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Extreme pK_a displacements at the active sites of FMN-dependent α-hydroxy acid-oxidizing enzymes

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Abstract

Flavocytochrome b_2 (or L-lactate dehydrogenase) from baker's yeast is thought to operate by the initial formation of a carbanion, as do the evolutionarily related α-hydroxy acid-oxidizing FMN-dependent oxidases. Previous work has shown that, in the active site of the unligated reduced flavocytochrome b_2, the group that has captured the substrate α-proton has a high pK_{app}, calculated to lie around 15 through the use of Eigen's equation. A detailed inspection of the now known three-dimensional structure of the enzyme leads to the conclusion that the high pK_a belongs to His 373, an active site group that plays the role of general base in the forward reaction and of general acid in the reverse direction. Moreover, consideration of the kinetics of proton transfer during the catalytic cycle suggests that the pK_a of the reduced FMN N5 position should be lowered by several pH units compared to its pK_a of 20 or more when free. The features of the three-dimensional structure possibly responsible for these pK shifts are analyzed; they are proposed to consist of a network of hydrogen bonds with the solvent and of a mutual electrostatic stabilization of anionic reduced flavin and the imidazolium ion. Finally, it is suggested that similar pK shifts affect the active sites of the α-hydroxy acid-oxidizing flavooxidases, which are homologous to flavocytochrome b_2. The functional significance of these pK shifts in terms of catalysis and semiquinone stabilization is discussed.

Keywords: α-hydroxy acid oxidation; flavocytochrome b_2; flavoenzymes; ionization constants; proton transfer

Flavocytochrome b_2 (L-lactate cytochrome *c* oxidoreductase, EC 1.1.2.3), a tetrameric bifunctional enzyme carrying one FMN and a protoheme IX on each subunit, catalyzes the oxidation of lactate to pyruvate (Appleby & Morton, 1954; Jacq & Lederer, 1974). The two-domain structure of the *Saccharomyces cerevisiae* protein shows a $\beta_8\alpha_8$ barrel fold for the flavodehydrogenase domain (Xia et al., 1987; Xia & Mathews, 1990), which is also present, among other proteins, in spinach glycolate oxidase (short chain L-α-hydroxy acid oxidase isozyme A, EC 1.1.3.15) (Lindqvist & Brändén, 1985; Lindqvist, 1989). Amino acid sequence data (Lederer et al., 1985; Cederlund et al., 1988; Black et al., 1989; Risler et al., 1989) show that these two proteins belong to a family of FMN-dependent α-hydroxy acid-oxidizing enzymes, which also includes long chain L-α-hydroxy acid oxidase (isozyme B) from higher organisms (EC 1.1.3.15) (Urban et al., 1988; Lê & Lederer, 1991), lactate oxidase from *Mycobacterium smegmatis* (L-lactate 2-monooxygenase EC 1.13.12.4) (Giegel et al., 1990), and mandelate dehydrogenase from *Pseudomonas putida* (Tsou et al., 1990). Reoxidation of the cofactor occurs for the oxidases at the expense of oxygen with formation of H_2O_2, whereas for flavocytochrome b_2 it occurs by intramolecular single electron transfer to heme b_2. On the other hand, for those members that are well studied, substrate dehydrogenation is believed to follow essentially the same course, namely substrate α-proton abstraction by an enzyme base, followed by electron transfer from the ensuing carbanion to FMN (Ghisla, 1982; Urban & Lederer, 1985; Urban et al., 1988). The so-called carbanion mechanism was initially based on experiments with 2-hydroxy-3-halogeno carboxylic acids (or 2-amino-3-halogeno acids as substrates for evolutionarily unrelated D-amino acid oxidase) and with 2-hydroxy-3-butynoate (for review see Ghisla, 1982). Although these studies did not strictly exclude the possibility of hydride transfer, such a mechanism was ruled out, at least for flavocytochrome b_2, by comparative deuterium isotope effect measurements on the reverse reaction (see below) and on the dehydrohalogenation reaction undergone by 3-bromopyruvate (Urban & Lederer, 1985). On

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the other hand, the nature of the electron transfer step from carbanion to flavin is still under debate. Two possibilities have been advocated: formation of a covalent intermediate between carbanion and flavin (for review see Ghisla, 1982) or two single electron transfer steps in quick succession (Bruice, 1980). Although two special cases of covalent intermediate formation have been reported (Porter et al., 1973; Massey et al., 1980), arguments against the formation of a covalent intermediate in the case of flavocytochrome b_2 are presented in Lederer (1991a).

Most members of the family have been shown to catalyze a nonphysiological reverse reaction, namely reduction by the reduced enzyme of α-keto acids to α-hydroxy acids (Massey et al., 1980; Urban et al., 1983, 1988; Lederer, 1984). The case of flavocytochrome b_2 was studied in particular detail. In the presence of [2-^{3}H]lactate and a halogenoketo acid, the enzyme catalyzes an intermolecular hydrogen transfer between the two compounds, which leads to formation of a 3-halogeno[2-^{3}H]lactate (Urban & Lederer, 1985). When extrapolated to infinite substrate concentration, transfer is total, indicating that the abstracted substrate α-proton never resides on a heteroatom carrying more than one proton. Experiments at varying keto acid concentrations led to a value of 2 s^{-1} for the first order rate constant for exchange with the solvent of the protein-bound substrate α-proton (Urban & Lederer, 1985), the solvent being 100 mM phosphate buffer, pH 7. Assuming buffer catalysis, this leads to a second order rate constant for exchange on the order of 50 $M^{-1} \cdot s^{-1}$. Using Eigen's equation (Eigen, 1964) one can derive from this figure a pK_a of around 15, which would be the ionization pK_a of the protein-bound substrate α-proton, assuming it were freely accessible to solvent. This value is much higher than that of any normal amino acid side chain and much lower than that of the N5 hydrogen bond in free reduced FMN. Indeed, the latter has been ascribed values of between 19 and 28 (Venkataram & Bruice, 1984; Ghisla et al., 1991).

At the time these results were obtained, the three-dimensional structure of the enzyme was still unknown. It was suggested that the pK_a of ~15 belonged to the active site base of the reduced enzyme, which plays the role of a general acid in the reverse reaction and, in order to rationalize this extreme pK shift, that the protonated side chain formed a strong hydrogen bond with another group, possibly the reduced flavin N5 position (Urban & Lederer, 1985). When the refined three-dimensional structure of the enzyme was published (Mathews & Xia, 1987), His 373 was identified as the active site base (Lederer & Mathews, 1987). The possibility that it was indeed the group with the high pK_a was briefly discussed then as well as in subsequent papers (Lederer, 1991a,b). The present publication analyzes in more detail the topography of the active site, which seems to make it impossible for His 373 to yield its proton to any other protein group and therefore concludes that this residue undergoes an important upward pK shift upon flavin reduction. In addition arguments are presented that support the idea that the pK_a of the FMN N5 position is markedly lowered in the reduced enzyme compared to free reduced flavin. It is suggested that the pK shift undergone by His 373 importantly contributes to catalysis of electron transfer, and that a similar shift also occurs during the reaction cycle in flavooxidases, which are evolutionarily related to flavocytochrome b_2.

Results and discussion

Does the substrate α-proton reside on His 373 while enzyme-bound?

The refined crystal structure of flavocytochrome b_2 from baker's yeast yields the picture of the active site in two different redox states (Xia & Mathews, 1990). In one subunit, a pyruvate molecule forms hydrogen bonds with a number of side chains close to the flavin, which is in the semiquinone state. In the other subunit, the cofactor is reduced and the ligand is replaced by a number of fixed water molecules that form a hydrogen bond network with one another and with side chains.

Based on these data and on previous mechanistic results, a role was assigned to the active site residues in lactate dehydrogenation (Fig. 1) (Lederer & Mathews, 1987; Lederer, 1991b). This scheme was subsequently accepted in its broad outline for glycolate oxidase (Lindqvist & Brändén, 1989) and lactate oxidase (Ghisla & Massey, 1989, 1991a,b; Giegel et al., 1990). Its validity is now being examined using site-directed mutants in combination with molecular graphics (Reid et al., 1988; Dubois et al., 1990; Lederer, 1991a). According to the proposed mechanism, in the forward direction His 373 acts as the general base, which abstracts the substrate α-hydrogen. In order to do this, it must be neutral in the oxidized enzyme, and its pK_a could be that of a normal imidazole side chain. When the reduced enzyme catalyzes the reverse reaction, His 373 plays the role of a general acid, therefore its pK_a must be raised at some point during the reaction. Thus, is His 373 the residue that has an apparent pK_a of about 15, the side chain from which exchange occurs with the solvent in the unligated reduced active site?

The answer is yes if one can show that His 373 cannot yield the acquired proton to another group in the active site, from which it would have to be totally transferred back during the reverse reaction. For example, it was suggested that the His 373 N^{ϵ} position can transfer a proton to FMN N5 (Lindqvist & Brändén, 1989; Ghisla & Massey, 1991a). A look at the three-dimensional structure shows that this is impossible for geometric reasons (Fig. 2). In both independently refined active sites of the crystal structure, the backbone amide of Ala 198 makes a hydrogen bond to the FMN N5 atom. The angle between

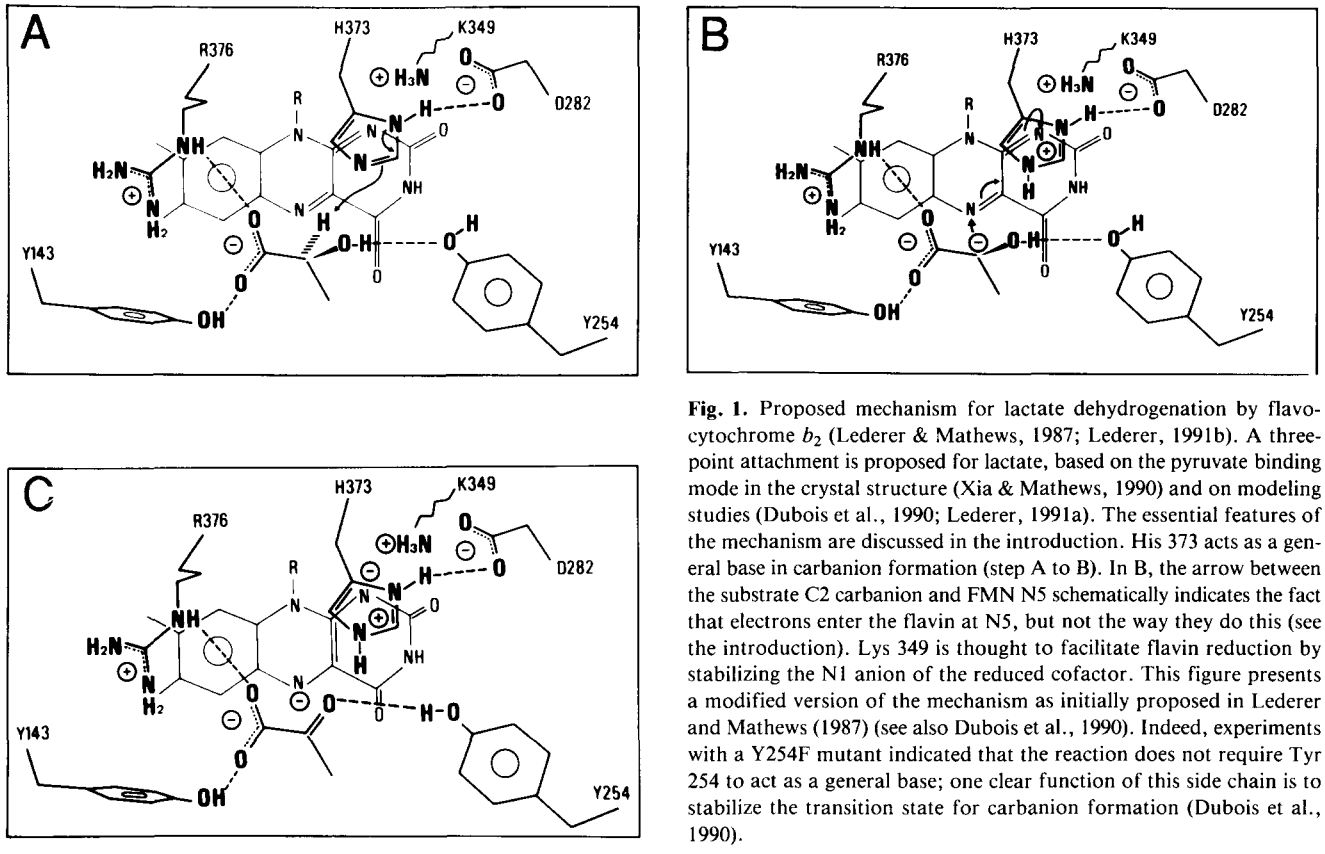

Fig. 1. Proposed mechanism for lactate dehydrogenation by flavocytochrome b_2 (Lederer & Mathews, 1987; Lederer, 1991b). A three-point attachment is proposed for lactate, based on the pyruvate binding mode in the crystal structure (Xia & Mathews, 1990) and on modeling studies (Dubois et al., 1990; Lederer, 1991a). The essential features of the mechanism are discussed in the introduction. His 373 acts as a general base in carbanion formation (step A to B). In B, the arrow between the substrate C2 carbanion and FMN N5 schematically indicates the fact that electrons enter the flavin at N5, but not the way they do this (see the introduction). Lys 349 is thought to facilitate flavin reduction by stabilizing the N1 anion of the reduced cofactor. This figure presents a modified version of the mechanism as initially proposed in Lederer and Mathews (1987) (see also Dubois et al., 1990). Indeed, experiments with a Y254F mutant indicated that the reaction does not require Tyr 254 to act as a general base; one clear function of this side chain is to stabilize the transition state for carbanion formation (Dubois et al., 1990).

this bond and the direction of a putative hydrogen bond linking the latter to His 373 N^{ϵ} atom is nearly 180° in both subunits. This is far from the tetrahedral geometry expected around the N5 atom, involving FMN atoms C4a and C5a as well as two other bonds or electron pairs more or less symmetrically located with respect to the flavin plane. Moreover, the distance of 4.3 or 4.5 Å (depending on the subunit) is too long. Therefore no hydrogen bond can be formed between His 373 N^{ϵ} and reduced FMN N5 atoms in the crystal structure. As hydrogen bonding is a prerequisite to proton transfer (Eigen, 1964; Hibbert, 1986), any direct proton transfer between the two atoms is ruled out.

Could protein mobility facilitate such a transfer during catalysis? Let us first remark that mobility of the histidine side chain should be restricted to movements of small amplitude owing to two factors. The first factor is the hydrogen bond between Asp 282 and the imidazolium N^{δ} atom. The second factor appears to be an internal bridge involving two symmetrical hydrogen bonds between the carboxamide group of Asn 372, the neighbor of His 373, and the carboxamide group of Asn 157. This hydrogen bonding arrangement appears to be structurally important as it is conserved in glycolate oxidase (Lê & Lederer, 1991). Therefore any His 373 movement would have to be limited in order to avoid transmission through

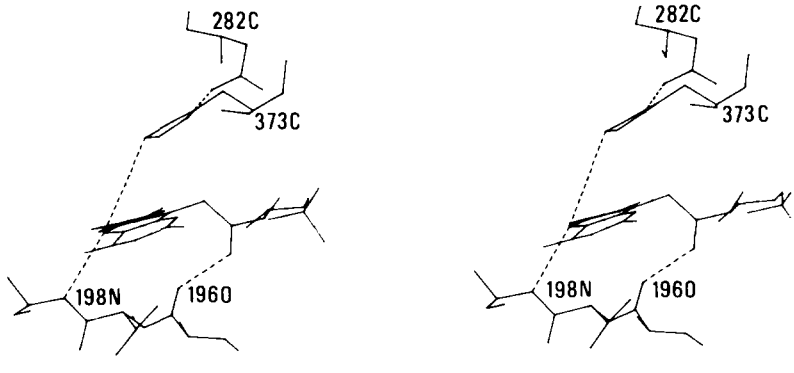

Fig. 2. Stereoview of the orientation of His 373 with respect to FMN in subunit 1 of flavocytochrome b_2. The peptide chain below the flavin, Ala 196–Thr 197–Ala 198, is also represented. Dashed lines show directions between Ala 198 NH and FMN N5, Ala 196 O, and FMN ribityl O_2 (both directions also represent H bonds) and FMN N5 and His 373 N^{ϵ}. The hydrogen bond between Asp 282 and His 373 N^{δ} is also represented.

the backbone to the side chain of Asn 372. Let us nevertheless assume that, within this context, a sliding movement parallel to the flavin plane could bring His 373 N^{ϵ} closer by at least 1 Å to the flavin N5 atom. This would ease the distance problem and make the geometry around N5 better although not necessarily good. On the other hand a hydrogen bond to an imidazole proton is expected to lie more or less in the ring plane (Baker & Hubbard, 1984); in this respect the situation is already not highly favorable in the crystal structure (Fig. 2), and the postulated movement would increase the angle of the putative hydrogen bond with the imidazole plane. In view of the proximity between the flavin and the imidazole planes (see Fig. 4), any other movement than the sliding movement seems to be precluded. It therefore appears that, even when taking mobility into account, no hydrogen bond can be formed at any time between the His 373 N^{ϵ} and flavin N5 atoms, and therefore any direct proton transfer between the two atoms is ruled out. Any transfer to another of the flavin heteroatoms, which all lie at distances $\geq$3.5 Å, is also ruled out for the same geometric reasons that prohibit transfer to N5. A direct hydrogen bond to the Tyr 254 phenol group appears also impossible as it would not present the required in-line geometry. No other side chain is within less than 5 Å of His 373 N^{ϵ}. Thus, the topography at the active site does not appear to favor the existence of the kind of multiple proton pool such as was described for some enzymes that catalyze proton abstraction from carbon (Kuo & Rose, 1987; Rose & Kuo, 1989; Rose et al., 1990).

The presence of a number of fixed water molecules at the active site of subunit S1 (no ligand present) might at first sight suggest the possibility of a water-mediated proton transfer. Indeed, in this active site, a water molecule (no. 662) occupies in space the same location as the pyruvate keto oxygen in the other active site and is similarly hydrogen bonded to the His 373 N^{ϵ} position and the Tyr 254 phenol group. It is also linked to two other water molecules, nos. 687 and 664. Although the former makes contacts with other fixed water molecules, the latter lies within hydrogen bonding distance of FMN O4 and N5, as well as of a heme propionate carboxylate oxygen (Fig. 3). Thus, a one-water bridge exists between His 373 and Tyr 254 and a two-water bridge between His 373 and FMN N5. Could these bridges mediate proton transfer from His 373 N^{ϵ}? The answer is clearly negative in view of the results of intermolecular hydrogen transfer experiments (Urban & Lederer, 1985). Indeed, these showed that the substrate α-proton resides at all times on a monoprotic heteroatom and can be totally transferred to the reducible substrate. On the contrary, any water-mediated transfer would entail isotope scrambling.

The inescapable conclusion is that the His 373 N^{ϵ} atom is the position from which the substrate α-proton is exchanged with the solvent in the unligated reduced active site. The apparent pK_a of ~15 calculated from the results of hydrogen transfer experiments should then belong to His 373. Can one, however, exclude the possibility of proton transfer between the latter and the carboxylate group of Asp 282 (Fig. 1)? The latter receives a hydrogen bond from the His 373 N^{δ} atom, with the favorable *syn* orientation (Gandour, 1981). In the case of a transfer between the two groups, both His 373 and Asp 282 would become neutral; the substrate α-hydrogen would still be

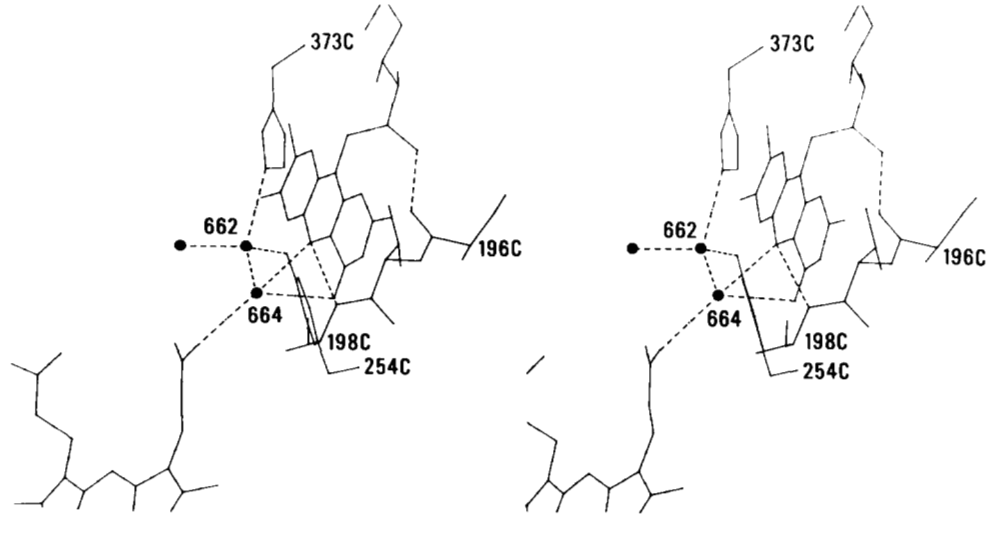

Fig. 3. Stereoview of the water network at the active site of flavocytochrome b_2. Dashed lines represent hydrogen bonds. WAT 662 is hydrogen bonded to His 373 N^{ϵ} and Tyr 254 phenol group, as well as to two water molecules. One of these, WAT 664, is itself hydrogen bonded to FMN N5 and O4, as well as to a heme propionate (bottom left). Black dots highlight the position of water molecules.

exchanged with the solvent from the His 373 N^{ϵ} position, but the high pK_a would have to be ascribed to Asp 282, implying an even larger pK shift than for a histidine. The idea is not totally unreasonable because, in nonaqueous solvents, the pK_a of a carboxyl group increases significantly and can exceed that of an imidazole (Komiyama et al., 1977; Halle & Simonnin, 1981). Nevertheless, in enzymes, known histidine–aspartate interactions are usually considered to involve ion pairs: in the classical serine proteases, lactate and malate dehydrogenases (Birktoft & Banaszak, 1983), phospholipase A_2 (Dijkstra et al., 1981), pancreatic lipase (Brady et al., 1990; Winkler et al., 1990), deoxyribonuclease I (Suck & Oefner, 1986), and thermolysin (Weaver et al., 1977). Direct experimental evidence indicates that His 57 in serine proteases does not transfer a proton to Asp 102 during catalysis (Kossiakoff & Spencer, 1981; Bachovchin, 1986, and references therein; Liang & Abeles, 1987). By extension, it is assumed that it behaves similarly in other hydrolases possessing a catalytic triad. In other cases, evidence concerning the role of the histidine in catalysis suggests that it remains charged (Weaver et al., 1977). In the present situation, considerations to be detailed below suggest that a positively charged side chain for His 373 in reduced enzyme has functional importance in catalysis, and therefore that in all likelihood the high apparent pK_a does belong to His 373. Furthermore, the fact that fluoropyruvate can affinity label His 373 in the oxidized enzyme but does not alkylate the reduced enzyme, although it is a substrate for it, can be easily explained if His 373 is in its acid form in the reduced enzyme (Urban & Lederer, 1988).

What is the pK_a of position N5 of flavocytochrome b_2-bound reduced flavin?

It seems that this group must also undergo an important pK shift relative to free flavin, although its exact value cannot be assessed at present. Figure 1C pictures the next to the last stage of the physiological reaction, namely the reduced enzyme–product complex, which is also the Michaelis complex for the reverse reaction. The reduced cofactor is represented as an N1–N5 dianion. The occurrence of such a highly charged species has been questioned (Ghisla & Massey, 1989, 1991a); nevertheless it cannot be avoided. Indeed, an examination of the three-dimensional structure suggests that as long as the product/substrate is bound at the active site, solvent has no access to the flavin N5 position. It is unreasonable to suppose the latter could abstract a proton from the backbone amide of Ala 198 to which it is hydrogen bonded; moreover, we have seen above that protonation by the imidazolium group of His 373 is excluded. Therefore protonation of the flavin N5 position could only occur after product dissociation.

With a pK_a similar to that of free $FMNH_2$, protonation at N5 should be extremely fast if it occurs. But correlatively deprotonation would be slow, and this step would have to take place before keto acid substrate binding in the reverse reaction. Using Eigen's equation, one can calculate that with a pK of 20, in 100 mM phosphate buffer at pH 7, the first order rate constant for N5-H deprotonation would be on the order of 10^{-4} s^{-1}, assuming buffer catalysis; with hydroxide ion catalysis the rate would be about 10^{-2} s^{-1}. These figures are too low, in view of experimentally determined k_{cat} values for the reverse reaction with various substrates: they range from 0.1 s^{-1} to 4 s^{-1} (Urban et al., 1983) and are limited by the rate of the carbanion protonation step (Urban & Lederer, 1985). Furthermore, during the normal physiological reaction, the next step after flavin reduction is electron donation from reduced flavin to heme b_2 with formation of the catalytically competent anionic semiquinone (Capeillère-Blandin, 1975), which is unprotonated at N5. The rate constant of this step has been estimated to lie in the range of 5×10^2 to 10^3 s^{-1} (Capeillère-Blandin et al., 1975; Pompon, 1980). It follows that the first order rate constant for flavin deprotonation must be higher than 10^3 s^{-1}, hence the pK_a at position N5 of bound reduced flavin must be lower by 5–7 pH units than the value of 20 assumed in the calculation.

Inspection of the three-dimensional structure (Xia & Mathews, 1990) actually suggests that reduced flavin may never be protonated at N5. The fixed water network at the unligated reduced active site has been described above. Whereas the geometry around most water molecules there is roughly tetrahedral, as it should be, the angle between the FMN N5-O(H_2) (664) and FMN O4-O(H_2) (664) directions is only 58°. This suggests the existence of a bifurcated hydrogen bond, in which WAT664 would be the H donor and unprotonated N5 and O4 the acceptors (Baker & Hubbard, 1984). Therefore there are hints from the crystal structure that the pK_a at N5 might even be lower than 7.

What is the origin of the large pK_a shifts in the active site of flavocytochrome b_2?

A possible rationale is afforded by the crystal structure. Two factors may be invoked to explain the large pK shifts observed: hydrogen bonding and direct electrostatic interactions.

The unligated active site, as mentioned above, shows a network of hydrogen-bonded water molecules. Although many of them will become loose when the enzyme is in solution, it is not unreasonable to assume that the positions of WAT662 and 664, owing to their anchoring to the protein structure, may retain a certain degree of occupancy; these molecules could thus play a dual role: that of affording electrostatic stabilization through hydrogen bonding to both the imidazolium group and the possibly negatively charged FMN N5 anion, as well as that of hin-

dering buffer approach to these atoms for deprotonation and protonation; the microenvironment around these atoms would be different from that provided by bulk solvent, and the pK_a of ~15 would thus indeed be an apparent pK_a. Assuming water catalysis, the calculated pK_a of His 373 in the reduced enzyme would drop to around 10. The weight of this hydrogen-bonding factor is being checked with a Y254F mutant of baker's yeast flavocytochrome b_2 (Dubois et al., 1990; A. Balme & F. Lederer, unpubl.).

The importance of direct electrostatic interactions is suggested by the peculiar topology around the flavin. The reduced FMN N1 anion is stabilized by ion pair formation with Lys 349 (Fig. 1). Nevertheless, it is striking to see that the His 373 imidazole plane and the flavin plane are nearly parallel and in van der Waals contact (Fig. 4A). The distance from the imidazole N^{δ} and N^{ϵ} atoms to flavin N1 is 4.2 and 3.6 Å, respectively (to flavin N5:6.3 and 4.5 Å, respectively). Thus, the flavin N1 anion faces approximately the center of the imidazolium ring. This arrangement might afford a strong electrostatic stabilization to both charged groups and would contribute to raise the pK_a of His 373. Furthermore, buffering the charge at FMN N1 by the imidazolium ring may contribute to lower the pK_a at N5 relative to free FMN by decreasing electrostatic repulsion between two negative charges that would lie within 3.6 Å of one another. Finally, a mutually stabilizing interaction might also exist between the charged imidazolium group and an anionic FMN N5, possibly relayed by the two-water bridge. An interesting observation related to the interaction between anionic flavin N1 and the charged imidazolium ion is to be found in Tegoni and Mathews (1988). The authors determined the structure of the flavin–sulfite complex in flavocytochrome b_2. In such a complex, sulfite is covalently attached to FMN N5, and the N1 position is anionic. The crystal structure shows one of the sulfite oxygens to lie 2.7 Å away from His 373 N^{ϵ} in subunit 1 and 2.2 Å in subunit 2. This implies hydrogen bonding between the two atoms, hence a protonated histidine. It is tempting

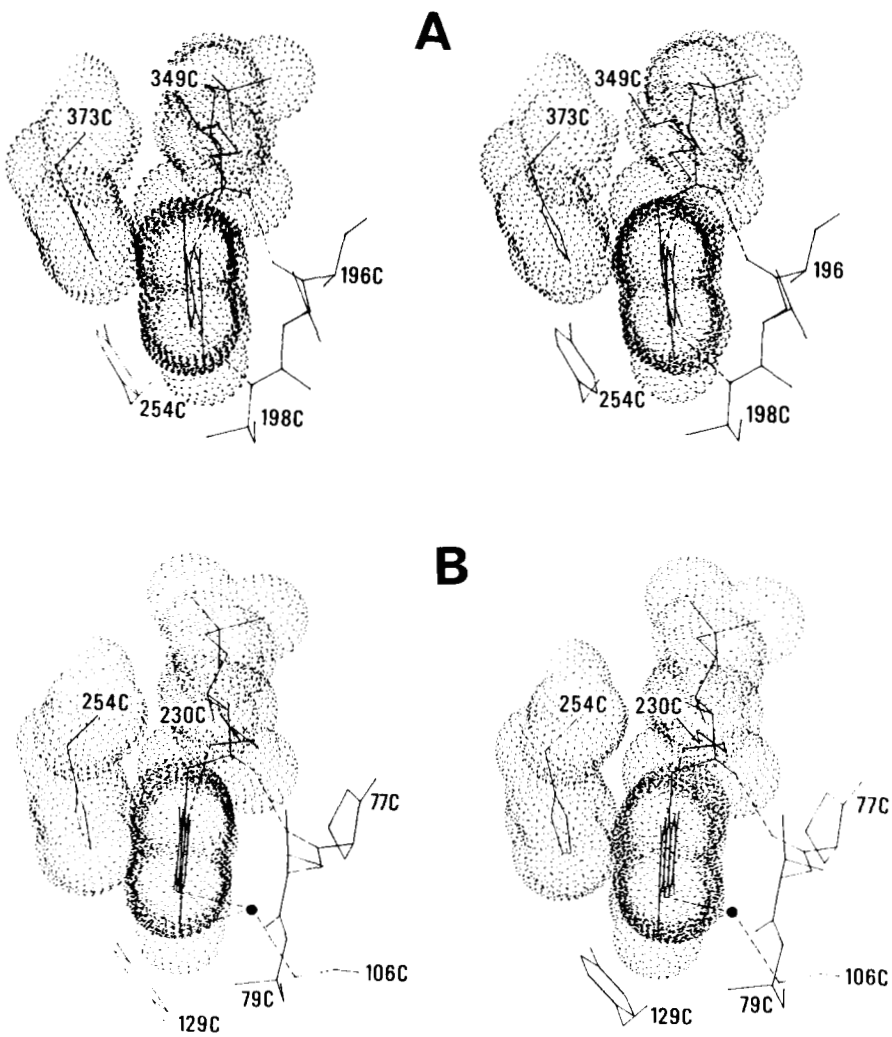

Fig. 4. Stereoview of van der Waals surfaces of FMN and histidine in flavocytochrome b_2 **(A)** and glycolate oxidase **(B)**. The hydrogen bonds between flavins and Ala 198 NH (b_2), Ala 196 O (b_2) or 77 (glycolate oxidase), and Lys 349 (b_2) or 230 (glycolate oxidase) are shown as landmarks, as well as those between WAT 394 (glycolate oxidase), FMN O4 and N5, and Ser 106 OH.

to speculate that the histidine pK shift was induced by the formation of a negative charge at FMN N1 as the result of sulfite addition at N5.

It is tempting to assign a functional significance in electron transfer to the arrangement of His 373 and the cofactor in the active site, which can be considered as a new catalytic device. These two groups constitute a kind of biological capacitor, with an imidazole plate and an isoalloxazine plate. After α-proton abstraction, the positive charge on the former should facilitate electron funneling from the carbanion into the flavin plate, thus charging it negatively. Furthermore, in the next step, after donation by the flavin of one electron to the heme, the His 373 plate is still positively charged, as suggested by the three-dimensional structure of the ligated active site, which is in agreement with the known stabilization in solution of the semiquinone by pyruvate binding (Tegoni et al., 1986; Walker & Tollin, 1991). It is only after the isoalloxazine plate is completely discharged that His 373 can lose its proton to the medium, or in the case of the reverse reaction to a keto acid-derived carbanion. It should be remarked that, strictly speaking, no proton is required at N5 at any stage of the mechanism. A proton is only required if the protein environment does not provide enough stabilization for the bound N1–N5 dianion.

What is the situation with respect to the related oxidases?

Much less evidence exists for the oxidases. In order to address the question, one has to rely on the crystal structure of glycolate oxidase, assuming that the structure of long chain hydroxy acid oxidase and lactate oxidase will show common similarities and differences with respect to that of flavocytochrome b_2 (Lindqvist et al., 1991), and on the extensive enzymological data published for lactate oxidase (Ghisla & Massey, 1991b).

The oxidized crystals of glycolate oxidase display the same mutual orientation as in flavocytochrome b_2 between the His 254/373 side chain and the isoalloxazine ring (distances from His N^{δ} and N^{ϵ} to FMN N1: 3.9 and 3.5 Å, respectively) (Fig. 4B) and a hydrogen bond is no more possible between His 254 and the FMN N5 position than in flavocytochrome b_2. Furthermore, lactate oxidase presents a number of similarities with flavocytochrome b_2. A particularly relevant one is that it also catalyzes intermolecular hydrogen transfer, even though the studies were not carried out all the way through to the calculation of a pK_a (Lederer, 1984). Lactate oxidase, as flavocytochrome b_2, shows stabilization of the anionic semiquinone upon pyruvate binding (Choong & Massey, 1980; Tegoni et al., 1986), which strongly suggests hydrogen bonding to the ligand keto oxygen from protonated His 290 (the homologue of His 373 [b_2] and His 254 [glycolate oxidase]) as well as from Tyr 151 (254 b_2). Moreover, studies of oxalate binding to lactate oxidase indicated that a group with $pK_a = 4.7$ undergoes a pK shift to a value of 9.8 upon ligand binding, and it was thus suggested that oxalate might act as a transition state analogue (Ghisla & Massey, 1977). It is tempting to assign these pK values to His 290 (Ghisla & Massey, 1989). This pK shift affects the ligated oxidized active site where the flavin is neutral. Finally, it is noteworthy that both glycolate oxidase and lactate oxidase bind sulfite very tightly, as does flavocytochrome b_2 (Massey et al., 1969; Lederer, 1978). The dissociation constant of sulfite for these enzymes is on the order of 10^{-6} M, whereas it is in the millimolar range for other flavoproteins tested (Massey et al., 1969). This low dissociation constant is probably attributable to interaction of the sulfite oxygens with Tyr 143 and 254 (b_2 numbering) as well as with the positively charged guanido groups of Arg 376 and imidazolium of His 373 (Tegoni & Mathews, 1988). In conclusion, the combined results suggest that the active site histidine in the oxidases indeed also acquires a high pK_a in the unligated reduced and semiquinone states when the flavin is anionic at N1.

The question is more open with respect to the pK_a of FMN N5. In the structure of glycolate oxidase, the backside of the flavin is accessible to solvent and has been proposed to be the oxygen attack side (Lindqvist et al., 1991). Hence protonation at N5 might be possible even in the presence of product. On the other hand, lactate oxidase and glycolate oxidase have been shown, as most oxidases (Massey et al., 1969), to form anionic semiquinones, which are unprotonated at N5. These semiquinones are catalytically incompetent and have never been observed as transients in normal catalysis. Model studies have led to the conclusion that reaction of reduced flavin with oxygen involves the formation of a 4a-hydroperoxy intermediate with transient formation of a radical pair (Eberlein & Bruice, 1983; Bruice, 1984). Nevertheless, such covalent intermediates have never been observed with oxidases of the type discussed here. The alternative is possibly a second single-electron transfer to the oxygen radical. Whatever the case, reduced flavin must lose its proton extremely rapidly in order to go through the anionic semiquinone stage; this is hardly compatible with a pK_a of 20 for the reduced flavin N5 position, for kinetic reasons similar to those presented above for flavocytochrome b_2. In conclusion, although it seems that in the oxidases the pK_a of reduced FMN N5 must be lowered relative to that of free $FMNH_2$, its exact value remains in doubt as it does for flavocytochrome b_2.

Finally, let us consider the fact that both flavocytochrome b_2 and the oxidases form an anionic semiquinone rather than a neutral one, which would have a protonated, positively charged N5 position. The direct flavin–protein interactions in the N5, C4a, C4, and O4 area of FMN are different in glycolate oxidase and fla-

vocytochrome b_2 (Fig. 4 and Lindqvist et al., 1991). In particular, the hydrogen bond from backbone 198 NH to FMN N5 is not present in glycolate oxidase. Therefore the stabilization of the anionic semiquinone in both proteins must have another origin elsewhere. This should be the presence of the charged imidazolium group, which would exclude a positive charge at N5.

Methods

The coordinates of flavocytochrome b_2 refined to 2.4 Å resolution (Xia & Mathews, 1990) were kindly communicated by Dr. F.S. Mathews before publication. They are identical with those subsequently deposited in the Brookhaven Protein Data Bank, from which the glycolate oxidase coordinates refined to 2 Å resolution (Lindqvist, 1989) were retrieved. The structures were examined with an Evans & Sutherland graphics system model PS390 using the software Manosk (Cherfils et al., 1988).

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References

Appleby, C.A. & Morton, R.K. (1954). Crystalline cytochrome b_2 and lactic dehydrogenase in yeast. *Nature 173*, 749–752.

Bachovchin, W.W. (1986). ^{15}N NMR spectroscopy of hydrogen-bonding interactions in the active site of serine proteases: Evidence for a moving histidine mechanism. *Biochemistry 25*, 7751–7759.

Baker, E.N. & Hubbard, R.E. (1984). Hydrogen bonding in globular proteins. *Prog. Biophys. Mol. Biol. 44*, 97–179.

Birktoft, J.J. & Banaszak, L.J. (1983). The presence of a histidine–aspartic acid pair in the active site of 2-hydroxy acid dehydrogenases. X-ray refinement of cytoplasmic malate dehydrogenase. *J. Biol. Chem. 258*, 472–482.

Black, M.T., Gunn, F.J., Chapman, S.K., & Reid, G.A. (1989). Structural basis for the kinetic differences between flavocytochromes b_2 from the yeasts *Hansenula anomala* and *Saccharomyces cerevisiae*. *Biochem J. 263*, 973–976.

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L., & Menge, U. (1990). A serine protease triad forms the catalytic center of a triacylglycerol lipase. *Nature 343*, 767–770.

Bruice, T.C. (1980). Mechanisms of flavoprotein catalysis. *Acc. Chem. Res. 13*, 256–262.

Bruice, T.C. (1984). Flavin oxygen chemistry brought to date. In *Flavins & Flavoproteins* (Bray, R.C., Engel, P.C., & Mayhew, S.G., Eds.), pp. 45–55. Walter de Gruyter, Berlin.

Capeillère-Blandin, C. (1975). Flavocytochrome b_2: Simulation studies of the electron transfer reactions among the prosthetic groups. *Eur. J. Biochem. 56*, 91–101.

Capeillère-Blandin, C., Bray, R.C., Iwatsubo, M., & Labeyrie, F. (1975). Flavocytochrome b_2: Kinetic studies by absorbance and electron-paramagnetic-resonance spectroscopy of electron distribution among prosthetic groups. *Eur. J. Biochem. 54*, 549–566.

Cederlund, E., Lindqvist, Y., Söderlund, G., Brändén, C.I., & Jörnvall, H. (1988). Primary structure of glycolate oxidase from spinach. *Eur. J. Biochem. 173*, 523–530.

Cherfils, J., Vaney, M.C., Morize, I., Surcouf, E., Colloc'h, N., & Mornon, J.P. (1988). Manosk: A graphics program for analyzing and modeling molecular structure and function. *J. Mol. Graphics 6*, 155–160.

Choong, Y.S. & Massey, V. (1980). Stabilization of lactate oxidase flavin anion radical by complex formation. *J. Biol. Chem. 255*, 8672–8677.

Dijkstra, B.W., Kalk, K.H., Hol, W.G.J., & Drenth, J. (1981). Structure of bovine pancreatic phospholipase A2 at 1.7 Å resolution. *J. Mol. Biol. 147*, 97–124.

Dubois, J., Chapman, S.K., Mathews, F.S., Reid, G.A., & Lederer, F. (1990). Substitution of Tyr254 with Phe at the active site of flavocytochrome b_2: Consequences on catalysis of lactate dehydrogenation. *Biochemistry 29*, 6393–6400.

Eberlein, G. & Bruice, T.C. (1983). The chemistry of a 1,5-diblocked flavin. 2. Proton and electron transfer steps in the reaction of dihydroflavins with oxygen. *J. Am. Chem. Soc. 105*, 6685–6697.

Eigen, M. (1964). Proton transfer, acid-base catalysis and enzymatic hydrolysis. Part I: Elementary processes. *Angew. Chem. Int. Ed. Engl. 3*, 1–19.

Gandour, R.D. (1981). On the importance of orientation in general base catalysis by carboxylate. *Bioorg. Chem. 10*, 169–176.

Ghisla, S. (1982). Dehydrogenation mechanism in flavoprotein catalysis. In *Flavins & Flavoproteins* (Massey, V. & Williams, C.H., Eds.), pp. 133–142. Elsevier North Holland, Amsterdam.

Ghisla, S., Macheroux, P., Sanner, Ch., Rüterjans, H., & Müller, F. (1991). Ionisation properties of reduced, 1,5-dihydroflavin, rates of N(5)-H exchange with solvent. In *Flavins & Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.), pp. 27–32. Walter de Gruyter, Berlin.

Ghisla, S. & Massey, V. (1977). Studies on the mechanism of action of the flavoenzyme lactate oxidase. Proton uptake and release during the binding of transition state analogs. *J. Biol. Chem. 252*, 6729–6735.

Ghisla, S. & Massey, V. (1989). Mechanisms of flavoprotein catalyzed reactions. *Eur. J. Biochem. 181*, 1–17.

Ghisla, S. & Massey, V. (1991a). L-Lactate monooxygenase, proposed active site structure and mechanism. In *Flavins & Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.), pp. 123–130. Walter de Gruyter, Berlin.

Ghisla, S. & Massey, V. (1991b). Lactate oxidase. In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 2 (Müller, F., Ed.), pp. 243–289. CRC Press, Boca Raton, Florida.

Giegel, D.A., Williams, C.H., & Massey, V. (1990). L-Lactate 2-monooxygenase from *Mycobacterium smegmatis*. Cloning, nucleotide sequence and primary structure homology within an enzyme family. *J. Biol. Chem. 265*, 6626–6632.

Halle, J.C. & Simonnin, M.P. (1981). Proton transfer in histidine hydrochloride induced by a dipolar aprotic solvent. *J. Biol. Chem. 256*, 8569–8572.

Hibbert, F. (1986). Mechanisms of proton transfer between oxygen and nitrogen acids and bases in aqueous solution. *Adv. Phys. Org. Chem. 22*, 113–212.

Jacq, C. & Lederer, F. (1974). Cytochrome b_2 from baker's yeast (L-lactate dehydrogenase). A double headed enzyme. *Eur. J. Biochem. 411*, 311–320.

Komiyama, M., Bender, M.L., Uteka, M., & Takeda, A. (1977). Model for "charge-relay": Acceleration by carboxylate anion in intramolecular general base-catalyzed ester hydrolysis by the imidazolyl group. *Proc. Natl. Acad. Sci. USA 74*, 2634–2638.

Kossiakoff, A.A. & Spencer, S.A. (1981). Direct determination of the protonation states of aspartic acid-102 and histidine-57 in the tetrahedral intermediate of the serine proteases: Neutron structure of trypsin. *Biochemistry 20*, 6462–6474.

Kuo, D.J. & Rose, I.A. (1987). Aconitase: Its source of catalytic protons. *Biochemistry 26*, 7589–7596.

Lê, K.H.D. & Lederer, F. (1991). Amino acid sequence of long chain α-hydroxy acid oxidase from rat kidney: A member of the family of FMN-dependent α-hydroxy acid oxidizing enzymes. *J. Biol. Chem. 266*, 20877–20881.

Lederer, F. (1978). Sulfite binding to a flavodehydrogenase, cytochrome b_2 from baker's yeast. *Eur. J. Biochem. 88*, 425-431.

Lederer, F. (1984). Dehydrohalogenation and hydrogen transfer reactions catalyzed by some lactate-oxidizing enzymes. In *Flavins & Flavoproteins* (Bray, R.C., Engel, P.C., & Mayhew, S.G., Eds.), pp. 513-526. Walter de Gruyter, Berlin.

Lederer, F. (1991a). On some aspects of the catalysis of lactate dehydrogenation by flavocytochrome b_2. In *Flavins & Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.), pp. 773-782. Walter de Gruyter, Berlin.

Lederer, F. (1991b). Flavocytochrome b_2. In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 2 (Müller, F., Ed.), pp. 153-242. CRC Press, Boca Raton, Florida.

Lederer, F., Cortial, S., Becam, A.M., Haumont, P.Y., & Perez, L. (1985). Complete amino acid sequence of flavocytochrome b_2 from baker's yeast. *Eur. J. Biochem. 139*, 59-65.

Lederer, F. & Mathews, F.S. (1987). Mechanism of L-lactate dehydrogenation catalyzed by flavocytochrome b_2 from baker's yeast. In *Flavins & Flavoproteins* (Edmondson, D. & McCormick, D.B., Eds.), pp. 133-142. Walter de Gruyter, Berlin.

Liang, T.C. & Abeles, R.H. (1987). Complex of α-chymotrypsin and *N*-acetyl-L-leucyl-L-phenylalanyltrifluoromethylketone: Structural studies with NMR spectroscopy. *Biochemistry 26*, 7603-7608.

Lindqvist, Y. (1989). Refined structure of spinach glycolate oxidase at 2 Å resolution. *J. Mol. Biol. 209*, 151-166.

Lindqvist, Y. & Brändén, C.-I. (1985). Structure of glycolate oxidase from spinach. *Proc. Natl. Acad. Sci. USA 82*, 6855-6859.

Lindqvist, Y. & Brändén, C.-I. (1989). The active site of spinach glycolate oxidase. *J. Biol. Chem. 264*, 3624-3628.

Lindqvist, Y., Brändén, C.-I., Mathews, F.S., & Lederer, F. (1991). Spinach glycolate oxidase and yeast flavocytochrome b_2 are structurally homologous and evolutionarily related enzymes with distinctly different function and flavin mononucleotide binding. *J. Biol. Chem. 266*, 3198-3207.

Massey, V., Ghisla, S., & Kieschke, K. (1980). Studies on the reaction mechanism of lactate oxidase. Formation of two covalent flavin-substrate adducts on reaction with glycolate. *J. Biol. Chem. 255*, 2796-2806.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P.A., Howell, L.G., Mayhew, S.G., Mathews, R.G., & Foust, G.P. (1969). The reactivity of flavoproteins with sulfite. Possible relevance to the problem of oxygen reactivity. *J. Biol. Chem. 244*, 3999-4006.

Mathews, F.S. & Xia, Z.-X. (1987). Three dimensional structure of baker's yeast flavocytochrome b_2. In *Flavins & Flavoproteins* (Edmondson, D. & McCormick, D.B., Eds.), pp. 123-132. Walter de Gruyter, Berlin.

Pompon, D. (1980). Flavocytochrome b_2 from baker's yeast. Computer-simulation studies of a new kinetic scheme for intramolecular electron transfer. *Eur. J. Biochem. 106*, 151-158.

Porter, D.J.T., Voet, J.G., & Bright, H.J. (1973). Direct evidence for carbanions and covalent N5-flavin-carbanion adducts as catalytic intermediates in the oxidation of nitroethane by D-amino acid oxidase. *J. Biol. Chem. 248*, 4400-4416.

Reid, G.A., White, S., Black, M.T., Lederer, F., Mathews, F.S., & Chapman, S.K. (1988). Probing the active site of flavocytochrome b_2 by site-directed mutagenesis. *Eur. J. Biochem. 178*, 329-333.

Risler, Y., Tegoni, M., & Gervais, M. (1989). Nucleotide sequence of the *Hansenula anomala* gene encoding flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase). *Nucleic Acids Res. 17*, 8381.

Rose, I.A., Fung, W.J., & Warms, J.V.B. (1990). Proton diffusion in the active site of triose phosphate isomerase. *Biochemistry 29*, 4312-4317.

Rose, I.A. & Kuo, D.J. (1989). The substrate proton of the pyruvate kinase reaction. *Biochemistry 28*, 9579-9585.

Suck, D. & Oefner, C. (1986). Structure of DNase I at 2.0 Å resolution suggests a mechanism for binding to and cutting DNA. *Nature 321*, 620-625.

Tegoni, M., Janot, J.M., & Labeyrie, F. (1986). Regulation of dehydrogenase/one-electron transferases by modification of flavin redox potentials. Effect of product binding on semiquinone stabilization in yeast flavocytochrome b_2. *Eur. J. Biochem. 155*, 491-503.

Tegoni, M. & Mathews, F.S. (1988). Crystallographic study of the complex between sulfite and baker's yeast flavocytochrome b_2. *J. Biol. Chem. 263*, 19278-19281.

Tsou, A.Y., Ransom, S.C., Gerlt, J.A., Buechter, D.D., Babbitt, P.C., & Kenyon, G.L. (1990). Mandelate pathway of *Pseudomonas putida*: Sequence relationships involving mandelate racemase, (*S*)-mandelate dehydrogenase and benzoylformate decarboxylase and expression of benzoylformate decarboxylase in *Escherichia coli*. *Biochemistry 29*, 9856-9862.

Urban, P., Alliel, P.A., & Lederer, F. (1983). On the transhydrogenase activity of baker's yeast flavocytochrome b_2. *Eur. J. Biochem. 134*, 275-281.

Urban, P., Chirat, I., & Lederer, F. (1988). Rat kidney L-2-hydroxy acid oxidase. Structural and mechanistic comparison with flavocytochrome b_2 from baker's yeast. *Biochemistry 27*, 7365-7371.

Urban, P. & Lederer, F. (1985). Intermolecular hydrogen transfer catalyzed by a flavodehydrogenase, baker's yeast flavocytochrome b_2. *J. Biol. Chem. 260*, 11115-11122.

Urban, P. & Lederer, F. (1988). Inactivation of flavocytochrome b_2 with fluoro-pyruvate. Reaction at the active-site histidine. *Eur. J. Biochem. 173*, 155-162.

Venkataram, U.V. & Bruice, T.C. (1984). On the mechanism of flavin-catalyzed dehydrogenation α,β to an acyl function. The mechanism of 1,5-dihydroflavin reduction of maleimides. *J. Am. Chem. Soc. 106*, 5703-5709.

Walker, M.C. & Tollin, G. (1991). Laser flash photolysis studies of the kinetics of electron-transfer reactions of *Saccharomyces* flavocytochrome b_2: Evidence for conformational gating of intramolecular electron transfer induced by pyruvate binding. *Biochemistry 30*, 5546-5555.

Weaver, L.H., Kester, W.R., & Matthews, B.W. (1977). A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substrates. *J. Mol. Biol. 114*, 119-132.

Winkler, F.K., D'Arcy, A., & Hunziker, W. (1990). Structure of human pancreatic lipase. *Nature 343*, 771-774.

Xia, Z.-X. & Mathews, F.S. (1990). Molecular structure of flavocytochrome b_2 at 2.4 Å resolution. *J. Mol. Biol. 212*, 837-863.

Xia, Z.-X., Shamala, N., Bethge, P.H., Lim, L.W., Bellamy, H.D., Xuong, N.H., Lederer, F., & Mathews, F.S. (1987). Three-dimensional structure of flavocytochrome b_2 from baker's yeast at 3.0 Å resolution. *Proc. Natl. Acad. Sci. USA 84*, 2629-2633.