Flavocytochrome b_2: Reactivity of Its Flavin with Molecular Oxygen†

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ABSTRACT: Flavocytochrome b_2, a flavohemoprotein, catalyzes the oxidation of lactate at the expense of the physiological acceptor cytochrome *c* in the yeast mitochondrial intermembrane space. The mechanism of electron transfer from the substrate to monoelectronic acceptors via FMN and heme b_2 has been intensively studied over the years. Each prosthetic group is bound to a separate domain, N-terminal for the heme, C-terminal for the flavin. Each domain belongs to a distinct evolutionary family. In particular, the flavodehydrogenase domain is homologous to a number of well-characterized L-2-hydroxy acid-oxidizing enzymes. Among these, some are oxidases for which the oxidative half-reaction produces hydrogen peroxide at the expense of oxygen. For bacterial mandelate dehydrogenase and flavocytochrome b_2, in contrast, the oxidative half-reaction requires monoelectronic acceptors. Several crystal structures indicate an identical fold and a highly conserved active site among family members. All these enzymes form anionic semiquinones and bind sulfite, properties generally associated with oxidases, whereas electron transferases are expected to form neutral semiquinones and to yield superoxide anion. Thus, flavocytochrome b_2 is a highly unusual dehydrogenase−electron transferase, and one may wonder how its flavin reacts with oxygen. In this work, we show that the separately engineered flavodehydrogenase domain produces superoxide anion in its slow reaction with oxygen. This reaction apparently also takes place in the holoenzyme when oxygen is the sole electron acceptor, because the heme domain autoxidation is also slow; this is not unexpected, in view of the heme domain mobility relative to the tetrameric flavodehydrogenase core (Xia, Z. X., and Mathews, F. S. (1990) *J. Mol. Biol. 212*, 837−863). Nevertheless, this reaction is so slow that it cannot compete with the normal electron flow in the presence of monoelectronic acceptors, such as ferricyanide and cytochrome *c*. An inspection of the available structures of family members does not provide a rationale for the difference between the oxidases and the electron transferases.

Saccharomyces cerevisiae flavocytochrome b_2 (L-lactate: cytochrome *c* oxidoreductase, EC 1.1.2.3) (Fcb2)[1] enables yeast to grow on lactate as the sole carbon source (*1*). It catalyzes the oxidation of L-lactate to pyruvate at the expense of cytochrome *c* in the mitochondrial intermembrane space (*2*). Each subunit of the tetrameric enzyme is composed of two domains: a heme-binding domain (residues 1−99) and an FMN-binding domain (residues 100−511) (*3*, *4*). In the crystallographic asymmetric unit (a dimer), one heme domain out of two is invisible, due to its mobility. Solution studies confirm that the heme domains are mobile relative to the flavodehydrogenase tetrameric core (FDH) (*5*, *6*).

During catalysis, lactate reduces the flavin, which yields the reducing equivalents one at a time to heme b_2 in the same subunit, with intermediate formation of a flavin semiquinone. Reduced heme b_2 is reoxidized by cytochrome *c* (*7*−*10*) (Scheme 1). Nonphysiological electron acceptors such as ferricyanide are often used in vitro.

The heme b_2 domain belongs to the family of b_5-like cytochromes (*11*, *12*), while the FDH domain is a member of the family of FMN-dependent L-2-hydroxy acid-oxidizing enzymes. The other well-characterized members of the latter family are lactate monooxygenase (LMO; previously called lactate oxidase) from *Mycobacterium smegmatis* (*13*), glycolate oxidase (GOX; or short-chain hydroxy acid oxidase) from spinach (*14*), long-chain hydroxy acid oxidase from rat kidney (LCHAO; a GOX isozyme) (*15*), lactate oxidase from *Aerococcus viridans* (LOX) (*16*), and mandelate dehydrogenase from *Pseudomonas putida* (MDH) (*17*). Crystal structures are known for all these homologues except for LMO (*18*−*23*). The Fcb2 FDH domain has been expressed independently, and its crystal structure is practically the same as that of the domain when in the holoenzyme

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[1] Abbreviations: AU, absorbance unit; Fcb2, flavocytochrome b_2; FDH, flavodehydrogenase; Fl_{ox}, oxidized flavin or FMN; Fl_{sq}, flavin semiquinone or $FMN^{\bullet -}$; Fl_{red}, reduced flavin or $FMNH^-$; GOX, glycolate oxidase; HRP, horse radish peroxidase; LCHAO, long-chain hydroxy acid oxidase; LMO, lactate monooxygenase; LOX, lactate oxidase; MDH, mandelate dehydrogenase; NBT, 4-nitro blue tetrazolium chloride; SOD, superoxide dismutase; WT, wild type; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium 5-carboxanilide, sodium salt.

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Scheme 1

(*24*). All these structures indicate an identical $\beta_8\alpha_8$ fold and a highly conserved active site, but there is evidence for mobility of a number of side chains; in particular, part of barrel loop 4 is invisible in the structures of Fcb2, GOX, and LCHAO (*18*−*20*, *24*).

The family comprises both dehydrogenases−oxidases and dehydrogenases−electron transferases, according to the accepted terminology (*25*). The former are reoxidized by oxygen with formation of hydrogen peroxide, while the latter (Fcb2 and MDH) use monoelectronic acceptors; in their reaction with oxygen, they should produce the superoxide anion radical (*25*, *26*). The distinction between oxidases and electron transferases was proposed to be correlated with a set of properties specific to each subclass (*25*, *27*). The oxidases are expected to form an anionic flavin semiquinone and to be able to yield a reversible covalent adduct with sulfite, while the electron transferases yield a neutral flavin semiquinone and do not react with sulfite. Nevertheless, a few exceptions have been documented, such as, for example, ETF (*28*), putrescine oxidase (*29*), and aryl alcohol oxidase (*30*).

In this respect, Fcb2 is an unusual dehydrogenase−electron transferase, insofar as it forms an anionic semiquinone (*8*) and reacts readily with sulfite (*31*). To our knowledge, Fcb2 is even one out of only two flavoproteins for which the crystal structure of a flavin−sulfite adduct has been determined (*32*−*34*). These properties combined with the structural similarity between Fcb2 and the homologous oxidases raise an intriguing question: How does the Fcb2 FDH domain react with oxygen when separated from its natural electron acceptor? In this work, we show that the domain reacts slowly with oxygen with formation of superoxide anion, whether isolated or included in the holoenzyme.

MATERIALS AND METHODS

Enzymes and Chemicals. S. cerevisiae recombinant WT Fcb2 and its recombinant FDH domain were prepared as described earlier in ref *35* and refs *36* and *37*, respectively, with a few modifications for the FDH domain, as follows. DEAE cellulose was replaced by DEAE Sepharose, and a chromatographic step on hydroxyapatite was added. Both preparations were stored at −70 °C in 0.1 M phosphate buffer, 1 mM EDTA, pH 7; 10 mM DL-lactate was added to the Fcb2 preparations to keep the enzyme in the reduced state. The holoenzyme was dialyzed back to the oxidized state just before the experiments. Its concentration was expressed as heme concentrations ($\epsilon_{413}^{ox} = 129$ mM^{-1} cm^{-1}, $\epsilon_{423}^{red} = 183$ mM^{-1} cm^{-1}); the FDH concentration was determined using $\epsilon_{452} = 11$ mM^{-1} cm^{-1} for FMN. The Fcb2 heme domain was prepared by tryptic proteolysis of the WT holoenzyme, as described in ref *38*. Lactate (lithium salt), bovine erythrocyte superoxide dismutase (SOD), bovine catalase, horse radish peroxidase (HRP), horse heart cytochrome *c*, *o*-dianisidine, NBT (4-nitro blue tetrazolium chloride), and XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium 5-carboxanilide, sodium salt) came from Sigma-Aldrich. Recombinant human GOX was purified in the laboratory as described by Vignaud et al. (*39*). All the experiments were carried out at 30 °C in 0.1 M phosphate buffer, pH 7, unless otherwise mentioned.

Steady-State Kinetics. Enzymatic activity was assayed with a Uvikon 943 spectrophotometer. Lactate and ferricyanide concentrations were 10 and 2 mM, respectively, for the holoenzyme and 20 and 13 mM, respectively, for the FDH domain. For ferricyanide, the following extinction coefficients were used: $\epsilon_{420}^{red-ox} = 1.04$ mM^{-1} cm^{-1} and $\epsilon_{440}^{red-ox} = 0.6$ mM^{-1} cm^{-1}.

Product Analysis during Turnover with Oxygen as an Acceptor. The holoenzyme and the FDH domain were incubated under the same conditions. Oxidized enzyme (10 μM) was incubated at 30 °C in the presence of 10 mM L-lactate in 200 μL aliquots in 2 mL Eppendorf plastic tubes. When present, SOD and catalase were at 50 μg/mL. The enzyme activity was followed over time by withdrawing aliquots which were immediately diluted (more than 100-fold) in the assay solution. For substrate disappearance and product formation, aliquots were similarly withdrawn and immediately mixed with an equal volume of 0.4 M HCl to stop the reaction. The protein was removed by filtration with a Micro-Pure EZ filter (Millipore). Lactate and products were then separated on an HPLC cation exchange column (AMINEX HPX 87H, BioRad) developed at 30 °C with 5 mM sulfuric acid at 0.3 mL/min. The elution profile was monitored at 210 nm.

Hydrogen Peroxide and Superoxide Anion Production Tests. For hydrogen peroxide assays, the HRP and *o*-dianisidine concentrations were 10 μg/mL and 0.48 mM, respectively. The reaction was monitored at 440 nm ($\epsilon_M = 11.6$ mM^{-1} cm^{-1}) in the presence of FDH and 10 mM L-lactate; recombinant human GOX (0.7 μM) with 10 mM glycolate or 30 mM L-lactate was used as a control. For superoxide assays, the reaction was initiated by adding 5−10 μL of enzyme (final concentration 20 μM FDH domain) to the cuvette which contained 20 mM L-lactate and the superoxide acceptor at the desired concentration in the presence or absence of SOD (50 μg/mL). The following acceptors were used: 100 μM cytochrome *c* ($\epsilon_{550}^{red-ox} = 21$ mM^{-1} cm^{-1}), 100 μM NBT ($\epsilon_{560}^{red-ox} = 12.6$ mM^{-1} cm^{-1}), 20 μM XTT ($\epsilon_{470}^{red-ox} = 21.6$ mM^{-1} cm^{-1}). For the assays in anaerobiosis, nitrogen purged on an Alltech Big Oxygen Trap was bubbled into buffer and reagent solutions; they were introduced into anaerobic cuvettes with a syringe through a septum, and so was the small enzyme volume.

Stopped-Flow Studies of the Reaction with Oxygen. Studies of flavin reoxidation were performed using a DX.17MV stopped-flow spectrophotometer (Applied Photophysics) at 30 °C. The oxidized domain (20−40 μM) in one syringe was mixed with an equimolar amount of L-lactate in air-equilibrated buffer at 30 °C in the other syringe in the presence or absence of 80 μg/mL SOD. The first few milliseconds showed the end of the reduction phase before reoxidation. The rate of flavin reoxidation was monitored at 452 and 380 nm. Typically, 2−4 kinetic traces were averaged and analyzed using single- or double-exponential equations. As a control, FDH domain reduction by saturating L-lactate was monitored at 452 nm; it was biphasic. The amplitude of the second phase was about 10−12% that of the first phase, as described for the reaction at 5 °C (*40*). The k_{red}

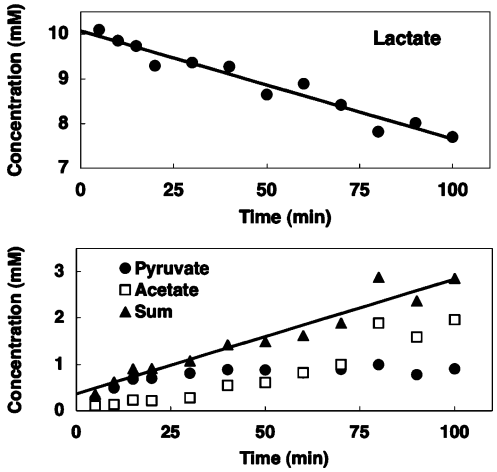

FIGURE 1: FDH domain turnover in the presence of oxygen as an acceptor. The reaction was carried out in 0.1 M phosphate buffer, pH 7, at 30 °C with 10 μM FDH domain and 10 mM L-lactate. Aliquots were withdrawn as a function of time and mixed with an equal volume of 0.4 N HCl. The substrate and products were quantified by analysis on a cation exchange HPLC column. For further details, see Materials and Methods. In this particular experiment, lactate disappeared at a rate of 40.4×10^{-3} s^{-1}; pyruvate + acetate appeared at a rate of 41×10^{-3} s^{-1}.

value was 468 ± 44 s^{-1}, compared to a k_{cat} of 260 s^{-1} in the steady state in the presence of ferricyanide at the same temperature (*40*). The rate of the second phase was less than 10% that of the fast phase. The Fcb2 reoxidation kinetics were monitored at 557 nm for the heme and at 438 and 350 nm, two heme isosbestic points, for the flavin. The first wavelength monitors global flavin reduction or oxidation (*8*), and the second one is specific for the semiquinone; at this wavelength, the semiquinone absorbance is higher than that of both oxidized and reduced flavin (*41*). The final enzyme concentrations were 3 μM for heme kinetics and 15 μM for following the flavin. The Fcb2 initial reduction was achieved in the same way as for the FDH domain. For the heme b_2 domain, it was achieved by mixing the oxidized domain, supplemented with 1/10 its concentration of holoenzyme, in one syringe with a 2-fold excess of lactate in the other syringe with or without SOD.

RESULTS

Steady-State Reaction of the FDH Domain in the Presence of Oxygen as an Acceptor. When the domain was mixed with saturating L-lactate in a test tube or a spectrophotometer cuvette, the flavin was reduced and remained so for a long period. Nevertheless, a reaction was occurring, as evidenced by an HPLC analysis for lactate disappearance and pyruvate formation. As shown in Figure 1, lactate disappeared linearly with time, whereas pyruvate formation slowed with time. This could be rationalized by the identification of a second product, which appeared after a time lag. It was identified as acetate by its elution time from the HPLC column. The sum of pyruvate and acetate formed was equal, within error, to the amount of lactate that disappeared.

Acetate can be formed by oxidative decarboxylation of pyruvate with hydrogen peroxide. This reaction actually takes place at the active site of LMO, and acetate is the normal product of lactate consumption by this enzyme, due to slow pyruvate dissociation from the active site (*42*). In the FDH

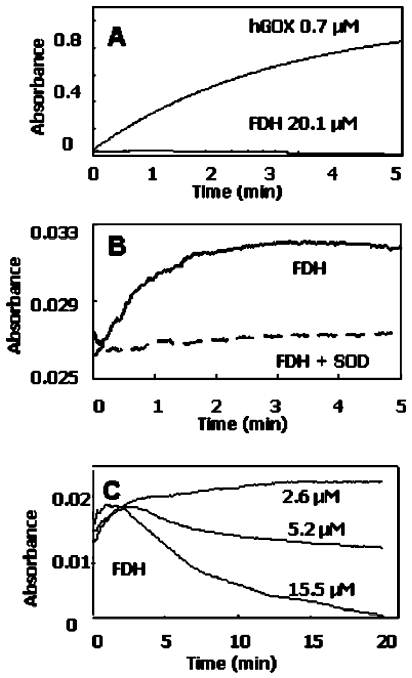

FIGURE 2: Hydrogen peroxide production tests. (A) The enzymes were mixed in a spectrophotometer cuvette with 10 μg of HRP and 0.48 mM *o*-dianisidine in 0.1 M phosphate buffer at 30 °C. H_2O_2 formation was monitored at 440 nm in the presence of 10 mM lactate for the FDH domain and 10 mM glycolate for GOX. (B) Experimental conditions were the same, except that the FDH domain concentration was 4.5 μM. The SOD concentation was 50 μg/mL. (C) The experimental conditions were identical, but the FDH domain concentration was varied, as indicated in the figure.

domain case, the time lag for acetate appearance suggested that the reaction was taking place after the products had diffused out of the active site. When the incubation was carried out in the presence of catalase, only pyruvate was formed in a linear fashion, with a rate identical within error to the lactate consumption rate. This confirmed the formation of hydrogen peroxide in the reaction mixture. Nevertheless, the experiment did not indicate whether H_2O_2 originated directly from the flavin reaction with oxygen or from superoxide dismutation.

What Is the Origin of the Hydrogen Peroxide? The classical assay for hydrogen peroxide uses HRP and one of its substrates, such as *o*-dianisidine. Figure 2A compares the result obtained when *o*-dianisidine and HRP were incubated with a mixture of substrate and either concentrated FDH or a much lower concentration of glycolate oxidase, an oxidase of the same enzyme family. Under the experimental conditions chosen, in view of the relative enzyme concentrations and respective rates (this work, *39*), we would have expected the absorbance variation for the FDH domain to be about half that observed for GOX, if it were producing hydrogen peroxide. This was clearly not the case. Nevertheless, upon closer inspection, there was a slight absorbance increase during the first minute, followed by a slow decay. When HRP was added after a 30 min incubation in the presence of *o*-dianisidine, at a time when acetate is formed at a detectable rate (Figure 1), the phenomenon was the same, namely, an initial small absorbance rise and then a slow decay. Product analysis by HPLC 90 min after HRP addition gave a pyruvate:acetate molar ratio of 7.1, while this ratio was 0.2 for the control without HRP. Thus, HRP was

Table 1: Superoxide Anion Production Tests[a]

acceptor	acceptor reduction rate ($k_{obs} \times 10^3$) (s^{-1}) aerobiosis	aerobiosis + SOD	anaerobiosis	inhibition by SOD (%)
cytochrome *c*	4.2 ± 0.9	2.7 ± 0.8	5.4 ± 0.2	36
NBT	11.6 ± 1.5	5.6 ± 1.1	8.5 ± 1.3	52
XTT	18.1 ± 0.1	7.5 ± 1.2	18.7 ± 3.5	58

[a] The reactions were carried out in 0.1 M phosphate buffer, pH 7, at 30 °C with 20 μM FDH domain and 20 mM L-lactate in the presence of either 100 μM cytochrome *c* or NBT or 20 μM XTT as described in Materials and Methods. The values are the average of three independent experiments for the reaction with cytochrome *c* in aerobiosis with or without SOD and of two independent experiments for the other reactions.

scavenging the hydrogen peroxide, but the expected *o*-dianisidine oxidation product was not detected.

A partial answer to this apparent inconsistency was obtained by analyzing the absorbance changes at varying FDH domain concentrations. The absorbance increase was more important at lower domain concentrations and the decrease slower ((Figure 2C). This suggests that the *o*-dianisidine oxidation product can be somehow reduced by the FDH domain. How this happens might deserve further investigation, but this is outside the scope of this work. In addition, when SOD was added to the incubation mixture at time zero, the initial absorbance increase was suppressed (Figure 2B). Altogether, the very different behavior of GOX and the FDH domain suggested that H_2O_2 was not the direct product of the reaction between E−FMNH$^-$ and oxygen, but arose from the dismutation of $O_2^{\bullet-}$. We therefore tried to directly demonstrate the formation of superoxide anion.

The reduction of cytochrome *c* by $O_2^{\bullet-}$ and its inhibition by SOD constitute the traditional test for superoxide anion production. In the present case, the inhibition was incomplete (Table 1). When the reaction was carried out in anaerobiosis, it became clear that cytochrome *c* behaves as a slow substrate for the FDH domain (eqs 1 and 2). In the holoenzyme, it is reduced only by heme b_2 at a much faster rate (as an example, the steady-state rate of cytochrome *c* reduction is on the order of 100−200 s^{-1}, depending on the temperature and buffer conditions (*7*, *35*, *43*)). Balme et al. (*36*) had already found that cytochrome *c* was a slow substrate for the Fcb2 FDH domain at a time when they were unaware of the possible superoxide anion generation by the flavin. The incomplete effect of SOD on cytochrome *c* reduction by the FDH domain can be easily rationalized. Reduced cytochrome *c* is known to autoxidize in the presence of oxygen, yielding $O_2^{\bullet-}$ (eq 3);

$$\text{E-FMNH}^- + \text{cytochrome } c^{3+} \rightarrow \text{E}-\text{FMN}^{\bullet-} + \text{cytochrome } c^{2+} + \text{H}^+ \quad (1)$$

$$\text{E}-\text{FMN}^{\bullet-} + \text{cytochrome } c^{3+} \rightarrow \text{E}-\text{FMN} + \text{cytochrome } c^{2+} \quad (2)$$

$$\text{cytochrome } c^{2+} + \text{O}_2 \rightleftarrows \text{cytochrome } c^{3+} + \text{O}_2^{\bullet-} \quad (3)$$

the latter can reduce cytochrome *c* back, and SOD prevents this back reaction by accelerating the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (*44*). We tested two other compounds known to be reduced by $O_2^{\bullet-}$, namely, NBT and XTT (*45*, *46*). Table 1 shows that they both behave as cytochrome *c*, since they act as acceptors for the reduced flavin (as shown by the experiments in anaerobiosis) and their reduction in aerobiosis is only incompletely suppressed by SOD. Altogether, these results did not enable a firm conclusion as to the production of superoxide anion by flavin autoxidation.

Table 2: Influence of Catalase and Superoxide Dismutase on Lactate Turnover by the FDH Domain with Oxygen as the Acceptor[a]

	$k_{obs} \times 10^3$ (s^{-1}) FDH	FDH + catalase	FDH + SOD	inhibition by SOD (%)
lactate consumption	45 ± 4	47 ± 7	16 ± 6	64
product formation	39 ± 5	41 ± 2[b]	14 ± 2	64

[a] The reaction was carried out with 10 μM enzyme in the presence of saturating L-lactate in air-saturated buffer at 30 °C. Aliquots were withdrawn and mixed with an equal volume of 0.4 N HCl to stop the reaction. They were then analyzed by HPLC for lactate, pyruvate, and acetate. Further details are described in Materials and Methods. The values are the average of 2−8 independent experiments. [b] Pyruvate was the only product.

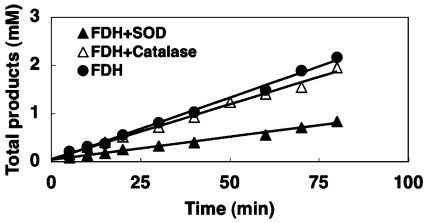

FIGURE 3: Influence of catalase and superoxide dismutase on the rates of total product formation during turnover of the FDH domain with oxygen as an acceptor. The experimental conditions are the same as those described for Figure 1.

Effect of Superoxide Dismutase on the Rate of Lactate Consumption with Oxygen as an Electron Acceptor. As mentioned above, when the FDH domain incubation with lactate was carried out in the presence of catalase, only pyruvate was formed. The rates of lactate consumption and of product formation were identical, within error, in the presence and absence of catalase. When the reaction was carried out instead in the presence of SOD, acetate was still produced, but the substrate disappearance and total product formation rates were slowed (Table 2 and Figure 3). Since, at lactate saturation, the turnover rate k_{cat} in the presence of ferricyanide at 30 °C is on the order of 250 s^{-1}, it is clear that, with oxygen as the sole electron acceptor, the slowest step during turnover is Fl_{red} oxidation, and it is this step that is affected by SOD. A likely scenario is that E−Fl_{red} reacts slowly with oxygen with production of E−Fl_{sq} and $O_2^{\bullet-}$. Superoxide then oxidizes E−FMNH$^-$ and E−FMN$^{\bullet-}$ faster than oxygen does, as happens with free flavins (*47*, *48*), and these reactions are suppressed by SOD. In this way, SOD slows the turnover in the presence of oxygen, because it slows flavin reoxidation. In contrast, when SOD was added to a glycolate oxidase and lactate solution in the presence of HRP and *o*-dianisidine, the reaction was not slowed. The initial rates of *o*-dianisidine oxidation were 19.0 ± 0.6 and 15.1 ± 0.6 min^{-1} in the presence and absence of SOD, respectively. These results thus lead us to conclude that the FDH domain does produce $O_2^{\bullet-}$ in its reaction with oxygen. The hydrogen peroxide responsible for the observed acetate formation must arise from the dismutation of $O_2^{\bullet-}$.

Table 3: Lactate Turnover by Flavocytochrome b_2 with Oxygen as the Acceptor[a]

	$k_{obs} \times 10^3$ (s^{-1})		
	Fcb2	Fcb2 + SOD	inhibition by SOD (%)
lactate consumption	54 ± 9	24 ± 2	56
product formation	58 ± 4	26 ± 4	55

[a] The experiments using 10 μM Fcb2 were carried out under the same conditions as for the FDH domain, as described in Table 2 and in Materials and Methods.

Stopped-flow experiments were carried out with the FDH domain at 452 nm (the Fl_{ox} maximum) and at 380 nm (the Fl_{sq} maximum) (*40*) to detect a possible semiquinone intermediate. The rates at the two wavelengths were identical, and no absorbance rise at 380 nm could be detected at the end of the dead time. Both rates were slowed in the presence of SOD and in that case were (12.2 ± 0.8) × 10^{-3} s^{-1} at 452 nm and (10.4 ± 2.1) × 10^{-3} s^{-1} at 380 nm, in fair agreement with steady-state rates of product formation. These results indicate that oxidation of E−Fl_{sq} by O_2 and/or by $O_2^{\bullet-}$ is much faster than the initial reaction of E−Fl_{red} with O_2, as is known for free flavin (*47*−*49*).

Turnover of Flavocytochrome b_2 with Lactate in the Presence of Oxygen as an Electron Acceptor. Since Fcb2 itself and the isolated heme b_2 domain are known to be slowly autoxidizable (*50*), the preceding results raised the following question regarding the site of reaction with oxygen in the holoenzyme: Is the reaction of the flavin with oxygen entirely suppressed when the FDH domain is incorporated in the holoenzyme?

A series of steady-state autoxidation experiments were carried out with the holoenzyme by analyzing for products and residual substrate, as for the FDH domain. Again, acetate was formed, with a time lag (not shown), and SOD decreased the rate of lactate consumption and product formation. Table 3 indicates that, with and without SOD, the rates were somewhat higher for Fcb2 than for the isolated FDH domain. If the reaction of the latter with oxygen was entirely suppressed in the holoenzyme, the steady-state rate would be that of the heme domain autoxidation, since the transfers from E−Fl_{red} and E−Fl_{sq} to heme b_2 are orders of magnitude faster than the steady-state autoxidation rates measured here (*51*, *52*).

To find out whether this was the case, we carried out stopped-flow experiments first with the isolated heme b_2 domain and then with Fcb2 itself. With the domain in the absence of SOD, a very slow absorbance increase at 557 nm after the oxidation phase suggested that superoxide could reduce the heme back. In the presence of SOD, reoxidation was monophasic, with a rate of (14 ± 2) × 10^{-3} s^{-1}, similar to the oxidation rate of the isolated FDH domain. This rate is somewhat faster than the autoxidation rate published for soluble microsomal cytochrome b_5 (2 × 10^{-3} s^{-1} at 30 °C, pH 7 (*53*)), with which the heme b_2 domain shares a strong structural similarity, including heme ligation (*4*, *53*, *54*).

Surprisingly, the heme oxidation rate in the holoenzyme, in the presence of SOD, turned out to be (122 ± 10) × 10^{-3} s^{-1}, faster than that of the isolated domain. In the presence of SOD, the absorbance increase at 438 nm, which monitors the reduced flavin overall oxidation, was monophasic, with a rate of 0.95 ± 0.13 s^{-1}. In contrast, the evolution of the semiquinone at 350 nm, an isosbestic point for the heme where the semiquinone absorbs more than both Fl_{ox} and Fl_{red} (*41*), was multiphasic. An initial rapid increase indicated Fl_{sq} formation from Fl_{red} ((17.4 ± 1.6) s^{-1}), correlated with heme reduction at 557 nm ((16.2 ± 0.3) s^{-1}) (Scheme 1). This rate is entirely limited by flavin reduction at the low substrate concentration used. The absorbance reached a maximum between about 1 and 2 s and then decreased with a rate of (270 ± 40) × 10^{-3} s^{-1}. All oxidation rates were faster in the absence of SOD.

These observations can in principle be interpreted in several ways. One could first assume that the heme oxidation rate is intrinsically higher in the holoenzyme. This assumption is however hardly tenable, since comparisons between the separated domains and the holoenzyme showed identical properties in terms of redox potentials, kinetics (for flavin reduction by lactate), and crystal structures (*4*, *9*, *24*, *36*, *40*). A reasonable explanation for this lack of difference lies in the mobility of the heme domain relative to the FDH domain tetrameric core, mentioned in the introduction. Furthermore, if the heme oxidation rate were intrinsically higher in the holoenzyme and the flavin had no access to oxygen in the holoenzyme, the holoenzyme heme oxidation rate would then be entirely limiting for Fl_{sq} disappearance, which does not appear to be the case.

The alternative hypothesis assumes that the individual reaction rates with oxygen of the two independent domains are unchanged when they are combined in the holoenzyme. The electron-transfer rates from both E−Fl_{red} and E−Fl_{sq} to oxidized heme b_2 are known to be several orders of magnitude faster than the oxidation rates found in this study (*8*, *51*, *52*). Nevertheless, the transfer from E−Fl_{sq} to heme would be dependent on the heme reoxidation, which we found to be as slow as that of the flavin in the FDH domain. This would leave an opportunity for the flavin to react with oxygen in the Fl_{sq}−$heme_{red}$ state. In turn, this would lead to a leak of electrons at the FDH domain level and would accelerate the heme return to the oxidized state in Fcb2 compared to the isolated heme b_2 domain, as is observed. This hypothesis is also consistent with the fact that, in the steady state, heme b_2 accelerates the turnover compared to that of the independent FDH domain (Tables 2 and 3); indeed, it contributes, together with oxygen, to flavin reoxidation. Altogether, the stopped-flow oxidation rates we measured are not rates of individual reactions, since electron transfers both to oxygen and between prosthetic groups are contributors.

In conclusion, our results indicate that, in the holoenzyme, both domains contribute to autoxidation, when oxygen is the sole electron acceptor. Nevertheless, the reaction with oxygen cannot take place in the presence of acceptors such as ferricyanide or cytochrome *c*, acceptors to which electron transfer is several orders of magnitude faster (*9*).

DISCUSSION

In what precedes, we have shown that GOX, an oxidase, and the Fcb2 FDH domain behave very differently in the classical test for hydrogen peroxide with HRP and *o*-dianisidine. Furthermore, SOD slows FDH turnover with oxygen as an acceptor, but has no effect on GOX turnover.

Table 4: Comparison of Rate Constants for the Reaction with Oxygen and of Redox Potentials among Members of the Family of 2-Hydroxy Acid-Oxidizing Enzymes[a]

	rate constant (M^{-1} s^{-1})	temp (°C)	ref	$E_{ox/red}$ (mV)	$E_{ox/sq}$ (mV)	$E_{sq/red}$ (mV)	ref
FDH domain	0.5×10^2	30	this work	−80[b]			*40*
MDH	8.6×10^3 [c]	20	*63*		−91		*63*
GOX	8.5×10^4	4	*72*[d]	−25	−33	−17	73[e]
LOX	1.9×10^6	25	*16*	−128	−95	−161	*74*
LMO	9×10^3	25	*42*	−149	−67	−231	*75*
LMO + pyruvate	1.8×10^6	25	*42*	−145	+80	−370	*13*

[a] The second-order rate constant for the FDH domain is derived from the turnover rate in the presence of SOD (Table 2). [b] According to ref *40*, the two midpoint potentials differ by 5−10 mV. [c] Calculated from the first-order rate constant determined at air saturation, assuming the oxygen concentration at 20 °C to be 290 μM. [d] Recombinant spinach enzyme. [e] Pig liver enzyme.

Thus, although the pyruvate oxidative decarboxylation indicates that the FDH domain turnover produces H_2O_2, the latter must arise from $O_2^{\bullet-}$ dismutation. Our results thus point to the formation of superoxide anion during the reaction of $E-Fl_{red}$ with oxygen, even though the enzyme forms an anionic semiquinone and not the neutral one expected for a dehydrogenase−electron transferase (*25*, *27*, *48*, *55*, *56*). The transfer of the first electron is the slow step, as it is for free flavin (*47*−*49*). Assuming an oxygen concentration of 220 μM in air-saturated buffer at 30 °C, a second-order rate of about 50 M^{-1} s^{-1} can be calculated for the FDH domain autoxidation from the first-order rate of its oxidation in the presence of SOD. This value is somewhat lower than that for free flavin (250 M^{-1} s^{-1} at pH 6.5 (*49*)). These results raise two related questions: What are the factors that may favor $O_2^{\bullet-}$ formation rather than H_2O_2? What are the structural features that distinguish the electron transferases from the oxidases in the family of L-2-hydroxy acid-oxidizing enzymes?

Several recent reviews discussed possible factors that may come into play in determining the differential reactivity of flavoproteins with molecular oxygen (*48*, *56*, *57*). The thermodynamic factor was considered as insufficient to rationalize the experimental results. Table 4 summarizes the known rates of reaction with oxygen and the corresponding redox potentials for several family members. The case of LMO and LOX has been discussed by Massey (*56*). Now adding the FDH domain, MDH, and GOX to the list does not contribute to a clarification.

An inspection of flavoprotein crystal structures suggested to Mattevi (*57*) that positive charges would favor the reaction with oxygen. Nevertheless, in this case, all the Fcb2 homologues present in the active site two arginines, a lysine, and a histidine which are invariant; the histidine in Fcb2 was shown to be protonated in the reduced enzyme (*58*), and this is probably also the case for the homologues, for mechanistic reasons.

Accessibility to oxygen was also discussed as a possible favorable factor, even though, as remarked by Mattevi (*57*), flavin accessibility may not be important for reactivity with oxygen, in view of its rapid diffusion rate into protein matrixes. Several structures have been published, in several oxidation states, with and without ligands, for three oxidases and two electron transferases of the enzyme family. The folds are highly similar, with the exception that part of β-barrel loop 4 is invisible for Fcb2, its isolated FDH domain, GOX, and LCHAO, due to mobility. At atomic resolution, MDH loop 4, which is responsible for attachment to the membrane, is entirely defined, but it is not the original one; it has been replaced by that of glycolate oxidase, a manipulation that rendered the protein soluble (*59*). In spite of mobility, available evidence suggests that loop 4 could form contacts with active-site residues on the flavin *si* face (*15*), and this has been recently demonstrated to be the case in the LOX crystal structure (*21*, *22*). In Fcb2, the only solvent-accessible atom of the reduced flavin in the unliganded active site is N5. The flavin accessibility to solvent in other structures, when the ligand is omitted, is also very low, whatever the reactivity with oxygen (see, for example, ref *20*).

A detailed analysis of the flavin hydrogen-bonding pattern in the various structures discloses a few differences. In Fcb2, K349 lies within H-bonding distance to ribityl 2′-OH, N1, and O2. Other hydrogen bonds of the isoalloxazine pyrimidine ring with the protein are those between O2 and the T250 hydroxyl, between N3 and the Q252 amide oxygen, and between O4 and the S228 hydroxyl (Figure 4A). These side chains are invariant in all the homologues studied to date. Furthermore, in Fcb2 the flavin forms two additional hydrogen bonds with the protein backbone. The isoalloxazine ring *re* side leans against an extended piece of peptide chain, ATA (196−198), immediately after the barrel β1 strand, with H bonds formed between the A196 peptide carbonyl and the 2′-OH as well as between the A198 peptide amide and flavin N5 (Figure 4B). All the hydrogen bonds with the flavin are maintained in the structures published to date, with two exceptions. First, in LOX, the invariant serine to O4 distance and that between N5 and the backbone appear too long for hydrogen bonds (*21*, *22*). Second, in the unliganded spinach GOX crystal structure, the isoalloxazine ring is tilted away from the protein compared to other structures (*60*). This position prevents formation of the hydrogen bonds of N3, O4, and N5 with the protein; O4 forms instead a hydrogen bond with Y129(GOX, Y254 Fcb2), and a water molecule is located close to C4a and N5 on the *re* side of the flavin in the space generated by the different flavin position (Figure 4B). It was proposed that this water molecule could be an oxygen mimick and that the flavin position would be specific to the oxidase function (*60*). Subsequently, in the structure of GOX in complex with inhibitors, the flavin was found in a position similar to that observed in the other structures (*19*), including that of liganded LCHAO (*20*). This observation led to the proposal that ligand binding to GOX and the other oxidases would be responsible for pushing the flavin back toward the backbone (*20*). But, for the time being, there is no experimental evidence that the flavin movement takes

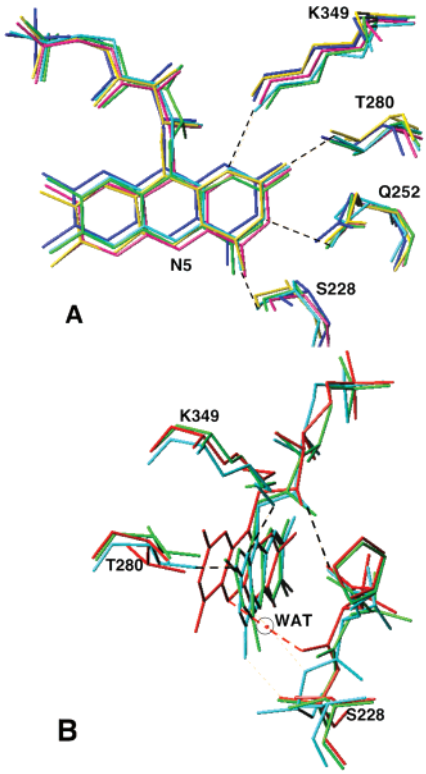

FIGURE 4: Flavin interactions with the protein. (A) Hydrogen bonds from invariant side chains to the ribityl chain and to the pyrimidine ring heteroatoms. The side chain numbering is that of Fcb2. Superposition of Fcb2 (pale blue, from 1FCB (PDB ID)), oxidized MDH (pink, from 1P4C (PDB ID)), LCHAO (yellow, from 1TB3 (PDB ID), subunit A), spinach GOX in complex with TKP (green, from 1al8 (PDB ID)), and LOX (dark blue, from 2DU2 (PDB ID), subunit A). (B) Comparison of the isoalloxazine ring orientation with respect to the backbone. Superposition of Fcb2 (blue, from 1FCB (PDB ID)), spinach GOX in complex with TACA (green, from 1al7 (PDB ID)), and free GOX (red, from 1GOX (PDB ID)). Shown is the backbone on the flavin *re* side (ATA(196−198) for Fcb2, PTA(77−79) for GOX) and its hydrogen bonds to 2′-OH and N5 in Fcb2 (black dashed lines). The invariant lysine, threonine, and serine side chains (Fcb2 numbering) form hydrogen bonds (black dashed lines) to N1, O2, and O4 in Fcb2 and liganded GOX. Q252, which is H bonded to N3 in these two structures, is not shown for the sake of clarity. In free GOX (red) the flavin movement breaks the bonds to N3, O4, and N5. The red dashed line illustrates the position of WAT394 (highlighted by a circle), halfway between N5 and the T78 carbonyl oxygen in this same structure.

place in family members other than GOX, except perhaps in LCHAO, its isozyme (see the discussion in ref *39*).

Thus, altogether, the structures of two oxidases with a free active site suggest a slightly (for LOX) or significantly (for GOX) better solvent accessibility in the oxidized flavin O4−N5 area. This accessibility does not exist when a ligand is bound in the GOX and LCHAO active sites. Since no structure exists for a reduced oxidase that could be compared to those of reduced Fcb2 and MDH (*4*, *23*, *24*), one cannot evaluate for these oxidases the interesting proposal by Wang and Thorpe (*61*) that desolvation of the active site could contribute to destabilization of the superoxide anion intermediate formed during the flavin reoxidation.

Another intriguing fact relating to the reactivity with oxygen in this enzyme family has to be mentioned. The residue that forms in some structures a hydrogen bond to N5 is often Ala, but is Gly in MDH and LMO. These residues were interchanged by site-directed mutagenesis. In LMO, the G to A mutation induced a 100-fold decrease in the reaction rate with oxygen of the free enzyme (*62*). In contrast, the same G to A mutation in MDH resulted in a 2−8-fold increase in the reaction rate with oxygen (*63*), and the inverse A to G mutation in LOX lowered this rate 8-fold (*64*). No structure exists for any of these mutant enzymes. That of the A196G mutant Fcb2 is published (*65*); it shows no significant difference with that of the WT enzyme, but to our knowledge the reactivity of this mutant enzyme with oxygen was not studied.

Enzymes that introduce a double bond adjacent to the carboxyl group in acyl-CoA derivatives of fatty acids form another evolutionary family encompassing both dehydrogenases−electron transferases and dehydrogenases−oxidases (*66*). Several structures have been published for both types of enzymes (*67*). Tokuoka et al. (*68*) suggested, from a comparison between the structures of mitochondrial medium-chain acyl-CoA dehydrogenase and that of peroxisomal acyl-CoA oxidase, that the latter had a more spacious active site which allowed easy accessibility to oxygen for the oxidative reaction. A somewhat different view was proposed by Mackenzie et al. (*69*). The comparison between the structures of glutaryl-CoA dehydrogenase and a plant short-chain-specific acyl-CoA oxidase (ACX4) led these authors to propose that a terminal loop in the oxidase would block solvent access to the FAD, preventing uncontrolled electron transfer, while at the same time a pocket behind the flavin in the C4a−N5 area would favor productive encounters with oxygen; this pocket does not exist in the glutaryl-CoA dehydrogenase. Altogether, it appears that, in the case of these enzymes, the structural data do not provide at present a clearer answer to the problem of oxygen reactivity than in the case of the L-2-hydroxy acid-oxidizing enzymes. Adding to the complexity of the situation, it should be recalled at this point that medium-chain acyl-CoA dehydrogenase, which, in the absence of active-site ligand, reacts with oxygen an order of magnitude faster than the Fcb2 FDH domain (*70*), is reported to form a neutral semiquinone, as expected (*71*), but yields essentially hydrogen peroxide, rather than superoxide anion (*61*).

In conclusion, the Fcb2 flavodehydrogenase domain remains an unusual dehydrogenase−electron transferase. Whether separated from the heme domain or linked to it, it forms an anionic semiquinone as related oxidases do, but reacts slowly with oxygen in the absence of other acceptors, with formation of superoxide anion. Analysis of available crystal structures of homologues, electron transferases and oxidases, does not give a clue as to the structural distinction between the two functions. An understanding of the electrostatic environment of the flavins and of the protein dynamics might shed light on the question.

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