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Cytochrome b_2, an electron carrier between flavocytochrome b_2 and cytochrome c

Rapid kinetic characterization of the electron-transfer parameters with ionic-strength-dependence

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The oxidation–reduction properties of free cytochrome b_2 isolated by controlled proteolysis from flavocytochrome b_2, i.e. the flavodehydrogenase-bound cytochrome b_2, were investigated by using stopped-flow spectrophotometry. The rapid kinetics of the reduction of cytochrome b_2 by flavocytochrome b_2 in the presence of L-lactate are reported. The self-exchange rate constant between reduced cytochrome b_2 bound to the flavodehydrogenase and free cytochrome b_2 was determined to be 10^5 $M^{-1} \cdot s^{-1}$ at 5 °C, I 0.2 and pH 7.0. The specific electron-transfer reaction between reduced cytochrome b_2 and cytochrome c was also studied, giving an apparent second-order rate constant of 10^7 $M^{-1} \cdot s^{-1}$ at 5 °C, I 0.2 and pH 7.0. This electron-exchange rate is slightly modulated by ionic strength, following the Debye–Hückel relationship with a charge factor $Z_1Z_2 = -1.9$. Comparison of these data with those for the reduction of cytochrome c by flavodehydrogenase-bound cytochrome b_2 [Capeillère-Blandin (1982) Eur. J. Biochem. **128**, 533–542] leads to the conclusion that the intramolecular electron exchange between haem b_2 and haem c within the reaction complex occurs at a rate very similar to that determined experimentally in presence of the flavodehydrogenase domain. The low reaction rate observed with free cytochrome b_2 is ascribed to the low stability of the reaction complex formed between free cytochrome b_2 and cytochrome c.

INTRODUCTION

The reactivities of cytochrome c with redox proteins have been extensively studied to obtain information on the mechanism of electron transfer (Wherland & Gray, 1976; Cusanovitch, 1978; Ferguson-Miller *et al.*, 1979; Marcus & Sutin, 1985). The cytochrome c from yeast *Hansenula anomala* has been used for electron-transfer studies with free radicals (Thomas *et al.*, 1986) and with flavocytochrome b_2, an L-lactate:cytochrome c oxidoreductase (Capeillère-Blandin, 1982) purified from the same organism. This enzyme is a tetramer, each chain (M_r 58000) being folded into two distinct domains possessing different functions, namely the L-lactate dehydrogenase and the flavodehydrogenase, and its electron acceptor, the cytochrome b_2 core (Iwatsubo *et al.*, 1977; Gervais *et al.*, 1979), which then acts as a one-electron donor to cytochrome c (Capeillère-Blandin *et al.*, 1980). Rapid-kinetic analyses enabled us to show that reaction rates between reduced flavocytochrome b_2 and cytochrome c are modulated by ionic strength, reaching a saturation behaviour at low ionic strength. These data lead to the conclusion that the electron-transfer step occurs within a flavocytochrome b_2–cytochrome c complex stabilized mainly by specific electrostatic interactions (Capeillère-Blandin, 1982).

The cytochrome b_2 core and the flavodehydrogenase can be isolated after cleavage of flavocytochrome b_2 by controlled proteolysis (Gervais *et al.*, 1977). Fluorescence studies demonstrated that cytochrome c can bind both to the flavodehydrogenase (Thomas *et al.*, 1983) and to the cytochrome b_2 domain (Thomas *et al.*, 1983; Albani, 1985), the affinity to the flavodehydrogenase domain ($K_d = 10^{-7}$ M) being higher than that to the cytochrome b_2 ($K_d = 10^{-6}$ M). In both cases the stoichiometry was 1:1, as previously demonstrated for the flavocytochrome b_2–cytochrome c complex (Prats, 1977). The equilibrium dissociation constant (K_d) of the latter complex was determined to be equal to 10^{-7} M. Thus it seems that the flavodehydrogenase domain plays an essential role in the stabilization of this complex.

In the overall electron transfer from L-lactate to ferricytochrome c are involved several electron-transfer steps, as follows (Capeillère-Blandin, 1982): from L-lactate to the flavodehydrogenase, then to cytochrome b_2, and finally to cytochrome c. To determine whether the electron-transfer efficiency between the enzyme and cytochrome c is affected or not by the absence of the stabilizing area pertaining to the flavodehydrogenase, the one-electron-transfer kinetics between reduced cytochrome b_2 and oxidized cytochrome c has now been investigated by stopped-flow spectrophotometry. In the present study the electron-exchange rate constant and its variations with concentration and ionic strength were determined. The data obtained are compared with the kinetic model previously proposed for flavocytochrome b_2–cytochrome c reaction. To characterize further the cytochrome b_2 as an electron carrier, the present study includes kinetics of its reduction by reduced flavocytochrome b_2.

MATERIALS AND METHODS

Proteins

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase) was purified from *Hansenula anomala* yeast essentially by the procedure of Labeyrie *et al.* (1978) with

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the modifications described by Capeillère-Blandin *et al.* (1986*b*). The concentrations of reduced flavocytochrome b_2, expressed in haem molarity, were determined spectrophotometrically with the use of an absorption coefficient of 183 $mM^{-1} \cdot cm^{-1}$ at 423 nm (Pajot & Groudinsky, 1970).

Cytochrome b_2 core or free cytochrome b_2 was prepared from flavocytochrome b_2 by proteolysis with trypsin [Tos-Phe-CH_2Cl-('TPCK'-)treated; Worthington] as described by Gervais *et al.* (1977) with the modifications detailed by Albani (1985). The haemoderivative thus obtained corresponded to a cytochrome form of low M_r (14000) without any bound flavin group. Its concentration was determined spectrophotometrically with the use of an absorption coefficient of 120 $mM^{-1} \cdot cm^{-1}$ at 412 nm for the oxidized form (Pajot & Groudinsky, 1970). The concentration of the reduced form is measured at 557 nm with the use of an absorption coefficient of 31 $mM^{-1} \cdot cm^{-1}$ (Capeillère-Blandin *et al.*, 1984).

Hansenula anomala cytochrome *c* was extracted as a by-product of flavocytochrome b_2 purification as described by Labeyrie *et al.* (1978) and purified by following conventional procedures (Margoliash & Walasek, 1967). The purity index was $A_{549}/A_{280} = 1.2$. To ensure that the cytochrome *c* used for the experiments was fully oxidized, a small amount of $K_3Fe(CN)_6$ was added to the cytochrome *c* and the excess of oxidant was removed by passing the mixture through a Sephadex G-25 (fine grade) column equilibrated and eluted with the appropriate buffer. Cytochrome *c* concentrations were determined spectrophotometrically after reduction by ascorbate by taking an absorption coefficient of 129 $mM^{-1} \cdot cm^{-1}$ for the reduced γ-band at 415 nm.

Spectrophotometric data were obtained with a Perkin–Elmer model 555 spectrophotometer with 1 cm-light-path cuvettes.

All experiments were carried out in a 0.1 M-K_2HPO_4/NaH_2PO_4 buffer, pH 7.0, unless otherwise noted.

Stopped-flow kinetics

Rapid-kinetic absorbance measurements were made with a modified Gibson–Durrum apparatus (Capeillère-Blandin *et al.*, 1986*a*), thermostatically maintained at 5 °C and coupled to a Tracor NS 570 analogue-to-digital converter, which stored 1024 points and then transferred the absorbance-versus-time data on to an *X–Y* recorder. With a 1.9 cm cell the instrument dead-time was measured to be 2.2 ± 0.2 ms. The wavelength calibration was checked as described by Capeillère-Blandin (1982). All the concentrations of components given in the text correspond to final concentrations after mixing.

Cytochrome *c* reduction

This was monitored at 416.5 nm, an isosbestic point of the cytochrome b_2. The position of this wavelength was controlled by rapid mixing of the oxidized free cytochrome b_2 with a dithionite solution freshly prepared as described by Capeillère-Blandin *et al.* (1986*a*) and by checking the absence of absorbance variations at this wavelength. At 416.5 nm the absorption coefficient difference for cytochrome *c* is 51 ± 6 $mM^{-1} \cdot cm^{-1}$ (Capeillère-Blandin, 1982).

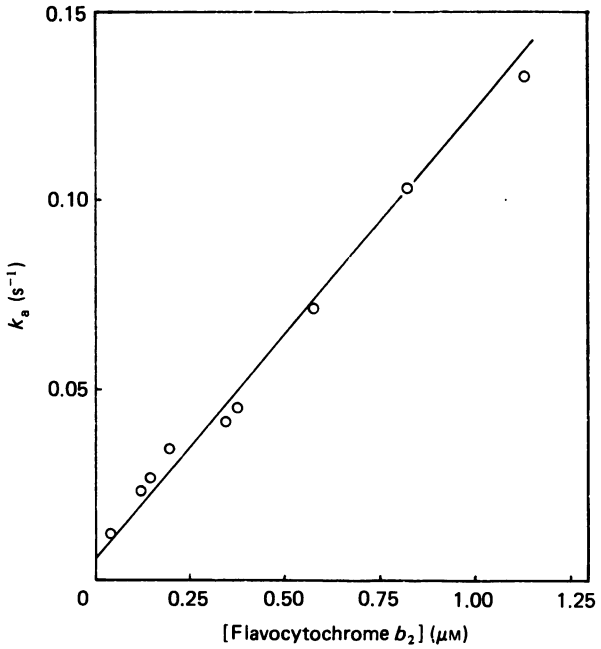

Fig. 1. Dependence of the observed first-order rate constant of free cytochrome b_2 reduction on reduced flavocytochrome b_2 concentration

The reduction of free cytochrome b_2 (0.5 μM) by various reduced flavocytochrome b_2 concentrations in the presence of 5 mM-L-lactate was monitored at 423 nm in a stopped-flow apparatus. The observed rate constants, k_a, were determined from the slopes of semi-logarithmic plots of the kinetic traces. From the second-order plot, the second-order rate constant, calculated by linear-regression analysis, was found to be 1.1×10^5 $M^{-1} \cdot s^{-1}$ in 0.1 M-phosphate buffer, pH 7.0, at 5 °C.

Reduced cytochrome b_2 sample

The isolated cytochrome b_2 does not possess an L-lactate-binding site, and thus the presence of a catalytic amount of an L-lactate dehydrogenase, such as flavocytochrome b_2, is necessary for the reduction by L-lactate. Moreover reduced cytochrome b_2 is auto-oxidizable. Thus to achieve complete reduction of the initially oxidized cytochrome b_2 in the syringe, the buffer was first fully deoxygenated, and then a catalytic amount of flavocytochrome b_2 (< 4% of total haem b_2) together with 1 mM-L-lactate and 3 mM-oxalate were added. Under these conditions cytochrome b_2 remains in the reduced state before the mixing with oxidized cytochrome *c*.

Data analysis

For every kinetic experiment at least two traces were recorded at the same time sweep. The data were analysed graphically through semi-logarithmic plots and least-squares regressions according to the equation:

$$y = y_0 + A_I e^{-k_I t} + A_{II} e^{-k_{II} t}$$

and by digital computer as follows: a set of 20–30 data points was used for multi-exponential (one to two) non-linear iterative regression based on a least-squares criterion as previously detailed (Capeillère-Blandin *et al.*, 1986*a*).

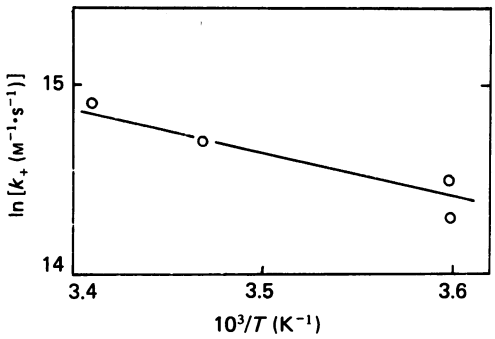

Fig. 2. Arrhenius plot of temperature-dependence of cytochrome b_2 reduction

The second-order rate constants for cytochrome b_2 reduction by reduced flavocytochrome b_2 were determined at different temperatures from 5 to 20 °C. The line through the data is a least-squares fit with a slope corresponding to an activation energy of 100 kJ/mol (24 kcal/mol).

RESULTS

Reduction of free cytochrome b_2 by reduced flavocytochrome b_2

Although cytochrome b_2 is unable to be reduced by L-lactate, we have found that flavocytochrome b_2 is able to reduce cytochrome b_2 in presence of L-lactate under steady-state conditions. The reduction of cytochrome b_2 observed at 423 nm followed monophasic kinetics. From the dependence of the first-order rate constant on flavocytochrome b_2 concentration a second-order plot was established and found to be linear (Fig. 1). The second-order rate constant of the electron transfer was calculated to be $k_+ = 1.1 \times 10^5$ $M^{-1} \cdot s^{-1}$ by linear-regression analysis in 0.1 M-phosphate buffer, pH 7.0, at 5 °C.

This second-order rate constant was found to be independent of L-lactate concentration between 1 and 10 mM, $k_+ = 1.8(\pm 0.6) \times 10^5$ $M^{-1} \cdot s^{-1}$. These results indicate that the reduction of the cytochrome b_2 core is clearly limited by the electron-exchange step between reduced flavocytochrome b_2 and cytochrome b_2.

The dependence of the second-order rate constants on temperature is illustrated in Fig. 2. From the linearity of the Arrhenius plot an activation energy of 100 kJ/mol (24 kcal/mol) is calculated.

It should be noticed that, under the standard conditions of concentrations used in the following study (see Fig. 3), the reduction rate of cytochrome b_2 was 0.01 s^{-1}, 100-fold lower than the rate of the overall reaction between L-lactate and cytochrome c as catalysed by flavocytochrome b_2 and 1000-fold lower than the electron-transfer process between reduced cytochrome b_2 and cytochrome c.

Reaction of reduced cytochrome b_2 with oxidized cytochrome c

Fig. 3(a) shows the time course of reduction of cytochrome c by reduced cytochrome b_2 in the presence of a small amount of flavocytochrome b_2. Multiphasic absorbance changes were observed. The initial phase, which occurs rapidly, is first-order ($k_I = 21$ s^{-1}) and its amplitude corresponds to 8–10% of the total absorbance

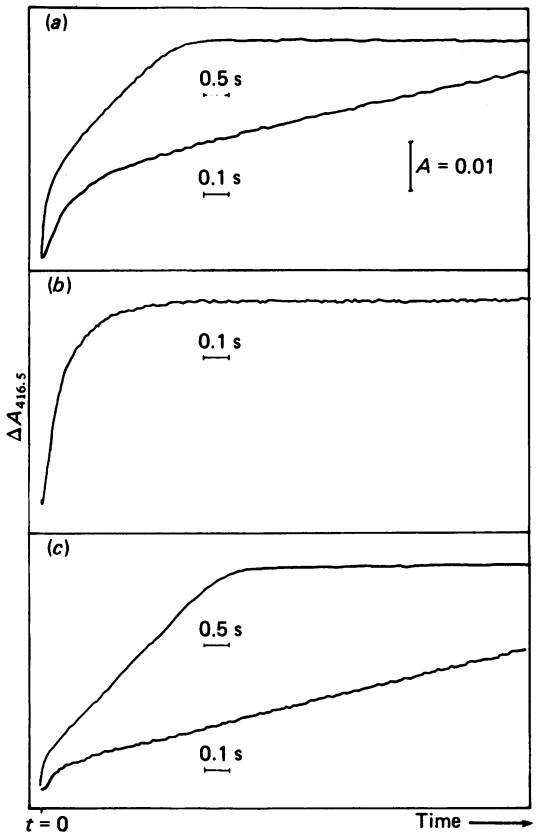

Fig. 3. Reaction of ferricytochrome c with reduced cytochrome b_2

Typical time courses recorded at 416.5 nm (an isosbestic wavelength of cytochrome b_2 oxidoreduction) for the reduction of cytochrome c. For (a) and (b) the recordings were obtained after mixing 0.75 μM-ferricytochrome c with free cytochrome b_2 reduced by 1 mM-L-lactate, in the presence of 3 mM-oxalate (a competitive inhibitor) and a small amount of flavocytochrome b_2 (0.03 μM), and at concentrations of 0.66 μM and 1.25 μM respectively. (c) This shows the result of a control experiment carried out on mixing ferricytochrome c with L-lactate, oxalate and flavocytochrome b_2 under the same condition of concentrations but in the absence of cytochrome b_2 (at I 0.21 and 5 °C).

change observed in the course of the reaction. This phase is followed by a slower first-order phase with a half-time of 110 ms and which is virtually complete within 0.6 s. The rate constant and amplitude of the second phase depend on the concentration of reduced cytochrome b_2. These two first-order phases are followed by a final pronounced turnover phase.

The total absorbance change observed in the course of the reaction is in agremeent with data from static differential spectrophotometry $\Delta\epsilon = 51(\pm 6)$ $mM^{-1} \cdot cm^{-1}$ (Capeillère-Blandin, 1982) indicating total reduction of cytochrome c.

The rate of the turnover phase (k_{III}) is found to be independent of the cytochrome b_2 concentration, $k_{III} = 4.5(\pm 0.7)$ s^{-1} at 5 °C obtained from six independent measurements, but is dependent on flavocytochrome b_2 concentration. This process can be assigned to the reduction of cytochrome c by L-lactate as catalysed by flavocytochrome b_2. Indeed, control experiments

Table 1. Comparison of kinetic data obtained from analysis of the different reaction phases involved in the reduction of cytochrome *c* at various concentrations of free cytochrome b_2

Rate constants were determined from typical data sets presented in Fig. 3. In the initial exponential phase k_I and k_{II} were defined by using non-linear least-squares fits to $A_I e^{-k_I t} + A_{II} e^{-k_{II} t}$. The turnover rate k_{III}, defined as μM-cytochrome *c* reduced/s per μM-flavocytochrome b_2, corresponds to the slope of the linear phase leading to the complete reduction of cytochrome *c*. The total absorbance change observed in the course of the reaction corresponds to the total reduction of 0.7 μM-cytochrome *c* in the absence and in the presence of free cytochrome b_2. Abbreviations: n.d., 'not defined', i.e. unresolved phase for calculation; n.o., 'not observed', i.e. no detection of such a phase.

[Cytochrome b_2] (μM) ...	0	0.5	0.7	1.2	2.7	4.4
First-order phases						
k_I (s^{-1})	21(±4*)	22	25	n.d.	n.d.	n.d.
k_{II} (s^{-1})	n.o.	4	6	11	32	44
Turnover phase						
k_{III} (s^{-1})	4.4(±1.0†)	5.2	4.4	n.o.	n.o.	n.o.

* Standard deviation from six independent measurements.
† Standard deviation from three independent measurements.

concerning the reduction kinetics of cytochrome *c* by the L-lactate/flavocytochrome b_2 mixture (Fig. 3*c*) indicated that in the absence of cytochrome b_2 two phases are observed. The initial phase has the same rate constant as the one observed in the presence of cytochrome b_2 (Fig. 3*a* and Table 1). The second phase is a turnover phase with an apparent rate of 4.4(±1.0) s^{-1} from three independent measurements. This turnover phase is only flavocytochrome b_2-concentration- and L-lactate-concentration-dependent. Moreover, this reaction is slow and has a negligible effect on the electron-transfer kinetics between initially reduced cytochrome b_2 (or flavocytochrome b_2) and cytochrome *c*.

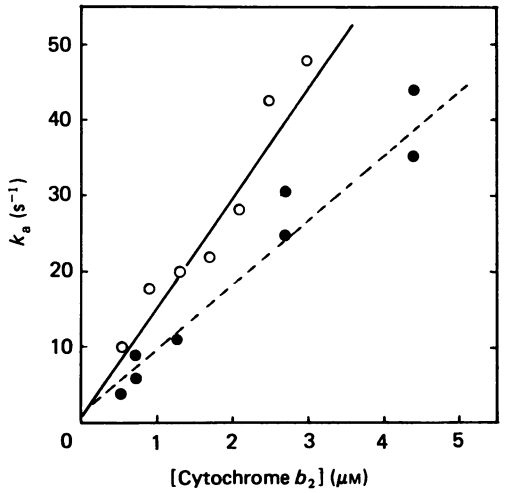

Fig. 4. Variation in reduction rate constant of cytochrome *c* with cytochrome b_2 concentration

The dependence of k_a, the apparent first-order rate constant of the exponential phase preceding the linear turnover phase of cytochrome *c* reduction, on free cytochrome b_2 concentration is shown, at two ionic strengths. From a linear-regression analysis, $k_+ = 1.5 \times 10^7$ and 0.8×10^7 $M^{-1} \cdot s^{-1}$ for *I* 0.12 and *I* 0.21 respectively at 5 °C.

Finally, the above data indicate that the initial rapid phase represents the reduction of cytochrome *c* by the initially reduced flavocytochrome b_2 (a three-electron donor), and the slow turnover phase the reduction by L-lactate as catalysed by flavocytochrome b_2. The second phase corresponds to the direct reduction of cytochrome *c* by reduced cytochrome b_2. As the concentration of reduced cytochrome b_2 increases, the rate constant of the second first-order phase, as well as its magnitude, increase (Fig. 3*b*). For cytochrome b_2/cytochrome *c* molar ratio equal to or higher than 1.6:1, apparently monophasic traces leading to the complete reduction of cytochrome *c* are observed. Fig. 4 illustrates the cytochrome b_2-concentration-dependence of the pseudo-first-order rate constant of the electron transfer between the cytochrome b_2 and cytochrome *c*. The linear relationship indicates that this portion of cytochrome *c* reduction is a second-order process with a rate constant $k_{II} = 0.8(\pm 0.3) \times 10^7$ $M^{-1} \cdot s^{-1}$ at *I* 0.21 and 5 °C.

Effect of ionic-strength variations

The ionic-strength-dependence of the electron-transfer reaction between reduced cytochrome b_2 and ferricytochrome *c* was investigated by the use of various NaCl concentrations in a constant 5 mM-phosphate buffer concentration, the pH being adjusted to 7.0. The pseudo-first-order rate constant increases with decreasing ionic strength, as expected for a reaction between oppositely charged proteins. Results obtained at two ionic strengths (0.21 and 0.12) are presented in Fig. 4.

The variation of the second-order rate constant with ionic strength is illustrated by the classical Debye–Hückel plot defined by $\log k_+$ versus the square root of ionic strength and presented in Fig. 5. A linear dependence is observed with a negative slope of 1.9 and an extrapolated value at zero ionic strength $k_{+,0} = 6 \times 10^7$ $M^{-1} \cdot s^{-1}$ at 5 °C.

DISCUSSION

The present study provides information on the functional competence of free cytochrome b_2, i.e. cytochrome b_2 core, to act as an external one-electron acceptor withdrawing electron from reduced flavo-

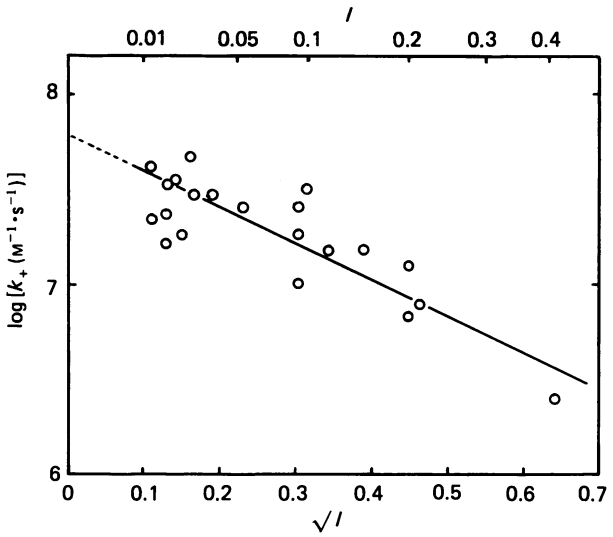

Fig. 5. Ionic-strength-dependence of the second-order rate constant for reduction of cytochrome c by free reduced cytochrome b_2

The logarithm of the second-order rate constant k_+ for cytochrome c reduction is plotted versus the square root of the ionic strength. The rate constants were calculated from analysis of recordings similar to the ones presented in Fig. 3 under the concentrations of 0.5 μM and 0.8 μM for cytochrome c and cytochrome b_2 respectively. The line corresponds to a least-squares linear regression based on data according to the classical Debye–Hückel expression:

$$\log k_+ = \log k_{+,0} + 2AZ_1Z_2\sqrt{I}$$

with a charge factor $Z_1Z_2 = -1.9$ and an extrapolated rate at I 0 of 6×10^7 $M^{-1} \cdot s^{-1}$.

dehydrogenase-bound cytochrome b_2, i.e. flavocytochrome b_2. The self-exchange rate determined between the reduced flavodehydrogenase-bound cytochrome b_2 and free cytochrome b_2 is 100-fold lower than the rate of specific electron exchange between reduced flavodehydrogenase-bound cytochrome b_2 and cytochrome c (Capeillère-Blandin, 1982). This difference in rate can be related first to the equilibrium constant for the electron exchange ($K = 7.7 \times 10^4$) largely in favour of the reduction of cytochrome c whereas a reversible electron exchange is expected between the two haem b_2 groups. Secondly, the charge interactions may well have a considerable influence on complex-formation. Indeed, the isoelectric points for free cytochrome b_2 and flavodehydrogenase-bound cytochrome b_2 are similar, being at pH 4.9 and 5.2 respectively. The two proteins will bear identical net negative charges at neutral pH, and hence have negligible coulombic global attraction. The high value determined for the activation energy illustrates the contribution of these two parameters in the reactivity of the two cytochromes and explains the weak electron-transfer efficiency.

It should be noted that the reduction of free cytochrome b_2 under steady-state conditions implies a series of inter- and intra-molecular electron-transfer processes between L-lactate and the prosthetic groups of flavocytochrome b_2. All these steps are rapid and well characterized in terms of rate constants (Capeillère-Blandin *et al.*, 1986*b*). Finally, in the course of the reaction, when reoxidized the haem b_2 group bound to flavocytochrome b_2 rapidly returns to the reduced state as the result of an intramolecular one-electron transfer provided by the nearby flavin hydroquinone ($k_{on} = 380$ s^{-1} at 5 °C; Capeillère-Blandin *et al.*, 1982) or flavin semiquinone ($k_{on} = 160$ s^{-1} at 16 °C; Tegoni *et al.*, 1984). So the concentration of electron donor, i.e. reduced cytochrome b_2 bound to the flavodehydrogenase, does not vary significantly in the course of the slow reaction with oxidized free cytochrome b_2 ($k_a < 1$ s^{-1} at 5 °C).

The data obtained for the reaction of reduced free cytochrome b_2 with oxidized cytochrome c indicate that the individual rate constants are all substantially less (two to three orders of magnitude) than the analogous reaction of the flavodehydrogenase-bound cytochrome b_2 with cytochrome c. The difference observed can be assigned to specific parameters, the free energy of reaction ΔG^0, or electrostatic interactions. The difference in oxidation–reduction potentials is known to be the same. Thus the cytochrome b_2 reactivity is markedly

Table 2. Ionic-strength-dependence of second-order rate constants and equilibrium dissociation constants for the reaction with cytochrome c

k_+ expressions were established according to the classical Debye–Hückel interpretation of ionic-strength effect for data presented in this paper for free cytochrome b_2 (Fig. 5) and obtained by us under the same conditions with bound cytochrome b_2. For the purpose of comparison the expressions of the K_D of the various cytochrome b_2 derivatives are given. Abbreviation: n.d., not determined.

		Flavodehydrogenase-bound cytochrome b_2	
	Free cytochrome b_2	Containing deflavocytochrome b_2*	Containing flavocytochrome b_2
$k_+ = k_{+,0} \times 10^{-2AZ_1Z_2\sqrt{I}}$ ($M^{-1} \cdot s^{-1}$)	$6 \times 10^7 \times 10^{-1.9\sqrt{I}}$	$7 \times 10^8 \times 10^{-4.4\sqrt{I}}$	$2 \times 10^{10} \times 10^{-5.6\sqrt{I}}$†
Reference	Present work	Capeillère-Blandin *et al.* (1980)	Capeillère-Blandin (1982)
$K_d = K_{d,0} \times 10^{2AZ_1Z_2\sqrt{I}}$ (M)	$2.5 \times 10^{-6} \times 10^{2.6\sqrt{I}}$	n.d.	$10^{-8} \times 10^{6.1\sqrt{I}}$
Reference	Albani (1985)	–	Thomas *et al.* (1983)

* Derivative corresponding to flavocytochrome b_2 molecule deprived of flavin coenzyme.
† The linear dependence is experimentally observed above $\sqrt{I} = 0.27$ ($I > 0.07$).

influenced by structural factors within the protein, and in particular the presence of the flavodehydrogenase domain enhances considerably the electron-exchange rate from cytochrome b_2 to cytochrome c. Moreover, the dependence of the reaction rates with ionic strength (see below) implies the involvement of attractive coulombic interactions, mainly achieved by the latter domain. A similar conclusion has been proposed on the basis of an increase by the factor 2-fold of the reactivity of bound cytochrome b_2 in an heterologous reaction with a copper protein, stellacyanin (Silvestrini *et al.*, 1986).

One obvious question about the low reactivity of free cytochrome b_2 is whether it arises from low affinity for cytochrome c or from a relatively low electron-exchange rate within the reaction complex. The present results show that the electron-transfer reaction behaves as a second-order process. However, this interpretation is limited by the fact that only a narrow range of concentration of the reductant, cytochrome b_2 core, was investigated, owing to the strong absorbance of the reactants. Thus, instead of the simple analysis of the reaction in terms of a bimolecular reaction, it is important to consider the formation of intermediate complexes with the possible intervention of monomolecular steps. Indeed, direct detection by equilibrium procedures have proved that these molecules might readily form a complex with the stoichiometry of one cytochrome c molecule per free cytochrome b_2 molecule (Albani, 1985). However, the affinity is not as high as in the case of flavodehydrogenase-bound cytochrome b_2 and cytochrome c (Table 2) (Thomas *et al.*, 1983).

In the present work the kinetic analysis fails to show evidence for such a complex, though this might be due to its short life-time. Such a conclusion does not exclude the possibility that free cytochrome b_2 (cyt. b_2) and cytochrome c (cyt. c) are in rapid binding equilibrium with their complex according to the following scheme:

$$\text{Cyt.}b_2^{2+} + \text{cyt.}c^{3+} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (\text{cyt.}b_2^{2+} - \text{cyt.}c^{3+}) \xrightarrow{k_{+2}} \text{cyt.}b_2^{3+} + \text{cyt.}c^{2+}$$

The irreversibility of the electron exchange (k_{+2}) is a consequence of the large redox potential difference ($\Delta E = 270$ mV; Capeillère-Blandin *et al.*, 1982) between the donor and acceptor groups. Assuming a rapid binding equilibrium between reduced cytochrome b_2, oxidized cytochrome c and the complex (i.e. $k_{-1} \gg k_{+2}$), such a two-step reaction scheme is associated to the following rate expression (Strickland *et al.*, 1975):

$$k_a = \frac{k_{+1}k_{+2}[\text{cyt.}b_2^{2+}]}{k_{+1}[\text{cyt.}b_2^{2+}] + k_{-1}} \quad (1)$$

In this case, the apparent second-order rate constant would be:

$$k_+ = \frac{k_a}{[\text{cyt.}b_2^{2+}]} = \frac{k_{+2}}{[\text{cyt.}b_2^{2+}] + K_d} \quad (2)$$

and thus proportional to the reciprocal equilibrium dissociation constant $K_d = k_{-1}/k_{+1}$.

The analysis of the dependence of the reaction rate on ionic strength and its comparison with previous data on flavocytochrome b_2 provide information on the relative importance of the protein binding capacity and the intrinsic catalytic efficiency in the reaction. The second-order rate constant slightly increases when the ionic strength decreases, as expected from an anion–cation interaction. This ionic-strength-dependence, although small, is well outside experimental error ($\pm 15\%$), and a charge factor of -1.9 is determined. This finding is in sharp contrast with the reaction between flavodehydrogenase-bound cytochrome b_2 and cytochrome c, which was found to be greatly dependent on ionic strength, with a charge factor of -5.7 (Capeillère-Blandin, 1982). Also, the reaction rate of deflavocytochrome b_2 (the flavin-free derivative) with cytochrome c is highly dependent on ionic strength, with a charge factor of -4.4 (Capeillère-Blandin *et al.*, 1980) (Table 2). Therefore the presence of the flavodehydrogenase domain increases the contribution of electrostatic interactions in the control of cytochrome b_2 reactivity.

From the consideration of the extrapolated second-order rate constant, $k_{+,0}$, at I 0 according to the Debye–Hückel relationship (Table 2), we can deduce that the presence of the flavodehydrogenase domain affords a great enhancement, at least two to three orders of magnitude, of the electron-transfer efficiency, which reaches a value theoretically calculated for a diffusion-controlled reaction occurring every time there is an encounter, as developed by Smoluchovski (see Moelwyn-Hugues, 1961). The high affinity observed between flavodehydrogenase-bound cytochrome b_2 and cytochrome c permits the increase in the number of effective collisions between the two proteins and the stabilization of the complex formed before the intramolecular electron transfer occurs. When the electrostatic interactions are weak, as in the free cytochrome b_2–cytochrome c reaction, the probability of effective collisions becomes small, as shown by $k_{+,0} = 6 \times 10^7$ M$^{-1}\cdot$s^{-1}. In this case the reaction is not controlled by the free diffusion of the molecules. Indeed, it has been suggested (Koppenol, 1980; Matthew *et al.*, 1983) that electrostatic interactions

Table 3. Comparison of calculated (k_c) and experimental (k_{+2}) data for the intramolecular electron-exchange rate between free and bound cytochrome b_2 and cytochrome c at various ionic strengths

From consideration of the two-step reaction model presented in the text $k_c = k_{+2}$ was calculated by the relationship (2) $k_c = k_+([\text{cyt.}b_2^{2+}] + K_d)$ for a concentration of electron donor equal to 0.5 μM. k_+ and K_d values are deduced from the known linear variations with ionic strength illustrated in Table 2. In the case of the flavodehydrogenase-bound cytochrome b_2 reaction a linear variation for k_+ is not defined below I 0.07.

I ...	0	0.04	0.06	0.36
$\sqrt{I}$ ...	0	0.2	0.4	0.6
Free cytochrome b_2 k_c (s^{-1})	180	213	296	395
Flavodehydrogenase-bound cytochrome b_2 or flavocytochrome b_2 k_c (s^{-1})	380*	380*	315	200

* At $I < 0.07$ k_+ becomes independent of I and a saturation effect is observed at 380 s^{-1}.

would pre-orient the proteins before they make physical contact, facilitating the formation of an optimal reaction complex. Thus when the interactions are weak the pre-orientation is not adequate at every collision and the probability of formation of a reaction complex is small. Moreover, these orientational constraints can decrease the association rate constant by several orders of magnitude by effectively decreasing the size of the target relative to the size of the diffusing molecules (Berg & Von Hippel, 1985). Finally, the decrease in free cytochrome b_2 reactivity may be related either to a very poor probability of electron transfer within the transient reaction complex or to the existence of such a complex only in very small amounts at equilibrium.

Indeed, as shown by the ionic-strength effects on the reactivities of free and bound cytochrome b_2 towards cytochrome c, the linear variation of the second-order rate constant, k_+, with ionic strength can be simply explained by the influence of salts on the equilibrium constant of the active encounter complex (Table 2). Thus, as predicted by the kinetic model developed above, the difference in second-order rate constants relies on the difference in stability of the complex. Indeed, the calculated product $k_+ \times ([\text{cyt.}b_2^{2+}] + K_d)$ is roughly constant whatever the ionic strength considered in the range 0–0.3 for free cytochrome b_2 (Table 3). This calculated value is found in the same range of magnitude as the intramolecular electron-transfer rate experimentally determined for the flavodehydrogenase-bound cytochrome b_2–cytochrome c reaction ($k_{+2} = 380\ s^{-1}$) (Capeillère-Blandin, 1982).

In spite of different individual variations for k_+ and K_d with ionic strength, i.e. a factor of 9-fold for free cytochrome b_2–cytochrome c reaction and a factor of 700-fold for the flavodehydrogenase-bound cytochrome b_2-cytochrome c reaction, it appears that the flavodehydrogenase domain in the native flavocytochrome b_2 exerts very little influence on the efficiency of the electron transfer between cytochrome b_2 and cytochrome c within the reaction complex. This result implies that similar transition states must be achieved in the reactions of cytochrome c with both free and bound cytochrome b_2. Consequently the electron transfer occurs via the same mechanism involving the same pathway through the intramolecular reaction complex. In both cases the relative distance and orientation of the haem b_2 and haem c planes are optimal to achieve the electron-transfer process. The site of electron exchange is not altered by modification of the protein environment by reason of absence of the flavodehydrogenase domain, although smaller electrostatic interactions contribute to the stability of the binding of free cytochrome b_2 to cytochrome c. However, it is certainly feasible that non-polar interactions might play an important role in defining the specific orientations and distance between the reactive groups. At the moment we do not have any specific information bearing on this point.

In conclusion, the results obtained are consistent with the view that in biological electron-exchange reactions with comparable redox-potential differences the apparent rate of the electron transfer is directly controlled by the stability of the reaction complex formed between the two proteins.

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