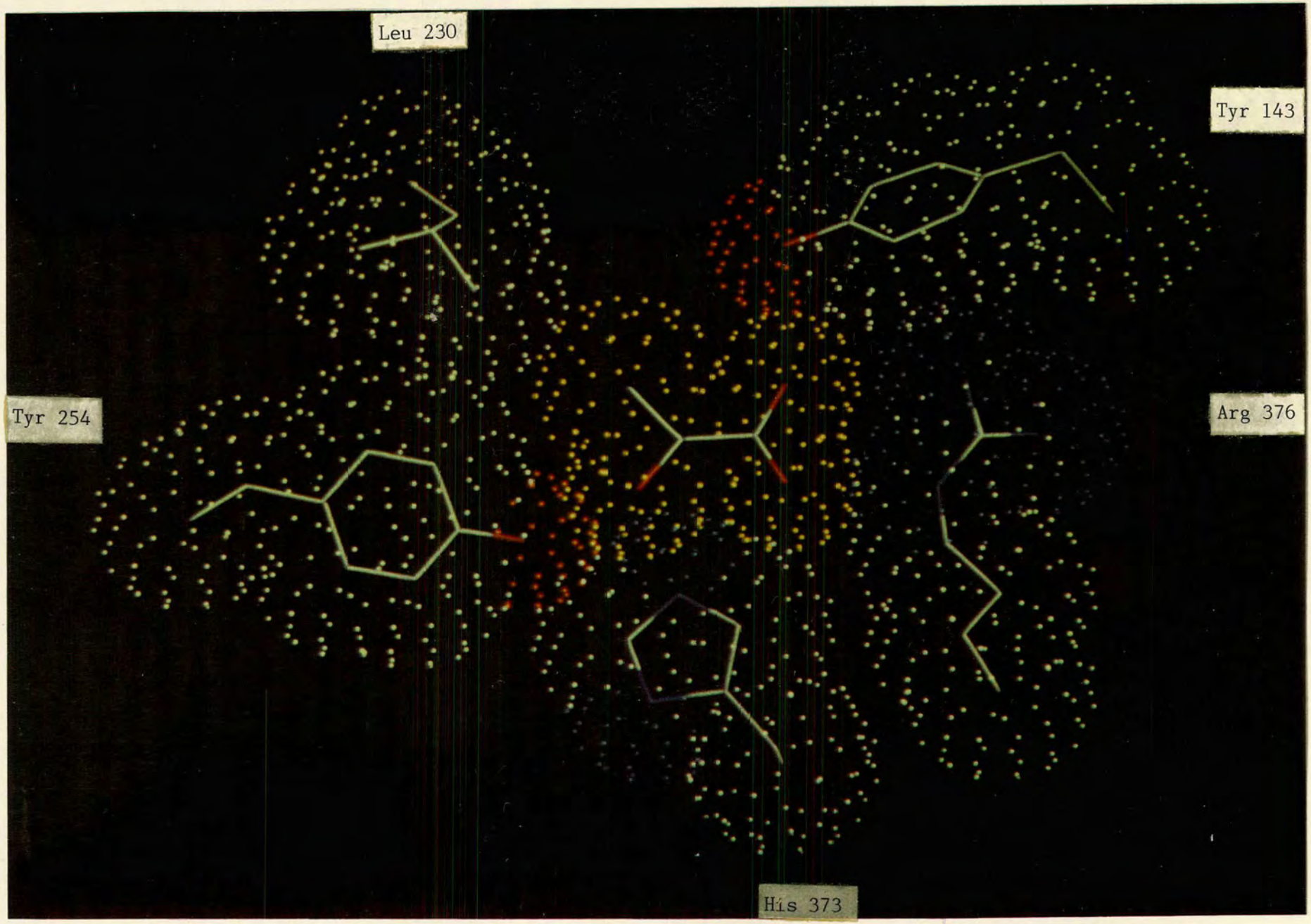


Figure 1.17 Structural comparison of the flavin-binding domains of flavocytochrome b_2 (*S.c.*b_2), spinach glycolate oxidase (SGO), and trimethylamine dehydrogenase (TMADH). α-helices are represented by rectangles and circles, and β-sheets by arrows.


Leu 230
Tyr 143
Tyr 254
Arg 376
His 373

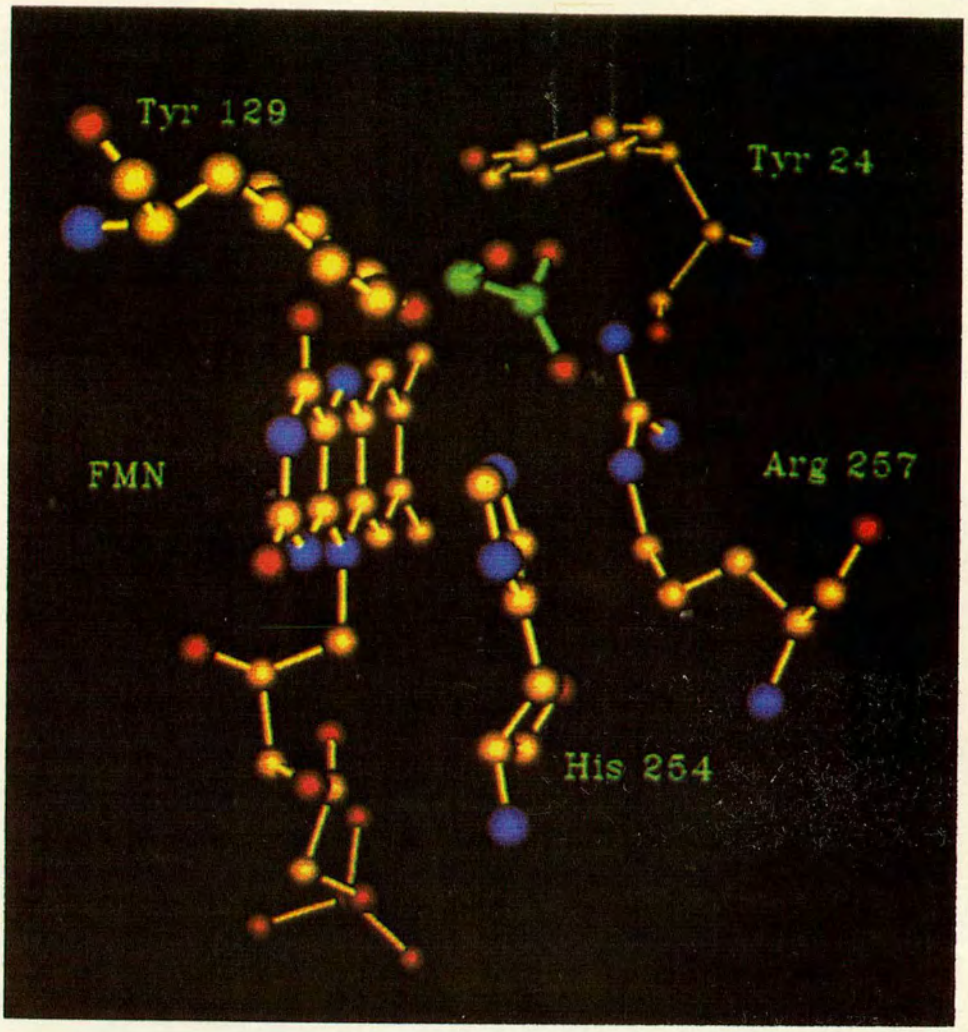


Figure 1.18 Comparison of the flavin active sites of flavocytochrome b_2 (preceding page) and glycolate oxidase.

The opposite page shows the amino-acid side-chains around the substrate molecule at the flavin active site of flavocytochrome b_2. The FMN prosthetic group (not shown) is situated behind the side-chains shown.

which catalyses the oxidation of L-α-hydroxy acids [147]. Mechanistic studies and the N-terminal sequence indicate that the enzyme may be homologous to glycolate oxidase and flavocytochrome b_2. Of the 26 postions considered, hydroxy acid oxidase shows about 25% identity with glycolate oxidase and about 33% identity with flavocytochrome b_2.

S. cerevisiae contains other α-hydroxy acid dehydrogenases [148]. D-lactate cytochrome *c* oxidoreductase from aerobically grown cells [149], contains FAD and zinc, both being essential for activity [150,151]. The enzyme is located in the inter membrane space of mitochondria where it catalyses the oxidation of D-lactate and passes the electrons directly to cytochrome *c* [11]. It is thought that the enzyme works in parallel to flavocytochrome b_2 within a short electron-transport chain involving D-lactate cytochrome *c* oxidoreductase, cytochrome *c* and cytochrome *c* oxidase. D-α-hydroxy acid dehydrogenase, isolated from anaerobically grown cells [152] is a metalloflavoprotein which does not reduce cytochrome *c* [153]. It is not certain which metal is present, but reconstitution of the apoenzyme with zinc gives the highest activity. An enzyme related to these two is the flavoenzyme D-lactate dehydrogenase from *Megasphaera elsdenii* [154]. The protein is a dimer of two apparently identical subunits of molecular weight 55kDa, each containing an FAD and a Zn^{II} ion.

Lactate oxidase from *Mycobacterium smegmatis* is an octameric protein of subunits with molecular weight ≈44kDa [155]. It is an FMN-binding protein and has been shown to react with lactate on the si face, in common with the other FMN-binding proteins oxidising α-hydroxy substrates [156]. As described in section 1.5.5, studies with β-chloroalanine [76] and glycolate [82] provided evidence for a carbanion type mechanism. It is proposed that the flavin forms an intermediate covalent bond with the substrate [82]. This is a similar situation to D-amino acid oxidase but is in contrast to flavocytochrome b_2.

Little is known about the protein structure.

Lactate can be reversibly oxidised to pyruvate by muscle lactate dehydrogenase (LDH). Three dimensional structures are available for several forms of this enzyme [157]. There is little in common functionally or structurally between LDH and flavocytochrome b_2. Muscle LDH catalyses the reaction using a hydride transfer mechanism as opposed to the carbanion mechanism described for flavocytochrome b_2 [158], and is a NAD (not FMN) dependent enzyme. In the active site of *B. stearothermophilus* LDH [157], the carboxylate of lactate binds electrostatically in a planar arrangement with the guanidinium group of an arginine. A histidine side-chain hydrogen bonds through its NE atom to the hydroxyl oxygen of lactate. The histidine is also hydrogen bonded to an aspartate carboxylate oxygen. This histidine aspartate pair has been shown to be important for activity [158]. Another active site arginine also hydrogen bonds with the substrate hydroxyl oxygen. The reaction is proposed to take place as follows: the histidine deprotonates the C2 hydroxyl group, the resulting oxyanion is stabilised by the histidine aspartate pair. The C2 hydrogen transfers to the nucleotide ring as a hydride to leave a carbocation which is neutralised by the oxyanion to give pyruvate.

1.7.4. Overall Quaternary Structure

Since the enzyme consists of two functionally distinct domains, which have been shown to be homologous to other cytochromes and flavoproteins, it has been suggested that flavocytochrome b_2 might be the result of a gene fusion of a b_5 type protein and a flavodehydrogenase [28,32,40].

The quaternary structure of flavocytochrome b_2 is unusual in that it has rotational four fold symmetry. This symmetry has been observed in only one other protein, the influenza virus glycoprotein antigen neuraminidase [159,160]. This protein, a tetramer of molecular weight 240kDa, is attached to the viral membrane by a hydrophobic N-terminal region. The box-shaped tetramer has

dimensions 100Å x 100Å x 60Å, very similar to flavocytochrome b_2. Perhaps flavocytochrome b_2 is also situated on or near to a membrane surface, possibly near to the membrane bound cytochrome *c* oxidase, so that cytochrome *c* would only have to move small distances or even just rotate to a different active site. Indeed, Cremel *et al* obtained some evidence to suggest formation of a complex between flavocytochrome b_2 and rat liver mitochondrial inter membrane [161]. Kinetic studies indicated that L-lactate respiration may be limited by this complex formation rather than by enzymatic activity. A tetrahedral structure would be more suited to tetrameric enzymes which operated in the bulk phase of the solution.

1.8. Summary

Flavocytochrome b_2 is a tetramer of identical subunits of molecular weight 57.5kDa. Each subunit consists of a single polypeptide chain folded into two functionally distinct domains, a heme-binding domain (residues 1-100) and an FMN-binding domain (residues 101-511). The three dimensional crystal structure has been solved and refined to 2.4Å. The enzyme is found in the intermembrane space of yeast mitochondria, where it catalyses the oxidation of L-lactate to pyruvate and the reduction of cytochrome *c*. It is part of a short electron-transfer chain also involving cytochrome *c* oxidase. The enzyme requires three electrons to become fully reduced but in catalysis it acts as a two-electron transferase. The oxidation of lactate is proposed to proceed via a carbanion mechanism where the abstraction of the lactate α-proton is the main rate-limiting step of the whole catalytic turnover process. The heme-binding domain is homologous to cytochrome b_5 and the flavin-binding domain is homologous to glycolate oxidase. It is proposed that flavocytochrome b_2 has evolved from a gene fusion of a b_5 type cytochrome and a flavodehydrogenase enzyme.

1.9. References

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Chapter Two

HIGH-LEVEL EXPRESSION OF FLAVOCYTOCHROME b_2 IN *ESCHERICHIA COLI*

2.1. Introduction

In probing the mechanism of enzyme catalysis, it is often desirable to study modified versions of the wild-type enzyme, for example, to focus on a particular amino acid or region of the protein. Modification of enzymes has been approached in two ways: 1) the development of a series of residue or site-specific chemical reactions [1,2], for example, neutralisation of charged residues by esterification or acid/base condensation reactions; 2) Site-directed mutagenesis which is one of various ways to bring about alterations in the amino-acid sequence of an enzyme by use of recombinant DNA techniques [3].

Chemical modifications have been used for some time and a large series of reactions has now been developed [1,2]. Although the technique has been useful in elucidating enzyme mechanisms [4-6], it has some serious disadvantages: 1) The chemical reaction may be specific to one type of residue; however, the enzyme may have more than one of these residues accessible to the modifying reagent. Consequently, an often difficult separation and identification of the various singly-modified enzymes is required. This may require large quantities of pure, unmodified enzyme. 2) The choice of modification reagent may be limited by the reaction conditions (eg pH or reagent and/or enzyme concentrations) which may be detrimental to the enzyme. 3) The most serious disadvantage is that the modification invariably introduces extra bulk to the enzyme, which may or may not alter protein conformation, block active sites and substrate pathways or interfere with interactions between other amino-acid side-chains. Therefore careful interpretation of the modification results is essential.

The second method of protein modification (site-directed mutagenesis) has only been available for around ten years [7,8] and already has been proven to be a very important technique in enzymology [9-12]. The DNA, encoding for the enzyme under study, can be manipulated so that when the protein is

expressed, there is a specific alteration to the amino-acid sequence [3]. Once the mutation has been carried out and the DNA plasmid is transformed into a host cell, production of the mutant enzyme is only limited by the level of protein expression by the host cell. Site-directed mutagenesis has been used to study, amongst other things, the role of particular amino acids and interactions between side-chains and substrate molecules, for example, in the mechanism of enzyme turnover [10-12, and references within] or molecular recognition [13,14]. The technique is slightly limited in that an amino-acid side-chain can only be changed to one of nineteen others at present; however, mutagenesis with unnatural amino acids can be carried out in small quantities *in vitro* [15], and it is hoped that in the future the number of possible side-chains will be increased

It was decided to use site-directed mutagenesis to probe functional aspects of flavocytochrome b_2, therefore several residues in flavocytochrome b_2 were chosen for modification (chapter 3). Some of these mutants were successfully expressed by a plasmid (pGR401) in a strain of Saccharomyces cerevisiae (GR20) which lacks the chromosomal flavocytochrome b_2 gene. However, the level of expression of flavocytochrome b_2 was such that enzyme quantities restricted the number of experiments possible. It was clear that for further characterisation of mutant enzymes to be feasible, a better expression system offering improved yields of enzyme was required. High-level expression systems have been developed for a number of enzymes. von Bodman *et al* obtained overexpression of wild-type and mutant forms of mammalian cytochrome b_5 in *E. coli* [16]. The wild-type cytochrome b_5 was fully incorporated with heme. A mutant soluble form, analogous to the trypsin solubilised form of cytochrome b_5, accounted for approximately 8% of the cell protein. Barstow *et al* expressed *B.stearothermophilus* L-lactate dehydrogenase in *E. coli* at levels upto 36% of

the *E. coli* soluble protein [17].

This chapter describes the expression of wild-type and mutant forms of flavocytochrome b_2 in GR20 *S. cerevisiae* (*S.c.*b_2) and *E. coli* (*E.c.*b_2) and compares some of the physical properties of the two wild-type enzymes. A comparison of the two expression systems and the purification of flavocytochrome b_2 from commercial yeast, GR20 yeast and *E. coli* is made.

2.2. Methods and Materials

All buffers and columns were prepared as described in sections 6.1 and 6.2. The enzyme activity using ferricyanide as the electron acceptor was measured as described in section 6.8.1. The concentrations of flavocytochromes b_2 were calculated using the following extinction coefficients: ε_{423}^{red} = 183mM^{-1}cm^{-1}, ε_{557}^{ox}= 9.4mM^{-1}cm^{-1} and ε_{557}^{red} 30.9mM^{-1}cm^{-1} [18]. The most accurate concentration measurements were made using $\varepsilon_{557}^{red-ox}$ 21.5mM^{-1}cm^{-1}. Kinetic analysis of the enzyme to obtain k_{cat} and K_M values is described in section 6.8.1. Protein molecular weights were measured using SDS polyacrylamide gel electrophoresis (section 6.4) or Sephacryl S-300 gel filtration (section 6.2.3) The N-terminal sequence was kindly carried out by Dr J van Beeumen, using an Applied Biosystems 477 Sequencer.

2.2.1. Isolation of *S.c.*b_2

Commercial yeast (Distillers Ltd, Menstrie, Scotland) was dried thoroughly (3-4 days) and ground to a fine talc-like powder using a porcelain ball mill. The powder was stored at -20°C until used. GR20 transformed yeast was grown in YPD media (section 6.6.1) for about 48 hours at 30°C. 1.0-1.4g of yeast (dry weight) were obtained per litre of YPD culture. Harvested yeast cells (8000g for 4 mins) were dried and ground using a porcelain mortar and pestle and stored as above. Flavocytochrome b_2 was isolated from commercial yeast by the method of Labeyrie *et al* with minor modifications [19], (section 6.5.2),

giving about 40mg of pure protein from 1kg dry weight of yeast. Site-directed mutant flavocytochrome b_2 was isolated from GR20 transformed yeast, based on the commercial yeast prep, but adapted for small scale (around 70g dry weight yeast cells, section 6.5.3). A typical yield of around 15-30mg of pure protein was obtained per kilogram of dry weight GR20 yeast.

2.2.2. Generation of pDSb_2 and Isolation of *E.c.*b_2

The entire flavocytochrome b_2 coding sequence, including the region encoding the 80 amino-acid presequence, was excised from the plasmid pGR401 [20] (M.T.Black) on a 1.8kb *Eco*R1-*Hin*dIII restriction fragment and inserted between the *Eco*RI and *Hin*dIII sites of the plasmid pDS6 [21] to generate pDSb_2 (figure 2.1). *E. coli* cells were grown in Luria broth (supplemented with 100µg/ml ampicillin) incubated at 37°C on an orbital shaker (250rpm) for about 12 hours. Harvested cells (10,000rpm for 3 mins) were stored wet at -20°C until used. Isolation from *E. coli* was carried out as follows:

Step 1) Frozen *E. coli* cells were resuspended in phosphate buffer 100mM, pH 7.0, containing 10mM EDTA and 50mM lactate. Lysozyme was added ca 0.2mg/ml and the suspension was incubated for 20-30 minutes at 4°C. The cell lysis mixture was centrifuged at 39,000g for 15 minutes to give an orange/red coloured supernatant (flavocytochrome b_2).

Step 2) The enzyme solution was precipitated with $(NH_4)_2SO_4$ (30% and 70%) The first pellet was discarded, the second was dissolved in a minimum volume of phosphate buffer 100mM, pH 7.0 containing 5mM lactate and 1mM EDTA, and dialysed overnight against 1l of the same solution at 4°C under nitrogen. For kinetic experiments the enzyme could be used after $(NH_4)_2SO_4$ precipitation (see discussion).

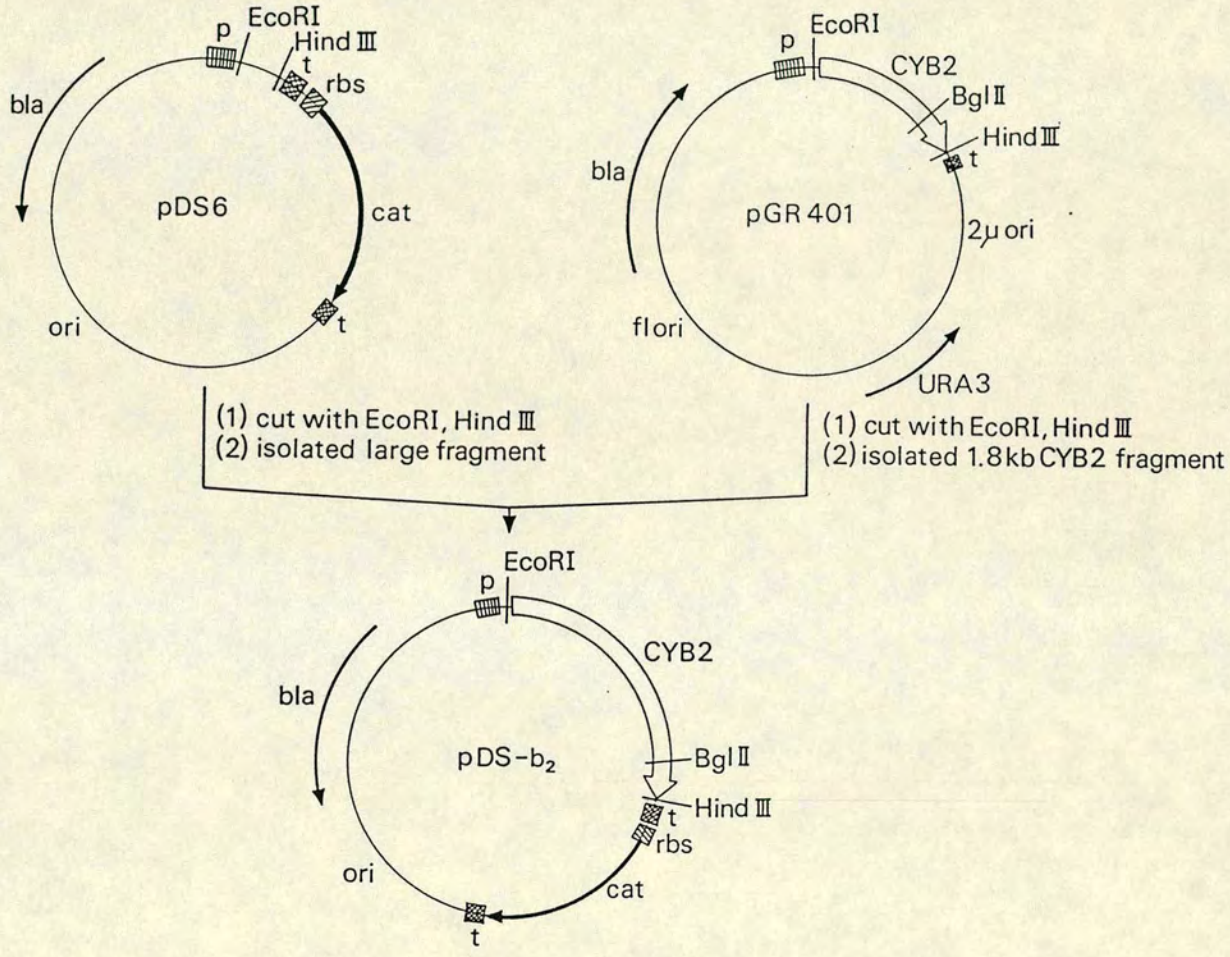

Figure 2.1 Construction of the plasmid pDSb_2 from pGR401 and pDS6. Abbreviations: p, promoter; t, terminator; rbs, ribosome-binding site; cat, chloramphenicol acetyltransferase gene; bla, β-lactamase gene; ori, origin of replication.

Step 3) The flavocytochrome b_2 solution from step 1) was applied to a hydroxyapatite column (section 6.2.1). Once the enzyme was bound, the column was washed with 2 column volumes of the phosphate/lactate solution. The column was then eluted with a linear gradient of a) 500ml of phosphate 100mM, pH 7.0, containing 5mM lactate and 10mM EDTA and b) 500ml of this buffer containg 35g of $(NH_4)_2SO_4$ (10% saturation). The fractions containing enzyme were pooled and precipitated with 70% $(NH_4)_2SO_4$. If step 4 was required the enzyme was dialysed overnight in phosphate 100mM, pH 7.0 containing 5mM lactate, and 10mM EDTA at 4°C under nitrogen.

Step 4) The dialysed flavocytochrome b_2 was applied to a derivatised sepharose column (section 6.2.2) and eluted with a linear gradient of a) 300ml of 100mM phosphate, 5mM lactate, 1mM EDTA and b) 300ml of 200mM phosphate, 5mM lactate and 1mM EDTA. The fractions containing flavocytochrome b_2 were pooled and precipitated at 70% $(NH_4)_2SO_4$. The enzyme was stored under nitrogen at 4°C.

2.3. Results and Discussion

2.3.1. Expression and Isolation of *S.c.*b_2 from GR20 Yeast

Mutant flavocytochrome b_2 was isolated from GR20 yeast by the method of Labeyrie *et al* [18], with the exception that the acetone precipitation step was omitted since there was little increase in purification at this step. Preparation conditions were adjusted to scale. Between 15 and 30mg of pure mutant flavocytochrome b_2 were obtained per kilogram of dried yeast (cf ≈40mg per kg commercial yeast), however, only 1.0–1.4g of dried yeast were harvested per litre of YPD culture. Isolation of flavocytochrome b_2 from GR20 yeast was usually carried out with an initial cell weight of 70g (60l culture), giving 1–2mg pure protein.

2.3.2. Expression and Isolation of *E.c.b*$_2$

E. coli cells were freeze-thawed before lysing. Sonication for periods of about 30s was tried as an alternative to lysozyme, but was found to cause the loss of activity. Although step 2 in the *E. coli* prep (ammonium sulphate precipitation) did not achieve a large purification in the enzyme, it did offer a convenient step in which the working volume of enzyme could be reduced dramatically, making column loading much quicker. Step 3, the hydroxyapatite column, gave the best purification (10fold) and comparatively little enzyme was lost to the column (ca 11%). With new column material and careful elution, the hydroxyapatite column achieved even better purification (giving pure enzyme on several occasions). Step 4, the derivatised sepharose column, took the enzyme to homogeneity, but this was usually an expensive step in that ≈45% of the enzyme bound irreversibly to the column material. The four-step procedure gave an overall purification of 21 fold as expected, with flavocytochrome b_2 accounting for approximately 5% of the total soluble (cell lysate) protein. The high-level expression of flavocytochrome b_2 in *E. coli* enables further characterisation and even makes X-ray structural analysis a possibility.

As can be seen from figure 2.2 the absorbance spectra of *S.c.b*$_2$ and *E.c.b*$_2$ are identical, therefore there must be full incorporation of flavin and heme. Purified flavocytochromes b_2 from both sources show as single bands on SDS-page gels giving apparent molecular weights of around 57kDa (figure 2.3). In non-denaturing conditions, *E.c.b*$_2$ has an apparent molecular weight of 230kDa (figure 2.4) indicating that the enzyme must be adopting the tetramer formation. The rate of oxidation of L-lactate under steady state conditions has been measured over a range of lactate concentrations. Typical saturation kinetics were observed for both *E.c.b*$_2$ and *S.c.b*$_2$ (figures 2.5 and 2.6). Data were fitted to a least squares program [22] to yield k_{cat} and K_M values. Table

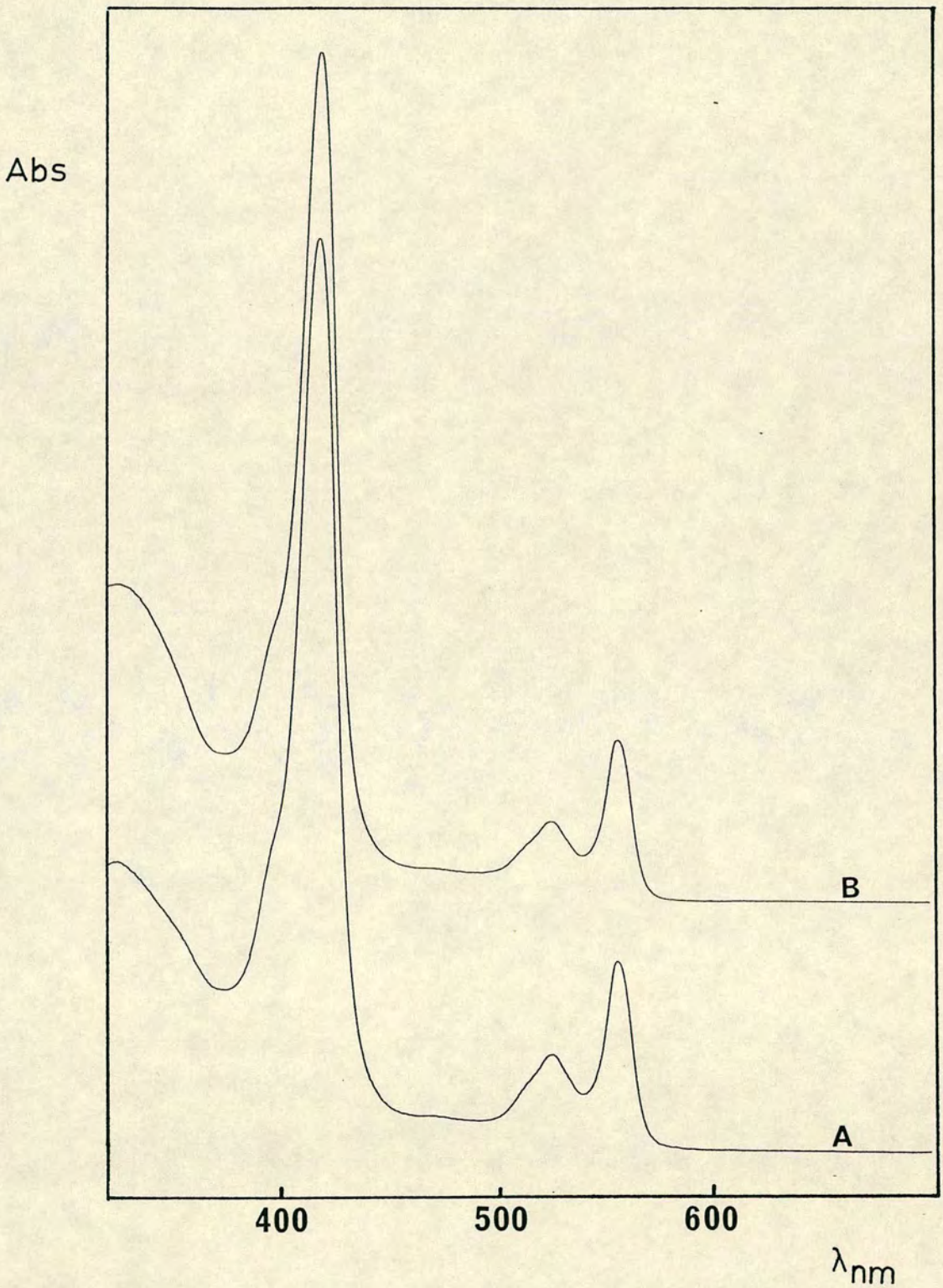

Figure 2.2 Electronic absorption spectra of (A) fully purified flavocytochrome b_2 expressed in yeast and (B) flavocytochrome b_2 expressed in *E. coli* and purified up to the 70%-saturated $(NH_4)_2SO_4$ step (offset on vertical axes).

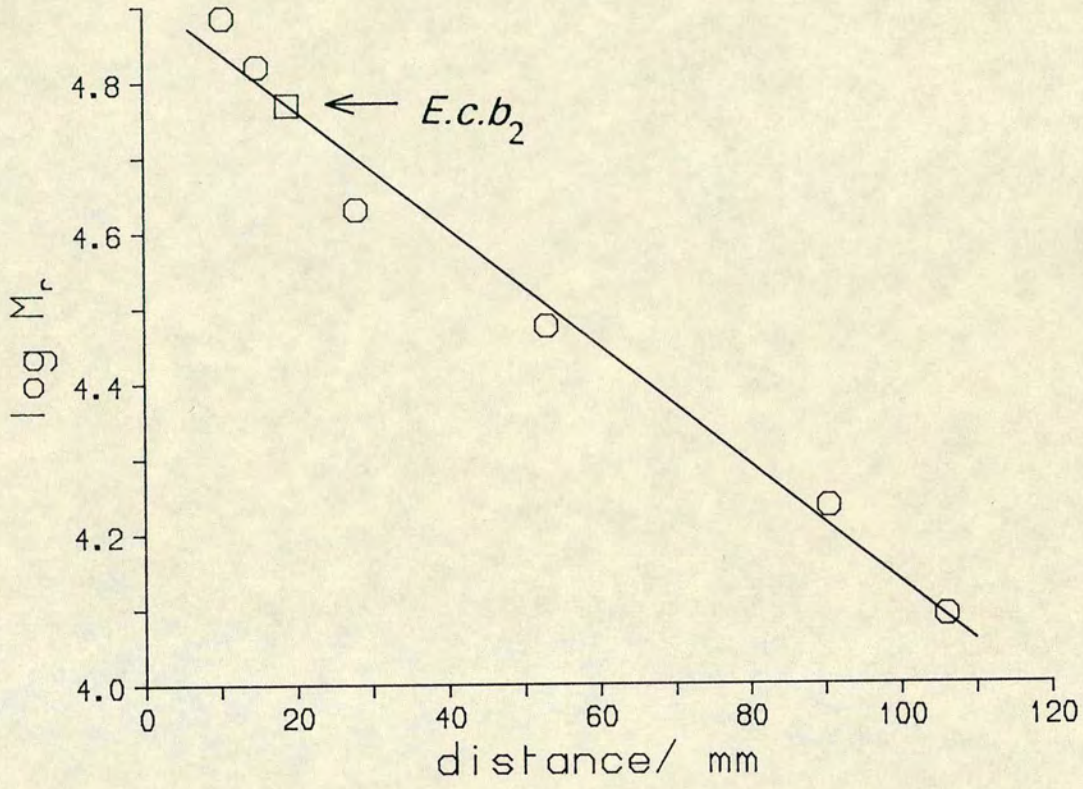

Figure 2.3 Molecular weight determination of *E.c.b*$_2$ by 12% SDS-page. The following molecular weight markers were used: cytochrome *c* (12.3kDa); myoglobin (17.2kDa); chymotrypsinogen (25.7kDa); ovalbumen (45kDa); albumen (66.2kDa) ovotransferrin (76-78kDa).

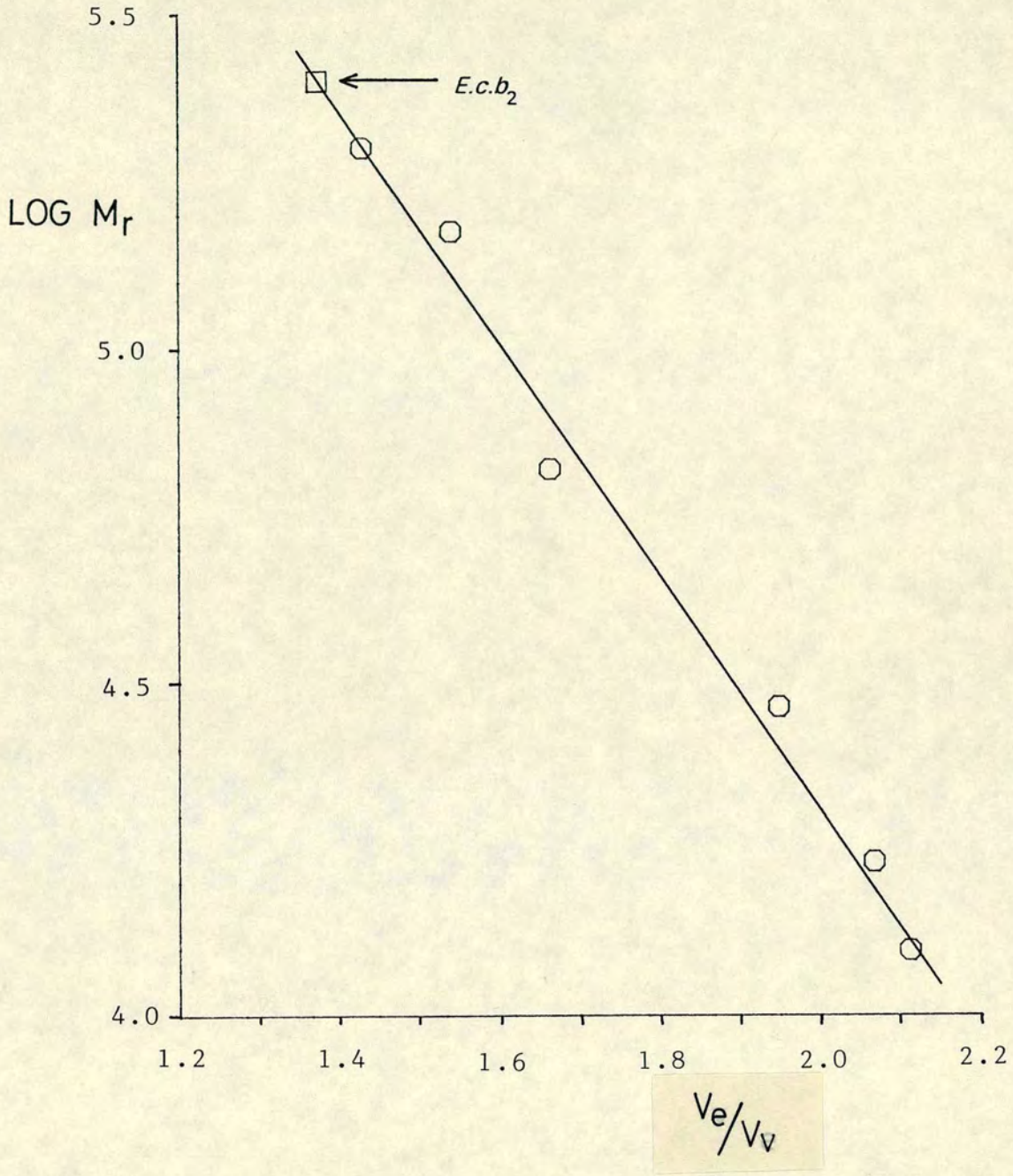

Figure 2.4 Molecular weight determination of *E.c.b*$_2$ by S-300 gel filtation column (150cm x 2.5cm). The following molecular weight markers were used: cytochrome *c* (12.3kDa); myoglobin (17.2kDa); carbonic anhydrase (29kDa); albumin (66.2kDa); alcohol dehydrogenase (150kDa); β-amylase (200kDa).

The experimental elution volumes fall outside the standardisation range and therefore require an unjustified extrapolation.

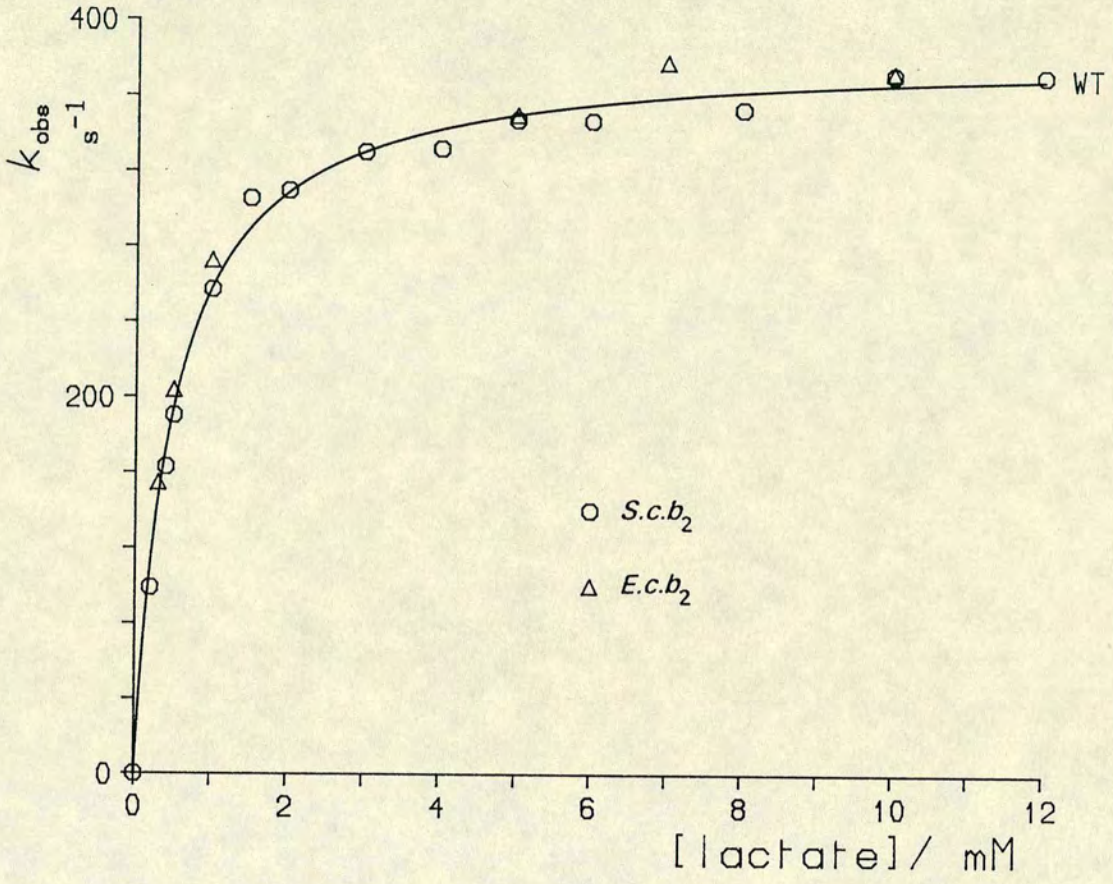

Figure 2.5 *E.c.*b_2 and *S.c.*b_2 saturation kinetics under steady-state conditions using $Fe(CN)_6^{3-}$ as the electron acceptor. Data were obtained at 25±0.1°C, in Tris.HCl buffer, pH 7.5, I = 0.1M.

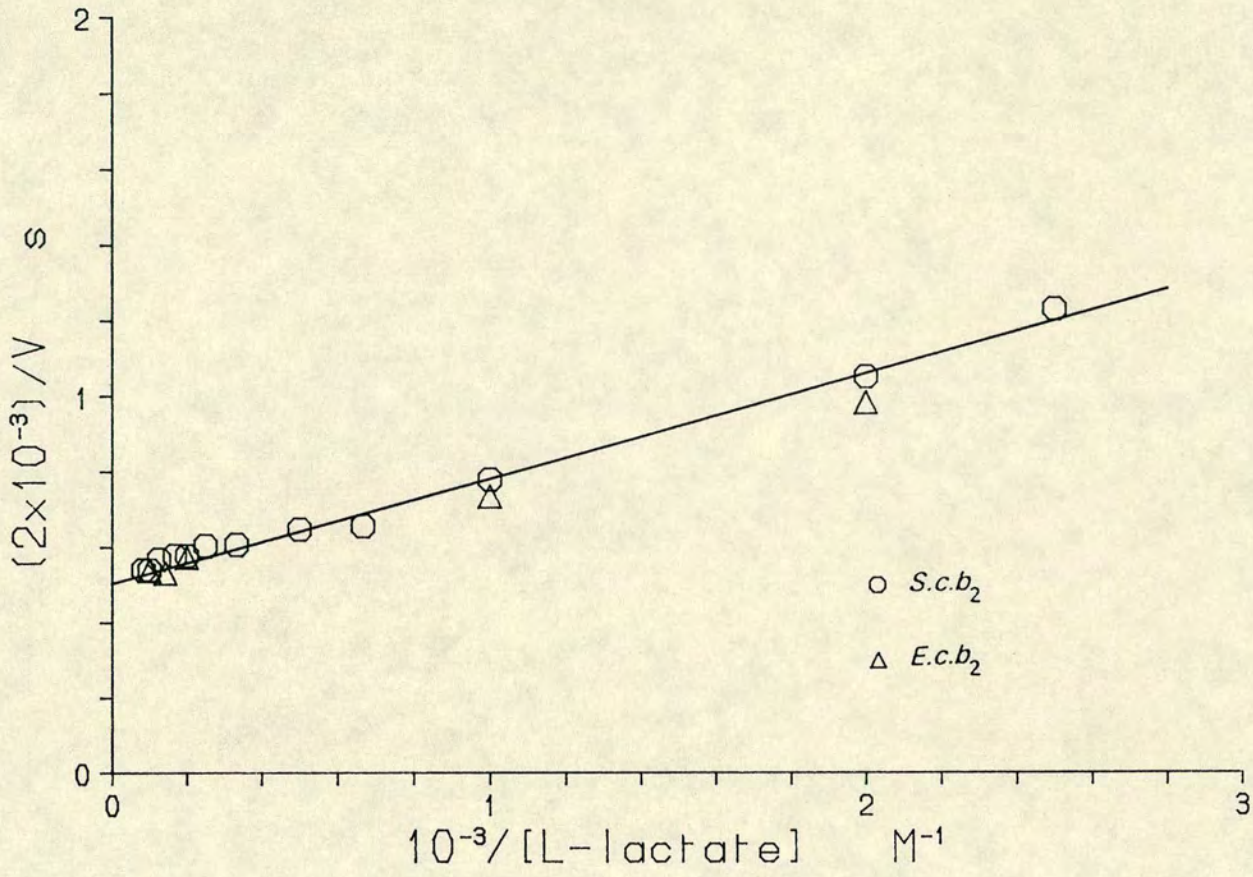

Figure 2.6 Double-reciprocal plot of the steady-state saturation kinetics of *E.c.b*$_2$ and *S.c.b*$_2$. To account for the reduction of two molecules of $Fe(CN)_6^{3-}$ per molecule of L-lactate oxidised, the observed rates of $Fe(CN)_6^{3-}$ reduction were halved (ie plotted as 2/v; unit s).

2.1 lists k_{cat} and K_M values for wild-type flavocytochrome b_2 expressed in yeast and *E. coli*. The values for wild-type *S.c.*b_2 and wild-type *E.c.*b_2 are identical within error limits, showing that *E.c.*b_2 is fully active. The value of k_{cat} for partially purified enzyme from *E. coli* was slightly higher than when fully purified. This is due to a small loss in activity occuring over the two days required for the chromatography. These values compare well with those previously determined [18]. Cell lysate from ordinary *E. coli* has no lactate ferricyanide oxidoreductase activity so kinetic data can be obtained using enzyme purified to the ammonium sulphate precipitation (step 1) providing that an accurate enzyme concentration can be measured. Since flavocytochrome b_2 is the major component of the ammonium sulphate precipitate and the enzyme absorbs very strongly in the visible region, accurate concentration measurements can be easily achieved.

The N-terminal sequence (figure 2.7) indicates that *E.c.*b_2 commences at position six (Met) of the mature flavocytochrome b_2 sequence [23]. The expression vector pDSb_2 contains a strong promoter, but does not have a ribosomal binding site at the beginning of the DNA encoding for flavocytochrome b_2. The factors which affect translational initiation have recently been reviewed [24], and it has been proposed that five components provide high level translational initiation in *E. coli*. In this case three factors appear to be particularly relevant: a Shine-Dalgarno region (consensus sequence AGGAGG) of four or five nucleotides is probably sufficient for complementary base-pairing with the 3′ end of 16S RNA; an ATG initiation codon is required, and a spacing of seven to nine nucleotides between these two components is probably optimal. The flavocytochrome b_2 gene contains a hexanucleotide sequence $^{5'}$ACGAGC$^{3'}$ commencing at nucleotide 239 and extending over the region corresponding to the first amino acid of the mature-protein sequence (figure 2.7); four out of the six nucleotides match the

Enzyme	k_{cat} (s^{-1})	K_M (mM)
S.c.b_2	192 ± 5	0.49 ± 0.03
E.c.b_2	189 ± 5	0.44 ± 0.05
* *E.c.*b_2	204 ± 10	0.46 ± 0.03

Table 2.3 Comparison of the k_{cat} and K_M values of *E.c.*b_2 and *S.c.*b_2. * = *E.c.*b_2 after 70% $(NH_4)_2SO_4$ precipitation. All data were obtained at 25±0.1°C, in Tris.HCl buffer, pH 7.5 and I = 0.1M, with $Fe(CN)_6^{3-}$ (1mM) as the electron acceptor.

	1				5					10
S.c.b_2.	Glu	Pro	Lys	Leu	Asp	Met	Asn	Lys	Gln	Lys
E.c.b_2						Met	Asn	Lys	Gln	Lys

Figure 2.7 Comparison of the N-terminal sequence of *E.c.*b_2 with the mature sequence of *S.c.*b_2.

consensus Shine-Dalgarno sequence. An ATG codon, corresponding to methionine 6, is present 11 bases down stream from the putative Shine-Dalgarno sequence. It is therefore likely that the nucleotides which code for amino acids -1 to +6 of the mature sequence act as a translational initiation site for flavocytochrome b_2 in *E. coli*. The absence of these five amino acids appears to have no effect on kinetic or structural properties. From the crystal structure, it can be seen that these five amino acids are on the heme domain at a large distance from the active site.

The level of flavocytochrome b_2 expression using *E. coli* transformed with pDSb_2 is high (approximately 5% of total soluble protein). Table 2.2 summarises the purification data for a typical enzyme prep. From 6g wet weight of *E. coli* cells, ≈10-20mg of enzyme was purified 21 fold to give ≈4-6mg of pure protein. *E. coli* growth was variable, but within any one set of experiments there was a direct correlation between cell doubling time and the lactate dehydrogenase activity of the flavocytochrome b_2 being expressed. The more active mutants had a higher tendency to lose expression of flavocytochrome b_2 yet retain ampicillin resistance within the cell. It is very important, especially for wild-type and the more active mutants, that reselection is repeated frequently (every 4-6 weeks).

2.3.3. Comparison Between The Two Systems

Isolation of flavocytochrome b_2 from *E. coli* was far simpler and faster than isolation from either commercial yeast or GR20 transformants. Yeast cells required 3-4 days preparation (drying and grinding) whereas *E. coli* cells were grown for ≈12 hours and required no drying. Approximately 15-30mg of pure flavocytochrome b_2 were obtained from 1kg dry weight of GR20 yeast compared to 40mg from commercial yeast. However, *E. coli* yields a much higher quantity of 700-1000mg pure enzyme from 1kg wet weight of cells.

When flavocytochrome b_2 is purified from yeast, proteolytic cleavage

Step	Yield of b_2 (%)	Loss from previous step (%)
Butanol extraction	100	-
Acetone precipitation	80	20
Ammonium sulphate precipitation	70	12.5
Chromatography	50	30

Table 2.2 Purification table for *S.c.*b_2 [29].

Step	Total Protein (mg)	Flavocytochrome b_2 (mg)	Yield (%)	Purification (fold)
Cell lysate	300	14	100	1
Ammonium sulphate precipitation	170	9-10	65-70	1.1-1.3
Hydroxyapatite	15	8-9	55-65	11-13
Derivatised sepharose	4-6	4-6	30-40	21

Table 2.1 Purification table for *E.c.*b_2.

occurs unless phenylmethyl sulphonyl fluoride, PMSF, is present in all solutions [25]. In the purification of *E.c.*b_2 there is no evidence (either kinetic or electrophoretic) to suggest that any proteolysis occurs even when there is no PMSF in working solutions. Presumably flavocytochrome b_2 is stable to *E. coli* proteases under the conditions employed during purification. Separation between flavocytochrome b_2 and cytochrome c can be a problem during the purification of flavocytochrome b_2 from yeast, particularly for GR20 transformants. This is normally achieved to a great extent by ammonium sulphate precipitation after the hydroxyapatite stage and completed by chromatography on the derivatised sepharose column [18]. However, because there is no cytochrome *c* present in aerobically grown *E. coli*, this is no longer a problem when purifying from *E. coli* [26].

2.4. Conclusions

Wild-type and site-directed mutants of flavocytochrome b_2 have been expressed at high level in *E. coli*. They have full incorporation of flavin and heme prosthetic groups and the wild-type enzyme has full activity compared to that expressed in *S. cerevisiae*. Flavocytochromes b_2 expressed in *S. cerevisiae* and *E. coli* have superimposable electronic spectra and apparently identical molecular weights, as measured by both SDS-page and S-300 gel filtration chromatography. The first five amino acids of the flavocytochrome b_2 mature sequence are absent in *E.c.*b_2, but this has little or no effect on the physical properties of the enzyme. The high level expression system in *E. coli* (5% of total soluble protein) offers a convenient and comparatively simple method of producing wild-type and site-directed mutants of flavocytochrome b_2 rapidly and in high yield.

2.5. References

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Chapter Three

KINETIC CHARACTERISATION OF FLAVOCYTOCHROME b_2 POINT MUTANTS

3.1. Introduction

In the crystal structure of flavocytochrome b_2, the asymmetric unit is a dimer (one half of the solution phase tetramer) [1]. The cytochrome domain is ordered in subunit 1 and disordered in subunit 2 (invisible in the electron density map). In subunit 2, there is a piece of exogenous electron density located at the flavin active site which has been identified as pyruvate on the basis of the affinity of pyruvate for the semiquinone state of the flavin and the lack of affinity of lactate for the hydroquinone state. EPR experiments using flavocytochrome b_2 crystals have provided evidence that the flavin is indeed in the semiquinone state [2]. In subunit 1, Y143 is hydrogen bonded to the heme propionate from ring A, whereas in in subunit 2, Y143 is hydrogen bonded to the substrate carboxylate oxygen and the cytochrome domain is disordered. As discussed in the introductory chapter, it is not certain whether the mobility of the cytochrome domain is functionally important or only an artefact of crystal packing. NMR linewidth studies of the enzyme in solution suggest that there is mobility within the enzyme [3].

Figures 3.1 and 3.2 show the active sites in subunits 1 and 2 and table 3.1 lists some of the important interatomic distances. Based upon this x-ray data and the solution studies described in chapter 1, Lederer and Mathews proposed a mechanism for lactate oxidation/pyruvate reduction [4] which predicts certain roles for some of the amino-acid side-chains. Site directed mutagenesis provides a useful tool for investigating the role of amino-acid residues by replacing one amino acid for another by manipulation at the DNA level. Consequently, site-directed mutants were generated to probe some of the amino acids which are: 1) involved in the Lederer and Mathews model; 2) located in the interdomain contact region; and 3) located in the surface exposed, crystallographically disordered, loop.

In the Lederer and Mathews model, the substrate is thought to be bound

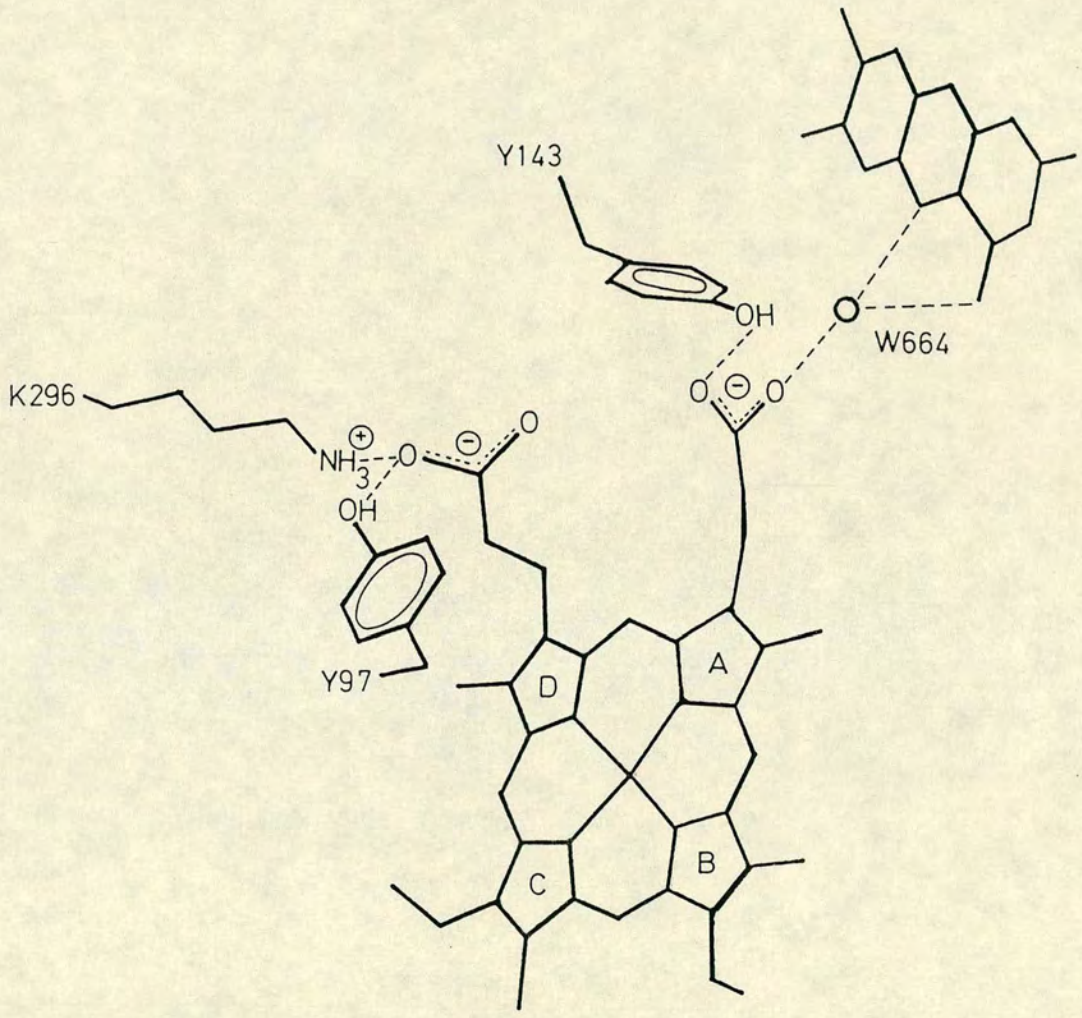

Figure 3.1 The active site in subunit 1. Interatomic distances are listed in table 3.1.

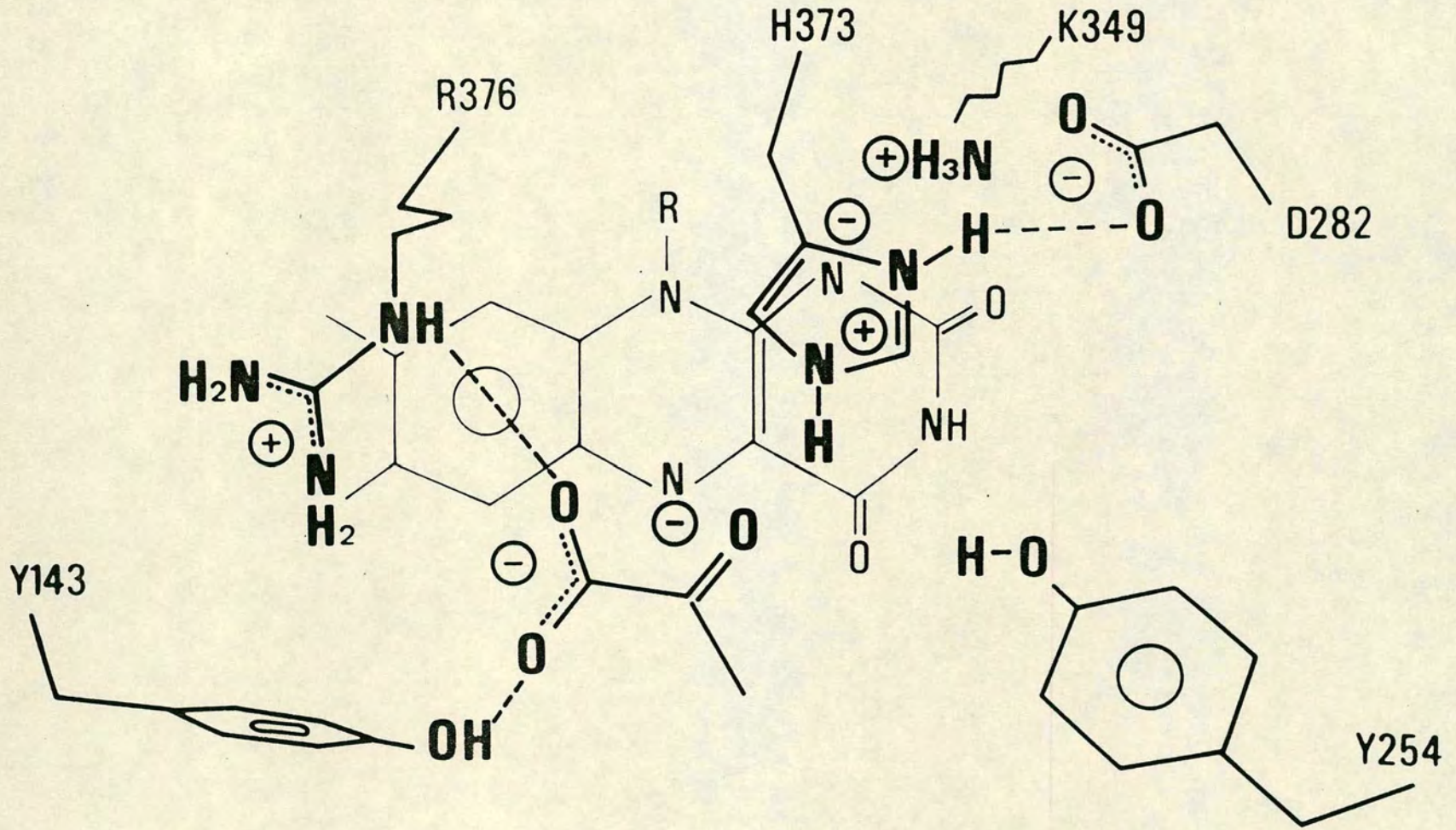

Figure 3.2 Schematic diagram of the chemical surroundings of the pyruvate molecule in subunit 2. See also figure 1.18.

Interatomic distances

FMN	Amino acid/ Water molecule	Subunit 1 (Å)	Subunit 2 (Å)
N1	K349 NZ	2.6	2.8
O2	K349 NZ	2.9	2.8
O4	Water 664	2.8	-
N5	Water 664	3.0	-
Heme			
O1D	Y97 OH	2.4	-
O2A	Y143 OH	2.4	-
O1D	K296 NZ	3.3	-
O1A	Water 664	3.0	-
Pyruvate			
O1B	Y143 OH	-	2.8
C2	Y254 OH	-	3.6
O2	Y254 OH	-	2.4
C3	Y254 OH	-	3.8
C2	H373 N3	-	3.6
O2	H373 N3	-	2.6
C3	H373 N3	-	5.0
O1A	R376 N	-	3.0
O1A	R376 NH1	-	4.0
O1B	R376 N	-	4.1
O1B	R376 NH1	-	4.5
C2	FMN N5	-	3.7
Other distances	D282 OD1 - H373 N1	2.4 Å	
	D282 OD2 - H373 N1	3.2 Å	

Table 3.1 List of the interatomic distances between atoms of the amino-acid residues shown in figures 3.1 and 3.2. All distances are in Å.

in the active site by electrostatic and hydrogen bonding to R376, and hydrogen bonding to Y143 and Y254 (figure 3.2). A tyrosine to phenylalanine change would create a mutant in which the hydrogen bonding capability of the side-chain is lost, but the hydrophobicity and a large proportion of the steric bulk is retained. This change has been made for Y143 and Y254.

Changing arginine to lysine creates a mutant where the positive charge is retained at the active site, but at a slightly different position. In the crystal structure K349 interacts with N1 of the flavin prosthetic group, presumably to facilitate the electron transfer to the flavin by stabilising the anionic form of the reduced flavin. Changing lysine to arginine creates a mutant designed to probe this interaction by shifting slightly the location of the positive charge in this area which may have effects on the flavin reduction potential and hence catalysis.

In the region between the two structural domains, there are certain residues and interactions which may be important in flavocytochrome b_2. Y97, from the heme domain, hydrogen bonds to the propionate from ring D and to E139. Replacing this tyrosine with a phenylalanine removes these hydrogen bonds. F325 from the flavin-binding domain points into a hydrophobic pocket consisting of F39, P44 and Y74 from the heme domain. Phenylalanine was replaced by alanine to remove the phenyl group from the methyl side-chain.

Little is known about the surface exposed loop (residues 300-315), since it is disordered in both subunits. Any experimental information comes indirectly from the kinetic characteristics of the cleaved flavocytochrome b_2 [5] where a number of these residues are missing due to proteolysis [6] and the newly generated peptide termini are free to adopt new conformations. The cleaved enzyme has lower binding affinity for lactate (K_M increases from 0.5mM to 1.6mM upon proteolysis, k_{cat} is about 2.5 fold lower). A sequence comparison with *H.a.*b_2 (figure 1.16) also suggests that this region may be functionally very

important [7]. The corresponding loop is much more negatively charged (-6 compared to +4) and it may be this charge difference which is responsible for the differences in kinetic characteristics between *H.a.*b_2 and *S.c.*b_2. It was therefore decided to replace A306 with a serine which introduces a new hydroxyl group in the disordered loop.

This chapter describes the generation of the site-directed mutants listed below and compares the kinetic characteristics of all of these enzymes with wild-type flavocytochrome b_2. The implications of these results are discussed in relation to the mechanism of L-lactate oxidation and the subsequent electron transfers to flavin and heme.

Y97→F	Y143→F	Y254→F
K349→R	R376→K	A306→S
F325→A		

3.2. Methods and Materials

3.2.1. Generation of Point Mutants (M.T.Black)

Site-directed mutant forms of flavocytochrome b_2 were generated using the double primer method of Zoller and Smith (see appendix II). The oligonucleotides (listed in table 3.2) were synthesised on an Applied Biosystems model 380B DNA synthesiser. The nucleotide sequences of the entire flavocytochrome b_2 gene were determined and found to contain no secondary mutations. *E. coli* strain HB101 was grown in L-broth (section 6.6.2) and transformed by the $CaCl_2$ method. Transformants were selected by ability to grow in the presence of ampicillin and by pink colouration due to the presence of the heme-containing flavocytochrome b_2.

Y97F	T CCT CCT TTT GCT CCT G
Y143F	G TGG GCC TTC TAT TCC T
Y254F	C CAA CTA TTT GTT AAC TC
A306S	GGT CCA AAA TCG ATG AAG
F325A	TA TCA AAG GCT ATT GAC C
K349R	GTT ATC AGA GGT GTT CAA
R376K	GT GGT AAA CAA TTA GAT

Table 3.2 List of the oligonucleotides used for site-directed mutagenesis. All oligonucleotides were synthesised on an Applied Biosystems model 380B synthesiser

3.2.2. Enzyme Preparation and Characterisation

Wild-type and the point mutant forms of flavocytochrome b_2 were isolated from GR20 transformed yeasts and/or *E. coli* as described in sections 6.5.3 and 6.5.4. When purified from *E. coli*, all the flavocytochromes b_2 showed as single bands on SDS-page and had electronic absorption spectra equivalent to the wild-type enzyme.

Kinetic analysis of the mutant flavocytochromes b_2 were carried out under conditions as described in section 6.8.1. Typical saturation kinetics were observed for Y254F, Y143F, A306S and F325A. Data were fitted to a non-linear least mean squares program [8] to yield k_{cat} and K_M values. In the case of the Y97F mutant k'_{cat} and K_M values were obtained by extrapolation back to 1/[lactate] = 0 in a double reciprical Lineweaver-Burk plot (see figure 3.4). The enzyme activity for wild-type and each mutant flavocytochrome b_2 was measured over the ferricyanide concentration range 0 to 2mM to check for any variation of rate with ferricyanide concentration.

The presence of flavin could be detected by monitoring the change in absorbance at 438.3nm (which is a heme isosbestic point [9]) upon reduction of the flavocytochrome b_2 with sodium dithionite. To further verify the presence of flavin, it was isolated from the R349 mutant enzyme by using procedures similar to those previously reported [10]. Precipitated (70% satd $(NH_4)_2SO_4$) mutant flavocytochrome b_2 was solubilised in the minimum volume of 35mM phosphate/4M urea/100mM 2-Mercaptoethanol/1mM PMSF at pH7.0. The mixture was incubated at 0°C for 50 minutes to allow complete dissociation of flavin. The mixture was then passed through a column of Sephadex G-25 (2x20cm) equilibrated in the same solution. Two coloured peaks were eluted from the column, the protein which still contained heme was eluted first and was red in colour, followed by FMN, which was oxidised and therefore yellow in colour.

3.3. Results and Discussion

Values of k_{cat} and K_M for L-lactate oxidation seen for wild-type and mutant enzymes are listed in table 3.3. k'_{cat} for Y97F represents a theoretical saturation rate (the maximum rate obtained if there were no substrate inhibition).

R376K - Replacing R376 with a lysine, results in a mutant enzyme with no detectable dehydrogenase activity at any stage during isolation. There was no observation of heme reduction (which is a very sensitive indicator of activity) even at high lactate concentrations. From figure 3.2, it appears that the substrate is bound and orientated by hydrogen bonding between one carboxylate oxygen and the guanidinium group of R376 and between the other carboxylate oxygen and the hydroxyl function of Y143. There would also be an electrostatic interaction between the guanidinium group and the substrate carboxylate. R376 also hydrogen bonds with polypeptide backbone carbonyl groups of three amino acids in α helix c, a short helix in the flavin domain situated between the cytochrome domain and the $\alpha_8\beta_8$ barrel (figure 1.17) [11]. It is therefore likely that the absence of activity in the R376K mutant enzyme is due to a combination of two effects: 1) a conformational change within the structure of the flavin domain due to the loss of several arginine-backbone hydrogen bonds and 2) poor binding and orientation of lactate giving an enzyme which cannot orientate the substrate molecule into a catalytically competent position. Guanidinium groups of arginine are commonly involved in binding the carboxylate groups of various substrates. In a functionally related enzyme, L-lactate dehydrogenase, from *Bacillus stearothermophilus*, Hart *et al* generated a similar mutant by altering R171 (which interacts with the substrate carboxylate) for a lysine [12]. This mutant enzyme retained about 10% of the wild-type activity with a large increase in K_M from 0.04mM to 125mM. Recently Luyten *et al* have generated R171Y and R171W mutant *B.s*LDH's which have

Enzyme	k_{cat} (s^{-1})	K_M (mM)
WT	200 ± 10	0.49 ± 0.03
Y97F	210 ± 10	0.46 ± 0.03
Y143F	70 ± 10	0.60 ± 0.10
Y254F	6.5 ± 0.5	0.51 ± 0.05
A306S	160 ± 3	0.80 ± 0.04
F325A	96 ± 5	0.87 ± 0.05

Table 3.3 Values of k_{cat} and K_M for wild-type and the point mutant enzymes. All values were determined at 25±0.1°C, Tris.HCl buffer, pH 7.5 and I = 0.1M, with $Fe(CN)_6^{3-}$ (1mM) as the electron acceptor.

surprisingly high activity (≈34% and ≈12% of wild-type) [13]. The K_M values also increase, but not to the degree seen for the R171K mutant (increases are from 0.04 to 58 and 51mM for R171Y and R171W respectively). So for *B.s*LDH at least, arginine is not essential for binding of substrates.

K349R - Replacing K349 with an arginine group also resulted in a mutant enzyme which had zero activity. In retrospect, it may seem quite unreasonable to introduce a large bulky guanidinium group into a densely packed area and expect the flavin to orientate correctly and participate in the catalysis. Indeed it is surprising that the flavin binds at all (see section 3.2 for verification of flavin incorporation). Lederer *et al* attempted to reincorporate flavocytochrome b_2 with 1-deaza-FMN in an alternative experiment to study this flavin N1-O2 interaction with K349 [14]. The failure of flavocytochrome b_2 to bind the flavin anologue was accounted for by the presence of an extra proton in the C1 site. In flavoprotein chemistry, the presence of a residue which hydrogen bonds to the N1-O2 region of the flavin is well known [15]. It is the charge on this residue which to a large extent determines the reduction potential of the flavin and hence affects catalysis.

Y254F - Replacing Y254 with phenylalanine results in a 30 fold lowering in the value of k_{cat}. K_M, however, remains the same within experimental error. Initially it would seem surprising that removal of the hydrogen bond does not alter the binding constant of the substrate by an order of magnitude.* However, recent studies by Dubois *et al* [16], find that Y254F mutant enzyme undergoes catalysis via a carbanion type mechanism as seen for wild-type enzyme. The authors suggest that the role of Y254 is not principally in substrate binding, but in orientating the substrate for αH abstraction by H373. Therefore the lowering of k_{cat} presumably arises because the substrate or the transition state substrate complex is less well orientated.

Y143F - The Y143F mutant enzyme has a lower value of k_{cat} and a slightly

* Although following the analysis of Fersht (A.R.Fersht, Biochem., 27, 1577-1580, (1988).) this result would be consistent with Y254 binding the substrate in the transition state and not the ground state.

higher value of K_M than the wild-type enzyme (table 3.3). Since Y143 hydrogen bonds to the substrate carboxylate in subunit 2 and to the heme propionate in subunit 1, it must be remembered that the change in kinetic characteristics may be as a result of losing one or both of these interactions. If the substrate is bound to the active site by R376 and Y143, it is surprising that there is not a larger increase in the value of K_M on going from tyrosine to phenylalanine. Perhaps the electrostatic interaction with R376, the hydrogen bond to Y254, and the van der Waal's contact with L230 is sufficient to keep the same affinity for lactate at the active site. This interpretation is somewhat supported by the fact that in an enzyme, where catalysis is proposed to proceed via a hydride transfer mechanism, for example *B.s*LDH, the substrate carboxylate is bound only by guanidinium group of an arginine. However, in the enzymes flavocytochrome b_2 and glycolate oxidase from spinach [17], where the mechanism is proposed to proceed via a carbanion intermediate, the substrate carboxylate is bound by both an arginine and a tyrosine. One can postulate that the principle role of Y143 is to protonate the carboxylate in order to facilitate the formation of a carbanion at the C2 position. Without this protonation, the substrate would be more unstable as a transitory carbanion, and this would account for the reduction in catalytic efficiency. From a result obtained using cytochrome *c* as the electron acceptor, there is evidence to suggest that the rate of electron transfer from flavin to heme is lower for Y143F than for wild-type, perhaps slow enough to become the rate-limiting step (see section 5.3.1). Since Y143 is situated between the flavin and the heme group, it is expected that it will be important in the electron transfer between the two prosthetic groups. If F143 is more mobile than Y143 due to the loss of the hydrogen bond, then electron transfer may be slower. Measurement of the isotope effect using 2-^{2}H-lactate should confirm whether there has been a change in the rate determining step or not. Spinach

* see footnote on p104

glycolate oxidase, which does not have a cytochrome domain, also has this tyrosine-substrate carboxylate hydrogen bond (see figure 1.18). A similar mutation with this enzyme would help determine whether it is the lack of substrate carboxylate protonation or whether it is the flavin to heme electron transfer which has brought about the kinetic differences between wild-type and Y143F.

As can be seen from figure 3.3, the activity of wild-type and all other mutant forms of flavocytochrome b_2 apart from Y143F, is independent of the ferricyanide concentration in the range 0.1-2.0mM. The Y143F mutant enzyme gives rise to a concentration dependence for ferricyanide giving an apparent K_M of 0.6mM. The value of k_{cat} reported in table 3.3 represents the rate at saturating ferricyanide concentration. Recent molecular modelling studies by Tegoni [18] have shown three possible sites for ferricyanide association on the enzyme surface. One of the sites is almost equidistant from the flavin and the heme and is situated towards the edge of the interdomain region. It is possible that association of ferricyanide at this position could promote electron transfer from flavin to heme by binding to both domains and compensating for the lack of the interdomain contact between Y143 and the heme propionate from ring D.

F325A - The K_M and k_{cat} values for the F325A mutant enzyme (table 3.3) are higher and lower respectively when compared to wild-type enzyme. The removal of the phenylalanine from the hydrophobic pocket has presumably caused a conformational change resulting in a less catalytically efficient enzyme.

Y97F - k'_{cat} for Y97F mutant enzyme is the same within experimental error to the wild-type enzyme (table 3.3) however this figure is based on an extrapolation from data measured below 4-5mM lactate to an infinite lactate concentration. The K_M value for Y97F is the same as the value for wild-type

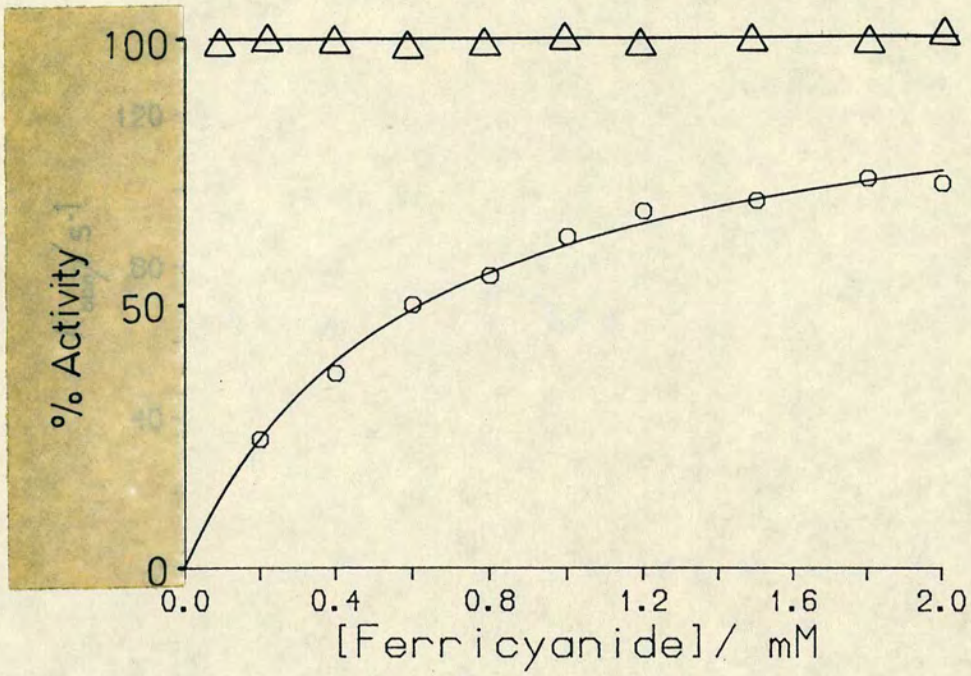

Figure 3.3 shows the $Fe(CN)_6^{3-}$ concentration dependence for Y143F mutant flavocytochrome b_2. The line at 100% activity represents values obtained for the wild-type and following mutant enzymes: Y97F, Y254F, A306S and F325A. All data were obtained in Tris.HCl buffer, pH 7.5, 0.1M ionic strength, 12mM L-lactate.

within experimental error (table 3.3). The Y97F mutant enzyme exhibits substrate inhibition (figure 3.4), with a maximum activity at 6mM lactate. It has been known for some time that wild-type enzyme also exhibits substrate inhibition, although higher concentrations of lactate are required to observe any significant effect on rate [19]. Substrate inhibition may be due to the presence of a low affinity binding site. Recent studies with *H.a.b*$_2$ [20] show that pyruvate has dissociation constants for oxidised, semiquinone and hydroquinone flavins of 8, 0.2 and 20mM respectively. It is quite probable that a similar situation exists for lactate at higher concentrations. It is difficult to imagine how a tyrosine to phenylalanine change at residue 97 could alter substrate affinities at the active site. Therefore a low affinity binding site must be favoured for the Y97F mutant enzyme. The exact location of this low affinity binding site is difficult to determine since tyrosine 97 is only ordered in subunit 1.

A306S - The A306S enzyme has a slightly reduced k_{cat} and a slightly higher K_M compared to the wild-type enzyme (table 3.3). These changes in kinetic parameters are consistent with a small conformational change transmitted through to the active site. Little is known about the interactions of A306 since it is part of the disordered loop in both subunits. Information from cleaved *S.c.b*$_2$ and sequence comparison with *H.a.b*$_2$ indicate that the mobility of this surface exposed loop may be important in catalysis; 1) passively by having to move from one conformation to another to allow mobility of the cytochrome domain; 2) actively by its mobility being involved in some sort of trigger mechanism. An alanine to serine mutation may reduce the mobility of the loop by introducing loop to surface hydrogen bonds which may account for the decrease in k_{cat}.

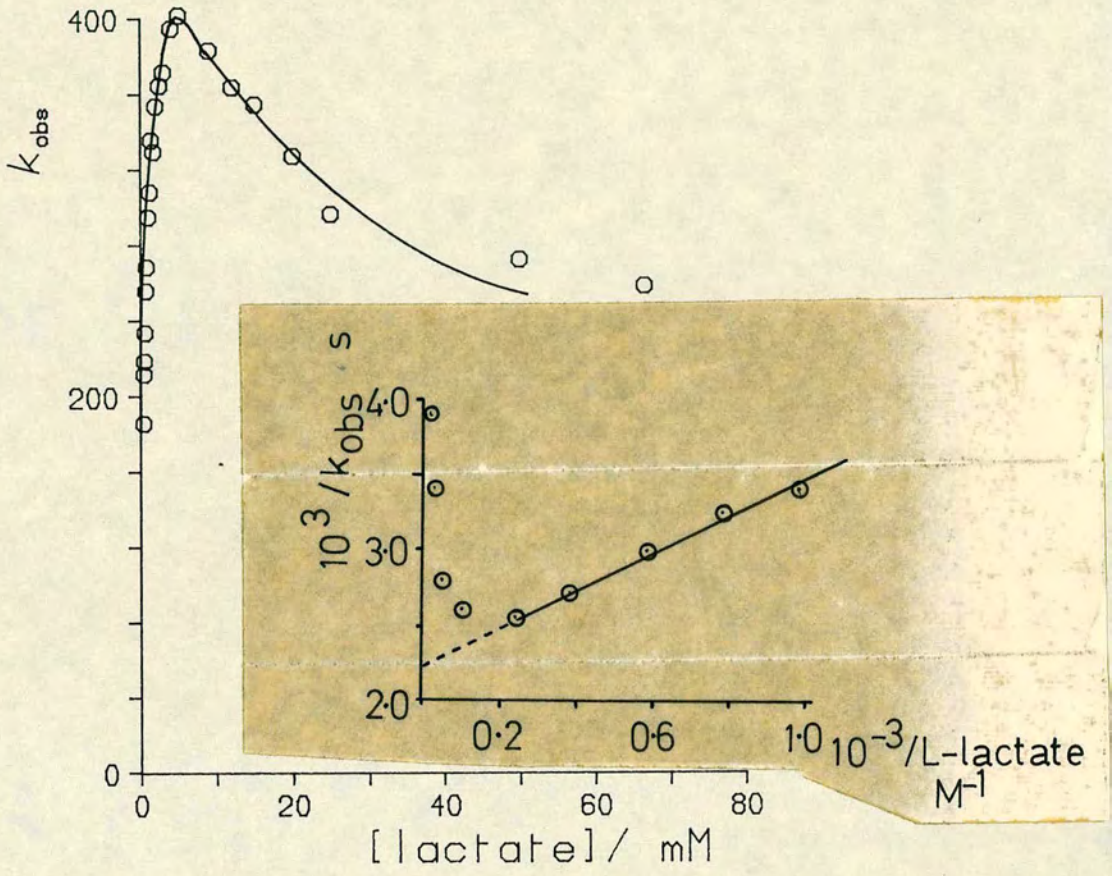

Figure 3.4 Substrate inhibition of Y97F by L-lactate giving an optimum activity at 6mM. Inset shows the double reciprocal plot. All data were obtained at 25± 0.1°C, in Tris.HCl buffer, pH 7.5, I = 0.1M.

3.4. Conclusions

From the studies on the point mutant forms of flavocytochrome b_2 the following conclusions can be drawn: 1) In the carbanion mechanism proposed by Lederer and Mathews, Y143 and Y254 were assigned substrate binding roles. It is remarkable therefore that there are no significant differences in the K_M values. Clearly, there is some discrepancy between these data and the model of the Michaelis-Menten complex proposed by Mathews and Lederer. 2) Y254 is important for catalytic efficiency as demonstrated by the marked difference in activity seen with the Y254F mutant and wild-type enzymes. 3) Y143 appears to have a role in the flavin to heme electron transfer as well as being involved in the catalysis of lactate oxidation. 4) Replacement of R376 with a lysine leads to a totally inactive enzyme. Whether it is the loss of interactions between R376 and other amino acids, or the inability for substrate to bind at the active site, R376 nevertheless appears to be an essential residue for enzyme activity. 5) A complete loss of lactate dehydrogenase activity was also observed when K349 was replaced by an arginine, consistent with the proposed involvement of this residue in catalysis. Although this substitution conserves charge, it also introduces substantial bulk in a tightly packed region close to the flavin. Although the presence of flavin has been verified in this mutant, gross structural alterations are expected around the flavin cofactor. 6) The observation that a substitution in the disordered loop can affect the kinetic parameters highlights the importance of this mobile region for catalytic efficiency and complements the results obtained for the cleaved form of the enzyme. 7) Replacement of Y97 with a phenylalanine does not affect the catalytic properties of the flavin active site. It does, however, significantly enhance substrate inhibition. This was unexpected considering that Y97 is located in the heme-binding domain. 8) Replacement of F325 with an alanine gives rise to a mutant enzyme which is less efficient in catalysis. This is

consistent with small conformational changes being transmitted through to the flavin active site.

3.5. References

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Chapter Four

THE ROLE OF THE C-TERMINAL TAIL

4.1. Introduction

In the stopped-flow studies of Capeillere-Blandin *et al* [1] and Pompon *et al* [2], the three-electron reduction of oxidised flavocytochrome b_2 proceeded in a biphasic manner in which phase I was a fast phase corresponding to the entry of the first two electron pairs per dimer (resulting in reduced heme and semiquinone flavin) and phase II corresponded to the slow intersubunit electron transfer allowing entry of the third electron pair per dimer (resulting in fully reduced flavocytochrome b_2 (section 1.5.4). These intersubunit electron-transfer steps (shown to be too slow to be involved in enzyme turnover [1,2]) complicate the stopped-flow studies. One way to simplify the electron transfer for stopped-flow studies would be to generate a monomeric flavocytochrome b_2.

From NMR linewidth and X-ray diffraction studies of flavocytochrome b_2 [3,4], there is evidence to suggest that there is mobility within the flavocytochrome b_2 tetramer. It is possible that mobility of the cytochrome domain allows the enzyme to adopt conformations which enhance electron transfer to the physiological partner, cytochrome *c* . In the crystal structure, the subunits related by a 2 fold rotational axis are disordered. It is possible that there is some degree of cooperativity between the flavocytochrome b_2 subunits. This could also be investigated by the production of monomeric flavocytochrome b_2.

From the crystal structure (figure 1.5), it can be seen that the flavocytochrome b_2 polypeptide chain can be divided into three sections: the cytochrome domain (residues 1-100); the flavin-binding domain (residues 101-488); and an extended C-terminal tail (residues 489-511). The observation that this tail wraps around each of the other three subunits in the holoenzyme would be consistent with the idea that the primary role of the C-terminal tail is to hold the subunits together as a tetramer. A tail-deleted mutant (hereafter

referred to as TD-b_2) was generated by site-directed mutagenesis in an effort to produce monomeric flavocytochrome b_2. However, the TD-b_2 was found to be tetrameric and exhibited some surprising and interesting kinetic properties which have given an insight to the role of the C-terminal tail.

4.2. Methods and Materials

4.2.1. Generation of TD-b_2, (M.T.Black)

The gene encoding tail deleted flavocytochrome b_2 (TD-b_2) was generated by the double primer method of Zoller and Smith (appendix II), using single stranded pGR401 as template. The following oligonucleotide synthesised on an Applied Biosystems model 380B DNA synthesiser, was used for mutagenesis: GAACAGTTTGAGTACCA. The nucleotide sequence of the entire TD-b_2 gene was determined and found to contain no secondary mutations. The TD-b_2 gene was excised form pGR401 as a 1.8kb *Eco*R1/*Hin*dIII restriction fragment and inserted into the polylinker region of the plasmid pDS6 [5]. *E. coli* strain HB101 was grown in L-broth (section 6.6.2) and transformed by the $CaCl_2$ method. Transformants were selected by ability to grow in the presence of ampicillin and by pink coloration due to the presence of the heme-containing TD-b_2.

4.2.2. Enzyme Preparation and Characterisation

Wild-type and tail-deleted flavocytochrome b_2 were isolated from *E. coli* as described in section 6.5.4. When fully purified the wild-type enzyme showed a single band at around 57.5kDa on an SDS-page gel, whereas the TD-b_2 showed as a single band at ≈55kDa. TD-b_2 had an electronic spectrum in the visible region that was superimposable on that of the wild-type enzyme. The molecular weight of TD-b_2 under non-denaturing conditions was determined using a Sephacryl S-300 column as described in section 6.4.2. Both active and deactivated enzyme were eluted with apparent molecular weights of 220 ±

20kDa (figure 4.1).

4.2.3. Michaelis-Menten Kinetics

Kinetic experiments involving enzymatic oxidation of L-lactate were carried out under steady-state conditions at 25 $\pm$ 0.1°C in 10mM Tris.HCl, pH 7.5, I = 0.10M (NaCl), using ferricyanide (1mM) as electron acceptor (section 6.8.1). Enzyme activity was observed to fall with time, long before lactate or ferricyanide were depleted. Treatment of the initial rates of enzyme turnover gave values of k_{cat} and K_M. Enzyme concentrations were determined using the published extinction coefficients of the wild-type enzyme (section 6.8.1). 2-^{2}H lactate was prepared enzymatically using yeast lactate dehydrogenase, alcohol dehydrogenase and NAD^+, and purified by chromatography on a DOWEX 1 x 8 column. Isotopic purity was confirmed by ^{1}H NMR (section 6.7.2). Enzyme turnover rates were calculated over the lactate range 1-12mM for wild-type and TD-b_2 using both the deuterated and undeuterated lactate. Kinetic isotope effects were calculated as $k_{cat}(H)/k_{cat}(D)$.

4.2.4. Deactivation Kinetics

The decrease in enzyme turnover was monitored by following the decrease in the rate of ferricyanide consumption with time (figure 4.2). Semilog plots of log(enzyme turnover rate) versus time gave biphasic curves (figure 4.3) which could be separated into two phases, $k_{f(deact)}$ and $k_{s(deact)}$. The rates of deactivation were calculated between 1 and 10mM lactate (as shown in figure 4.4).

4.2.5. Verification of Flavin

The presence of flavin was detected using difference visible spectroscopy by the method of Lederer *et al* (figure 4.6) [6]. Identical solutions of oxidised TD-b_2 in Tris/HCl buffer, pH 7.5, were placed in the reference and observation cells of a Perkin-Elmer λ9 spectrophotometer. To the reference cell was added

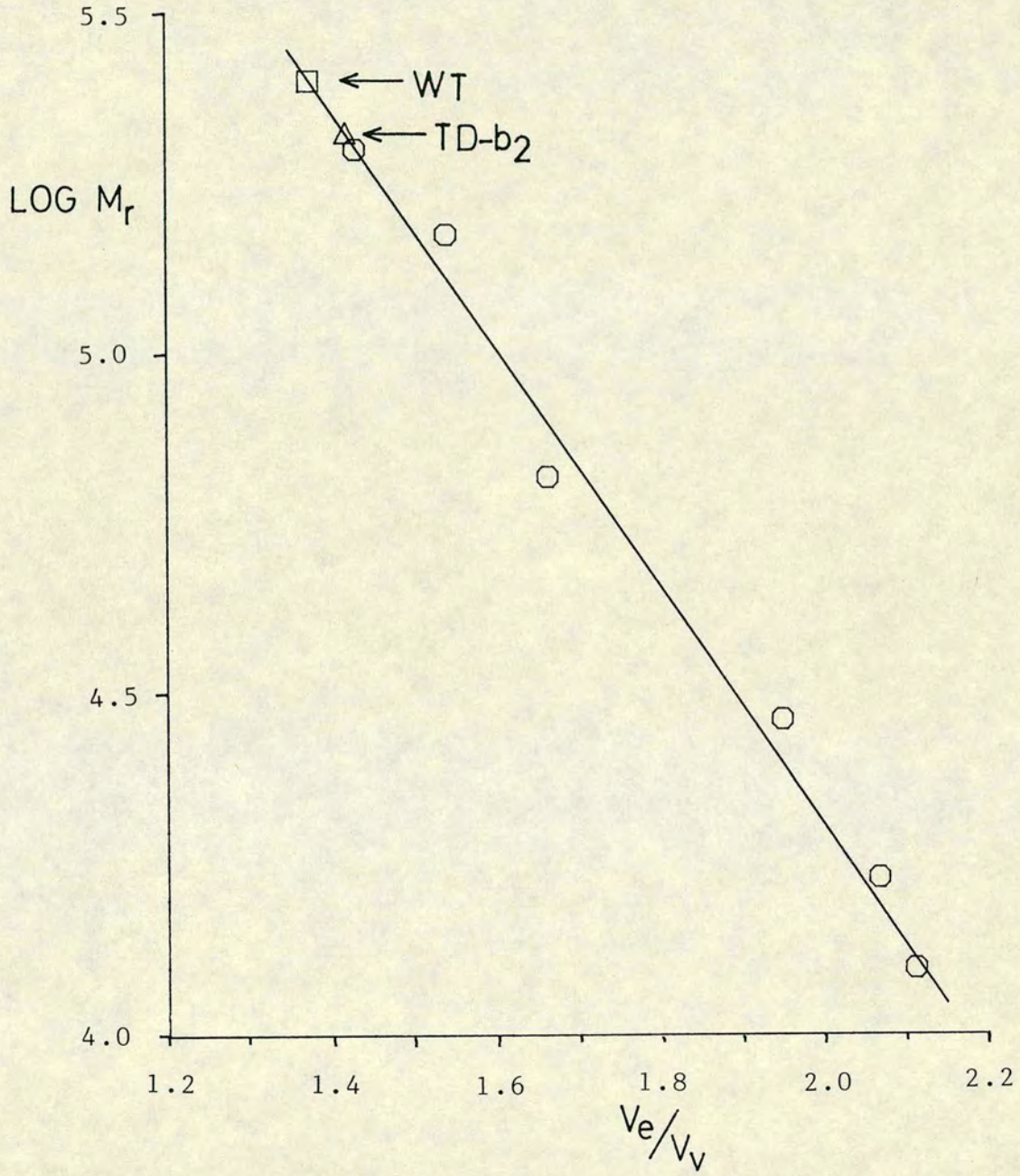

Figure 4.1 Molecular weight determination of TD-b_2. Molecular markers as before, figure 2.4.

The experimental elution volumes fall outside the standardisation range and therefore require an unjustified extrapolation.

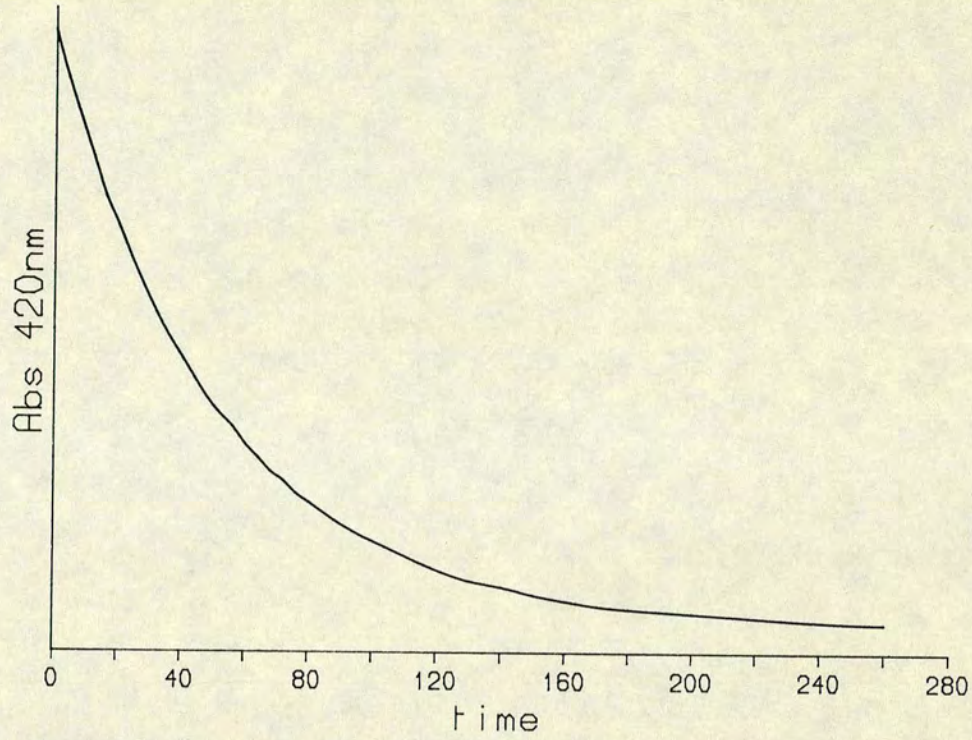

Figure 4.2 Consumption of $Fe(CN)_6^{3-}$ versus time for TD-b_2, showing the deactivation of TD-b_2. Time traces were recorded at 25±0.1°C, in Tris.HCl buffer, pH 7.5, and I= 0.10M.

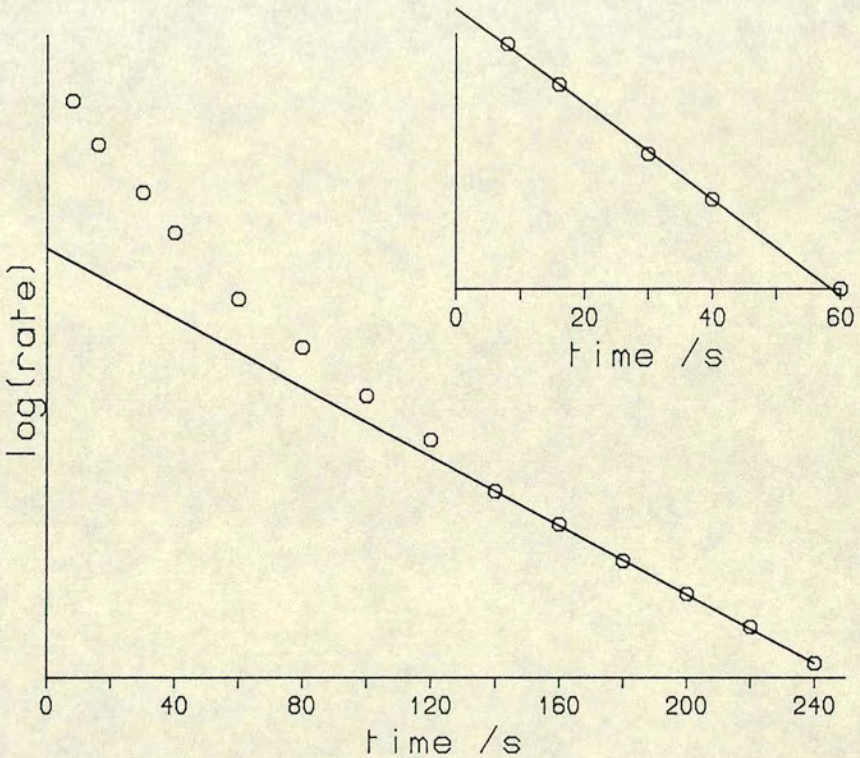

Figure 4.3 Semilog plot of $Fe(CN)_6^{3-}$ consumption versus time, showing the biphasic nature of TD-b_2 deactivation.

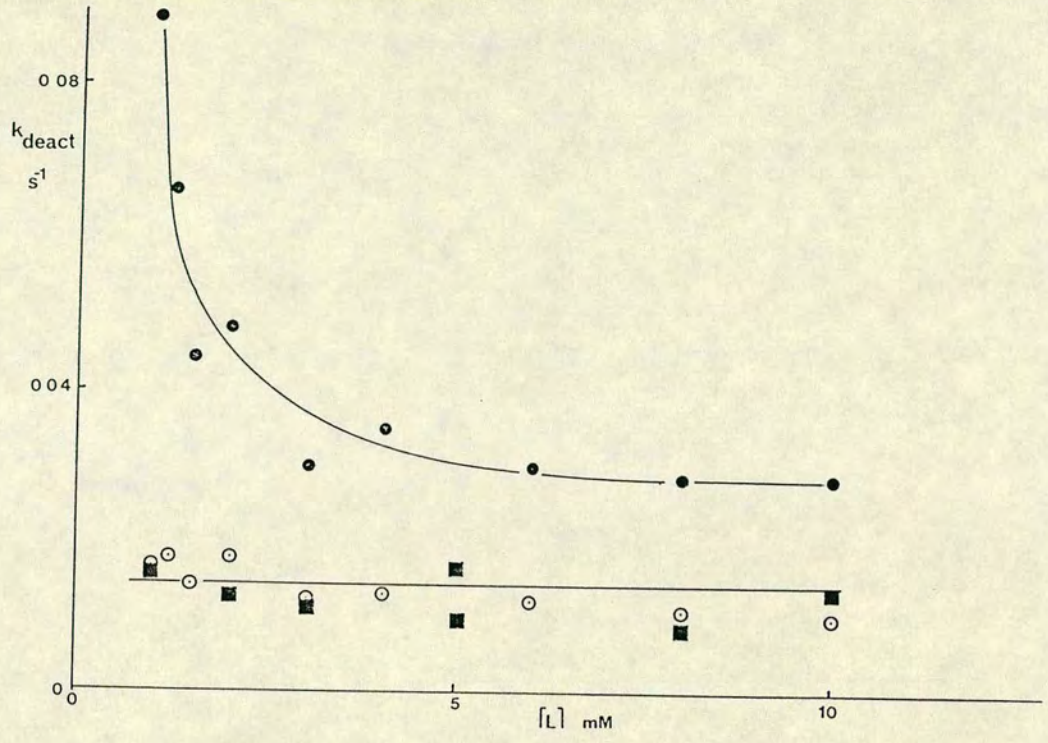

Figure 4.4 The dependence of deactivation rate constants on L-lactate concentration. ● = $k_{f(deact)}$, ⊙ = $k_{s(deact)}$ and ■ = k_{diss} All data shown were determined at 25±0.1°C, in Tris.HCl buffer, pH 7.5 and I = 0.10M.

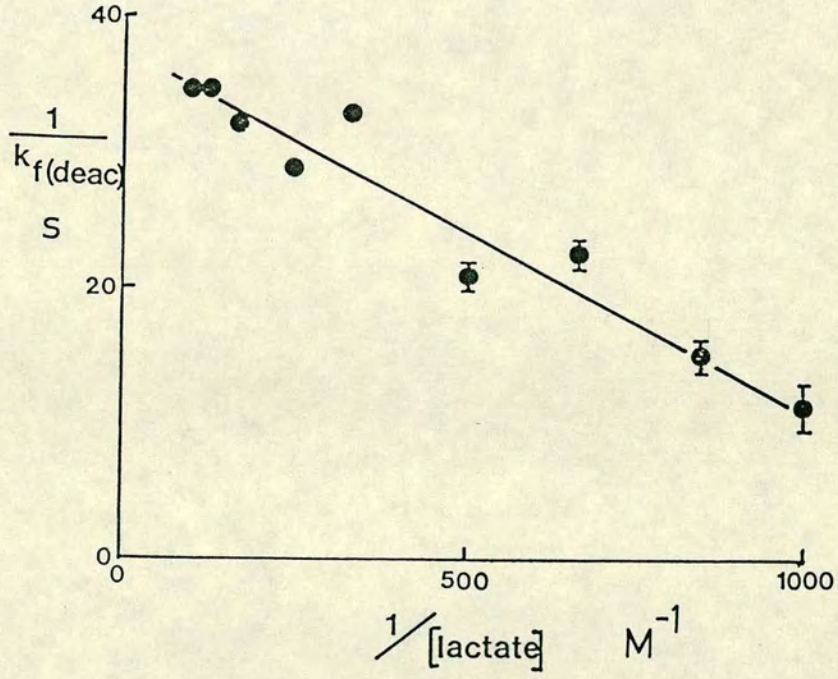

Figure 4.5 Double-reciprocal plot demonstrating the linear relationship of $1/k_{f(deact)}$ against 1/[L-lactate].

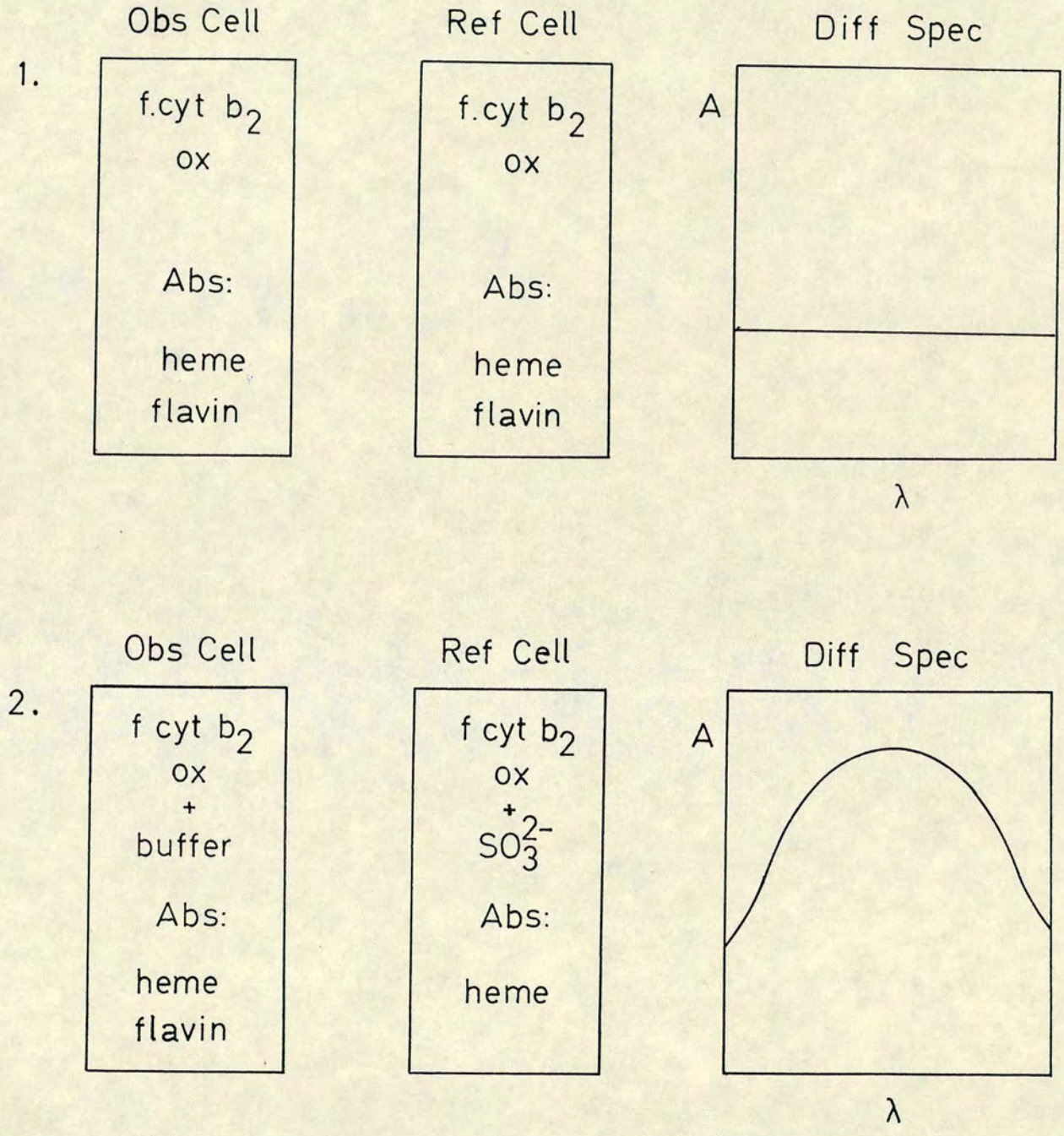

Figure 4.6 The use of sulphite to obtain an electronic absorbance spectrum of enzyme-bound FMN. see Fig 4.9, for example

10µl of 0.3M Na_2SO_3 (which bleaches the flavin absorbance) and to the observation cell 10µl of buffer. The resulting difference spectrum corresponds to bound flavin.

4.2.6. Flavin Loss

The dissociation of flavin during turnover was monitored as an increase in fluorescence at 520nm on a Perkin Elmer LS-5 Luminescence spectrometer with an emission bandwidth of 2.5nm and with the excitation wavelength set at 450nm with an excitation bandwidth of 10nm. The intensity of the fluorescence as a function of FMN concentration is known to be linear in the concentration range of 10^{-10}–10^{-5}M [7], and consequently all experiments were carried out with the enzyme concentration between 0.1 and 0.01µM. The ferricyanide concentration was 8mM and the lactate concentration was varied between 1 and 10mM (figure 4.4).

4.2.7. Flavin Reincorporation

Reincorporation of flavin into the flavin defficient enzyme was carried out according to the following procedure. Deactivated TD-b_2 was partially denatured in solution-A (150mM phosphate buffer, pH 7.0, containing 4M urea, 100mM 2-mercaptoethanol, 100mM Lactate and 1mM EDTA). A ten-fold excess of flavin mononucleotide (Sigma) was added and renaturation facilitated by dialysis of the TD-b_2/FMN mixture firstly against solution A in the absence of urea and then against solution A in the absence of urea and 2-mercaptoethanol. Each dialysis wass carried out for 20 hours at 4°C under nitrogen. After the final dialysis the reconstituted protein showed similar kinetics to the original TD-b_2. The procedure for the wild-type protein was as above except that flavin dissociated from the protein during denaturation.

4.3. Results and Discussion

The 3-dimensional structure of a subunit of flavocytochrome b_2 shows very clearly an extended C-terminal tail which projects from the main body of the molecule. In the tetramer the tail of each subunit wraps around each of the other three making many intersubunit contacts (figures 4.7 and 4.8). Therefore, the most obvious role one might suggest for this C-terminal tail is that it holds the four subunits together. To test this, and to hopefully generate monomeric enzyme, a stop codon was introduced into the flavocytochrome b_2 gene at a position corresponding to amino-acid residue 489. This mutation resulted in production of a mutant enzyme (TD-b_2) lacking 23 amino-acid residues at the C-terminal portion of the molecule which showed, as expected, a molecular weight of ≈55kDa on SDS-PAGE. However, TD-b_2 exhibited a molecular weight (under non-denaturing conditions) of 220 $\pm$ 20kDa corresponding to a tetramer (figure 4.1).

The kinetic studies indicated that the tail-deletion had significantly affected the stability of the enzyme. Under steady state conditions, (normal assay mix, with 1mM ferricyanide and lactate) the rate of ferricyanide (or cytochrome *c*) reduction decreased with time, reaching zero within a few minutes (figure 4.2). Addition of extra TD-b_2 to the same assay mix showed that ferricyanide and lactate were not exhausted and that the enzyme was deactivating to give a totally inactive enzyme. However, initial rates of enzyme turnover could be determined and values of k_{cat} and K_M were calculated to be 165 $\pm$ 6s^{-1} and 0.96 $\pm$ 0.06mM, compared to 200 $\pm$ 10s^{-1} and 0.49 $\pm$ 0.04mM for the wild-type enzyme.

The changes in kinetic parameters are consistent with a conformational change taking place at the active site arising from the tail-deletion. To check if this conformational change had any effect on the rate-limiting step of the lactate oxidation (known to be the αH abstraction of lactate to form a

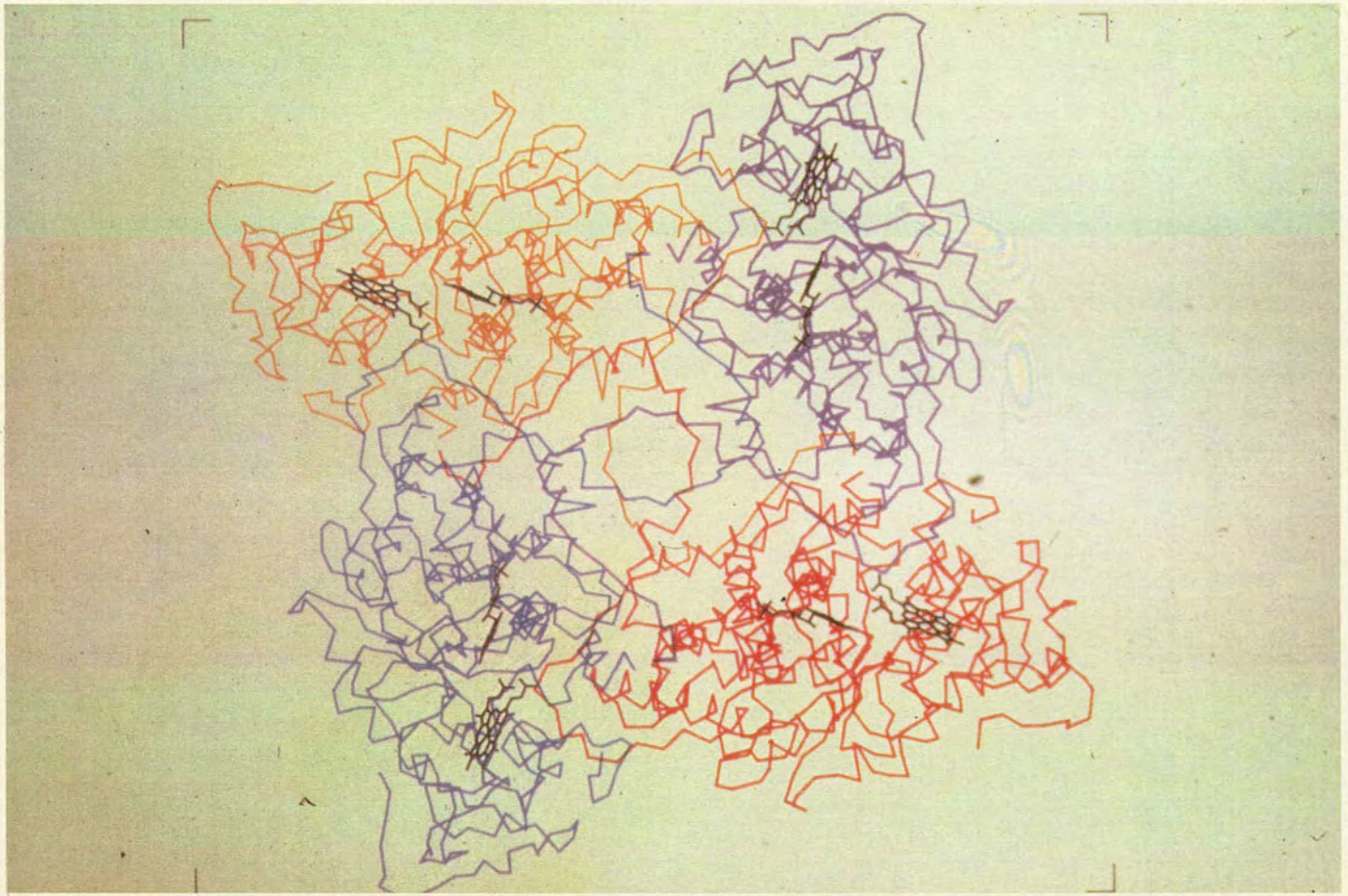

Figure 4.8 Theoretical structure of the flavocytochrome b_2 tetramer generated by rotation of a single heme containing subunit.

carbanion) the kinetic isotope effects of wild-type and TD-b_2 were measured using lactate deuterated at the Cα position. Wild-type enzyme gave a kinetic isotope effect of 4.2 ± 0.2, in agreement with the value published previously [8] and TD-b_2 gave a value of 3.6 ± 0.4, indicating that the rate-limiting step had not significantly changed upon tail-deletion. The molecular weight of totally deactivated TD-b_2 was determined to see if the deactivation was a result of the breaking up of the tetramer into monomers during turnover, however, the molecular weight was found to be exactly the same as the active TD-b_2 enzyme (figure 4.1). Thus the tetramer is stable under assay conditions.

The rate of lactate oxidation has been found to correlate with the level of flavin incorporation [9]. The presence of flavin can be determined by the method of Lederer *et al* [6], in which a difference spectrum of oxidised flavocytochrome b_2 with sodium sulphite present in the reference cell (see methods and materials and figure 4.5) produces an absorption spectrum of the oxidised flavin. With active TD-b_2, the flavin absorbance spectrum was the same as that of the wild-type enzyme (figure 4.9a). With deactivated TD-b_2, however, there was no flavin absorbance at all (figure 4.9b), indicating that the flavin must have been lost under enzyme turnover, resulting in the deactivation of TD-b_2. Thus, it appears that the C-terminal tail is essential for stability of flavin at the active site. Denaturation of TD-b_2 (and wild-type as a control) followed by renaturation in the presence of a ten-fold excess of flavin, achieved reincorporation of flavin at the active site with ≈60% of the original activity (≈50% for wild-type). The values of K_M and k_{cat} were higher and lower respectively than the wild-type enzyme (table 4.1), consistent with the fact that the reconstituted enzyme was slightly less efficient in catalysis (as previously observed for wild-type enzyme [9]). The deactivation kinetics of reconstituted TD-b_2 were found to be the same within experimental error as the original TD-b_2 indicating that the flavin dissociates from the original and

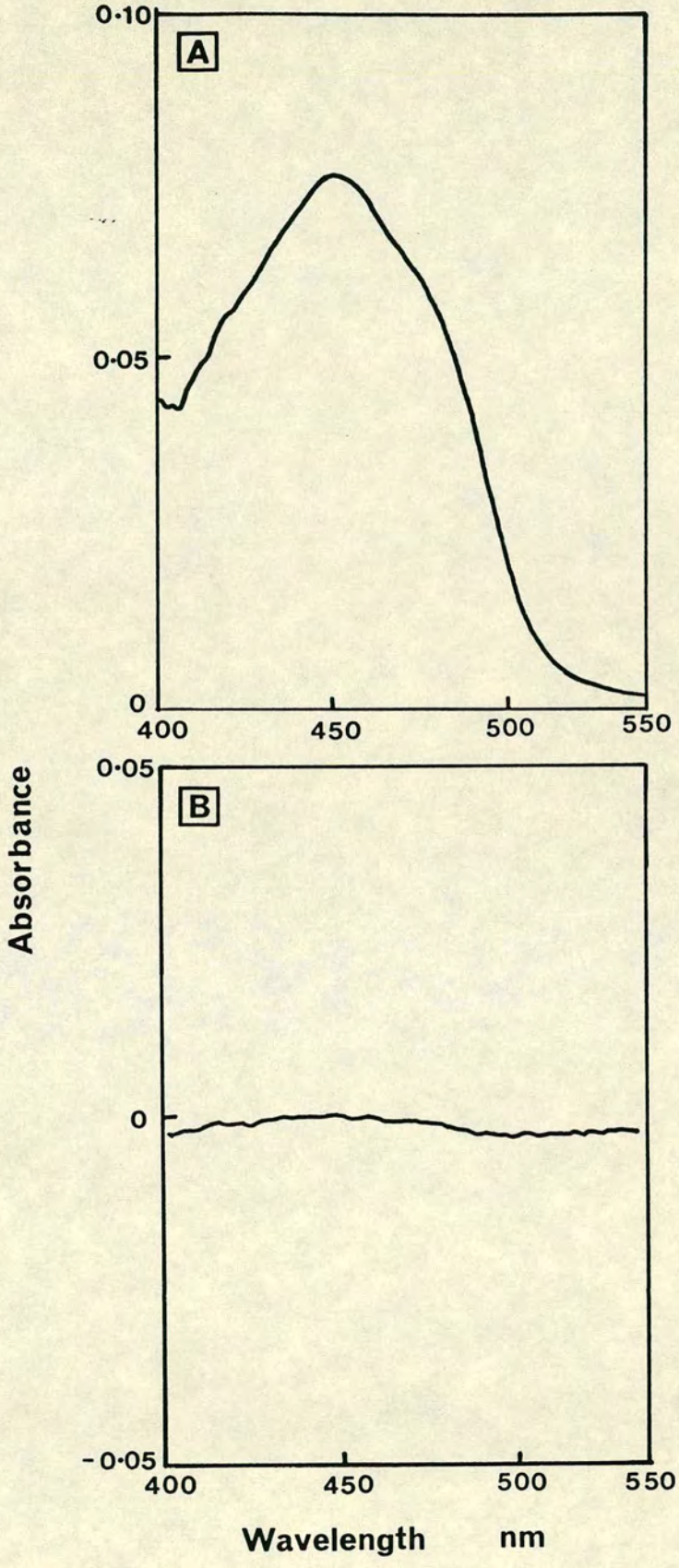

Figure 4.9 Electronic absorbance difference-spectra obtained by using the sulphite method of Lederer *et al* [6], described in section 4.2.5. Spectrum A is of TD-b_2 before deactivation. Spectrum B is of fully deactivated TDb_2. The zero absorbance in spectrum B indicates that the FMN group has dissociated from TD-b_2 during deactivation.

reactivated TD-b_2's in the same way.

Analysis of activity versus time traces showed that the deactivation process was biphasic in nature. The faster of the two phases gave a first-order rate constant, $k_{f(deact)}$ dependent on L-lactate concentration (figure 4.4). Increasing the L-lactate concentration decreased the value of $k_{f(deact)}$ until a limiting value was reached (table 4.1). From the double reciprocal plot (figure 4.5) an apparent K_d for L-lactate of 0.8 $\pm$ 0.1mM was determined, which is very close to the K_M value of 0.96 $\pm$ 0.06mM. The similarity of these values is consistent with an intimate association between deactivation during the fast phase and L-lactate binding. A first-order rate constant, $k_{s(deact)'}$ was determined for the slower phase of the biphasic deactivation and was essentially independent of L-lactate concentration (figure 4.4).

The fluorescence of free flavin is known to be directly proportional to the flavin concentration [7], and is quenched upon binding to the enzyme [11]. Thus, the loss of flavin can be monitored during enzyme turnover, by following the increase of fluorescence with time. Analysis of the fluorescence-time traces gave a first-order rate constant for flavin dissociation, k_{diss} (table 4.1), which did not vary with the concentration of L-lactate. It can be seen from figure 4.4 that the slower phase of deactivation corresponds exactly with the dissociation of flavin and that the fast phase corresponds to some substrate dependent change in the enzyme which is less catalytically efficient and can lead to flavin dissociation. The mechanism might possibly be represented by the following equation:

	ENZYME			
	wild-type	TD-$\underline{b}_2$	reconstituted wild-type	reconstituted TD-$\underline{b}_2$
k_{cat} s^{-1}	200±10	165±6	98±3	97±8
K_m mM	0.49±0.04	0.96±0.06	0.65±0.05	1.76±0.26
$k_{f(deac)}$ s^{-1}	-	0.029±0.002	-	0.028±0.006
$k_{s(deac)}$ s^{-1}	-	0.010±0.001	-	0.008±0.001
k_{diss} s^{-1}	-	0.012±0.002	-	-
Kinetic Isotope Effect	4.2±0.2	3.6±0.4	-	-

Table 4.1 Kinetic properties of wild-type and tail-deleted (TD-b_2) flavocytochromes b_2. All values were determined at 25±0.1°C, Tris.HCl buffer, pH 7.5 and I = 0.1M, with $Fe(CN)_6$ (1mM) as the electron acceptor. The kinetic isotope effect was determined with 2-^{2}H-lactate. The value of k_{diss} was determined by monitoring the increase in fluorescence (caused by dissociation of FMN during turnover) with time; $Fe(CN)_6$ concentration was 8mM in this case.

$$E \xrightarrow{k_{f(deact)}} E_c \xrightarrow{k_{s(deact)}} E_i + \text{free FMN}$$

where: E = TD-b_2,

E_c = a conformationally altered TD-b_2,

E_I = a totally inactive TD-b_2.

With the apparent K_d for lactate being so close in value to the K_M (0.8 and 0.96 respectively), the fast phase of deactivation, $k_{f(deact)}$ could be dependent upon either the rate of lactate oxidation or the level of substrate occupancy at the active site. However, TD-b_2 and reconstituted TD-b_2 have different values of k_{cat} ($165s^{-1}$ and $97s^{-1}$ respectively) yet have the same value of $k_{f(deact)}$ ($0.029 \pm 0.002s^{-1}$ and $0.028 \pm 0.006s^{-1}$), therefore, the most likely explanation of the lactate dependence on $k_{f(deact)}$ is that occupancy at the active site inhibits the movement of flavin to produce the conformationally altered, partially deactivated enzyme. Further studies measuring the effect of competitive inhibitors on the deactivation kinetics should verify this since the effect of increasing inhibitor concentration should cause a decrease in $k_{f(deact)}$ if the level of active site occupancy is important, and an increase in $k_{f(deact)}$ if rate of substrate oxidation is important.

The kinetics of cytochrome *c* reduction by TD-b_2 were also significantly affected by the tail deletion and will be described in chapter 5.

4.4. Conclusions

The generation of a tail-deleted flavocytochrome b_2 has failed to produce a monomeric enzyme, despite the fact that the tail appears to form many intersubunit contacts. However, the C-terminal tail has been shown to be essential for the structural integrity of the flavin active site. Deletion of the tail section results in the formation of a less catalytically efficient enzyme, with a poorer affinity for flavin. The deactivation of TD-b_2 during turnover occurs in a biphasic process with a fast phase which is lactate concentration dependent and a slow phase which is lactate concentration independent and which corresponds to the dissociation of flavin. Any conformational change occuring at the active site does not lead to a change in the rate-limiting step in the mechanism of lactate oxidation.

4.5. References

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Chapter Five

CYTOCHROME *c* AS THE ELECTRON ACCEPTOR

5.1. Introduction

Cytochrome *c* was first suggested as an electron acceptor for flavocytochrome b_2 by Appleby and Morton [1], and was later shown to be the physiological partner of flavocytochrome b_2 in a short electron-transfer chain which also includes cytochrome *c* oxidase [2]. Stopped-flow kinetic studies with flavocytochrome b_2 and cytochrome *c* both from *H. anomala* showed that the rate of cytochrome *c* reduction was ionic-strength dependent [3]. The rate increased as the ionic strength decreased, reaching a saturation value at low ionic strength. The Debye-Huckel plot (linear above $0.27M^{1/2}$ ionic strength) gave a charge product of -5.7, whereas a similar study with cytochrome b_2 core gave a linear ionic-strength dependence with a charge product of -1.9, and no saturation effect [4]. Fluorescence studies demonstrated that cytochrome *c* has a higher affinity for the flavin-binding domain than the cytochrome domain [5,6]. However kinetic studies have shown that the b_2 heme was essential for cytochrome *c* reductase activity [7,8]. Thus it appears that cytochrome *c* reduction occurs by formation of an ionic-strength dependent complex between flavocytochrome b_2 and cytochrome *c*, with subsequent intracomplex electron transfer between the two hemes.

Tegoni *et al* observed complex formation in flavocytochrome b_2 crystals which were washed in buffers of low ionic strength containing cytochrome *c* [9]. When the ionic strength of the buffer was increased, the ratio of cytochrome *c* to flavocytochrome b_2 decreased and cytochrome *c* diffused out of the crystals.

Steady-state kinetic studies were undertaken with mutant flavocytochromes b_2 to determine whether any of the mutants affected the rate of cytochrome *c* reduction. These studies show that k_{cat} and K_M are limited by the rate of lactate oxidation and indicate that deletion of the extended C-terminal tail has a profound effect upon the interaction with

cytochrome *c* pointing toward a possible location of the cytochrome *c* association site.

5.2. Methods and Materials

Wild-type and mutant flavocytochromes b_2 were isolated from *E. coli* as described in section 6.5.4. Horse heart cytochrome *c* (Sigma, type VI) was used without further purification. Cytochrome *c* concentrations were measured spectrophotometrically using the extinction coefficients $\varepsilon_{550}^{ox} = 9{,}400 M^{-1}cm^{-1}$ and $\varepsilon_{550}^{red-ox} = 22{,}640 M^{-1}cm^{-1}$. Rates of cytochrome *c* reduction by flavocytochrome b_2 in the presence of a saturating concentration of L-lactate (10mM) were measured under steady-state conditions at 25°C in Tris.HCl buffer, 0.1M ionic strength as described in section 6.8.1. The concentration of cytochrome *c* was varied between 0 and 80µM. Data were analysed to give values of maximum rate, k_{cat} , and apparent Michaelis-Menten constants, $K_{M(app)}$. The rates of deactivation of tail-deleted flavocytochrome b_2 (TD-b_2) were measured as described in section 4.2.4. 2-^{2}H-lactate was prepared and purified as described in section 6.7.2. Rates of enzyme turnover, *v*, were measured over the lactate range 1-12mM for wild-type and TD-b_2 using both deuterated and undeuterated lactate as substrates and cytochrome *c* as the electron acceptor. Kinetic isotope effects were calculated as v_H/v_D.

5.3. Results and Discussion

5.3.1. Rates of Cytochrome *c* Reduction

As can be seen from table 5.1, the value of k_{cat} varies from $5.3s^{-1}$ (Y254F) to $200s^{-1}$ (WT) and, with the exception of Y143F and Y254F, is approximately half the value of k_{cat} obtained using ferricyanide as the electron acceptor ($k_{cat(ferri)}$). This ratio of 1:2 is in agreement with ratios previously determined (0.5-0.6, 13mM phosphate buffer, pH 7.3 [10]) but in contrast to recent results obtained by Lederer who found a ratio of 1.0 (100mM phosphate buffer [11]). It

Enzyme	K_M (μM)	$k_{cat(cyt\ c)}$ (s^{-1})	$k_{cat(ferri)}$ * (s^{-1})
WT	10 ± 1	109 ± 10	200 ± 10
Y143F	1.5 ± 0.2	14.5 ± 1.0	70 ± 10
Y254F	0.28 ± 0.10	5.3 ± 0.3	6.5 ± 0.5
A306S	7.1 ± 0.1	78 ± 6	160 ± 3

* Data from Table 3.3.

Table 5.1 Kinetic rates for wild-type and point mutant enzymes. Cytochrome *c* was used as the electron acceptor (0-100μM). All data were obtained at 25±°C, in Tris.HCl buffer, pH 7.5, I = 0.1M. L-lactate was present as the substrate (10mM). Data from table 3.3 are also shown for comparison.

is likely that the differences are due to the nature of the buffer and the ionic strength. Dissociation of cytochrome *c* may be partially rate-limiting; thus at higher ionic strengths the cytochrome *c* reduction rate would approach that of ferricyanide.

The observation that $k_{cat(ferri)}$ is not twice the value of $k_{cat(cyt\ c)}$ for Y143F suggests that for this mutant enzyme there is a difference in the rate of electron transfer from flavin to heme, since cytochrome *c* can only be reduced by the b_2 heme. Iwatsubo *et al* found that dehemoflavocytochrome b_2 had a lowered ferricyanide reductase activity [8], attributed to the fact that flavin hydroquinone is a poor one-electron donor, and electron transfer from flavin to heme would give two highly efficient one-electron donors (flavin semiquinone and reduced heme). The differences in $k_{cat(ferri)}$ and $k_{cat(cyt\ c)}$ between wild-type and Y143F flavocytochromes b_2 are consistent with a reduced flavin to heme electron-transfer rate for Y143F. A measurement of the 2-^{2}H-lactate kinetic isotope effect with Y143F using ferricyanide and cytochrome *c* should confirm this.

Figure 5.1 shows a plot of $k_{cat(cyt\ c)}$ versus $K_{M(cyt\ c)}$ for wild-type and three mutant flavocytochromes b_2. The value of K_M is proportional to $k_{cat(cyt\ c)}$ and therefore appears to be limited by the rate of lactate oxidation. Therefore the K_M's cannot be considered as true K_M values and are thus referred to as $K_{M(app)}$'s. At low concentrations of substrate the rate, *v*, of enzyme turnover is given by $v = (k_{cat}/K_M)[E0][S]$, therefore k_{cat}/K_M can be thought of as an apparent second-order rate constant, k_2'. The values of k_2' found for the wild-type and mutant enzymes are listed in table 5.1.

Unlike wild-type and the point mutations of flavocytochrome b_2, saturation kinetics were never observed for TD-b_2 in the cytochrome *c* concentration range studied. The rate of cytochrome *c* reduction has been dramatically lowered as shown in figure 5.2. From the slope of k_{obs} versus the

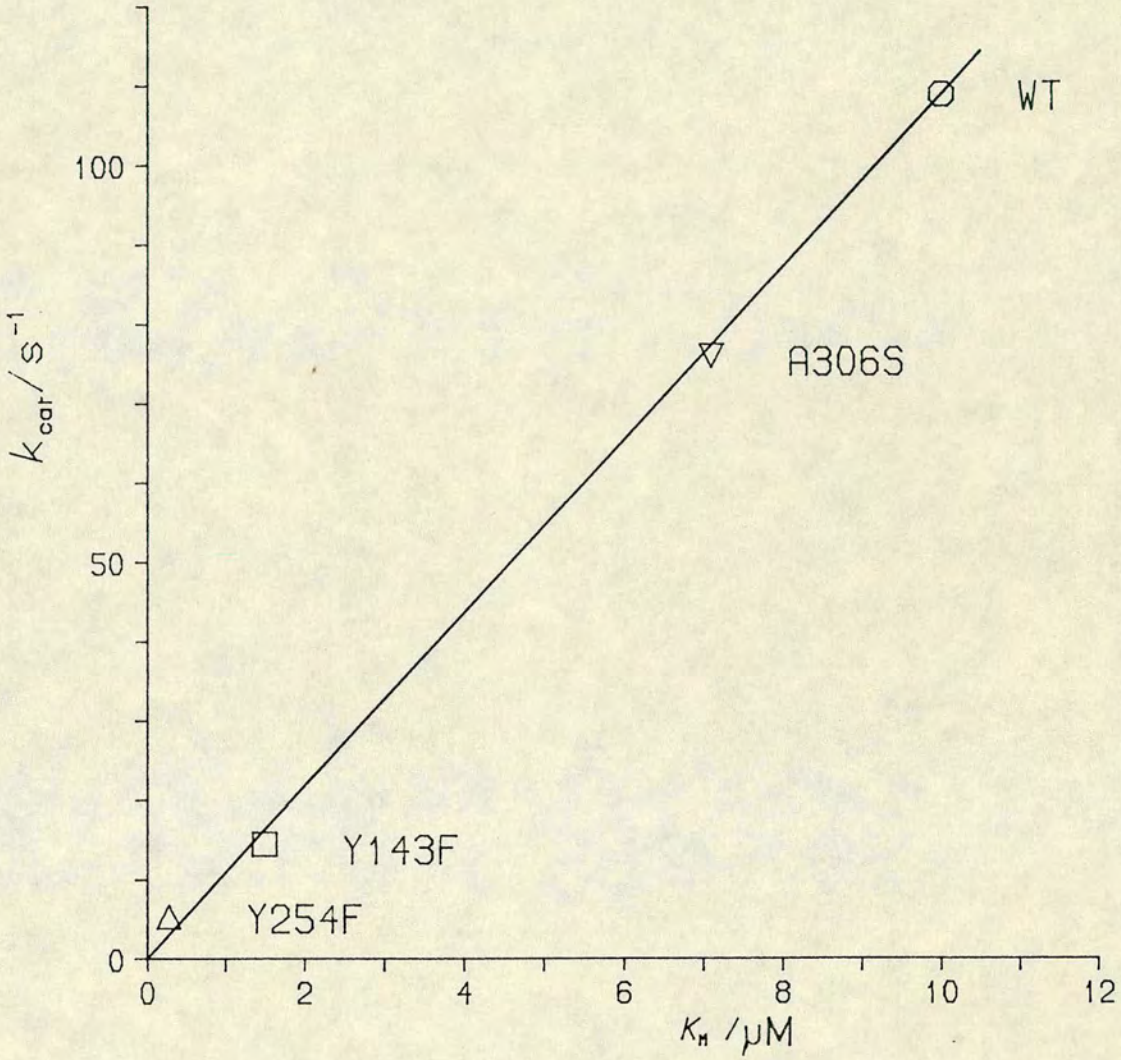

Figure 5.1 k_{cat} versus K_M for wild-type and mutant enzymes. Cytochrome *c* was used as the electron acceptor. All data shown were determined at 25± 0.1°C, in Tris.HCl buffer, pH 7.5 and I = 0.10M.

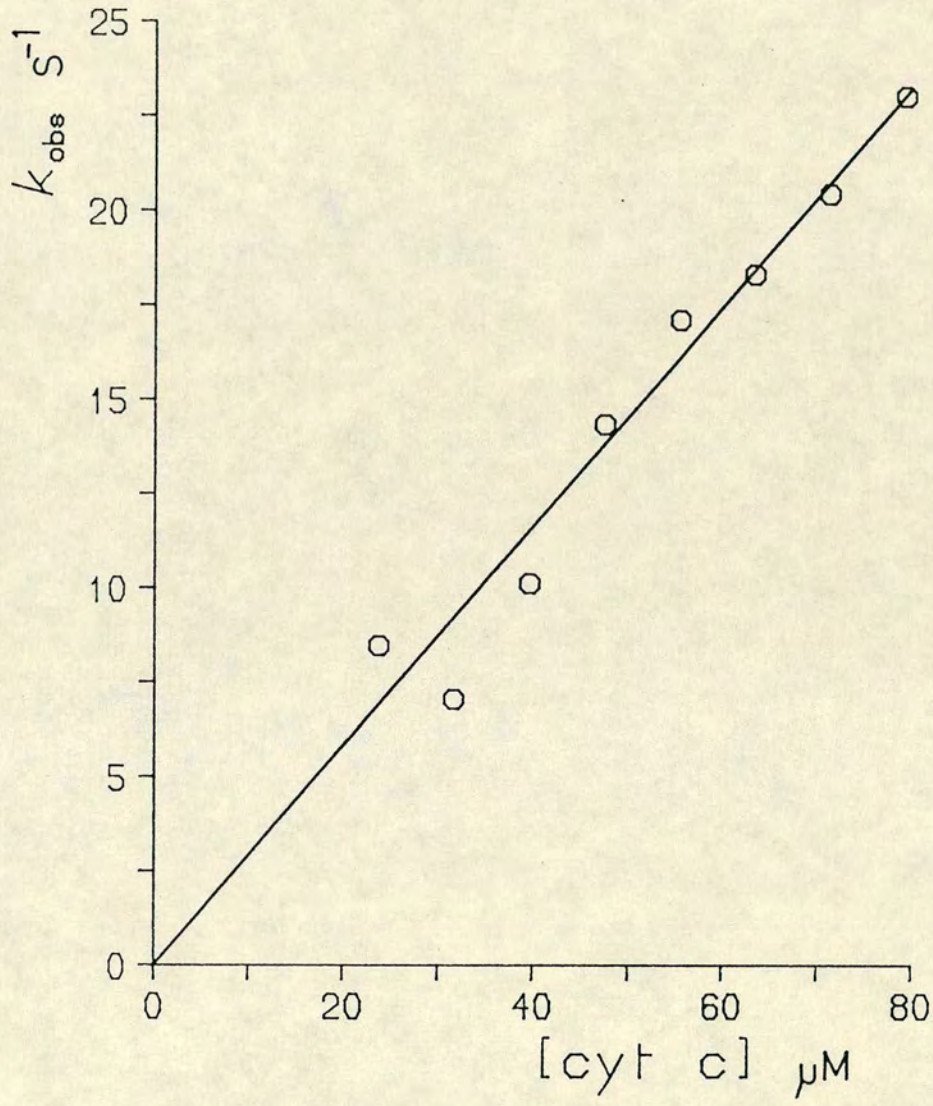

Figure 5.2 k_{obs} versus the concentration of cytochrome *c* as seen for TD-b_2. Values of k_{obs} were determined from the initial slopes of activity.

concentration of cytochrome *c*, a second-order rate constant of $2.87 \times 10^5 M^{-1}s^{-1}$ was obtained, two orders of magnitude lower than the pseudo-second-order rate constant calculated for the wild-type enzyme. This implies that the flavocytochrome b_2/cytochrome *c* interaction has been significantly affected by the tail-deletion, and since the rate of ferricyanide reduction, $k_{cat(ferri)}$ for TD-b_2 is about 80% that of wild-type, the difference in rate must be due to a large change in the structure or stability of the flavocytochrome b_2/cytochrome *c* complex. From the equation $k_2 = k_{cat}/K_M$, and assuming that $k_{cat(cyt\ c)}$ is likely to be half the value of $k_{cat(ferri)}$ ($83s^{-1}$) a value of $K_{M(app)}$ for TD-b_2 can be estimated to be about 0.58mM, sixty-fold higher than the value of $K_{M(app)}$ for wild-type flavocytochrome b_2 and about seventy-fold higher than the value of $K_{M(app)}$ that would have been predicted for TD-b_2 from figure 5.2.

The 2-^{2}H-lactate kinetic isotope effects (KIE) for cytochrome *c* reduction were measured under normal assay conditions. The values obtained were 2.3 for wild-type and 1.4 for TD-b_2. The KIE for wild-type using ferricyanide as the electron acceptor was 4.2 indicating that there is a partially rate-limiting step in the reduction of cytochrome *c* after the initial αH abstraction from lactate. With TD-b_2, the KIE of cytochrome *c* reduction is even smaller, suggesting that this partially rate-limiting step has become even more important.

As yet, no area of the flavocytochrome b_2 surface has been found to be complementary to cytochrome *c*, but molecular modelling studies have been restricted to the flavocytochrome b_2 subunit only. A flavocytochrome b_2/ cytochrome *c* complex similar to that for Salemme's model of cytochrome b_5/ cytochrome *c* seems unlikely in view of the fact that most of the "front face" of the b_2 cytochrome domain is covered by the flavin-binding domain. Therefore a large reorganisation energy would be required to permit movement of the cytochrome domain to allow such a complex to form.

Evidence cited above for flavocytochrome b_2 from *H. anomala* suggest that cytochrome *c* has a higher affinity for the flavin-binding domain than the cytochrome domain. As can be seen in figures 4.7 and 4.8, the C-terminal tail from one subunit extends toward the cytochrome domain of the subunit diagonally opposite. The C-terminal tail may participate in cytochrome *c* complex formation by forming part of a complementary surface (figure 5.3). Both *Saccharomyces* and *Hansenula* flavocytochromes b_2 have two glutamates and an aspartate in the last section of the C-terminal tail, which may interact electrostatically with part of the cytochrome *c* surface. Any complex which does form would probably have the two hemes aligned in such a way as to allow optimal electron transfer. Alternatively, formation of any complex may be more difficult with TD-b_2 due to the large decrease in steric bulk around the C-4 axis.

It appears from previous studies that at low ionic strength the flavocytochrome b_2/cytochrome *c* complex is more stable and that cytochrome *c* reduction may be partially limited by the dissociation of cytochrome *c*. At higher ionic strengths the cytochrome *c* dissociation constant increases due to the weaker electrostatic interaction and the cytochrome *c* reduction rate is less limited. With tail-deletion there are two possibilities: 1) the flavocytochrome b_2/cytochrome *c* complex does not form at all and electron transfer only occurs at the limit of molecular collision ie a simple second-order mechanism; or 2) a complex (inferior to the one with wild-type) does form, with cytochrome *c* spending less time in the optimum position for electron transfer, thus slowing down the rate of reduction. It is difficult to distinguish between the two with the kinetic data available (figure 5.2), since at the maximum cytochrome *c* concentration studied (80μM) there would only be a difference of 12% in the two calculated rates (assuming a K_M of 0.58mM for Michaelis-Menten kinetics and a k_2 of 2.87 x $10^5 M^{-1}s^{-1}$ for the

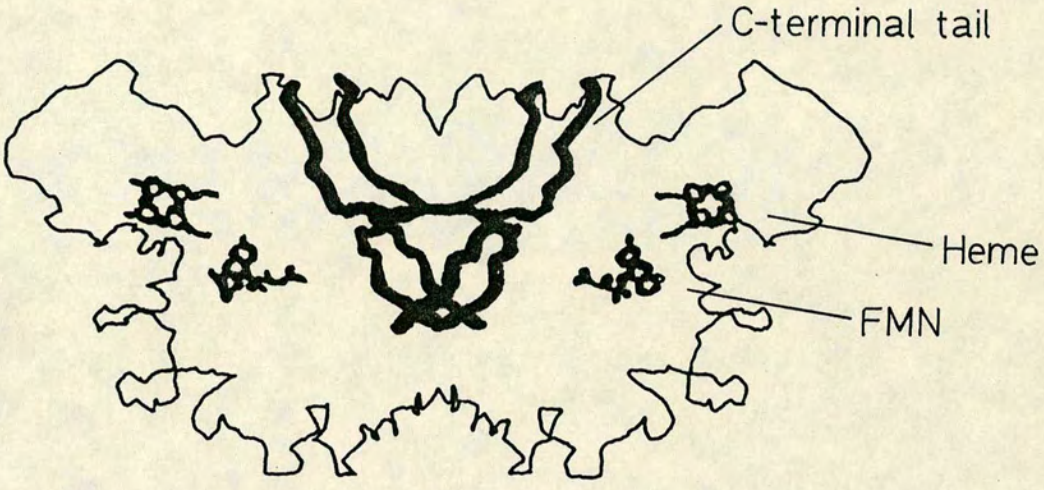

Figure 5.3 Schematic diagram of the flavocytochrome b_2 tetramer viewed from the side with the molecular 4-fold axis vertical.

bimolecular kinetics). Rate measurements at higher concentrations of cytochrome *c* are necessary to help decide which mechanism describes the TD-b_2/cytochrome *c* interaction more accurately. However, these results clearly imply that the C-terminal tail is involved in some way with the interaction of cytochrome *c*. Further experiments with smaller tail-deletions or point mutations within the tail coupled with ionic-strength dependence studies should provide further information on the nature of this interaction.

5.3.2. Deactivation of TD-b_2

The previous chapter described the deactivation kinetics of TD-b_2 using ferricyanide as the electron acceptor. The enzyme was found to lose activity in a biphasic process with the slower of the two phases corresponding to dissociation of the flavin. The fast phase was found to be dependent upon the concentration of L-lactate and was thought to correspond to some sort of alteration in the flavin active site leading to a less efficient enzyme. The experiment was repeated using cytochrome *c* as the electron acceptor, to see how the slow rate of cytochrome *c* reduction affected the deactivation kinetics. Again, TD-b_2 was observed to deactivate in a biphasic process. Values of $k_{f(deact)}$ and $k_{s(deact)}$ at saturating concentrations of cytochrome *c* were found to be identical within experimental error to the values obtained with ferricyanide (table 5.2). Results suggest that $k_{s(deact)}$ is still independent of the concentration of lactate and that $k_{f(deact)}$ will have a similar dependence as previously observed. The deactivation kinetics appear to be the same despite the fact that the rate of lactate oxidation has been dramatically reduced. This evidence adds weight to the argument that it is the level of occupancy by substrate at the active site and not the rate of lactate oxidation which regulates the conformational change at the active site ($k_{f(deact)}$).

Electron acceptor	$k_{s(deact)}$ (s^{-1})	$k_{f(deact)}$ (s^{-1})
Cytochrome c	0.009 ± 0.001	0.028 ± 0.006
ferricyanide	0.010 ± 0.001	0.029 ± 0.002

Table 5.2 Kinetic rates of deactivation for TD-b_2 using cytochrome *c* as the electron acceptor. All data were obtained at 25±0.1°C, in Tris.HCl buffer, pH 7.5, I = 0.1M. L-lactate was present as the substrate (10mM). Data from table 4.1 (ferricyanide) are also shown for comparison.

5.4. Conclusions

The following conclusions can be drawn from experiments using cytochrome *c* as the electron acceptor for wild-type and various mutants of flavocytochrome b_2: 1) The K_M value for cytochrome *c* does not represent a true K_M or dissociation constant since it is limited by the rate of lactate oxidation. 2) The kinetic isotope effects measured for wild-type and TD-b_2 show that the reduction of cytochrome *c* is partially rate limiting. 3) The studies with TD-b_2 clearly indicate that the C-terminal tail is somehow involved in the interaction with cytochrome *c*. In the absence of the tail, either the stability and/or conformation of the flavocytochrome b_2/cytochrome *c* complex has changed, or electron transfer is limited to the rate of successful molecular collisions. 4) The low rate of lactate oxidation and the presence of a much larger electron acceptor does not alter the rate of TD-b_2 deactivation and confirms that the stability of the flavin active site is determined by the level of substrate occupancy.

5.5. References

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Chapter Six

METHODS AND MATERIALS

6.1. Preparation of Buffer Solutions

6.1.1. General

The pH of buffers was measured using a WPA CD620 Digital pH meter fitted with a WPA glass electrode. The pH meter was calibrated at pH 7.0 and either pH 4.0 or 10.0 using standard colourkey buffer solutions (BDH, electrometrically checked). All pH measurements were made at 25°C. Deionised water purified to a resitivity of 18.3MΩ by reverse osmosis and ion exchange (Millipore Milli-QSP reagent water system) was used throughout to make all buffers.

6.1.2. Tris.HCl Buffer

Tris(hydroxymethyl)aminoethane (Sigma, reagent grade) hereafter referred to as Tris was used, with HCl (Fisons, SLR), to prepare Tris.HCl buffer; to 500ml of 0.02M HCl was added 5.2596g of NaCl (Fisons, SLR). The solution was titrated with Tris to pH 7.50 and the volume adjusted, to yield 1 litre of Tris.HCl buffer, 0.1M ionic strength, pH 7.50. All kinetic experiments were carried out in Tris.HCl buffer.

6.1.3. Phosphate Buffer

Monobasic and dibasic sodium phosphate, NaH_2PO_4 and Na_2HPO_4 (Sigma, reagent grade) were used to prepare phosphate buffers for the pH range 5.7 to 8.0. An appropriate volume and concentration of NaH_2PO_4 was titrated with an appropriate amount of Na_2HPO_4. These buffers were only used in the purification of proteins.

6.1.4. Acetate Buffer

A 0.02M sodium acetate (Fisons, SLR) solution was titrated with glacial acetic acid (Fisons, SLR) to give buffers of pH range 3 - 5. Acetate buffers were used only in the preparation and recovery of column materials.

6.1.5. General Stock Solutions

Lactate:- 200g of D-L lactic acid (BDH, analar) was mixed with 80ml of deionised water. 80g of NaOH (May and Baker, LR) was added slowly to avoid overheating. The solution was kept at 70°C with a slight excess of alkali for 1 hour. After cooling to room temperature, the volume and pH were adjusted to 1 litre and pH 7.0.

PMSF:- Phenylmethanesulphonylfluoride (BDH, analar) was added to buffers used in protein purification to inhibit protease activity [1]. A 0.3M PMSF solution in ethanol was made immediately before use and pipetted in below the surface of the buffer to give a final PMSF concentration of 1mM.

6.2. Columns

All columns were equilibrated and used in a cold cabinet kept at 4°C. The column materials were prepared (sections 6.2.1-6.2.3) and washed with the appropriate buffer until the effluent solution had the same pH as the elutant. The column dimensions are reported as diameter x height. Columns stored for long periods between use were equilibrated in the appropriate buffer with 0.02% azide. Deionised water was used throughout. When required fractions were collected using a Biorad 2110 fraction collector.

6.2.1. Ion Exchange Columns

Carboxymethyl Sephadex cation exchanger (C-50-120)(Sigma)

Column material, obtained in granular bead form, was swollen with an excess amount of the appropriate buffer and then washed with two or three column volumes of buffer until equilibrated. CM columns were always made with fresh column material.

Diethylaminoethyl (DEAE) anion exchanger

Two DEAE column materials were used, DEAE (Sigma) and DE52 (Whatman). For DEAE, the following preliminary treatment was carried out. The

dry ion-exchange material was stirred into 15 volumes (volumes per dry weight of ion exchange material) of 0.5M HCl and left to settle. The supernatant liquid was decanted off and replaced with an equal volume of deionised water with stirring. The decanting procedure was repeated until the pH was > 4.0. The ion-exchange material was then treated with 15 volumes of 0.5M NaOH, and then washed with deionised water, as already described, until the pH was < 8.0. After the preliminary treatment, the ion-exchange slurry was equilibrated in the desired buffer. Column material was recovered by the same process as the preliminary treatment.

DE52 was obtained pre-swollen and needed no preliminary treatment. The column material was washed with two to three column volumes of the desired buffer until equilibrated.

DOWEX 1 x 8-200 (Chloride form) (Sigma) was prepared for use by stirring in an excess of deionised water and washing until the column material was equilibrated. Column material was recovered by washing with a large excess of deionised water.

Hydroxyapatite [$Ca_5(OH)(PO_4)_3$] (Fluka, fast flow) was prepared for use by stirring with a large excess of the desired buffer, then washing until equilibrated. Column material was recovered by washing with a large excess of 1M NaCl, then stored in a 1M NaCl solution with 0.02% azide present.

6.2.2. Affinity Column

Derivatised sepharose (Aminohexaethyl-sepharose coupled with oxalate using 1-ethyl-3-(3-dimethylyaminopropyl) carbodiimide)

Commercial aminohexaethyl sepharose (15g) was swollen in an excess of 0.5M NaCl. To remove any low molecular weight solutes, eg lactose and dextran, the column was washed with 0.5M NaCl (200 ml/g powder) followed with deionised water to remove the NaCl. To the sepharose slurry, 3% disodium oxalate (75ml) was added and the pH adjusted to 4.7. The mixture

was stirred gently at room temperature and 10ml of 1.46M carbodiimide were added dropwise, to give a final concentration of 0.1M. The pH was maintained at 4.7 for 24 hours by the addition of 4M HCl. After 1 hour, there was little change in pH. The gel material was then washed thoroughly with a large excess of phosphate buffer pH 10.0, containing 1M NaCl, followed by acetate buffer at pH 4.0 containing 1M NaCl. This process was repeated several times followed by a final wash with deionised water. The column material was recovered by washing with the alkaline and acidic NaCl solutions described above and stored at 4°C in the acidic buffer with 0.02% azide.

6.2.3. Gel Filtration

Sephadex gel-filtration beads (Sigma) were prepared for use by swelling the gel in excess buffer and washing with buffer until equilibrated. Column material was recovered by washing with a large excess of deionised water and stored as a slurry at 4°C with 0.02% azide. Sephacryl gel-filtration (S-300) was also prepared by this method.

6.3. Dialysis

Seamless dialysis tubing (Sigma) was soaked in either deionised water or the appropriate buffer before use. All dialyses were carried out at 4°C under an atmosphere of nitrogen. The volume contained in the dialysis sack was never more than 5% of the dialysis buffer and was changed every 12 hours if required.

6.4. Molecular Weight Determination

6.4.1. S-300 Gel-Filtration Chromatography

Native molecular weight (non-denatured) determinations were carried out on an S-300 gel-filtration column (150 x 2.5cm). The column material was prepared as described in section 6.2.3 and the column was stored and ran at

4°C. The void volume (V_e) was measured using Blue-Dextran (average molecular weight 2,000kDa, Sigma). The column was calibrated using the following enzymes: β-amylase (200kDa); alcohol dehydrogenase (150kDa); bovine serum albumen (66kDa); carbonic anhydrase (29kDa); myoglobin (17.5kDa) and cytochrome *c* (12.5kDa). Elution profiles were recorded as V/V_e.

6.4.2. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis [2] was carried out in denaturing conditions using sodium dodecyl sulphate, hereafter referred to as SDS. The various buffers and solutions were prepared as follows.

Sample buffer:- 1% SDS, 8M Urea (BDH, analar), 1% 2-Mercaptoethanol (Sigma), 0.01M H_3PO_4 adjusted to pH 6.8 with Tris base.

Acrylogel 5 premix (BDH) was stored either as a solid or a 36% (w/v) solution.

Reservoir buffer:- 0.025M Tris.HCl pH 8.8, 0.19M Glycine (Sigma) and 1% SDS (Sigma). Reservoir buffer was usually stored as a five fold concentrate.

Resolving Buffer:- 0.75M Tris.HCl, pH 8.8 and 0.2% SDS. Resolving buffer was usually stored as a two fold concentrate.

Stacking Buffer:- 0.25M Tris.HCl, pH 8.8, 0.19M Glycine and 0.1% SDS. Stacking buffer was usually stored as a two fold concentrate.

Fixer Solution:- 11.4% (w/v) Trichloroacetic acid (Sigma), 3.4% (w/v) 5-Sulphosalicylic acid (Aldrich) and 30% (v/v) Methanol.

Stain:- 25% (v/v) Isopropanol, 10% (v/v) Acetic acid and 0.24% (w/v) Coomassie Brilliant Blue (Sigma, Dye content 65%).

Destain:- Solution 1. 10% (v/v) Acetic acid and 25% (v/v) Isopropanol. Solution 2. 10% (v/v) Acetic acid and 10% (v/v) Isopropanol. Solution 3. 10% (v/v) Acetic acid.

Polyacrylamide gel electrophoresis was carried out using a vertical gel box system (BRL). A potential was applied across the gel and controlled either by an LKB 2103 power supply or a Biorad 200/2.0 power supply. The resolving gel

solution was prepared with resolving buffer, an appropriate amount of acrylamide and deionised water. The solution was filtered and degassed then polymerised using a 10% ammonium persulphate solution (2.5µl/ml) (Sigma) and N,N,N',N'-tetramethyl ethylenediamine (approx. 1µl/ml) (Sigma) hereafter referred to as TEMED. The stacking gel solution was prepared from stacking buffer, acrylamide (half the corresponding amount used for the resolving gel) and water. The gel solution was polymerised using ammonium persulphate and TEMED as with the resolving gel.

The percentage of acrylamide used for the resolving gels depended on the protein molecular weight or protein separation required. Typical percentages for flavocytochrome b_2 (subunit molecular weight of 57,500) and cytochrome *c* (12,400) would be 12% and 18% respectively.

6.5. Protein Purification

6.5.1. Buffer Solutions For Protein Preparations

Buffer solutions A-E were prepared with the concentrations as shown in table 6.1.

6.5.2. Commercial Yeast

Flavocytochrome b_2 was extracted and purified from commercial bakers' yeast (*Saccharomyces cerevisiae*) essentially by the method of Labeyrie *et al* [3].

Yeast Preparation:- Approximately 2-3 kg of high activity yeast (DCL Ltd) were air dried (2-3 days) and ground using a porcelain ball mill (16-24 hours) to give 1 kg of a fine talc-like powder.

Extraction:- n-Butanol (600ml) was added to 1 kg of dried yeast. After 20 minutes the butanol-yeast paste was homogenized with solution A (5l), stirred at room temperature for 20 minutes, then centrifuged at 14,000g for 50 minutes at 0-4°C.

Buffer	Phosphate (mM)	DL-lactate (mM)	EDTA (mM)	PMSF (mM)	pH
A	-	170	0.25	1	5.1
B	100	1	1	1	7.0
C	100	5	1	1	7.0
D	50	5	1	1	7.0
E	200	5	1	1	7.0
F	100	100	-	-	8.0
G	100	100	-	-	7.0

H 35 mM phosphate, 45% saturated with ammonium sulphate, pH 7.0

I 35 mM phosphate, 4 M urea, 100 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.0

Table 6.1 Concentrations of buffers used in the isolation of *S.c.*b_2 and *E.c.*b_2.

Acetone Precipitation:- The supernatant was filtered through a glass wool plug and stored at -5 to -10°C. Precooled acetone (-20°C) was added (final concentration 20%) and the suspension was centrifuged at 14,000g for 40 minutes at 0-4°C. The supernatant was then brought to 33% acetone and centrifuged at 1,000g for 10 minutes, -5°C, to produce a dark red viscous precipitate.

Ammonium Sulphate Precipitation:- The viscous pellet from the acetone precipitation was dissolved in solution B (100-150ml) and immediately centrifuged at 39,000g for 15 minutes at 0-4°C. The supernatant was precipitated with $(NH_4)_2SO_4$ (45% and 70%) centrifuging each time at 39,000g for 15 minutes. The major components of the second pellet were flavocytochrome b_2 and cytochrome *c* (iso-1). This pellet was dissolved in the minimum volume of solution B and dialysed overnight against 1 litre of solution B, at 4°C, under nitrogen.

Chromatography:- The crude flavocytochrome b_2/cytochrome *c* solution was filtered through a short DE52 column (ca 5 x 4 cm) directly onto a hydroxyapatite column (2.5 x 30 cm). Both columns were equilibrated in solution B. After washing the hydroxyapatite column (1-2 column volumes of solution B) a linear gradient was set up with 1 litre of solution B and 1 litre of solution B containing 70g of $(NH_4)_2SO_4$. The red fractions containing a mixture of cytochromes b_2 and *c* were pooled, precipitated with $(NH_4)_2SO_4$ (70%) and centrifuged at 39,000g for 15 minutes at 4°C. The pellet, which contained the flavocytochrome b_2 and some of the cytochrome *c*, was dissolved in the minimum volume of solution B and dialysed overnight against 1 litre of solution C at 4°C, under nitrogen. The flavocytochrome b_2 solution was diluted to half the concentration with deionised water and then adsorbed onto a derivatised sepharose column (2.5 x 10cm, equilibrated in solution D). The enzyme was eluted with a linear gradient of solutions C (100ml) and E (100ml)

at a flow rate of 30–40 ml/hour. An elution profile was recorded and the fractions containing the flavocytochrome b_2 with an absorbance ratio (A_{269}:A_{423}) of 0.5 were pooled. The enzyme was stored as an $(NH_4)_2SO_4$ precipitate (70%) at 4°C, under nitrogen.

The purified flavocytochrome b_2 gave a single band on an SDS polyacrylamide gel, and an A_{269}/A_{423} ratio of 0.5 as previously reported.

6.5.3. GR20 Mutant Yeasts

Site-directed mutant forms of flavocytochrome b_2 were generated (M.T.Black) using the double primer method of Zoller and Smith (see appendix II). Mutant flavocytochrome b_2 was extracted from GR20 yeast cells by essentially the same method as the commercial yeast preparation (section 6.5.2) carried out on a smaller scale but omitting the acetone precipitation step.

6.5.4. *Escherichia coli*

Frozen *E. coli* cells were suspended in phosphate buffer 100mM, pH 7.0, containing 10mM EDTA, 50mM lactate. Lysozyme was added 0.2 mg/ml and the solution was incubated for 20–30 minutes at 4°C. The cell lysis mixture was centrifuged at 39,000g for 15 minutes to give an orange/red coloured supernatant (flavocytochrome b_2). After $(NH_4)_2SO_4$ precipitation (30% and 70%) the pellet was dissolved in a minimum volume of solution B and dialysed overnight under nitrogen. The flavocytochrome b_2 was then purified by chromatography as in section 6.5.2 .

The flavocytochrome b_2 from *E. coli* gave a single band on an SDS polyacrylamide gel and a UV–VIS spectrum identical to flavocytochrome b_2 from yeast (6.5.2).

6.6. Growth Media

The following media were made for growing cultures. All solutions were autoclaved prior to use.

6.6.1. Yeast Cultures.

YPD:- 2% glucose (BDH, analar), 2% bactopeptone (Difco), and 1% yeast extract (Difco)

6.6.2. *E. coli* Cultures.

L.broth:- 1% tryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl (Fisons, SLR), pH adjusted to 7.0. Ampicillin (100µg/ml) was added after autoclaving.

6.6.3. Culture Plates.

3% agar (Difco) was added to double strength media to make plates and stabs.

6.7. Substrates

6.7.1. L-Lactate

Pure L-lactate was obtained as the lithium salt (Sigma) and stored dessicated at 0-4°C. Fresh L-lactate solutions were prepared in Tris.HCl buffer pH 7.5, 0.1M ionic strength, prior to the kinetic experiments.

6.7.2. Deuterated L-Lactate

2-^{2}D-L-(+)-Lactate was made using a coupled enzyme system essentially by the method of Shapiro *et al* [4].

To 50ml of 90mM hexadeuteroethanol, 70mM pyruvate and 1.8mM NAD^{+} in 10mM phosphate buffer pH 8.0 at 37°C, were added 2mg of yeast alcohol dehydrogenase and 12mg of beef heart lactate dehydrogenase. After 18 hours, the reaction was stopped by boiling the mixture for 3 minutes. The labelled

lactate was purified on a DOWEX 1 X 8 ion exchange column (2 x 8cm, equilibrated in deionised water) by eluting with a linear gradient of deionised water (400ml) and 3.6M formic acid (400ml). The 2-^{2}H-L-(+)-Lactate product was free of pyruvate and 2-^{1}H-L-(+)-Lactate (not detectable by nmr, see figure 6.1.

6.8. Kinetic Analysis

6.8.1. Steady-State Kinetics

In the presence of excess L-lactate and acceptor (cytochrome *c* or ferricyanide) and a known amount of enzyme, the reaction velocity, *v*, can be measured by calculating the rate of reduction of acceptor. Over a range of substrate concentrations the reaction velocity follows saturation kinetics [5]. Experimental data can be fitted to either the Michaelis-Menten equation or the Lineweaver-Burk equation (using least mean squares programs) [6] (see Appendix I for the derivation of these equations) to yield two parameters, k_{cat}, the velocity of reaction per unit enzyme at saturating substrate concentration and K_M, the Michaelis-Menten constant. Since for flavocytochrome b_2, two moles of ferricyanide are reduced for every mole of L-lactate oxidized, k_{cat} (lactate) = 1/2 k_{cat} (acceptor). All turnover rates are reported as k_{cat} (lactate) and measured with ferricyanide as acceptor, unless otherwise stated.

Assays were carried out using 3ml quartz cuvettes. The velocity of turnover, *v*, was measured by following the change in absorbance due to the reduction of ferricyanide or cytochrome *c* with time. Difference extinction coefficients ($\varepsilon_{ox} - \varepsilon_{red}$) are 1,010$M^{-1}cm^{-1}$ for ferricyanide at 420nm and -20,640$M^{-1}cm^{-1}$ for cytochrome *c* at 550nm. Flavocytochrome b_2 concentrations were calculated using the following extinction coefficients [7]: ε^{red}_{423} = 183,000$M^{-1}cm^{-1}$; ε^{red}_{557} = 30,000$M^{-1}cm^{-1}$; ε^{ox}_{413}= 129,500$M^{-1}cm^{-1}$; and ε^{ox}_{557} = 9,400$M^{-1}cm^{-1}$. The most accurate enzyme concentration measurements were

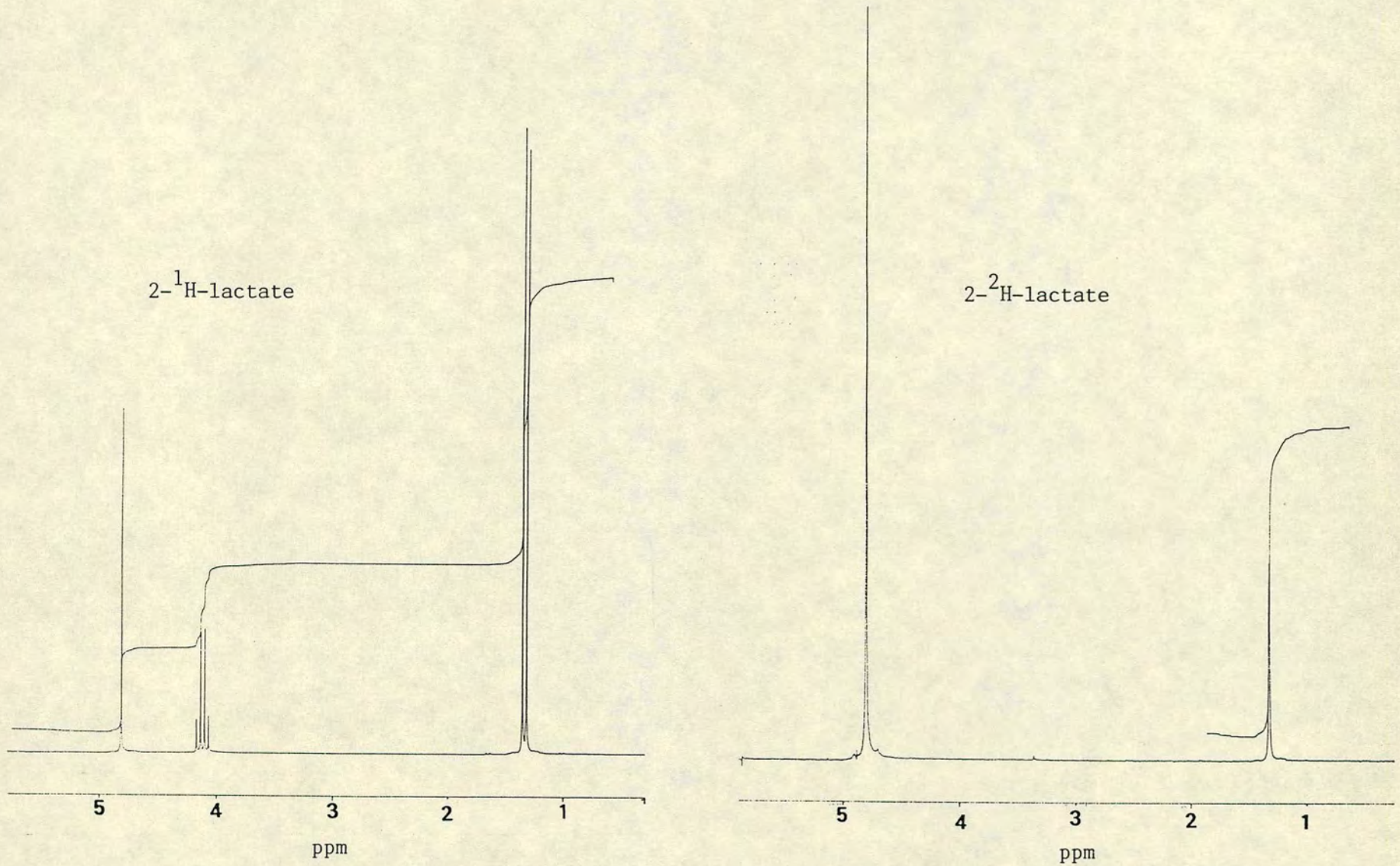

Figure 6.1 nmr spectra of 2-^{2}H-lactate and 2-^{1}H-lactate, showing the isotopic purity of the deuterated substrate.

made by using a difference absorbance (A_{ox}-A_{red}) at 557nm. Only pure L-lactate (lithium salt, Sigma) was used for quantitative analysis. Ferricyanide (potassium salt, Fisons) and cytochrome *c* (Horse heart Type VI, Sigma) solutions were freshly prepared prior to assay experiments. All absorbance and assay measurements were made using either a Beckman DU-62 or a PYE Unicam SP8-400 UV-VIS spectrophotometer. Both machines were thermally equilibrated to 25 $\pm$ 0.1°C. Tris.HCl buffer pH 7.5, 0.1M ionic strength was used throughout.

For substrate K_M measurement, the ferricyanide concentration was kept constant at 1mM and lactate concentrations varied between 0 and 15mM. For the cytochrome *c* apparent K_M measurement, lactate concentration was kept constant and cytochrome *c* concentration varied from 0 to 100µM. For inhibitor K_I measurement, the reaction velocity was measured over a range of substrate concentrations with a constant concentration of inhibitor.

6.8.2. Stopped-Flow Kinetics

Stopped-flow kinetic experiments were carried out at 25 $\pm$ 0.1°C in Tris.HCl buffer pH 7.5, 0.1M ionic strength, under pseudo-first-order conditions using a Hiteck SF 30-A stopped-flow spectrophotometer interfaced to a Commodore Pet desk top computer. Data were treated with a simple program to yield pseudo-first-order rate constants.

6.9. References

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APPENDICES

I. Derivation of Kinetic Equations

I.I. Michaelis-Menten

The Michaelis-Menten equation is the basic equation of enzyme kinetics [1]. The equation can be derived as follows:-

Consider the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_2} E + P$$

Where E and S are enzyme and substrate, E.S is an enzyme substrate complex and P is the product formed. The rate of product formation v, is given by

$$v = \frac{d[P]}{dt} = k_2[E.S] \qquad (1)$$

The concentration of enzyme at a time shortly after the start of the reaction is

$$[E] = [E_0] - [E.S] \qquad (2)$$

Briggs and Haldane [2] postulated that shortly after the mixing of enzyme and substrate, the concentration of the enzyme-substrate complex will reach a constant value so that the steady state approximation can be applied.

$$\frac{d[E.S]}{dt} = 0 = k_1[E][S] - k_{-1}[E.S] - k_2[E.S] \qquad (3)$$

Substituting (2) into (3)

$$0 = k_1([E_0] - [E.S]) - (k_{-1} + k_2)[E.S]$$

$$(k_{-1} + k_2)[E.S] = k_1[E_0][S] - k_1[E.S][S]$$

$$[E.S](k_{-1} + k_2) + k_1[S] = k_1[E_0][S]$$

$$[E.S] = \frac{k_1[E_0][S]}{k_{-1} + k_2 + k_1[S]}$$

substituting into equation (1)

$$v = \frac{k_2 k_1[E_0][S]}{k_{-1} + k_2 + k_1[S]}$$

$$= \frac{k_2[E_0][S]}{[(k_{-1} + k_2)/k_1] + [S]}$$

$$= \frac{k_{cat}[E_0][S]}{K_M + [S]} \qquad \text{(Michaelis-Menten equation)}$$

or

$$k_{obs} = \frac{k_{cat}[S]}{K_M + [S]} \qquad \text{where } k_{obs} = v/[E_0]$$

where the Michaelis-Menten constant, $K_m = (k_{-1} + k_2)/k_1$ and the catalytic turnover number, $k_{cat} = k_2$.

When $k_{-1} >> k_2$ ie a rapid equilibrium, then $K_m = k_{-1}/k_1$ ie Kdiss

I.II. Lineweaver-Burk

The Michaelis-Menten equation can be transformed into a more useful linear form as suggested by Lineweaver and Burk [3]

$$k_{obs} = \frac{k_{cat}[S]}{K_M + [S]}$$

$$\frac{1}{k_{obs}} = \frac{K_M}{[S]k_{cat}} + \frac{1}{k_{cat}}$$

Therefore a plot of $1/k_{obs}$ versus [S] yields a straight line with intercept $1/k_{cat}$ and slope of K_m/k_{cat}.

I.III. Treatment for Biphasic Kinetics

The type of biphasic kinetics that were described in chapter 4, can be seen in figure A.1. The first-order rate constant for the second (slow) stage, $k_{s(deact)}$, was determined by plotting ln R (where R = enzyme turnover rate) against time and taking the slope of the latter linear section of the plot. This gave a slope of $k_{s(deact)}$ (s^{-1}), and an intercept of ln x, see figure A.1. The first-order rate constant of the first (fast) phase stage, $k_{f(deact)}$, was then determined by plotting $\ln(R-x.e^{-k_s t})$ against time, see the inset to figure A.1. This then effectively subtracts the second stage from the first, enabling $k_{f(deact)}$ (s^{-1}) to be determined.

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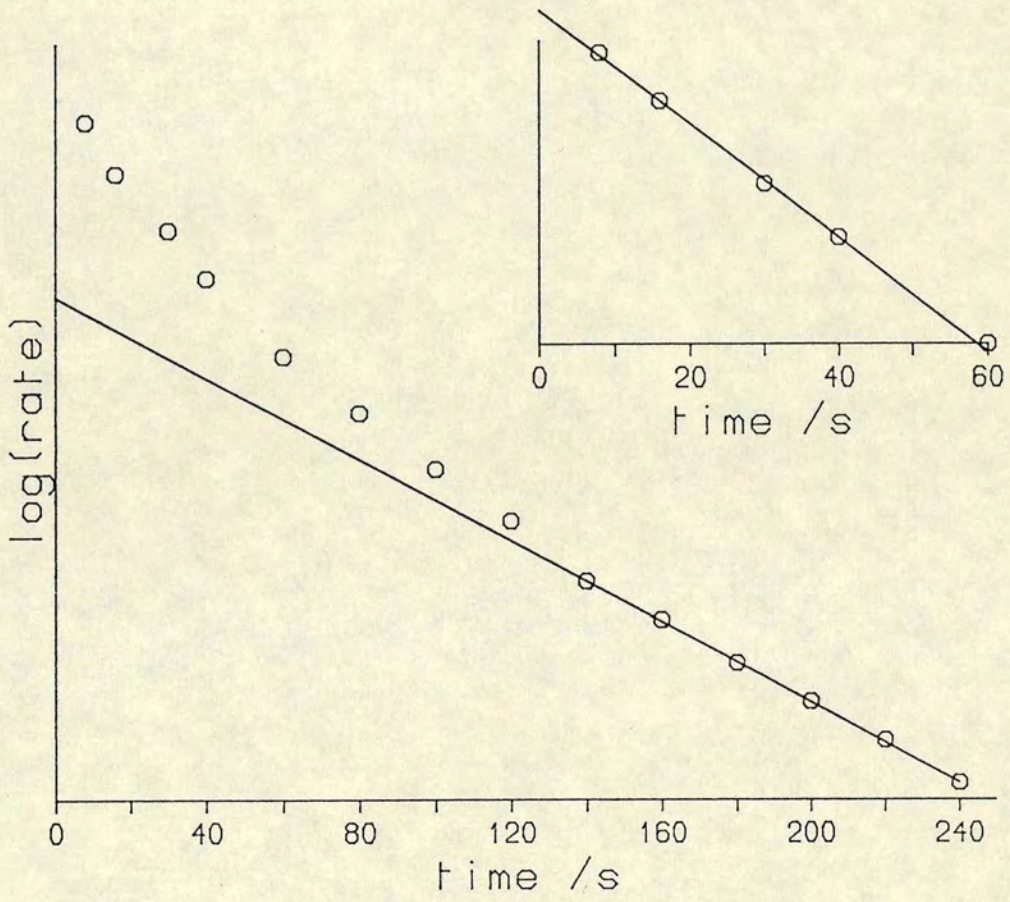

Figure A.1 A biphasic plot of the type described in chapter 4. The two stages of the reaction can be seen in the main plot. The inset corresponds to the first stage, after subtraction of the second stage, as described in section A.I.III.

II. Site-Directed Mutagenesis

II.I. The Method of Zoller and Smith

Site-directed mutagenesis is the most specific and general form of mutagenesis available. Any chosen nucleotide in a long DNA fragment can be specifically changed to any of the three other possible nucleotides. The basic principle of site-directed mutagenesis using two primers is shown in figure A.2. An oligonucleotide (usually 17 base) complementary to the single-stranded template, except for a single base mismatch, is annealed along with a second oligonucleotide (perfectly matched) to the single-stranded template DNA and then extended using *E. coli* DNA polymerase I "Klenow" fragment in the presence of T4 DNA ligase. This results in a double-stranded closed-circular DNA molecule, which contains a single base pair mismatch at the mutation site. This heteroduplex molecule will, following transformation of a suitable host cell, give rise to mutant progeny. In order to pick out mutants, the clones can be screened by nucleic acid hybridisation with ^{32}P-labelled oligionucleotide as probe.

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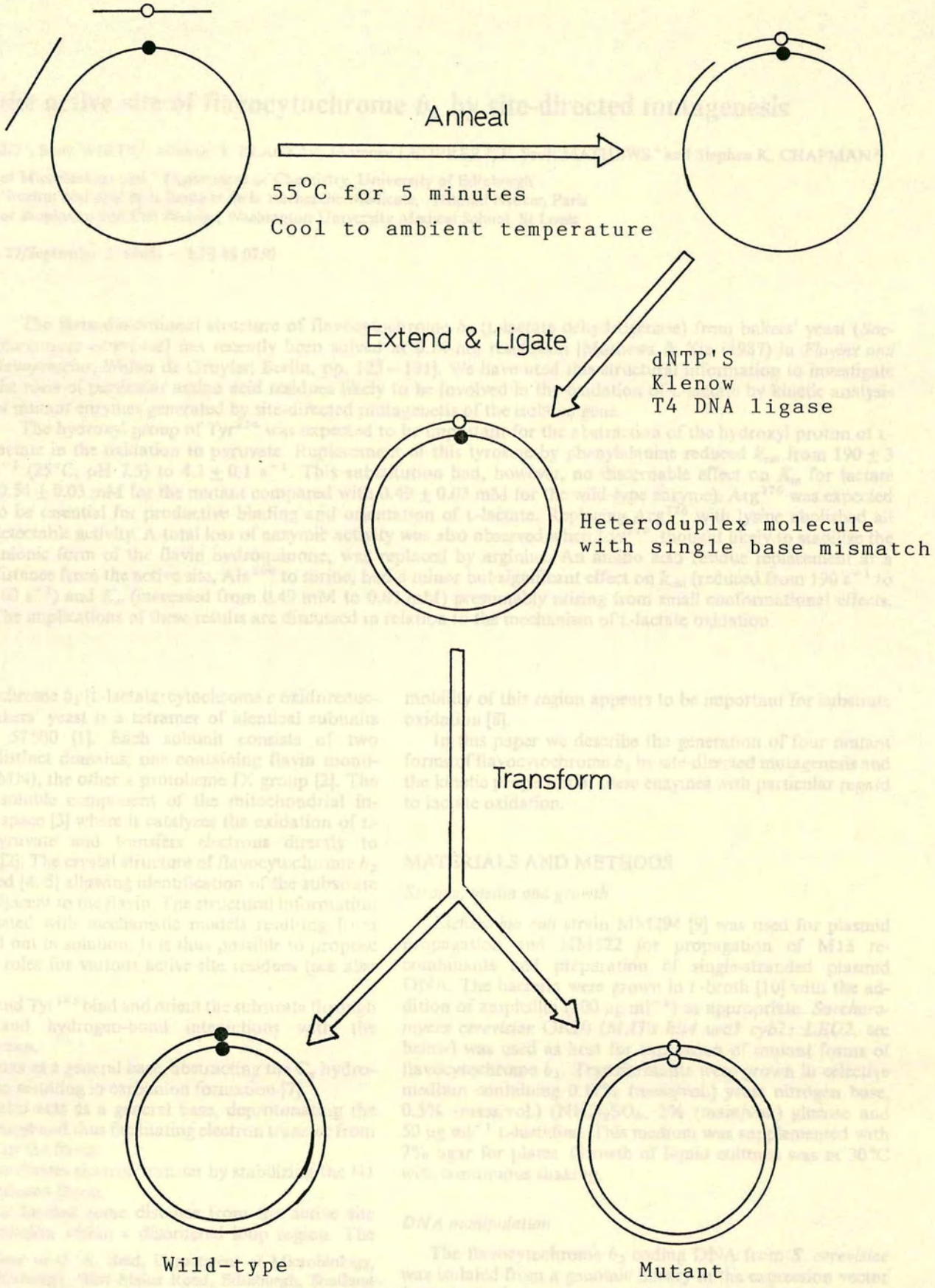
Anneal
55°C for 5 minutes
Cool to ambient temperature
Extend & Ligate
dNTP'S
Klenow
T4 DNA ligase
Heteroduplex molecule
with single base mismatch
Transform
Wild-type
Mutant
Segregation upon
transformation to
yield both wild-type
and mutant progeny.

Figure A.2.

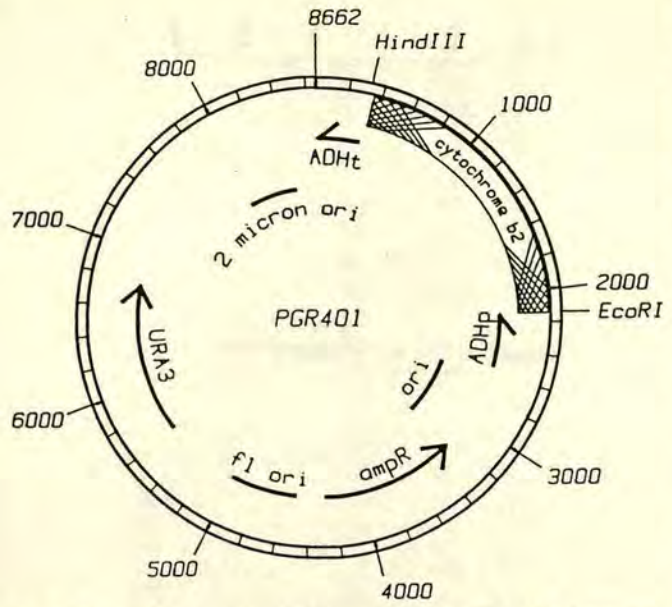

Fig. 1. *Map of the yeast expression vector pGR401.* The flavocytochrome b_2 coding sequence, shown as a hatched block, lies between the promoter (ADH_p) and terminator (ADH_t) of the *S. cerevisiae ADH1* gene. The plasmid also contains origins of replication and selectable markers for maintenance in *E. coli* and in yeast. The presence of the origin of replication from bacteriophage f1 (f1 ori) also allowed isolation of single-stranded DNA for site-directed mutagenesis and DNA sequence determination

determined. To simplify manipulation of the gene an *Eco*RI restriction site was introduced immediately upstream from the ATG initiation codon by addition of a synthetic linker after *Bal*31 exonuclease digestion from an upstream *Sac*I site. The entire coding region plus 0.5 kb of 3′ untranslated DNA was transferred to M13mp19 [11] as an *Eco*RI – *Hin*dIII fragment to generate M13-Z1. Single-stranded DNA from M13-Z1 was used as template for site-directed mutagenesis in the construction of the Arg^{349} and Lys^{376} mutants. The Phe^{254} and Ser^{306} mutations were generated with a single-stranded plasmid template. This plasmid, pGR401 (Fig. 1), was constructed from the yeast expression vector pVT102-U [12] by first destroying the *Eco*RI site and then introducing an *Eco*RI site suitable for cloning in the polylinker region. pVT102-U was cleaved with *Eco*RI, made blunt-ended by the Klenow filling-in reaction and ligated. A recombinant plasmid was isolated and shown to lack an *Eco*RI cleavage site. This plasmid was then cut with *Bam*HI, made blunt-ended with the Klenow reaction and ligated with phosphorylated *Eco*RI linkers (pGGAATTCC). After cleavage with *Eco*RI and religation a recombinant plasmid with the expected structure, pVT102-A, was isolated. The flavocytochrome b_2 coding sequence was inserted into this plasmid by cleaving the vector with *Eco*RI plus *Pvu*II and ligated with the 1.8-kb *Eco*RI-*Dra*I cytochrome b_2 DNA fragment to generate pGR401. Single-stranded plasmid DNA was isolated from transformants superinfected with M13KO7 (Pharmacia) as described [12].

Site-directed mutagenesis was performed using the double-primer method [13]. Mutants were detected by plaque- or colony-hybridization with M13 and plasmid vectors respectively using nylon discs (Amersham Hybond-N) according to the manufacturer's instructions. The following oligonucleotides, synthesized on an Applied Biosystems model 380B DNA synthesizer, were used for mutagenesis: CCAACTA-TTTGTTAACTC (Tyr^{254} → Phe), GGTCCAAAATCGA-TGAAG (Ala^{306} → Ser), GTTATCAGAGGTGTTCAA (Lys^{349} → Arg) and GGTGGAAACAATTAGAT (Arg^{376} → Lys). The mutant clones were subjected to nucleotide sequence determination along the entire length of the flavocytochrome b_2 coding region using a series of eight oligonucleotide primers. Apart from the oligonucleotide-directed mutation, a second substitution was found in the Ser^{306} mutant DNA. This was removed by replacing the *Eco*RI – *Kpn*I fragment from the mutant with the corresponding wild-type fragment. The mutated DNA in M13 was transferred to pGR401 as a 0.8-kb *Kpn*I – *Dra*I fragment, replacing the equivalent wild-type fragment.

Flavocytochrome b_2 *gene disruption*

To construct a yeast host lacking wild-type flavocytochrome b_2, the chromosomal gene encoding this enzyme was disrupted by insertion of the *S. cerevisiae LEU2* gene [14]. The 3.0-kb *Bgl*II *LEU2* fragment from YEp13 [15] was inserted at the *Bcl*1 site in the cytochrome b_2 coding sequence on a plasmid derived from pSP64 [16]. This recombinant plasmid was cleaved at each end of the flavocytochrome b_2 coding sequence and used directly to transform the haploid *S. cerevisiae* strain SF747-19D (*MATα his4 ura3 leu2*) to leucine prototrophy. A transformant, GR20, was isolated which was shown by Southern blot hybridization [10, 17] of chromosomal DNA to have undergone the expected gene distruption and which failed to synthesize immunologically detectable flavocytochrome b_2 polypeptide (Fig. 2). GR20 was transformed with pGR401 and derivatives by the lithium acetate method [18] and selected for uracil prototrophy. Protein was extracted from these yeast strains as described previously [19] and flavocytochrome b_2 detected by Western blotting [20].

Enzyme preparation

Flavocytochrome b_2 was prepared either from commercial bakers' yeast (United Yeast Co. Ltd, Menstrie, Scotland) or from GR20 transformants as described previously [21]. Phenylmethylsulphonyl fluoride (1 mM) was present throughout the preparation to yield the intact enzyme [21]. Enzyme preparations were stored under nitrogen at 4 °C as precipitates in 70% $(NH_4)_2SO_4$. The enzyme was maintained in the reduced state, where possible, by the presence of L-lactate (5 mM) in all buffers. Enzyme purity was determined by SDS/polyacrylamide gel electrophoresis (single band at M_r = 57500) and protein to heme ratio ($A^{red}_{269}/A^{red}_{423}$ = 0.5 [21]). Except for the Arg^{349} enzyme (which was purified only to the hydroxyapatite column step [21] due to a poor yield) all mutant enzymes showed a single band on SDS/PAGE identical to wild-type enzyme and exhibited identical ultraviolet/visible absorption spectra to wild-type enzyme with expected protein/heme ($A^{red}_{269}/A^{red}_{423}$ = 0.5) and flavin/heme ($A^{red}_{328}/A^{red}_{423}$ = 0.21 and $A^{ox}_{362}/A^{ox}_{413}$ = 0.27) ratios.

Kinetic analysis

All kinetic experiments were carried out in Tris/HCl buffer at pH 7.5 and at 25 ± 0.1 °C. The buffer concentration was 10 mM with the ionic strength adjusted to 0.1 M by addition of NaCl. Flavocytochrome b_2 concentrations were determined spectrophotometrically using the following molar ab-

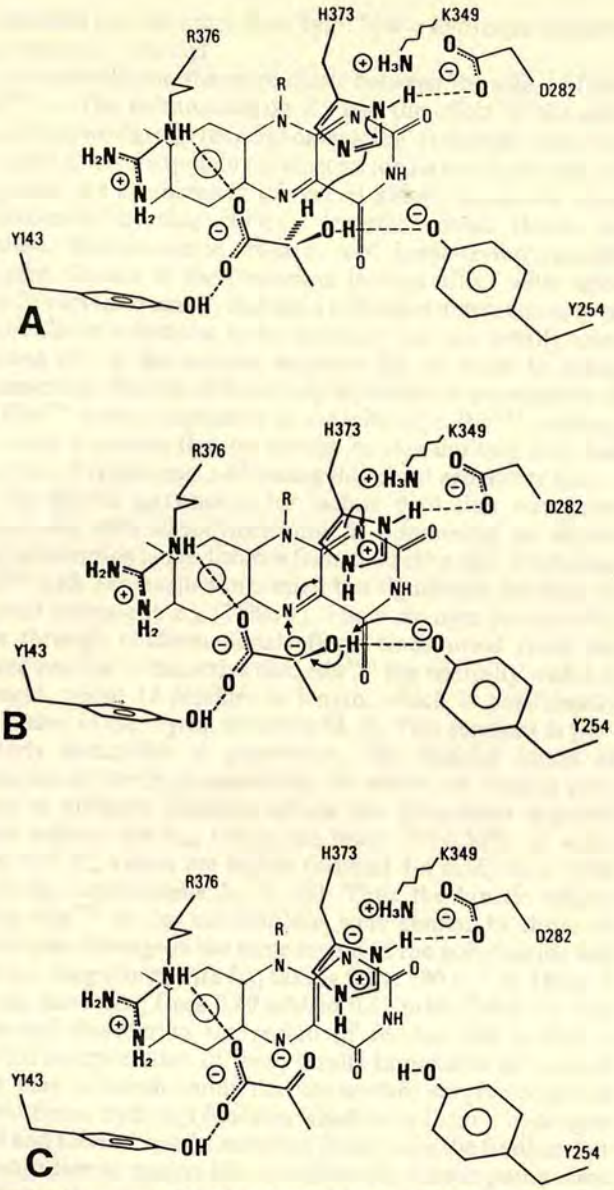

Fig. 4. *The proposed rôle for side chains in the carbanion mechanism of oxidation of* L-*lactate by flavocytochrome* b_2. (A) Substrate is bound via an electrostatic and hydrogen-bond interaction with Arg^{376} and by hydrogen-bonding to Tyr^{143}. His^{373} is poised to abstract the C_α hydrogen as a proton. (B) The resultant imidazolium ion is stabilized by Asp^{282}. Removal of the hydroxyl proton by Tyr^{254} facilitates electron transfer from the carbanion to the flavin. (C) The resultant N1 anion of the reduced flavin is stabilized by Lys^{349}. Note that the structure gives no indication of how electrons transfer between lactate and flavin and that a covalent intermediate in the transition state cannot be ruled out. The mechanism is discussed more fully in the text

site [6] though it now appears more likely to be that of pyruvate stabilizing the flavin semiquinone [25] (and Y. Henri, F. Lederer and F. S. Mathews, unpublished experiments). In any case there are no significant structural differences between the reduced and fully oxidized forms of the enzyme (M. Tegoni and F. S. Mathews, unpublished experiments). It can be assumed that the carboxylate of the substrate lactate is bound in the same way as pyruvate while its hydroxyl group would be hydrogen-bonded to the phenol or phenolate group of Tyr^{254} (Fig. 4). It was proposed [6] that the C_α hydrogen of L-lactate is removed as a proton by His^{373}, leaving a substrate carbanion. The resultant imidazolium ion at His^{373} is stabilized by Asp^{282}. Electron transfer to the flavin is facilitated by removal of the substrate hydroxyl proton to Tyr^{254} and by stabilization of the anionic form of the reduced flavin by electrostatic interaction with Lys^{349} (Fig. 4B). We have used site-directed mutagenesis to test a number of points raised by this model.

Replacing Arg^{376} with Lys abolished enzymic activity completely. It is interesting to compare this result with studies of a nicotinamide-linked lactate dehydrogenase in which replacement of Arg^{171}, which interacts with the substrate carboxylate, by Lys did not result in inactive enzyme, though k_{cat} was lowered about 4×10^4-fold [26]. In this enzyme the interaction between Arg^{171} and the substrate carboxylate involves the two terminal nitrogens of the guanidinium group and is symmetrical [27]. This contrasts with flavocytochrome b_2, in which the interaction involves the δN and one of the terminal nitrogens of Arg^{376}; the distance between the latter and the carboxylate oxygens appears to preclude a hydrogen bond at the present stage of refinement. Moreover Tyr^{143} also contributes to substrate carboxylate binding. Since interaction between the Lys^{376} and the lactate negative charge would not exactly match that of Arg^{376} one would not be surprised by weaker binding and possibly lowered activity because of a less favourable substrate orientation; one might, however, expect still to find some activity. A possible explanation for the total loss of activity (the lack of heme reduction is a very sensitive indicator) may be that the Arg → Lys substitution removes interaction between the guanidinium group and neighbouring atoms of the protein which would be essential for the integrity of the active site. This point will be addressed in further work.

A complete loss of lactate dehydrogenase activity was also observed when Lys^{349} was replaced by Arg, consistent with the proposed involvement of this residue in catalysis. Although this substitution conserves charge, it also introduces substantial bulk in a tightly packed region close to the flavin. We have shown that flavin is present at normal levels in the Arg^{349} enzyme expressed in *E. coli* (M. T. Black, S. White, G. A. Reid and S. K. Chapman, unpublished results) but cannot exclude the possibility of gross structural alterations around the cofactor.

More information can be gained from analysis of the Phe^{254} mutant enzyme which lacks the hydroxyl function of the normal tyrosine residue at this position. According to the proposed catalytic mechanism Tyr^{254} accepts the hydroxyl proton from L-lactate and its replacement by Phe would be expected to reduce the catalytic efficiency of the enzyme. In fact k_{cat} is reduced almost 50-fold (Table 1) whereas the K_m for lactate is identical within experimental error in the wild-type and mutant enzymes. For the wild-type enzyme the steady-state K_m value is believed to be close to that of K_s [8]. The results therefore suggest at first sight that Tyr^{254} is important not in substrate binding but in transition-state stabilization, i.e. it makes no interaction with the substrate hydroxyl group in the Michaelis complex. This is somewhat surprising in view of the observation that propionate behaves as a competitive inhibitor of lactate dehydrogenation with $K_i = 28 \pm 1$ mM (R. Genet and F. Lederer, unpublished results), implying that the hydroxyl group contributes about 10.5 kJ/mol to substrate binding. This figure seems compatible with the existence of a hydrogen bond between the neutral alcohol group and a charged side chain [28]. If the postulated mechanism is correct, then the crystal structure offers

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High-level expression of fully active yeast flavocytochrome b_2 in *Escherichia coli*

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Wild-type flavocytochrome b_2 (L-lactate dehydrogenase) from the yeast *Saccharomyces cerevisiae* and three singly substituted mutant forms (F254, R349 and K376) have been expressed in the bacterium *Escherichia coli*. The enzyme expressed in *E. coli* contains the protohaem IX and flavin mononucleotide (FMN) prosthetic groups found in the enzyme isolated from yeast, has an electronic absorption spectrum identical with that of the yeast protein and an identical M_r value of 57500 estimated by SDS/polyacrylamide-gel electrophoresis. *N*-Terminal amino-acid-sequence data indicate that the flavocytochrome b_2 isolated from *E. coli* begins at position 6 (methionine) when compared with mature flavocytochrome b_2 from yeast. The absence of the first five amino acid residues appears to have no effect on the enzyme-catalysed oxidation of L-lactate, since K_m values for the yeast- and *E. coli*-expressed wild-type enzymes were identical within experimental error. The F254 mutant enzyme expressed in *E. coli* also showed kinetic parameters essentially the same as those found for the enzyme from yeast. The R349 and K376 mutant enzymes had no activity when expressed in either yeast or *E. coli*. The yield of flavocytochrome b_2 from *E. coli* is estimated to be between 500- and 1000-fold more than from a similar wet weight of yeast (this high level of expression results in *E. coli* cells which are pink in colour). The increased yield has allowed us to verify the presence of FMN in the R349 mutant enzyme. The advantages of *E. coli* as an expression system for flavocytochrome b_2 are discussed.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase; EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a tetramer of identical subunits with M_r 57500 [1]. Each subunit consists of two functionally distinct domains, one containing protohaem IX and the other flavin mononucleotide (FMN) [2]. The protein is a soluble component of the mitochondrial intermembrane space [3], where it catalyses the oxidation of L-lactate to pyruvate and transfers electrons directly to cytochrome *c* [2]. The protein is synthesized as a precursor form with an 80-amino-acid leader sequence [4] which is proteolytically removed during translocation to the mitochondrial intermembrane space [5]. The crystal structure of flavocytochrome b_2 has been solved [6,7], allowing structural information to be correlated with models of the catalytic mechanism. It was on this basis that site-directed mutagenesis of the flavocytochrome-b_2 gene was carried out to probe the active site of the enzyme [8]. Among the mutations generated were Y254→F, K349→R and R376→K [8]. Kinetic characterization of the wild-type and mutant enzymes gave results consistent with a mechanism for L-lactate oxidation as proposed by Lederer & Mathews [9]. One technical limitation with this work, however, was that yields of the mutant enzymes expressed in yeast were low, preventing extensive characterization. This was a particular problem for one mutant enzyme, namely K349→R, which was obtained in low yield, preventing verification of the presence of the FMN prosthetic group [8]. Clearly a better expression system, offering improved yields of enzyme, was desirable for further work to proceed. In the present paper we describe the expression of wild-type and mutant forms of flavocytochrome b_2 in *Escherichia coli* and the characterization of these enzymes with respect to those isolated from yeast.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

E. coli strains MM294 [10] or HB101 [11] were used for plasmid propagation and expression of flavocytochrome b_2. Plasmid-bearing cells were grown at 37 °C in Luria Broth [11] supplemented with ampicillin at 100 μg/ml. The entire flavocytochrome b_2 coding sequence, including the region encoding the presequence, was excised from the plasmid pGR401 [8] on a 1.8 kb *Eco*RI–*Hin*dIII restriction fragment and inserted between the *Eco*RI and *Hin*dIII sites of the plasmid pDS6 [12] to generate $pDSb_2$ (see Fig. 1). Mutant forms of the flavocytochrome-b_2 gene were excised from appropriate pGR401 derivative plasmids [8] as *Eco*RI–*Bgl*II fragments and inserted into $pDSb_2$ after cleavage with *Eco*RI and *Bgl*II. *E. coli* were transformed by the $CaCl_2$ method [11], and positive transformants were selected according to ability to grow in the presence of ampicillin and by the presence of a pink coloration due to high-level expression of a haem-containing protein, flavocytochrome b_2.

Abbreviations used: Y254→F (etc.), a mutation from the wild-type-enzyme's tyrosine-254 to phenylalanine (one-letter amino acid notation); PAGE, polyacrylamide-gel electrophoresis.

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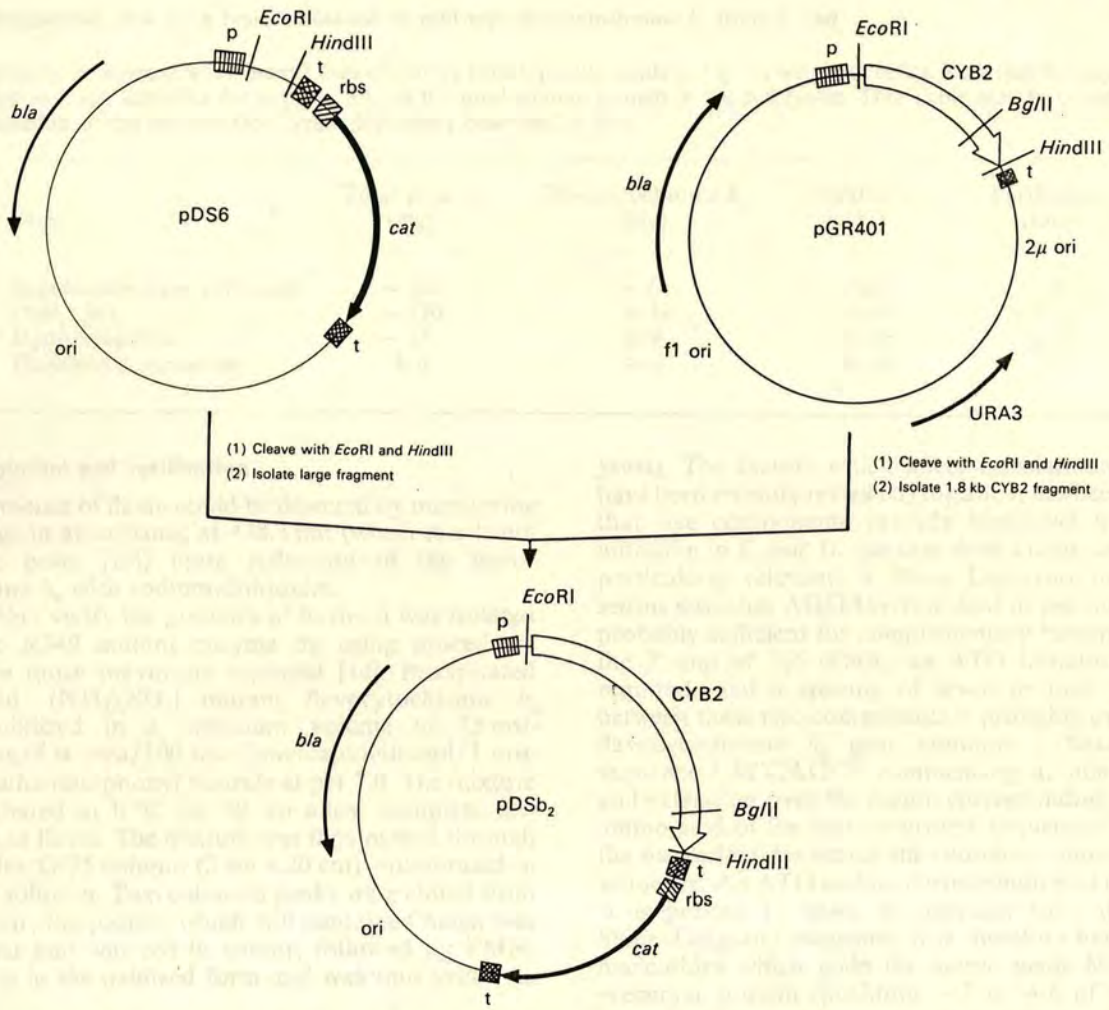

Fig. 1. Construction of a plasmid for expression of flavocytochrome b_2 in *E. coli*

The DNA encoding flavocytochrome b_2 was excised from pGR401 [8] on an *Eco*RI–*Hin*dIII restriction fragment and inserted into pDS6 [12]. Mutant flavocytochrome b_2 DNA sequences were transferred as *Eco*RI–*Bgl*II restriction fragments as described in the Materials and methods section. Abbreviations: p, promoter; t, terminator; rbs, ribosome-binding site; *cat*, chloramphenicol acetyltransferase gene; *bla*, β-lactamase gene; ori, origin of replication.

Enzyme isolation

From yeast. Flavocytochrome b_2 was prepared either from commercial baker's yeast (United Yeast Co. Ltd., Menstrie, Scotland) or from GR20 transformants [8] as described previously [13]. Phenylmethanesulphonyl fluoride (1 mM) was present throughout the preparation to yield intact enzyme [13].

From *E. coli*. Frozen *E. coli* cells were resuspended in 10 mM-Tris/HCl buffer (*I* adjusted to 0.10 with NaCl), pH 7.5, containing 10 mM-EDTA at 4 °C. Lysozyme (Sigma) was added to approx. 0.2 mg/ml and the mixture was incubated on ice for 30 min. The solution was centrifuged at 39000 *g* for 10 min to remove cell debris etc. The supernatant, which had a strong orange–red colour due to the presence of flavocytochrome b_2, was adjusted to 30% $(NH_4)_2SO_4$ saturation (the pH was maintained at 7.5 if necessary by addition of NaOH) and centrifuged at 39000 *g* for 10 min. The pellet was discarded, and the supernatant was adjusted to 70% $(NH_4)_2SO_4$ saturation to precipitate the flavocytochrome b_2 and centrifuged at 39000 *g* for 10 min. The pellet was redissolved either in the appropriate buffer (10 mM-Tris/HCl, pH 7.5; *I* adjusted to 0.1 with NaCl), for preliminary kinetic measurements, or in 0.1 M-phosphate buffer, pH 7.0, containing 1 mM-EDTA and 1 mM-D,L-lactate, for further purification. The purity of the enzyme after the 70%-satd.-$(NH_4)_2SO_4$-precipitation step was such that a characteristic flavocytochrome b_2 spectrum in the visible region could be obtained (see Fig. 2 below). All subsequent purification steps, involving dialysis and chromatography on hydroxyapatite and derivatized-Sepharose columns, were as previously described [13]. Enzyme prepared from *E. coli* or yeast was stored under N_2 at 4 °C as a 70%-satd.-$(NH_4)_2SO_4$ precipitate and maintained in the reduced state, where possible, by the presence of L-lactate (5 mM). When fully purified the enzyme showed a single band at M_r 57500 on SDS/PAGE and a protein/haem ratio (A_{269}/A_{423}) when reduced of 0.5 [13]. Further details of the purification procedure are given in Table 1.

Fig. 2. Visible spectra of (A) fully purified flavocytochrome b_2 expressed in yeast and (B) flavocytochrome b_2 expressed in *E. coli* and purified up to the 70%-satd.-$(NH_4)_2SO_4$ step as described in the Materials and methods section

determinations carried out on the enzyme isolated from *E. coli* immediately after purification to the 70%-satd.-$(NH_4)_2SO_4$ step always showed slightly higher $k_{cat.}$ values. It is known that flavocytochrome b_2 does lose some activity with time, and it would appear that the 2 days required for further purification result in this small loss of activity. This observation shows the benefit of obtaining kinetic parameters at as early a stage as possible. K_m and $k_{cat.}$ values for the F254 mutant enzyme are also shown in Table 2. Again the $k_{cat.}$ value for the *E. coli*-expressed enzyme after the 70%-satd.-$(NH_4)_2SO_4$ step is slightly higher than that after full purification. For a detailed discussion of the role of tyrosine-254 in the mechanism of L-lactate oxidation and the effect of replacing this residue with a phenylalanine residue, see [8].

The K376 and R349 mutant enzymes show no detectable activity whether isolated from yeast or *E. coli*. Possible explanations for this total loss of activity have already been put forward [8]. One such explanation in the case of the R349 mutant was that replacing the original lysine residue with an arginine residue in a tightly packed region close to the flavin group may have prevented incorporation of the flavin itself. The yield of this mutant enzyme from yeast was so small that it was

Table 2. Kinetic properties of wild-type and mutant flavocytochrome b_2 for the oxidation of L-lactate to pyruvate

All constants were determined from steady-state kinetic measurements at 25±0.01 °C, pH 7.5, with ferricyanide (1 mM) as electron acceptor. The K349→R and R376→K mutant enzymes expressed in either yeast or *E. coli* had no activity.

Enzyme and source	$k_{cat.}$ (s^{-1})	K_m (mM)
Wild-type		
Yeast	192±5	0.49±0.03
E. coli		
Fully purified	189±5	0.44±0.05
After 70%-satd.-$(NH_4)_2SO_4$ step	204±10	0.46±0.03
Y254→F mutant		
Yeast	4.3±0.1	0.54±0.03
E. coli		
Fully purified	3.8±0.1	0.52±0.04
After 70%-satd.-$(NH_4)_2SO_4$ step	6.5±0.2	0.51±0.03

impossible to verify the presence or absence of flavin. However, with the improved yields from expression in *E. coli* we have now been able to show unequivocally that flavin is indeed present in the R349 enzyme. Spectrophotometric measurements at haem isosbestic points show the expected content of flavin, and this has been further verified by isolating and assaying the FMN after denaturing the protein as described in the Materials and methods section. Both experiments indicate the expected flavin content in the R349 mutant enzyme within experimental error.

In conclusion, we have shown that *E. coli* transformed with $pDSb_2$ provides an improved expression system for the yeast protein flavocytochrome b_2. Advantages over expression of the protein in yeast include vastly improved yields and quicker and easier purification. Wild-type and mutant proteins expressed in *E. coli* have been shown to exhibit the same kinetic properties as the protein from yeast. The increased yields have allowed more extensive characterization of these enzymes and make possible experiments requiring large quantities of protein, including crystallographic structure determination of the mutant enzymes.

We thank Dr. J. Van Beeumen and Professor R. P. Ambler for advice and for determining the *N*-terminal sequence, and C. Miles for some preliminary kinetic studies. This work was funded by a research grant from the Science and Engineering Research Council (SERC), a SERC research studentship to S. A. W. and by the Research Corporation Trust.

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The role of the *C*-terminal tail of flavocytochrome b_2

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A flavocytochrome b_2 (L-lactate dehydrogenase) mutant was constructed in which the *C*-terminal tail (23 amino acid residues) had been deleted (Gly-489 →Stop). This tail appears to form many intersubunit contacts in the tetrameric wild-type protein, and it was expected that its removal might lead to the formation of monomeric flavocytochrome b_2. The isolated tail-deleted mutant enzyme (TD-b_2), however, was found to be tetrameric (M_r 220000). TD-b_2 shows K_m and $k_{cat.}$ values (at 25 °C and pH 7.5) of 0.96±0.06 mM and 165±6 s^{-1} respectively compared with 0.49±0.04 mM and 200±10 s^{-1} for the wild-type enzyme. The kinetic isotope effect with [2-^{2}H]lactate as substrate seen for TD-b_2, with ferricyanide as electron acceptor, was essentially the same as that observed for the wild-type enzyme. TD-b_2 exhibited loss of activity during turnover in a biphasic process. The rate of the faster of the two phases was dependent on L-lactate concentration and at saturating concentrations showed a first-order deactivation rate constant, $k_{f(deact.)}$, of 0.029 s^{-1} (at 25 °C and pH 7.5). The slower phase, however, was independent of L-lactate concentration and gave a first-order deactivation rate constant, $k_{s(deact.)}$, of 0.01 s^{-1} (at 25 °C and pH 7.5). This slower phase was found to correlate with dissociation of FMN, which is one of the prosthetic groups of the enzyme. Thus fully deactivated TD-b_2, which was also tetrameric, was found to be completely devoid of FMN. Much of the original activity of TD-b_2 could be recovered by re-incorporation of FMN. Thus the *C*-terminal tail of flavocytochrome b_2 appears to be required for the structural integrity of the enzyme around the flavin active site even though the two are well separated in space.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a tetramer of identical subunits with M_r 57500 [1]. Each subunit contains FMN, which is non-covalently bound, and protohaem IX prosthetic groups in two functionally distinct domains [2]. The enzyme is a soluble component of the mitochondrial intermembrane space [3], where it catalyses the oxidation of L-lactate to pyruvate [2]. In the proposed mechanism for L-lactate oxidation, hydrogen abstraction at C-2 is the major rate-limiting step [4]. Studies with [2-^{2}H]lactate as substrate with ferricyanide as electron acceptor are consistent with this proposal, showing a deuterium isotope effect of between 4 and 5 [5]. The crystal structure of flavocytochrome b_2 has been solved to 0.24 nm resolution [6,7] and reveals that each subunit of the protein consists of three readily identified regions: a haem-containing domain, a flavin-containing domain and an extended *C*-terminal tail. The observation that this tail wraps around each of the other three subunits in the holoenzyme (see Fig. 1) would be consistent with the idea that the primary role of the *C*-terminal tail is to hold the subunits together as a tetramer. One experimental approach to test this idea was to investigate the properties of the enzyme lacking the tail. In the present paper we describe the generation of a *C*-terminal-tail-deleted mutant of flavocytochrome b_2 (referred to below as TD-b_2) by site-directed mutagenesis and report the kinetic and physical properties of this mutant enzyme.

MATERIALS AND METHODS

Strains, media, growth and DNA manipulation

The gene encoding tail-deleted flavocytochrome b_2 (TD-b_2) was generated by the double-primer method of Zoller & Smith [8], with single-stranded plasmid pGR401 [9] as template. The following oligonucleotide, synthesized on an Applied Biosystems model 380B DNA synthesizer, was used for mutagenesis: GAACAGTTTGAGTACCA. The nucleotide sequence of the entire TD-b_2 gene was determined and found to contain no secondary mutations. The TD-b_2 gene was excised from plasmid pGR401 as a 1.8 kb *EcoRI–HindIII* restriction fragment and inserted into the polylinker region of the plasmid pDS6 [10]. *Escherichia coli* strain HB101 was grown in L-broth and transformed by the $CaCl_2$ method [11]. Transformants were selected by ability to grow in the presence of ampicillin (100 μg/ml) and by pink coloration due to the presence of the haem-containing TD-b_2.

Enzyme preparation and characterization

Wild-type and mutant flavocytochromes b_2 were isolated from *E. coli* as previously described [10]. When fully purified the wild-type enzyme showed a single band at M_r 57500 on SDS/polyacrylamide-gel electrophoresis, whereas the tail-deleted mutant enzyme showed a single band at M_r 55000, as expected. TD-b_2 had an electronic absorption spectrum in the visible region that was superimposable on that of the wild-type enzyme. The M_r of TD-b_2 in the native state was determined by gel

Abbreviation used: TD-b_2, *C*-terminal-tail-deleted mutant of flavocytochrome b_2.
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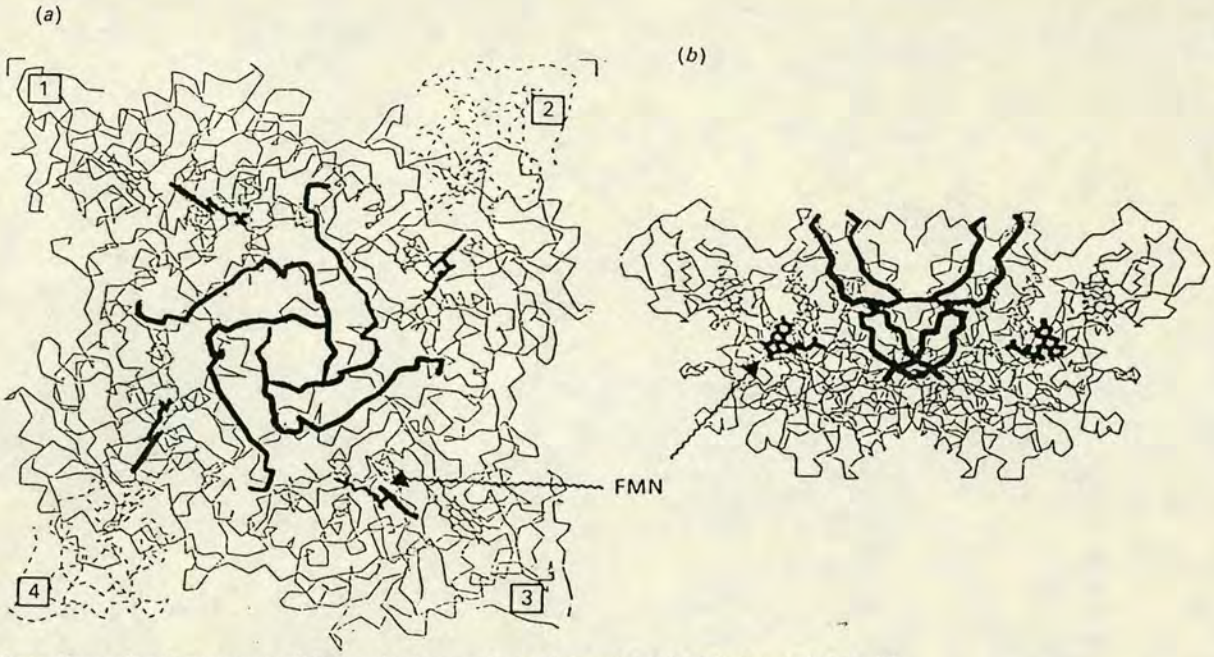

Fig. 1. Three-dimensional structure of flavocytochrome b_2 as determined by Mathews & Xia [7]

The *C*-terminal tails and FMN prosthetic groups of the four subunits are highlighted in view (*a*). A side view (*b*) is also shown. The four subunits are numbered 1 to 4 in view (*a*). The shaded portions seen in subunits 2 and 4 represent the two haem-containing domains, which are disordered in the X-ray crystal structure.

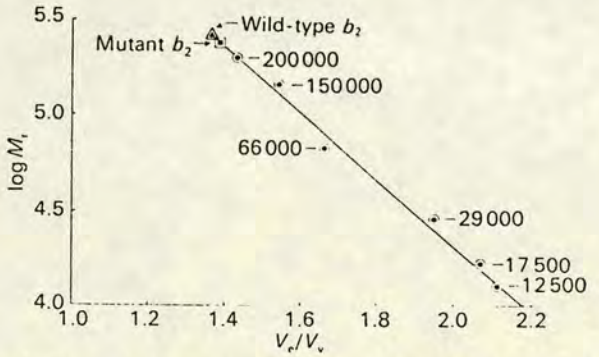

Fig. 2. Determination of the M_r of the TD-b_2 by gel filtration on a Sepharose S300 column (150 cm × 3 cm)

The markers were wild-type flavocytochrome b_2 (M_r 230000), β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 66000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17500) and cytochrome *c* (M_r 12500). The calculated M_r of TD-b_2 was 220000, confirming it as a tetramer. Deactivated (FMN-deficient) TD-b_2 was also eluted with an apparent M_r of 220000.

filtration on a Sepharose S300 column (150 cm × 3 cm) calibrated with the following markers (Sigma Chemical Co.): β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 66000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17500) and cytochrome *c* (M_r 12500) (see Fig. 2). Wild-type flavocytochrome b_2 (M_r 130000) was also used as a marker.

Bound flavin was detected by using the sulphite method of Lederer [12]. Identical solutions of oxidized TD-b_2 (8 μM) in Tris/HCl buffer, pH 7.5, were placed in the reference and observation cells of a Perkin–Elmer λ9 spectrophotometer. To the reference cell was added 10 μl of 0.3 M-Na_2SO_3 (which bleaches the flavin absorbance) and to the observation cell 10 μl of buffer. The resulting difference spectrum corresponds to bound flavin and is illustrated in Fig. 3.

Flavin re-incorporation

Under turnover conditions TD-b_2 was found to lose flavin, resulting in fully deactivated enzyme (see the Results section). Re-incorporation of flavin into the flavin-deficient enzyme was carried out according to the following procedure. Deactivated TD-b_2 was partially denatured in 20 ml of solution A (150 mM-phosphate buffer, pH 7.0, containing 4 M-urea, 100 mM-2-mercaptoethanol, 100 mM-lactate and 1 mM-EDTA) to yield a final protein concentration of 8 μM. A 10-fold excess of FMN (Sigma Chemical Co.) was added, and renaturation was facilitated by dialysis of the TD-b_2/FMN mixture firstly against solution A in the absence of urea and then against solution A in the absence of both urea and 2-mercaptoethanol. Each dialysis was carried out for 20 h at 4 °C under N_2. After the final dialysis the reconstituted protein showed similar kinetics to the original TD-b_2. The procedure for the wild-type protein was as above except that flavin was first removed from the protein by denaturation as previously described [10].

Kinetic analysis

Kinetic experiments involving enzymic oxidation of L-lactate were carried out under steady-state conditions at 25 ± 0.1 °C in 10 mM-Tris/HCl buffer, pH 7.5 and *I* 0.10 (NaCl), with ferricyanide (1 mM) as electron acceptor as previously described [9,10]. Flavocytochrome b_2 concentrations were determined spectrophotometrically by

using published molar absorption coefficients [9]. Kinetic isotope effects were measured by using [2-^{2}H]lactate prepared and purified as previously described [5]. Isotopic purity of [2-^{2}H]lactate was confirmed by ^{1}H n.m.r. [13].

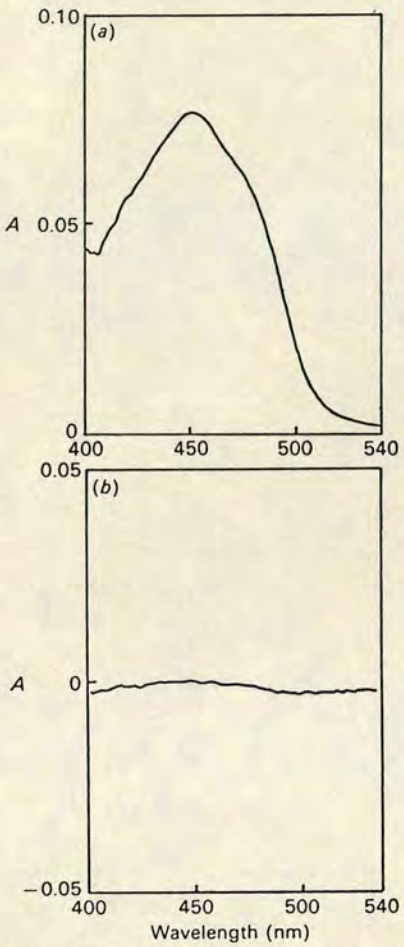

Fig. 3. Spectra determined by using the sulphite method (described in the Materials and methods section) for (*a*) active TD-b_2 and (*b*) deactivated TD-b_2

TD-b_2 concentration was 8 μM and 10 μl of 0.3 M-Na_2SO_3 was added. It is clear that the deactivated TD-b_2 has no bound flavin.

The dissociation of FMN during turnover was monitored as an increase in fluorescence at 520 nm on a Perkin–Elmer LS-5 luminescence spectrometer with an emission bandwidth of 2.5 nm and with the excitation wavelength set at 450 nm with an excitation bandwidth of 10 nm. The intensity of the fluorescence as a function of FMN concentration is known to be linear in the concentration range 0.1 nM–10 μM [14], and consequently all experiments were carried out with the enzyme concentrations between 0.1 and 0.01 μM. The ferricyanide concentration was 8 mM and the L-lactate concentration was varied between 1 and 10 mM.

RESULTS

Determination of M_r

The subunit M_r of TD-b_2 was 55000 according to SDS/polyacrylamide-gel electrophoresis, as expected for flavocytochrome b_2 lacking 23 amino acid residues. The native M_r of TD-b_2, determined by gel filtration on a calibrated Sepharose S300 column, was found to be 220000 (see Fig. 2). This indicates that TD-b_2 in the native state is tetrameric. TD-b_2 remained tetrameric in the active and inactive (flavin-deficient) states. The behaviour of TD-b_2 was very similar to that of the wild-type enzyme, and this can also be seen in Fig. 2.

Kinetics of L-lactate oxidation

The steady-state rate of oxidation of L-lactate by TD-b_2 was found to decrease long before L-lactate or ferricyanide was depleted. However, initial rates at several L-lactate concentrations were determined and did give rise to typical saturation behaviour, yielding the K_m and $k_{cat.}$ values listed in Table 1. These values can be compared with those found for the wild-type enzyme, which are also listed in Table 1. The decrease in rate of reaction seen with TD-b_2 proceeded in a biphasic manner, leading eventually to complete loss of enzyme activity. This biphasic process was independent of ferricyanide concentration, and was also observed if cytochrome *c* was used as an alternative electron acceptor (results not shown). TD-b_2 was perfectly stable for long periods [months at 4 °C in the reduced state as an $(NH_4)_2SO_4$ precipitate, and hours at 25 °C in solution at pH 7.5 in either reduced or oxidized state]; rapid deactivation was only observed under turnover conditions. The faster of the two phases was found to be dependent on L-lactate

Table 1. Kinetic properties of wild-type and tail-deleted (TD-b_2) flavocytochromes b_2

All values, unless otherwise stated, were determined at 25 °C, pH 7.5 and I 0.10, with ferricyanide (1 mM) as terminal electron acceptor. Values of $k_{f(deact.)}$ and $k_{s(deact.)}$ were determined at saturating L-lactate concentrations. The kinetic isotope effect was determined with [2-^{2}H]lactate. The value of $k_{diss.}$ was determined by monitoring the increase in fluorescence (caused by dissociation of FMN during turnover) with time; ferricyanide concentration was 8 mM in this case.

	Wild-type enzyme	TD-b_2 enzyme	Reconstituted wild-type enzyme	Reconstituted TD-b_2 enzyme
$k_{cat.}$ (s^{-1})	200 ± 10	165 ± 6	98 ± 3	97 ± 8
K_m (mM)	0.49 ± 0.04	0.96 ± 0.06	0.65 ± 0.05	1.76 ± 0.26
$k_{f(deact.)}$ (s^{-1})	–	0.029 ± 0.002	–	0.028 ± 0.006
$k_{s(deact.)}$ (s^{-1})	–	0.010 ± 0.001	–	0.008 ± 0.001
$k_{diss.}$ (s^{-1})	–	0.012 ± 0.002	–	–
Kinetic isotope effect	4.2 ± 0.2	3.6 ± 0.4	–	–

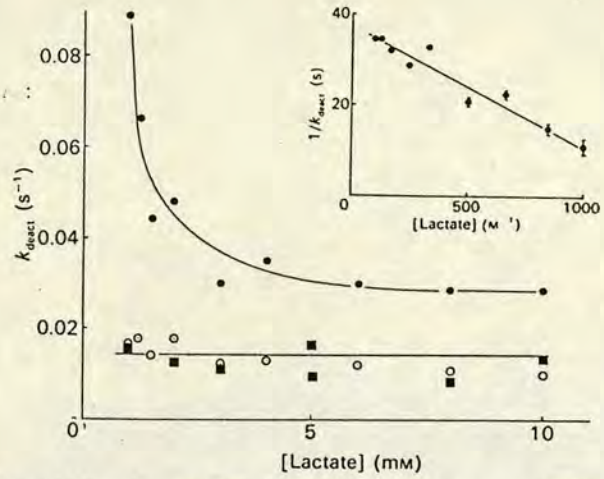

Fig. 4. Dependence of the deactivation rate constants for TD-b_2 on the concentration of L-lactate

The biphasic nature of TD-b_2 deactivation gave rise to first-order rate constants for a fast phase, $k_{f(deact.)}$, and a slow phase, $k_{s(deact.)}$. Note that whereas $k_{f(deact.)}$ shows a marked dependence on L-lactate concentration $k_{s(deact.)}$ is essentially L-lactate-independent. $k_{diss.}$ was determined by spectrofluorimetric measurements and corresponds to $k_{s(deact.)}$, indicating it is this phase in which FMN is lost. ●, $k_{f(deact.)}$; ⊙, $k_{s(deact.)}$; ■, $k_{diss.}$. Inset: double-reciprocal plot demonstrating the linear relationship of $1/k_{f(deact.)}$ against 1/[L-lactate]. All data shown were determined at 25±0.1 °C, in Tris/HCl buffer, pH 7.5 and *I* 0.10 (NaCl).

concentration, whereas the slow phase was L-lactate-concentration-independent (Fig. 4). The first-order deactivation rate constants at saturating L-lactate concentrations for the fast ($k_{f(deact.)}$) and slow ($k_{s(deact.)}$) phases are listed in Table 1. A plot of $1/k_{f(deact.)}$ against 1/[L-lactate] yielded a straight line (Fig. 4 inset), from which an estimate of the apparent dissociation constant, K_d, for L-lactate could be made. This was found to be 0.8±0.1 mM, in close agreement with the K_m value listed in Table 1. Kinetic parameters were also obtained for FMN-reconstituted TD-b_2 (which again showed a biphasic deactivation process), and are listed in Table 1.

Kinetic isotope effect with [2-^{2}H]lactate

With ferricyanide as electron acceptor, the deuterium isotope effect seen for the TD-b_2 enzyme was the same, within experimental error, as that for the wild-type enzyme. Values are shown in Table 1. This indicates that hydrogen abstraction at C-2 of L-lactate remains the rate-limiting step in TD-b_2.

FMN dissociation and re-incorporation

The deactivation of TD-b_2 during turnover was found to lead eventually to complete loss of the FMN prosthetic group. Before turnover a typical spectrum for bound FMN could be observed by using the sulphite method described in the Materials and methods section. After deactivation this bound FMN spectrum was found to be completely absent (Fig. 3), indicating loss of FMN. Absence of FMN was also shown by an inability to extract it under denaturing conditions as previously described [10]. The rate of dissociation of FMN was determined directly by monitoring the increase in fluorescence with time during enzyme turnover (the fluorescence intensity of bound FMN is quenched by the protein). These measurements gave a first-order rate constant for FMN dissociation, $k_{diss.}$, of 0.012±0.002 s^{-1}, which was independent of L-lactate concentration (Fig. 4). The value of $k_{diss.}$ and its independence of L-lactate concentration correlate (within experimental error) with the slow phase of deactivation already mentioned (Table 1), indicating that it is the slow phase only in which FMN is lost. Confirmation that activity loss was due to flavin absence was obtained by restoring up to 60% of the activity by re-incorporation of flavin. The reconstituted protein showed similar kinetic behaviour to the original TD-b_2 (see Table 1).

DISCUSSION

The three-dimensional structure of a subunit of flavocytochrome b_2 shows very clearly an extended *C*-terminal tail that projects from the main body of the molecule. In the tetramer the tail of the subunit wraps around each of the other three, making many intersubunit contacts (Fig. 1) [7]. Therefore the most obvious role one might suggest for this *C*-terminal tail is that it holds the four subunits together. To test this and hopefully to generate monomeric enzyme (in order to simplify kinetic analysis), a stop codon was introduced into the flavocytochrome b_2 gene at a position corresponding to amino acid residue 489. This mutation resulted in production of a mutant enzyme (TD-b_2) lacking 23 amino acid residues at the *C*-terminal portion of the molecule, which showed, as expected, an M_r of 55000 according to SDS/polyacrylamide-gel electrophoresis. However, TD-b_2 exhibited an M_r (under non-denaturing conditions) of 220000, corresponding to a tetramer (Fig. 2).

Kinetic studies indicated that deletion of the *C*-terminal tail had significantly affected the stability of the enzyme under turnover conditions. Investigations of L-lactate oxidation by TD-b_2 revealed that this mutant enzyme became totally deactivated during turnover. This deactivation did not occur as a result of dissociation of TD-b_2 into monomers during turnover: the enzyme remained tetrameric. Further studies revealed that the fully deactivated TD-b_2 totally lacked FMN and that a substantial amount (60%) of the original activity could be restored by flavin re-incorporation. The kinetics of the deactivation process were investigated in an attempt to elucidate the mechanism by which the non-covalently bound flavin had been lost from TD-b_2.

The rate of deactivation proceeded in a biphasic manner. The faster of the two phases gave a first-order rate constant, $k_{f(deact.)}$, that was dependent on L-lactate concentration (Fig. 4). Increasing the L-lactate concentration decreased the value of $k_{f(deact.)}$ until a limiting value was reached (Table 1). From the double-reciprocal plot (Fig. 4 inset) an apparent K_d for L-lactate of 0.8±0.1 mM was determined, which is very close to the K_m value of 0.96±0.06 mM. The similarity of these values is consistent with an intimate association between deactivation during the fast phase and L-lactate binding. A first-order rate constant, $k_{s(deact.)}$, was determined for the slower phase of the biphasic deactivation and was essentially independent of L-lactate concentration. This slower phase was found to correlate (within experimental error) with the rate of dissociation of FMN from the enzyme as determined by fluorescence measurements

(Table 1), with the rate of FMN dissociation also being independent of L-lactate concentration. A possible explanation for the biphasic deactivation process is that there is an initial, substrate-dependent, fast phase that leads to a partially deactivated conformationally altered enzyme. This conformational alteration is sufficient to allow dissociation of FMN, which occurs in the slow phase and is independent of substrate concentration. This process might be represented as shown in the equation below, where E_C is the conformationally altered enzyme and E_I is the fully inactive (FMN-deficient) enzyme, and k_f and k_s are the rate constants for the fast and slow phases respectively:

$$E \xrightarrow{k_f} E_C \xrightarrow{k_s} E_I + \text{free FMN}$$

After FMN has been lost the enzyme is completely inactive. TD-b_2 that had been reactivated by flavin reincorporation underwent flavin loss again by a biphasic process, resulting in $k_{f(deact.)}$ and $k_{s(deact.)}$ values identical with the original values, within experimental error (see Table 1). From these results we suggest that deletion of the *C*-terminal tail allows an alteration in the active-site conformation to occur during turnover that reduces the ability of the enzyme to bind FMN sufficiently tightly to prevent its loss.

Although TD-b_2 loses activity during turnover, it was possible to use initial-rate data from steady-state experiments to determine K_m and $k_{cat.}$ values for L-lactate. The difference in the values of K_m and $k_{cat.}$ between TD-b_2 and wild-type enzymes (Table 1) are of an order that would be consistent with a general destabilization of the enzyme resulting from a conformational difference. The possibility that such a change could affect the rate-limiting step, which is known to be hydrogen abstraction at C-2 of L-lactate [5], was considered. To explore this, the kinetic isotope effect seen with [2-^{2}H]lactate was investigated and found to be essentially the same, within experimental error, for both TD-b_2 and wild-type enzymes. Thus, although K_m and $k_{cat.}$ values are affected by tail deletion, this would not appear to be due to any change in the rate-limiting step.

From our studies on TD-b_2 we draw the following conclusions: (i) although it appears to be involved in many intersubunit contacts, the *C*-terminal tail is not essential for holding the subunits together as a tetramer; (ii) the *C*-terminal tail acts as a conformational 'anchor' influencing the structure of the protein in regions distant from it, particularly the FMN active site; (iii) TD-b_2 is deactivated during turnover in a biphasic process of which the slow phase corresponds to FMN dissociation; (iv) any alteration in the structural integrity of the flavin active site, caused by tail deletion, does not result in any significant change in the rate-determining step for L-lactate oxidation.

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