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Rapid kinetic studies of the electron-exchange reaction between cytochrome c_3 and ferredoxin from *Desulfovibrio desulfuricans* Norway strain and their individual reactions with dithionite

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The electron-exchange reaction between *Desulfovibrio desulfuricans* Norway cytochrome c_3 and ferredoxin was investigated by rapid-mixing techniques under anaerobic conditions. Their reduction by dithionite is biphasic when several redox centres are present in the molecule. The observed first-order rate constant of the rapid and slow phases are both attributed to the reduction by the radical anion SO_2^-. The reduction rate constants for cytochrome c_3 (10^7 $M^{-1} \cdot s^{-1}$) are higher than for ferredoxin (10^5 $M^{-1} \cdot s^{-1}$) at 5°C. These results are compared with data also obtained with *Desulfovibrio vulgaris* cytochrome c_3 and discussed in terms of kinetic models implying inter- and/or intramolecular electron exchange. The electron-transfer kinetics between reduced cytochrome c_3 and oxidized ferredoxin is monophasic with a rate-limit of 160 s^{-1} at 5°C. The amplitude detected corresponds to the oxidation of one heme per molecule. A reaction mechanism involving the formation of one intermediate complex, followed by an intramolecular electron exchange leading to the oxidation of the heme of lowest oxidation–reduction potential is proposed. The reverse reaction (i.e., mixing the oxidized cytochrome c_3 with reduced ferredoxin) is still monophasic and of an apparent second-order character with a rate constant of $7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 5°C. At extremely low ferredoxin concentrations the reaction is limited by the rate of the reverse reaction ($k_- = 150$ s^{-1}). The reduction of four hemes is observed. This extent of reduction reveals that the apparent equilibrium constant for the electron-exchange reaction is greater than 1. Nevertheless, the data support the view that a complex is formed rapidly between the two proteins and is followed by a rapid intramolecular transfer of electrons in either direction. The rate constants for the reduction of cytochromes c_3 by ferredoxins, both from different species, are also reported. It is concluded that cytochrome c_3 is not equally efficient in both directions of the electron exchange. Depending on the relative oxidation-reduction states and concentrations, one or two hemes and four hemes might be involved in the electron exchange with ferredoxin. The influence of oxidation–reduction potentials, electrostatic interactions and charge distribution on the proteins are taken into account for the interpretation of the electron-exchange mechanism.

Introduction

Desulfovibrio desulfuricans Norway strain cytochrome c_3 has been the subject of extensive studies. This protein is a member of a class of small (molecular weight, about 13000) soluble proteins containing four hemes associated with a single polypeptide chain [1]. Characteristic of cytochrome c_3 is the four distinct low oxidation–reduction potentials which are spread between −400 and −165 mV as shown from electrochemical

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Abbreviation: HiPIP, high-potential iron protein.

studies [2–4]. This is opposed to other classes of heme *c* proteins, such as mammalian cytochrome *c* and bacterial cytochrome c_2 and cytochrome *c*-551 which are of high oxidation reduction potential, about 250 mV, and have been shown to have substantial structural homology [5]. The physiological role of cytochrome c_3 is to act as an electron carrier between hydrogenase and ferredoxin. In the sulfate-reducing pathway, cytochrome c_3 accepts electrons from hydrogenase and transfers them to ferredoxin [6]. In the pyruvate oxidating or phosphoroclastic pathway it donates electron taken from ferredoxin to hydrogenase [6]. Two ferredoxins have been isolated from *Desulfovibrio desulfuricans* Norway strain [7,8]. Both were found to be active in these reactions. They differ by the presence of one or two 4Fe-4S clusters per subunit found in ferredoxin I and II, respectively. The protein molecules are well characterised on structural features with the amino-acid sequences established [9] and with some cristallographic data reported [10].

Considerable physico-chemical work has been completed on *D.desulfuricans* cytochrome c_3 with the amino-acid sequence [11] and 3-dimensional structure [12,13] determined. From the structural studies each heme centre has been characterised and found in a distinct environment. Although each heme unit in the protein has two imidazole groups as fifth and sixth ligands, the local environment and extent of exposure to water of each heme is somewhat different [4,12].

In view of the structural information available it appears interesting to investigate the oxidation-reduction properties of cytochrome c_3. Previous pre-steady state studies using stopped-flow spectrophotometry [14,15] and pulse radiolysis [16] on *Desulfovibrio vulgaris* cytochrome c_3 have been concerned with electron transfer from small reductants, dithionite and methyl-viologen radical, to the protein. Data show that the four hemes are equivalent two by two with regard to their reduction rates [14], and that the possibility of random reduction of hemes with equal reactivities can be excluded [15].

Indeed, the structural differences noticed between the hemes may be reflected in the different capacities of the heme to accept electrons. Therefore in an effort to further our understanding of biological electron transfer, we are reporting here rapid kinetic studies on the interaction of cytochrome c_3 with ferredoxin both from *D.desulfuricans*. Taking advantage of natural modifications of cytochrome c_3 and ferredoxin, we are also reporting the comparative studies of the reduction of cytochrome c_3 from two different microorganisms *D.desulfuricans* and *D.vulgaris* by ferredoxin from *D.desulfuricans* and *Clostridium thermocellum*.

The goals of the studies reported here are twofold: (1) to obtain information on the mechanism of electron transfer to and from the hemes of cytochrome c_3, including the participation, if any, of specific amino-acid side chains; (2) to investigate the rapid electron exchange between two well-defined proteins (cytochrome c_3 and ferredoxin) and relate these data to those obtained with non-physiological oxidants and reductants. Thus the present study includes the rapid kinetics of reduction and oxidation of the various proteins using dithionite and oxygen, respectively.

Materials and Methods

Proteins

Cytochrome c_3 and ferredoxin I from *Desulfovibrio desulfuricans* Norway strain were prepared by the methods described by Bruschi et al. [1] with a purity index $A_{553} - A_{570}/A_{280} = 3.2$ for cytochrome c_3 and $A_{390}/A_{305} = 0.80$ for ferredoxin.

Ferredoxin II from *D.desulfuricans* Norway strain was prepared as described by Guerlesquin et al. [7] with a purity index of 0.72.

Ferredoxin from *Clostridium thermocellum* was purified as described previously in Ref. 17 with a purity index of 0.76.

Cytochrome c_3 from *Desulfovibrio vulgaris* was isolated as described by Le Gall et al. [18] with a purity index of 2.8.

Protein concentrations were determined spectrophotometrically with a Perkin Elmer 555 spectrophotometer using the following millimolar absorption coefficients: for cytochrome c_3 *D.desulfuricans* $\varepsilon^{red}_{552} = 130\ mM^{-1} \cdot cm^{-1}$; $\varepsilon^{ox}_{408} = 570\ mM^{-1} \cdot cm^{-1}$; for cytochrome c_3 *D.vulgaris* $\varepsilon^{red}_{552} = 159\ mM^{-1} \cdot cm^{-1}$, $\varepsilon^{ox}_{408} = 687\ mM^{-1} \cdot cm^{-1}$; for ferredoxin *D.desulfuricans*: Fd I $\varepsilon^{ox}_{390} = 17.5\ mM^{-1} \cdot cm^{-1}$, Fd II $\varepsilon^{ox}_{390} = 31.5\ mM^{-1} \cdot cm^{-1}$; for ferredoxin *C.thermocellum* $\varepsilon^{ox}_{390} = 31.5\ mM^{-1} \cdot cm^{-1}$.

Spectral data acquisition

To compare the magnitude of the difference in absorbance observed in the course of rapid-kinetic studies with static differential spectrophotometric measurements, performed at the same wavelengths, it is necessary first to determine the individual absorption coefficients ε^{ox}, ε^{red}, ε^{red-ox} of cytochrome c_3 and ferredoxins. Therefore, spectral data processing were carried out using a microcomputer as previously described in Ref. 19. Series of absorbance spectra of oxidized and dithionite-reduced species were routinely scanned from 600 to 350 nm, using a 555 Perkin Elmer spectrophotometer, with a slit width of 1 nm, connected to a SP 4100 Spectra Physics calculator which compute at each wavelength absolute absorption coefficients and/or absorption difference coefficients (red − ox). In the course of static spectrophotometry, the mean value of the difference absorption coefficients at 552 nm calculated from n independent determinations (standard deviations given in parentheses) are the following: 91(∓5) $mM^{-1} \cdot cm^{-1}$ ($n = 6$) and 116 (∓4) $mM^{-1} \cdot cm^{-1}$ ($n = 3$) for *D.desulfuricans* and *D.vulgaris* cytochrome c_3, respectively.

Preparation and standardization of dithionite solutions

Concentrate dithionite solutions (0.2–0.4 M) were freshly prepared by addition of a weighted quantity of solid Merck sodium hydrosulfite to a deaerated 0.2 M phosphate buffer at pH 8, then kept under argon. Dilute solutions were prepared under argon and used within a few hours of being prepared. Dithionite solutions were standardized by anaerobic spectrophotometric titration against ferricyanide monitored at 420 nm ($\Delta\varepsilon = 1.02$ $mM^{-1} \cdot cm^{-1}$) [20].

Stopped-flow kinetics

Rapid absorbance kinetic measurements were conducted in a thermostated Durrum–Gibson stopped-flow apparatus fitted with a 1.9 cm cell, having a 2.2 ms dead-time and with improvements as previously reported in Ref. 21. To minimize oxygen leaks, all parts of the apparatus were submerged in a thermostatically controlled circulating water bath, continuously bubbled with N_2 gas at 5 (∓0.1)°C. The stopped-flow system was thoroughly flushed with anaerobic buffer immediately before the experiments were started. To perform quantitative absorbance measurements (a) baselines were established at each wavelength with anaerobic buffer, (b) the initial absorbance level of the chromophore (cytochrome c_3 or ferredoxin) was determined after mixing the protein solution with buffer, (c) absolute absorbance changes were followed as the reaction proceeded. Finally the slit opening was adjusted to the same extent as that of the Perkin Elmer 555 spectrophotometer. The absolute absorbance values obtained from these measurements were typically within 5% of those obtained from the absorbance spectra of the same solutions recorded on the Perkin Elmer 555 spectrophotometer (see Results). All the concentrations of components given in the text correspond to final concentrations after mixing.

Preparation of anaerobic solutions for kinetics

Unless noted otherwise, all solutions were prepared in 0.1 M monosodium-dipotassium phosphate buffer (pH 7.0). Buffer solutions in glass tonometers were made anaerobic by at least six cycles, of evacuation and flushing with oxygen-free argon gas. A concentrated aliquot of protein or reductant was added to the anaerobic solution with gas tight syringes and the mixture was left to incubate at 4°C under slight argon flushing. This solution was then transferred to a storage syringe of the flow system under a positive pressure of argon gas.

Data analysis

For every kinetic experiment, at least two sets of 1024 points were recorded at the same time-sweep then transferred on an $X-Y$ recorder with an analog-to-digital converter, Tracor NS 570. The data were analysed graphically (through semilogarithmic plots and least-squares regressions) and by digital computer as follows. A set of 20–30 data points was used for multiexponential (1 to 2) non-linear iterative regression based on a least-squares criterion. The programme, adapted to an Apple IIe by Drs. Mispelter and Favaudon, includes adjustments for the amplitude of the reaction and the first-order rate constant and gave their related standard deviations. The theoretical equation (i.e., resulting curve) was plotted on the

same scale as the original experimental data points to allow visual inspection and check for systematic deviation. The reaction rates were the mean values of analyzing 4–6 traces. The standard deviation was found to be 5–12%, the greater for faster processes.

Results

Reaction of cytochrome c_3 with dithionite

The reduction of cytochrome c_3 with dithionite under pseudo-first-order conditions (0.1–30 mM), observed at 552 nm, followed biphasic kinetics (Fig. 1). These absorption changes may be adequately fitted by the sum of two exponentials.

Whatever dithionite concentration, the total amplitude of the reaction was constant. It corresponded to a kinetic absorption difference coefficient $\Delta\varepsilon = 95$ (∓ 6) $mM^{-1} \cdot cm^{-1}$ ($n = 19$) for *D.desulfuricans* Norway cytochrome c_3 and $\Delta\varepsilon = 119$ (∓ 6) $mM^{-1} \cdot cm^{-1}$ ($n = 10$) for *D.vulgaris* cytochrome c_3, in agreement with the values obtained for full reduction of cytochrome c_3 in static differential spectrophotometry $\Delta\varepsilon = 91$ (∓ 5) $mM^{-1} \cdot cm^{-1}$ ($n = 6$) and 116 (∓ 4) ($n = 3$), respectively. The initial phase consisted of a fast process which made the major contribution to the absorbance change, i.e. 86 (∓ 6)% (with $n = 27$ independent traces obtained at different dithionite concentration) for cytochrome c_3 *D.desulfuricans* Norway and 72 (∓ 4)% ($n = 13$) for cytochrome c_3 *D.vulgaris*. The relative amplitude of the two phases is maintained whatever the dithionite concentration (0.1–30 mM) and the cytochrome c_3 concentration (0.1–5 μM). At low dithionite concentrations (below 0.2 mM) the reaction exhibited an induction period whose length increased with

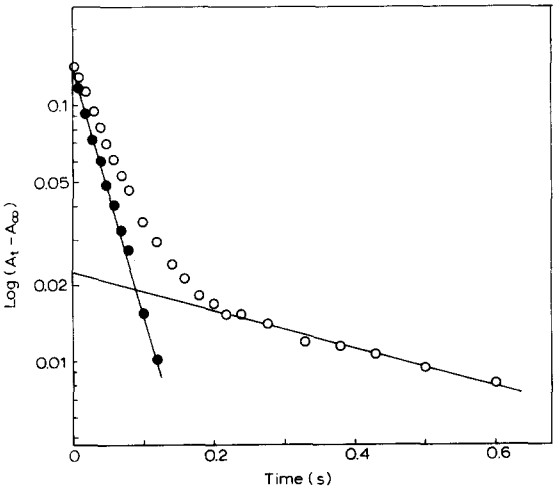

Fig. 1. Logarithmic plot to show the biphasic nature of the reaction between *D.desulfuricans* cytochrome c_3 and dithionite. A logarithmic plot of data obtained during a flow experiment in which 0.43 μM cytochrome c_3 was mixed with 9 mM dithionite (final concentrations) in the 1.9 cm path-length cell of the stopped-flow apparatus. The reaction was followed at 552 nm in the presence of 0.1 M phosphate buffer (pH 7.0) and at 5°C, ○, Data points from the reaction trace; ●, points obtained after substracting the slow phase of the reaction (———) from the points of the reaction trace. The slopes yielded the apparent first-order rate constant $k_I = 23\ s^{-1}$ and $k_{II} = 1.6\ s^{-1}$ of the reaction.

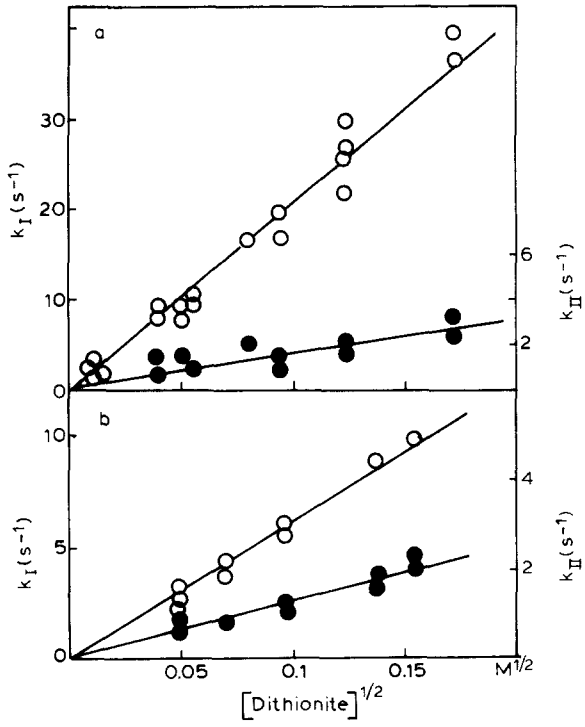

Fig. 2. Dependence of the pseudo-first-order rate constants k_I and k_{II} on the square root of the dithionite concentration for the reaction with *D.desulfuricans* and *D.vulgaris* cytochrome c_3. (a) *D.desulfuricans* cytochrome c_3 concentration, 0.43 μM; dithionite concentration varied from 0.12 to 30 mM. The continuous lines were fitted to Eqn. 1; $k_a = k_+ (S_2O_4^{2-} \cdot K)^{1/2}$ leading to $k_+ = 1 \cdot 10^7\ M^{-1} \cdot s^{-1}$, $k_{II+} = 6.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$. (b) *D.vulgaris* cytochrome c_3 concentration, 0.22 μM; dithionite concentration varied from 2.4 to 24 mM. The fit of the data points leads to $k_{I+} = 3.2 \cdot 10^6\ M^{-1} \cdot s^{-1}$, $k_{II+} = 6.3 \cdot 10^5\ M^{-1} \cdot s^{-1}$.

decreasing dithionite concentration and also when oxygen concentration (> 10 μM) was still present in the solution (see below Fig. 6c). Therefore, the first point of the time course was taken at the end of the induction period.

For both cytochromes c_3 species, the observed first-order rate constants for each phase exhibited a complex dependence on the concentration of dithionite, but a simple linear dependence on its half-power (Fig. 2a, b). Lambeth and Palmer [22] showed that dithionite can act as a reductant by two independent pathways with the $S_2O_4^{2-}$ anion and with the SO_2^- free radical anion, the monomer being the stronger reducing agent [23]. Since the equilibrium between these two species $S_2O_4^{2-} \rightleftharpoons 2\ SO_2^-$, is very fast and the concentration of SO_2^- is proportional to $(S_2O_4^{2-})^{1/2}$, the fact that the observed rate constant depends on $(S_2O_4)^{1/2}$ and extrapolates strictly to zero with confidence from regression analysis of these experimental data indicates that the reduction of cytochrome c_3 occurs mainly by the radical SO_2^-. Moreover, such a fact indicates that the reduction of cytochrome c_3 by $S_2O_4^{2-}$ is negligible. The experimental pseudo-first-order-rate constants k_a are related to the true second-order constants k_+ by:

$$k_a = k_+ \left(S_2O_4^{2-} \cdot K\right)^{1/2} \qquad (1)$$

where K is the equilibrium constant for the dissociation of $S_2O_4^{2-}$ to SO_2^-.

The second-order rate constant k_+, for the reduction of cytochromes c_3 from *D.desulfuricans* Norway and *D.vulgaris* were calculated from Eqn. 1 and are given in Table I using a value of $0.43 \cdot 10^{-9}$ M for the equilibrium constant, K, of the $S_2O_4^{2-}$ monomerisation at $\mu = 0.02$ M, pH = 7.0 and 5°C, taking into consideration its variations with the ionic strength [24] and an enthalpy change of 89 kJ/mol [25].

At a given concentration of dithionite the observed pseudo-first-order rate constants were checked to be independent of cytochrome c_3 concentration between 0.17 and 4.7 μM (not shown here). Thus the rate constants were clearly first-order with respect to cytochrome c_3 and half-order with respect to $Na_2S_2O_4$ concentrations.

Reactions of ferredoxins with dithionite

For comparative purposes we have also conducted similar experiments with the two ferredoxins which interact with *D.desulfuricans* Norway cytochrome c_3. These results are given in Fig. 3a and b. The reduction of ferredoxin II (containing two clusters per subunit) with dithionite, observed at 410 nm, followed biphasic kinetics. The fast initial phase extended over 77 (∓6)% ($n = 10$) of the reaction whatever the dithionite concentration.

Monophasic time-courses were observed for the reduction of ferrodoxin I (one cluster per subunit) by dithionite.

All first-order rate constants were dependent on the square root of the dithionite concentration. This is interpreted as the reaction with sulfite radical, SO_2^-, as the reductant. Second-order rate constants for these phases were calculated from Eqn. 1 by linear regression analysis and are listed in Table I.

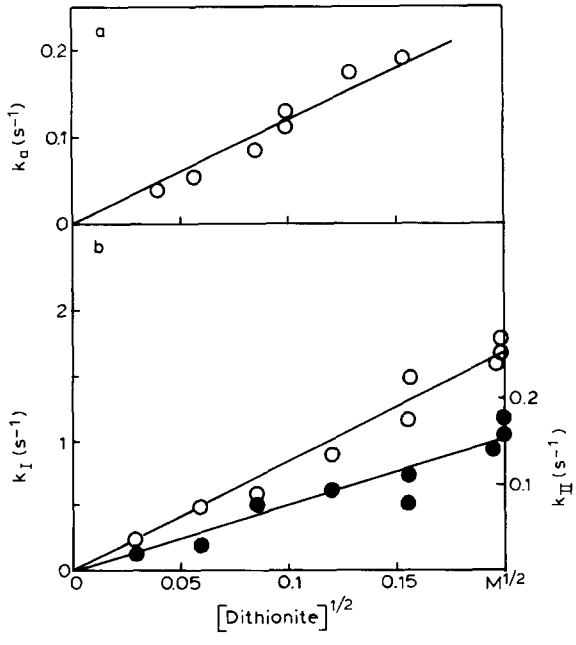

Fig. 3. Dependence of the pseudo-first-order rate constant k_a or k_I or k_{II}, on the square root of the dithionite concentration for the reactions with *D.desulfuricans* ferredoxin I and ferredoxin II. (a) *D.desulfuricans* ferredoxin I concentration, 3.5 μM; dithionite concentration varied from 1.6 to 24 mM. The fit leads to $k_+ = 0.6 \cdot 10^5\ M^{-1} \cdot s^{-1}$. (b) *D.desulfuricans* ferredoxin II concentration, 3.6 μM; dithionite concentration varied from 0.9 to 24 mM. The fit leads to $k_{I+} = 4 \cdot 10^5\ M^{-1} \cdot s^{-1}$, $k_{II+} = 0.38 \cdot 10^5\ M^{-1} \cdot s^{-1}$.

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE REACTION WITH SULFITE ANION RADICAL, SO_4^-

k-Values were determined from the experimental data presented in Figs. 2 and 3, using an equilibrium constant of monomerisation of dithionite equal to $0.43 \cdot 10^{-9}$ M at pH 7 and 5°C. For purpose of comparison the values of the oxidation-reduction potentials of the various redox centres are given, together with the values of the isoelectric points of the proteins.

	Oxidation-reduction potential values at pH = 8, 25°C (mV)	pI	Reduction rate-constant k with SO_2^- at 5°C ($\times 10^5$ $M^{-1} \cdot s^{-1}$)	
			Phase I	Phase II
D.desulfuricans Cytochrome c_3	−400; −365; −305; −165	7.0	100	6.5
D.vulgaris Cytochrome c_3	−375; −345; −335; −290	9	32	6.3
D.desulfuricans Ferredoxin[II]	−500; −450	3.7	4	0.4
D.desulfuricans Ferredoxin[I]	−375	3.9	0.6	

Reaction of reduced cytochrome c_3 with oxygen

Reduced cytochrome c_3 is known to be auto-oxidizable. In order to quantify this reaction we studied the kinetics of oxidation of reduced cytochrome c_3 with oxygen. Upon mixing reduced cytochrome c_3 maintained in presence of excess dithionite with a buffer solution equilibrated at atmospheric pressure with air, a slow reaction takes place (Fig. 4). The time-course recorded at 552 nm indicated that the oxidation of the hemes occurred through a first-order process. A partial oxidation (75%) is observed due to the competition with the rereduction of the hemes by the dithionite concentration present in excess at a rate constant of 0.9 s^{-1}. The apparent rate of oxidation of cytochrome c_3 by air was calculated to be equal to 2.5 (∓ 0.4) s^{-1} at 5°C, thus corresponded to a second order rate constant of 3.1 (∓ 0.5) $\cdot 10^4$ $M^{-1} \cdot s^{-1}$.

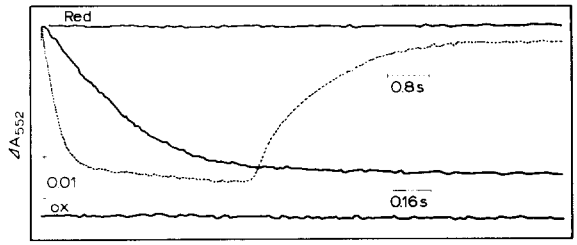

Fig. 4. Reaction of *D.desulfuricans* ferrocytochrome c_3 with oxygen. Reaction trace observed at 552 nm on mixing in the stopped-flow apparatus 0.26 μM ferrocytochrome c_3 with buffer equilibrated with air under atmospheric pressure at 5°C, i.e., 0.08 mM oxygen (concentration after mixing). Cytochrome c_3 was maintained under reduced state in its syringe by adding small amount of dithionite (0.15 mM, concentration after mixing). The oxidation of cytochrome c_3 was monitored on a short time scale (0.16 s per unit), while the reduction of oxidized cytochrome c_3 was detected on a longer time scale (0.8 s per unit). Vertical scale: 0.01 DO per unit.

Reaction of reduced cytochrome c_3 with oxidized ferredoxin

When dithionite-reduced cytochrome c_3 was mixed anaerobically in the stopped-flow apparatus with variable concentrations of oxidized ferredoxin, monophasic reaction traces were observed at 552 nm (Fig. 5a). Increasing the ferredoxin over cytochrome c_3 molar protein ratio up to 20 did not affect the amplitude of the reaction. The absorbance changes are much small (21–25%) than expected for total oxidation of cytochrome c_3 and reflect the oxidation of one heme per molecule. The kinetic titration close to stoichiometry for 1 FeS/1 heme shown in Fig. 5a, inset, indicates that the reaction between oxidized ferredoxin and reduced cytochrome c_3 proceeds to completion as far as one heme per molecule is concerned. This result is not unlikely, since one heme has a lower oxidation-reduction potential (−400 mV) than ferredoxin [2,8] which favours complete oxidation of this heme by ferredoxin.

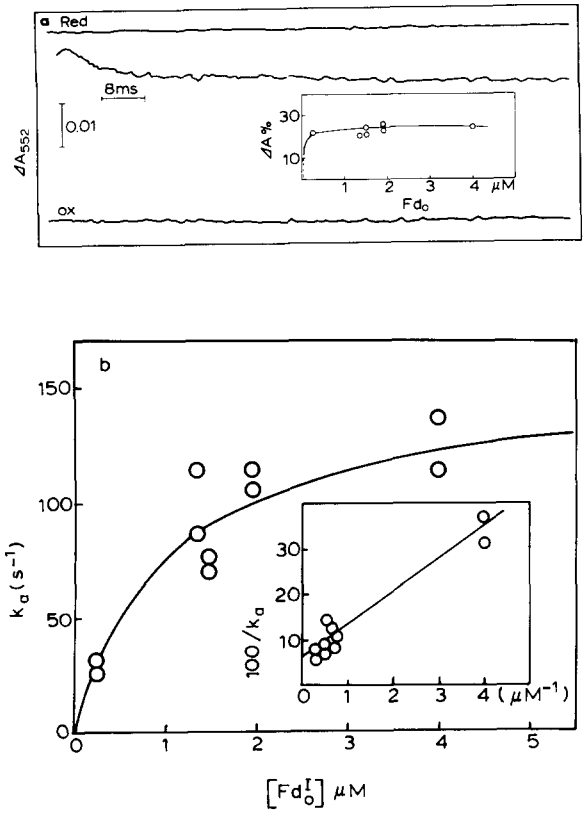

Fig. 5. Reaction of ferrocytochrome c_3 with oxidized ferredoxin I both from *D.desulfuricans*. (a) The figure shows the time-course of the oxidation of one heme centre per molecule of cytochrome c_3 obtained on mixing 0.26 μM ferrocytochrome c_3 with 1.9 μM ferredoxin I (final concentrations). The vertical scale corresponds to a ΔA of 0.01 per unit and the horizontal one to a time sweep of 8 ms per unit. On a longer time scale (up to 10 s, not shown here) the rereduction of the partly oxidized cytochrome c_3 by dithionite (0.11 mM) can be detected. Inset: Amplitude of the oxidation (expressed in percentage of total oxidation for four hemes) as a function of ferredoxin concentration. The figure shows the amplitude of the process $\Delta A = (A_{100\%\,red} - A_t)$ observed in the stopped-flow apparatus by mixing reduced cytochrome c_3 with various amounts of oxidized ferredoxin. (B) Second-order plot for the oxidation of ferrocytochrome c_3 by ferredoxin I. The solid line was obtained by fitting the data to a hyperbolic saturation curve calculated from the parameters of the reciprocal plots presented below. Inset: Double reciprocal plot of the same data points. A linear dependence of $1/k_a$ on 1/[Fd] is observed. The extrapolation of the plot to the Y axis gives the maximum oxidation rate limit (k_{max}) of 160 s^{-1}. The dissociation equilibrium constant K_d is found from the ratio of slope to intercept, here $K_d = 1.1$ μM.

The absorbance changes were accurately analysed as a single exponential process. The form of the dependence of the rate of oxidation on ferredoxin concentration shows an hyperbolic character with the rate constant reaching a limit of 160 s^{-1} at 5°C as determined from double-reciprocal plots (Fig. 5b). The present data (amplitude of the reaction and rate constant variations) are consistent with the kinetic mechanism for the electron transfer reaction which includes a complex formation step before the unimolecular electron transfer from cytochrome c_3 to ferredoxin as described below:

$$C_3(4Fe^{2+}) + Fd_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_3(4Fe^{2+}) - Fd_0$$

fast

$$\xrightarrow{k_2} C_3(3Fe^{2+}, Fe^{3+}) + Fd_r$$

slow

Scheme I

Furthermore, the data indicate that the rate of complex formation, thus of equilibrium, is considerably faster than the rate of electron transfer ($k_{-1} > k_2$), since little if any lag phase in absorbance changes was observed after mixing the reactants. Analysis of the data on the basis of Scheme I, according to Strickland et al. [26] yielded, as illustrated in Table II:

$$k_2 = 160\ s^{-1} \text{ and } K_d^1 = \frac{k_{-1}}{k_1} = 1.1 \cdot 10^{-6}\ M$$

It has to be noticed that in general the experiments conducted in the direction of the specific oxidation of cytochrome c_3 by ferredoxin are technically difficult because of the problem of maintaining strict anaerobic conditions which can affect the analysis of the oxidation of further hemes by ferredoxin.

On a longer time range the re-reduction of the heme was observed up to 6 s, due to the presence of excess of dithionite (not shown). This process corresponded to an apparent first-order rate constant of 0.9 (∓ 0.2) s^{-1}.

TABLE II

KINETIC PARAMETERS OF THE ELECTRON-EXCHANGE REACTION BETWEEN CYTOCHROME c_3 AND FERREDOXIN I

The values of the microscopic rate constants defined in Scheme I and III were determined from the data presented in Figs. 5b and 7. The initial slopes of the second-order plots yield second-order rate constants k_+ for oxidation (Fig. 5b) and reduction (Fig. 7) of *D.desulfuricans* cytochrome c_3 with rate equation $k_+ = k_{max}/K_d$. The limiting first-order rate constant, k_{max}, obtained at high concentrations allows to determine the intramolecular rate constant for oxidation, k_2, (Fig. 5b inset). The dissociation equilibrium constant is determined in the case of oxidation of cytochrome c_3 (K_d^1) from the graph presented in (Fig. 5b, inset), using the ratio of slope to intercept. In the case of the reduction of cytochrome c_3 (K_d^4) is estimated assuming a lower limit of 1000 s^{-1} for the intramolecular electron exchange, k_5. The rate constant corresponding to the reverse reaction, k_-, is determined from the *Y*-intercept.

	Oxidation by ferredoxin I				Reduction by ferredoxin I			
	$k_+ = k_{max}/K_d^1$ ($M^{-1} \cdot s^{-1}$)	$k_{max} = k_2$ (s^{-1})	K_d^1 (M)	$k_- = k_2$ (s^{-1})	$k_+ = k_{max}/K_d^4$ ($M^{-1} \cdot s^{-1}$)	$k_{max} = k_5$ (s^{-1})	K_d^4 (M)	$k_- = k_{-5}$ (s^{-1})
D.desulfuricans cytochrome c_3	$1.5 \cdot 10^8$	160	$1.1 \cdot 10^{-6}$	≈ 0	$6.6 \cdot 10^7$	$\geqslant 1000$	$1.5 \cdot 10^{-5}$	150
D.vulgeris cytochrome c_3	not determined				$1.4 \cdot 10^7$	$\geqslant 1000$	$7 \cdot 10^{-5}$	140

Reaction of reduced ferredoxin with oxidized cytochrome c_3

The mixing of dithionite-reduced ferredoxin with oxidized cytochrome c_3, under anaerobic conditions, produces biphasic absorbance changes observed at 552 nm, at low reductant concentration ($< 8 \times$ cytochrome c_3 molar concentration). Fig. 6a shows the time-course of reduction of 0.25 μM cytochrome c_3 by 0.24 μM ferredoxin as followed at 552 nm. The rapid initial phase has a half-time of 4 ms and is virtually complete within 25 ms. The slower phase, on the other hand, has a half-time of at least 450 ms and requires 6 s to reach completion. The total absorbance change observed in the course of the reaction corresponds to a molar absorbance coefficient of 93 (∓ 4) $mM^{-1} \cdot cm^{-1}$ ($n = 5$). This value is in agreement with the coefficient determined by static differential spectrophotometry for the total reduction of cytochrome c_3 from *D.desulfuricans*.

Analysis of the data indicates that the initial rapid phase represents the reduction of cytochrome c_3 by ferredoxin and the slower phase the reduction by excess of dithionite. As the concentration of reduced ferredoxin is increased, the initial rate as well as the magnitude of the initial absorbance change increases (Fig. 6b). For molar ratio of ferredoxin to cytochrome c_3 equal to 10, monophasic traces leading to a magnitude of reduction of cytochrome c_3 representing 97 (∓ 5)% of that obtained with dithionite alone are observed. Concerning the slower phase, when it exists, its rate is found to be independent of the ferredoxin concentration, $k_a = 1.6$ (∓ 0.4) s^{-1} at 5°C (for six independent measurements). This process is assigned to the slower reduction of the remaining oxidized cytochrome c_3 by the slight excess of dithionite (0.14 mM) used to maintain ferredoxin in the reduced state. Control experiments on the dithionite reduction kinetics of cytochrome c_3 presented in Fig. 6c have shown that the reaction is very slow (apparent first-order rate constant $k_a = 1.5$ (∓ 0.3) s^{-1} ($n = 7$), under the same experimental conditions) thus of negligible effect on the electron-transfer kinetics between reduced ferredoxin and cytochrome c_3 at the concentrations of dithionite likely to be present.

From variations of the amplitude of the initial fast reduction phase as a function of the ratio of reduced ferredoxin to oxidized cytochrome c_3 concentrations (expressed in FeS cluster per heme) (Fig. 7a), a kinetic titration is observed with the stoichiometry of 5 FeS/4 hemes in the case of *D.desulfuricans* cytochrome c_3, indicating that the equilibrium constant for the electron exchange is higher than 1, large in favour of the formation of reduced cytochrome c_3. The complete reduction occurs only for 10 FeS/4 hemes. The deviation from a 4:4 stoichiometry is perfectly reasonable considering that, whereas the redox potentials of

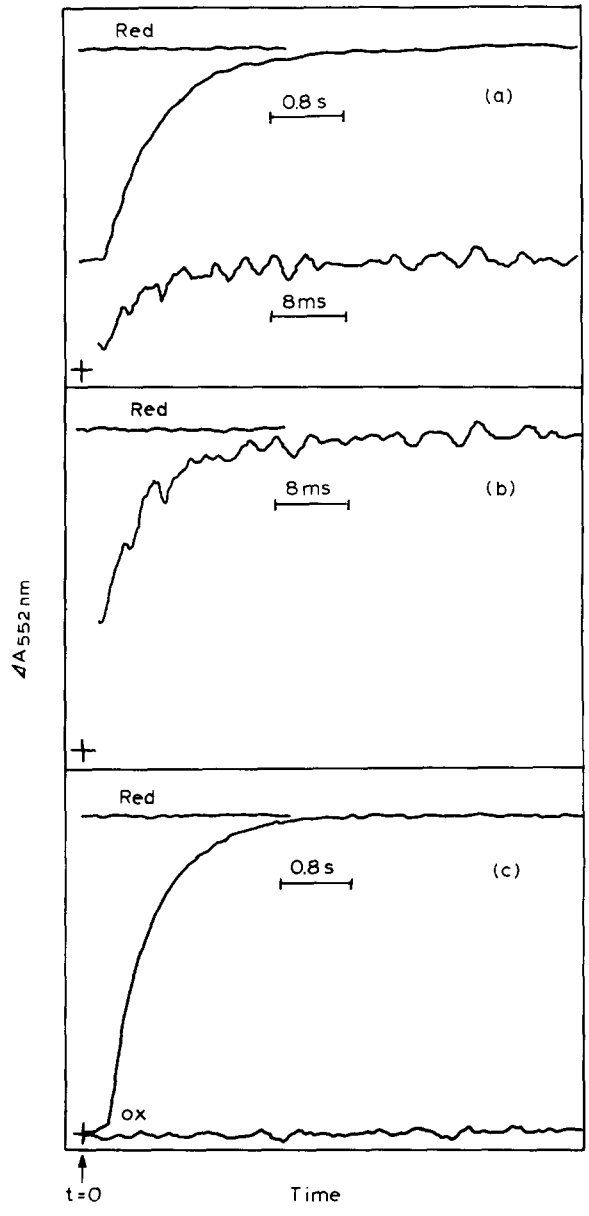

Fig. 6. Reaction of ferricytochrome c_3 with reduced ferredoxin I both from *D.desulfuricans*. Typical time-courses recorded at 552 nm for the reduction of cytochrome c_3 by reduced ferredoxin. For (a) and (b), the recordings were obtained after mixing 0.25 μM ferricytochrome c_3 with ferredoxin reduced by 0.14 mM dithionite and at respective concentrations of 0.24 and 2.6 μM. (c) This shows the result of a control experiment carried out on mixing ferricytochrome c_3 with 0.14 mM dithionite in absence of ferredoxin.

three of the hemes of cytochrome c_3 molecular are higher than that of the ferredoxin cluster, the heme of lowest potential (-400 mV) can reversibly exchange electron with ferredoxin.

Fig. 7b shows the ferredoxin concentration-dependence of the pseudo-first-order rate constant for the initial phase associated with the electron transfer between the two proteins. Such a plot can be satisfactory fitted to the relationship: $k_a = k_+[Fd_r] + k_-$; where $[Fd_r]$ is the concentration of the species in excess. Considering the two partners from *D.desulfuricans* (Fig. 7b) k_+ may be obtained from the slope of k_a versus $[Fd_r]$ plots

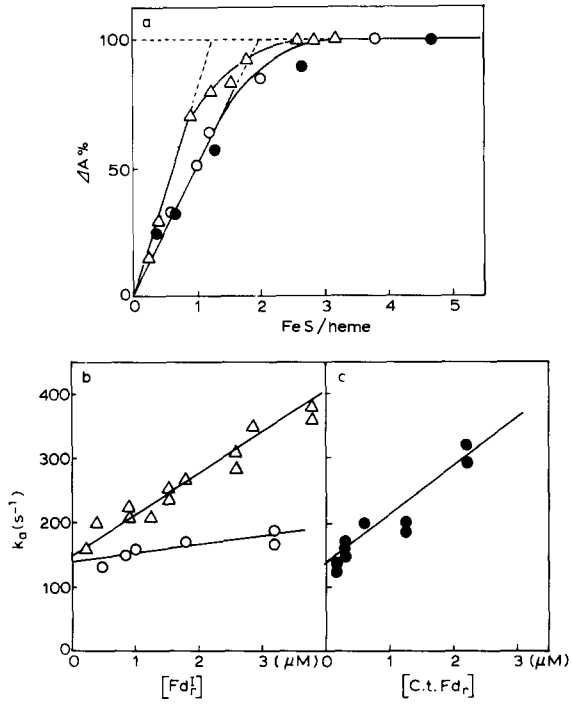

Fig. 7. Reaction of ferricytochrome c_3 with reduced ferredoxin. (a) The amplitude of the fast initial reduction is shown as a function of the ratio of ferredoxin-to-cytochrome c_3 concentrations (expressed in FeS cluster per heme). Various cross reactions were investigated and correspond to the data points Δ, *D.desulfuricans* cytochrome c_3 and ferredoxin I; ○, *D.vulgaris* cytochrome c_3 and *D.desulfuricans* ferredoxin I; ●, *D.desulfuricans* cytochrome c_3 and *C.thermocellum* ferredoxin. (b) The dependence of k_a, the pseudo-first-order rate constant of the fast initial reduction phase of cytochrome c_3 from *D.desulfuricans*, (Δ) and *D.*vulgaris (○) on ferredoxin I concentration are shown. (c) The dependence of k_a the pseudo-first-order rate constant of the fast initial reduction phase of *D.desulfuricans* cytochrome c_3 on *C.thermocellum* ferredoxin concentration are shown.

($k_+ = 7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, at 5°C) and the intercept at $[Fd_r] = 0$ gives a measure of k_- ($k_- = 150$ s^{-1} at 5°C).

Repeating experiments of the type described above were performed with ferredoxin and cytochrome c_3 from different sources. Data are presented in Fig. 7b and the corresponding kinetic parameters k_+ and k_- thus obtained are listed in Table II. From the various cross-reactions studied it can be concluded that the rate constants of the cytochrome c_3 with its specific partner is much higher than *D.desulfuricans* cytochrome c_3 with *C.thermocellum* ferredoxin and *D.vulgaris* cytochrome c_3 with *D.desulfuricans* Norway ferredoxin I.

Discussion

Reactions with non-physiological reactants

Reduction of cytochromes c_3 by dithionite

The present results show that the addition of dithionite to ferric *D.desulfuricans* cytochrome c_3 produces a rapid reaction as a first stage followed by a slower phase representing 14% of the reaction. This biphasic character may be due to heterogeneity, i.e., to the presence of either two reducing entities or two protein species, or changes in the rate-limiting step. The former could be consistent with the well-documented equilibrium between $S_2O_4^{2-}$ and SO_2^- [22]. However, the observed dependences of the reduction rate constants, k_a, upon the square root of the dithionite concentration account for the reaction with SO_2^- known to be the more reactive species ($E_{m,7} = -650$ mV [23]). The presence of a heterogeneous population of cytochrome c_3 molecules is not consistent with our observation of a monophasic progress curve when ferricytochrome c_3 was reduced with ferredoxin. The relative contribution of the two phases and the rate constants are independent of cytochrome c_3 concentration. This observation implies that the slow phase cannot be ascribed to a slow intermolecular electron transfer between cytochrome c_3 molecules.

Since cytochrome c_3 has been shown to be a four redox-centre molecule with four hemes with four different oxidation-reduction potentials, it is possible that these biphasic time-courses represent combinations of concurrent or consecutive bimolecular and unimolecular processes, involving either conformation changes and/or redistribution of electrons between the four redox centres of each cytochrome c_3 molecule.

Among the possible reaction schemes, there are two obvious kinetic models which give rise to this behavior. First, it can be proposed that the reactivity of one particular heme with rate-constant equal to k_{II} ($6.5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$) is smaller than that of the other three hemes which react with an individual rate-constant equal to one third of k_I ($33 \cdot 10^5$ $M^{-1} \cdot s^{-1}$). The reactivity of each heme could depend on its redox potential and its local environment. However, the biphasicity is still observed with *D.vulgaris* cytochrome c_3 where the four redox potentials are close to each other ranging over 100 mV (Table I) and the position and relative orientation of the hemes are very similar to that in *D.desulfuricans* cytochrome c_3 [27]. This difference in heme reactivities could be explained by heme–heme interactions as already noticed from circular dichroism observations [15]. Moreover, pulse-radiolysis results on *D.vulgaris* cytochrome c_3 [16] have suggested that the redox properties of the individual heme are dependent on the degree of reduction of the other hemes of the molecule. Thus the reactivity of one heme can be decreased by the presence of other reduced hemes in the molecule or be modulated by the structural interactions involved in the complex formation between ferredoxin and cytochrome c_3.

Second, the tentative scheme presented in Fig. 8 can also account for the biphasicity. It involves the following assumptions. (i) The bimolecular electron exchange between SO_2^- and the protein occurs at one specific reduction site. (ii) Three heme centres are in a rapid equilibrium with one another and the intramolecular distribution of electron density among them is determined solely by their relative oxidation-reduction potentials. Thus it leads first to the full reduction of the two hemes of highest redox potentials. (iii) The apparent change in rate-limiting step (final slow phase) is related to a relatively slow electron distribution between the reduction site i.e. heme (−400 mV) and the heme (−365 mV). This view is supported by the interpretation of X-rays data on *D.desulfuricans* cytochrome c_3 molecule in the crystal [12,13]. Up to

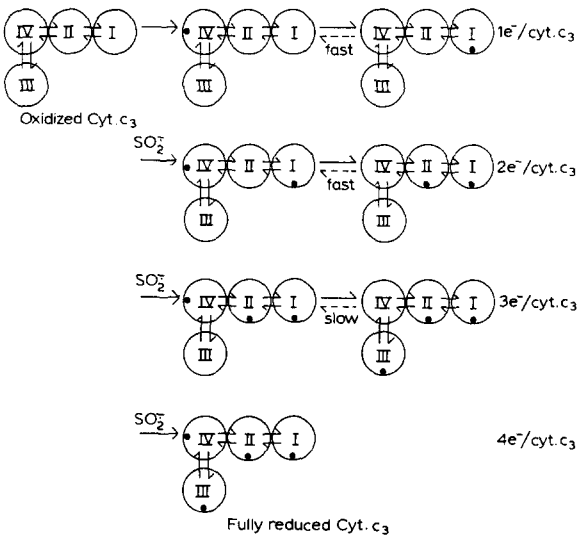

Fig. 8. Reaction scheme for the reduction of *D.desulfuricans* cytochrome c_3 by dithionite. The four hemes of cytochrome c_3 are schematized as four circles. Hemes IV (−400 mV), II (−305 mV) and I (−165 mV) are in rapid equilibrium, while hemes IV (−400 mV) and III (−365 mV) are in slow equilibrium. The reaction site is assumed to be located at the level of heme No. IV, the more accessible to the solvent [12,13], where four consecutive second-order one-electron reduction occur. The black dot corresponds to the presence of one electron on the heme.

now the correlation between the hydrophobicity of the heme environment and the redox potentials [28] would allowed to identify the heme (−400 mV) as the more accessible to the solvent [12,13]. Thus this heme can afford the attack by small molecules like the sulfite anion radical. Moreover, the distances and relative orientations between the hemes and the π-conducting bands of aromatic amino-acids would be in favour of rapid electron exchange at least between three hemes of the same molecule, and rule out the slow exchange (2 s^{-1} at 20°C) estimated in previous work [14]. Nevertheless, at equilibrium on the NMR time-scale [29] no intramolecular electron exchange was observed in *D.desulfuricans* cytochrome c_3 probably due to the large redox potential difference between the individual hemes (−250 mV). If it exists, this transfer would be very fast. It is tempting to put a lower limit on its rate-constant. The pseudo-first order rate-constants observed in the reaction with dithionite are on the order of 40 s^{-1} and in the case of the reaction with ferredoxin on the order of 400 s^{-1}. If these reactions proceed with the same mechanism, any true unimolecular process must be greater than 600–1000 s^{-1} (at 5°C).

At the present state of knowledge concerning the electron transfer processes involving cytochrome c_3 and its tertiary structure it is not possible to go very much further in choosing the 'best' kinetic model. Furthermore, at the light of the fact that the assignment of the redox potential value to each structurally defined heme is still speculative and not clearly established, there remain unresolved problems in particular on the independent reactivity of each heme with external reactants and on the role of intramolecular electron exchange between the hemes.

To ascertain if these observations with dithionite were unique we have investigated the reaction of dithionite with *D.vulgaris* cytochrome c_3. The main kinetic features are essentially the same as those observed for *D.desulfuricans* cytochrome c_3: a biphasic time-course with a slow phase representing 28% of the reaction and smaller second-order rate constants equal to $3 \cdot 10^6$ and $6 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ at 5°C. Previous studies on the reduction of *D.vulgaris* cytochrome c_3 found pluriphasic kinetics. For some authors a biphasicity 50:50 was noticed [14], whereas, for others, four successive equal phases were clear-cut [15]. Discrepancies were noted in the rate-constant values ranging $2 \cdot 10^7$–$2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ due to different experimental conditions of temperature (20°C) and pH. However, in agreement with our interpretations, Tabushi et al. [15] exclude the possibility of random reduction of the four hemes with equal reactivities.

Comparison between the reactivities of cytochromes c_3 and ferredoxins with dithionite

Table I summarizes the rate constants obtained for the reaction of cytochromes c_3 and ferredoxins with the sulfite radical anion. The individual rate-constants for the ferredoxins (with one or two clusters per monomer) are all substantially less (2–3 orders of magnitude) than the analogous reactions with cytochromes c_3. The difference observed can be assigned to specific parameters, the oxidation-reduction potential difference or electrostatic interactions. It is known that the rate con-

stant value of a bimolecular electron exchange involving a radical anion, SO_2^-, will be influenced by the nature of the net electrostatic charge of the other reactant. Indeed, as far as redox proteins with oxidation-reduction potential values higher than +200 mV are considered, the cytochrome *c* family (*c*, c_2, *c*-551) [30] and Cu proteins, plastocyanin [22], stellacyanin and azurin [31] proteins, have been shown to have positive-charged sites predominant in the distribution of charged residues in the molecule, and their redox centre is close to the surface and exposed to the solvent. Hence these proteins react rapidly with the anionic reactants. On the contrary the net electrostatic charge on ferredoxins, HiPIP [32] and oxidase [31] is less favourable to the SO_2^- attack. Moreover, the iron-sulfur cluster is appreciably buried. More precisely it can be proposed that the reactivity differences of hemes and iron–sulfur clusters with SO_2^- are related to the net electrostatic charge localised in the vicinity of the reaction site which is considered in the case of cytochrome *c* [30] to direct the reactivity with oppositely charged redox reactants at the heme edge. This suggestion agrees with the strong interaction observed between cytochrome c_3 and ferredoxin as evidenced by the rapid electron exchange noticed.

Reaction of ferrocytochrome c_3 with oxidized ferredoxin

The present results strongly indicate that rapid electron exchanges occur between cytochrome c_3 and ferredoxin. In the case of the reaction of oxidation of reduced cytochrome c_3 the hyperbolic character of the observed rate constants, when plotted as a function of oxidized ferredoxin concentration, suggest that these molecules interact specifically via a complex formation mechanism. The involvement of an active cytochrome c_3-ferredoxin complex is not surprising. Previous studies indicated that cytochrome c_3 and ferredoxin form a stable complex in their fully oxidized state, as determined by NMR [34] and microcalorimetric (unpublished results) studies, with a 1:1 stoichiometry and a dissociation constant of 10^{-5} M.

The simplest possible kinetic mechanism that can be used to described the electron transfer is given by Scheme II.

$$C_3(4Fe^{2+}) + Fd_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} C_3(3Fe^{2+}, Fe^{3+}) + Fd_r$$

Scheme II

in which X_1 and X_2 represent forms of a molecular complex between cytochrome c_3 and ferredoxin. At low cytochrome c_3 concentrations the first two steps become rate limiting and this scheme reduces to the previously presented Scheme I. Under rapid equilibrium assumption ($k_{-1} > k_2$) [26] the observed oxidation rate-constants, k_{ox}, is related to the individual constants by Eqn. 2

$$k_{ox} = \frac{k_1 k_2 [Fd_0]}{k_{-1} + k_1 [Fd_0]} \qquad (2)$$

The initial slope of the second-order plot yield the second-order rate constant for oxidation $k_+ = k_2/K_d^1$ (Fig. 5b). From double reciprocal plots it is possible to assign values of 160 s^{-1} to k_2, the rate-limiting transfer of electrons within the complex, and $1.1 \cdot 10^{-6}$ M to $K_d = k_{-1}/k_1$. These results suggest that the rate of the reaction between reduced cytochrome c_3 and ferredoxin is close to that expected for a diffusion-controlled reaction between molecules of small size and oppositely charged: $k_1 = 10^9$ $M^{-1} \cdot s^{-1}$ [21,35]. This would confer a value of approx. 10^3 s^{-1} for k_{-1} the dissociation rate constant of oxidized ferredoxin. So the criterion of rapid equilibrium, i.e., $k_{-1} > k_2$ is satisfied.

It is of interest to compare the kinetic results obtained here with thermodynamic data reported in Refs. 4 and 8. The kinetic titration observed predicts that the equilibrium constant, K_{eq}, for the oxidation of one heme is largely higher than 1. The rough estimation of K_{eq} (obtained by extrapolation of the double reciprocal plot of the amplitude at infinite concentration of reactant) [26] is found equal to 15 (∓ 5). This value can be correlated to the difference in oxidation-reduction potentials, ΔE, between oxidized ferredoxin and one heme component of cytochrome c_3 by the Nernst equation. We have calculated at pH 7 and 5°C $\Delta E = RT(\ln K_{eq})/nF = 65$ (∓ 10) mV. The present data are not out of line with the thermodynamic findings calculated from EPR and cyclic voltammetry measurements of ferredoxin and cytochrome c_3 [4,8,36], carried out at pH 8 and 25°C. When

considering the heme of lowest oxidation reduction potential one obtains $\Delta E = 45$ mV (including the largest limits of uncertainty). It implies an equilibrium constant $K_{eq} = 7$.

As expected from the equilibrium constants calculated on the basis of the known oxidation-reduction potentials for the three other hemes (K_{eq} values. 0.6; 0.05; 0.0001) the reaction of oxidation of ferrocytochrome c_3 by ferredoxin, a weak oxidant for it, lies mainly to the left (see Scheme II), in favour of the reduction of cytochrome c_3. In the present study we found no evidence for the oxidation of a second heme centre. However, this fact does not exclude the possibility of oxidation of heme (-365 mV) considering that only a narrow range of concentration of the excess protein was investigated due to limited quantities of ferredoxin I.

Reaction of ferricytochrome c_3 with reduced ferredoxin

Kinetic studies on the reverse reaction show that oxidized cytochrome c_3 react much more rapidly than the reduced form with ferredoxin. As the measurements were restricted to low concentration of reactants the data are not strictly comparable with Scheme II (reverse reaction) and we found no evidence for any change in the rate-limiting step. We have no reason to think that the mechanism impling binding reaction before electron exchange is not operative for the reverse transfer, even though no complexes for the interaction of oxidized cytochrome c_3 and reduced ferredoxin were kinetically detected due to their short lifetime. Despite two steps the reaction behaves as a second-order process.

Formely the reduction of cytochrome c_3 can be illustrated by Scheme III.

$$C_3(4Fe^{3+}) + Fd_r \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} X \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} C_3(4Fe^{2+}) + Fd_0$$

Scheme III

It may be shown [26] that the kinetic equation for a two-step reaction reduced to a single-exponential process with an apparent rate constant k_a proportional to $[Fd_r]$, according to the rate equation (Eqn. 3):

$$k_a = \frac{k_4[Fd_r](k_5 + k_{-5}) + k_{-4}k_{-5}}{k_4[Fd_r] + k_{-4} + k_5} \quad (3)$$

Given the assumptions that oxidized cytochrome c_3 and reduced ferredoxin are in rapid equilibrium with the complex X (i.e., $k_{-4} > k_5$), which is consistent with the absence of lag-phase detection, and that, at relatively low concentration of reduced ferredoxin, $k_4[Fd_r]$ is lower than k_{-4}, the kinetic behaviour would be as we found in the simple analysis described above with $k_+ = k_5/K_d$ and $k_- = k_{-5}$. As the maximum pseudo-order rate constants observed were of the order of 400 s^{-1} at 5°C, i.e., near the limit of stopped flow, any true first-order process must be greater than 600–1000 s^{-1} and would not be detectable by available mixing methods. So the criterion $k_4[Fd_r] < k_5$ is satisfied. Unfortunately, we cannot slow down the electron-exchange rate, since temperature cannot be decreased below 5°C and ionic-strength effects are not to be expected on an intramolecular electron exchange involving electron tunnelling. An estimation of the dissociation equilibrium constant can be obtained: $K_d^4 = k_5/k_+ = (0.8\text{–}1.4) \cdot 10^{-5}$ M.

When the reaction is monitored, the operative rate-limiting step is the electron exchange between reduced ferredoxin bound at the reaction site, postulated to be close to one of the four hemes. Thus Scheme III assumes a rapid electron distribution between the four heme centres. A lower limit to the intramolecular electron-exchange rate between the four hemes may correspond to 3–5 times the limit ascribed to k_5 that is $(3\text{–}5) \cdot 10^3$ s^{-1} at 5°C. In that regards NMR studies have revealed fast electron exchange for at least two of the hemes in *D.vulgaris* cytochrome c_3 at a rate higher than $5 \cdot 10^4$ s^{-1} at 25°C [37].

The consideration of the amplitude of the reaction can afford substantial information on the degree of irreversibility of step 5. The amplitude remains constant and equal to the total reduction of cytochrome c_3 down to $2.5 \cdot 10^{-6}$ M of reduced ferredoxin. Then the following relationship is verified $2.5 \cdot 10^{-6} \gg K_d/K_{eq}$, where K_{eq} is the equilibrium constant for the electron transfer from the reduced ferredoxin to cytochrome c_3 in the complex [38]. With $k_d^4 = 10^{-5}$ M and equating the inequality sign with a factor of 5 (from the minimum detected change in amplitude) then $K_{eq} \approx 20$.

This value may be converted into a redox potential difference between the two proteins by using the Nernst equation. A value of $\Delta E = 72$ mV is calculated assuming a one-electron process. The value of -302 mV is therefore the apparent mid-point potential of the heme c_3 which kinetically controls the electron exchange with ferredoxin via a rate-limiting step. Although our evidence for this is tenuous recent NMR studies localized the interaction site between cytochrome c_3 and ferredoxin near the heme (-305 mV) and the heme (-165 mV) [34]. Thus it appears still speculative to define the starting point for the electron entry in the cytochrome c_3 molecule. However, possibly the most attractive at the light of the available data, the heme (-305 mV) could be taken as a hypothesis to establish a detailed electron-transfer pathway.

The mechanistic significance and nature of the observed non-zero intercept may be due to the appropriate rate-limiting reverse reaction. The result of $k_{-5} = 150\ s^{-1}$ compares favourably with the value of 160 s^{-1} obtained in the study of the oxidation of cytochrome c_3 by ferredoxin. In the cross-reactions studied between cytochromes c_3 and ferredoxins from different species the kinetic parameter k_{-5} is comparable, while the apparent second-order rate constant (i.e., the product of the equilibrium constant for complex formation and the limiting first-order rate constant: $k_5 \cdot K^4$) is different. Since the X-rays structure of the two cytochrome c_3 is similar on the point of view of exposure of the hemes to the solvent and their orientations, the intramolecular electron exchange rate constant, k_5, would be considered as the same for the three systems. Consequently, K^4 is the characteristic parameter of the various systems, and a higher affinity is observed when deasling with redox proteins from the same species, here *D.desulfuricans*. The formulation of a molecular complex stabilized by specific interactions can account for the observed efficiency of electron transfer. Electrostatic attraction between the complementary charges present at the surface of cytochrome c_3 and ferredoxin could be one of the parameters involved in the interaction as already suggested from analysis of sequence data [39].

Cytochrome c_3 and ferredoxin I from *Desulfovibrio desulfuricans* are all soluble redox proteins. The two metabolic pathways, i.e., reducing sulfate and oxidating pyruvate, are coupled at their levels. The present kinetic results strongly indicate that rapid electron transfers occur between cytochrome c_3 and ferredoxin which interact specifically. However, it is evident that the process is not a simple single reaction. The complexity is increased by the presence of four redox centres each characterized by a specific oxidation-reduction potential. Depending on the relative redox states of ferredoxin and cytochrome c_3, the affinity of the proteins and the number of heme centres participating to the reaction are modulated. In the cell this may constitute a regulatory mechanism for the electron-transfer leading either to sulfate reduction or hydrogen cycling [40].

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