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L-Lactate cytochrome *c* reductase: rapid kinetic studies of electron transfers within the flavocytochrome b_2-cytochrome *c* assembly

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This study is part of a series aimed at the characterization of individual steps of electron transfer taking place between prosthetic flavin, heme b_2, heme *c* within active sites and complexes. After rapid mixing of ferricytochrome *c* with partially reduced flavocytochrome b_2, the reaction is followed at the level of two reactants, cytochrome b_2 and cytochrome *c*. In order to define the proper reactivity of flavosemiquinone, conditions under which this form is highly stabilized (presence of pyruvate) have been chosen. With the help of simulations, it has been possible to characterize a rapid step of electron transfer from cytochrome b_2 to cytochrome *c* within a complex (at approx. 70% saturation) and a slow step $k = 5\ s^{-1}$ assigned to cytochrome b_2 reduction by flavosemiquinone within the active site of the pyruvate-liganded enzyme.

Introduction

The purpose of this kinetic study was to determine the rate constants of one-electron transfers taking place between two biological specific partners strongly associated together. The system considered is the assembly of *Hansenula anomala* cytochrome *c* (final acceptor) with *Hansenula anomala* flavocytochrome b_2 in a partially reduced state. The latter is itself a two-domain enzyme formed of a cytochrome b_5-like moiety and of a L-lactate flavodehydrogenase moiety [1,2].

The two proteins are known to form very stable complexes, particularly at low ionic strength [3–5], which are competent for the heme b_2 to heme *c* electron transfer, but not for the flavin to heme *c* process [6–10]. The interest in this system mainly comes from the knowledge of the three-dimensional structure of these two protein partners, established at 2.4 Å resolution for their *Saccharomyces cerevisiae* homologues [2,11].

Finally, two kinds of intramolecular one-electron transfer take place in turnover with cytochrome *c* as an external acceptor: (i) from flavin (semiquinone or hydroquinone) to ferricytochrome b_2 [12–14]; (ii) from ferrocytochrome b_2 to ferricytochrome *c* [5,15].

In the present study, our aim was to investigate, using stopped-flow techniques, the behavior of the semiquinone form of the flavin as a donor to cytochrome b_2. To that end, we have chosen conditions where pyruvate is present at the near-saturation concentration of 10 mM [12,16]. Under such conditions, the semiquinone proportion is close to 95% of total flavin for a large range of heme reduction levels [16–18]. A priori, the rapid mixing of ferricytochrome *c* with partially reduced flavocytochrome b_2 could allow us to characterize kinetically the transfer from flavosemiquinone to cytochrome b_2. We actually detected a slow process with a rate constant of $5\ s^{-1}$ at 5 °C in which the donor was unambiguously assigned to the flavosemiquinone. This phase is rate-limiting for cytochrome *c* reduction in such rapid-mixing experiments as well as in turnover with cytochrome *c*, in the presence of pyruvate as has been recently shown in our laboratory [19]. This value is in agreement with previous T-jump estimates (calculated with a lower accuracy than in the present study) in which the assignment of the relaxation to an F_{sq} to cytochrome b_2 transfer was precisely supported [12,16].

Abbreviations: ferricytochrome b_2 or ferrocytochrome b_2: oxidized or reduced cytochrome b_2; ferricytochrome *c* or ferrocytochrome *c*, oxidized or reduced cytochrome *c*; *H.a.* flavocytochrome b_2, *Hansenula anomala* flavocytochrome b_2.

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Material and Methods

Yeast-Hansenula anomala flavocytochrome b_2

The pure enzyme, with optimal molar activity of 1000 s^{-1} (standard units at 30° C), was prepared, stored and assayed as described in Ref. 20 with the modifications introduced by Naslin and described by Gervais et al. [21]. Solutions of the fully oxidized form were prepared in 100 mM Na/K_2 phosphate buffer (pH 7) just before each series of experiments. Dilutions were made and kept in an ice-box in closed vessels with a water-saturated argon flow in order to eliminate oxygen. The enzyme is known to be stable under these conditions even for several days. However, activity was always checked at the end of experiments to make sure that it had not significantly decreased. Flavin adduct formation with oxalate [22] and with pyruvate (Blazy and Baudras, personal communication), known to occur on illumination of the enzyme in aerobic conditions and leading to total inactivation, does not take place under our conditions. Concentrations were determined at the maximum of the γ peak after reduction with lactate (423 nm) with $\varepsilon = 183\ mM^{-1} \cdot cm^{-1}$ [23].

Yeast-Hansenula anomala cytochrome c

The pure protein, currently isolated as a byproduct of the flavocytochrome b_2 preparation [20], was purified by conventional procedures as detailed in Ref. 24; the concentrated solution was stored in a cold room. For experiments, a diluted solution was prepared in the same buffer as for flavocytochrome b_2. In order to ensure total oxidation it was mixed with increasing amounts of ferricyanide until a minimal optical absorbance at 550 nm was reached, and poured on a Sephadex G-25 column to remove ferro- and ferricyanide before being diluted to a convenient level and kept in ice. Controls were carried out at the end of each experiment in order to check that no autoreduction had taken place. The experiment was discarded if autoreduction was detected. Concentrations were determined after reduction by dithionite taking $\varepsilon = 129\ mM^{-1} \cdot cm^{-1}$ for the reduced γ band at 415 nm [25].

Stopped-flow experiments

An improved Durrum-Gibson apparatus connected to a Tracor multichannel analyser (M.C.A.) as described in Ref. 5 was used. After digitalization of the absorbance outputs the signals were transferred to an Apple II microcomputer for memorization and data treatment. Special attention has been paid to obtaining optimal performances. The water-bath housing the valve block and the drive syringes is maintained at 5 ± 1° C and bubbled with a nitrogen flow.

All the experiments in each series were carried out with the same concentrations of cytochrome c and of total flavocytochrome b_2. Just before anaerobic transfer of the diluted reactants to the reservoir syringes, pyruvate was added to each of them (except as specified for certain controls) at a final concentration of 10 mM. FMN, EDTA and occasionally L-lactate were added to the flavocytochrome b_2 dilutions as specified below in the Photoreduction section. In the final solution, after rapid mixing of equal volumes from the two drive syringes, concentrations of cytochrome c and flavocytochrome b_2 were always equal to 10 (± 0.1) and 5 (± 0.1) μM, respectively. The reduction level of flavocytochrome b_2, defined as the proportion, R, of cytochrome b_2 in the reduced form, was varied in the experiments presented between 0 and 95%, as described below.

Signal intensities were measured relative to the buffer, for each solution of cytochrome c and flavocytochrome b_2, mixed together or with the buffer, in the fully oxidized (by addition of ferricyanide) and fully reduced (by addition of dithionite) states (in the absence and in the presence of pyruvate). Optical absorbances were calculated from the differences between signal intensities of these solutions and that of the buffer at the same wavelength. All these measurements were carried out at each wavelength used throughout the complete study. These determinations gave differential absorbances ($mM^{-1} \cdot cm^{-1}$) of: $\underline{7.5} \pm 0.3$ for cytochrome c at 544.8 nm; 0 ± 0.3 for cytochrome b_2 at 544.8 nm (isosbestic); < 0.05 for cytochrome c at 555.8 nm (isosbestic); $\underline{23.2} \pm 2$ for cytochrome b_2 at 555.8 nm. The values underlined are the estimates used for calculations of ferrocytochrome c and ferrocytochrome b_2 concentrations at the appropriate wavelengths throughout this study.

A comparison of the whole differential spectra (red − ox) for *H.a.* cytochrome c and *H.a.* flavocytochrome b_2 (as obtained with the current wavelength adjustment of our stopped-flow equipment) with published data for Horse-Heart cytochrome c and *S.c.* flavocytochrome b_2 showed that: (a) The wavelengths corresponding to the maxima of the α peaks of the reduced forms for *H.a.* cytochrome c and cytochrome b_2 were found to be at 550 and 556 nm, respectively, identical to published values for horse-heart cytochrome c [25] and cytochrome b_2 [23]. (b) However, the left wing of the differential spectrum of *H.a.* cytochrome c was blue-shifted by 1 nm relative to published values, resulting in the higher $\Delta\varepsilon$ value of 7.5 instead of 5 $mM^{-1} \cdot cm^{-1}$ at 544.8 nm. (c) The $\Delta\varepsilon$ value for cytochrome b_2, at 555 nm is 10% higher than published values.

It should be noted that pyruvate had been omitted from the solutions of flavocytochrome b_2 when full oxidation or full reduction was needed. Indeed, as stressed by Tegoni et al. [17], the presence of pyruvate does not allow total oxidation to be reached without the addition of ferricyanide and does not allow total reduc-

tion to be attained, even in the presence of dithionite, which becomes a donor to pyruvate in a reverse turnover.

Treatment of data

The kinetics, recorded in 1024 channels for each sweeptime, were transferred from the M.C.A. to floppy disks for memorization. Such files were used for further computation, involving a smoothing procedure (yielding 100 averaged points starting at 3 ms from mixture time), correction for the dead time of 2 ms [5], calculations in terms of optical densities and of increment or decrement of absorbance ($A_t - A_0$) relative to the fully oxidized mixture, calculations in terms of concentration of the reduced forms taking into consideration the optical path (1.9 cm) and the $\Delta\varepsilon$ values listed above. The corresponding new files, with each set of data, 't, [ferrocytochrome c]$_t$, [ferrocytochrome b_2]$_t$', were also memorized on disks and used for further analyses.

Partial reduction of flavocytochrome b_2 before rapid mixing and determination of R, initial reduction level

The reduction of the deaerated enzyme solution before mixing experiments was achieved by means of one of the two following procedures:

Photoreduction by illumination of the drive syringe containing the flavocytochrome b_2 solution by flashes provided by a photographic device using an ultraviolet filter; the cytochrome c syringe was protected from light. 30 to 50 flashes were often necessary. FMN and EDTA were previously added to the solution in the reservoir syringe at final concentrations of 2 μM and 1 mM. This method, first described by Massey et al. [26], has been used in the present study in order to reach low reduction levels.

Reduction by L-lactate added to the reservoir syringe. Final concentrations of up to 250 μM were used to reach the higher reduction levels ($R \approx 95\%$). Progressive re-oxidation, because of slight air diffusion, takes place very slowly allowing us to reach increasingly lower reduction levels.

The kinetic results do not depend upon the procedure used to reach a particular reduction level.

The initial reduction level, R, of flavocytochrome b_2 at time zero was estimated for each experiment by three different methods:

(a) $R_a = (A - A_{ox})/(A_{red} - A_{ox})$ was calculated from the absorbance (A) values, measured when the observation chamber was filled with the flavocytochrome b_2 dilution contained in the drive syringe, without mixing with the second syringe, A_{red} and A_{ox} having been previously measured by the same procedure in the absence of pyruvate.

(b) $R_b = [\text{ferrocytochrome } b_2]_{t=0}/[\text{cytochrome } b_2]_{tot}$, the upper term being derived from extrapolation to time zero of the first-order plot of cytochrome b_2 oxidation after mixing with cytochrome c (after correction of the dead time).

(c) R_c is the R value that allows the best fit between experimental and simulated data corresponding to the biphasic cytochrome c time-course after mixing.

Results

Kinetics of electron transfers from a multidonor system, partially reduced flavocytochrome b_2 to ferricytochrome c, have been thoroughly investigated at 5 °C, after rapid-mixing of the two reactants by following (i) cytochrome c reduction at an isosbestic wavelength of the cytochrome b_2 absorption spectrum (544.8 nm), (ii) cytochrome b_2 reduction at an isosbestic wavelength of the cytochrome c absorption spectrum (555.8 nm). The corresponding $\Delta\varepsilon_{red-ox}$ values were precisely determined as described in the Material and Methods section.

It is known (as reviewed in Refs. 12, 14, 15) that within the active sites of flavocytochrome b_2, one-electron exchanges occur between heme and flavin prosthetic groups. These exchanges which are involved in the catalytic turnover are rapid enough to result in complete redox equilibration within 1 min or less after addition of a reductant such as the substrate L-lactate. Once the equilibrium is reached, the proportions of flavin present in the three redox states (F_o the quinone, F_s the semiquinone and F_h the hydroquinone) for a reduced state, R, of the cytochrome b_2 can be calculated using Eqn. 1.

$$R = [\text{ferrocytochrome } b_2]/[\text{cytochrome } b_2]_{tot}$$

$$K_1 = \frac{[\text{ferrocytochrome } b_2]\cdot[F_o]}{[\text{ferricytochrome } b_2]\cdot[F_s]} = 0.05$$

$$K_2 = \frac{[\text{ferrocytochrome } b_2]\cdot[F_s]}{[\text{ferricytochrome } b_2]\cdot(F_h)} = 40 \qquad (1)$$

with [ferrocytochrome b_2]/[ferricytochrome b_2] = $R/(1 - R)$.

The equilibrium constant values, K_1 and K_2, given are the best estimates previously determined under conditions very similar to those of the present study (10 mM pyruvate) but at 18 °C [17].

The initial level of cytochrome b_2 reduction, R, is varied between 0 and 95%. As detailed in the Material and Methods section, three procedures were used for R estimations, leading to values, R_a, R_b and R_c, which are all slightly different (cf. Table I).

The three phases of ferrocytochrome b_2 and ferrocytochrome c progress curves

Three different phases can be resolved when mixing flavocytochrome b_2 at different reduction levels (R) with cytochrome c. The fastest process, *phase I*, can be

TABLE I

Reaction of ferricytochrome c with partially reduced flavocytochrome b_2: Analysis of the three phases involved in the cytochrome c and flavocytochrome b_2 time-courses

Typical results for one series of experiments are presented. Ferricytochrome *c* and partially reduced flavocytochrome b_2 (both from the yeast *H. anomala*) are, after rapid-mixing, respectively 10 and 5 μM in a 100 mM phosphate buffer (pH 7) 10 mM pyruvate at 5 ± 1° C containing small amounts of FMN, EDTA and L-lactate.

Expt. No.		0	1	2	3	4	56	
(A) Initial state of flavocytochrome b_2 [a]								
Cytochrome b_2 reduc. level (%)								
R_a		0	8	28	30	70	92	95
R_b		0	5	13	20	63	78	80
R_c		0	7	13	43	79	87	93
Donor concn. (μM)								
$[B_r]$		0	0.35	0.65	2.15	4.0	4.35	4.57
$[F_s]$		0	3.0	3.7	4.6	4.5	4.25	3.75
$[F_h]$		0	0	0.01	0.08	0.4	0.7	1.25
$[e.\ equiv.]_{tot}$ (μM)		0	3.4	4.4	6.9	9.3	10	10.9
(B) Phase parameters								
Phase I: [b]								
$[b_2]$ vs. t, k	s^{-1}	0	170	150	165	220	180	190
$[c]$ vs. t, $k_{app,I}$		0		12	27	67	77	84
$[c]$ vs. t, $k_{I,corr}$		0			40	250	150	200
$[c]/[b_2]$, mol/mol						1.4	1.5	1.6
Δ[ferrocytochrome c]$_I$	μM		0.25	0.5	1.6	4.3	4.8	5.6
Phase II: [c]								
$[c]$ vs. t, $k_{app,II}$	s^{-1}	–		1.2	2.8	1.9	4.2	5.6
$C_{II,o}$	μM	10	9.8	9.5	8.4	5.7	5.2	4.3
Δ[ferrocytochrome c]$_{II}$	μM		2.0	3.0	4.7	5.7	5.2	4.3
Phase III: [d]								
$[b_2]$ vs. t, k	s^{-1}	–		–	–		0.3	
$[c]$ vs. t, k	s^{-1}	–	–	0.1	0	–	–	–
$C_{III,o}$	μM	10	7.8	6.5	3.7	0	0	0

[a] The initial cytochrome b_2 reduction level, $R = B_r/5$, is estimated by three different methods as R_a, R_b, R_c (cf. Material and Methods). Initial concentrations of F_s and F_h are calculated with Eqn. 1).

[b] k is the slope of the first-order plot corresponding to cytochrome b_2 oxidation (cf. Fig. 2A) in the relevant time interval 3 to 6 ms. k_{app} is the initial slope of the first-order plot corresponding to cytochrome *c* reduction (cf. Fig. 3A). $k_{I,corr}$ is the true rate constant of the process taking place in phase I, computed taking into account values of k_{II} and $C_{II,o}$ defined by analysis of phase II (see below). $[c]/[b_2]$ is the slope of the plot cf. Fig. 5. Δ[ferrocytochrome c]$_I$ is the amount of cytochrome *c* reduced in phase I, i.e., $10-C_{II,o}$.

[c] $k_{app,II}$ is the slope of the first-order plot of cytochrome *c* reduction cf. Fig. 3 in the time interval 50 to 100 ms. Extrapolation of the line to $t = 0$ yields the $y_{II,o}$ intercept and $C_{II,o} = \exp(-y_{II,o}) \cdot \Delta[\text{ferrocytochrome } c]_{II} = C_{II,o} - C_{III,o}$ (see below).

[d] Since the first-order plot for phase III is not linear (Fig. 3B), the slope at 2 s is taken into consideration for $k_{III} \cdot C_{III,o}$ residual concentration of ferricytochrome *c* at the beginning of phase III is taken equal to C_o at time 1 s.

followed on both cytochromes by (i) cytochrome b_2 oxidation (Fig. 1, part A) and (ii) cytochrome *c* reduction (Fig. 1, part B) using a 100 ms sweeptime. Cytochrome b_2 oxidation is complete within approx. 30 ms and follows an apparently first-order process (Fig. 2) with a rate constant close to 200 s^{-1} (Table I), whatever the initial cytochrome b_2 reduction level. First-order analyses of cytochrome *c* reduction (Fig. 3) show that the latter takes place with an apparent rate constant, $k_{app,I}$. These k_{app} values are proportional to R, that is, to the initial amount of ferrocytochrome b_2 (cf. Fig. 4). The amplitude of phase I, i.e., the amount of ferrocytochrome *c* formed, Δ[ferrocytochrome c]$_I$, is approximately equal to the initial amount of ferrocytochrome b_2, over the whole range of R values explored (Fig. 4). Once corrected for phase II (taking into consideration its amplitude and rate constant following classical procedures), the rate constant of phase I, $k_{I,corr}$, is approx. 250 s^{-1} (cf. Table I). This estimation is possible only for R values above 40%. At lower R values, the resolution of phase I is too poor for an accurate measurement.

When the amount of ferrocytochrome *c* produced (symbol *c*) and the amount of residual ferrocytochrome b_2 (symbol b_2) are compared at each reaction time along the course of this phase I (Fig. 5), the slope c/b_2 is found to be equal to 1.55 ± 0.1 μM/μM.

No process faster than phase I escapes detection at the cytochrome *c* level since, when rigorous controls were achieved (for most of the experiments presented), it has been verified that the extrapolation to time zero

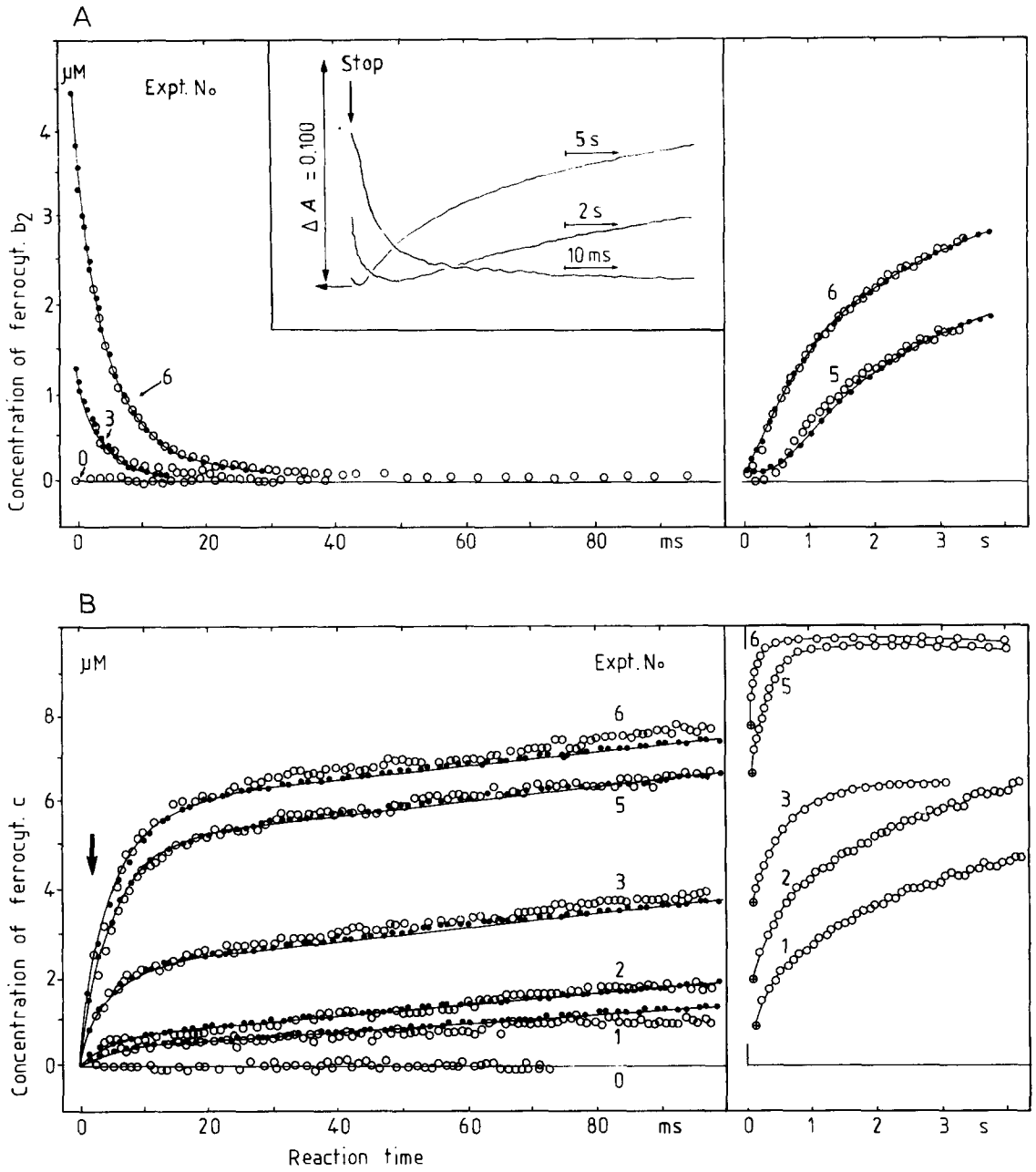

Fig. 1. Reaction of ferricytochrome *c* with partially reduced flavocytochrome b_2 showing the time-courses of cytochrome b_2 oxidation (top) and of cytochrome *c* reduction (bottom). Before rapid-mixing with ferricytochrome *c*, flavocytochrome b_2 (in the reservoir syringe) is partially reduced to reach a proportion, *R*, of cytochrome b_2 in the reduced form. Estimates of *R* by three methods (yielding R_a, R_b and R_c) for each experiment are listed in Table I. 10 mM pyruvate is present in both reactant solutions. Total concentrations, after mixing, are 10 and 5 μM for cytochrome *c* and flavocytochrome b_2, respectively. Time-courses in terms of concentrations of the reduced forms vs. time from mixing are computed from records of absorbance variations at 555.8 nm (for ferrocytochrome b_2, part A) and at 545.8 nm (for ferrocytochrome *c*, part B). All procedures for acquisition and treatment of data are detailed in the Material and Methods section. A typical series of experiments, 0 to 6, corresponding to *R* values varying from zero to approx. 95% is presented; in part A, certain points have been omitted for clarity: experimental points are presented as open circles, and simulated points, computed as detailed below and in the text, as black circles. Data are presented for two sweeptimes, 100 ms on the left and 10 s on the right. Simulations: For the 100 ms sweeptime (in parts A and B), scheme 2 (cf. Table II) was used with $k_1 = 250\ s^{-1}$, $k_5 = 6\ s^{-1}$, $k_6 = 400\ s^{-1}$ for all experiments, and R_c values were adjusted for each of them as listed in Table I. For the 10 s sweeptime, the simulations presented for cytochrome b_2 re-reduction (part A) were carried out using scheme 2′ with $k_L = 0.02\ \mu M^{-1}\cdot s^{-1}$, $(L)_o = 18\ \mu M$ for Expt. 5, and scheme 3 with $k_5 = 6\ s^{-1}$, $k_6 = 400\ s^{-1}$, $k_8 = k_{-8} = 1000\ \mu M^{-1}\cdot s^{-1}$, $k_L = 0.015\ \mu M^{-1}\cdot s^{-1}$ and $[L] = 130\ \mu M$, for Expt. 6. Simulations of cytochrome *c* reduction for this time range are presented for first-order plots in Fig. 3. Inset: Direct absorbance records of ferrocytochrome b_2 oxidation (initial concentration is 3.1 μM) and re-reduction, followed at 555.8 nm from stop time, for a same reaction mixture at three sweeptimes. Time and absorbance scales are indicated for each curve.

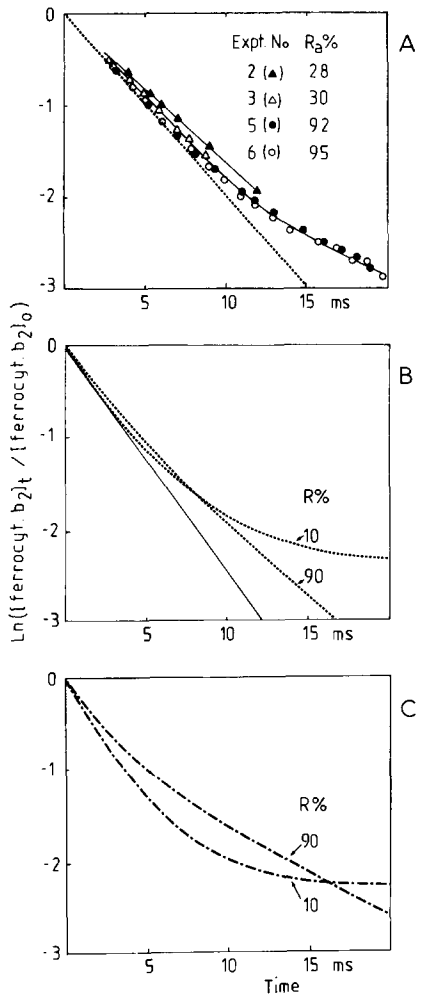

Fig. 2. First-order analysis of ferrocytochrome b_2 time-courses in phase I. Same series of experiments as in Fig. 1. (A) Experimental data. For each experiment, values of (ferrocytochrome b_2)$_t$ were used in a preliminary first-order graph to estimate the initial value at $t = 0$ through a linear extrapolation of data in the time range 3 to 6 ms. This estimate is used to determine R_a ((ferrocytochrome b_2)$_o$/5) and to plot the normalized curves (solid lines) presented here. Points correspond to Expt. No. and R_a values of 2 and 28% (▲), 3 and 30% (△), 5 and 92% (●), 6 and 95% (○). The dotted line corresponds to a first-order process with $k = 200$ s^{-1}. (B) Simulations with scheme 2 (cf. Table II, monomolecular process for reaction 1). Simulations for two extreme values of R, with $k_1 = 250$ s^{-1}, $k_5 = 5$ s^{-1}, $k_6 = 400$ s^{-1} are shown as dotted lines. For comparison, we have traced in a solid line the simulated curve for a single monomolecular reaction with $k = 250$ s^{-1}. (C) Simulations with scheme 2′ (bimolecular process for reaction 1). Rate constants used were $k_1' = 30$ μM^{-1}·s^{-1}, $k_5 = 5$ s^{-1}, $k_6 = 400$ s^{-1}.

of the first-order plots corresponding to the absorbance progress curves yielded an absorbance level identical to the corresponding sum of the contributions of the initial reactants, accounting for the low but significant contribution of the three flavin species. Similar controls at 555.8 nm showed that the plateau level reached by cytochrome b_2 after 30 ms corresponds to, at least, 97% of oxidation.

All these results demonstrate that ferrocytochrome b_2 is the main effective electron donor to cytochrome c in phase I. However, the value of the stoichiometric ratio close to 1.5 in the 3 to 10 ms time range, significantly different from 1, suggesting that another donor, flavohydroquinone and/or flavosemiquinone, also participates in cytochrome c reduction in this phase. The participation of these two donors will be further analysed below.

Phase II. This phase becomes dominant after 20 ms, when cytochrome b_2 has reached full oxidation and remains at a plateau level up to about 1 s (Fig. 1A). During this period, cytochrome c reduction proceeds slowly, according to a first-order behavior (Fig. 3), with $k \approx 5$ s^{-1} (Table I). When ferricytochrome c is initially in excess over the three donors available in flavocy-

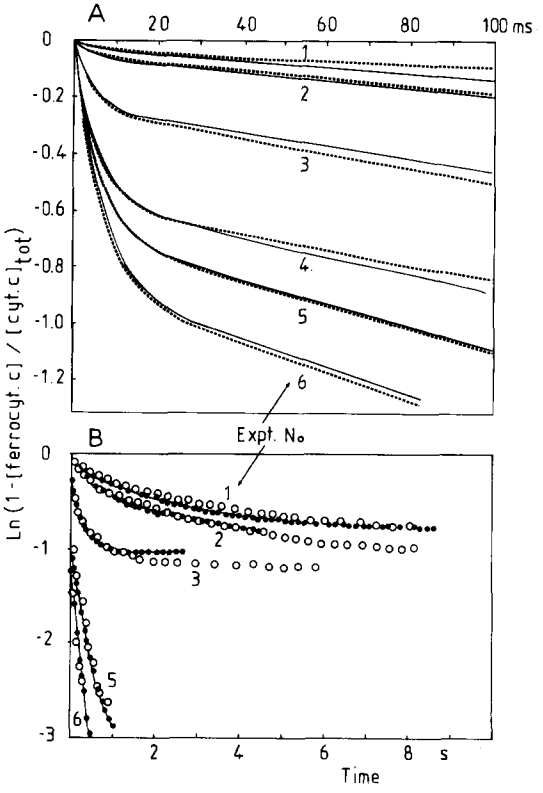

Fig. 3. First-order analysis of cytochrome c reduction. (Expt. numbers cf. Table I and Figs. 1 and 2). (A) 100 ms sweeptime. Plots from experimental data are shown as dotted lines. Corresponding simulated date (solid lines) result from simulations of scheme 2 with $k_1 = 250$ s^{-1}, $k_5 = 5$ s^{-1}, $k_6 = 400$ s^{-1}. R values leading to satisfactory fits (R_c in Table II) were respectively 13%, 43%, 79%, 87% and 93% for Expts. 1 to 6. It should be noted that they have to be accurately adjusted. (B) 10 s sweeptime. Open circles correspond to experimental data. Black circles are computed values from simulations of scheme 1 with the additional reaction (4), using $k_1 = k_3 = 250$ s^{-1}, $k_2 = 4$ s^{-1}, $k_4 = 0.015$ μM^{-1}·s^{-1}. The values of the variable R_c and [L] parameters for each experiment are: 5% and 4 μM (No. 1), 9% and 5 μM (No. 2), 40% and 0 μM (No. 4), 80% and 10 μM (No. 5) and 88% and 0 μM (N. 6). Expt. No. 2 was as well simulated using scheme 3 with $k_1 = 250$ s^{-1}, $k_5 = 2$ s^{-1}, $k_6 = 400$ s^{-1}, $k_L = 0.02$ μM^{-1}·s^{-1} and [L] = 2 μM.

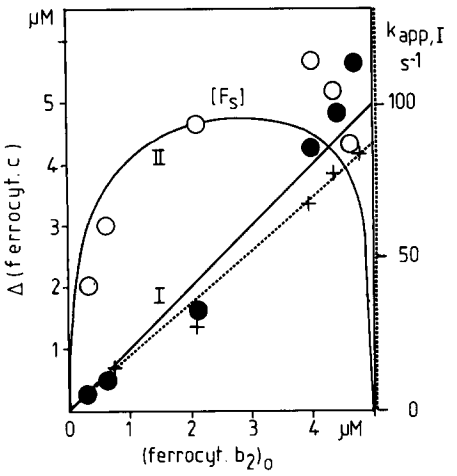

Fig. 4. Correlations between the amplitudes of phases I and II in cytochrome *c* reduction and the initial amounts of cytochrome b_2 and flavosemiquinone donors. Abscissae are [ferrocytochrome b_2] at time zero, taken equal to $R_a \times 5$ μM. Black circles are Δ[ferrocytochrome *c*]$_I$ (cf. Table I), i.e., the amount of cytochrome *c* reduced in phase I in ordinates. The solid line I corresponds to expectation for a stoichiometric reduction of cytochrome *c* by ferrocytochrome b_2. Open circles are Δ[ferrocytochrome *c*]$_{II}$, i.e., the amount of cytochrome *c* reduced in phase II. The solid line II is a plot of the calculated amount of F_s at equilibrium with ferrocytochrome b_2 at time zero (using Eqn. 1); it corresponds to expectation if the flavosemiquinone is the effective donor to cytochrome *c* in phase II. Crosses and the dotted line (with the dotted scale at right) show the variation of k_{app} in phase I (cf. Table I) with the initial ferrocytochrome b_2 concentration. This variation is a consequence of the variation of the relative amplitude of phase I and not of its true rate constant, $k_{I,corr.}$ that remains constant and equal to 250 s^{-1} (cf. Table I).

tochrome b_2 (for $R < 80\%$), the residual amount of ferricytochrome *c* at the end of phase II is $C_{III,o}$. As an approximation, we take this value equal to the amount present at 1 s. The amplitude of phase II (Δ[ferrocytochrome *c*]$_{II}$) is large whatever the initial cytochrome b_2 reduction level. As shown in Fig. 4, it is clear that these values are not significantly different from the amounts of flavosemiquinone present in the initial mixtures. This fact entirely supports the conclusion that the flavosemiquinone is the effective donor to cytochrome *c* during phase II.

Phase III. This is a very slow process detected with a 10 s sweeptime. When the initial reduction level of flavocytochrome b_2 is lower than 80%, the total concentration of intrinsic donors remains lower than 10 μM (Table I) and ferricytochrome *c* remains in excess after total heme and flavin flavocytochrome b_2 oxidation. Then phase III is seen as a slow step of cytochrome *c* reduction by an external reductant (Fig. 1B).

In the opposite case, for $R > 80\%$, ferricytochrome *c* is exhausted at the end or before the end of phase II. Then the final step in phase III is the rereduction of cytochrome b_2 (Fig. 1A). The corresponding semilog plot for cytochrome *c* is shown in Fig. 3B and the non-linearity suggests that the reaction is not first-order but probably second-order. The apparent first-order rate constants at 2 s are listed in Table I. This reduction can be achieved only by an extrinsic donor. There is good evidence that small amounts of L-lactate are responsible for this effect, as shown in the simulation studies presented below.

Simulation studies

Successful simulations (not reported extensively since they are beyond the scope of the present study), have been carried out. The purpose of this approach was: (a) to simulate all experiments considered here at various *R* values with a logical scheme using a same set of *k* values; (b) to discriminate the kinds of scheme that cannot satisfactorily fit the experimental data. The main study concerned phases I and II up to 100 ms after mixing. The schemes considered in the simulation stud-

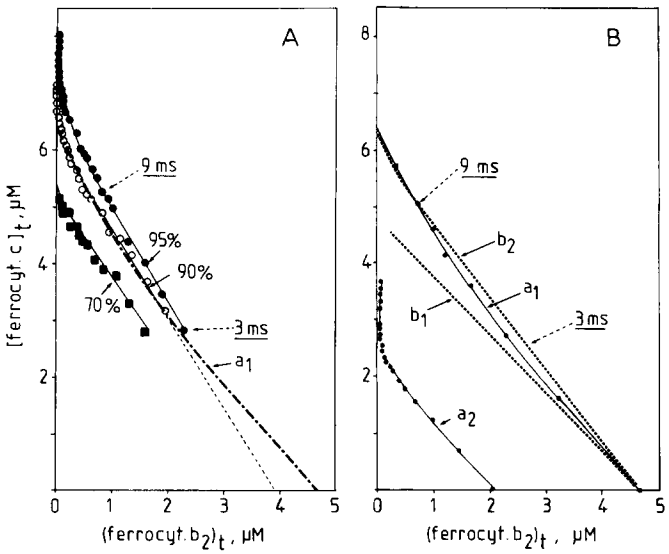

Fig. 5. Stoichiometry of the c/b_2 electron exchange along phase I. (A) Experimental results. At same reaction times, *t*, correlation between values of [ferrocytochrome b_2]$_t$ (abscissae) and [ferrocytochrome *c*]$_t$ (ordinates). The first relevant point for each experiment corresponds to 3 ms. Time intervals between points are of 1 ms. Slopes of the segments between 3 and 9 ms are respectively 1.45, 154 and 1.65 for Expts. 4 (■), 5 (○) and 6 (●), (i.e., for $R_{approx} = 70$, 90, 95%), respectively. For Expt. No. 5, extrapolation of this segment to the abscissae intercept (fine dashed line) gives an estimate of (ferrocytochrome b_2) at time zero of 3.9 μM (i.e., $R = 78\%$); for sake of comparison is reproduced the simulated curve a_1, also drawn to the right in panel B, obtained with scheme 2 and $R = 93\%$, which perfectly fits data between 3 and 10 ms, but not the value of the *x* intercept (4.65 μM) for that experiment. (B) Simulations: comparison of schemes 1 and 2 (Table II). Solid lines a_1 and a_2: points (dark dots) calculated each 1.5 ms from time zero with scheme 2, for $R = 93\%$ (a_1) and 43% (a_2) with $k_1 = 250$ s^{-1}, $k_5 = 5$ s^{-1} and $k_6 = 400$ s^{-1}. They are not straight lines: the slope for a_1 in the useful time range 3 to 9 ms is 1.6. Dotted lines b_1 and b_2: these are calculated with scheme 1, for R = 93%, with $k_1 = 250$ s^{-1}, $k_2 = 5$ s^{-1} and $k_3 = 0$ (for b_1) or 200 s^{-1} (for b_2). They are straight lines with slopes respectively equal to 1.3 and 1.0.

TABLE II

Schemes used for computed simulations

The schemes are expected to allow the simulations of: (1) The fast processes (phases I and II) taking place up to 100 ms at the level of ferrocytochrome b_2 (species B_r) and ferrocytochrome c (species C_r). Two pairs of assumptions are considered: (a) C_o reacts directly with the three possible donors intrinsic to flavocytochrome b_2, that is, B_r, F_s and F_h (schemes 1 and 1′) or C_o reacts directly with the single donor B_r, B and F species reacting together according to reversible intramolecular exchanges (reactions 5 and 6 in schemes 2 and 2′, plus reaction 8 in scheme 3). (b) C_o has a saturation behavior relatively to the donor(s) species considered (schemes 1 and 2) or C_o has a bimolecular behavior relatively to the donor(s) considered (schemes 1′ and 2′). (2) The slow processes (phase III) taking place after 1–2 s, at the level of ferrocytochrome b_2 (species B_r) or ferrocytochrome c (species C_r). Approximately at that time, with the reactant concentrations used, two kinds of reaction alternatively take place depending on R values (see text). If $R \leqslant 80\%$, there remains an excess of ferricytochrome c after complete oxidation of flavocytochrome b_2. The slow cytochrome c reduction is assumed to be indirect, via reaction 7 added to scheme 2. If $R > 80\%$, there remains an excess of reduced forms on flavocytochrome b_2 (B_r, F_s) after complete reduction of cytochrome c. The slow production of B_r at equilibrium with F_o, F_s, F_h is assumed to be due to reactions 7 and 8. The latter, with an equilibrium constant of 1 and a high value of k_8 allows equilibration of all flavin and heme species within flavocytochrome b_2 to be achieved. Indices o and r correspond to the oxidized and reduced forms, respectively of cytochrome c (symbol C) and of cytochrome b_2 (symbol B) or of flavin (symbol F); indices s and h correspond for the latter to the semiquinone and hydroquinone forms.

For simulation of phases I and II:

(A) ELECTRON TRANSFER TO FERRICYTOCHROME c LIMITED BY MONOMOLECULAR PROCESSES

Scheme 1: Hyp: direct transfers to C_o from the three possible donors.

(1) $B_r \rightarrow C_r + B_o$, rate constant k_1 in s^{-1}

(2) $F_s \rightarrow C_r + F_o$, rate constant k_2 in s^{-1}

(3) $F_h \rightarrow C_r + F_s$, rate constant k_3 in s^{-1}

Note: Reactants at left are supposed quantitatively complexed with C_o; the sum of their initial concentrations must be no greater than 10 μM ($R < 80\%$).

Scheme 2: Hyp: direct transfer to C_o only from B_r.

(1) $B_rF_0 \rightarrow C_r + B_oF_o$, rate constant k_1 in s^{-1}
$B_rF_s \rightarrow C_r + B_oF_s$, rate constant k_1 in s^{-1}
$B_rF_h \rightarrow C_r + B_oF_h$, rate constant k_1 in s^{-1}

(5) $B_oF_s \leftrightarrow B_rF_o$, rate constant k_5, $k_{-5} = k_5/0.05$

(6) $B_oF_h \leftrightarrow B_rF_s$, rate constant k_6, $k_{-6} = k_6/40$

Note: All reactants carrying the species B_r are supposed quantitatively complexed with C_o.

(B) ELECTRON TRANSFER TO FERRICYTOCHROME c LIMITED BY BIMOLECULAR PROCESSES

Scheme 1′: Hyp: direct transfer to C_o from the three possible donors. Reactions (1), (2) and (3) are as in scheme 1 except that, to the left, reactants are $C_o + B_r$, $C_o + F_s$, etc. The rate constants are then expressed in $\mu M^{-1} \cdot s^{-1}$. Initial $[C_o]$ is always 10 μM.

Scheme 2′: Hyp: direct transfer to C_o only from B_r.
All reactions are the same as in scheme 2 except that reactions (1) are written, to the left as $C_o + B_rF_o$, etc.

For the simulation of phase III

The schemes involve bimolecular reduction by an additional bielectronic donor: L (for the substrate, L-lactate). Several procedures have been used:

TABLE II (continued)

Scheme 1 or 1′ with an additional process:
(only for phase III of cytochrome c reduction) for $R < 80\%$. The additional process is the following:

(4) $1/2\ L + C_o \rightarrow C_r$, rate constant: k_L, $\mu M^{-1} \cdot s^{-1}$

Note: For scheme 1, C_o at time zero is taken equal to $10 - B_r - F_s - F_h \cdot 2$.

Scheme 2 with an additional process:
L is supposed to react only with the species F_o to yield F_h. Since C_o is supposed in excess, this scheme works only for cytochrome c at $R < 80\%$.

(7) $B_oF_o + L \rightarrow B_oF_h$, rate constant k_L
$B_rF_o + L \rightarrow B_rF_h$, rate constant k_L

Scheme 2′ with the addition process 7:
This scheme works in all conditions for phase III of cytochrome b_2 ($R > 80\%$) or cytochrome c ($R < 80\%$) reduction.

Scheme 3:
For the slow phase of cytochrome b_2 re-reduction after consumption of ferricytochrome c, and total oxidation of the flavocytochrome in B_oF_o, the scheme can be simplified into the four following processes:

(7) $B_oF_o + L \rightarrow B_oF_h$, rate constant: k_L

(5) $B_oF_s \leftrightarrow B_rF_o$, rate constant: k_5, k_{-5}

(6) $B_oF_h \leftrightarrow B_rF_s$, rate constant: k_6/k_{-6}

to which must be added:

(8) $B_rF_s + B_oF_o \leftrightarrow B_oF_s + B_rF_o$, rate constant: $k_8 = k_{-8}$

in order to reach equilibration of the three flavin species. Note that the lag in cytochrome c_2 re-reduction corresponding to phases I and II is not accounted for with this scheme.

ies are presented in Table II. The initial proportions of the donors, ferrocytochrome b_2 (B_r), flavosemiquinone (F_s) and flavohydroquinone (F_h) were calculated using Eqn. 1 for each R value.

Two main questions have been considered: (1) Are schemes 1 and 1′, which involve three independent direct transfers (from F_h, F_s, B_r) to cytochrome c (C_o), as valid as schemes 2 and 2′, which involve B_r as a specific donor? In the latter case, regeneration of B_r is considered to take place by means of reversible intramolecular electron exchanges at the expense of F_h and F_s. (2) Are electron transfers to cytochrome c monomolecular (schemes 1 and 2) or bimolecular (schemes 1′ and 2′)?

The answer to the first question was ambiguous: Schemes involving the independent direct monomolecular transfers from the three donors to cytochrome c at saturation (scheme 1) can approximately account for the various curves. To that end, k_1 and k_2 values were respectively taken equal to 200 and 5 s^{-1}, and the k_3 value had to be taken close to 200 s^{-1}. It thus appears that B_r and F_h are consumed with similar rate constants. With such rate constants within scheme 1, the stoichiometry c/b_2 reached along phase I (slope in Fig. 5) is 1.3, i.e., not significantly lower than the experimental values (1.55 ± 0.1).

Similarly, it is possible to consider scheme 2 (Table II) involving only B_r as the direct donor to C_o at saturation (mono-molecular behavior) and reversible intramolecular exchanges between flavin and heme within the various 'FB' species that can exist in active sites. Such a scheme also allows perfect fits of all experimental curves; to that end, correct R_c values had to be precisely adjusted in successive assays with a same set of rate constants. For the latter, best fits were obtained with k_1, k_5 and k_6 respectively equal to 250, 5 and $\geqslant 400$ s^{-1}. Examples of these fits are shown in Figs. 1, 2 and 3 for the series of experiments presented. For the c/b_2 stoichiometry, the slope reached in the relevant part (3 to 10 ms) is 1.6 (Fig. 5, part B). In such a case, two facts have to be noted. Firstly, along phase I of cytochrome b_2 oxidation, the apparent rate constant of simulated curves, $k = 200$ s^{-1}, is lower than the value $k_1 = 250$ s^{-1} used in the simulation. This effect results from the fact that steady-state B_r levels are lower than the amount produced in reactions 1, due to concomitant reduction through reactions 5 and 6. Secondly, the phase II process with $k = 5$ s^{-1} is entirely limited by the rate constant, k_5, corresponding to the intramolecular transfer from F_s to B_o within active sites.

The answer to question 2 is clearer. Scheme 2', similar to scheme 2 but with bimolecular instead of monomolecular reactions 1 between C_o and B_r is able to fit C_r time-courses but *not* the B_r time-course (Fig. 2). Indeed, if the reaction were bimolecular, at the reactant concentrations used, the apparent rate constant should decrease significantly at high R values, since initial C_o concentrations are no longer in large excess over B_r concentration.

It should be noted that simulation assays carried out with other estimates of K_1 and K_2 (0.1 and 16 instead of 0.05 and 40) also led to satisfactory results but with slightly different adjusted R_c values. This finding is related to the fact that in the whole R range from 30 to 80%, the amount of F_s which controls the amplitude of phase II is approx. equal to 1, and does not depend on the precise K_1 and K_2 values but rather on their product. The amplitude of phase I, corresponds a priori to the sum of B_r and of F_h at time zero. Only the latter is strongly influenced by the K_2 value used; but, as it remains always a minor component in the range explored, an error in the K_2 value used for simulations will not be significant.

If we now turn our attention to phase III, it has been clearly shown that it is possible to simulate cytochrome c reduction for low R values (Fig. 3B). To that end, we used, in addition to reactions 1, 5 and 6 in scheme 2', two other reactions: firstly reaction 7, between an external 2e donor (the substrate lactate) and the species containing F_o, and secondly reaction 8, that allows the equilibration between the various 'FB' couples. A high k_8 was chosen so that the corresponding process is not a limiting one.

The same scheme allows the simulation of cytochrome b_2 rereduction (Fig. 1A) (formation of B_r) for R values higher than 80%, after exhaustion of ferricytochrome c near 1–2 s. With $k_L = 0.02$ $\mu M^{-1} \cdot s^{-1}$, the known value of the lactate binding rate constant in the same experimental conditions in the presence of 10 mM pyruvate [19], adequate [L] values are respectively 2 and 130 μM for Expts. 1 and 6. Very similar values for L-lactate concentrations (1.4 and 110 μM) can be calculated as the amount at equilibrium with 10 mM pyruvate and the ferri/ferrocytochrome b_2 system at respectively 10 and 90% reduction levels if the $E_{m,7}$ values for the pyruvate/L-lactate couple and the cytochrome b_2 couple were taken respectively as -100 mV and -20 mV. Note that, for simulations, large variations of k_L and [L] are tolerated if their product is kept constant.

It is not possible to use scheme 2 itself for such simulations of phase III, since it is based on the assumption that C_o remains always in excess, an assumption that is no longer valid after 1 s at $R > 80\%$. Then B_r production from the fully oxidized species F_oB_o, the form that is present at 1–2 s, can be stimulated using the simple scheme 3, in which requilibration between the various redox centers is also taken into consideration (reactions 5, 6 and 8).

The conclusions of the simulation study are therefore the following:

(1) There is no support for, but no opposition to, the former assumption that cytochrome c is able to react directly exclusively at the cytochrome b_2 level.

(2) Under our experimental conditions, the electron transfer from ferrocytochrome b_2 (< 5 μM) to ferricytochrome c (10 μM) has a kinetic behavior quasi-monomolecular with $k = 250$ s^{-1}. In fact, a saturation level of 70% for the cytochrome c/flavocytochrome b_2 complex can not be distinguished from total saturation due to experimental uncertainty on cytochrome b_2 time-courses.

(3) The reaction that limits phase II, with $k = 5$ s^{-1}, can be a direct transfer from F_s to cytochrome c (scheme 1) as well as the transfer from F_s to cytochrome b_2 (scheme 2) if the latter is followed by faster reactions of redox equilibration between prosthetic groups and a faster transfer from ferrocytochrome b_2 to cytochrome c.

(4) The reaction between F_h and cytochrome c which takes place in phase I could be either direct with $k = 200$ s^{-1} or indirect via an F_h to ferricytochrome b_2 step and a ferrocytochrome b_2 to cytochrome c step if the rate constant for the former is greater than 400 s^{-1}.

(5) The assumption that L-lactate, present in minute amounts, is the donor in phase III is very well sup-

ported, since it yields good simulations of that phase using the known value of the rate constant for binding of lactate to the enzyme in the presence of pyruvate.

Discussion

The comparison of ferrocytochrome *c* and ferrocytochrome b_2 time-courses after rapid mixing of the reactants – (partially reduced flavocytochrome b_2 and ferricytochrome *c* in slight excess (2-fold)) – has allowed a clear analysis of two kinetic steps. One of them can be assigned to the electron transfer from ferrocytochrome b_2 to ferricytochrome *c* in quasi-saturation conditions. The other, much slower, corresponds to cytochrome *c* reduction at the expense of the flavosemiquinone and involves an intramolecular electron transfer from flavosemiquinone to cytochrome b_2, within active sites of the pyruvate-liganded enzyme.

Under the conditions used (that is, in 100 mM phosphate buffer (pH 7) with 10 mM pyruvate, and at partial reduction of flavocytochrome b_2) when the heme of the cytochrome b_2 domain is reduced to the level *R*, two other intrinsic donors are present: flavohydroquinone (minor species in the range studied) and flavosemiquinone (major species that reaches 95% of the total flavin in the presence of 10 mM pyruvate) [17]. Indeed, redox equilibria between heme and flavin species are known to take place rapidly [12,14]. It has been shown that pyruvate, lying on one side of the isoalloxazine ring [2], drastically modifies the redox properties of the flavin prosthetic groups, markedly increasing the proportion of semiquinone [14], and decreases the rate of the F_s to cytochrome b_2 electron transfer as seen using T-jump relaxation technology [12].

A part from these three intrinsic donors pertaining to flavocytochrome b_2, we observed that an extrinsic donor, L-lactate present in minute amounts, provides a very slow final reduction detected either at the cytochrome *c* or at the cytochrome b_2 level as detailed in the Results section.

We will now discuss the interpretation of the results presented above concerning the global reactions of *H.a.* cytochrome *c* with the three intrinsic donors present in partially reduced flavocytochrome b_2.

Reduction of cytochrome c by ferrocytochrome b_2

This process takes place in fastest phase I, with a first-order rate constant near 250 s^{-1}. The behavior at the level of the donor indicates that it is quasi-saturated by cytochrome *c*. Since the maximal rate at saturation, precisely determined at the same temperature, is 380 s^{-1} [5], it appears that, in fact, the saturation level was 70% in the concentration and ionic strength conditions used. The latter were deliberately chosen high enough to slow down the reaction and allow a more precise analysis. The stoichiometry, in terms of μM of ferrocytochrome *c* formed per μM of ferrocytochrome b_2 oxidized along phase I, is 1.5 ± 0.1 along the relevant time range (3 to 10 ms). Thus, another donor (F_s and/or F_h) contributes significantly to cytochrome *c* reduction, as will be discussed below.

Oxidation of flavin species by cytochrome c

A priori, the oxidation of the flavin groups by ferricytochrome *c* could be a direct process or a process mediated by cytochrome b_2, the latter being reduced by the semiquinone (or hydroquinone) as it is oxidized by ferricytochrome *c*. In fact, as detailed below, all previous data entirely support the second mechanism, corresponding to the assumption made by Morton et al. [6] that cytochrome *c* does not react with the flavin prosthetic groups in flavocytochrome b_2, but reacts specifically with the cytochrome b_2 moiety. (a) Cytochrome *c* is able to accept an electron from the isolated cytochrome b_2 core domain [15]. In such a case, the rate constant at saturation seems similar to that of the native flavocytochrome b_2 [5,15]. (b) Ferricytochrome *c* does not accept electrons from heme-free derivatives [7,8] or from the isolated flavodehydrogenase domains [9,10]. This lack of reactivity between cytochrome *c* and flavin groups is likely to be due to a steric and not to an energetic factor. Indeed, (i) the gap in midpoint potentials is favorable and large ($E_{m,7}$ values are respectively +250 mV for cytochrome *c* and approx. −20 mV and −60 mV for the two flavin couples under normal conditions [14,17]) and (ii) stable complexes, cytochrome *c*/flavodehydrogenase (incompetent in electron transfer), are formed [4]. These data can be understood in the light of the three-dimensional structure of flavocytochrome b_2, since it seems that the flavin is buried in the flavodehydrogenase domain and that the position near the flavin is covered, at least in the resolved stable configuration, by the cytochrome b_2 domain [2].

The flavohydroquinone donor, in the experiments presented, remains always at a low level relative to that of ferrocytochrome b_2 (< 20%). The corresponding phase of cytochrome *c* reduction cannot be resolved, but appears concomitant with the phase I of cytochrome *c* reduction by ferrocytochrome b_2. Considering that F_h oxidation is achieved via the ferri-ferrocytochrome b_2 shuttle, the step of F_h to ferricytochrome b_2 electron transfer should not be rate-limiting. Its rate constant should be markedly higher than that corresponding to the ferrocytochrome $b_2 \rightarrow$ ferricytochrome *c* transfer. By simulation, it was found that a minimal value of 400 s^{-1} gave correct fits to experimental data. As shown elsewhere [12], this transfer is very fast in the presence of pyruvate.

The flavosemiquinone donor makes the major contribution in phase II of cytochrome *c* reduction, since its concentration remains near 4.5 μM for initial ferrocyto-

chrome b_2 concentrations between 1 and 4.5 μM. The experiments presented have shown that it slowly reduces cytochrome *c* in a distinct phase II with $k = 5$ s^{-1}, detected after total consumption of ferrocytochrome b_2. Actually, the amplitude of phase II, in terms of increment of ferrocytochrome *c*, corresponds to the amount of F_s initially present (shown in Fig. 4). Since the time-course is first-order, we assume without relevant experimental support that the rate-limiting process is monomolecular.

We will now comment on the nature of the step that limits, in our rapid-mixing experiments, this F_{sq} to ferricytochrome *c* global transfer. In fact, several successive steps are involved after the first series of reactions taking place in phase I. First of all, ferrocytochrome *c* dissociation from its complex with flavocytochrome b_2, then ferricytochrome *c* binding, electron transfer from F_s to cytochrome b_2 and *e*-transfer from cytochrome b_2 to cytochrome *c*. Obviously, dissociation of ferrocytochrome *c* from its prime complex with flavocytochrome b_2 could be a priori the rate-limiting step; however, this assumption is not supported by available data, since such a step should also limit the turnover with the *H.a.* cytochrome *c* acceptor, which is a contradiction with the V_{max} value of 80 s^{-1} [19,14] found under identical experimental conditions except for the absence of pyruvate. Estimates of 200 s^{-1} for such a dissociation step have been suggested [5]. None of the steps of cytochrome *c* binding and of cytochrome b_2 to cytochrome *c* transfer can be limiting in phase II, since it would also limit phase I. Finally, the limiting step in the F_{sq} to cytochrome *c* transfer observed here in the presence of 10 mM pyruvate appears to be identical to that limiting the turnover of the enzyme in the presence of cytochrome *c* and pyruvate where, the steady-state rate, V_{max}, was found to be approx. 9 s^{-1} under the same conditions [19], a value not significantly different taking into account the difference in conditions and technology used.

It is interesting to comment on the slowness of this $F_s \rightarrow$ cytochrome b_2 electron transfer in the case of the pyruvate-liganded enzyme. The fact that the rate was markedly changed in the presence of this ligand was previously demonstrated in T-jump determinations, yielding at 16 °C rate constants of 140 ± 70 s^{-1} without pyruvate and, with 1 mM pyruvate [12], down to 12 s^{-1} (recent re-estimate cf. Ref. 19). The estimate afforded in the present study, i.e. 5 s^{-1} at 5 °C under pyruvate-saturating conditions, agrees with these former results and gives a much more accurate value. It should be kept in mind that this step takes place in energetically unfavorable conditions, since the midpoint potential of the F_o/F_s couple is 80 mV higher than that of the ferri/ferrocytochrome b_2 couple.

A detailed analysis of the pyruvate binding to the three redox states of the flavoprotein and of its effect on the steady-state enzyme reactivity with respect to the different acceptors, ferricyanide and ferrocytochrome *c* has been recently carried out in our laboratory (Tegoni et al. [19]). The inhibition observed – essentially non-competitive – can be interpreted by the formation of a pyruvate flavosemiquinone complex ($K_d \approx 0.2$ mM). This behaves as a dead-end complex and has a very low electron transfer reactivity with respect to its cytochrome b_2 partner as shown here, and also, more unexpectedly, with respect to ferricyanide. This behavior led to question the real nature of the rate-limiting step in the presence of pyruvate: electron transfer from the pyruvate flavosemiquinone transient or pyruvate dissociation regenerating the active free form? Further analyses of the T-jump relaxations support the former assumption (Silvestrini, personal communication).

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