Electron-transfer steps involved in the reactivity of *Hansenula anomala* flavocytochrome b_2 as deduced from deuterium isotope effects and simulation studies

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The L-lactate–flavocytochrome b_2 –ferricyanide electron-transfer system from the yeast *Hansenula anomala* was investigated by rapid-reaction techniques. The kinetics of reduction of oxidized flavocytochrome b_2 by L-lactate and L-[^2H]lactate were biphasic both for flavin and haem prosthetic groups and at all concentrations tested. The first-order rate constants of the rapid and slow phases depended upon substrate concentrations, a saturation behaviour being exhibited. Substitution of the C $^{\alpha}$ -H atom by ^2H was found to cause appreciable changes in the rate constants for the initial reduction of flavin and haem (phase I), which were respectively about 3-fold and 2-fold less than with L-lactate. In contrast, no significant isotope effect was noted on the apparent reduction rate constants of the slow phase, phase II. Under steady-state conditions an isotope effect of 2.0 was found on the overall electron transfer from L-lactate to ferricyanide. These transient reduction results were discussed in terms of a kinetic model implying intra- and inter-protomer electron exchanges between flavin and haem b_2 , all of which have been experimentally described. Computer simulations indicate that the reaction scheme provides a reasonable explanation of the fast-reduction phase, phase I (in absence of acceptor). The pseudo-first-order rate constant for oxidation of reduced haem b_2 in flavocytochrome b_2 increased with increasing ferricyanide concentration in a hyperbolic fashion. The limiting value at infinite ferricyanide concentration, which was attributed to the intramolecular electron-transfer rate from ferroflavocytochrome b_2 to the iron of ferricyanide within a complex, was $920 \pm 50 \text{ s}^{-1}$ at pH 7.0 and 5 °C. Stopped-flow and rapid-freezing measurements showed haem b_2 and flavin to be 90 and 44 % oxidized respectively under steady-state conditions in presence of ferricyanide. Simulation studies were carried out to check the participation of the proposed reduction sequence in the overall catalytic reaction together with the role of reduced haem b_2 (H_r) and flavin semiquinone (F_{sq}) as electron donors to ferricyanide. When the rate of the intramolecular electron-transfer exchange between F_{sq} and ferricyanide was adjusted to 200 s^{-1} , simulated data accounted for molar activities defined under various conditions of L-lactate, [^2H]lactate and ferricyanide concentrations. Simulation studies were extended to data obtained using cytochrome c as acceptor and reaction catalysed by *Saccharomyces cerevisiae* flavocytochrome b_2 . The differences in reactivity observed for H_r and F_{sq} with ferricyanide and cytochrome c were discussed in terms of redox potentials, electrostatic interactions, distances and accessibility of the participating groups.

INTRODUCTION

Intramolecular electron transfers are of central importance to the understanding of the role of multiple redox centres of biological systems in electron transport. Flavocytochrome b_2 , an L-(+)-lactate:cytochrome c oxidoreductase (EC 1.1.2.3) from the yeast *Hansenula anomala*, provides an interesting model to investigate oriented coupled intramolecular electron transfer. Convenient properties are that its flavin and haem b_2 are tightly bound to each protomer of molecular mass 56.1 kDa as deduced from gene sequence [1,2]. This polypeptide chain is folded into two distinct domains [3–5] which are involved in two different functions, L-lactate:flavin dehydrogenase and its electron acceptor, the cytochrome b_2 , which then reacts with external acceptors such as yeast cytochrome c [6–8] and ferricyanide [6,9–11].

Several electron-transfer steps have been characterized in the reduction process of flavocytochrome b_2 by a two-electron donor, L-lactate, conducted at equilibrium [12,13] and in the course of rapid-mixing-and-freezing kinetic studies [14]. The electron-transfer sequence starts by two-electron reduction of flavin by

bound L-lactate within the flavodehydrogenase moiety, as illustrated in Scheme 1. The second step is reversible intramolecular one-electron transfer between reduced flavin and haem b_2 , the latter belonging to the cytochrome b_2 domain, taking place within a protomer, with the concomitant transient formation of flavin semiquinone. Further reversible intramolecular electron transfer from flavin semiquinone to haem b_2 has been demonstrated from T-Jump studies on partially reduced enzyme [15]. In turnover reaction with *Hansenula anomala* cytochrome c as external acceptor, intramolecular one-electron transfers take place from reduced cytochrome b_2 to cytochrome c within the reaction complex [7,8].

The purpose of the present work was first to obtain additional information on the kinetic parameter values assigned to intramolecular electron transfers, both between bound substrate and flavin, and between flavin and haem b_2 prosthetic groups, and secondly to document their participation and rate limitation effect on the overall reaction between L-lactate and ferricyanide. First, rapid reduction kinetics of flavin and haem b_2 in *Hansenula anomala* flavocytochrome b_2 (referred to below as H-enzyme or

Abbreviations used: H-enzyme, *Hansenula anomala* flavocytochrome b_2 ; S and/or S_x -enzymes, intact and/or cleaved forms of *Saccharomyces cerevisiae* flavocytochrome b_2 respectively; F_o , oxidized flavin; F_{sq} , flavin semiquinone; F_r , reduced flavin hydroquinone; H_o , oxidized haem; H_r , reduced haem; A_o and A_r , oxidized and reduced acceptor; C_o and C_r , oxidized and reduced cytochrome c ; red-ox, reduced minus oxidized.

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Scheme 1. Starting electron-transfer sequence from L-lactate (S) to flavin (F_o) and haem b_2 (H_o) prosthetic groups within a flavocytochrome b_2 protomer

Scheme 2. Overall electron-transfer sequence from L-lactate (S) to acceptors (A_o or C_o) catalysed by flavocytochrome b_2

H.a. flavocytochrome b_2) were investigated at various L-lactate and L-[2H]lactate concentrations, together with steady-state measurements. Secondly, rapid kinetics of oxidation of reduced haem b_2 in H-enzyme were investigated at various concentrations of ferricyanide, together with the observation of the redox state of haem b_2 and flavin species under steady-state conditions. The results reported constitute a direct observation of the electron transfer between reduced flavocytochrome b_2 and ferricyanide mediated by a 1:1 complex between the two reactants.

Finally, to interpret the kinetic data obtained under steady-state conditions, computer-simulation studies have been performed on the basis of a kinetic scheme (Scheme 2) involving intramolecular electron-transfer steps within flavocytochrome b_2 . A two-electron distribution per protomer is allowed (illustrated above in Scheme 1), together with steps of oxidation of flavin semiquinone and reduced haem b_2 by monoelectronic acceptors. Scheme 2 was investigated with rate-constant values for the most part experimentally determined in the present study. It accounted for experimental results obtained under multiple conditions of substrate and acceptor concentrations for *Hansenula anomala* flavocytochrome b_2 , as well as for its counterpart in *Saccharomyces cerevisiae* (baker's yeast). Simulation studies of Scheme 2 allowed one to conclude that a sequence of intramolecular electron transfers is involved as rate-limiting steps in the overall reaction, with ferricyanide as well as with cytochrome c . Furthermore, this study provides a basis for improving the full reaction sequence (Scheme 3) previously proposed for electron distribution within fully reduced flavocytochrome b_2 in absence of external acceptors. Scheme 3 implied, besides intraprotomer electron exchanges between flavin and haem b_2 (as in Scheme 1), inter-protomer electron-exchange steps, all of which have been experimentally described. By using the set of parameters ascribed above in reduction steps, Scheme 3 allowed the best fits of the reduction time courses obtained both with L-lactate and L-[2H]lactate and established for flavin, flavin semiquinone and reduced haem b_2 in H-enzyme.

MATERIALS AND METHODS

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase, EC 1.1.2.3) was purified from *Hansenula anomala* essentially according to [16], with the modifications reported in [14]. Its concentration expressed as the molarity of haem b_2 in solution was measured with a Perkin-Elmer 555 spectrophotometer from the γ -band absorbance of dithionite-reduced enzyme by using $\epsilon_{423}^{423} 183 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [17].

L-[2H]Lactate) was kindly given by Dr. D. Pompon (CGM du CNRS, Gif-sur-Yvette, France), who synthesized it by a coupled enzymic procedure followed by a purification on Dowex ion-exchange column [18]. Substrate concentrations (i.e. L-lactate and L-[2H]lactate, two-electron donors) were determined by titration of excess ferricyanide (a one-electron acceptor) monitored at 420 nm in the presence of catalytic amounts of flavocytochrome b_2 .

Enzymic activities were measured spectrophotometrically by monitoring the initial decrease of ferricyanide absorbance as a function of time at 420 nm ($\Delta\epsilon 1.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The assay solution contained a saturating concentration of ferricyanide (0.9 mM) and various concentrations of L-lactate and L-[2H]lactate in 0.1 M-sodium/dipotassium phosphate buffer, pH 7.0. The molar activities were expressed as the molar concentration of L-lactate oxidized per molar haem b_2 concentration. The standard molar activity defined in presence of 10 mM-L-lactate and 0.9 mM-ferricyanide corresponded to $120 \pm 15 \text{ s}^{-1}$ ($n = 7$), at 5 °C, as listed in Table 2 (below). When *Hansenula anomala* cytochrome c (*H.a.* cytochrome c) was used as electron acceptor, its reduction was monitored at 550 nm ($\Delta\epsilon 19 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and studied at variable concentration (0.5–20 μM). The corresponding standard molar activity was defined in presence of 10 mM-L-lactate and, for extrapolated cytochrome c concentration, to infinite value, yielding $85 \pm 10 \text{ s}^{-1}$ for three independent determinations, with a K_m of $2.4 \pm 0.3 \mu\text{M}$, at 5 °C (Table 2 below). With both acceptors, assays on duplicate samples generally agreed to better than 5%.

Stopped-flow experiments were carried out with a modified Gibson-Durrum apparatus coupled to an analog-digital multichannel converter (Tracor NS 570) and improved as previously reported in [19], having a 2.2 ms dead time and thermostatically controlled at $5 (\pm 0.1) ^\circ\text{C}$. The wavelengths used to monitor the various redox reactions studied in this work were carefully determined from difference absorbance spectroscopy carried out on cytochrome b_2 , flavocytochrome b_2 and cytochrome c as described by us previously in [8], [14] and [20] respectively. The relative contribution of haem b_2 and the various flavin redox species at these wavelengths were previously determined in [14]. Briefly, haem b_2 reduction was monitored in the α -band at 557 nm ($\Delta\epsilon_{\text{red-ox}} 21.8 \pm 0.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Flavin reduction was monitored at 438.3 nm ($\Delta\epsilon_{\text{red-ox}} -8.6 \pm 0.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), a haem isosbestic point determined precisely on the apparatus before each series of experiments as described in [21]. At 438.3 nm the absorbance-coefficient values for the flavin redox species were $\epsilon_{F_o} = 10 \pm 1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_{F_{sq}} = 3 \pm 0.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{F_r} = 1.2 \pm 0.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. At 480 nm, both haem ($\Delta\epsilon -4.6 \pm 0.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and flavin ($\Delta\epsilon -7.5 \pm 0.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) contributed to the absorbance [14]. As deduced from the absorbance spectra of the flavodehydrogenase isolated from *H.a.* flavocytochrome b_2 published in [22], at 480 nm the absorbance coefficient values for the flavin redox species are taken equal to $\epsilon_{F_o} = 8.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_{F_r} = 1.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{F_{sq}} = 4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Ferricyanide reduction was recorded at 415.6 nm ($\Delta\epsilon 1.02 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), an isosbestic point of oxidized and reduced forms of flavocytochrome b_2 .

Rapid-freezing experiments were conducted by the rapid-

mixing and freezing technique [23] performed at Dr. R. C. Bray's Laboratory in the School of Chemistry and Molecular Sciences of the University of Sussex (Falmer, Brighton, Sussex, U.K.). The e.p.r. detection of haem iron and free-radical signals, together with their quantification, was carried out as described previously [14].

All the concentrations of components given in the text correspond to final concentrations after mixing. All kinetic studies were performed in 0.1 M-sodium/dipotassium phosphate buffer at pH 7.0 and 5 °C, unless otherwise specified.

All data analyses were performed on an Apple II computer. For every kinetic experiment at least two traces were recorded at the same time-sweep. These data were then fitted to an analytic expression containing one or two exponential terms by application of non-linear iterative regression based on a least-squares criterion, as previously detailed [19]. The reaction rates were the mean values of analysing four to six traces. The s.d. was found to be 6–15 %, the highest values being those for the faster processes. Complex kinetic models were examined using the fourth-order Runge-Kutta method [24] to perform the integration of the appropriate set of differential equations, thus providing the concentrations of the different species involved as a function of time. Then, at each wavelength, the time course was reconstituted using the appropriate set of absorbance coefficients. Semi-quantitative fits were then obtained by placing tracings of the experimental data over the computed time courses.

RESULTS

Reaction of flavocytochrome b_2 with L-lactate and L-[^2H]lactate.

Reduction of flavocytochrome b_2 prosthetic groups. The kinetics of the reduction of flavocytochrome b_2 by L-lactate and L-[^2H]lactate were studied by monitoring the absorbance changes at 438.3 nm (i.e. flavin reduction) as well as at 557 nm (haem reduction). The reduction by substrate under pseudo-first-order conditions (0.1–12 mM) followed biphasic kinetics (Fig. 1). These absorption changes, for both flavin and haem, adequately fit with the sum of two exponential terms, leading to the determination of reduction rate constants k_1 and k_{11} for phases I and II respectively. High reduction rates obtained at 10 mM-L-lactate concentration are illustrated in Fig. 1(a), together with simulated time courses. Typical time courses with L-[^2H]lactate are presented in Fig. 1(b).

Whatever the substrate concentration, the total amplitude of the reaction was constant and in agreement with data from static differential spectrophotometry, indicating total reduction of flavin and haem ($\Delta\epsilon_{\text{red-ox}} -8.6 \pm 0.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\Delta\epsilon_{\text{red-ox}} 21.8 \pm 0.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [14] respectively). The initial phase (phase I) consisted of a fast process which was the major contribution to the absorbance change. The relative amplitude of the two phases was found to be essentially independent of L-lactate or L-[^2H]lactate concentration (0.4–12 mM) and flavocytochrome b_2 concentration (0.9–10 μM). The amplitude of the slow phase (phase II) in the flavin-reduction time course corresponded to 19 (± 2) % and 25 (± 4) %, mean values obtained for different concentrations of L-lactate ($n = 20$ independent traces) and L-[^2H]lactate ($n = 16$) respectively. In the haem-reduction time course, the amplitude of the slow phase corresponded to 20 (± 3) % and 22 (± 6) % for L-lactate ($n = 18$) and L-[^2H]lactate ($n = 12$) respectively.

Variations of the first-order rate constant, k_1 , of the initial phase of reduction of flavin and haem b_2 with concentration of substrate were investigated. Results obtained for L-lactate and L-[^2H]lactate are presented in Figs. 2 and 3 respectively. In both

cases, and for both flavin and haem, typical hyperbolic saturation curves were obtained. The best fits for parameters K_m and k_{max} are given in Table 1. With L-lactate there was a marked difference between the apparent reduction rate constants of flavin and haem, reaching respective saturation limits of 360 s^{-1} and 180 s^{-1} . With L-[^2H]lactate, the individual rate constants in the initial phase of reduction of flavin and haem were all substantially less than the analogous reactions with L-lactate and were substantially reduced to 100 s^{-1} , as listed in Table 1.

From analyses of the variations of the first-order rate constant of the slow phase, k_{11} , with substrate concentrations (Fig. 2), it appears that both flavin- and haem-reduction rates depend on L-lactate concentrations and have a hyperbolic character, with rate constants reaching limits of 15 s^{-1} and 13 s^{-1} respectively. After isotopic substitution, k_{11} values for flavin and haem reached limits of 12 s^{-1} and 10 s^{-1} respectively (Fig. 3).

Overall catalytic reaction and ^2H substitution. Under steady-state conditions, as described in the Materials and methods section, the initial velocity of ferricyanide reduction was determined for a series of initial concentrations of substrate,

Scheme 3. Reduction mechanism of flavocytochrome b_2

The enzyme is considered as a dimer, each protomer containing one haem b_2 (H) and one flavin mononucleotide (F). The relative position of the prosthetic groups is based on the three-dimensional structure of the tetrameric molecular determined at 0.24 nm (2.4 Å) resolution [35]. The Scheme involves 12 species related basically by a limited number of well-documented intramolecular electron-transfer steps [14,15]. The six irreversible two-electron-transfer steps from substrate to flavin (including substrate-binding step) are represented by the double arrows ($\Rightarrow$). The three reversible intraprotomer electron transfer from F_r to H_o are represented by thick single arrows pointing each way ($\rightleftharpoons$). The reversible electron-transfer from F_{sq} to H_o , performed either inter-protomer or intra-protomer, is represented by thin single arrows ($\rightarrow$) and broken arrows ($\dashrightarrow$) respectively. Rate constants used to simulate kinetic data illustrated in Fig. 1 are given. The two patterns differ by the value ascribed to k_r . At each substrate concentration the rate constant was modulated by the percentage of saturation of the complex obtained by Michaelis relationship: $k_r = k_{\text{max}} \times [S]/K_m + [S]$.

Fig. 1. Comparison of experimental reduction time courses of *H.a.* flavocytochrome b_2 prosthetic groups established at two extreme electron-entry rates

(a) The reduction time courses of *H.a.* flavocytochrome b_2 by 10 mM-L-lactate concentration at 5 °C, published in [14], were illustrated here as a shadowed area indicating (i) the reproducibility of the stopped-flow absorbance recorded tracings observed at 557 nm for haem b_2 and 438.3 nm for flavin (a haem b_2 isosbestic point) in an independent series of experiments (numbers indicated in parentheses below) performed at enzyme concentrations of 2.2 (4), 10 (2) μ M and 2.2 (2), 4.5 (1) and 10 (2) μ M respectively. The fit of the flavin and haem time courses to a sum of two exponential processes led to the determination of the apparent reduction rate constants for phases I and II. At 10 mM-L-lactate, values were 350 ± 25 s $^{-1}$ and 14 ± 2 s $^{-1}$ respectively for flavin, and 168 ± 13 s $^{-1}$ and 12 ± 2 s $^{-1}$ respectively for haem. Data from e.p.r. rapid-freezing experiments were presented as solid circles (●) for reduced haem b_2 (H_r) and circles (○) for flavin semiquinone (F_{sq}) obtained at 94 μ M-flavocytochrome b_2 concentration. The broken lines (-----) corresponded to computed time courses obtained with Scheme 3. The kinetic parameters used are given in Table 3 and, at the substrate concentration used, $k_F = k_{max} \times [S]/(K_m + [S]) = 350$ s $^{-1}$. The flavin absorbance time course (ΔA_t) at 438.3 nm was reconstituted knowing absorbance coefficients given in the Materials and methods section and simulated time courses, $[F_{o,t}]$ and $[F_{sq,t}]$, according to the relationship:

$$\Delta A_t = [F_{o,t}] + (0.22 \times [F_{sq,t}])$$

(b) Continuous lines correspond to typical stopped-flow recordings obtained in the course of the reduction of flavocytochrome b_2 by 0.25 mM-L-[2 H]lactate concentration at 5 °C for haem b_2 and flavin at 1.8 and 4.9 μ M enzyme concentration respectively. The fit of the

L-lactate and L-[2 H]lactate, in the presence of a catalytic amount of flavocytochrome b_2 . These data were then fit to the rectangular hyperbola by a least-squares non-linear iterative regression program, and the parameters K_m and V_{max} thus obtained. Results from four and two independent series of measurements for L-lactate and L-[2 H]lactate respectively are presented in Table 1. Comparison between the rate-constant values determined in phases I and II and the overall reaction rate indicates that phase II is much too slow to be part of the catalytic turnover.

Estimation of kinetic isotope effects. Data summarized in Table 1 allow calculation of the kinetic 2 H isotope effect on the maximum reduction rate constants (2Hk) and on the specificity constant ($^2Hk/K_m$) defined by the respective ratios $^2Hk = ^1Hk_{max}/^2Hk_{max}$, and $^2Hk/K_m = ^1H(k_{max}/K_m)/^2H(k_{max}/K_m)$ of experimental values determined for flavin and haem b_2 , and under steady-state conditions with ferricyanide as electron acceptor. The comparison of the kinetic isotope effect 2Hk of 3.2 (± 0.4) and 1.8 (± 0.1) on flavin- and haem-reduction rates respectively, defined in the initial phase I, indicate that: (i) flavin reduction precedes that of the haem b_2 in agreement with previous data [14,21,25], and (ii) the reduced flavin to oxidized haem b_2 transfer, as illustrated in Scheme 1, is not extremely rapid compared with overall flavin reduction. These values, compared with a 2Hk of 2.0 for turnover rate, suggest no rate limitation of the transfer to ferricyanide. As stated above, the reduction of *Saccharomyces cerevisiae* flavocytochrome b_2 (referred to below as S_x -enzyme for the cleaved form of *S.c.* flavocytochrome b_2) by L-[2 H]lactate has already been studied and kinetic isotope parameters, 2Hk , were 8, 6, 5 for flavin, haem and ferricyanide [18].

Concerning isotope effects defined on the specificity constant $^2Hk/K_m$, no significant difference was noted between values, almost equal to 2, determined either at the level of flavin or haem b_2 during a single turnover, or at the level of ferricyanide during turnover. This observation held true for published S_x -enzyme kinetic isotope data [18] when we calculated $^2Hk/K_m$. Values of 3.3, 4.1 and 3 were found respectively for flavin, haem and steady state. The comparison of the 2Hk and $^2Hk/K_m$ values for H-enzyme and S_x -enzyme brought further support to the conclusion that the main difference between the two counterpart flavocytochrome b_2 species is at the first step, namely the electron exchange between bound substrate and flavin, as evidenced in earlier reports.

Calculation of L-lactate binding parameters. 2 H substitution data obtained at the level of flavin reduction by L-lactate provide an interesting relationship between the isotope effects defined above, which can be rearranged to give the kinetic binding parameters, k_s and k_{-s} , involved in the substrate binding step:

$$^2Hk/K_m = ^2Hk \times (k_{-s} + ^2Hk_{max}) / (k_{-s} + ^1Hk_{max})$$

We thus obtain $k_{-s} = 320$ s $^{-1}$ and $k_s = 360$ mM $^{-1}$ s $^{-1}$, with $K_m = k_{-s} + k_{max}/k_s$, for the substrate binding parameters at 5 °C, values which lead to $K_d = k_{-s}/k_s = 0.90$ mM. In the present study, the lack of identity between K_m (2 mM) on the substrate, defined at the level of the first electron acceptor, and K_d , the equilibrium thermodynamic constant of the substrate binding to complex,

flavin and haem time courses to a sum of two exponential processes led to apparent reduction rate constants, for phases I and II, of 17 s $^{-1}$ and 2 s $^{-1}$ for flavin and 23 s $^{-1}$ and 3 s $^{-1}$ for haem. E.p.r. rapid-freezing results, given as ● for H_r and ○ for F_{sq} , were obtained at 90 μ M enzyme concentration. The broken lines correspond to computed time courses obtained with Scheme 3. The kinetic parameters used are given in Table 3 and, at the substrate concentration used, $k_F = 18$ s $^{-1}$.

Fig. 2. Dependence of the pseudo-first-order rate constants, k_I and k_{II} , of the two successive reduction phases of *H.a.* flavocytochrome b_2 on L-lactate concentration

Each data point represents the mean value of at least two determinations in a single experiment. Data for haem and flavin reduction are presented as ● and ○ respectively and correspond to six and five independent series of experiments (numbers indicated in parentheses below) performed at enzyme concentrations of 2.2 (4), 10 (2) μM for haem b_2 and 2.2 (2), 4.5 (1) and 10 (2) μM for flavin. The continuous lines drawn through the data points were obtained by fitting the data to a hyperbolic saturation curve with an iterative procedure as indicated in the Materials and methods section. The best-fit parameters for K_m and k_{max} are listed in Table 1. (a) Shows variations of k_I for the rapid reduction phase, with maximum rate limits of $393 \pm 35 \text{ s}^{-1}$ and $183 \pm 6 \text{ s}^{-1}$. (b) Shows variations of k_{II} for the slow reduction phase.

established that the flavin reduction rate, k_{max} , is comparable with, or higher than, k_s . In the case of the counterpart S_x -enzyme, the corresponding value for K_a was 0.44 mM, compared with 1.29 mM for the K_m observed at the level of flavin and 0.66 mM for the steady-state value [18].

Rapid kinetic studies of electron exchanges between reduced flavocytochrome b_2 and ferricyanide

In an effort to furnish experimental support to the interpretation of the kinetic isotope effect in the catalytic cycle, we investigated the electron-transfer reaction between reduced flavocytochrome b_2 and ferricyanide as an external acceptor.

Determination of the electron exchange rate between haem b_2 and ferricyanide. To quantify the electron exchange rate between reduced flavocytochrome b_2 and ferricyanide, the oxidation of reduced haem b_2 , monitored at 557 nm, was studied under

pseudo-first-order conditions (5–60 μM -ferricyanide). After rapid mixing, an apparent first-order time course was observed. The second-order plot of k_a (the apparent pseudo-first-order rate constant) versus ferricyanide concentration is illustrated in Fig. 4. Only a narrow range of concentration of the oxidant was investigated, owing to the rapidity of the recorded process. Indeed, when more than 60 % of the reaction occurs during the 2.2 ms dead time, the resolution is too poor for accurate measurement of the apparent first-order rate constant. Therefore, processes occurring at a rate higher than 450 s^{-1} were not adequately analysed.

At very low ferricyanide concentration ($< 10 \mu\text{M}$) the reaction appeared second-order, and the initial slope yielded an estimate of the second-order rate-constant for haem b_2 oxidation: $k_{\text{ox}} = (1.2 \pm 0.2) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 5°C . Above 20 μM , k_a values became relatively independent of oxidant concentration. As strengthened by control experiments previously carried out to check the performance limits of our stopped-flow equipment [7], the deviation from linearity observed in Fig. 4 did not arise from a failure to monitor the reactions accurately. Thus the electron-transfer reaction between reduced flavocytochrome b_2 and ferricyanide occurred by a simple two-step mechanism. A saturation effect of the apparent rate constant with respect to ferricyanide concentration can be shown by fitting the experimental data to a hyperbolic saturation curve. Thus the electron exchange rate between ferrocycytochrome b_2 and ferricyanide would proceed at a maximum rate, reaching $920 \pm 50 \text{ s}^{-1}$, within a protein complex characterized by a dissociation equilibrium constant, K_d , of $61 \pm 6 \mu\text{M}$.

Redox states of the prosthetic groups under steady-state conditions. According to previously obtained data, the external monoelectronic acceptors react with transient reduced forms of the enzyme [6,7,14] characterized by the presence of one-electron reduced redox centres, such as reduced haem or flavin semiquinone. To provide information on their involvement in rate-determining steps in the overall catalytic reaction, the extent of oxidation of haem b_2 and flavin redox species was compared by stopped-flow measurements performed at two wavelengths under steady-state conditions, during the reduction of ferricyanide by excess L-lactate. This approach was formerly established for S_x -enzyme and revealed no significant difference between the time courses recorded at two wavelengths [10].

As evidenced by the typical experiments presented in Fig. 5, haem b_2 absorbance remained mainly in an oxidized state corresponding to a constant level of $90 \pm 5\%$ (Table 2), as long as steady-state conditions were maintained. This observation confirms that, for haem b_2 , the reduction process is slower than the oxidation step. At a wavelength of 480 nm interpretation of transient absorbance variation was further complicated by the overlap between absorbance spectra of haem b_2 , oxidized flavin and flavin semiquinone [14,22] and the initial absorbance of ferricyanide ($\Delta\epsilon$ 0.02 $\text{mm}^{-1} \cdot \text{cm}^{-1}$). The time course was distinctly different from that recorded at 557 nm: after rapid mixing, a significant fall in absorbance was detected, followed by a continuous absorbance decrease related to ferricyanide consumption during steady state. At the end, the absorbance variation of the enzyme corresponded to $34 \pm 6\%$, as compared with the total absorbance variation determined when complete reduction of the enzyme was performed by L-lactate in the absence of ferricyanide. Nevertheless, the resulting time course at 480 nm was apparently synchronized with that at 557 nm. When ferricyanide was almost entirely consumed, i.e. at 1.8 s, as observed at 415.6 nm (isosbestic point of oxidized and reduced forms of flavocytochrome b_2), then the reduction rate of the individual groups began to exceed the oxidation rate and an absorbance

Table 1. ^2H isotope effects

After rapid mixing of oxidized H-enzyme with L-[^1H]lactate or L-[^2H]lactate the values of K_m and k_{max} for flavin and haem reduction, defined in the rapid phase I and slow phase II, were derived from the fit of the dependence of k_i and k_{ii} on substrate concentration, to a hyperbolic relation (illustrated in Figs. 2 and 3). The steady-state parameters defined with 0.9 mM-ferricyanide, as external electron acceptor, corresponded to results from four and two independent series of measurements performed with L-[^1H]lactate and L-[^2H]lactate respectively. The k_{cat} value corresponds to a maximum value, k_{max} , extrapolated at infinite substrate concentration and expressed as mol of substrate consumed/s per mol of enzyme. Results are means $\pm$ s.d.

	L-[^1H]Lactate			L-[^2H]Lactate			Isotope effects	
	K_m (mM)	k_{max} (s^{-1})	k_{max}/K_m ($\text{mM}^{-1}\cdot\text{s}^{-1}$)	K_m (mM)	k_{max} (s^{-1})	k_{max}/K_m ($\text{mM}^{-1}\cdot\text{s}^{-1}$)	$^2\text{H}k^*$	$^2\text{H}k/K_m^\dagger$
Reduction phase I								
Flavin	2.0 ± 0.2	393 ± 35	200 ± 40	1.3 ± 0.2	124 ± 6	95 ± 19	3.2 ± 0.4	2.0 ± 0.8
Haem b_2	0.85 ± 0.09	183 ± 6	220 ± 30	0.8 ± 0.1	100 ± 3	120 ± 20	1.8 ± 0.1	1.7 ± 0.5
Steady-state reaction								
Ferricyanide	1.1 ± 0.2	130 ± 15	120 ± 40	0.9 ± 0.1	64 ± 8	70 ± 20	2.0 ± 0.5	1.7 ± 0.8
Reduction phase II								
Flavin	0.94 ± 0.25	16 ± 1	17 ± 6	0.86 ± 0.23	12 ± 0.8	14 ± 5	1.3 ± 0.2	1.2 ± 0.8
Haem b_2	0.76 ± 0.16	13 ± 0.7	17 ± 5	0.51 ± 0.19	10 ± 0.9	20 ± 9	1.3 ± 0.2	0.9 ± 0.6

* Defined as $^1\text{H}k_{\text{max}}/^2\text{H}k_{\text{max}}$.

† Defined as $^1\text{H}(k_{\text{max}}/K_m)/^2\text{H}(k_{\text{max}}/K_m)$.

Fig. 3. Dependence of the pseudo-first-order rate constants, k_i and k_{ii} , of the two successive reduction phases for flavin and haem in *H.a.* flavocytochrome b_2 on L-[^2H]lactate concentration

Each data point represents the mean value of at least two determinations in a single experiment. Data for flavin (a) and haem b_2 (b) reduction are presented as $\bullet$ and $\circ$ respectively and correspond to independent series of experiments (numbers indicated below in parentheses) performed at enzyme concentrations of 4.9 (1), 10 μM (2) and 1.8 (1) and 2.6 μM (1) respectively. The continuous lines drawn through the data points were obtained by fitting the data to a hyperbolic saturation curve. The best-fit parameters for K_m and k_{max} are listed in Table 1. (a) Shows variations of k_i with maximum rate limits of $124 \pm 6 \text{ s}^{-1}$ and $100 \pm 3 \text{ s}^{-1}$ for the flavin and haem b_2 respectively. (b) Shows variations of k_{ii} for slow reduction phases for flavin and haem b_2 .

Fig. 4. Dependence of the pseudo-first-order rate constant, k_s , for oxidation of reduced haem b_2 by ferricyanide

Typical stopped-flow traces recorded at 557 nm were obtained after the rapid mixing of ferricyanide, varying from 5 to 60 μM , with flavocytochrome b_2 (1.4 μM) maintained under fully reduced state by 0.05 mM-L-lactate in the presence of an inhibitor (5 mM-sodium oxalate). Each data point represents the mean value of two determinations in a single experiment. The fit of the data points to a hyperbolic saturation curve leads to the determination of the maximum oxidation rate limit of reduced haem b_2 , k_{max} , of $920 \pm 50 \text{ s}^{-1}$ and a dissociation equilibrium constant, K_d , of $61 \pm 6 \mu\text{M}$.

spite experimental uncertainties, a reasonably constant level of $18 \pm 10\%$ was maintained during steady state. Taking into consideration the variation in absorbance coefficients, $\Delta\epsilon$, for flavin species and haem b_2 as depicted in the Materials and methods section, the radical estimate allowed computation of an oxidized flavin fraction of $44 \pm 16\%$ from the absorbance variation detected at 480 nm in steady-state conditions.

Simulation studies

Reaction scheme for steady-state conditions. In the light of the present data, we simulated the steady-state data in Table 2 obtained in the overall electron transfer from L-lactate to

level corresponding to a complete reduction of the enzyme prosthetic groups was finally reached.

The semiquinone radical was estimated in the course of parallel rapid-mixing-and-freezing experiments (result not shown). De-

Table 2. Experimental and simulated steady-state parameters

Experimental steady-state kinetic parameters were determined in the present study at 10 mM or at saturating L-lactate concentration conditions, either with ferricyanide or *Hansenula anomala* cytochrome *c*, for *H.a.* and *S.c.* flavocytochrome b_2 , in 0.1 M-sodium/dipotassium phosphate buffer, pH 7.0 at 5 °C. All the kinetic results at saturating substrate concentration were collected in Table 1. Data at 5 mM-L-lactate and 0.41 mM-ferricyanide were taken from Fig. 5. Other determinations were previously published in [14]. Simulated values (written in italic in parentheses) were predicted by analysis of Scheme 2, using rate constants listed in Table 3.

Conditions	Molar activities (s ⁻¹)	Redox levels of prosthetic groups (%)		
		H _o	F _{sq}	F _o
At 5 mM-L-lactate				
<i>H.a.</i> flavocytochrome <i>b</i> ₂ 0.41 mM-Ferricyanide	90 (87)	90 ± 6 (85)	18 ± 10 (24)	44 ± 16 (50)
At 10 mM-L-lactate				
<i>H.a.</i> flavocytochrome <i>b</i> ₂ 0.9 mM-Ferricyanide	120 ± 15* (117)	(81)	(29)	(35)
Cytochrome <i>c</i> †	83 ± 10 (75)	(64)	(44)	(21)
<i>S.c.</i> flavocytochrome <i>b</i> ₂ 0.9 mM-Ferricyanide	32 ± 5* (34)	(94)	(8.5)	(81)

At infinite substrate concentration	<i>k</i> _{cat} (s ⁻¹)	Redox levels of prosthetic groups (%)			<i>K</i> _m (mM)‡
		H _o	F _{sq}	F _o	
<i>H.a.</i> flavocytochrome <i>b</i> ₂ 0.9 mM-Ferricyanide and:					
L-Lactate	130 ± 15 (124)	(79)	(31)	(30)	1.1 ± 0.2 (1.23)
L-[² H]Lactate	64 ± 8 (68)	(89)	(17)	(64)	0.9 ± 0.1 (1.07)

ren and four determinations for H- and S_x-enzyme respectively [14].
 values at infinite cytochrome *c* concentrations from three determinations with *K*_m = 2.4 ± 0.3 μ

assuming *k*_{-s} + *k*_{cat}./*k*_s.

* Mean values from seven and four determinations for H- and S_x-enzyme respectively [14].

† Extrapolated mean values at infinite cytochrome *c* concentrations from three determinations with $K_m = 2.4 \pm 0.3 \mu\text{M}$ in 0.1 M-phosphate buffer, pH 7.0, 5 °C.

‡ K_m values simulated assuming $k_{-S} + k_{cat}/k_S$.

ferricyanide. For this purpose we extended to turnover conditions the range of simulation studies carried out previously using the kinetic model (here presented as Scheme 2), which we proposed for the flavocytochrome b_2 -cytochrome *c* reaction during a single turnover [7].

Indeed, in rapid-kinetic analyses we showed that two kinds of intramolecular one-electron transfers take place in the turnover with cytochrome *c*: (i) from flavin hydroquinone or semiquinone to ferricytochrome b_2 [14,15], i.e. respectively from three to four and from five to six species, (ii) from ferrocycytochrome b_2 to ferricytochrome *c* [7], i.e., from four to five and from six to one species [7,8]. The rate of the latter process, here termed ' $k_{ox}(\text{cytc})$ ', was experimentally determined to reach a value of $380 \pm 30 \text{ s}^{-1}$ in the protein complex [7].

By contrast, when ferricyanide was considered as external acceptor, an additional donor, flavin semiquinone, was implied in the electron transfer [6,9–11]. The value, still unknown, of the rate constant of such a process would have to be adjusted. Moreover, under turnover conditions it was necessary to add to the previous Scheme compulsory steps of flavin reduction by L-lactate in order to describe the reduction of the flavin group, alternatively oxidized by haem b_2 and/or acceptor. When data obtained under non-saturating-substrate conditions were fitted, the substrate-binding parameters determined in the present study

were applied to steps 1→2, and 6→7. These steps were followed by intramolecular electron transfer between bound L-lactate and flavin (steps 2→3 and 7→8), performed at maximum rate ($k_{max} = 400 \text{ s}^{-1}$). On the basis of a detailed analysis of the pyruvate product binding to the three redox states of the flavin in the *H.a.* flavocytochrome b_2 [26], the dissociation of the product after L-lactate oxidation from F_r species can be considered to be very fast and thus does not appear on the Scheme.

Table 2 shows simulated steady-state values compared with the corresponding experimental data obtained under different conditions for substrate concentration, for different donors (L-lactate and L-[²H]lactate) and different acceptors (cytochrome *c* and ferricyanide). Basically, Scheme 2 was investigated by using rate constants listed in Table 3, based on the experimental values determined in the present study and taken within the interval of accuracy. Only two of them were adjusted as detailed below.

Careful analysis of Scheme 2 enabled us to discriminate the particular rate-constant values which cannot satisfactorily fit the whole set of experimental data. These rate constants were relative to the oxidation of F_{sq} by H_o within the same protomer and/or by external acceptor. (i) The intramolecular electron transfer between flavin semiquinone and haem b_2 (step 5→6) is compulsory when the acceptor is cytochrome *c* and not fer-

Table 3. Kinetic parameters used in Scheme 2 providing best estimates for the steady-state parameters collected in Table 2

(a) Optimized values chosen in the uncertainty range of experimentally determined kinetic parameters for *H.a.* and *S.c.* flavocytochrome b_2 reactions

Reaction	Parameter and value	Reference
Substrate binding	$k_{6,7} = k_{1,2} = 360 \text{ mM}^{-1} \cdot \text{s}^{-1}$	Present study
Flavin reduction by substrate for H-enzyme	$k_{7,6} = k_{2,1} = 320 \text{ s}^{-1}$	Present study
Flavin reduction by substrate for S_x -enzyme	$k_{7,3} = k_{2,3} = 400 \text{ s}^{-1}$ (for L-[^1H]lactate)	Present study
Oxidation of reduced haem b_2 by acceptors at saturating concentration*	$k_{7,3} = k_{2,3} = 124 \text{ s}^{-1}$ (for L-[^2H]lactate)	Present study
	$k_{7,3} = k_{2,3} = 44 \text{ s}^{-1}$	[14]
	$k_{4,5} = k_{6,1} = 920 \text{ s}^{-1}$ (for Ferricyanide)	Present study
	$k_{4,5} = k_{6,1} = 400 \text{ s}^{-1}$ (for <i>H.a.</i> cytochrome <i>c</i>)	[7]

(b) Adjusted parameters

Reaction	Parameter and value
Oxidation of flavin semiquinone by ferricyanide at saturating concentration*	$k_{4,6} = k_{5,1} = 200 \text{ s}^{-1}$
Intramolecular electron exchange between: Flavin hydroquinone and haem b_2	$k_{3,4} = 400 \text{ s}^{-1}$
	$k_{4,3} = 180 \text{ s}^{-1}$
Flavin semiquinone and haem b_2	$k_{5,6} = 400 \text{ s}^{-1}$
	$k_{6,5} = 200 \text{ s}^{-1}$

* For non-saturating acceptor concentration the rate constant was modulated by the percentage of saturation level of the complex obtained by consideration of the Michaelis relationship: $k_{\text{max.}} \times [\text{S}]/K_m + [\text{S}]$, with $K_m = 61 \text{ } \mu\text{M}$.

ricyanide, since it can be shunted through processes 4→6 and 5→1. Thus its parameters would rather be adjusted when considering cytochrome *c* data. By simulations we found here that this step should not be rate-limiting. Correct fits were obtained with $400 \text{ s}^{-1}/200 \text{ s}^{-1}$, a ratio in agreement with the equilibrium constant deduced from redox titrations ($K = 1.5 \pm 0.7$) [14]. Clearly these absolute values are in disagreement with T-Jump estimates ($140 \pm 70 \text{ s}^{-1}$) as recalculated with the equilibrium constant equal to 1 ± 0.5 in [13]. (ii) The turnover rate of L-lactate to ferricyanide was higher than to cytochrome *c*. Thus an additional electron flow from F_{sq} to ferricyanide was considered. The oxidation rate of F_{sq} by ferricyanide was assumed to be performed within a reactant complex and adjusted to 200 s^{-1} . This value made possible correct fits to experimental kinetic data obtained under multiple conditions of substrate concentrations, for H-enzyme as well as for S_x -enzyme (Table 2). Indeed, the analysis of Scheme 2 underlined the absence of one single rate-determining step, as identified in the initial flavin reduction in S_x -enzyme overall reaction. In contrast, the H-enzyme mechanism illustrated an unexpected situation where a sequence of steps of similar rates participated in the overall reaction rate by splitting the initial electron-transfer rate. The results obtained here suggested that these catalytic differences between the two flavocytochrome b_2 species might be associated with the localized structural difference in the folding of the polypeptide chain underlined in [1] from gene-sequence analysis.

Moreover, the whole set of rate constants allowed us to predict steady-state redox states of prosthetic groups close to the experimental ones and account for the steady-state rates determined in different conditions of substrate or acceptor concentration. K_m values defined at saturating L-lactate or L-[^2H]lactate concentration could be simulated as calculated from substrate-binding parameters. It is worthwhile remarking here that the binding rate-constant parameters, k_s and k_{-s} , calculated from the isotope-effect approach, and here found equal to $360 \text{ mM}^{-1} \cdot \text{s}^{-1}/320 \text{ s}^{-1}$, suggest that earlier estimates of $100 \text{ mM}^{-1} \cdot \text{s}^{-1}/70 \text{ s}^{-1}$, deduced from the analysis of the depen-

dence of the molar activities with low L-lactate concentration ($< 100 \text{ } \mu\text{M}$), and assuming K_d equivalent to K_m to calculate k_{-s} , were likely to have been underestimated [26]. Furthermore we verified that earlier-obtained parameters were not appropriate for predicting the steady-state parameters.

Reduction of flavocytochrome b_2 in the absence of external acceptors. Simulations of reduction data presented in Fig. 1 were investigated with Scheme 3, which is based on the formalism developed by Pompon [27] to interpret *S.c.* flavocytochrome b_2 kinetic data [18]. As in other reaction schemes previously proposed for the reduction processes occurring in S_x -enzyme [28–30], Scheme 3 implies the two basic initial steps, presented in Scheme 1 (first flavin reduction then electron transfer to haem) besides the one-electron-exchange steps. The reversibility of the electron exchanges between flavin and haem was underlined in [28] to account for the thermodynamic and kinetic properties we observed for S_x -enzyme prosthetic groups [21] and now determined for H-enzyme [12–14]. Moreover, Scheme 3 has specific improvements. Among them are: first, the involvement of all the twelve possible redox states of two flavins and two haems in a dimer, thus allowing all kinds of electron exchanges between them, in particular one-electron exchange between protomers. Second, the consideration of a two-step reduction for the two flavins of a dimer instead of a coupled reaction such as we used previously [28], a simplification valid only at high rates of electron entry. However, in the present study, only three types of one-electron exchanges between protomers were selected: two rapid transfers involving F_i or F_{sq} and H_o within a protomer, and a slower inter-protomer electron exchange between F_{sq} and H_o . The kinetic parameters for the best fits were detailed in Scheme 3. The same values provided acceptable fits for steady-state parameters and therefore were listed in Table 3. The corresponding simulated curves, illustrated in Fig. 1, for rapid reduction at 10 mM -L-lactate and slow reduction at 0.25 mM -L-[^2H]lactate account for the main characteristics of the reduction pattern, i.e. the description of the lag time between flavin and

Fig. 5. Redox states of flavocytochrome b_2 during steady state and the return to the reduced state

Typical recordings of haem b_2 (trace *a* at 557 nm) and flavin (trace *b* at 480 nm) absorbance changes were obtained after the rapid mixing of oxidized enzyme (1.3 μ M) with L-lactate (5 mM) and ferricyanide (0.42 mM). The reduction of ferricyanide (trace *c*) was monitored at 415.6 nm and corresponded to a molar activity of 90 s^{-1} . After its consumption, the enzyme prosthetic groups returned to the fully reduced state by reduction with excess L-lactate. In steady-state conditions, transient enzyme species reached a constant level corresponding to 90 $\pm$ 6% for oxidized haem b_2 . At 480 nm the initial decrease in absorbance estimated after consumption of ferricyanide corresponds to 34 $\pm$ 6%. Taking into consideration, first, the difference absorbance coefficients at 480 nm for haem b_2 , ferricyanide, and flavin species and, secondly, the semiquinone content of 18 $\pm$ 10% determined by e.p.r. measurements in the course of rapid-mixing-and-freezing experiments performed during the catalytic cycle, the computed fraction of oxidized flavin would be 44 $\pm$ 16%.

haem time courses at high electron entry and the biphasic form of the time courses.

The initial reduction of F_o by substrate was performed at a rate constant defined experimentally by its dependence on the concentration (Figs. 2 and 3). The parameters of the intramolecular electron exchange between F_r and H_o were shown to control the correct fits of the reduction time courses of haem b_2 . Values and limits of 400 $\pm$ 50 s^{-1} and 180 $\pm$ 30 s^{-1} , giving a ratio still compatible with our redox titration data ($K = 2.8 \pm 0.7$) [14], were the best estimates both for reduction processes and steady-state simulations.

Analysis of patterns of simulated time courses indicated that the one-electron transfer from F_{sq} to H_o allowed one to control both the maximal amount of F_{sq} , lower than H_r , and its time scale, as experimentally observed. The individual rate constants of this exchange were adjusted at 200 s^{-1} /100 s^{-1} . Such a transfer was directly observed by T-jump on partially reduced H-enzyme [15]. Values considered for the inter-protomer exchange in the simulation, adjusted at 200 s^{-1} /100 s^{-1} , were included in the limits 140 $\pm$ 70 s^{-1} ascribed to the kinetic parameters as recalculated with an equilibrium constant equal to 1 $\pm$ 0.5 in [13]. Simulation studies indicated that further reversible exchange between F_{sq} and H_o of the same protomer had to be performed with a non-limiting rate. Correct fits were obtained with 400 s^{-1} /200 s^{-1} , which provided rapid-equilibration distribution of two or four electrons over the four prosthetic groups present in a dimer according to their redox potentials ($K = 1.5 \pm 0.57$) [14].

The preceding one-electron exchange led to the formation of one F_o per dimer required to uptake the third couple of electrons from bound L-lactate. Simulation studies indicated that the final reduction should be performed at a rate lower than the initial flavin reduction ($k_F/3$). It thus contributed to account for the slowness of flavin and haem reduction in phase II.

DISCUSSION

Initial steps of electron exchange within flavocytochrome b_2

The results presented here on the reduction of *H.a.* flavocytochrome b_2 by L-lactate and L-[2H]lactate confirm and extend those reported previously on S_x -enzyme [18,21,25,27,28] and on H-enzyme [12,14] concerning the sequence of steps involved in the enzyme reduction and the catalytic cycle. They provide further information on the relative rate constant values controlling the intramolecular electron-transfer steps established by other approaches.

Hence the present data entirely support the viewpoint that, after the binding of substrate, the reduction of flavin precedes that of haem b_2 . Indeed, the substitution of the α -hydrogen atom by 2H has a greater effect on the rate of flavin reduction than on the apparent reduction rate of haem b_2 . However, the value of 2Hk of 3.2 found for flavin reduction was less than the theoretical value of the primary isotope effect of C–H bond breaking, calculated to be equal to 6.8 [31,32]. This finding is explained in two ways. First, the maximum velocity can be limited in part by steps that are not related to the α -proton abstraction. Indeed the mechanism of flavin reduction in flavocytochrome b_2 implies an electron transfer from the resulting carbanion to the flavin [25]. Thus, in H-enzyme, this electron-transfer step will not be very fast compared with that which involves breaking of the substituted bond. Secondly, the lower value of the 2Hk can be related to the extent of breaking of the C α –H bond in the transition state of the enzyme reaction [32,33].

The reduction of the haem proceeds by an intramolecular one-electron transfer from F_r to H_o , producing F_{sq} [14,21]. The present data confirm this proposal by providing new evidence. The observed decrease in isotope effect, 2Hk , between the initial transfer to flavin (3.2 $\pm$ 0.4) and the overall transfer to haem (1.8 $\pm$ 0.1) implies the existence of an additional rate-determining step involved in the haem reduction, to a rate that is not extremely high as compared with the initial flavin reduction. Indeed, at saturating L-lactate concentration, a lag time of 1.3 $\pm$ 0.5 ms for haem reduction becomes significantly detectable (Fig. 1). This condition would allow better definition of the absolute values of the rate constants for the flavin-to-haem electron transfer than previously done for S_x -enzyme. Indeed, Scheme 3 predicted fits for flavin- and haem-reduction time courses of H-enzyme reasonably well, under both substrate conditions, when the electron distribution from F_r to H_o , synchronous with semiquinone (F_{sq}) formation, was performed at a rate with the same order of magnitude (400 s^{-1} /180 s^{-1}) as the flavin reduction rate seen when distribution was performed at maximal rate of 400 s^{-1} in the L-lactate-liganded F_o complex. We checked that these values were also able to account for S_x -enzyme data obtained at 10 mM-L-lactate [14,18]. Such an agreement was very likely, since the initial step of flavin reduction was rate-limiting for the following electron-transfer steps.

Another point of interest is that Schemes 2 and 3 predicted the involvement of rapid intramolecular electron exchange between F_{sq} and H_o of the same protomer. Values of 400 s^{-1} /200 s^{-1} ascribed to the compulsory intramolecular electron transfer between F_{sq} and haem b_2 gave correct fits to experimental data, both with cytochrome *c* and ferricyanide as electron acceptor. Furthermore, this transfer afforded a rapid electron equilibration among the four prosthetic groups in a dimer which accounted for the transient maximal formation of F_{sq} both in time and concentration. The existence of such a rapid transfer was suspected in the course of T-jump studies, but could not be proven, owing to its rapidity at 16 $^{\circ}C$ ($> 2000 s^{-1}$) [15]. Indeed the rapidity of these intra-protomer electron exchanges is compatible with the close distance (1 nm edge to edge) found between

flavin and haem in the three-dimensional structure of the counterpart enzyme from *Saccharomyces cerevisiae* [34,35].

Slow final reduction stages of flavocytochrome b_2

The present data clearly established that: (i) the fast reduction phase I for flavin and haem correlated with F_{sq} development, reaching a maximal level at its end, corresponded to the distribution of four electrons per dimer [21,14] and (ii) was involved in the catalytic cycle. Now the significance of the following slow phase II could be questioned. In Scheme 3 the slowness of phase II is related to the slow uptake of the third electron pair by flavin ($k_F/3$) in a two-thirds-reduced enzyme. The nature of the step that modified the reactivity of flavin could be raised. The pre-reduction of three groups over four in a dimer could modified the structure of the assembly and sterically hindered the approach of the substrate near the assumed N(5) reactive site of the flavin [33]. On the other hand, dissociation of pyruvate from its prime complex with F_r species, resulting from one substrate turnover per protomer, could be, *a priori*, the rate-limiting step in phase II. Both assumptions would be in agreement with the absence of isotope effect noted on the rate of the slow reduction phase, phase II. The slowness of product dissociation would directly affect the turnover rate, which was in contradiction with the available data. Indeed, recent studies on the inhibition behaviour of pyruvate on the catalytic activity of flavocytochrome b_2 provided information on the dependence of the dissociation rate of pyruvate from its flavin-binding site on the redox state of flavin, but performed at a non-limiting rate from F_r species [26].

Another important fact which has to find an explanation in terms of steps in the reaction scheme is that amounts of F_{sq} do not substantially decrease in phase II. This behaviour was not easy to rationalize with Scheme 3. However, recent detailed studies on the role of pyruvate have demonstrated that this ligand binds to F_{sq} intermediate species ($K_d = 0.2$ mM) [13,26]. The resulting complex behaved as a dead-end complex in steady-state kinetics [26]. The reactivity of pyruvate-liganded F_{sq} species towards H_o in H-enzyme was determined to be reduced to 5 s^{-1} , instead of 200 s^{-1} , for non-liganded F_{sq} species by stopped-flow measurements at 5°C [36]. These results provide an explanation for the steady-state plateau reached by F_{sq} and continued over 200 ms, when two electrons per protomer have been delivered to the enzyme [14]. Indeed, the experimental data showed that the initial reduction processes detected at the level of haem (from 4 to $94\text{ }\mu\text{M}$) and at the level of flavin (from 2 to $10\text{ }\mu\text{M}$) were independent of flavocytochrome b_2 concentration. However, at the high enzyme concentration used for e.p.r. measurements of flavin semiquinone, a single substrate turnover per protomer led to the formation of approx. 0.1 mM-pyruvate, which can re-bind to the enzyme, leading to a 60% saturated complex with F_{sq} transient species. The half-time of 0.5 s we experimentally detected for the semiquinone decrease [14] would thus correspond to the low reactivity of such a pyruvate-liganded- F_{sq} complex. The unexpected stability of F_{sq} in the final stages of reduction could not be explained by this kinetic model and requires further investigation.

Electron-transfer reactions between flavocytochrome b_2 and external acceptors

Previous studies led to the characterization of the L-lactate-flavocytochrome b_2 -cytochrome c system from *H.a.* yeast [7,8,37]. Rapid-kinetic investigations showed that the pseudo-first-order rate constant of electron transfer between reduced flavocytochrome b_2 and oxidized cytochrome c decreased with increasing ionic strength, as expected for a reaction facilitated by electrostatic interactions. However, this rate reached a saturation behaviour of $380 \pm 30\text{ s}^{-1}$ at low ionic strength [7]. Such an

intramolecular transfer was recently confirmed to take place in reaction between *S.c.* flavocytochrome b_2 and horse heart cytochrome c derivatives, with a first-order rate constant $700 \pm 100\text{ s}^{-1}$ at 25°C [38]. The present paper reports a parallel study with ferricyanide as acceptor, which affords valuable information on the reactivity of the two possible one-electron donors, haem b_2 and flavin semiquinone.

Data on kinetic ^2H isotope effects allowed a qualitative analysis of the relative rate values for the intramolecular electron-transfer steps occurring in the catalytic reaction (Scheme 2). No significantly different isotope effects on $^2\text{H}k$ were noted in haem reduction and in the overall catalytic reaction to ferricyanide. This observation suggested that similar rate-determining steps participate in both types of reaction sequences. All additional steps, i.e. oxidation of transient reduced redox centres by external acceptors, proceed at a rate high enough to introduce no additional limitation. This is in agreement both with the observation that haem b_2 remained mainly oxidized in steady-state conditions in the presence of both ferricyanide and L-lactate, and with the determination of an electron exchange rate as high as $920 \pm 50\text{ s}^{-1}$ between H_r and ferricyanide mediated by a 1:1 complex between the two reactants. Moreover, the finding that most of the flavin remained in reduced forms under the same conditions suggested that the oxidation of the flavin by ferricyanide occurred at a rate much lower than haem b_2 oxidation. Indeed, the flavin oxidation should proceed at a rate equivalent to, or lower than, the flavin reduction rate. Through critical analysis of computer-simulation studies based on Scheme 2, we obtained information on the range and variations to be ascribed to the rate constants of oxidation of F_{sq} . In particular, the electron exchange between F_{sq} and ferricyanide was assumed to be performed in a transient-competent electron-transfer complex at a rate lower than that of the flavin-reduction process and optimized at 200 s^{-1} .

Several lines of evidence have emphasized the importance of protein or reactant-protein complex in the mechanism of electron exchange. Kinetic data obtained on the electron transfer involving H_r and F_{sq} and external acceptors suggest that flavocytochrome b_2 provided a most interesting system for illustrating the factors which control biological electron-transfer processes. Possible controlling factors of the electron-transfer rate include the difference in redox potentials between participating electron carriers, distances, relative orientations and degrees of solvent exposure of redox centres, and site-specific and overall charge distribution, as reviewed in [39–41]. A comparison of data obtained for the reaction of reduced haem b_2 with ferricyanide or cytochrome c indicates that, in terms of second-order rate constant ($k = 1.2 \times 10^7\text{ M}^{-1}\cdot\text{s}^{-1}$), the reaction is substantially slower with ferricyanide than the analogous exchange with oxidized *H.a.* cytochrome c [$k = 7 \times 10^7\text{ M}^{-1}\cdot\text{s}^{-1}$ (in conditions of 0.2 M ionic strength, pH 7.0 and 5°C)] [7]. This difference in rate is in agreement with a repulsive electrostatic interaction involved with the negatively charged (-3) ferricyanide. Once the intermediate complex is formed, ionic-strength effects are not expected to be manifested on the first-order rate constant. Indeed, the higher oxidation rate limit for the oxidation of haem b_2 by ferricyanide (920 s^{-1}) than by cytochrome c (380 s^{-1}) is consistent with the expected greater potential difference towards ferricyanide ($\Delta E = 420\text{ mV}$) than towards cytochrome c ($\Delta E = 250\text{ mV}$).

Simulation studies of Scheme 2 provide comparative data on the reactivity of flavin semiquinone and haem b_2 with ferricyanide and/or cytochrome c . The first-order rate constants for the reactivity of semiquinone with external acceptor are uniformly lower than those involving H_r , in spite of the implication of a similar difference in redox potential [13,14]. This suggests that

substantial steric effects are operative. A similar difference in reactivity of the prosthetic groups in flavocytochrome b_2 was earlier observed in the course of pulse-radiolysis studies with carboxylate radicals [42]. The results led to the conclusion that, whereas the haem b_2 crevice would be accessible to the solvent, the methylbenzene end of the flavin isoalloxazine ring (presumably the site of electron transfer [33]) would be buried, prevented from solvent contact by a compact folding of the polypeptide chain [43]. This assumption is now supported by data on the three-dimensional structure of the *S.c.* flavocytochrome b_2 molecule [34,35]. Presumably this increases the distance over which electron transfer must proceed and is the cause of the lower reactivity of semiquinone as compared with haem b_2 .

It is interesting to note that the difference in rate-constant magnitude observed for electron transfer from F_{sq} or H_r to cytochrome c or ferricyanide could be a consequence of the relative distances and orientations of the participating redox centres within the electron-transfer complex. In the flavocytochrome b_2 -cytochrome c complex, the main association area is known to be localized on the flavodehydrogenase domain [37]. However, comparison of the reactivity of haem b_2 in free cytochrome b_2 or in flavodehydrogenase-bound cytochrome b_2 towards cytochrome c leads to the conclusion that the intramolecular electron exchange between haem b_2 and haem c occurs at a very similar rate, thus implying a similar transition state for the competent reaction complex [8]. Finally, electrostatic interactions in the flavocytochrome b_2 -cytochrome c complex may not orient the two proteins in a manner as favourable to electron transfer from F_{sq} as from H_r . The relatively small ferricyanide ion is less sterically hindered than a cytochrome c molecule, and for this reason is less influenced by the steric constraints on the flavin exposure. This is consistent with the reactivity noted between F_{sq} and ferricyanide, in contrast with its complete absence of reactivity towards cytochrome c .

Finally, the kinetic characterization of multiple electron transfer involving flavin, haem b_2 and external acceptors were reported in *H.a.* flavocytochrome b_2 . It is hoped that progress in the elucidation of the role of strategic amino acid residues in the electron-carrier function of flavocytochrome b_2 will now be performed both by the generation of mutants of flavocytochrome b_2 with the help of site-directed mutagenesis [1,2] and analysis of computer-generated molecular models of the acceptor-flavocytochrome b_2 complex [44].

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