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Comparison of the processes involved in reduction by the substrate for two homologous flavocytochromes b_2 from different species of yeast

Chantal CAPEILLÈRE-BLANDIN,*‡ Michael J. BARBER†§ and Robert C. BRAY†

*Groupe d'Enzymologie Physicochimique, Centre de Génétique Moléculaire du C.N.R.S., 91190 Gif-sur-Yvette, France, and ‡School of Chemistry and Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.

A detailed study of the electron exchanges involved between FMN and haem b_2 groups within flavocytochrome b_2 of yeast *Hansenula anomala* (H-enzyme) was performed. The results were compared with those for the homologous enzyme of yeast *Saccharomyces cerevisiae* (S_x-enzyme) re-investigated at 5 °C. The mid-point reduction potentials of FMN and haem were determined by two complementary methods: potentiometric titration with substrate, L-lactate, in the presence of dye mediators with quantification of the reduced species performed by spectrophotometry at suitable wavelengths; anaerobic titration of the enzyme by its substrate by monitoring the e.p.r. signals of the semiquinone and Fe^{3+} species. Values of $E_{m,7} = -19$, -23 and -45 V were determined respectively from the data for the three redox systems H_o/H_r, F_o/F_{sq} and F_{sq}/F_r in the H-enzyme instead of $+6$, -44 and -57 mV respectively in the S_x-enzyme [Capeillère-Blandin, Bray, Iwatsubo & Labeyrie (1975) Eur. J. Biochem. **54**, 549–566]. Parallel e.p.r rapid-freezing and absorbance stopped-flow studies allowed determination of the time courses of the various redox species during their reduction by L-lactate. The flavin and the haem reduction time courses were biphasic. In the initial fast phase the reduction of flavin monitored by absorbance measurements is accomplished with a rate constant $k_F = 360\ s^{-1}$. The reduction of the haem lags the reduction of flavin with a rate constant $k_H = 170\ s^{-1}$. The appearance of flavin free radical is slower than the reduction in flavin absorbance and occurs with a rate constant close to that of the reduction of the haem. At saturating L-lactate concentration the initial rapid phase (up to 15 ms) involved in the overall turnover can be adequately simulated with a two-step reaction scheme. The main difference between the enzymes lies especially at the level of the first step of electron exchange between bound lactate and flavin, which for the H-enzyme is no longer the rate-limiting step in the haem reduction and becomes 8-fold faster than in the S_x-enzyme. Consequently in the H-enzyme for the following step, the intramolecular transfer from flavin hydroquinone to oxidized haem, a reliable evaluation of the rate constants becomes possible. Preliminary values are $k_{+2} = 380\ s^{-1}$ and $k_{-2} = 120\ s^{-1}$ at 5 °C. These are believed to be close to the true values, though the slow phase of reduction has not so far been taken into consideration in the simulation, so that minor revision may be needed.

INTRODUCTION

Flavocytochrome b_2 is an L(+)-lactate:cytochrome *c* oxidoreductase (EC 1.1.2.3) found in mitochondria of aerobically grown yeasts. It is a tetrameric enzyme, containing one FMN and one protohaem IX group per protomer (Labeyrie *et al.*, 1978). Each chain is folded into two distinct domains assuming different functions, an L-lactate flavodehydrogenase and its electron acceptor the cytochrome b_2 core, which then reacts with cytochrome *c* (Iwatsubo *et al.*, 1977; Gervais *et al.*, 1979; Capeillère-Blandin, 1982). Few data are available on the rates of the intramolecular electron-transfer processes involved between multi-redox centres in biological systems. One of the aims of rapid kinetic study of flavocytochrome b_2 is to obtain values for the rate constants involved in the intramolecular electron exchange between the prosthetic groups. Previous studies on the reduction of baker's yeast flavocytochrome b_2 (a nicked form, symbol S_x) brought information on the thermodynamic and kinetic parameters of the electron exchanges (Capeillère-Blandin *et al.*, 1975, 1976). These data were obtained by measuring the extent of reduction of the various redox centres (oxidized flavin, F_o, flavin semiquinone, F_{sq}, and oxidized haem, H_o) by means of their optical absorption and their e.p.r. spectra on frozen samples at equilibrium and in the course of the reduction by L-lactate at high concentration.

Several electron-transfer steps were characterized. The first step is a two-electron transfer from bound L-lactate to flavin, shown to be the main rate-determining step involved in the overall reaction ($k = 120\ s^{-1}$ at 24 °C).

Abbreviations used: H-enzyme, *Hansenula anomala* flavocytochrome b_2; S_x-enzyme, *Saccharomyces cerevisiae* flavocytochrome b_2; F_o, oxidized flavin; F_{sq}, flavin semiquinone; F_r, reduced flavin hydroquinone; H_o oxidized haem; H_r, reduced haem.

‡ To whom correspondence should be addressed at present address: Hôpital Necker, Nephrologie I.N.S.E.R.M. U25, 5è Étage, 161 Rue de Sèvres, 75743 Paris Cedex 15, France.

§ Present address: Department of Biochemistry, Box 7, University of South Florida, College of Medicine, Tampa, FL 33612, U.S.A.

The second step is a reversible intramolecular one-electron transfer between reduced flavin hydroquinone (F_r) and oxidized haem b_2 (H_o). Computer-simulated fits of the data allowed the determination of the limits for the kinetic rate constant of the later intramolecular electron exchange ($k > 600\ s^{-1}$ at 24 °C; Capeillère-Blandin, 1975). The involvement of these two basic initial steps was confirmed in rapid-kinetic studies of the reduction of S_x-enzyme by [^{2}H]lactate at 5 °C (Pompon *et al.*, 1980; Pompon, 1980). Moreover, further reversible intramolecular electron transfer between F_{sq} and H_o was demonstrated in temperature-jump studies on partially reduced enzyme (Tegoni *et al.*, 1984). One-electron-reduced centres (F_{sq}, H_r) could be involved in successive processes taking place in the overall catalytic reaction when the external acceptors cytochrome *c* and/or ferricyanide were additionally present (Morton & Sturtevant, 1964; Suzuki & Ogura, 1970; Forestier & Baudras, 1971; Baudras *et al.*, 1972; Capeillère-Blandin *et al.*, 1980; Capeillère-Blandin, 1982).

The present paper reports studies analogous to those by Capeillère-Blandin *et al.* (1975) on a homologous enzyme, namely flavocytochrome b_2 from *Hansenula anomala* yeast (H-enzyme), which differs from that from *Saccharomyces cerevisiae* (S_x-enzyme) in the values of a number of steady-state kinetic parameters, in particular a 5-fold-higher molar activity (Baudras, 1971). It was of particular interest to determine the rate of the electron exchanges within H-flavocytochrome b_2 prosthetic groups and to provide a basis for interpreting the differences between the two enzyme species. For that purpose parallel and detailed e.p.r. rapid-freezing and absorbance stopped-flow studies were carried out. Moreover, direct measurements of the mid-point potentials of the centres in H-enzyme were performed by means of potentiometric titrations by using mediators and a platinum electrode.

The study also included a detailed comparison between the characteristics of H-enzyme and those of S_x-enzyme, kinetically re-investigated for that purpose at 5 °C. Finally, to improve the understanding of the electron exchange within the prosthetic groups of flavocytochrome b_2, we aimed to put forward a reaction scheme and to test its agreement with the experimental kinetic data by simulation studies. Part of this work was presented in preliminary form by Capeillère-Blandin *et al.* (1982).

MATERIALS AND METHODS

Proteins

H-flavocytochrome b_2 was purified from the yeast *Hansenula anomala* by the procedure of Labeyrie *et al.* (1978) with modifications introduced by Naslin and described by Gervais *et al.* (1980).

S_x-flavocytochrome b_2 was prepared from dried commercial baker's yeast (Springer strain) by the modified method of Appleby & Morton (1959) as reviewed by Labeyrie *et al.* (1978). It corresponds to the crystallized (or nicked) enzyme form as defined previously (Labeyrie *et al.*, 1978).

Both enzyme species were stored as $(NH_4)_2SO_4$ precipitates in L-lactate-containing phosphate buffer under N_2 at 4 °C. Working oxidized solutions were prepared from the suspension by centrifugation (18000 *g* for 10 min) with the use of different buffers. First, to eliminate L-lactate the pellet was washed twice in 0.1 M-sodium/potassium phosphate buffer, pH 7.0, containing $(NH_4)_2SO_4$ at 40% saturation. When oxidized the pellet was dissolved in the minimum volume of phosphate buffer and centrifuged (30000 *g* for 10 min). The supernatant corresponds to a concentrated solution of flavocytochrome b_2 (approx. 600 μM) with an optimal A_{280}/A_{423} absorbance ratio equal to 0.49. Flavocytochrome b_2 concentrations, always expressed as the molarity of haem b_2 in solution, were measured with a Perkin–Elmer 555 spectrophotometer by using $\epsilon^{423}_{red.} = 183\ mM^{-1}\cdot cm^{-1}$ (Pajot & Groudinsky, 1970).

Enzymic activity was measured spectrophotometrically at 30 °C by monitoring the decrease of ferricyanide absorbance as a function of time ($\Delta\epsilon^{420} = 1.04\ mM^{-1}\cdot cm^{-1}$); the standard assay solution contained 0.9 mM-ferricyanide and a saturating concentration of L-lactate, either 10 mM for H-enzyme or 40 mM for S_x-enzyme, in 0.1 M-sodium/potassium phosphate buffer, pH 7.0. The molar activities, expressed as the molar concentration of one-electron acceptor reduced/s per molar haem concentration (i.e. protomer concentration) were close to $1000 \pm 50\ s^{-1}$ and $220 \pm 10\ s^{-1}$ for H-enzyme and S_x-enzyme respectively at 30 °C.

Redox-potential measurements

Oxidation–reduction-potential titrations were performed in a special absorbance cuvette with a side arm for addition of reductant and a Kel-F top for insertion of the electrode so that the potential could be monitored during spectrophotometric measurements. A combined platinum/calomel electrode (Radiometer PK 149) was used, connected to a digital voltmeter (Tacussel mini 5000) indicating the solution potential. The standard potential of the reference electrode was checked either against the ferricyanide/ferrocyanide couple or against a saturated quinhydrone solution (Clark, 1960) yielding an average value $E_c = 217 \pm 10$ mV at 10 °C (mean ± S.D. for five determinations). Then all redox potentials were referred to the normal hydrogen electrode.

The reaction mixture, containing 1.5 μM of the dyes pyocyanine ($E_{m,7} = -35$ mV at 30 °C) and Methylene Blue ($E_{m,7} = 11$ mV at 30 °C) (Clark, 1960) in 0.1 M-sodium/potassium phosphate buffer, pH 7.0, was made anaerobic by repeated cycles of gas evacuation and equilibration with an atmosphere of argon purified by bubbling through a vanadyl sulphate column (Labeyrie, 1963). Then a concentrated oxidized flavocytochrome b_2 solution was added to give a final concentration of 24 μM. The titration was conducted by addition of lactate from a 10 μl Hamilton syringe with a continuous flushing with argon. To achieve complete reduction when necessary, a small amount of dithionite solution was added at the end of the titration. A magnetic stirrer placed in the cuvette allowed a uniform diffusion of the reductant. After equilibration for 1 min the potential was measured and the absorbance spectrum recorded between 580 and 430 nm, as illustrated in Fig. 1(*a*). All titration experiments were carried out at 10 °C and pH 7.0.

Stopped-flow experiments

All rapid kinetic experiments were carried out with a modified Gibson–Durrum apparatus (Capeillère-Blandin,

1982), thermostatically maintained at 5 °C, and coupled to a Tracor NS 570 analogue-to-digital converter, which stored 1024 points, then transferred the absorbance-versus-time data on to an $X-Y$ recorder. The instrument dead-time was measured to be 2.2 ± 0.2 ms and the wavelength calibration was checked as described by Capeillère-Blandin *et al.* (1975). The optical pathlength was 1.9 cm. Haem reduction was monitored in the α-band, at 557 nm ($\Delta\epsilon_{red.-ox.} = 21.8\pm0.9$ mM$^{-1}\cdot$cm^{-1}). Flavin reduction was monitored at 438.3 nm ($\Delta\epsilon_{red.-ox.} = -8.6\pm0.9$ mM$^{-1}\cdot$cm^{-1}), a haem isosbestic point determined precisely on the apparatus before each series of experiments as described by Capeillère-Blandin *et al.* (1975).

Evaluation of the concentration of different redox species from absorbance measurements at various wavelengths

In particular in the region 430–520 nm, where the individual spectra overlap considerably, the absorbance spectra of flavocytochrome b_2 correspond to the additive contributions of the two chromophores, bound haem b_2 and bound flavin (Capeillère-Blandin *et al.*, 1975). Therefore, as discussed previously (Capeillère-Blandin *et al.*, 1984), the individual absorbance coefficients $\epsilon_{ox.}$, $\epsilon_{red.}$ and $\Delta\epsilon_{red.-ox.}$ of bound haem b_2 and bound flavin are obtained from analysis of a series of oxidized and reduced spectra of flavocytochrome b_2 and of cytochrome b_2 core, the deflavo derivative. These spectra were routinely scanned with a Perkin–Elmer 555 spectrophotometer, then memorized into a Spectra Physics SP 4100 calculator, allowing the storage of the individual spectra on magnetic tapes and the calculation at each wavelength of absolute absorbance coefficients and/or absorbance difference coefficients (reduced − oxidized).

Relative contributions of haem b_2 and flavin. The flavin parameters were obtained by subtraction of the haem contribution, calculated from cytochrome b_2 data, from the spectrum of flavocytochrome b_2. For example, at 480 nm in the course of redox titrations the flavin redox contribution ($\Delta\epsilon^{480}_{F_r-F_o} = -7.5\pm0.5$ mM$^{-1}\cdot$cm^{-1}) was obtained after correction for the haem b_2 redox change; the latter were estimated by taking into account the known haem variations detected at 557 nm (Fig. 1) (where the flavin absorbance is assumed to be zero) divided by the haem absorbance difference coefficients ratio $\Delta\epsilon^{557}_{H_r-H_o}/\Delta\epsilon^{480}_{H_r-H_o} = 21.6/4.6 = 4.7$.

Relative contributions of the various flavin redox species. The other absorbance coefficient values of interest were those at 438.3 nm, where $\epsilon_{F_o} = 10\pm1$ mM$^{-1}\cdot$cm^{-1} and $\epsilon_{F_r} = 1.2\pm0.3$ mM$^{-1}\cdot$cm^{-1}. This wavelength corresponds to one of the isosbestic points of the bound haem b_2 (Capeillère-Blandin *et al.*, 1975). The absorbance coefficient value for the red semiquinone (identified by its e.p.r. signal bandwidth of 1.5 T) were those established for a red semiquinone bound to L-amino acid oxidase (Massey & Curti, 1967), D-amino acid oxidase (Yagi *et al.*, 1969; Yagi, 1975) and 'old yellow enzyme' (Matthews & Massey, 1971): at 438.3 nm $\epsilon^{438}_{F^{sq}} = 3\pm0.5$ mM$^{-1}\cdot$cm^{-1} and at 480 nm $\epsilon^{480}_{F^{sq}} = 4\pm0.5$ mM$^{-1}\cdot$cm^{-1}. These values agree with the absorption coefficients deduced from the absorbance spectrum of the red semiquinone recently established in the course of redox titrations of flavocytochrome b_2 in the presence of pyruvate (Tegoni *et al.*, 1986).

Rapid-freezing experiments

These experiments were conducted by the rapid-mixing-and-freezing technique (Bray, 1961*a*,*b*) under anaerobic conditions as described by Bray *et al.* (1973) (see also Gutteridge *et al.*, 1978). Substrate and enzyme solutions were freed from O_2 before transfer into the syringes. Under our experimental conditions the reaction times, corrected for a 2 ms dead time, could be varied from 6 ms to 30 s. The anaerobic titration was carried out with the same equipment, at a reaction time of 21 s.

E.p.r. spectroscopy

E.p.r. spectra were recorded at a microwave frequency of 9.1 GHz on a Varian E 9 spectrometer at 118 K for free radical and at 25 K for haem iron, under non-saturating conditions.

The characteristics of the e.p.r. signals detected in a partially reduced H-flavocytochrome b_2 were indistinguishable from those for the S_x-enzyme (Capeillère-Blandin *et al.*, 1975). At 25 K a low-spin ferric haem iron signal is detected with g values at 2.99, 2.22 and 1.47, and is not saturated at 100 mW microwave power with a modulation amplitude of 2 mT. Upon progressive reduction of the enzyme the heights of the sharper peaks, g_1 and g_2, were used to estimate the amount of decrease of oxidized haem, the signal keeping a constant linewidth. At 118 K the free-radical signal, at $g = 2.004$, of the flavin semiquinone is readily detected; the peak-to-peak linewidth of 1.5 ± 0.1 mT is characteristic of a red semiquinone, as previously observed in the S_x-enzyme (Capeillère-Blandin *et al.*, 1975). The linewidth of the signal is affected neither by the intensity of the microwave power (between 10 μW and 100 mW, with a modulation amplitude of 0.5 mT) nor by the extent of reduction of the haem, as shown from the saturation curves established at 115 K (Fig. 2*a*). The saturation behaviour ($P_{\frac{1}{2}} = 150$ μW) is quite similar to that of the FMN radical of flavodoxin from *Azotobacter chroococcum* (results not presented, but cf. Capeillère-Blandin *et al.*, 1975), which contains only one flavin group per molecule instead of four FMN and four haem groups per tetramer of flavocytochrome b_2. Thus the radical quantification as detailed below will be free of systematic errors due to spin–spin interactions.

E.p.r. signal intensities were corrected for variations in e.p.r.-tube diameters and normalized to the same gain, microwave power and enzyme concentration (cf. Capeillère-Blandin *et al.*, 1975). For absolute quantification of the radical signals double integrations of the spectra were performed with Cu(II)–EDTA complex as standard, with correction for differences in g values (Aasa & Vänngård, 1970). Results obtained with flavodoxin as a standard agreed with these values. The presence of isopentane in the frozen enzyme samples was taken into consideration by using a dilution factor of 1.75 ± 0.2 (16 determinations) estimated in this work. Quantification of oxidized haem was based on comparison with fully oxidized samples, frozen under similar conditions, and therefore did not depend on such corrections.

Data analysis

A Wang 2200 computer was used for all calculations. Data were fitted with a non-linear iterative regression program based on a least-squares criterion using various analytical expressions: in the kinetic case, a single

exponential or a sum of exponential terms $Y = a_I e^{-k_I t} + a_{II} e^{-k_{II} t}$. The program included adjustments for the amplitude of the phase and the first-order rate constant and gave their standard deviations. In the case of redox titrations, the potentiometric haem data were fitted to the theoretical Nernst plot for a one-electron reduction process:

$$E = E_H + 55\log[(1-\alpha)/\alpha] \quad (1)$$

where E is the potential of the solution and E_H the mid-point reduction potential for the haem, both expressed in mV relative to the standard hydrogen electrode. α corresponds to the percentage of reduced haem. The mid-point potential values E_1 and E_2 for the two half-reactions F_o/F_{sq} and F_{sq}/F_r were obtained by fitting the semiquinone peak to the following equation:

$$1/[F_{sq}] = 1 + 10^{(E-E_1)/55} + 10^{(E_2-E)/55} \quad (2)$$

E referring to the potential of the solution, assuming the mid-point potential of the haem, E_H, relative to which all the values were calculated to be -19 mV, as determined in this work.

Computer simulation studies were undertaken to test some reaction schemes. A fourth-order Runge–Kutta method was used to perform the integration of the appropriate set of differential equations providing the concentrations of the different species as a function of time.

RESULTS

Oxidation–reduction equilibrium between prosthetic groups in H-flavocytochrome b_2

Absorbance titrations. The features of the spectral changes detected in the course of an anaerobic potentiometric titration of flavocytochrome b_2 by L-lactate are reported in Fig. 1(*a*). Full reduction is obtained when 1.5 mol of L-lactate, i.e. 3 electron equivalents, is added per mol of enzyme protomer, leading to reduced haem b_2 and flavin hydroquinone, as previously demonstrated for the S_x-flavocytochrome b_2 (Capeillère-Blandin *et al.*, 1975). However, the absence of discrete isosbestic points at 535 and 517 nm on both sides on the β-band indicates the presence of a third absorbing species, namely the one-electron-reduced or flavin semiquinone state, which coexists transiently in equilibrium in the course of the titration of flavocytochrome b_2.

Typical absorbance variations of the haem b_2 (at

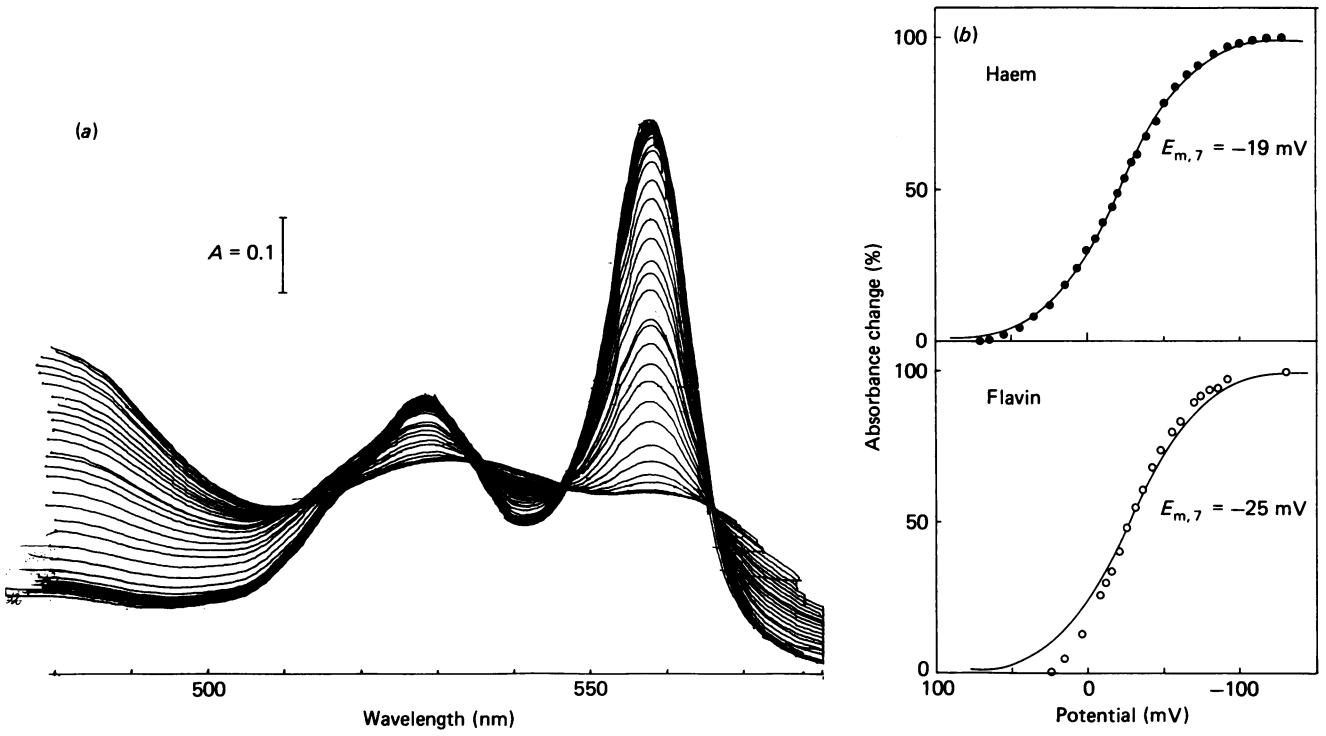


Fig. 1. Spectrophotometric potentiometric titration of H-flavocytochrome b_2 with L-lactate

(*a*) A solution of flavocytochrome b_2 (24 μM) in the presence of Methylene Blue (1.5 μM) was maintained under strict anaerobic conditions (see the Materials and methods section). After addition of each portion (4 μl) of L-lactate (0.8 μM), when the potential had stabilized, the spectrum was recorded. Experimental conditions: 0.1 M-phosphate buffer, pH 7.0, and 10 °C. (*b*) Typical haem b_2 (top) and flavin (bottom) titration curves: the percentage of reduction deduced from absorbance variations was plotted as a function of the potential in the solution. The reduction of haem was monitored by absorbance variations at 557 nm. The reduction of flavin was obtained from total absorbance variations at 480 nm after correction for absorbance haem changes as described in the Materials and methods section. The continuous lines through the experimental points are calculated for one-electron reduction processes ($n = 1$) with the following parameters: $E_{m,H} = -19$ mV for the haem and $E_{m,F} = -25$ mV for the flavin. Though a best fit is obtained for the haem, a deviation from a one-electron reduction reaction is observed for the flavin. The value of the semiquinone formation constant, K_{sq} is calculated from the slope of the tangent at the mid-point, and then the maximal percentage of free radical can be deduced (Elema, 1933). Here data found correspond to $K_{sq} = 1.8$ and $F_{sq} = 40\%$ respectively.

557 nm) and flavin (at 480 nm) in the course of the titration with L-lactate are plotted as a function of the redox potential (Fig. 1*b*). A quantitative analysis of these titration curves indicates the following. First, the haem shows an apparent simple reduction process. The best curve through the points was calculated for a one-electron reduction ($n = 1$). The mid-point potential values are $E_{m,H} = -19 \pm 5$ mV (mean $\pm$ S.D. for four determinations). Secondly, the flavin does not show either a one-electron or a two-electron reduction process, owing to the presence of the semiquinone at the same time as the quinone and hydroquinone forms. The apparent redox potential is determined to be -27 ± 5 mV (mean $\pm$ S.D. for three determinations) with corresponding n values $n_F = 1.1 \pm 0.1$.

By applying Elema's (1933) method, as a first approximation to estimate the maximal amount of semiquinone, from the flavin titration curve (Fig. 1*b*) a value of the order of $45 \pm 5\%$ is found and corresponds to a stability constant $K_{sq} = [F_{sq}]^2/[F_o][F_r]$, $K_{sq} = 2.7 \pm 1.5$, from three determinations. Consequently the difference between the redox potentials for the F_o/F_{sq} and F_{sq}/F_r systems would be equal to 25 ± 15 mV.

The uncertainty in measurements of redox potentials due to electrode errors, curve-fitting etc. for a simple redox curve like that of the haem is probably about ± 10 mV; however, reproducibility from one experiment to another was generally better than this (± 5 mV). With the flavin data there is an additional uncertainty in the error for the semiquinone estimate from such absorbance measurements since values depend on the estimation of the relative absorbance coefficients for haem and flavin at the chosen wavelength, here 480 nm.

E.p.r. titrations. To measure the semiquinone content, $[F_{sq}]$, stoichiometric anaerobic titrations with L-lactate were performed with the use of e.p.r. spectroscopy to monitor the signals of the semiquinone and of the paramagnetic oxidized ferric haem in frozen samples. The latter data for haem permit comparison with the results obtained by potentiometry.

As shown in Fig. 2(*b*), the redox behaviour of the haem b_2 deduced from e.p.r. measurements at low temperature (28 K) on concentrated samples (75 μM-flavocytochrome b_2) is fully consistent with the data obtained from spectrophotometric potentiometric determinations at 10 °C.

Concerning the semiquinone redox data (Fig. 2*b*), the radical content increases from zero to a maximal value near 40% of the total flavin content observed when about 1 mol of L-lactate per mol of enzyme protomer was added. The uncertainty in the estimation of the radical signal intensity depends on the reproducibility, on the estimation of the packing factor in isopentane (1.75 ± 0.2) and on the integrated e.p.r. signal intensity. Thus an uncertainty of $\pm 10\%$, i.e. $[F_{sq}]_{max.} = 40 \pm 4\%$, leads to uncertainty in the redox potential difference between F_o/F_{sq} and F_{sq}/F_r, i.e. $\Delta E = E_1 - E_2 =$

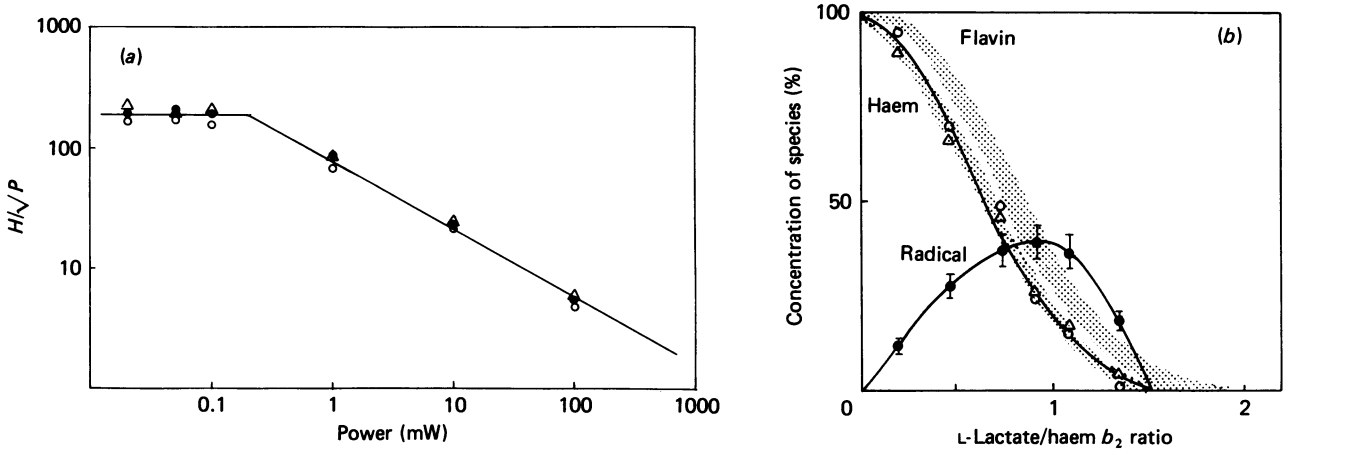


Fig. 2. (*a*) Microwave-power-dependence of the semiquinone radical signals in the presence of the ferric species within flavocytochrome b_2 in the course of redox titrations, and (*b*) e.p.r. titrations of H-flavocytochrome b_2 with L-lactate, showing haem b_2 and flavin semiquinone

(*a*) Samples were poised in the course of the reaction of 94 μM-flavocytochrome b_2 with L-lactate. They correspond to the simultaneous presence of two paramagnetic species, semiquinone and ferric haem, in relative amounts expressed in percentages 30:50 (○), 36:20 (●) and 40:8 (△). All the radical spectra were recorded at 118 K at variable microwave powers. In the log–log plot the amplitude of the semiquinone signal estimated from the peak height at $g = -2.0039$ divided by the gain setting and by the square root of the incident microwave power, P, is expressed as a function of the incident microwave power, P. (*b*) Experimental points correspond to e.p.r. measurements carried out on a 75 μM (final concentration) flavocytochrome b_2 solution anaerobically mixed with stoichiometric concentrations of L-lactate at 5 °C (0.1 M-phosphate buffer, pH 7.0) and frozen in isopentane 21s later, in the rapid-mixing-and-freezing apparatus. For each L-lactate/protomer concentration ratio, experimental points correspond to the average value for two separate samples. Signals for ferric haem and semiquinone radical were recorded under conditions described in the Materials and methods section. △ and ○ symbols correspond to normalized amplitudes of the g_1 and g_2 features of the Fe^{3+} signal respectively. ● symbols correspond to the amount of semiquinone radical; error bars take into consideration the uncertainty on the isopentane packing factor. The stippled area is related to spectrophotometric determination of the oxidized haem and flavin amounts performed at 557 nm and 480 nm (left and right) respectively. The continuous lines are the theoretical curves fitting the e.p.r. experimental points for oxidized haem, H_o, and semiquinone, F_{sq}, calculated with $E_{m,H} = -19$ mV and $E_{m,pyr/lact} = -190$ mV (Labeyrie *et al.*, 1960), with the following best parameters for the flavin systems: $E_1 = E_{m,F_o/F_{sq}} = -23 \pm 10$ mV and $E_2 = E_{m,F_{sq}/F_r} = -45 \pm 12$ mV.

Table 1. Mid-point reduction potentials for H-flavocytochrome b_2, together with semiquinone equilibrium parameters

Values were obtained by computer fitting of the titration data (Figs. 1 and 2) obtained from either absorbance or e.p.r. measurements. Potentials are expressed in mV relative to the standard hydrogen electrode. Values are given as means ± S.D. for four and two independent determinations for absorbance and e.p.r. determinations respectively. The data are compared with the corresponding values for S_x-flavocytochrome b_2 (Capeillère-Blandin *et al.*, 1975).

	H-enzyme		S_x-enzyme	
	Absorbance measurements	E.p.r. determinations	Absorbance measurements	E.p.r. determinations
$[F_{sq}]_{max.}$ (%)	45±5*	40±5	35±8*	40±4
$K_{sq} = \frac{[F_{sq}]^2}{[F_o][F_r]}$	2.7±1.5	1.8±0.8	1.2±0.6	1.8±0.8
$E_1 - E_2 = E_{F_o/F_{sq}} - E_{F_{sq}/F_r}$	25±5	14±10	10±8	14±10
E_H (mV)	−19±5		6±2	
$E_1 = E_{F_o/F_{sq}}$		−23±10		−44±8
$E_2 = E_{F_{sq}/F_r}$		−45±12		−57±9
Experimental conditions		0.1 M-Phosphate, pH 7.0		0.2 M-Phosphate, pH 7.05
Temperature	10 °C	5 °C		21 °C

* Predicted values obtained from Elema's (1933) analysis of the absorbance measurements.

14±10 mV. If the e.p.r. and absorbance titration data for flavin are also compared it is clear that there is a good agreement for the semiquinone estimates (Table 1).

By combining potentiometric data for haem and e.p.r. titrations for haem and semiquinone we can get the plot of concentration of semiquinone as a function of the redox potential (not presented here). Then the best estimates of the mid-point potential values for the two half-reactions E_1 and E_2 were obtained by fitting the data to eqn. (2) as described under 'Data analysis' in the Materials and methods section. They corresponded to −23±10 mV and −45±12 mV respectively.

Kinetic analysis of the electron-transfer processes

The H-enzyme is characterized by a high standard molecular activity, especially at 24 °C (molar activity 900 s^{-1}) where previous fast kinetic experiments on S_x-enzyme were conducted (molar activity 175 s^{-1}). Therefore it appeared necessary to lower the temperature to get lower electron-transfer rates compatible with a precise detection of the reduction time courses for flavin and haem. To determine the best temperature setting a quantitative analysis of the temperature effect under steady-state conditions was first carried out.

Effect of temperature on steady-state kinetics. This was studied as described in the Materials and methods section. The initial velocity of ferricyanide reduction measured over a range of temperature from 4 °C to 38 °C at saturating concentration of L-lactate (10 mM) and ferricyanide (0.9 mM) gives a linear Arrhenius plot, as presented in Fig. 3. A least-squares fit yields an activation energy of 38.5 kJ/mol (9.2 kcal/mol). Thus the lowest temperature convenient to rapid mixing, 5 °C, was chosen, at which temperature the electron-transfer rate is expected to be lowered 5-fold. Under this

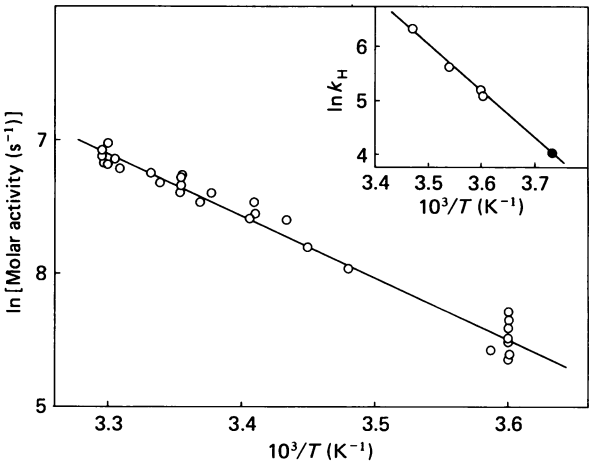


Fig. 3. Variation in overall reaction rate with temperature for turnover, by H-flavocytochrome b_2, of lactate in the presence of ferricyanide

Molar activities were measured under standard steady-state conditions as described in the Materials and methods section at different temperatures from 5 to 30 °C. The continuous line is a linear least-squares fit of the data to the Arrhenius equation with a slope corresponding to an activation energy of 38 kJ/mol (9.2 kcal/mol). The inset shows the variation in haem reduction apparent rate constant with temperature. The apparent rate constant for haem reduction (k_H) was determined from a semi-logarithmic analysis of phase I of the absorbance recordings obtained by stopped-flow after mixing oxidized flavocytochrome b_2 with 10 mM-L-lactate between 5 and 15 °C. The data at −5 °C were obtained from analysis of e.p.r. rapid-freezing measurements carried out with enzyme and lactate dissolved in an ethylene glycol/water (3:17, v/v) solution. Over the whole temperature range the data follow the Arrhenius law corresponding to an activation energy of 73 kJ/mol (17.4 kcal/mol).

condition the overall reaction rate corresponds to 240 ± 30 electrons/s per H-enzyme protomer.

Characteristics of the time courses of the various redox species during reduction. The reduction of flavin and haem by 10 mM-L-lactate was studied in parallel, but at different enzyme concentrations, with the use of the stopped-flow apparatus and the rapid-mixing-and-freezing system as described in the Materials and methods section. The pattern of concentration changes for the different redox species (Fig. 4) was established at 5 °C (0.1 M-phosphate buffer, pH 7.0) by pooling absorbance stopped-flow recordings at two wavelengths, 557 nm to monitor haem reduction, H_r, and 438 nm to monitor flavin reduction, with e.p.r. rapid-freezing results (experimental points) at a series of reaction times giving the amount of reduced haem, H_r, and flavin semiquinone, F_{sq}.

As in our previous work (Capeillère-Blandin *et al.*, 1975), this pattern did not change with the flavocytochrome b_2 concentration. Thus the e.p.r. and absorbance results for H_r are superimposed, indicating excellent agreement despite the 10-fold difference in enzyme concentration. Therefore the electron-distribution processes under study very probably result from intramolecular electron-exchange processes.

Biphasic time courses were detected at the level of both flavin and haem b_2. They are not synchronous. The parameters for the best fits of several determinations are presented in Table 2. The corresponding rate-constant values for flavin and for haem are well separate on the time scale of the experiments. During the stopped-flow dead-time the apparent reduction of flavin is already extending over 50%, whereas only 15% of the haem is reduced.

The flavin initial reduction phase I, corresponding to 74% of the total absorbance change and extrapolated to time zero, is followed by a slower phase II, which brings the reaction to completion. At the level of the haem the rapid phase I corresponds to the reduction of 80–85% of the amount of initially oxidized species and is followed by a first-order slower phase II leading to 100% reduction. When phase I semilogarithmic plot is extrapolated backwards for the 2.2 ms dead-time and down to the initial oxidized level, i.e. before the beginning of the recording, a lag phase can be detected extending over 1.3 ± 0.5 ms. It has to be noticed that rate-constant values can also be estimated from the e.p.r. data, although more roughly because of the rather large scattering of individual points. Nevertheless they are not significantly different from those determined from the absorbance data ($k_I = 160 \pm 30\ s^{-1}$).

As for the semiquinone time course, the amount of radical reaches its maximal level, $38 \pm 4\%$ of the total flavin content, at the time where the transition between phases I and II takes place. Its initial rapid formation is difficult to analyse precisely because of a 5 ms shortest time for the rapid-mixing-and-freezing system, so that nothing could be established about the existence of a lag phase. Nevertheless the estimated rate constant for the semiquinone formation $k_{sq} = 180 \pm 40\ s^{-1}$ is very close to the value determined for the haem reduction.

Taking into consideration the absorbance variations detected at the level of the flavin, which include the simultaneous absorbance contributions of oxidized flavin and semiquinone, and correspond at time t to:

$$\Delta A_t = \Delta\epsilon_{F_r-F_o} \cdot [F_o]_t + \Delta\epsilon_{F_{sq}-F_r} \cdot [F_{sq}]_t$$

and the F_{sq} time course determined from e.p.r. measurements, the F_r time course can be calculated. The resulting curve (----- in Fig. 4*a*) is complex and obtained with a large uncertainty because of the

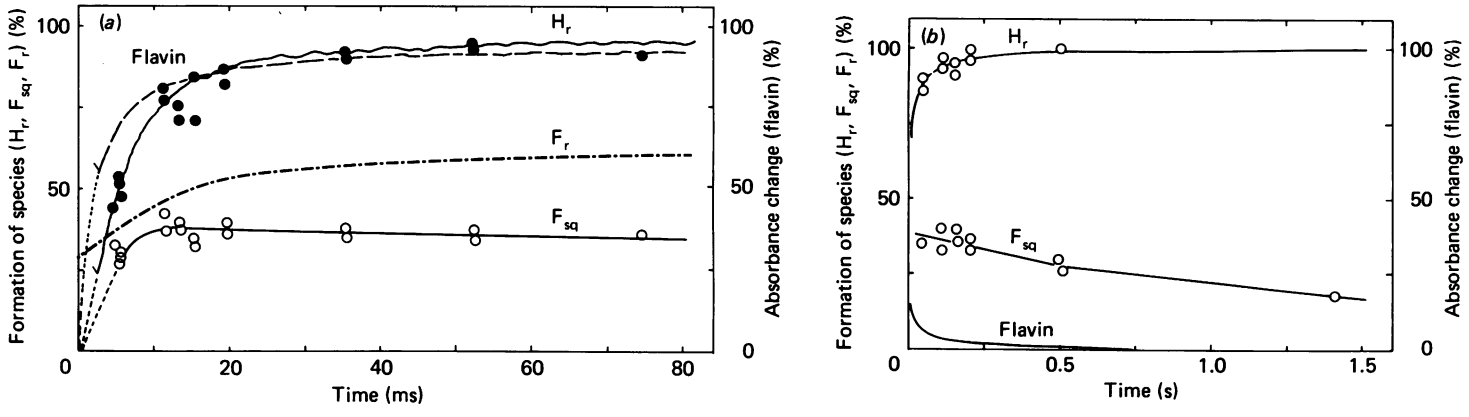


Fig. 4. Reaction time course for the different redox species during H-flavocytochrome b_2 reduction at 5 °C

(*a*) Along with typical stopped-flow absorbance recorder tracings at two wavelengths, 557 nm for haem and 438.3 nm for flavin (a haem isosbestic point) (noting that all flavin absorbance changes are shown in the reverse direction for comparison to the haem ones), obtained at 10 μM and 10 mM final concentration of enzyme and L-lactate respectively, e.p.r. rapid-freezing results for haem (●) and flavin semiquinone (○) obtained at 94 μM and 10 mM final concentrations of enzyme and L-lactate respectively, are given, with the calculated curve for hydroquinone, F_r (-----, calculated from $F_r = F_T - F_o - F_{sq}$; see the Results section). From fitting of the data sets to $a_I e^{-k_I t} + a_{II} e^{-k_{II} t}$, the flavin and haem time courses are biphasic and the rate constant values in the fast phase I correspond to 380 and 190 s^{-1} respectively; an initial burst of 30% amplitude is apparently detected in the initial part of the F_r curve. At the point $t = 10$ ms the total number of electrons delivered to each enzyme protomer by the substrate and calculated according to $1 \times (H_r) + 1 \times (F_{sq}) + 2 \times (F_r)$ is equal to 2. (*b*) Experimental conditions and symbols are as in (*a*), but observation time was up to 1.5 s.

Table 2. Comparison of kinetic data for reduction of H-flavocytochrome b_2 and S_x-flavocytochrome b_2 at saturating L-lactate concentration and 5 °C

Rate constants and amplitudes were determined from typical data sets presented in Figs. 4 and 6 by using non-linear least-squares fits to $a_{I}e^{-k_{I}t}+a_{II}e^{-k_{II}t}$. Values for flavin and haem derived from stopped-flow measurements are means ± S.D. for six and two independent determinations for H-enzyme and S_x-enzyme respectively. Data on the flavin semiquinone were obtained from e.p.r. rapid-freezing experiments (Figs. 4 and 6). Hydroquinone time course is calculated by the relation $F_r = F_T - F_o - F_{sq}$ and analysed by use of the Guggenheim (1926) method.

	Flavin			Haem, H_r		
	Recorded reaction (%)	Rate constant (s^{-1})		Recorded reaction (%)	Rate constant (s^{-1})	
		Phase I	Phase II		Phase I	Phase II
H-enzyme	50±8	360±40	30±7	80±5	170±15*	15±6
S_x-enzyme	86±4	42±4	2±1.5	95±3	35±4	1.5±0.3

	Semiquinone, F_{sq}			Hydroquinone, F_r		
	$[F_{sq}]_{max.}$ (%)	$t_{\frac{1}{2}}$† (ms)	Decay rate (s^{-1})	Burst (%)	Formation rate constant‡ (s^{-1})	Molar activity§ (s^{-1})
H-enzyme	38	3±0.5	0.6	32±8	100±10	120±15
S_x-enzyme	40	20±2	0.2	15±5	24±4	32±5

* A lag of 1.3±0.5 ms was observed.
† Increase.
‡ Exponential increase.
§ Defined as molecules of substrate consumed/s per haem protomer; means ± S.D. for seven and four determinations for H-enzyme and S_x-enzyme respectively.

scattering of the e.p.r. semiquinone data. Through a Guggenheim (1926) analysis of the best tracing a significant initial burst is detected corresponding to 32±8% of the flavin content. The uncertainty would be further increased if the rapid freezing time was in error. The burst is followed by an exponential process from 4 to 40 ms ($k_a = 100\ s^{-1}$), then by a very slow process leading to full reduction (Fig. 4*b*). These data tend to show that the flavin hydroquinone is formed in several different reaction steps of the sequential process: in particular, the initial burst corresponds to the fast reduction of flavin to the F_r state preceding the formation of F_{sq}.

From this pattern (Fig. 4*a*) it can be seen that the electrons from four lactate molecules per tetrameric flavocytochrome b_2 have been introduced and distributed over the different reduced species (H_r, F_{sq} and F_r) within 10 ms. At this time the flavin radical reaches its maximal value and the second slow phase in haem reduction is starting. The electrons from the fifth and sixth lactate molecules, which are needed for the full reduction as predicted from titration results, extend over the slow phase II. Comparison between the rate-constant values determined in phases I and II and the overall reaction rate (Table 2) indicates that phase II is much too slow to be part of the catalytic turnover.

In the slow stages of the reaction up to 1.5 s (Fig. 4*b*), further slow changes are occurring. At 0.5 s the haem is completely reduced (97%); the semiquinone radical is still present in relatively high amount (30%) and is slowly decreasing, and a corresponding parallel increase of F_r is noticeable.

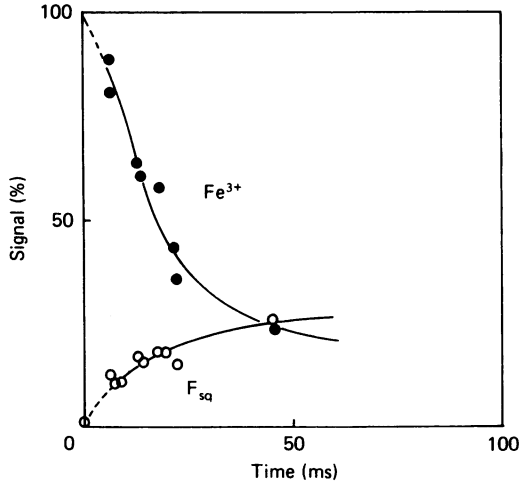


Fig. 5. Time courses of changes in intensity of the Fe^{3+} and radical e.p.r. signals during H-flavocytochrome b_2 rapid reduction at −5 °C

The reaction between H-enzyme and L-lactate, at 91 μM and 10 mM final concentration respectively, was performed in an ethylene glycol/buffer (3:17, v/v) mixture at −5 °C. E.p.r. rapid-freezing data are shown by ● (H_r) and ○ (F_{sq}) symbols. The H_r time course remains biphasic ($k_a = 54\ s^{-1}$) and the radical reaches a maximal level of 30% of the total flavin content.

Subzero-temperature reduction. To improve initial detection of semiquinone formation with the rapid-mixing-and-freezing system, an investigation at subzero temperature was performed in fluid aqueous organic solvent mixtures (Douzou, 1974, 1977). Preliminary

assays on the enzyme stability in terms of molar activities and flavin dissociation constant indicated the absence of modification of H-flavocytochrome b_2 reactivity when water/ethylene glycol or water/dimethyl sulphoxide systems with convenient ratios up to 4:1 (v/v) and 7:3 (v/v) respectively at −5 °C were used (C. Capeillère-Blandin, unpublished work).

On mixing L-lactate with H-enzyme at different reaction times in the presence of 15% (v/v) ethylene glycol at −5 °C and analysing the e.p.r. signals for haem and semiquinone, the results, presented in Fig. 5, show a significant decrease of the reduction rate. Development of the semiquinone is clearly apparent, reaching a maximal level of about 25% with a reaction-time $t_{\frac{1}{2}} = 10$ ms. The synchronous appearance of reduced haem and flavin semiquinone is supported by the constant ratio of their concentrations, the radical being always lower in concentration than the reduced haem.

The biphasic aspect of the H_r time course is maintained and the first-order rate constant of phase I corresponds to $k_{H_I} = 58\ s^{-1}$. This value fits the data obtained for the variations with temperature of the apparent reduction rate constant of the haem in the 5–15 °C range (Fig. 3*b*), thus corresponding to an activation energy of 72.8 kJ/mol (17.4 kcal/mol).

DISCUSSION

Redox equilibrium between flavin and haem prosthetic groups

The prosthetic groups have redox potentials close to each other and they rapidly reach equilibrium at partial reduction in the absence of O_2. In H-enzyme the mid-point potential values E_1 and E_2 for the two half-reductions F_o/F_{sq} and F_{sq}/F_r and for the haem were obtained by combining absorbance potentiometric (oxidized haem and flavin) and e.p.r. (oxidized haem and flavin semiquinone) titrations. The best estimates for the $E_{m,7}$ of the various systems are found to be -19 ± 5 mV for haem and -23 ± 10 and -45 ± 12 mV for E_1 and E_2 respectively, as summarized in Table 1. The fact that liquid-solution data at 10 °C (Fig. 1) and at 20 °C (Tegoni *et al.*, 1986) agree with the frozen-solution data (Fig. 2) suggests that changes of apparent pH and equilibrium constants on freezing (Williams-Smith *et al.*, 1978) are not important in the present work.

The mid-point potentials may be compared (Table 1) with the corresponding values reported for S_x-enzyme at 20 °C (Capeillère-Blandin *et al.*, 1975). The haem potential in H-enzyme has shifted to more negative potentials, whereas the potentials of flavin redox couples have shifted to more positive potentials for all of them, keeping contant the F_{sq} stability constant close to 2. Therefore the most striking difference between the two enzymes is that the equilibrium formation constant K_1 for the reaction $F_{sq} + H_o \rightarrow H_r + F_o$ is 3–5-fold smaller in H-enzyme than in S_x-enzyme. This effect is possibly characteristic of the H-enzyme or due to a rather strong temperature effect (−3 mV/degree) affecting mainly the H_o/H_r and F_o/F_{sq} equilibrium.

Reaction pattern during flavocytochrome b_2 reduction

The main features of the time courses established at saturating substrate concentrations for H-enzyme are essentially the same (except when otherwise mentioned) as those previously observed for S_x-enzyme at 24 °C (Capeillère-Blandin *et al.*, 1975). The latter enzyme was re-investigated for comparison in the present study at 5 °C, as illustrated in Fig. 6.

(*a*) The reaction is biphasic for flavin and haem (Table 2). Neither of the two phases is controlled by intermolecular electron exchange. A total of two electrons per protomer is distributed among H_r, F_{sq} and F_r at 10 and 80 ms for H-enzyme and S_x-enzyme respectively at the end of the rapid phase I, and the entry of the third electron per protomer occurs in the much slower phase II (slower by a factor of 10-fold) at a rate

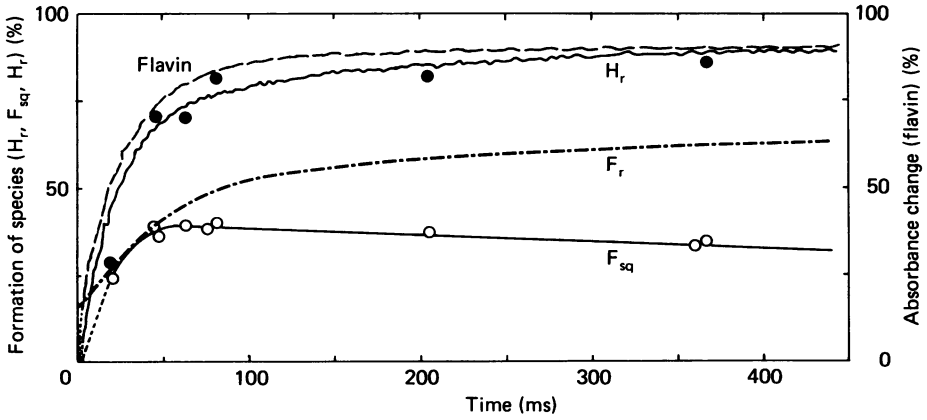


Fig. 6. Reaction pattern for the different redox species during S_x-flavocytochrome b_2 reduction at 5 °C

The experiments were carried out as described in Fig. 4(*a*), but with S_x-enzyme. Along with typical stopped-flow absorbance recordings determined as indicated in Fig. 4 legend for flavin and haem (at 9.5 μM-enzyme and 12.5 mM-L-lactate final concentrations) e.p.r. rapid-freezing results are given for H_r (●) and F_{sq} (○) (30 μM and 12.5 mM final concentrations of enzyme and L-lactate respectively) and the calculated curve for F_r (-----). The rate constant values of the initial phase for flavin and haem correspond to 44 and 40 s^{-1} respectively. A burst of 15% of F_r can be estimated in the initial part of the F_r curve. A total of two electrons per protomer is delivered at $t = 80$ ms.

constant irrelevant to its involvement in the turnover (Table 2). This result confirms that only the rapid processes involved in phase I can participate in the overall catalytic reaction.

(*b*) A common feature for both enzyme forms is the time course of F_{sq}, which exhibits a rapid initial increase along with the phase I defined at the level of flavin and haem, with an approximately constant F_{sq}/H_r ratio (lower than 1), then reaches a plateau near 40% in phase I.

(*c*) In contrast with the data reported on S_x-enzyme, there is a marked difference between the apparent reduction rate constants k_{F_I} and k_{H_I} for F_o and H_r, in the first rapid phase I, as illustrated in Table 2. The time courses observed for F_o and H_r, instead of being synchronous as observed for S_x-enzyme, are distinct for H-enzyme and well separated on the time scale with a significant lag time for the haem reduction in the region of 1.3 ± 0.5 ms.

The desynchronization with $k_F \gg k_H$ and the lag phase between flavin and haem reduction detected in H-enzyme constitute important additional direct evidence for the view that the flavin is the primary acceptor for the reducing equivalents, and are a powerful confirmation of the sequential electron-transfer mechanism already proposed by various authors (Hiromi & Sturtevant, 1966; Suzuki & Ogura, 1970; Lederer, 1974; Capeillère-Blandin *et al.*, 1975; Pompon *et al.*, 1980) to interpret the reduction kinetics of flavocytochrome b_2 by lactate. After the reversible substrate binding, the following steps taking place successively are, first, a lactate-to-flavin two-electron transfer, yielding F_r, and, secondly, a one-electron transfer from F_r to H_o, yielding F_{sq} and H_r, as illustrated in Scheme 1, characterized by an elementary rate constant k_2 that is not very high as compared with that of the first step, k_{+1}, for H-enzyme.

(*d*) Moreover, by considering the inital burst of F_r that precedes the appearance of F_{sq} and H_r at the very beginning of phase I ($t < 4$ ms for H-enzyme), an estimation of the values of the relative rate constants for the rapid formation and consumption of F_r can be obtained. In the case of H-enzyme a burst of 35% is observed instead of 15% for S_x-enzyme; this result is consistent with a faster formation process for F_r in the H-enzyme on the assumption that the rate of the following steps is unchanged.

Initial steps of electron exchange within flavocytochrome b_2

Considering these data, summarized in Table 2, it appears that the main difference between the two enzyme forms lies at the level of the first step, namely substrate binding and the electron-transfer step between bound substrate and flavin, a step that is much faster in H-enzyme. Consequently this system allows a reliable evaluation of the rate constants implied in the following step, the intramolecular electron transfer from flavin hydroquinone to oxidized haem. In previous simulation studies on S_x-enzyme kinetic data, obtained at 24 °C, only minimal estimations of these parameters were provided, owing to the apparent synchronization observation between the flavin and haem absorbance time courses.

The properties of the two-step model presented in Scheme 1 were analysed by computer simulations. A reversible electron distribution between F_r and H_o was assumed, as supported by the very small redox potential difference between the two redox systems. Concerning H-enzyme kinetic data, with the rate constants of 360, 380 and 120 s^{-1} for k_{+1}, k_{+2} and k_{-2} respectively, this scheme leads to a reasonable fitting of the flavin reduction in the initial time period and of the haem reduction which reaches its equilibrium level at the end of phase I at around 10 ms reaction time (Fig. 7*a*). It also accounts for the lag time between flavin and haem time courses. For S_x-enzyme kinetic data, the agreement between predicted and observed profiles (Fig. 7*b*) is obtained by applying to k_{+1} the value experimentally determined in the present work ($k_{+1} = 42$ s^{-1}) and using for the intramolecular electron exchange the parameters as previously adjusted for H-enzyme data.

However, in both cases, the simulated time course of H_r is not biphasic and the simulation does not take into account the experimental finding that the maximum concentration of F_{sq} is always lower than that of H_r. Also, for the H-enzyme an overshoot of F_r, for which there is no experimental evidence, is predicted. Therefore

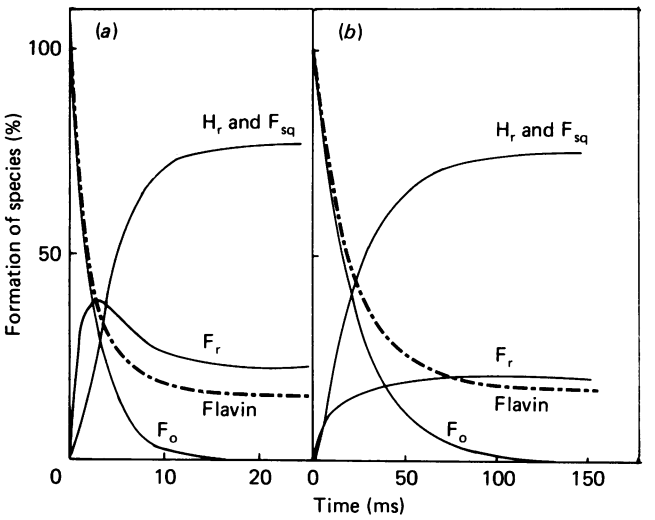
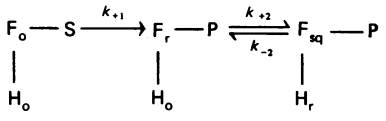


Fig. 7. Best simulated data obtained with Scheme 1 for the initial reduction phase I of (*a*) the H-enzyme and (*b*) the S_x-enzyme

The lines have been computed with Scheme 1 and the following best parameters: for H-enzyme $k_{+1} = 360$ s^{-1}, $k_{+2} = 380$ s^{-1} and $k_{-2} = 120$ s^{-1}; for S_x-enzyme $k_{+1} = 42$ s^{-1}, $k_{+2} = 380$ s^{-1} and $k_{-2} = 120$ s^{-1}. With such a kinetic model the time courses of H_r and F_{sq} are identical. The flavin absorbance time (-----), here presented upside down (cf. Figs. 4 and 6) for clarity, was reconstituted knowing F_o and F_{sq} time courses according to the relationship:

$$\Delta A_t = F_o + F_{sq}(\epsilon_{F_{sq}} - \epsilon_{F_r})/(\epsilon_{F_o} - \epsilon_{F_r})$$

with $\epsilon_{F_o} = 10$, $\epsilon_{F_r} = 1$ and $\epsilon_{F_{sq}} = 3$.



Scheme 1. Two-step model of the electron-transfer mechanism of the reduction of flavocytochrome b_2 by L-lactate

we must try to interpret in terms of elementary steps phase II (beyond 10 ms for H-enzyme), which leads to the total reduction of the enzyme. However, detailed simulation studies based on a full scheme are not included in the present paper.

In conclusion, the simulation study emphasizes the existence of an intramolecular electron transfer from flavin hydroquinone to oxidized haem occurring at similar rate in the two homologous enzymes. It is tempting to relate such a rate to the distance between the two groups pertaining to two distinct structural domains, the flavodehydrogenase moiety and the cytochrome b_2 core (Gervais *et al.*, 1983). From electron-tunnelling theories (De Vault, 1980, 1984) an edge-to-edge distance of 0.95 nm between the haem and flavin planes can be estimated by using Hopfield's treatment (Potasek & Hopfield, 1977). At such a close distance spin–spin interactions between the paramagnetic species ferric haem and semiquinone can be predicted. However, from our power-saturation study the interactions escape detection, presumably owing to the statistical presence of less than 15% of the Fe^{3+}–F_{sq} couples, or to unfavourable orientations of their planes.

Involvement of the initial reduction steps in the catalytic cycle

According to previous data, in the catalytic cycle of the H-enzyme the external mono-electronic acceptors react rapidly with transient reduced forms of the enzyme (Capeillère-Blandin, 1982). These transient reduced species are first characterized by the presence of one-electron reduced redox centres, such as reduced haem and flavin semiquinone, and secondly generated over a time scale compatible with the turnover rate, i.e. in the initial reduction phase I. From the comparison between the turnover rate and the initial reduction rate of flavin and haem groups (Table 2) it appears that a sequence of two successive electron-transfer steps (Scheme 1), which leads to the electron-pair splitting between prosthetic groups with formation of H_r and F_{sq} ($k_H = 170\ s^{-1}$), would be part of the rate-limiting sequence in the overall electron transfer catalysed by H-flavocytochrome b_2 (molar activity $120\ s^{-1}$). In contrast, data on S_x-enzyme show that the reduction rate of flavin essentially determines the rate of all the following sequence of steps, which are not rate-limiting in the catalytic cycle.

In the case of H-enzyme, among the possible slow steps whose rate might be postulated to affect directly the turnover rate are the product (pyruvate) dissociation and other types of intramolecular one-electron exchange leading to the reduction of one-electron donors, i.e. H_r and F_{sq}.

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