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Rapid Kinetic Studies of Partial Reactions in the Heme Free Derivative of L-Lactate Cytochrome *c* Oxidoreductase (Flavocytochrome b_2); the Flavodehydrogenase Function†

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ABSTRACT: The flavodehydrogenase, the "heme-free" derivative of flavocytochrome b_2 (L-lactate cytochrome *c* oxidoreductase, EC 1.1.2.3, present in aerobic yeast), has been successfully reconstituted from the apoprotein. Stopped-flow experiments show that the behavior of this flavodehydrogenase toward the substrate L-lactate is indistinguishable from that of the flavocytochrome b_2 containing its heme groups (both have the same lactate K_m and V_m values). In contrast, its behavior with a one-electron acceptor such as ferricyanide is very different: the apparent bimolecular rate constant for the reoxidation of the fully reduced enzyme by ferricyanide is 200 times lower for the flavodehydrogenase than for the flavocytochrome b_2 itself. Analysis of the reoxidation by ferricyanide of the reduced flavodehydrogenase in terms of two successive steps of one-electron transfer indicates that this acceptor reacts at least 20 times faster with the flavin semiquinone than with the hydroquinone. The reactions of ferricyanide with native flavocytochrome b_2 and its heme-free derivative are compared and discussed to shed light on the role of the heme within that multicomponent enzyme. The role of the cytochrome b_2 moiety when the enzyme reacts with an acceptor such as ferricyanide could be to act as the best acceptor of one electron from the substrate-reduced flavin, and allow the transformation of a poorly reactive electron donor (flavin hydroquinone) into two much more reactive one-electron donors (ferroheme and flavin semiquinone). The cytochrome b_2 moiety acts as the specific donor to cytochrome *c*. The assumption that heme does not modify the kinetic parameters of the flavodehydrogenase, but simply, by its presence, adds new possibilities, especially when cytochrome *c* is the acceptor, is discussed.

During the last 20 years, yeast mitochondrial flavocytochrome b_2 or L-lactate cytochrome *c* reductase has been the object of numerous investigations which have shed light on several facets of its structure and function. Catalysis leads to electron transfer from L-lactate to various acceptors, such as ferricyanide, dyes, and cytochrome *c*, the latter being its natural acceptor. The specific role of each prosthetic group—flavin mononucleotide and protoheme—in the catalytic function and the interactions between them are very intriguing problems. The protoheme and flavin groups, because of their much higher redox potentials, can both be completely reduced by L-lactate (Baudras, 1965c; Iwatsubo et al., 1968; Capeillère-Blandin et al., 1975); the enzyme then accepts a total of three electrons per protomer, i.e., 12 for the stable active tetramer. Both kinds of prosthetic groups are quantitatively reoxidized by external acceptors (Morton and Sturtevant, 1964). Thus a priori, both are able to take part directly in the transfer of the electrons from L-lactate to the external acceptors during the catalytic reaction.

To elucidate the role of each prosthetic group in the overall electron transfer, two experimental approaches were used. The first approach consisted of detailed kinetic studies of the holo(FMN[1]-heme)enzyme. Combined stopped-flow absorbance and rapid freezing EPR experiments were carried out on the

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[1] Abbreviations used are: F_{HQ}, flavohydroquinone; F_{SQ}, flavosemiquinone; F_{ox}, flavoquinone; H_{ox}, oxidized heme; H_{red}, reduced heme; EDTA, (ethylenedinitrilo)tetraacetic acid; FMN, flavin mononucleotide.

"crystalline, type II", enzyme from bakers' yeast by Capeillère-Blandin et al. (1975). These authors advanced strong experimental evidence supporting the following scheme. In step 1 after the first round of L-lactate binding (one per protomer), two electrons are transferred from this bound substrate to the flavin which becomes fully reduced (hydroquinone). In step 2, one of the electrons is then accepted by one prosthetic heme of the same enzyme molecule, generating one reduced heme and one flavin semiquinone per protomer. The slower of these two steps is step 1, i.e., the intramolecular two-electron transfer from L-lactate to flavin within the Michaelis enzyme–substrate complex, which has a rate constant of 110–120 s^{-1} at 24 °C. The four protomers apparently have the same reactivity. The rate constant of step 2, i.e., the intramolecular one-electron transfer from reduced flavin to heme, is much higher. Values ranging from 400 to 1200 s^{-1} at 24 °C were proposed by Capeillère-Blandin (1975) as being necessary to obtain a "satisfactory" simulation of stopped-flow recordings of both the flavin and the heme reduction time courses. A better estimate now seems to be 800 s^{-1} at 15 °C (Iwatsubo, unpublished data).

In the absence of external acceptors, these steps are followed by slower steps, possibly involving a disproportionation between two semiquinones, regenerating two oxidized flavins per tetramer and a second round of binding of two additional lactate molecules required to fulfill total reduction.

In the presence of external acceptors, the important question is: which of these sequential steps are involved in the catalytic process? The only answers given by that study were: (1) The two first steps leading to the formation of semiquinone and reduced heme are certainly involved. However, the slow steps leading to total reduction (5 s^{-1}) are certainly not, being much too slow. The enzyme does not turn over as an acceptor of three electrons but rather of two electrons per protomer. (2) Various one-electron intramolecular exchanges between pairs of flavins, or between heme and flavin, might be involved. In conclusion, this study did not yield any information on which form(s) (flavohydroquinone, flavosemiquinone, or reduced heme) is(are) the favored donor(s) to the one-electron external acceptors such as ferricyanide and ferricytochrome *c*.

The second approach consisted of attempts to prepare flavin- and heme-free derivatives and to study their functional characteristics by means of steady-state and rapid kinetic investigations. It was shown that, when the flavin group is removed (the whole protein moiety being conserved), the enzyme loses its catalytic activity and, furthermore, the heme group can no longer be reduced by lactate (Baudras, 1965a). These two properties are restored upon specific flavin rebinding. These studies demonstrated the essential role of the flavin in the catalytic process, but did not unambiguously demonstrate that flavin is the first electron acceptor. The bindings of FMN and lactate were indeed found to be highly cooperative (Baudras, 1965b; Iwatsubo and di Franco, 1965), and the lack of lactate binding when flavin is absent (Capeillère-Blandin, 1974) could account for the lack of electron transfer from lactate either to heme or to external acceptors.

The removal of the heme groups required much more drastic treatment. After a number of unsuccessful attempts using conventional methods, treatment with guanidine hydrochloride was found to result in removal of the heme and flavin with reasonable reversibility (Mével-Ninio et al., 1971). With a series of reconstituted flavo-derivative samples containing various amounts of heme (10–100%), Forestier and Baudras (1971) found a linear relationship between heme content and activity. Extrapolation to [heme] = 0 indicated that the heme-free flavo derivative had completely lost its cytochrome *c* reductase activity but retained a part of its ferricyanide reductase activity. The prosthetic heme, therefore, appeared to be essential for transfer to cytochrome *c* but not to ferricyanide.

In a parallel investigation, we succeeded in preparing an apoprotein containing all of the original protein moiety but devoid of both prosthetic groups. This apoprotein could be largely reactivated by incubation with protoheme and FMN (Mével-Ninio et al., 1971). In the present study, we describe the preparation of a flavo derivative devoid of the heme group, obtained by renaturation of the apoprotein in the presence of FMN only. A detailed comparison of the characteristics of the flavocytochrome enzyme and of the heme-free flavoprotein was undertaken by steady-state and rapid kinetic methods. The results are reported and discussed here.

Experimental Procedures

Enzymes

Flavocytochrome b_2, termed "cleaved", DNA-free, was prepared from commercial bakers' yeast according to Spyridakis et al. (1971) and stored at 0 °C as an ammonium sulfate precipitate. This procedure is a slight modification of the original methods of Appleby and Morton (1959) and Morton and Shepley (1961). This type of enzyme has suffered a modification in the course of its preparation (Somlo and Slonimski, 1966) due to a proteolytic cleavage (Jacq and Lederer, 1972).

Apoprotein. After centrifugation of the suspension of flavocytochrome b_2 in ammonium sulfate, the precipitate was dissolved in 3 mL of 0.1 M phosphate, pH 7.2, 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol and 20 μM EDTA, and dialyzed for 3 h against the same buffer at room temperature. Under these conditions, the protoheme dissociated from the protein and crystallized. It was removed by a centrifugation at 27 000*g* for 15 min. Residual protoheme and FMN were removed by sieving the supernatant (3 mL) through a Sephadex G-25 column (1.5 × 10 cm) equilibrated with buffer identical to the above buffer except that the concentration of guanidine hydrochloride was 3 M.

Flavoprotein. The apoprotein solution was then mixed with a tenfold excess of FMN (Sigma) and dialyzed for 3 days against several changes of 2 L of 0.15 M phosphate, pH 7.2, 50 mM lactate, 0.1 M β-mercaptoethanol, and 20 μM EDTA buffer at 4 °C under nitrogen in the dark. Since FMN passes slowly through the dialysis bag, the binding of the flavin to the apoprotein takes place before excessive dilution of the flavin by the dialysis buffer. After 3 days, a colorless flavoprotein (which is in the reduced state because of the presence of lactate) is obtained at about 40 μM concentration. This flavoprotein is stable and can be kept for 3 weeks at 4 °C without changes in its catalytic properties. When oxidized, however, it cannot be kept more than 1 day without inactivation.

Before each series of experiments, a 2-mL sample was passed through a 1.5 × 10 cm Sephadex G-25 column equilibrated with 0.1 M phosphate, pH 7.2, 20 μM EDTA to eliminate the lactate and β-mercaptoethanol, as well as unbound flavin. The flavoprotein was immediately oxidized by air to the fully oxidized form. The protein concentration was calculated from the tryptophan content measured fluorometrically in 6 M guanidine hydrochloride as described by Mével-Ninio et al. (1971) and Pajot (1976).

Flavin (bound, free, total) contents were measured fluorometrically; bound flavin in flavocytochrome b_2 is not fluorescent (Iwatsubo and di Franco, 1965). The comparison of fluorescence intensities of two aliquots, one diluted in 0.1 M

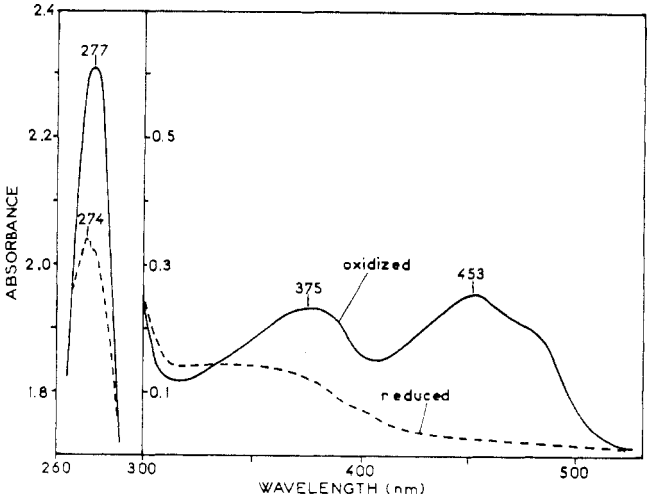


FIGURE 1: Absorbance spectra of the flavoprotein. The reconstituted flavoprotein was prepared as described under Materials and Methods. Concentrations of bound flavin and apoprotein protomers were 22 and 35 μM. After recording the spectrum of the fully oxidized form, the solution was reduced by L-lactate (30 μL of a 600 mM solution was added to 850 μL of the flavoprotein solution in 0.1 M phosphate, pH 7) and the spectrum of the fully reduced form was recorded.

phosphate buffer, pH 7.2, and the other one in 6 M urea (after full dissociation), provides the (free)/(total) ratio in the diluted sample. The total flavin content is obtained from a calibration curve either with commercial FMN (Sigma) which has a fluorescence of 97% as compared with that of pure riboflavin (five times crystallized, Wako Pure Chem. Ind. Ltd, Osaka, Japan) (a confirmation of Wassink and Mayhew (1975)) or directly with pure riboflavin.

Methods

Rapid kinetic measurements were carried out in a modified commercial stopped-flow apparatus (Durrum-Gibson) equipped with a Tracor NS 570 digital signal averager (12 bits, 1024 points) using a Tektronic memory oscilloscope as monitor. The recorded kinetic results were plotted on a fast response XY recorder. For absorption measurements, a calibrated stainless-steel cuvette of 19-mm optical path length was used. The dead time of this observation chamber was 1.8–2.0 ms when the pneumatic actuator was used at the pressure of 5 bars. All experiments were carried out at 15 ± 0.1 °C.

Steady-state kinetic measurements were performed on a spectrophotometer (Zeiss MQ II) combined with a Sargent log-scale recorder.

The simulation studies for the transient kinetic experiments were carried out using a Wang 2200 laboratory computer by numerical integration of the appropriate set of differential equations corresponding to the reaction scheme indicated in the text.

The circular dichroic measurements were performed on a dichrograph II (Roussel-Jouan). Absorption spectra were recorded with a Cary Model 15 spectrophotometer. Fluorescence emission spectra were recorded with a grating spectrofluorimeter described by Iwatsubo and di Franco (1965). The temperature was kept at 22 ± 0.2 °C during these measurements.

Results

Characteristics of the Flavoprotein Reconstitution. In previous studies, it was found that heme binding to the apoprotein takes place with a 100% yield by simply diluting the 6 M guanidine denaturing medium sixfold (Mével-Ninio et al., 1971). Under such conditions, FMN does not rebind at all. A long renaturing dialysis in the presence of FMN at 4 °C, as described under Methods, gives the best yield of binding: after 3 days, it was about 65%. In sedimentation studies, the flavoprotein was shown to behave as a homogeneous system with a $s_{20,w} = 7$. This value, similar to the value found for flavocytochrome b_2 itself (Armstrong et al., 1963), suggests that the flavoprotein probably has the same quaternary structure.

Optical Properties. The absorption spectrum of the oxidized flavoprotein in the 300–500-nm range is typical (Figure 1). Maxima are located at 375 and 453 nm with a shoulder at 480 nm. The extinction coefficients (in mM^{-1} cm^{-1}) of the bound flavin have been calculated for six different flavoprotein preparations: at 453 nm, ϵ_{ox} 11.1 ± 0.5, ϵ_{HQ} 1.1 ± 0.2; at 375 nm, ϵ_{ox} 10.6 ± 0.5, ϵ_{HQ} 5.9 ± 0.5; at 470 nm, ϵ_{ox} 9.8 ± 0.5, ϵ_{HQ} 1.08 ± 0.2; at 490 nm, ϵ_{ox} 7.2 ± 0.4, ϵ_{HQ} 0.9 ± 0.2. These last two wavelengths were used for the experiments of reoxidation of the fully reduced enzyme by ferricyanide as described later. The other values of interest are those at 438.5 nm: where $\epsilon_{ox} = 10.2 \pm 0.5$ mM^{-1} cm^{-1}, $\epsilon_{HQ} = 1.5 \pm 0.3$ mM^{-1} cm^{-1}. This wavelength corresponds to one of the isosbestic points of the bound heme in the redox titrations of flavin-free derivatives of flavocytochrome b_2. It is used as observation wavelength to observe the redox changes of the bound flavin in flavocytochrome b_2 (Iwatsubo et al., 1968).

We observed the formation of small quantities of red semiquinone (<10%) during anaerobic oxidative titration of the fully reduced flavoprotein derivative with ferricyanide (Iwatsubo and Risler, unpublished results). However, we could not determine the value of the extinction coefficient because of the very low concentration of the semiquinone. On the other hand, an EPR signal characteristic of red semiquinone (bandwidth of 14 G) was detected in the course of anaerobic photoreduction of the flavoprotein in the presence of 50 mM EDTA (Capeillère-Blandin, unpublished results).

The extinction coefficient values of the pure red semiquinone at 470 and 490 nm, which were used in the calculation of the simulated curve presented in Figure 4, were those established for L-amino acid oxidase (Massey and Curti, 1968), D-amino acid oxidase (Yagi, 1975), and the uncomplexed Old Yellow Enzyme (Matthews and Massey, 1971): i.e., at 470 nm ϵ_{SQ} = 4.3 mM^{-1} cm^{-1}, and at 490 nm ϵ_{SQ} = 4.7 mM^{-1} cm^{-1}.

Circular dichroism spectra were recorded. The absorption band of the oxidized form at 375 nm has a negative ellipticity $\theta = -3 \pm 0.5 \times 10^4$ deg·cm^2/dmol of bound flavin. In the reduced form, this CD band is centered at 380 nm with an ellipticity $\theta = 2 \pm 0.5 \times 10^4$ deg·cm^2/dmol. The absorption band at 453 nm has an ellipticity at least four times lower. These results are indistinguishable from those obtained for native flavocytochrome b_2 (Iwatsubo and Risler, 1969). Note that the signs of the ellipticities are the same as in free flavin (Yamaji et al., 1968; Edmonson and Tollin, 1971).

The fluorescence of flavin excited at 450 nm is completely quenched (>98%) in the flavo derivative as it was found in flavocytochrome b_2 (Iwatsubo and di Franco, 1965). This means that flavin quenching in flavocytochrome b_2 is due to the binding to the protein and not to an interaction with the heme group. A fluorometric study of the dissociation of bound flavin as a function of flavoprotein concentrations indicated that the dissociation constant of the reconstituted flavoprotein was $K_d \leq 10^{-8}$ M.

A heme-free flavo derivative of flavocytochrome b_2 has already been described (Morton and Shepley, 1963). This derivative lacked dehydrogenase activity and its flavin was fully fluorescent. In light of the present results, it seems likely that the flavin was dissociated or the protein was denatured.

TABLE I: Kinetic Parameters of the Flavoprotein Derivative. Comparison with Flavocytochrome b_2.[a]

		Flavoprotein	Flavocytochrome b_2
Steady-State Studies			
Rate under stand. assay conditions (L-lactate)$_\infty$			
With cyt c	v	$\leqslant 2\ s^{-1}$	$90\ s^{-1}$
With ferricyanide	v	$70\ s^{-1}$	$110\ s^{-1}$
Max. rates at (L-lactate)$_\infty$ and (ferricyanide)$_\infty$, in electron-equiv mol^{-1} s^{-1}	V_m	$100 \pm 10\ s^{-1}$ [b]	$125\ s^{-1}$
For ferricyanide	$K_{1/2}$ exptl	0.5 mM	0.011 mM[c], 0.005 mM[d]
	$K_{1/2}$ calcd	0.47–0.5 mM	
Stopped-Flow Studies			
Reduction by L-lactate			
Lactate binding	k_1	$\geqslant 2.2 \times 10^5\ M^{-1}\ s^{-1}$ *	$1.1 \times 10^5\ M^{-1}\ s^{-1}$ [e]
Lactate dissociation	k_{-1}	$\geqslant 200\ s^{-1}$ *	$\geqslant 100\ s^{-1}$, $\leqslant 250\ s^{-1}$ [f]
Lactate-FMN transfer, $k_{red,max}$	k_2	$64\ s^{-1} - 70\ s^{-1}$ *	$70\ s^{-1}$ [d]
For L-lactate	$K_{1/2}$	1.1 mM	0.8 mM[g]
Reoxidation of reduced enzyme			
bimolecular rate constants			
FMN by ferricyanide			
Overall reaction	k_{ox}'	$1.1 \times 10^5\ M^{-1}\ s^{-1}$	
Oxidn of $FMN_{HQ} \rightarrow FMN_{SQ}$	k_3'	$1.1 \times 10^5\ M^{-1}\ s^{-1}$ *	
Oxidn of $FMN_{SQ} \rightarrow FMN_{ox}$	k_4'	$\geqslant 20 \times 10^5\ M^{-1}\ s^{-1}$ *	
Heme			
By ferricyanide	k_5'		$1 \sim 2 \times 10^7\ M^{-1}\ s^{-1}$ [h]
By cytochrome c			$0.5 \times 10^7\ M^{-1}\ s^{-1}$
First-order (or apparent) rate constants			
FMN_{HQ} by 1 mM ferricyanide	(1 mM) × (k_3')	$110\ s^{-1}$	
FMN_{HQ} by prosth. Heme	k_H		$800\ s^{-1}$ * [d]
FMN_{SQ} by 1 mM ferricyanide	(1 mM) × (k_4')	$\geqslant 2000\ s^{-1}$	
$Heme_{red}$ by 1 mM ferricyanide	(1 mM) × (k_5')		10 000–20 000 s^{-1} [h]

[a] Unless otherwise mentioned, all kinetic data were obtained at 15 °C, pH 7.2, in 0.1 M phosphate buffer. The symbols for rate constants are those used in Schemes I, II, and III, and in the text. The results listed for the flavoprotein are those established in the present work; those adjusted with the help of simulation studies (cf. Figures 2 and 4) are indicated by an asterisk. [b] Value estimated by extrapolation from a range of ferricyanide concentrations small enough to eliminate the inhibition by excess ferricyanide. [c] Hiromi and Sturtevant (1966) under different conditions. [d] Precise experimental reinvestigations at 15 °C (which showed a lag of 1.3–1.5 ms for the heme reduction) with improved simulation studies led recently to these estimates (Iwatsubo, unpublished results). They are considered more precise than previous determinations in our laboratory (Capeillère-Blandin, 1974). [e] Results from transient kinetic studies at low lactate concentration, 25 °C (Iwatsubo and Capeillère, 1967). [f] Results from steady-state kinetics at 20 °C (Blazy et al., 1976). [g] Results from transient kinetic studies (Capeillère-Blandin, 1974). [h] Iwatsubo et al. (1968).

Kinetic Studies

Activities in the L-Lactate External Acceptor Test. The activities of the flavoprotein and of flavocytochrome b_2 are presented in Table I. The flavoprotein has no activity at all when cytochrome c is the acceptor, and the activity is 64% that of the initial flavocytochrome when ferricyanide is the acceptor. The conditions of the assays, however, are those devised for flavocytochrome b_2 itself, and the ferricyanide concentration (0.66 mM) is no longer saturating for the flavoprotein. Lineweaver-Burk plots indicate apparent saturation with increasing ferricyanide concentrations: the extrapolated V_m = $100 \pm 10\ s^{-1}$ (electron-equiv/mol of FMN) and $K_{m,ferricyanide}$ = 0.5 mM at 15 °C. For flavocytochrome b_2, under the same conditions, $K_{m,ferricyanide}$ = 0.005 mM and V_m = $125\ s^{-1}$ (Iwatsubo et al., unpublished results).

At first sight, it seems that the difference between the steady-state kinetic parameters of the flavocytochrome b_2 and of the flavoprotein derivative lies essentially at the level of the $K_{m,ferricyanide}$ values. It should be emphasized, however, that, in complex-catalyzed reactions involving two substrates and an electron donor and acceptor, the meaning of V_m and K_m is not simple. In particular, $K_{m,acceptor}$ generally is not a dissociation constant; a finite value does not imply the formation of a Michaelian complex. Rapid kinetic studies, reported below, of the partial reactions of enzyme reduction by lactate and enzyme reoxidation by ferricyanide will permit better interpretation of steady-state data. These studies will also show that the major modification resulting from the absence of the heme lies at the level of the overall apparent rate constant of the bimolecular electron exchange between free ferricyanide and the reduced enzyme, either flavoprotein or flavocytochrome (see Discussion).

Stopped-Flow Studies of the Partial Reactions

Reduction. After mixing the oxidized flavoprotein with L-lactate, the reduction apparently takes place as a single exponential process (Figure 2). The first-order plot (log ($A_t - A_\infty$) as a function of time) shows neither a lag nor an initial burst; extrapolation to time zero ($A_{0,ext}$) corresponds to the level of absorbance, A_0, of controls without lactate. Furthermore, the total absorbance change corresponds to that expected for the total reduction of the flavin (within 4%) to the fully reduced form. The accuracy of the determinations of A_0 and $A_{0,ext}$ values, however, is not very good (±5%) due to the relatively low flavoprotein concentrations we used in this particular experiment. Consequently, we are only able to conclude that (1) any possible lag should be shorter than 2 ms, (2) a possible burst should correspond to less than 5% of the absorbance change in the first rapid phase, and (3) a possible amount of slow-reacting material should correspond to less than 15% of the total absorbance change.

The apparent first-order reduction constant, $k_{red,app}$, measured at different L-lactate concentrations, exhibits a slightly upward concave relationship in the double-reciprocal plot:

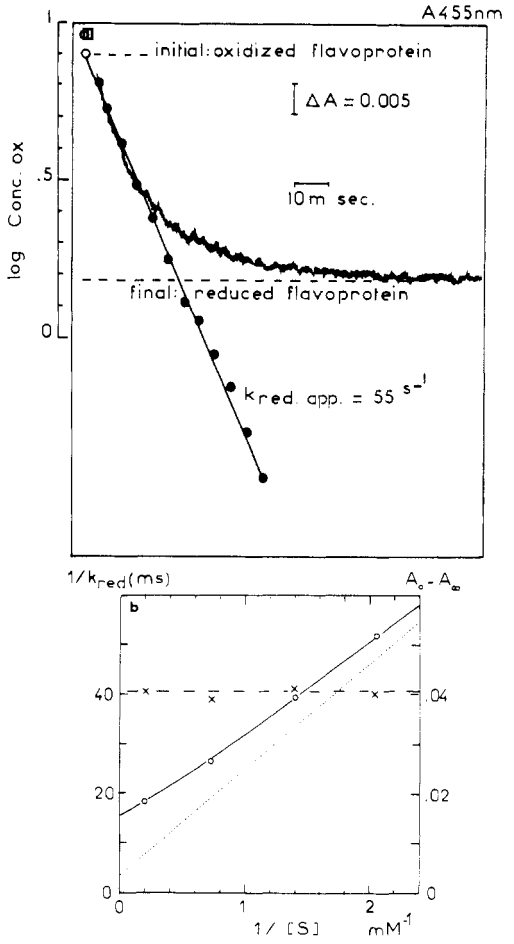


FIGURE 2: Reduction of the oxidized flavoprotein by L-lactate at 15 °C. (a, top) Oscilloscopic trace at 455 nm and first-order plot giving k_{red}. After mixing in the stopped-flow apparatus, the final concentrations were 3.3 μM bound FMN, 5 mM L-lactate, 0.1 M phosphate, pH 7.2, 20 μM EDTA. The first-order plot is traced taking $(A_0 - A_t)$ as the value of the concentration of oxidized flavoprotein at time t. Its slope multiplied by 2.3 gives $k_{red,app} = 55\ s^{-1}$ under these conditions. (b, bottom) Dependence of $k_{red,app}$ on the concentration of L-lactate. (O) Values of $k_{red,app}$ were determined as just described, at least two times, at six different L-lactate concentrations (5, 1.37, 0.71, 0.48, 0.15, and 0.05 mM). In these determinations, the value of $A_0 - A_\infty$ ((- -x- -) with the absorbance scale presented at right) always remained within the range 0.040 ± 0.0015. (—) Theoretical curve derived from the differential equations corresponding to Scheme I (see text) using the following values for the rate constants: $k_{+1} = 220\ mM^{-1}\ s^{-1}$, $k_{-1} = 200\ s^{-1}$, and $k_2 = 64\ s^{-1}$. Linear extrapolation for 1/[S] → zero, of the plot: $1/k_{red,app}$ as a function of 1/S, as established at very low substrate concentrations (between 0.15 and 0.05 mM). This part of the plot is not presented in this figure because it is too far off the scale.

$1/k_{red,app}$ as a function of 1/[L-lactate] in the range explored (Figure 2b). The extrapolated maximal value of k_{red} varies from 64 to 70 s^{-1} at 15 °C with three different flavoprotein preparations. The concentration giving half-saturation, $K_{1/2}$, = 1.1 mM.

The interpretation of such data must take into account the considerations and formulations developed by Strickland et al. (1975); in a two-step reaction, such as that presented in Scheme I, the observable time course (the absorbance change due to the formation of the final product F_{HQ}) should present a lag under certain conditions. Indeed, the reactions studied obey the reversible two-step Scheme I.

SCHEME I

$$F_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} S \cdot F_{ox} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} P \cdot F_{HQ}$$

In our case, the second step can be considered irreversible; i.e., k_{-2} is negligible; this assumption is supported by the marked difference in the redox potentials of the bound flavin in flavocytochrome b_2 (−50 mV) (Capeillère-Blandin et al., 1975) and of the lactate-pyruvate system (−190 mV) (Labeyrie et al., 1960; Burton and Wilson, 1953).

To give the best interpretation of our experimental data, we tried to simulate them, using for k_2 the experimentally determined value of 64 s^{-1}. The simulation studies indicated the following requirements for a correct fitting of all the data (absence of lag and correct double-reciprocal relationship). (a) The value taken for k_2 cannot be modified at all (±1 s^{-1}). (b) With the published values of $1.1 \times 10^5\ M^{-1}\ s^{-1}$ for k_1 (Hinkson and Mahler, 1963; Iwatsubo and Capeillère, 1967; Blazy et al., 1976) and of 80 s^{-1} for k_{-1} according to Hinkson and Mahler (1963), both established for flavocytochrome b_2, the simulated time course presented a lag of 4 ms for 1.3 mM L-lactate. Since the experimental first-order plot showed no lag outside the 2-ms dead time, this set of k_1 and k_{-1} values is not correct. With smaller values of k_{-1}, in particular with $k_{-1} = 3\ s^{-1}$ (Iwatsubo and Capeillère, 1967) the fit of the data is even less satisfactory. (c) In order to decrease the initial lag to less than 2 ms for a concentration of substrate close to the $K_{1/2}$ value, the value of k_{-1} must be larger than 200 s^{-1}. Blazy et al. (1976), to explain quantitatively the kinetic behavior of oxalate as an inhibitor under steady-state conditions, estimated for k_{-1} lower and upper limits of 100 and 250 s^{-1} at 20 °C. When the value $k_{-1} = 200\ s^{-1}$ is introduced into the simulation, we can derive a best estimate for k_{+1} of $2.2 \times 10^5\ M^{-1}\ s^{-1}$. However, k_{-1} and k_{+1} can be increased at least three to four times if their ratio remains constant, but for higher values the double-reciprocal plot becomes linear. (d) With the set of values proposed in c, $k_1 = 2.2 \times 10^5\ M^{-1}$, $k_{-1} = 200\ s^{-1}$, $k_2 = 64\ s^{-1}$, the double-reciprocal plot, $1/k_{red,app}$ as a function of 1/(L-lactate), was actually nonlinear. $k_{red,max}$ extrapolated from data between 0.5 and 1.3 mM L-lactate is overestimated by about 40%. The value extrapolated from 1.3 to 5 mM lactate, however, was not far from the correct one.

We therefore can conclude that the presence of the heme on the enzyme does not significantly affect the L-lactate dehydrogenase kinetic parameters, i.e., the L-lactate dehydrogenase function on the substrate side, since neither the intramolecular rate constant of bound lactate to bound flavin transfer $k_{red,max}$ (that is k_2) nor the $K_{1/2,L\text{-lactate}}$ are significantly different in the flavocytochrome and in its flavoprotein derivative. Both values are very close to those found for flavocytochrome b_2 under the same conditions, i.e., 70 s^{-1} and 0.8 mM, respectively (Capeillère-Blandin (1974) and Iwatsubo et al., unpublished results).

Reoxidation. We will now consider the reoxidation of fully reduced flavoprotein by ferricyanide. In this study, the reduced flavoprotein was obtained as described in the legend to Figure 3. We chose 470 and 490 nm as observation wavelengths where the relative contributions of the oxidized and semiquinone forms, in the total absorbance change, are different. Both wavelengths lie outside the absorption band of ferricyanide (maximum at 420 nm) and away from the isosbestic points between oxidized and red semiquinone flavin, near 400 and 500 nm.

After mixing with ferricyanide, an apparent first-order time course was also observed (Figure 3). The semilogarithmic plots extrapolate to initial levels (within 3%) for time zero, without a detectable lag or absorbance burst. The linearity extends to 93% of the total absorbance change expected for the quantitative production of the fully oxidized form. The slope of the first-order plot (in natural logarithms) gives $k_{ox,app}$. The linear

relationship between $k_{ox,app}$ and ferricyanide concentrations, the slope of which is $k_{ox}' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$, indicated bimolecular reactions without saturation behavior up to 1 mM ferricyanide.

Characteristics of the Two Successive One-Electron Steps. The oxidation of the reduced flavoprotein by ferricyanide has to be interpreted as two successive irreversible one-electron steps yielding a flavosemiquinone as an intermediate (Scheme II). If k_3' and k_4' represent the second-order rate constants for the first and second steps, respectively, multiplied by the ferricyanide concentration, they yield the apparent first-order rate constants $k_3 = k_3'$[ferricyanide] and $k_4 = k_4'$[ferricyanide].

SCHEME II

$$F_{HQ} \xrightarrow{k_3} F_{SQ} \xrightarrow{k_4} F_{ox}$$

We shall derive the relative absorbance time course corresponding to Scheme II, that is, to a reaction scheme involving two successive irreversible steps. The general analytical solution of such a scheme was established by Rodiguin and Rodiguina (1964) in terms of the instantaneous concentrations of each species, C_0, C_1 and C_2, as a function of the initial concentration $C_0°$ and of the rate constants involved in the two processes. Using our own notations, it would be written:

$$\frac{[F_{HQ}]_t}{[F_{tot}]} = 1 - e^{-k_3 t}$$

$$\frac{[F_{SQ}]_t}{[F_{tot}]} = \frac{k_3}{(k_4 - k_3)}(e^{-k_3 t} - e^{-k_4 t})$$

$$\frac{[F_{ox}]}{[F_{tot}]} = 1 - \frac{[F_{SQ}] + [F_{HQ}]}{[F_{tot}]}$$

Expressing the instantaneous absorbance as the sum of the three contributions,

$$A_t = \epsilon_{HQ}[F_{HQ}]_t + \epsilon_{SQ}[F_{SQ}]_t + \epsilon_{ox}[F_{ox}]_t$$

we derive the general equation:

$$\frac{\Delta A_t}{\Delta A_{tot}} = \frac{(A_t - A_0)}{(A_\infty - A_0)} = 1 - Xe^{-k_3 t} - Ze^{-k_4 t} \qquad (1)$$

under conditions where $k_3 \neq k_4$:

$$X = \frac{k_4}{(k_4 - k_3)}\left(1 - \frac{k_3}{k_4}\frac{(\epsilon_{SQ} - \epsilon_{HQ})}{(\epsilon_{ox} - \epsilon_{HQ})}\right)$$

$$Z = -\frac{k_3}{(k_4 - k_3)}\left(\frac{(\epsilon_{ox} - \epsilon_{SQ})}{(\epsilon_{ox} - \epsilon_{HQ})}\right)$$

Because of the formulation used, this equation is meaningless when $k_3 = k_4$. The solution in this particular case is:

$$X = k_3 t\left(1 - \frac{(\epsilon_{SQ} - \epsilon_{HQ})}{(\epsilon_{ox} - \epsilon_{HQ})}\right) \text{ and } Z = 1 \qquad (2)$$

Theoretically, the variation of absorbance as a function of time is never a single exponential process from time zero to the end. However, it becomes so in certain cases so that the form of the semilog plot near time zero may be informative of the ratio of the rate constants involved.

There are several solutions which will give a single exponential decrease from time zero with Scheme II. Two of them are mathematical solutions of the equation; they are $X = 0$ or $Z = 0$, implying a particular relationship between ϵ and k values, and therefore giving k_3 or k_4 values appearing to depend on the observation wavelength. These are artifactual solutions. The interesting solutions are those affording an estimation of the k_3/k_4 ratio; they correspond to the fact that one of the exponential terms in eq 1 becomes, with a certain k_3/k_4 ratio, negligible as compared to the others.

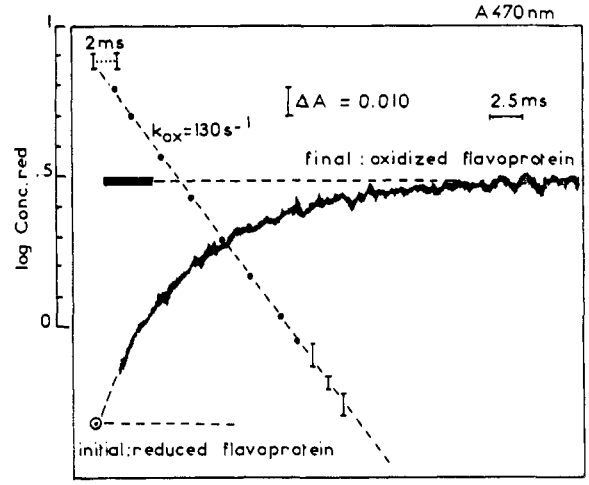


FIGURE 3: Reoxidation of the reduced flavoprotein by ferricyanide at 15 °C. Oscilloscopic trace at 470 nm and first-order plot (arbitrary units). The reduced flavoprotein solution introduced into the stopped-flow syringe was prepared by first diluting the stock solution with 0.1 M phosphate, pH 7.2, 20 μM EDTA solution, both solutions being deaerated with a water pump, and then adding L-lactate (50–150 μM). This small excess of lactate is required to avoid a partial reoxidation by the residual oxygen dissolved in the solution. Under such conditions, the apparent rate constant for the reduction of the flavoprotein by lactate is smaller than 5 s^{-1} so that this process does not disturb the observation of the reaction of reoxidation by ferricyanide. The final concentrations of bound FMN and ferricyanide after mixing in the stopped-flow apparatus were 4.6 μM and 1.1 mM. The first-order plot is traced taking $(A_\infty - A_t)$ as the value of the concentration of reduced flavoprotein at time t. Its slope multiplied by 2.3 gives $k_{ox,app}$. The same experiment has been carried out varying only the ferricyanide concentration from 33 μM to 1.1 mM and adapting the length of the optical path. Values of $k_{ox,app}$ were found to be proportional to ferricyanide concentration over the whole range explored; their ratio, $k_{ox}' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$.

In order to understand the reoxidation of the flavodehydrogenase by ferricyanide, we tried to determine the limiting step in this reaction and thus to distinguish among the three cases, $k_3 \sim k_4$, $k_3 \ll k_4$, $k_3 \gg k_4$, and also to approximate the k_3/k_4 ratio. To that end, a series of simulations of the plot: ln $(1 - \Delta A_t/\Delta A_{tot})$, as a function of time, was carried out as presented in Figure 4. The two artifactual solutions, which give a linear semilog relationship from the beginning, are $k_3'/k_4' = 2.7$ with $k_4' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$ at 470 nm and $k_3'/k_4' = 1.7$ with $k_4' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$ at 490 nm. The unique solution concerning k_3' and k_4', whatever the observation wavelength, is $k_3' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$ and $k_4'/k_3' > 20$. Under such conditions, the semiquinone does not accumulate.

In summary, with the help of detailed simulation studies, it has been possible to establish the rate constants involved in the partial reactions of reduction of the flavoprotein by lactate and of reoxidation by ferricyanide. This study has yielded data showing that the first electron delivered to ferricyanide is given much more slowly from the hydroquinone form than from the semiquinone form of the flavoprotein.

It should be recalled that, in certain flavoenzymes, the situation is the same as in the flavodehydrogenase derivative of flavocytochrome b_2; i.e., the reactivity of the semiquinone to ferricyanide is higher than that of the hydroquinone. Such a case was noted by Yagi (1975) for D-amino acid oxidase. The contrary was observed for NADH cytochrome b_5 flavoreductase (Strittmatter, 1965), for NADPH cytochrome c reductase, and for NADPH sulfite reductase (Kamin, 1975).

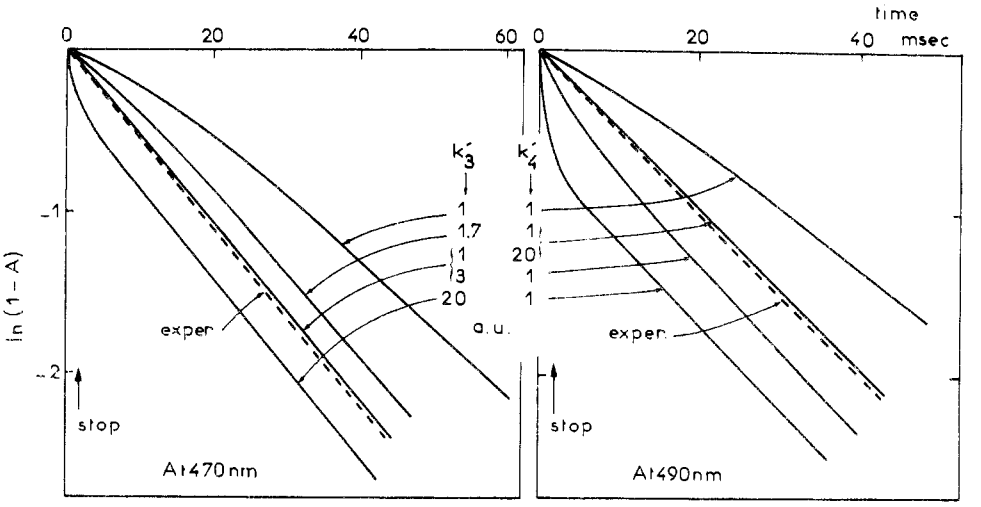


FIGURE 4: Analysis of the two one-electron steps (Scheme II) in the reoxidation of the reduced flavoprotein by ferricyanide. Comparison of simulated data with experimental data allows the estimation of the two rate constants. On the ordinate, $A = \Delta A_t/\Delta A_{tot}$ represents the normalized absorbance change at time t. On the abscissas, the reaction time consists of the sum of the dead time (2 ms) and of the time from the stop. (- - -) (Dashed lines) Experimental data. Reoxidation experiments were carried out essentially as described in Figure 3. Ferricyanide concentrations (after mixing) were 0.55 mM in the experiments observed at 470 nm (at left), giving $k_{ox,app} = 62\ s^{-1}$, and 0.50 mM in the experiments observed at 490 nm (at right) giving $k_{ox,app} = 52\ s^{-1}$. (Solid lines) Simulated data according to Scheme II and eq 1 and 2 for different sets of k_3' and k_4' parameters, as indicated on the graph in arbitrary units. One a.u. equals $1.1 \times 10^5\ M^{-1}\ s^{-1}$. The following ϵ $mM^{-1}\ cm^{-1}$ values were introduced (see optical properties section): 6.3 at 490 nm and 8.7 at 470 nm for $\epsilon_{ox} - \epsilon_{HQ}$, on one hand, and 3.8 at 490 nm and 3.2 at 470 nm for $\epsilon_{SQ} - \epsilon_{HQ}$, on the other hand. The calculations were carried out with a laboratory computer Wang 2200 ps. Comparison of simulated and experimental data indicates that the only set of k' parameters giving a perfect fit at both wavelengths from time zero is $k_3' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$ and $k_4' \geq 22 \times 10^5\ M^{-1}\ s^{-1}$. At each wavelength, there is also another solution which is artifactual (see text). For the sake of clarity, the dashed lines of the experimental curves were traced along side the corresponding simulated curve, although they are precisely superimposed.

Discussion

To understand the steady-state catalytic reaction mechanism of the flavoprotein component of flavocytochrome b_2, we should first compare the results of stopped-flow studies of the partial reactions with those of steady-state studies and examine their correlation. We will then discuss the role of the heme.

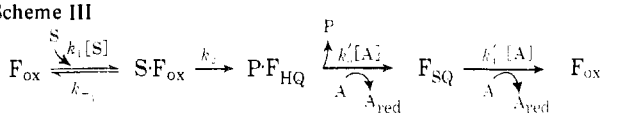
It should be emphasized that, at the present time, this entire discussion concerns the flavocytochrome b_2 referred to as baker's yeast, "cleaved" form (which corresponds to the Appleby-Morton (1959) type of preparation), and the corresponding flavoprotein, i.e., its heme-free derivative, with the same protein moiety.

However, preparations of such a flavoprotein, which involve a complete denaturation in 6 M guanidine hydrochloride followed by a long renaturation process, do not lead to a perfect reconstitution of flavin binding sites. Indeed, both the binding yield of the flavin (50–65%) and the stability of the reconstituted flavoprotein are not always exactly the same and are markedly different from those corresponding to the native flavocytochrome b_2 (this question has been discussed by Mévl-Ninio et al., 1977): for example, the K_d value for the oxidized flavoprotein is $\sim 10^{-8}$ M instead of $\sim 10^{-10}$ M for the oxidized flavocytochrome (Iwatsubo and di Franco, 1965). Also, the existence of species (~10%) reacting very slowly with lactate ($k_{red} \leq 1\ s^{-1}$) indicates the presence of some nonreactive flavin. Such nonreactive species are present only in the flavoprotein in its oxidized form, which is particularly labile. The deterioration takes place during the several-hour interval between the oxidation step and the end of the experiment.

Nevertheless, the k_{red} and k_{ox} values of the flavoprotein are fairly constant for all the preparations examined. Therefore, we will consider in the following discussion that the kinetic parameters observed with the flavoprotein represent the "native" flavoprotein part of flavocytochrome b_2.

Reaction Scheme of the Flavoprotein Enzyme. From the experimental results reported above concerning the two kinds of partial reactions (reduction of oxidized flavoprotein by lactate, reoxidation by ferricyanide of reduced flavoprotein), and from their best simulations, we have been able to calculate the rate constants involved in the two partial Schemes I and II. When the reaction is carried out in the presence of substrate, S, and acceptor, A, we can propose a unique scheme grouping these two partial schemes:

Scheme III



At substrate saturation, the steady-state assumption and conventional relationships enable the rate equation to be derived. It will be:

$$\frac{[E]_t}{v} = \frac{1}{2k_2} + \frac{1}{2[A]}\left(\frac{1}{k_3'} + \frac{1}{k_4'}\right) \qquad (3)$$

if v is expressed in electron-equiv/s and [E] in mol/L. Estimates of $K_{m,A}$ (equal to $k_2(1/k_3' + 1/k_4')$ and $V_m/[E]_t$ (equal to $2k_2$) can be calculated if we introduce in this formula the values established for k_2, k_3', and k_4' in partial reactions. In such a calculation, it was assumed that the maximal rate is not limited by the dissociation of pyruvate.

The comparison of experimental and calculated values of $K_{m,A}$ and V_m, as presented in Table I, indicates that there is a satisfactory fit. Therefore, the best and simplest assumption is that the catalytic reaction between L-lactate and ferricyanide in the presence of the flavoprotein actually follows Scheme III with the rate constants established here.

The fact that the parameters $K_{1/2,L\text{-lactate}}$ and k_2, i.e., those parameters which characterize the L-lactate-flavoprotein interactions, are almost the same for the flavoprotein and for the flavocytochrome shows that the L-*lactate dehydrogenase function,* on the substrate side, is not affected by the presence

of the heme within flavocytochrome b_2.

On the acceptor side, it was shown here that this L-lactate dehydrogenase flavoprotein is characterized by its unreactivity with cytochrome c (as already suggested by the studies of Forestier and Baudras, 1971). However, with ferricyanide, it can have a fairly high molar activity similar to that of flavocytochrome b_2 when this acceptor reaches saturating concentrations. The main difference in steady-state parameters between the flavoprotein and the flavocytochrome lies at the level of the $K_{m,ferricyanide}$ value, 100 times higher for the former. This high $K_{m,A}$ value corresponding to the flavoprotein can be understood by Scheme III and the corresponding rate equation (eq 3). The flavoprotein turnover rate, v, measured under the usual standard conditions used for flavocytochrome b_2 is limited, not only at the lactate-flavin transfer, but also at the step by which F_{HQ} is reoxidized by ferricyanide to F_{SQ} ($k_3' = 1.1 \times 10^5\ M^{-1}\ s^{-1}$). As shown by eq 3, the lower k_3' is, the higher is $K_{m,A}$. In the case of the flavocytochrome b_2, this slow step is overcome by the rapid electron transfer ($k_H = 800\ s^{-1}$) between F_{HQ} and the heme which itself reacts very rapidly with ferricyanide ($k_5' = 10^7\ M^{-1}\ s^{-1}$) so that the $K_{m,A}$ values are much smaller (cf. Table I).

Role of the Prosthetic Heme within Flavocytochrome b_2. The following possible role of the heme b_2 within flavocytochrome b_2 is proposed. Accepting rapidly one electron from the flavin hydroquinone, it should yield two species which, themselves, are very reactive toward ferricyanide, the flavin semiquinone and the reduced heme. To understand more precisely the validity of such an assumption, we can try to make a few estimates; we will assume a *noninterference* within flavocytochrome b_2 between heme and flavin, i.e. that the presence of the heme would not modify at all the intrinsic properties of the prosthetic flavin. The heme would add only two new possibilities: (a) heme would compete with an external acceptor such as ferricyanide to withdraw the first electron which can be donated by the flavin hydroquinone, (b) reduced heme would be an additional site able to react with ferricyanide. We now shall compare the apparent first-order rate constants (15 °C) for the one-electron transfer from F_{HQ} to H_{ox} within flavocytochrome b_2 (the best estimate is $k_H = 800\ s^{-1}$ according to Iwatsubo (cf. Table I)) and for the one-electron transfer from F_{HQ} to ferricyanide. At 1 mM ferricyanide, taking the values k_3' determined for the flavoprotein, the second reaction would be characterized by $k_3 = 1.1 \times 10^5 \times 10^{-3} = 110\ s^{-1}$. Therefore, even with very high concentrations of an acceptor of high reactivity such as ferricyanide, the prosthetic heme b_2 would take an electron from the hydroquinone at least seven times faster than would ferricyanide. With such a noninterference assumption, prosthetic heme b_2 appears, therefore, as the best one-electron acceptor for prosthetic flavin hydroquinone. This holds even more strongly for cytochrome c, since the latter cannot react with this reduced flavin.

The data concerning the actual reactivities of the three possible donors of reduced flavocytochrome b_2 to external acceptors will be presented in a future paper (Iwatsubo et al.); they tend to support the validity of the "noninterference" assumption presented here. Therefore, they tend to show that the complex flavocytochrome enzyme should behave merely as an association between a flavodehydrogenase (a flavocytochrome b_2 reductase) with its specific acceptor, the cytochrome b_2, with which cytochrome c reacts specifically.

A number of indications, including very recent ones (Gervais et al., 1975) indicate that these two functions, flavodehydrogenase on one hand, cytochrome b_2 on the other hand, are associated with different globular portions formed by the folding of a single polypeptide chain. These portions have very likely been associated by an event of gene fusion. The evolutive interest of such a fusion results from two facts. (1) The flavodehydrogenase and the cytochrome b_2 portions react together very slowly when they are not organized in a stable association. (2) Their mutual affinity is very low ($K_d > 1$ mM) so that they dissociate when the peptide bond between them is broken.

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Escherichia coli Dihydrofolate Reductase: Isolation and Characterization of Two Isozymes†

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ABSTRACT: A combination of affinity column chromatography and preparative gel electrophoresis has been used to purify to homogeneity the two isozymes of dihydrofolate reductase from a trimethoprim-resistant strain of *Escherichia coli* B (RT 500). These enzyme forms are noninterconvertible and are present in crude cell lysates, but other electrophoretic species can be generated during purification if sulfhydryl-protecting agents, such as dithiothreitol, are not present. The two isozymes, numbered form 1 and form 2 with respect to their decreasing electrophoretic mobilities, have similar molecular weights (18 500), molecular radii (21 Å), and apparent K_m values for reduced nicotinamide adenine dinucleotide (NADH) and NADH phosphate (NADPH). Both forms contain 2 mol of sulfhydryl/mol of enzyme which can be oxidized to intramolecular disulfide bonds. However, forms 1 and 2 differ physically in their electrophoretic mobility and isoelectric point and kinetically in their pH–activity profile, specific activity, K_m for dihydrofolate, and their affinity toward a number of inhibitors.

Multiple forms of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3.) have been isolated from a variety of bacterial and mammalian sources. In some cases the nature of the multiplicity is understood. For example, the two forms of dihydrofolate reductase from *Lactobacillus casei* (Gundersen et al., 1972), chicken liver (Huennekens et al., 1971), and L1210 cells (Perkins et al., 1967) that were observed on electrophoresis and column chromatography were identified as the free enzyme and the enzyme-NADPH[1] binary complex. The multiple forms of dihydrofolate reductase isolated from several strains of *Streptococcus faecium* var. durans A have been shown to be genetically determined isozymes with different physical and kinetic properties (Nixon and Blakley, 1968; Albrecht et al., 1969), and it has been recently shown (Amyes and Smith, 1975; Sköld and Widh, 1975) that R factors can introduce into *Escherichia coli* a trimethoprim-resistant enzyme that is significantly different from the chromosomal enzyme. However, in other cases, such as with the dihydrofolate reductase from hamster kidney cells (Hänggi and Littlefield, 1974), *Diplococcus pneumoniae* (Sirotnak, 1973), and bovine liver (Kaufman and Kamerer, 1976; Baumann and Wilson, 1975), the occurrence of polymorphism is well documented, while the cause of the multiplicity is not completely understood.

We previously reported the *E. coli* enzyme can exist as aggregates and monomers with different electrophoretic mobilities (Baccanari et al., 1975a). The purpose of the present study was to determine the basis for this polymorphism and to characterize the multiple enzyme forms. This report shows two of the multiple forms are isozymes with different physical and kinetic properties. These isozymes can exist in a variety of sulfhydryl oxidation states which results in a complex electrophoretic pattern. A mechanism for the sulfhydryl interconversions and the relationship of these findings to other studies of dihydrofolate reductase are also presented.

Materials and Methods

Iodoacetic acid, Nbs_2, $PhCH_2SO_2F$, and soybean trypsin inhibitor were from Sigma. Acrylamide and bisacrylamide were purchased from Eastman Kodak. Folate and DTT were from Calbiochem, and NADPH was from P-L Biochemicals. Ampholine-carrier ampholytes were supplied by LKB. The protein standards used in the Ferguson analysis were from several commercial sources. The α-lactalbumin (mol wt

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; $NADP^+$, NAD phosphate; NADPH, reduced NAD phosphate.