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FLAVOCYTOCHROME b_2

STEPHEN K. CHAPMAN,* SCOTT A. WHITE,* and GRAEME A. REID**

* Department of Chemistry and ** Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JJ, Scotland

I. Introduction

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC. 1.1.2.3) is a soluble component of the intermembrane space of yeast

mitochondria (*1*), where it catalyzes the oxidation of L-lactate to pyruvate with subsequent transfer of electrons to cytochrome *c* (*2*). The enzyme is a tetramer of identical subunits (*3*), with each subunit containing both flavin mononucleotide (FMN) and protoheme IX prosthetic groups (*2*) (Fig. 1) in two functionally distinct domains.

A short electron transport chain involving flavocytochrome b_2, cytochrome *c*, and cytochrome *c* oxidase allows yeast to respire on L-lactate even if the main electron transport chain is blocked, for example, by antimycin (*3*). The topological arrangement of this respiratory pathway is shown diagrammatically in Fig. 2.

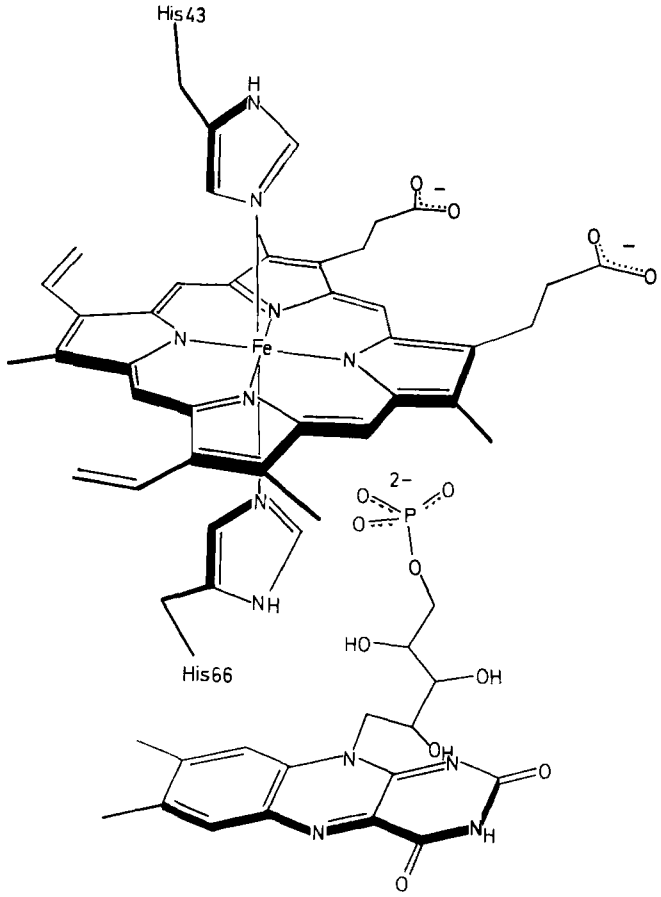

FIG. 1. Structure of the flavocytochrome b_2 prosthetic groups—protoheme IX and flavin mononucleotide.

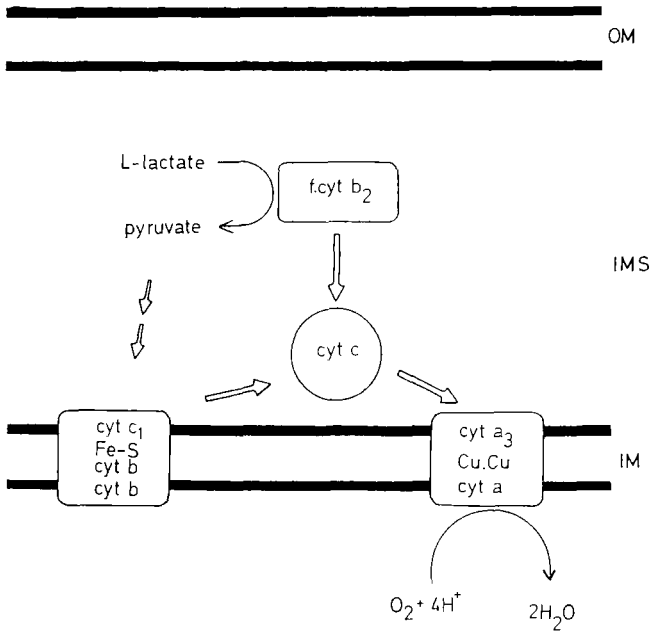

FIG. 2. The topological arrangement of the short electron transport chain involving flavocytochrome b_2 in yeast mitochondria; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; cyt, cytochrome; f.cyt b_2, flavocytochrome b_2.

HISTORICAL PERSPECTIVE

In 1915, Harden and Norris observed that dried yeast, when mixed with lactic acid, reduced methylene blue and formed pyruvic acid (*4*). Thirteen years later Bernheim prepared an extract from acetone-dried baker's yeast, which had lactate dehydrogenase activity (*5*). Bach and co-workers demonstrated that the lactate dehydrogenase activity was associated with a *b*-type cytochrome, which they named cytochrome b_2 (*6*). In 1954, the enzyme was crystallized, enabling the preparation of pure material and the identification of flavin mononucleotide as a second prosthetic group (*2*). Since then, significant advances have been made in the analysis of the structure and function of the enzyme. Much of the earlier work on flavocytochrome b_2 has already been summarized in previous review articles (*7–10*). In this article we shall describe recent developments in the study of this enzyme, ranging from kinetic, spectroscopic, and structural data to the impact of recombinant DNA technology.

II. Isolation and Purification of Flavocytochrome b_2

Purification procedures have been reported for flavocytochromes b_2 from the yeasts *Saccharomyces cerevisiae* (baker's yeast) and *Hansenula anomala*. Each of these enzymes has subsequently been subjected to extensive characterization.

A. From *Saccharomyces cerevisiae*

The first breakthrough in the purification of the enzyme from *S. cerevisiae* was achieved by Appleby and Morton, who used crystallization of the enzyme as a major step in the procedure (*2*). Around 1 μmol of what was termed type I enzyme was produced per kilogram of dried yeast. The crystals were later found to contain DNA (*11*), which could be removed by dialysis (*12*) or chromatography (*13*) to yield type II DNA-free flavocytochrome b_2. The enzyme purified in this way was found to be unstable and enzyme activities reported by various groups were inconsistent (*11–14*). Nichols *et al.* (*15*) observed changes in the properties of the enzyme during crystallization and Somlo and Slonimski noted that a "modification" within the enzyme was necessary before it would crystallize (*16*). Analysis of the crystallized enzyme by polyacrylamide gel electrophoresis showed that it consisted of two polypeptide chains of unequal length (36 and 21 kDa) (*17*). In 1972 it was demonstrated that this was due to selective proteolytic cleavage of the enzyme, by yeast proteinases, during the crystallization step (*18*). To prepare an "intact" form of the enzyme, Jacq and Lederer purified the enzyme in the presence of phenylmethylsulfonyl fluoride (PMSF, a known inhibitor of serine proteinases) (*18*). New purification procedures involving column chromatography and the use of PMSF in all steps have been developed (*18–20*). These procedures lead to the production of a single polypeptide of M_r 57,500. The enzymes prepared by crystallization or in the presence of PMSF will be referred to as "cleaved" or "intact" flavocytochromes b_2, respectively.

The intact enzyme can be kept for up to 4 months without major loss of activity, if stored as an ammonium sulfate precipitate in the presence of lactate, PMSF, and EDTA at 4°C under an atmosphere of nitrogen. The best preparations of intact enzyme yield around 1 μmol kg^{-1} of dried yeast (*20*).

B. From *Hansenula anomala*

Flavocytochrome b_2 from *H. anomala* is stable to endogenous proteinases, and purification does not lead to a cleaved form of the enzyme

(*21*). Thus PMSF is not required to protect the enzyme (*20*). As a result, the problems associated with purification of the *S. cerevisiae* enzyme have not been encountered. A high-yield purification procedure for the *H. anomala* enzyme has been reported (*22*). A comparison of the procedures for the preparation of flavocytochromes b_2 from both yeasts can be found in Ref *20*.

III. Structural Studies

Understanding molecular mechanisms of enzymatic catalysis depends on a detailed knowledge of the structural framework within which substrate recognition and catalysis occur. Determination of the X-ray crystal structure of *S. cerevisiae* flavocytochrome b_2 by Mathews and colleagues (*23–25*), coupled with the availability of the complete amino acid sequence (*26, 27*), has allowed detailed analysis of substrate recognition and catalysis in this enzyme.

A. Quaternary Structure

Flavocytochrome b_2 is composed of a single polypeptide chain with a molecular weight, as estimated by SDS–polyacrylamide gel electrophoresis, of 57,500 in the case of the *S. cerevisiae* enzyme (*28*) and 58,000 for the enzyme from *H. anomala* (*29*). The native enzymes behave as oligomers with molecular weights around 230,000 (*29–31*), suggesting that each is a homotetramer. This has been confirmed for *S. cerevisiae* flavocytochrome b_2 by X-ray crystallography, which shows the subunits to be related by a four-fold axis of symmetry (Fig. 3) (*23–25*). The tetrameric structure is important for activity because dissociation of the *H. anomala* enzyme at low ionic strength yields inactive monomers that can be reassociated with recovery of activity (*32*).

B. Primary Structure

The complete amino acid sequence of *S. cerevisiae* flavocytochrome b_2 has been determined (*26*). The mature form of the enzyme is composed of 511 amino acids (Fig. 4). The DNA sequence has also been determined (*27*), revealing the presence of an 80-residue N-terminal presequence. This N-terminal extension directs the enzyme into the mitochondrion, where it is processed in two proteolytic steps that result in mature flavocytochrome b_2, which is located in the intermembrane space (*1, 33–36*).

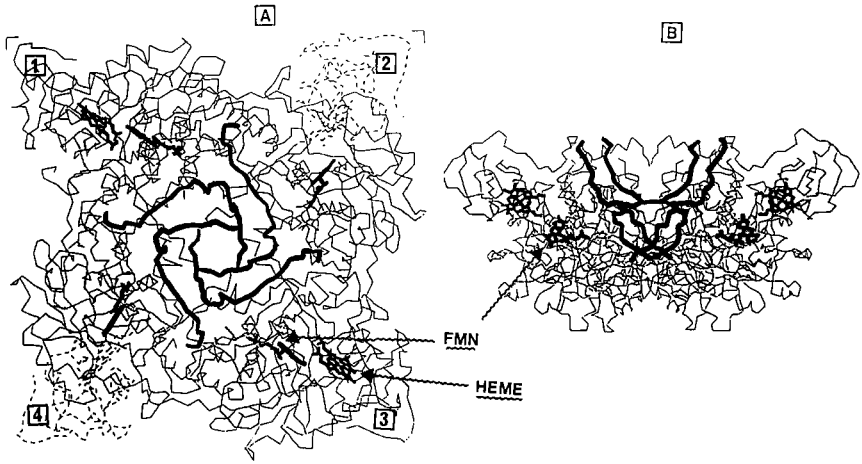

FIG. 3. The three-dimensional structure of *Saccharomyces cerevisiae* flavocytochrome b_2 as determined by Xia and Mathews (*25*). (A) The C-terminal tails and flavin mononucleotide (FMN) and heme prosthetic groups are highlighted in this view, which is looking down the fourfold axis of symmetry. The four subunits are numbered 1 to 4; the shaded portions seen in the subunits labeled 2 and 4 represent the two heme domains, which are disordered in the structure. (B) A side view, perpendicular to view A, is also shown.

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Scb2  EPKLDMNKQK ISPAEVAKHN KPDDCWVVIN GYVYDLTRFL PNHPGGQDVI KFNAGKDVTA IFEPLHAPNV IDKYIAPEKK   80
Hab2  .DVPHWKDIE LTPEIVSQHN KKDDLWVVLN GQVYDLTDFL PNHPGGQKII IRYAGKDATK IFVPIHPPDT IEKFIPPEKH   79
SpGO  .......... .......... .......... .......... .......... .......... .......... ..........

Scb2  LGPLQGSMPP ELVCPPYAPG ETKEDIARKE QLKSLLPPLD NIINLYDFEY LASQTLTKQA WAYYSSGAND EVTHRENHNA  160
Hab2  LGPLVGEFEQ E.....EEE LSDEEIDRLE RIER.KPPLS QMINLHDFET IARQILPPPA LAYYCSAADD EVTLRENHNA  152
SpGO  .......... .......... .......... .........M EITNVNEYEA IAKQKLPKMV YDYYASGAED QWTLAENRNA   41
Con   ---------- ---------- ---------- ---------- ---N--DFE- -A-Q-L---- --YY-S-A-D --T--EN-NA

Scb2  YHRIFFKPKI LVDVRKVDIS TDMLGSHVDV PFYVSATALC KLGNPLEGEK DVARGCGQGV TKVPQMISTL ASCSPEEIIE  240
Hab2  YHRIFFNPKI LIDVKDVDIS TEFFGEKTSA PFYISATALA KLGHP.EGEV AIAKGAGRE. .DVVQMISTL ASCSFDEIAD  229
SpGO  FSRILFRPRI LIDVTNIDMT TTILGFKISM PIMIAPTAMQ KMAHP.EGEY ATARAASAAG TIMT..LSSW ATSSVEEVAS  118
Con   Y-RIFF-P-I LIDV--VD-- T--LG----- P--I--TAL- KL--P-EGE- --A------- -------S-- A--S-EEI--

Scb2  AAPSDKQIQW YQLYVNSDRK ITDDLVKNVE KLGVKALFVT VDAPSLGQRE KDMKLKFL... ...SNTKAGP KAMKKTNVEE  314
Hab2  ARIPGQQ.QW YQLYVNADRS ITEKAVRHAE ERGMKGLFIT VDAPSLGRRE KDMKMKF... .EADS..DVQ G..DDEDIDR  300
SpGO  TGPG...IRF FQLYVYKDRN VVAQLVRRAE RAGFKAIALT VDTPRLGRRE ADIKNRFVLP PFLTLKNFEG IDLGKMD...  192
Con   ---------W YQLYV--DR- I----V---E --G-K----T VD-P-LG-RE -D-K--F--- ---------- ----------

Scb2  SQGASRALSK F....IDPSL TWKDIEELKK KTKLPIVIKG VQRTEDVIKA AEIGVSGVVL SNHGGRQLDF SRAPIEVLAE  390
Hab2  SQGASRALSS F....IDPSL SWKDIAFIKS ITKMPIVIKG VERKEDVLLA AEHGLQGVVL SNHGGRQLDY TRAPVEVLAE  376
SpGO  .KANDSGLSS YVAGQIDRSL SWKDVAWLQT ITSLPILVKG VITAEDARLA VQHGAAGIIV SNHGARQLDY VPATIMALEE  271
Con   -------LS- F----ID-SL -WKDI----- -T-LPI-IKG V---ED---A ---G--GVV- SNHG-RQLDY --A-I--L-E

Scb2  TMPILEQRNL KDKLEVFVDG GVRRGTDVLK ALCLGAKGVG LGRPFLYANS CYGRNGVEKA IEILRDEIEM SMRLLGVTSI  470
Hab2  VMPILKERGL DQKIDTFVDG GVRRGTDVLK LLCLAAKGVG LGRPFLYAMS SYGDKGVTKA IQLLKDEIEM NMRLLGVNKI  456
SpGO  VV.....KAA QGRIPVFLDG GVRRGTDVFK ALALGAAGVF IGRPVVFSLA AEGEAGVKKV LQMMRDEFEL TMALSGCRSL  346
Con   ---------- ------F-DG GVRRGTDVLK -L-L-A-GV- -GRP--Y--- --G--GV-K- ---L-DE-EM -M-L-G----

Scb2  AELKPDLL.. DLSTLKARTV GVPNDVLYNE VYEGPTLTEF EDA...   511
Hab2  EELTPELL.. DTRSIHTRAV PVAKDYLYEQ NYQRMSGAEF RPGIED  500
SpGO  KEISRSHIAA DWDGPSSRAV ARL.....  .......... ......  369
Con   -E-------- D------R-V ---------- ---------- --------
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FIG. 4. Sequence alignment of flavocytochromes b_2 and glycollate oxidase. The amino acid sequences of the mature forms of flavocytochromes b_2 from *Saccharomyces cerevisiae* (Scb2) and *Hansenula anomala* (Hab2) and of glycollate oxidase from spinach (SpGO) are shown along with a consensus (Con) wherein all three sequences are identical. The interdomain hinge region and the proteinase-sensitive loop of flavocytochromes b_2 are boxed.

The gene encoding *H. anomala* flavocytochrome b_2 has recently been cloned and sequenced (*37*, *38*) and the corresponding amino acid sequence, with its N-terminal extension, has been deduced (*37*). The amino acid sequences of flavocytochromes b_2 from *S. cerevisiae* (Scb2) and *H. anomala* (Hab2) are compared in Fig. 4. There is an overall identity of around 60% in the two sequences, with many areas well conserved (*37*). All of the residues identified as functionally important by Lederer and Mathews (*39*) are identical. There are two regions in which the sequences differ greatly, i.e., residues 92–103 and 298–314 (Scb2 numbering scheme). The first of these comprises the hinge region, which joins the cytochrome and flavodehydrogenase domains of the enzyme together (see Section III,C). The second corresponds to the proteinase-sensitive region in Scb2 (*40*), which forms a mobile loop on the enzyme surface and is disordered in the crystal structure (*23–25*). In each case the Hab_2 sequence is both shorter and considerably more acidic than the corresponding segment from Scb2 (*37*).

C. Domain Structure

1. The Cytochrome Domain

Labeyrie *et al.* (*41*) isolated a trypsin fragment of 11 kDa from *S. cerevisiae* flavocytochrome b_2 that contained heme but was devoid of flavin and had no lactate dehydrogenase activity. The fragment, which was referred to as cytochrome b_2 core, was found to have spectral properties very like those of microsomal cytochrome b_5 (*41*). This similarity with cytochrome b_5 is borne out by comparisons of amino acid sequence (*42–44*). The sequence similarity extends to related heme domains of sulfite oxidase (*45*, *46*) and assimilatory nitrate reductase (*47*). The existence of a protein family with a common "cytochrome b_5 fold" was suggested by Guiard and Lederer (*48*) and this is supported by the close similarity between the three-dimensional structures of microsomal cytochrome b_5 (*49*) and the cytochrome domain of flavocytochrome b_2 (*23–25*).

Biochemical and biophysical characterization of the cytochrome b_2 core has been extremely useful in analysis of its structure and function. In particular, its small size makes it very amenable to techniques such as NMR spectroscopy (see Section IV,B).

The cytochrome b_2 core has been shown by amino acid sequence determination to be located at the N-terminus of the flavocytochrome b_2 polypeptide chain (*43*). It is clearly seen as a distinct domain in the crystal structure, in close contact with the much larger FMN-containing domain (*23–25*) (Fig. 5). The cytochrome domain consists of resi-

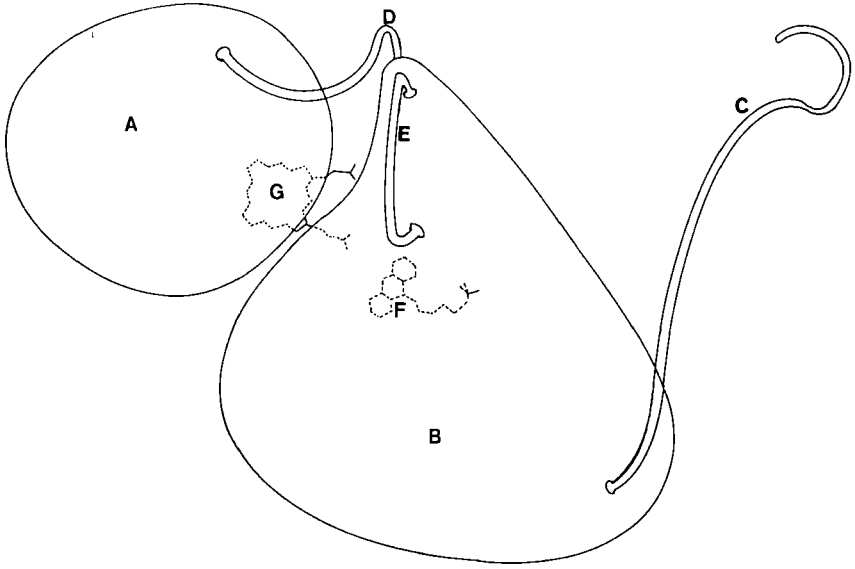


FIG. 5. Schematic representation of the structure of a flavocytochrome b_2 subunit. A, The heme domain; B, the flavodehydrogenase domain; C, the C-terminal tail; D, the hinge region linking the two domains; E, the proteolytically sensitive loop; F, flavin mononucleotide; G, protoheme IX.

dues 1–100, and residues 101–488 comprise the FMN-containing, or flavodehydrogenase, domain. Residues 489–511 form a C-terminal tail that wraps around the center of the tetramer (Fig. 3), making extensive contacts with each of the three other subunits (*25*), and may be important in stabilizing the quaternary structure.

2. *The Flavodehydrogenase Domain*

It has not been possible to isolate the intact *S. cerevisiae* flavodehydrogenase domain, independently of the cytochrome domain, by proteolytic cleavage methods. However, in the case of the enzyme from *H. anomala,* the flavodehydrogenase domain has been isolated by careful treatment with *Staphylococcus aureus* V8 proteinase (*50*). This procedure did not yield large amounts of the flavodehydrogenase moiety and so an alternative method using an *H. anomala* proteinase was developed (*51*), which gave a 25% yield (10 times that seen for the original method). The isolated flavodehydrogenase domain was found to be tetrameric ($M_r = 4 \times 39$ kDa) and had 70% of the L-lactate:ferricyanide reductase activity seen for the native enzyme but no cytochrome *c* reductase activity (*51*).

The flavodehydrogenase domain of the enzyme has been shown to be structurally related to other FMN-containing enzymes, including glycolate oxidase from spinach (*52*) and trimethylamine dehydrogenase from the methylotrophic bacterium W_3A_1 (*53*). All three of these enzymes contain a parallel $\beta_8\alpha_8$ barrel structure, with the α-carbon chains of flavocytochrome b_2 and glycolate oxidase particularly similar in conformation (*25, 54*) and the trimethylamine dehydrogenase structure more divergent. The amino acid sequence of the latter enzyme has not yet been determined, but those of flavocytochrome b_2 (*26*) and glycolate oxidase (*55*) are about 40% identical (Fig. 4).

Limited amino acid sequence information has shown that long-chain α-hydroxyacid oxidase from rat kidney is also related to these FMN-containing oxidoreductases (*56*). It is likely that several further members of this family remain to be identified. The flavodehydrogenase domain shows no sequence similarity to the lactate dehydrogenase from bacteria and higher eukaryotes that utilize NAD as a substrate. Yeasts lack such an enzyme and the substrate specificity of flavocytochrome b_2 has presumably evolved independently of the NAD-linked dehydrogenases.

D. The Active Site

The active site in *S. cerevisiae* flavocytochrome b_2 was readily identified in the crystal structure by locating electron density attributable to the product, pyruvate (*23–25*). In fact, pyruvate is present in only two of the four subunits of each tetramer, giving rise to two, crystallographically distinguishable, types of subunits (*23–25*). The active sites in these two types of subunit (labeled subunits 1 and 2) are illustrated in Fig. 6. Several amino acid residues around the active site appear capable of making chemically significant interactions with the substrate and/or FMN (Fig. 6). Arg 376 is well positioned to interact with the substrate carboxylate both electrostatically and by forming a hydrogen bond between NE of Arg 376 and one of the carboxylate oxygen atoms. In subunit 2 (Fig. 6), the hydroxyl of Tyr 143 is hydrogen bonded to the other carboxylate oxygen of pyruvate, and the keto oxygen of the pyruvate can interact with both the hydroxyl group of Tyr 254 and the NE2 group of His 373. Asp 282 appears to orientate the imidazole ring of His 373 by interacting with the histidine NE1.

The methyl group of pyruvate is in van der Waals contact with Leu 230 (not shown in Fig. 6), but is in an otherwise relatively uncrowded environment. This is borne out by the fact that flavocytochrome b_2 can

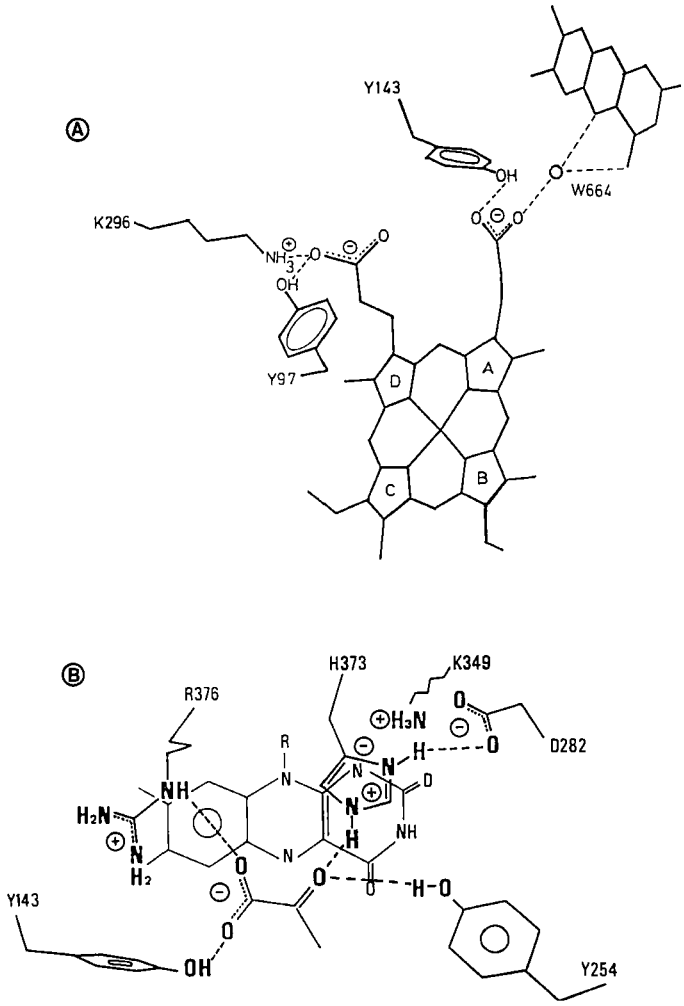


FIG. 6. The active site structure in (A) subunit 1 (pyruvate absent) and (B) subunit 2 (pyruvate present).

efficiently oxidize a number of alternative substrates in which the methyl group is replaced by bulkier groups (see Section IV,C).

Several residues involved in interactions with the FMN have been identified (*25, 39*). Of particular note is the interaction of Lys 349 with N1 and O2 of the isoalloxazine ring. Such interactions have been suggested to stabilize the anionic form of the FMN semiquinone and hydroquinone and to account for the enhanced reactivity of flavin N5

with sulfite as observed in several flavoenzymes (*57*). The anionic flavosemiquinone is known to be stabilized in flavocytochrome b_2 (*58, 59*) and the enzyme binds sulfite at the N5 position with high affinity (*60*).

The active site architecture of flavocytochrome b_2 (*25*) is very similar to that seen in glycolate oxidase (*54*), with residues equivalent to those mentioned above presumably performing equivalent functions in the latter enzyme.

E. Interdomain Interactions

As described above, two crystallographically discernable types of subunits can be seen in the crystal structure of *S. cerevisiae* flavocytochrome b_2. In the subunits that contain pyruvate at the active site (subunit 2), the cytochrome domain is disordered and cannot be located in the electron density map (*25*). This degree of structural disorder is consistent with substantial mobility of the cytochrome domain. It has been suggested that the ordering of the cytochrome domain in subunit 1 is imposed by crystal packing forces (*61*). Indeed, it may be that in crystals of the native enzyme, the ordering of the cytochrome domain in one subunit and not the other results in asymmetry of pyruvate binding by affecting the affinity for the ligand (*61*).

The interface between the flavodehydrogenase and cytochrome domains in subunit 1 is dominated by nonpolar contacts. Ten residues of the flavodehydrogenase domain are involved in nonpolar van der Waals interactions with nine amino acid side chains and the heme group of the cytochrome domain (*25*). A single interdomain salt bridge is formed between Lys 296 and one of the heme propionate groups. These groups also participate in one of the six hydrogen bonds between the domains (*25*).

The interaction between Tyr 143 and the carboxylate of pyruvate in subunit 2 has already been mentiond (Section III,D). In subunit 1, however, where pyruvate is absent, Tyr 143 is now hydrogen bonded to an oxygen of one of the heme propionate groups (*25*). Thus it would appear that this residue is involved in both lactate dehydrogenation and interdomain communication. The possible central role of Tyr 143 will be discussed further in Section VI,D,3.

F. Structural Studies on the *Hansenula anomala* Enzyme

Attempts to crystallize flavocytochrome b_2 from *H. anomala* have been unsuccessful (*62*), but the amino acid sequence has recently been

deduced (Fig. 4) from the DNA sequence of the isolated gene (*37*) and this has allowed some interesting inferences to be made. Flavocytochromes b_2 from *H. anomala* and *S. cerevisiae* exhibit 60% sequence identity (Fig. 4), with all of the active site residues identified in the *S. cerevisiae* enzyme structure conserved in the *H. anomala* enzyme (*37*). The residues in the eight β-strands of the β-barrel of the flavodehydrogenase domain are more highly conserved, with 83% identity. Surface loops are far less well conserved and the C-terminal tails are quite different in sequence. The N-terminal mitochondrial targeting sequences are only weakly conserved, with 24% identity, but this is not surprising given the well-documented variability of targeting sequences (*63*). The structure of these targeting sequences and the requirement for localization of flavocytochrome b_2 to the mitochondrial intermembrane space are not discussed in detail here, but recent work is summarized by Hartl and Neupert in Ref. *36*.

Within the sequences of mature flavocytochromes b_2 from *S. cerevisiae* and *H. anomala*, two surface loops are surprisingly and dramatically different (see Fig. 4 and discussion in Section III,B) (*37*). These differences are quite probably associated with differences in catalytic behavior of the two enzymes (Section V).

IV. Biophysical and Biochemical Properties of Flavocytochrome b_2

A. Reduction Potentials

The reduction potentials for the heme and FMN prosthetic groups of flavocytochromes b_2 from *S. cerevisiae* and *H. anomala* are listed in Table I. Values for various modified forms of the enzyme, such as the flavin-free (deflavo) derivative, and the isolated cytochrome domain (the cytochrome b_2 core) are also reported in Table I (*64–69*). The reduction potentials for the heme group are as expected for a b_5-type cytochrome (*70*), with little difference in the values for different forms of protein, e.g., the deflavo-derivative of the holoenzyme and the isolated cytochrome b_2. The reduction potentials for the FMN group are not too different from those of the heme (about 50 mV difference), consistent with reversible electron transfer between the two prosthetic groups (*10*).

In the *H. anomala* enzyme the monoelectronic reduction potentials for the FMN (F) group are altered in the presence of pyruvate (*58, 59, 71*) to such an extent that the value for F_{ox}/F_{sq} lies above that of the heme (Table I). This effectively means that flavosemiquinone → heme

TABLE I

REDUCTION POTENTIALS FOR PROSTHETIC GROUPS IN VARIOUS FORMS OF FLAVOCYTOCHROME b_2[a]

Enzyme[b]	Reduction Potentials[c] (mV)				Note[d]	Ref.
	H_{ox}/H_{red}	F_{ox}/F_{sq}	F_{sq}/F_{red}	F_{ox}/F_{red}		
S_X	0 ± 3	—	—	—	—	*64*
S_X	6	−50	—	—	i	*65*
S_X	6 ± 2	−44 ± 8	−57 ± 9	−51 ± 16	ii	*66*
Deflavo-S_X	0 ± 3	—	—	—	—	*64*
S_X-b_2-core	−28	—	—	—	—	*33*
H_I	−19 ± 5	−23 ± 10	−45 ± 12	−34 ± 10	—	*66*
H_I	−16 ± 5	−16	−60	−54 ± 10	iii	*58*
H_I + pyruvate	−18 ± 6	74 ± 4	−133 ± 7	−31 ± 5	iv	*58*
Deflavo-H_I	−5 ± 5	—	—	—	v	*66*
H_I-b_2-core	−10 ± 5	—	—	—	—	*67*

[a] All values are reported in millivolts and the conditions are 30°C, pH 7 unless otherwise stated. Other relevant potentials: lactate/pyruvate = −190 mV (Ref. *68*); ferri/ferrocytochrome c = 273 mV (Ref. *69*).

[b] Abbreviations: S_X, the cleaved form of flavocytochrome b_2 from *S. cerevisiae*; deflavo-S_X, S_x in which the FMN prosthetic group has been removed; S_X-b_2-core, the isolated cytochrome domain (or core) of S_X; H_I, the intact flavocytochrome b_2 from *H. anomala*; deflavo-H_I, H_I in which FMN has been removed; and H_I-b_2-core, the isolated heme domain (or core) of H_I.

[c] Abbreviations: H, heme; F, FMN; sq, semiquinone.

[d] Data collected at (i) 21°C, (ii) 20°C, (iii) 18°C, (iv) 18°C and in 10 m*M* pyruvate, and (v) 18°C.

electron transfer is blocked in the presence of pyruvate. The possibility that this may be physiologically relevant in the control of lactate dehydrogenation has been suggested (*58, 59*).

B. SPECTROSCOPIC PROPERTIES

1. Electronic Absorption Spectroscopy

Electronic absorption spectra of flavocytochrome b_2 were first reported in 1942 (*72*). Since then, visible absorption has been an important tool for measuring enzyme concentration. The electronic absorption spectra for *S. cerevisiae* flavocytochrome b_2 in both oxidized and reduced states are shown in Fig. 7. The sharp peaks at 557, 528, and 423 nm (reduced) are characteristic of a *b*-type cytochrome. The ratio of the absorbances at 269 and 423 nm (reduced) provides a useful indication of enzyme purity (*20*). The absorption coefficients generally accepted as most accurate are listed in Table II. There are no significant

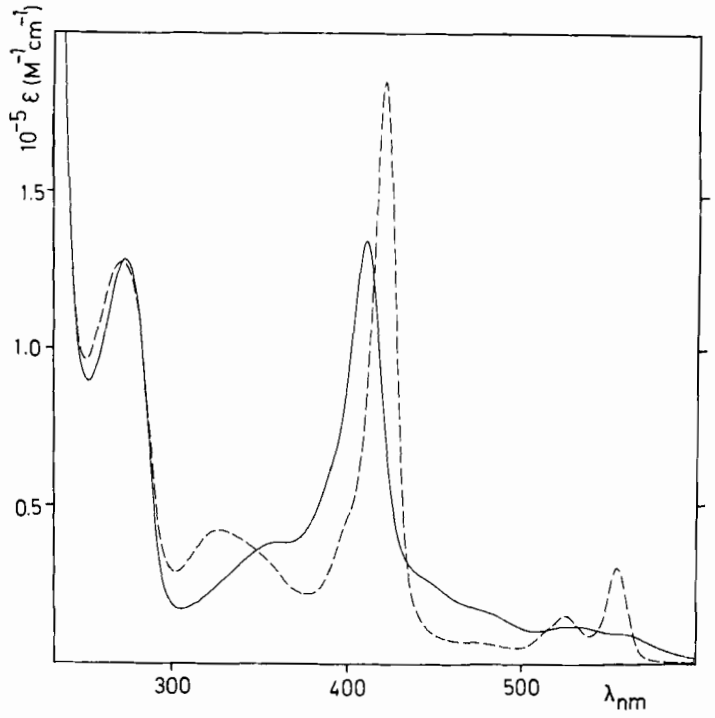


FIG. 7. The electronic absorption spectrum of *Saccharomyces cerevisiae* flavocytochrome b_2; the oxidized and reduced states are denoted by solid and dashed lines, respectively. The relevant absorption coefficients are listed in Table II.

TABLE II

DETAILS OF PEAK POSITIONS AND MOLAR ABSORPTION COEFFICIENTS IN UV–VISIBLE ABSORPTION SPECTRA OF OXIDIZED AND REDUCED FLAVOCYTOCHROME b_2[a]

	Oxidized form[b]		Reduced form	
Band	λ_{max} (nm)	ε(mM^{-1} cm^{-1})	λ_{max} (nm)	ε(mM^{-1} cm^{-1})
α	560	9.2	557	30.9
β	530	11.3	528	15.6
γ	413	129.5	423	183.0
δ	362	34.4	328	39.0
UV	275	89.0	269	88.0

[a] From Ref. *28*.

[b] Oxidized cytochrome b_2 core; λ_{max} = 413 nm, ε = 121.5 mM^{-1} cm^{-1}.

differences in these values for the various types of flavocytochrome b_2, e.g., the cleaved and intact forms from *S. cerevisiae*.

2. *Circular Dichroism and Magnetic Circular Dichroism*

Circular dichroism (CD) spectroscopy has been used to study the cleaved (*73*), deflavo (*74*), and intact (*19, 60*) forms of flavocytochrome b_2 from *S. cerevisiae* and the intact enzyme from *H. anomala* (*73, 75*). Magnetic circular dichroism (MCD) has been used to probe the holo- and deflavo-cleaved enzyme from *S. cerevisiae* (*76*), as well as the cytochrome b_2 core (*77*). It was noted that bands in the CD and MCD spectra ascribed to the heme were affected by removal of the FMN group, providing direct evidence of an interaction between the two prosthetic groups (*73*). Circular dichroism experiments on the intact and cleaved forms of *S. cerevisiae* flavocytochrome b_2 led Jacq and Lederer (*19*) to conclude that there were differences in the heme environment between these two forms of the enzyme.

3. *EPR Studies*

EPR signals for both the flavosemiquinone radical and the low-spin ferric heme have been reported (*65, 78–82*). The flavosemiquinone signal, which is easily observed at 123 K, shows a typical g value of 2.0039 ± 0.002 (*65*). The bandwidth, which is around 15 G, is very like that of an anionic, or red, semiquinone (*65*). The EPR signal of the low-spin ferric heme can be observed at low temperatures (~28 K) and shows g values of 2.99, 2.22, and 1.47 (*65*), which are similar to those found for cytochrome b_5 (*81*). EPR rapid freezing studies have allowed the amounts of semiquinone and ferric heme to be monitored during reduction of the enzyme by L-lactate (*66*). This has proved to be extremely useful in the development of kinetic schemes to describe the flow of electrons in the enzyme. The distance between the prosthetic groups in *H. anomala* flavocytochrome b_2 has been estimated from EPR experiments and spin–lattice relaxation measurements (*82*). Pyruvate was used to stablize the flavosemiquinone and the effect on the signal of this species from oxidized and reduced heme was measured. The results indicated a minimum intercenter distance of 18–20 Å (*82*).

4. *NMR Spectroscopy*

NMR studies of the cytochrome b_2 core have been carried out at 220 (*83*), 400 (*84, 85*), and 600 MHz (*85*). A comparison of the ^{1}H NMR spectra, at 220 MHz, of the cytochrome b_2 core with that reported for cytochrome b_5 indicates strong similarities among the two proteins (*83*). Recently the cytochrome b_2 core was investigated at 600 MHz and

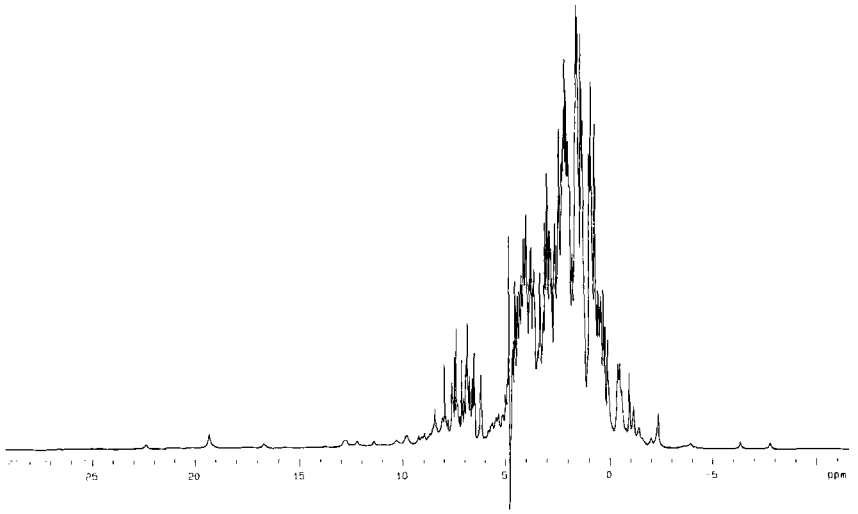


FIG. 8. The 600-MHz NMR spectrum of the oxidized cytochrome b_2 core (*85*). The paramagnetism of the ferric iron causes large shifts, resulting in peaks well upfield of 10 ppm and downfield of 0 ppm. Most of these peaks correspond to heme resonances, for example, from methyl and vinyl protons.

the NMR spectrum of the oxidized form is shown in Fig. 8. Peaks upfield of 10 ppm and downfield of 0 ppm are clearly visible and correspond to heme resonances that are shifted due to the paramagnetism of the ferric iron.

^{1}H NMR at 400 MHz has been used to probe the mobility of the cytochrome domain within the holoenzyme (*84*). The linewidths of heme resonances downfield of +12 ppm and upfield of −4 ppm have been compared for the holoenzyme and the cytochrome b_2 core and indicate that the cytochrome domains of the holoenzyme are markedly mobile (*84*). NMR has also been used to investigate the nature of the interaction between flavocytochrome b_2 and cytochrome *c* (*85, 86*) with the ultimate aim of defining the binding site on flavocytochrome b_2 used by its physiological partner. The extensive NMR studies on the interaction between cytochrome b_5 and cytochrome *c* (*87, 88*) provide a useful background to the work with cytochrome b_2.

C. SUBSTRATE SPECIFICITY

Flavocytochrome b_2 has been shown to be capable of oxidizing a broad range of α-hydroxy acids (*89–91*). The highest turnover rate is obtained, not surprisingly, with L-lactate. The "best" estimates of mo-

TABLE III

ENZYME MOLAR ACTIVITY AND K_M FOR L-LACTATE WITH VARIOUS FORMS OF FLAVOCYTOCHROME b_2[a]

Flavocytochrome b_2	Source	Molar Activity (sec^{-1})	K_M (mM)
Cleaved form	*S. cerevisiae*	210 ± 10	1.6
Intact form	*S. cerevisiae*	550	0.4
Intact form	*H. anomala*	1000 ± 100	1.3

[a] Determinations are best estimates (*20*) under steady-state conditions. Usual assay conditions are ferricyanide (1 mM) as electron acceptor, 30°C, 0.1 M phosphate buffer, pH 7.0. The molar activity, which corresponds to k_{cat}, is expressed as the number of mole electron equivalents transferred per mole of enzyme per second. Note that k_{cat} values are often expressed in terms of "moles of substrate consumed"; L-lactate is a two-electron donor, therefore the activity values listed above may be halved to express them in this way.

lar activity and K_M for L-lactate, according to Labeyrie *et al.* (*20*), are shown in Table III. Reaction rates found with alternative substrates are listed in Table IV. It can be concluded that the minimum requirement for a substrate is a carboxylate with an α-hydrogen and an α-hydroxyl group.

TABLE IV

ALTERNATIVE SUBSTRATES FOR FLAVOCYTOCHROME b_2 FROM *S. cerevisiae*[a]

Substrate	Relative rate
L-Lactate	1.00
DL-Chlorolactate	0.35
DL-Fluorolactate	0.10
DL-Bromolactate	0.91
L-Glycerate	0.50
DL-3-Phosphoglycerate	0.25
Glycolate	0.04
DL-α-Hydroxybutyrate	0.30
DL-α-Hydroxy-*n*-caproate	0.18
DL-α-Hydroxy-*i*-caproate	0.17
DL-Isocitrate	0.01
L-Malate	0.01
L-Tartrate	0.05

[a] For details of conditions, enzyme preparations, etc., see Refs. *89–91*. All values are expressed as relative rates, with that for L-lactate being 1.00.

As well as alternative substrates, there have been a number of studies on inhibitors of flavocytochrome b_2. Known inhibitors include D-lactate (*16, 92–95*), pyruvate (*16, 58, 60, 96*), propionate (*96*), DL-mandelate (*90, 91*), sulfite (*60*), and oxalate (*16, 60, 97*). Values of K_i for these inhibitors and the conditions and types of enzyme used can be found in the papers referenced above. All of the above inhibitors show typical competitive inhibition except pyruvate and oxalate, for which mixed inhibition has been observed (*60, 97*). Inhibition has also been reported for excess substrate with the intact enzymes from both *S. cerevisiae* (*16*) and *H. anomala* (*92*), though not apparently with the cleaved enzyme from *S. cerevisiae* (*16*). It is possible that inhibition by excess substrate arises either from different binding modes at the active site or from a second lower affinity binding site elsewhere on the enzyme.

D. Dependence on pH

The results from several pH-dependence studies on flavocytochrome b_2 are summarized in Table V (*98–100*). It is evident from this table that the enzyme has a pH optimum of between 7 and 8. It would appear that the free enzyme has pK_a values of around 6.5 and 9, which are shifted upon substrate binding to around 5.5 and 10. Because free lactate has a pK_a of 3.86 (*101*), the pK_a values must arise from groups on the enzyme at, or near to, the active site. From an inspection of the structure at the active site (Fig. 6), it would appear that there are three residues to which these pK_a values might possibly be assigned; these

TABLE V

Results from pH Dependence Studies on Flavocytochrome b_2 from *S. cerevisiae*

Enzyme[a]	pH optimum	pK_a	Temp. (°C)	Ref.
E	7.2	6.0, 8.8	25	*98*
E–S	—	5.3, 9.7	25	*98*
E	—	6.7	20	*95*
E–S	8.0	5.68	20	*95*
E–S	7.5	5.65	20	*99*
E–S	7.3	—	25	*100*
E–S	8.0	—	20	*2*

[a] E, Values for the free enzyme; E–S, values for the enzyme–substrate complex (determined at saturating L-lactate concentrations and with ferricyanide as the electron acceptor).

are His 373, Tyr 143, and Tyr 254. The possibility of a contribution from histidine has already been suggested (*98*), although as yet this is still only conjecture.

In a detailed pH-dependence study of the enzyme by Hinkson and Mahler (*95*), an apparent pK_a of 6.7 was measured for a group involved in L-lactate binding. There was no corresponding pK_a value in this region for the binding of D-lactate. Because D- and L-lactate are identical except for the orientation at the α-carbon, it was suggested that the residue on the enzyme responsible for the pK_a of 6.7 was interacting with one of the groups attached to the α-carbon of L-lactate, most probably the hydroxyl group.

The same workers looked at the pH dependence of pyruvate inhibition and reported pK_a values of 7.3 and 8.0 (*95*). They suggested that the pK_a of 7.3 was essentially that of the same group involved in L-lactate binding for which the pK_a was determined as 6.7. This would require a residue capable of binding to both an α-hydroxyl and an α-keto group!

V. Catalysis and Electron Transfer

A. Overview

The physiological pathway of electron transfer in flavocytochrome b_2 is from bound lactate to FMN, then FMN to b_2–heme, and finally b_2–heme to cytochrome *c* (Fig. 9) (*2, 11, 80, 102*). The first step, oxidation of L-lactate to pyruvate with concomitant electron transfer to FMN, is the slowest step in the enzyme turnover (*103*). With the enzyme from *S. cerevisiae*, a steady-state kinetic isotope effect (with ferricyanide as electron acceptor) of around 5 was obtained for the oxidation of DL-lactate deuterated at the C_α position, consistent with the major rate-determining step being cleavage of the C_α–H bond (*103*). Flavocytochrome b_2 reduction by [2-^{2}H]lactate measured by stopped-flow spectrophotometry resulted in isotope effects of 8 and 6 for flavin and heme reduction, respectively, indicating that C_α–H bond cleavage is not totally rate limiting (*104*).

FMN, the first acceptor in the electron-transfer pathway, has been clearly shown to be essential for lactate dehydrogenase activity (*105*). What is the role of the heme? Forestier and Baudras found a linear relationship between heme content and enzyme activity; extrapolation to zero heme content indicated zero cytochrome *c* reductase activity and a lowered ferricyanide reductase activity (*106*). Thus electron

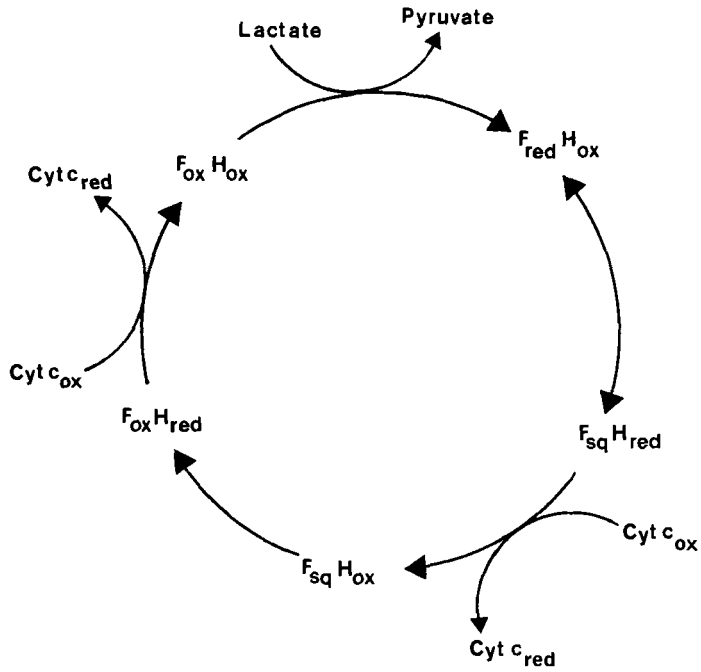
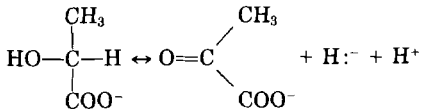


FIG. 9. The physiological pathway of electron transfer in flavocytochrome b_2. F_{ox}, Oxidized FMN; F_{sq}, flavosemiquinone; F_{red}, reduced FMN; H_{ox}, oxidized heme; H_{red}, reduced heme; Cyt c, cytochrome *c*.

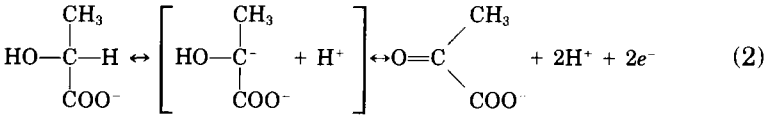
transfer from flavocytochrome b_2 to cytochrome *c* proceeds via the b_2–heme. We shall now describe the individual steps in the electron transfer pathway in detail.

B. LACTATE DEHYDROGENATION AND FMN REDUCTION

The conversion of L-lactate to pyruvate is a two-electron redox process. One could consider this occurring as two one-electron steps (a radical mechanism) or as one two-electron step. There are two options for a single two-electron step, and these are hydride transfer (H^-) or proton (H^+) abstraction followed by a two-electron transfer from a carbanion intermediate. These two alternatives for lactate are shown formally in Eqs. (1) and (2) for hydride transfer and the carbanion mechanism, respectively.



(1)



(2)

In the case of flavocytochrome b_2 and related flavoenzymes, there is a sufficient body of evidence to indicate that the carbanion mechanism operates. The formation of a carbanion is not, of course, an oxidation and two electrons need to be transferred from the carbanion intermediate to the flavin cofactor. This could occur possibly via a covalent intermediate or by sequential one-electron transfers. These possibilities will be discussed in detail later in this section.

The Carbanion Mechanism

To understand the carbanion mechanism in flavocytochrome b_2 it is useful to first consider work carried out on related flavoenzymes. An investigation into D-amino acid oxidase by Walsh *et al.* (*107*), revealed that pyruvate was produced as a by-product of the oxidation of β-chloroalanine to chloropyruvate. This observation was interpreted as evidence for a mechanism in which the initial step was C_α-H abstraction to form a carbanion intermediate. This intermediate would then be oxidized to form chloropyruvate or would undergo halogen elimination to form an enamine with subsequent ketonization to yield pyruvate. The analogous reaction of lactate oxidase with β-chlorolactate gave similar results (*108*) and it was proposed that these flavoenzymes worked by a common mechanism. Further evidence consistent with these proposals was obtained by inactivation studies of flavin oxidases with acetylenic substrates, wherein the carbanion intermediate can lead to an allenic carbanion, which can then form a stable covalent adduct with the flavin group (*109*). Finally, it was noted that preformed nitroalkane carbanions, such as ethane nitronate, acted as substrates of D-amino acid oxidase (*110*). Thus three lines of experimental evidence were consistent with a carbanion mechanism in flavoenzymes such as D-amino acid oxidase.

To elucidate the mechanism in flavocytochrome b_2, a series of reactions similar to those mentioned above were carried out. Urban *et al.* (*111*) studied the reverse reaction in flavocytochrome b_2 and demonstrated that dehydrohalogenation did indeed occur with bromo- and chloropyruvate, but not with fluoropyruvate. A partition ratio of 500 was found for oxidation versus elimination during the forward reaction (i.e., 1 mol of pyruvate formed for every 500 mol of halopyruvate) compared with a partition ratio of 2 for the reverse reaction (*111*).

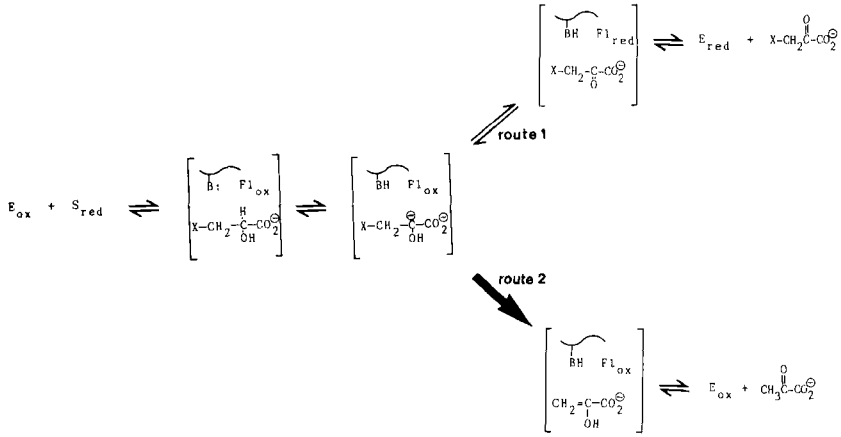


FIG. 10. Proposed mechanism for halosubstrate oxidation (route 1) or elimination (route 2) via a carbanion intermediate. The formation of the enzyme–substrate complex is followed by abstraction of the hydrogen at C-2 by the active site base. The carbanion intermediate can then undergo oxidation to form halopyruvate via route 1, or can eliminate halide to form pyruvate via route 2. E, Enzyme; S, substrate; Fl, flavin; B, active site base.

Figure 10 shows the proposed scheme that would explain halide elimination from a carbanion intermediate in flavocytochrome b_2.

If for α-hydroxyacid oxidation the C_α-H bond cleavage is rate limiting, then, according to the principle of microreversibility, formation of a C_α-H bond in the reduction of α-ketoacids (i.e., the reverse reaction) should also be rate limiting. Deuterium kinetic isotope effects have been measured for the reduction of β-halogenopyruvate by flavocytochrome b_2 that had previously been reduced with [2-^{2}H] lactate (*112*). A kinetic isotope effect of 4.4 was obtained, in 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 (*112*). This is explained by the fact that the carbanion intermediate can partition between an isotope-sensitive route (protonation/deuteration, route 1 in Fig. 10) and an isotope-insensitive route (elimination, route 2 in Fig. 10). This would certainly not be expected for a hydride mechanism.

When transhydrogenation reactions were carried out with [2-^{3}H] lactate and halogenopyruvate, tritium was found in water and on the resulting halogenolactate at the C-2 position (*112*). When the halogenopyruvate underwent halide elimination, tritium was found at the C-3 position (*112*). These results can be explained if the tritium resides

on a monoprotic active site base after it has been abstracted to form the carbanion intermediate. Exchange with this active site group was found to correlate with the level of free reduced enzyme (*112*).

Studies with the suicide inhibitor 2-hydroxy-3-butynoate (*103, 113*) have provided further support for a carbanion mechanism operating in flavocytochrome b_2. The 2-hydroxy-3-butynoate results in a carbanion intermediate that can resonate to an allenic carbanion, which can then form a covalent adduct with the flavin cofactor (Fig. 11). This adduct is highly reactive and cyclizes to form an inactive modified flavin group (*113, 114*).

With D-amino acid oxidase, one of the diagnostic tests for a carbanion mechanism was that ethane nitronate acted as a substrate (*110*). However, with flavocytochrome b_2 there is no evidence of electron transfer between ethane nitronate and the enzyme, rather this compound behaves as a competitive inhibitor (*96*). The reason why there is no electron transfer in the case of flavocytochrome b_2 is unclear, but one possibility is that the carbanion is not correctly oriented. This absence of electron transfer in no way disproves a carbanion mechanism. Indeed, other flavoenzymes, such as long-chain hydroxyacid oxidase also fail to utilize ethane nitronate (*115*). So, apart from the ethane nitronate result, there is substantial evidence to support a carbanion mechanism in flavocytochrome b_2. The question now is how do the electrons transfer to the oxidized FMN? There are essentially three possibilities: (1) there is a nucleophilic attack by the substrate carbanion at flavin N5, forming a covalent bond, with subsequent cleavage resulting in reduced flavin; (2) there is a one-electron transfer to the flavin followed by collapse of the radical pair to again form a

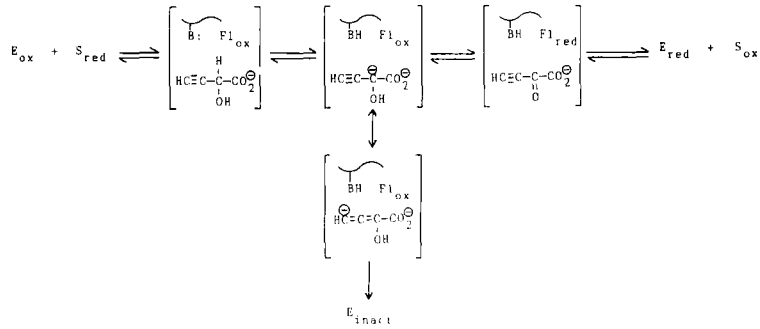


FIG. 11. Oxidation of the suicide substrate 2-hydroxybutynoate. Inactivation arises from nucleophilic attack at the flavin mononucleotide by the highly reactive allenic anion. E, Enzyme; S, substrate; Fl, flavin; B, active site base.

covalent adduct; or (3) there are two one-electron transfers with no covalent adduct formation. These possibilities are illustrated in Fig. 12. Differentiation between direct nucleophilic attack (A) and radical formation (B) of a covalent intermediate would be difficult if the collapse of the radical pair was fast.

By considering the above-mentioned solution studies and the refined three-dimensional structure of the *S. cerevisiae* flavocytochrome b_2 active site, Lederer and Mathews proposed a scheme for the reverse reaction (the reduction of pyruvate) (*39*). They did not discuss how the transfer of electrons took place except to say that the structure did not rule out the possibility of a covalent intermediate (*39*). Ghisla and Massey (*116*) considered the anionic flavin N5 to be too close to the pyruvate carbonyl (3.7 Å) without the formation of a covalent adduct taking place. Covalent intermediates between substrate and flavin have been observed for lactate oxidase (*117, 118*) and D-amino acid

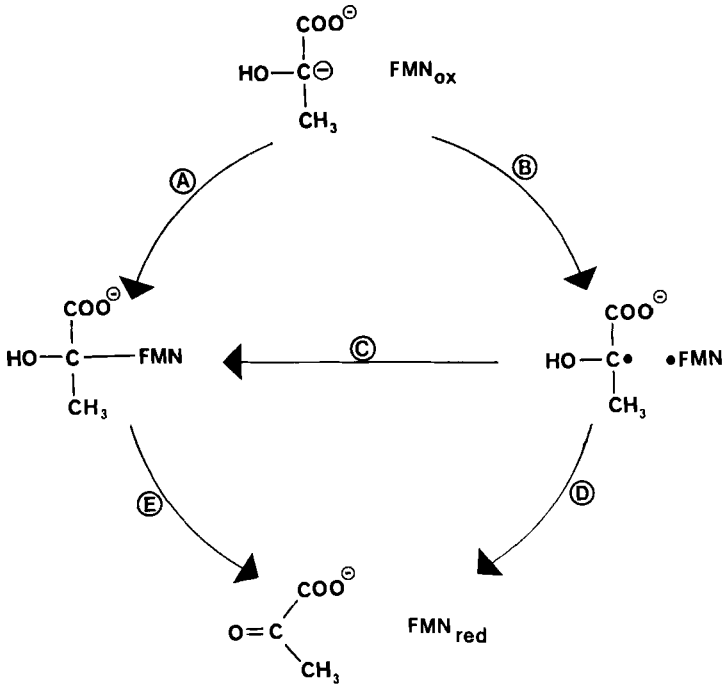


FIG. 12. Alternative routes of electron transfer from the substrate carbanion to the FMN. Oxidation of substrate can occur by a two-electron transfer via a covalent intermediate (A + E), by formation of a radical pair followed by a second one-electron transfer to give the covalent intermediate (B + C + E), and by formation of a radical pair directly followed by a second one-electron transfer to give reduced FMN (B + D).

oxidase (*110*), but to date there is no direct evidence for such an intermediate in flavocytochrome b_2. Even so, Ghisla and Massey (*116*) have proposed a mechanism in which a transient covalent intermediate (analogous to those seen with lactate oxidase and D-amino acid oxidase) is formed in flavocytochrome b_2.

At this stage it is worth considering a set of experiments, the results of which are inconsistent with a carbanion mechanism. The deazaflavins are a series of synthetic flavins in which one of the nitrogens has been replaced by a methylene bridge (*114, 119, 120*). The mechanism of flavocytochrome b_2 was probed with 5-deaza-FMN (*121*). The enzyme incorporated with 5-deaza-FMN was used to distinguish between hydride and carbanion mechanisms. If a covalent intermediate was formed along the reaction pathway, then a covalent bond with 5-deaza-FMN should be unable to undergo cleavage to yield products. Any incorporation of the C_α hydrogen onto the FMN would support a hydride rather than a carbanion mechanism. It was found that flavocytochrome b_2 reconstituted with 5-deaza-FMN was reducible by lactate (very slowly) and oxidizable by pyruvate. Furthermore, labeling studies with [2-^{3}H]lactate showed tritium incorporated onto the flavin analog (*121*), a result that would be consistent with hydride transfer. How can these observations be explained? Lederer and Mathews suggested that the enzyme may switch from a carbanion to a hydride mechanism when FMN is replaced by 5-deaza-FMN (*39*). It has also been suggested that deazaflavins should be regarded as analogs of flavin-shaped pyridine nucleotides and not actual flavins (*119, 122*). Whatever the explanation, the deazaflavins pose many problems as probes for those flavoenzymes thought to utilize a carbanion mechanism.

Taking an overall view of all of the solution studies mentioned above, and considering the three-dimensional structure of the enzyme, a mechanism for substrate oxidation/flavin reduction can be proposed and is shown in Fig. 13. In this mechanism a number of residues have specific substrate-binding and catalytic roles. His 373 is the active site base that abstracts the C_α hydrogen, and Asp 282 stablizes the resultant imidazolium ion. Tyr 254 facilitates electron transfer by abstracting the hydroxyl proton. Lys 349 stabilizes the anion at N1 of the reduced flavin. Arg 376 and Tyr 143 would appear to be involved in binding the substrate via electrostatic and hydrogen bonding interactions. All of these interactions are clearly shown in Fig. 13. In the mechanism as shown, the rate-limiting step is at k_2/k_{-2}. The existence of a covalent adduct (Fig. 13) is at this time conjecture and its supposed formation could equally well be considered as two one-electron transfer steps.

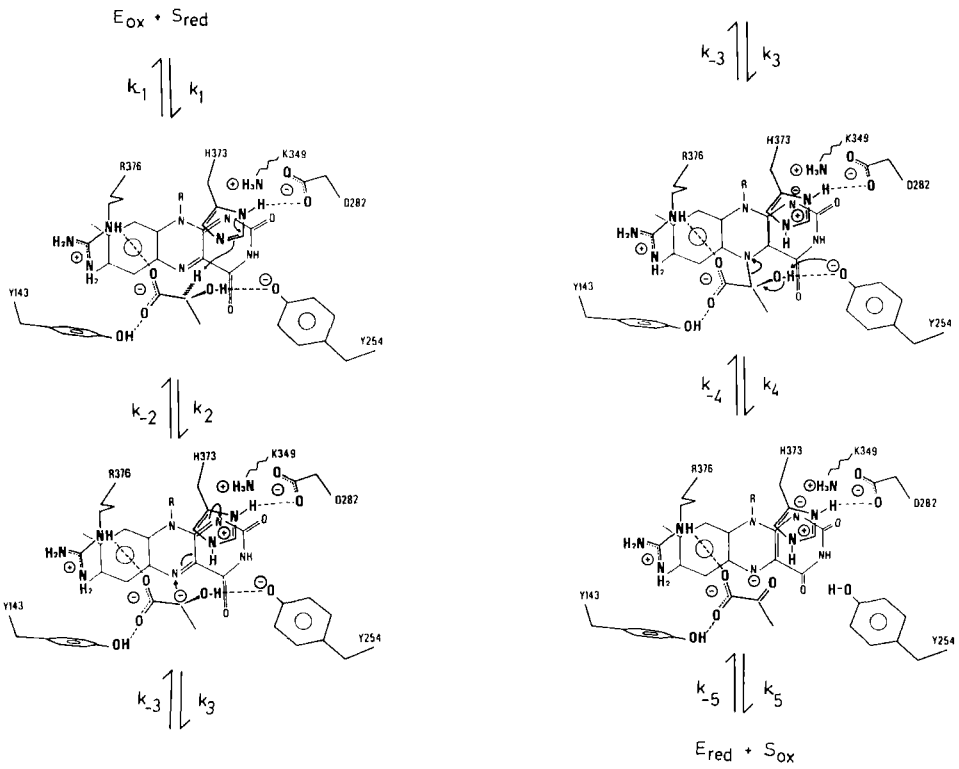


FIG. 13. Proposed mechanism of L-lactate oxidation by flavocytochrome b_2. The rate-determining step of the reaction is k_2/k_{-2}. Electron transfer from substrate carbanion to FMN is postulated as proceeding via a covalent intermediate; alternatives to this are shown in Fig. 12. E, Enzyme; S, substrate.

C. FURTHER STEPS IN THE ELECTRON TRANSFER PATHWAY

What are the electron transfer processes that occur after the initial flavin reduction? In 1975, Capeillère-Blandin *et al.* (*65*) carried out combined stopped-flow and EPR rapid-freezing experiments on cleaved flavocytochrome b_2. Simulation studies were used to derive an electron transfer scheme that would correlate with all the data (*123*). The time courses for FMN and heme reduction, which were biphasic in nature, were superimposable. The duration of phase 1 was 30–35 msec and accounted for approximately 85% of the total absorbance change observed. Phase 2 accounted for the remaining 15% and was some 20-fold slower than phase 1 (*65*). From EPR it was found that at the end of phase 1 up to 80% of the heme was reduced and up to 50% of the FMN

was flavosemiquinone, with 25–35% being flavohydroquinone. The electron distribution was equivalent to two electrons per protomer (*65*). Phase 2 corresponded to the entry of a third electron. A model scheme was proposed to account for these observations (Fig. 14) in which there was reversible FMN → heme electron transfer and an interprotomer electron transfer between two flavosemiquinones, which would allow the entry of a third electron pair per dimer, resulting in fully reduced flavocytochrome b_2 (*123*). Interprotomer electron transfer was also investigated by Pompon *et al.* (*104, 124*). The high isotope effect seen for the reduction of the enzyme with [2-^{2}H]lactate allowed heme and flavin reduction to be studied at a very low rate of electron entry. A model was proposed that allowed three variations of interprotomer electron transfer (*124*): (1) FMN → FMN, (2) FMN → heme, and (3) heme → heme. (For detailed schemes of these variations, see Fig. 1 of Ref. *124*). The Capeillère-Blandin and Pompon models agree on (1) kinetic rate constants for the initial reduction of flavin and heme, (2) reversible electron transfer between flavin and heme, and (3) that each protomer of the enzyme is a three-electron acceptor, but acts as a two-electron transferase during turnover. The main differences between the two models are (1) the initial FMN reduction is synchronized for any given dimer in the Capeillère-Blandin model, whereas Pompon assumes this step is random; (2) there is an order-of-magnitude difference in interprotomer electron transfer rates between flavosemi-

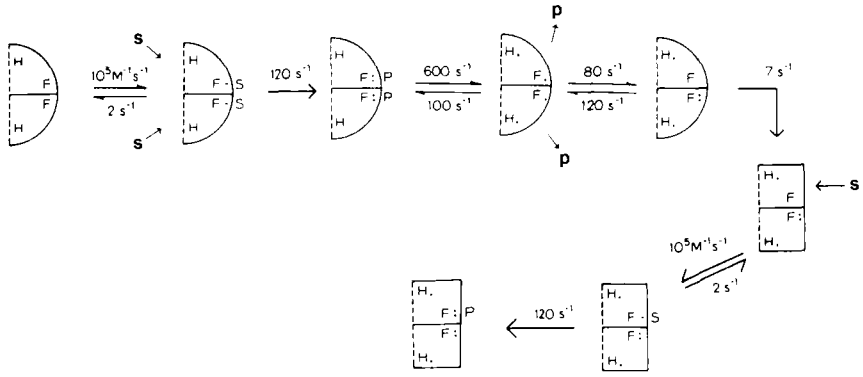


FIG. 14. A kinetic model (*123*) of electron transfer in flavocytochrome b_2. Each semicircle represents two of the four subunits of the enzyme during phase 1. Phase 1 corresponds to the entry of the first two electron pairs per dimer. After interprotomer electron transfer to give one fully reduced FMN and one flavosemiquinone, the dimer undergoes a conformation change (rectangles) to allow entry of the third electron pair per dimer (phase 2) to give fully reduced flavocytochrome b_2. H, Heme; F, FMN; S, substrate; P, product.

quinones; (3) only the Pompon model includes interprotomer electron transfer between heme pairs and FMN and heme; and (4) the slow phase-limiting step is a conformational change in the Capeillère-Blandin model, whereas Pompon interprets this as an interprotomer electron transfer. Thus the two models (*123, 124*) agree in the description of the steps involved in enzyme turnover but differ on the electron transfer steps that 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* because cytochrome *c* would always be available as an electron acceptor. It seems unlikely in view of the three-dimensional structure of the enzyme that interprotomer electron transfer from heme to heme would be possible, considering the distance involved (~52.8 Å), and this rules out some of the variations described in the Pompon model (*124*).

For *H. anomala* flavocytochrome b_2 there is a detectable lag phase between flavin and heme reduction, indicating that intramolecular electron transfer from FMN to heme is partly rate limiting (*66*). Values for rate constants (at 5°C) for phase 1 and phase 2 in both *H. anomala* and *S. cerevisiae* enzymes (*66*) are listed in Table VI. Using temperature-jump methods, Tegoni *et al.* (*125*) studied the electron transfer from flavosemiquinone to heme and determined a rate constant (at 16°C) of 160 sec^{-1}. This value was thought to correspond to the value of k_{cat}, which is 225 sec^{-1} (at 16°C) (*125*). Tegoni *et al.* (*125*)

TABLE VI

RATES OF ELECTRON TRANSFER FOR FLAVOCYTOCHROMES b_2 FROM *S. cerevisiae* AND *H. anomala*[a]

	FMN reduction rate constant (sec^{-1})		Heme reduction rate constant (sec^{-1})	
Enzyme[b]	Phase 1	Phase 2	Phase 1	Phase 2
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

[a] From Ref. *66*. Phase 1 is the fast electron transfer corresponding to the entry of the first two electron pairs per flavocytochrome b_2 dimer. Phase 2 corresponds to the entry of the third electron pair per dimer giving fully reduced flavocytochrome b_2. A lag phase of 1.3 ± 0.5 msec was observed for *H. anomala* flavocytochrome b_2 heme reduction. All values were determined at 5°C.

[b] H.a.b_2, *H. anomala* flavocytochrome b_2; S.c.b_2, *S. cerevisiae* flavocytochrome b_2.

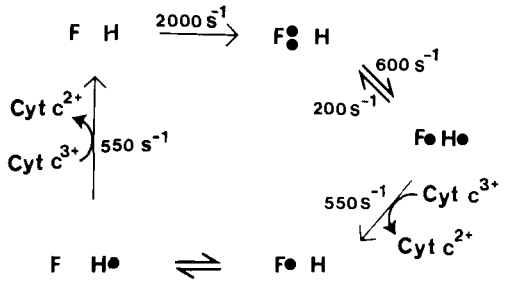


FIG. 15. Scheme of electron transfer in *H. anomala* flavocytochrome b_2. All constants were determined at 16°C (*125*). F, FMN; H, heme.

went on to describe the whole electron transfer scheme in *H. anomala* flavocytochrome b_2 and this is shown schematically in Fig. 15.

D. Electron Acceptors

1. Cytochrome c

As mentioned in Section V,A, heme-deficient (dehemo) flavocytochrome b_2 has no cytochrome *c* reductase activity (*126*), but retains some ferricyanide reductase activity. These results are consistent with b_2–heme being essential for cytochrome *c* reduction and also indicate that ferricyanide and cytochrome *c* are reduced by different mechanisms.

Cytochrome *c* is known to form a stable complex with flavocytochrome b_2 in solution and in the crystalline state (*32*, *127–130*). The complex stability is strongly dependent on ionic strength and pH, suggesting that the interaction is dominated by electrostatic effects. Baudras *et al.* (*128*, *129*) reported a flavocytochrome b_2 : cytochrome *c* heme ratio of 4 (i.e., one cytochrome *c* per flavocytochrome b_2 tetramer) in experiments with the cleaved *S. cerevisiae* enzyme both in solution and in cocrystals of the two proteins. Tegoni *et al.* (*127*) diffused cytochrome *c* through crystals of intact *S. cerevisiae* flavocytochrome b_2 and obtained a heme ratio of 1. Rates of electron transfer between flavocytochrome b_2 and a series of chemically modified cytochromes *c* have been measured and indicate that lysine residues on cytochrome *c* located near the exposed heme edge play a role in the interaction between the two proteins (*131*). However, finding the region on flavocytochrome b_2 where cytochrome *c* binds has yet to be accomplished. In a fluorescence study by Vanderkooi *et al.* (*132*), it was shown that the metalloporphyrin fluorescence in Zn(II)- and Sn(IV)-substituted cyto-

chromes *c* was affected by the flavocytochrome b_2 heme upon complexation. The changes in fluorescence allowed a distance of 18 Å between b_2 and *c* hemes to be calculated (*132*). Recently Janot *et al.* (*133*) have carried out rapid kinetic studies of electron transfer within the *H. anomala* flavocytochrome b_2–cytochrome *c* assembly. They estimated a flavocytochrome $b_2 \rightarrow$ cytochrome *c* electron transfer rate constant of 250 sec^{-1} (at 5°C) (*133*).

Cytochrome *c* has often been used as an external electron acceptor in steady-state experiments with flavocytochrome b_2 and usually gives rise to lower specific activities than would be found if ferricyanide was used as acceptor. The differences between cytochrome *c* and ferricyanide as electron acceptors are discussed further in the next section. Values of K_M for cytochrome *c* from steady-state measurements are very dependent on the nature of the buffer used, with values ranging from 10 μM (10 m*M* Tris–HCl, pH 7.5, I = 0.10 *M* NaCl) (*134*) to 180 μM (100 mM phosphate, pH 7.2) (*135*) seen with intact flavocytochrome b_2 from *S. cerevisiae*. It is perhaps not surprising that the K_M for cytochrome *c* is markedly affected in phosphate buffer considering the propensity for phosphate to bind to cytochrome *c* (*136*).

2. *Ferricyanide*

Ferricyanide is the most commonly used electron acceptor in steady-state kinetic experiments on flavocytochrome b_2. How is ferricyanide reduced by the enzyme? Ogura and Nakamura suggested that ferricyanide could accept electrons only from the b_2 heme (*79*). This is clearly incorrect, because dehemoflavocytochrome b_2 and the isolated flavodehydrogenase domain can still function as ferricyanide reductases, though at somewhat lower efficiency (*51*, *126*). These results imply that ferricyanide can accept electrons from both flavohydroquinone and flavosemiquinone as well as heme. In heme-free cleaved enzyme from *S. cerevisiae* it was calculated that ferricyanide was reduced around 20 times faster by flavosemiquinone than by flavohydroquinone (*126*). This would mean that in the holoenzyme, reduction of ferricyanide would occur rapidly from heme and flavosemiquinone. The fact that ferricyanide is reduced by both b_2 heme and flavosemiquinone, and that cytochrome *c* is reduced only by b_2 heme, might be an explanation for the observation that specific activities of the enzyme determined with cytochrome *c* are usually somewhat lower than those determined with ferricyanide.

Steady-state kinetic experiments indicate that the rate of ferricyanide reduction by the enzyme is essentially independent of ferricyanide concentration in the range 0.2–3.0 m*M*. This is another reason

why ferricyanide is ideal as an electron acceptor. Apparent K_M values for ferricyanide have been reported to be 0.15 m*M* with intact *S. cerevisiae* enzyme (*135*) and 0.1 m*M* with the *H. anomala* enzyme (*50*).

VI. Protein Engineering Studies

The ability to design and construct altered forms of enzymes has been extremely useful in furthering our understanding of enzyme catalysis, structure, and stability (*137–142*). The technology for protein engineering is now commonplace, but its application is dependent upon the availability of the DNA that encodes the protein to be altered, a high-resolution three-dimensional structure of the protein, and a system allowing expression and isolation of altered forms. Each of these conditions has been met in the case of *S. cerevisiae* flavocytochrome b_2, and several mutant enzymes have already proved to be extremely valuable in furthering our understanding of the relationship between structure and function in this enzyme (*143–145*).

A. The Flavocytochrome b_2 Genes

The *S. cerevisiae* gene encoding flavocytochrome b_2 was isolated independently in two laboratories (*27*, *146*) using antibodies directed against flavocytochrome b_2 to detect expression of antigenic material in *Escherichia coli* transfected with a λgt11 clone bank of yeast genomic DNA fragments. The isolated gene has been engineered to allow transfer of the flavocytochrome b_2 coding region between different cloning vectors (*143*, *146*), in particular by introducing unique restriction enzyme cleavage sites at either end, without modification to the coding region. This has been valuable in, for example, transferring mutated forms of the coding sequence, from vectors designed for *in vitro* mutagenesis and sequence determination, into vectors directing expression of the enzyme in either yeast (*143*) or *E. coli* (*147*).

B. Expression of Cloned Flavocytochrome b_2

1. In Yeast

Saccharomyces cerevisiae is quite commonly used as a host for expression of proteins from cloned genes from yeasts and other organisms. Because flavocytochrome b_2 is a yeast enzyme and procedures for isolation of intact enzyme from yeast had already been developed, Reid *et al.* (*143*) developed a system for expression of *S. cerevisiae* flavocy-

tochrome b_2 in yeast at relatively high levels using a multicopy plasmid with expression directed by the promoter from the alcohol dehydrogenase (ADH1) gene. Although this resulted in substantially increased yields of enzyme compared with native enzyme from non-engineered yeast, the level of expression appeared to be limited because of the need to transport flavocytochrome b_2 into mitochondria. When a modified form of the gene, encoding only the mature polypeptide (i.e., lacking the mitochondrial targeting sequence), was expressed in yeast, the level of expression increased further (*148*). The yeast expression system has now been superseded by a considerably more efficient system allowing expression of fully active flavocytochrome b_2 in *E. coli* (*147*).

2. *In Escherichia coli*

The *E. coli* harboring a plasmid, pDS-b_2 (Fig. 16), designed for *in vitro* transcription and translation, were noticeably pink in color (*147*). This results from constitutive expression of flavocytochrome b_2 at up to 5% of the total soluble protein in these cells. Expressed enzyme contains full stoichiometric amounts of FMN and heme (*147*). Expression of flavocytochrome b_2 *in vivo* was not expected with this vector because there is no *E. coli* ribosome-binding site (rbs). It appears that a fortuitous rbs exists within the region of DNA encoding the mitochondrial targeting sequence and this leads to initiation of translation at Met 6 of the mature flavocytochrome b_2 (*147*). The absence of residues 1–5

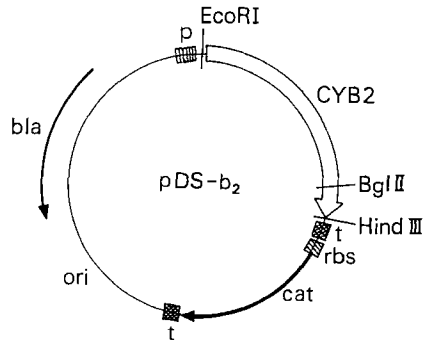


FIG. 16. Map of the plasmid used for expression of flavocytochrome b_2 in *Escherichia coli*. The flavocytochrome b_2 coding region (CYB2) is located between a strong *E. coli* promoter (p) and terminator (t). Selection is based on ampicillin resistance conferred by the β-lactamase gene (bla). *Eco*RI, *Bgl*II, and *Hind*III cleavage sites are indicated; ori, origin of replication; cat, chloramphenicol acetyltransferase coding sequence; rbs, ribosome-binding site.

TABLE VII

COMPARISON OF KINETIC PROPERTIES FOR OXIDATION OF L-LACATE BY WILD-TYPE *S. cerevisiae* FLAVOCYTOCHROME b_2[a]

Enzyme source	k_{cat} (sec^{-1})	K_M (m*M*)
Yeast	192 ± 5	0.49 ± 0.03
E. coli	200 ± 10	0.49 ± 0.05

[a] As isolated from yeast or after expression in *E. coli*. All constants were determined from steady-state kinetic measurements at 25°C, 10 m*M* Tris–HCl, pH 7.5, I = 0.10 *M* NaCl. Ferricyanide (1 m*M*) was used as electron acceptor. Values of k_{cat} correspond to the number of moles of L-lactate oxidized per mole of enzyme per second (these values can be doubled to express them in mole electron equivalents).

appears to have no effect on the spectroscopic and kinetic properties of the enzyme (Table VII).

It is fortunate that *E. coli* responds to expression of *S. cerevisiae* flavocytochrome b_2 by synthesizing sufficient FMN and heme to yield fully active enzyme. This capacity is apparently not infinite because attempts to express the enzyme at higher levels using a temperature-inducible λP_L promoter have resulted in the enzyme polypeptide constituting up to 30% of total cell protein, but this was found to be almost exclusively as inclusion bodies and was devoid of lactate dehydrogenase activity (*149*). Expression of flavocytochrome b_2 substantially reduces growth rate in *E. coli* such that transformants must be continually selected for efficient expression; mutations leading to loss of expression confer a strong selective advantage. The rate of growth and yield of enzyme from *E. coli* transformants appear to be related to enzyme activity; mutant forms with low activity have less effect on growth and give higher yields than do forms with higher activity (*150*).

C. SITE-DIRECTED MUTAGENESIS

Several point mutations in the *S. cerevisiae* flavocytochrome b_2 coding sequence have been constructed by oligonucleotide-directed site-specific mutagenesis of the cloned gene (*143*). The enzyme-coding region was transferred to a plasmid designed both for expression of

cloned DNA in yeast and for production of single-stranded DNA. This latter property was used to generate a single-stranded template for mutagenesis and for DNA sequence analysis of mutated clones. Mutations have been introduced by site-directed mutagenesis using chemically synthesized oligonucleotides (*143*) with mutated genes completely sequenced to ensure no secondary mutations had been introduced. Expression of mutant forms of the enzyme in yeast (*143*) or *E. coli* (*147*) has allowed isolation of substantial quantities of enzyme with a typical yield from *E. coli* of 50 mg from a 10-liter culture.

D. Characterization of Mutant Flavocytochromes b_2

Each of the mutant forms of flavocytochrome b_2 constructed in our laboratory has been purified and subjected to steady-state kinetic analysis. This simple first approach has often given us, very quickly, an impression of the mechanistic effects of the mutation, but perhaps more importantly has dictated which mutant enzymes deserved further characterization and in which way they should be analyzed.

1. Arg 376 and Lys 349

The R376K mutant (Arg 376 → Lys) was constructed to retain the positive charge of this residue, which binds the substrate carboxylate. Because the lysine side chain is shorter than that of arginine, it was expected that the substrate would no longer be optimally positioned for proton abstraction and electron transfer and this may account for the greatly decreased activity (see Table VIII). It is interesting to compare this result with those from studies of a nicotinamide-linked dehydrogenase in which replacement of Arg 171 (similar in function to Arg 376 in flavocytochrome b_2) by Lys resulted in a k_{cat} value some 10^4-fold lower (*151*). In this enzyme the interaction between Arg 171 and the substrate carboxylate involves the two terminal nitrogens of the guanidinium group and is symmetrical (*152*). This is in contrast with flavocytochrome b_2, in which the interaction involves the δ nitrogen and one of the terminal nitrogens of Arg 376; the distance between the latter and the carboxylate oxygens appears to preclude a hydrogen bond. Because the interaction between Lys 376 (in the mutant enzyme) and the carboxylate negative charge would not exactly match that of Arg 376, one would not be surprised by weaker binding and lowered activity because of a less favorable substrate orientation.

Substitution of Lys 349, which stabilizes the anion at N1 of the reduced FMN, by Arg resulted in an enzyme with only very low residual activity [this activity was too low to be detected when the enzyme

TABLE VIII

KINETIC PROPERTIES OF WILD-TYPE AND MUTANT FLAVOCYTOCHROMES b_2 FOR THE OXIDATION OF L-LACTATE TO PYRUVATE[a]

Enzyme	k_{cat} (sec^{-1})	K_M (mM)	Comments
Wild type	200 ± 10	0.49 ± 0.05	—
Tyr254 → Phe	6.5 ± 0.5	0.51 ± 0.05	Kinetic isotope effect identical to wild type
Tyr143 → Phe	230 ± 20[b]	0.60 ± 0.10	FMN → heme electron transfer appears to be rate limiting
TD-b_2 (Gly 489 → STOP)	165 ± 6	0.96 ± 0.06	Enzyme looses FMN during turnover
Ala 306 → Ser	160 ± 3	0.83 ± 0.04	—
Tyr 97 → Phe	210 ± 10	0.46 ± 0.03	Marked substrate inhibition is observed
Phe 325 → Ala	96 ± 5	0.87 ± 0.05	—
Arg 376 → Lys[c]	~2	~70	—[d]
Lys 349 → Arg[c]	—	—	Very low residual activity[d]

[a] All constants were determined at 25°C, 10 mM Tris–HCl, pH 7.5, I = 0.10 M NaCl. Ferricyanide (1 mM) was used as electron acceptor unless otherwise stated. Values of k_{cat} are expressed as moles of L-lactate oxidized per mole of enzyme per second. All enzymes were isolated from *E. coli* except the Ala 306 → Ser mutant enzyme, which was isolated from yeast.

[b] The Tyr 143 → Phe mutant enzyme shows a marked rate dependence on ferricyanide, therefore this value refers to that obtained at saturating ferricyanide concentrations.

[c] No activity was found for these mutant enzymes extracted from yeast (*143*).

[d] C. S. Miles, unpublished results.

was originally extracted from yeast (*143*)]. Although this substitution conserves positive charge, it also increases bulk in a tightly packed region close to the FMN, raising the possibility that FMN binding might be altered. The K349R mutant enzyme isolated from *E. coli* has been shown to contain FMN (*147*); however, the possibility of gross structural changes around this cofactor cannot be excluded.

2. *Tyr 254*

Tyr 254 is an active site residue (Section III,D, Fig. 6) that interacts with the hydroxyl group of L-lactate (and keto group of pyruvate). Based on the active site structure, Lederer and Mathews (*39*) proposed that the Tyr 254 hydroxyl group forms a hydrogen bond to the substrate O2 throughout the catalytic cycle and facilitates electron transfer from substrate carbanion to FMN by deprotonating the substrate hydroxyl group. To probe the function of the Tyr 254 hydroxyl, Reid *et*

al. (*143*) constructed a mutant enzyme in which Tyr 254 was replaced by Phe (Y254F), retaining the steric bulk and aromatic character of the side chain but removing the hydroxyl function. Preliminary studies of the mutant enzyme isolated from yeast revealed a k_{cat} some 40- to 50-fold lower than that seen for the wild-type enzyme (4.3 sec^{-1} for Y254F versus 190 sec^{-1} for wild type, at 25°C, 10 m*M* Tris–HCl, pH 7.5, $I = 0.10$ *M* NaCl). The K_M for lactate, however, was close to that of the wild-type enzyme (0.54 m*M* for Y254F versus 0.49 m*M* for wild type, conditions as above) (*143*). This latter result was somewhat surprising, because the Michaelis complex was thought to be stabilized by hydrogen bond formation between Tyr 254 and the L-lactate α-hydroxyl group. The importance of hydrogen bond formation is further indicated by the finding that propionate, which lacks the hydroxyl group, is a competitive inhibitor with a K_i value of 28 m*M* (*96*), implying a contribution to substrate binding by the hydroxyl group of around 10 kJ/mol. Because K_M for L-lactate is dependent on a number of kinetic parameters, interpretation of effects on its value is not simple, particularly because several distinct electron transfer steps are involved in the catalytic cycle of the enzyme.

It was considered that the rate-limiting step, α-hydrogen abstraction in the wild-type enzyme, might have been altered by the Y254F substitution. However, the steady-state kinetic isotope effect with [2-^{2}H] lactate seen for the mutant enzyme was identical to that for the wild-type enzyme, indicating that the rate-limiting step is unaltered (*145*).

Lederer and colleagues (*145*) investigated the possibility that removal of the Tyr 254 hydroxyl leads to hydrogen bond formation between the substrate hydroxyl and an alternative active site group. They concluded that His 373 was the only alternative hydrogen-bonding partner. An alternative orientation of the substrate, with the hydroxyl hydrogen bonded to N3 of His 373, could be achieved by simply rotating the C2 substituents around the C1–C2 axis and appeared to indicate the possibility for a switch to a hydride mechanism. The possibility that the Y254F change had induced a change in mechanism was probed using methods already outlined in Section V. The mutant enzyme behaved similarly to the wild type in that (1) it was inactivated by 2-hydroxybutynoate, (2) it catalyzed the dehydrohalogenation of bromopyruvate under transhydrogenase conditions, (3) it catalyzed intermolecular hydrogen transfer between [2-^{2}H] lactate or [2-^{3}H] lactate and bromopyruvate (*145*). It can be concluded then that the Y254F enzyme, like the wild type, operates via a carbanion mechanism.

This still leaves the lack of an effect on the K_M by the Y254F mutant enzyme unexplained. It has been suggested (*145*) that energetic com-

pensation for the loss of the Tyr 254–substrate hydrogen bond in the mutant enzyme might come from an interaction between the substrate O2 and the aromatic ring of Phe 254. Associated reorientation of the substrate would be expected to reduce the propensity for carbanion formation and might explain the decrease in k_{cat}. This possibility will be tested by characterization of a Tyr 254 → Leu mutant enzyme.

3. Tyr 143

In subunit 1 (pyruvate absent) the Tyr 143 hydroxyl is hydrogen bonded to a heme proionate, whereas in subunit 2 it is hydrogen bonded to the pyruvate carboxylate group (Fig. 6). The possible pivotal role of Tyr 143 in substrate recognition and in communication between flavodehydrogenase and heme domains has been investigated by substituting this residue with Phe (*153*). Determination of kinetic isotope effects with deuterolactate by both steady-state and rapid kinetic methods has demonstrated that FMN-to-heme electron transfer appears to be the major rate-determining step in the Y143F mutant enzyme and not C_α hydrogen abstraction as seen in the wild-type enzyme. Miles *et al.* (*153*) have observed that the rate of ferricyanide reduction by the Y143F mutant enzyme shows a clear dependence on ferricyanide concentration, which is in marked contrast to the wild-type enzyme, which is essentially independent of ferricyanide concentration greater than 0.2 m*M*. This ferricyanide dependence has been interpreted as arising from an increased contribution to the overall rate of reaction by electron transfer from flavohydroquinone to ferricyanide due to the shift in rate-limiting step; this is illustrated schematically in Fig. 17. Further characterization of the Y143F mutant enzyme is in progress.

4. The Role of the C-Terminal Tail

The extreme C-terminus of each subunit of flavocytochrome b_2 contains a 23-residue tail that wraps around the center of the tetramer, making successive contacts with each of the other subunits (Figs. 3 and 5). It would appear then that the tail may be primarily responsible for maintaining quaternary structure. To test this possibility and with the aim of constructing a monomeric form of the enzyme (which would simplify kinetics by abolishing interprotomer electron transfer), White *et al.* (*144*) constructed a mutant enzyme in which the C-terminal 23 residues were absent. This was achieved by introducing a stop codon in place of Gly 489. The isolated mutant enzyme was found to have a subunit molecular weight of 55 kDa from SDS–PAGE compared to 57.5 kDa for wild-type enzyme (*144*). This tail-deleted enzyme (TD-b_2)

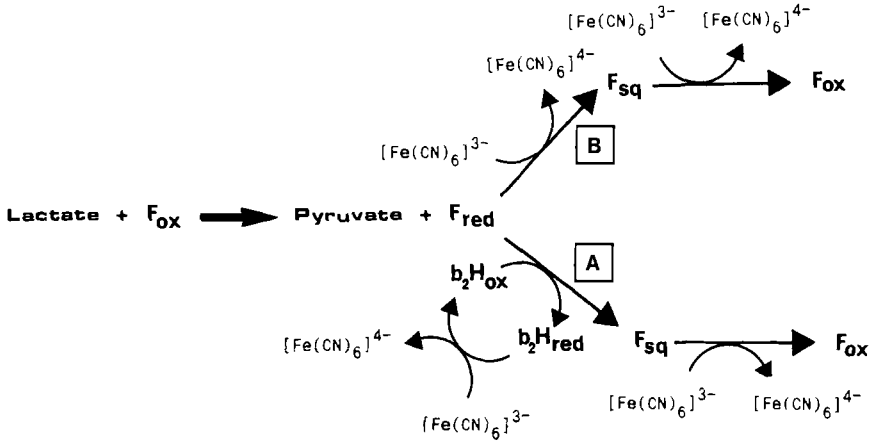


FIG. 17. Scheme describing electron transfer in the Tyr 143 → Phe mutant flavocytochrome b_2. In wild-type enzyme, route A is rapid. In the Y143F mutant enzyme, however, route A is substantially slowed down and it is suggested that route B becomes important. A significant contribution to the overall electron transfer rate from route B would explain the marked dependence of reaction rate on ferricyanide concentration, because electron transfer to ferricyanide is fast from heme and flavosemiquinone but is slow from flavohydroquinone.

was not, however, monomeric in solution, but behaved on gel filtration as a tetramer eluting immediately after the wild-type enzyme as expected (*144*). Thus it appears that although the C-terminal tail makes a substantial contribution to intersubunit contacts, there must be additional interactions between the bodies of the flavodehydrogenase domains to maintain the tetrameric structure.

Kinetic analysis of TD-b_2 under steady-state conditions with ferricyanide as electron acceptor showed that k_{cat} and K_M values for L-lactate (165 sec^{-1} and 0.96 m*M*, at 25°C, 10 m*M* Tris–HCl, pH 7.5, I = 0.10 *M* NaCl) were not dramatically different from the values determined for wild-type enzyme (200 sec^{-1} and 0.49 m*M*, conditions as above) (*144*). However, the behavior of TD-b_2 under assay conditions was unusual in that the rate of lactate oxidation decreased long before either L-lactate or ferricyanide became depleted. The decrease in reaction rate occurred as a biphasic process, leading eventually to complete loss of activity. This deactivation of TD-b_2 was only observed under turnover conditions. Deactivation was independent of ferricyanide and was also observed when cytochrome *c* was used as electron acceptor (*150*).

The faster of the two deactivation phases was dependent on L-lactate concentration, whereas the slower phase showed no such dependence. The first-order rate constants for deactivation at saturating L-lactate

concentrations (25°C, 10 m*M* Tris–HCl, pH 7.5, I = 0.10 *M* NaCl) were 0.029 and 0.01 sec $^{-1}$ for the fast ($k_{f(deact)}$) and slow ($k_{s(deact)}$) phases, respectively (*144*). It was noted that deactivation of TD-b_2 during turnover was associated with loss of FMN. FMN loss was followed spectrofluorimetrically and was found to be independent of L-lactate concentration, with a first-order rate constant of 0.012 sec^{-1} (conditions as above). Thus loss of FMN appears to be associated with the slow phase of deactivation. Inactive, FMN-deficient TD-b_2 could be reactivated by reconstitution with excess FMN. Activity was restored up to 60% of the original value, with the reconstituted enzyme undergoing deactivation as before.

White *et al.* (*144*) suggested that the biphasic deactivation of TD-b_2 could be explained by an initial substrate-dependent fast phase that leads to a partially deactivated, conformationally altered enzyme (E_c). The conformation adopted would then allow dissociation of FMN in a substrate-independent manner leading to completely inactive enzyme (E_I):

$$E \xrightarrow{k_f} E_c \xrightarrow{k_s} E_I + FMN$$

It is, of course, possible that the initial fast phase is reversible. It was concluded that removal of the C-terminal tail allows an active-site conformation to occur during turnover, in which the affinity for FMN is decreased, allowing the cofactor to dissociate from the enzyme. Thus the C-terminal tail is not essential for tetramer formation but appears to act as a conformational "anchor," exerting an effect on the structure of the enzyme in regions distant from it, particularly the FMN active site.

5. *Further Amino Acid Substitutions*

The work described above illustrates the immense value of the protein engineering approach to molecular enzymology. Further point mutants of flavocytochrome b_2 have also shown interesting, but rather less dramatic, results. Values of k_{cat} and K_M for the various mutant flavocytochromes b_2 (together with additional information) are compared in Table VIII.

Substitution of Ala 306, a residue in the proteinase-sensitive loop of *S. cerevisiae* flavocytochrome b_2, by Ser results in small but significant changes in k_{cat} and K_M for L-lactate (*143*) (Table VIII). Thus, a rather minor alteration to a surface residue quite remote from the active site has an effect on lactate dehydrogenation. The result is similar to that obtained by proteolytic cleavage of the region in which the substitution

was made. Presumably the mobility of this proteinase-sensitive loop is altered by the introduction of Ser, which provides the possibility of hydrogen bonding. Properties of further mutant enzymes, such as Tyr 97 → Phe and Phe 325 → Ala, which are still undergoing detailed analysis, are described in Table VIII.

E. Independent Expression of the Domains of Flavocytochrome b_2

By introducing a termination codon immediately after the Gly 100 codon of the *S. cerevisiae* flavocytochrome b_2 coding region, it has been possible to express the cytochrome b_2 core with the pDS-b_2 vector that was used for efficient expression of the holoenzyme in *E. coli*. This has provided high yields of the core domain, which has been used for biochemical and biophysical characterization, e.g., by high-field NMR (see Section IV, B). A procedure for independent expression of the flavodehydrogenase domain in *E. coli* has also been devised (*154*). An ATG initiation codon was introduced immediately preceding the codon for Gly 101 along with a restriction enzyme cleavage site to allow the flavodehydrogenase coding region to be transferred to an expression vector, pRC23 (*155*), under the control of the λP_L promoter. Expression of the flavodehydrogenase domain, in an *E. coli* host expressing the cI repressor, was induced by a shift from 30 to 37°C. This resulted in high yields of active protein. The isolated flavodehydrogenase domain is an efficient L-lactate : ferricyanide oxidoreductase but is unable to reduce cytochrome *c*. It is also unable to transfer electrons to the cytochrome b_2 core at a detectable rate, indicating the importance of the covalent linkage of the two domains for efficient interaction.

VII. Future Directions

It is quite clear that protein engineering will contribute substantially to future investigations of electron transfer in flavocytochrome b_2. To date protein engineering has been used to generate a number of single amino acid substitutions and has allowed the independent expression of the two functionally distinct domains of the enzyme. These two approaches can be readily combined, for example, to express the flavodehydrogenase domain with an active site mutation, thereby simplyfing analysis of electron transfer to FMN without interference from the cytochrome domain.

The two flavocytochromes b_2 that have been characterized to date, namely, from *H. anomala* and *S. cerevisiae,* have undergone consider-

able divergence but retain 60% sequence identity. Because the kinetic properties of these two enzymes are different in several respects, it is of interest to relate structural differences to function. Because both genes have been isolated and sequenced (*27*, *37*), it will be possible to engineer hybrid enzymes in which part of one flavocytochrome b_2 is substituted by the corresponding region from the enzyme from the other species. This could be done, for example, to transfer whole domains or to replace a surface loop. Characterization of such hybrid enzymes should help to elucidate the functional importance or otherwise of particular structural elements.

Protein engineering is of very wide interest, offering the potential to provide new or "better" enzymes. As we begin to understand the process of molecular recognition and catalysis in flavocytochrome b_2 in more detail, we should increase the scope for rational redesign of this enzyme.

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