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REDOX POTENTIALS AND KINETIC PROPERTIES OF FUMARATE REDUCTASE COMPLEX FROM *VIBRIO SUCCINOGENES*

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The isolated menaquinol: fumarate oxidoreductase (fumarate reductase complex) from *Vibrio succinogenes* was investigated with respect to the redox potentials and the kinetic response of the prosthetic groups. The following results were obtained. (1) The redox state of the components was measured as a function of the redox potential established by the fumarate/succinate couple, after freezing of the samples (173 K). From these measurements, the midpoint potential of the [2Fe-2S] cluster (−59 mV), the [4Fe-4S] cluster (−24 mV) and the flavin/flavosemiquinone couple (about −20 mV) was obtained. (2) Potentiometric titration of the enzyme in the presence of electron-mediating chemicals gave, after freezing, apparent midpoint potentials that were 30–100 mV more negative than those found with the fumarate/succinate couple. (3) The rate constants of reduction of the components on the addition of succinate or 2,3-dimethyl-1,4-naphthoquinol were as great as or greater than the corresponding turnover numbers of the enzyme in quinone reduction by succinate or fumarate reduction by the quinol. In the oxidation of the reduced enzyme by fumarate, cytochrome *b* oxidation was about as fast as the corresponding turnover number of quinol oxidation by fumarate, while the [2Fe-2S] and half of the [4Fe-4S] cluster responded more than 2-times slower. The rate constant of the other half of the 4-Fe cluster was one order of magnitude smaller than the turnover number.

Introduction

Fumarate reductase complex (menaquinol: fumarate oxidoreductase, EC 1.3.99.-) from *Vibrio succinogenes* is an enzyme bound to the cytoplasmic membrane that enables the bacterium to use fumarate as terminal electron acceptor during the oxidation of formate or H_2, a process which is coupled to the synthesis of ATP [1–10]. The essential components for the formate : fumarate oxidoreductase reaction catalyzed by the membrane preparations of *V. succinogenes* are formate dehydrogenase (0.1 μmol/g protein), a cytochrome *b* with an oxidation-reduction potential of −200 mV (0.7–0.75 μmol/g protein), menaquinone (8.7 μmol/g protein), a cytochrome *b* with a potential of −20 mV (0.7–0.75 μmol/g protein) and Fe-S clusters and flavin of fumarate reductase (0.83–0.96 μmol/g protein) [1,2].

From these membranes, a menaquinol : fumarate oxidoreductase can be purified that consists of three polypeptides [4] with discernible molecular weights (M_r 79000, 31000 and 25000), which are present in a molar ratio of 1 : 1 : 2. This enzyme contains five prosthetic groups in equal amounts [4,8]: FAD, a [4Fe-4S] cluster, a [2Fe-2S] cluster, a cytochrome *b* with a E_m value of −20 mV, hereafter referred to as the high-potential cytochrome *b*, and a cytochrome *b* with a E_m value of −200 mV, further referred to as the low-poten-

tial cytochrome *b*. The FAD is covalently linked, via the 8α-position of the flavin ring system, to the N^3 atom of a histidine residue [9] in the M_r 79 000 subunit, in which also the [4Fe-4S] cluster is located [8]. The M_r 31 000 subunit [10] contains the [2Fe-2S] cluster [8], while each of the cytochromes *b* represents one of the M_r 25 000 peptides. Sulphide analysis of pure preparations exclude the possibility of additional Fe-S clusters [8]. The low-potential cytochrome *b* was found to be not essential for the oxidation of menaquinol by fumarate, whereas all other components were.

In this communication, the results of redox titrations with the enzyme complex are reported. Furthermore, the kinetics of reduction (by succinate or a menaquinol analogue) and oxidation (by fumarate) of the components were investigated using the freeze-quench technique.

Methods

Menaquinol : fumarate oxidoreductase was purified as described previously [4].

Redox titration. Redox titrations with electron-mediating chemical were carried out in a suitable 10 ml plexiglass vessel under oxygen-free nitrogen. The enzyme (about 20 μM) was dissolved in 20 mM potassium-2-(*N*-morpholino)propanesulfonate/0.1% Triton X-100/1 mM sodium malonate (final pH 7.5). Two different mediator mixtures were used.

Mixture I comprised 2,3-dimethyl-1,4-naphthoquinone (0.1 mM/menadione (0.4 mM)/2-hydroxy-1,4-naphthoquinone (12 μM)/duroquinone (0.2 mM)/ anthraquinonesulfonate (0.2 mM)/diaminodurol (0.1 mM)/phenazine methosulphate (50 μM)/phenazine ethosulphate (50 μM)/$FeCl_3$ (1 mM)/phenosafranine (12.5 μM)/ safranine T (12.5 μM)/Neutral red (12.5 μM)/ benzyl viologen (80 μM)/methyl viologen (80 μM).

Mixture II comprised 2,3-dimethyl-1,4 naphthoquinone (40 μM)/indigosulfonate (40 μM)/2-hydroxy-1,4-naphthoquinone (40 μM)/safranine T (40 μM)/methyl viologen (60 μM)/benzyl viologen (60 μM)/triquat (60 μM). The potential was adjusted by aliquots of anaerobic solutions of 100 mM $Na_2S_2O_4$ or 50 mM $K_3Fe(CN)_6$ and measured with a Pt-electrode, using a Calomel-electrode as reference. The potential was calibrated against a quinhydrone standard. Samples were anaerobically transferred into EPR tubes, by applying an overpressure to the titration vessel, and then rapidly frozen by immersing the tube in cold (173 K) petroleum ether.

The titration with the fumarate/succinate couple was carried out as follows. A set of EPR tubes was anaerobically (N_2) filled with 250 μl of the anaerobic enzyme in 85 mM Tris-HCl/0.1% Triton X-100/0.2 mM dithiothreitol/0.7 mM sodium malonate (pH 8.1). Then 25 μl of an anaerobic mixture of 1 M sodium succinate/1 M sodium fumarate (pH 8.1, the ratio of which varied) was added and the contents of the EPR tubes was mixed (under N_2) for 15 s and then frozen by immersion of the tube in cold (173 K) petroleum ether. All samples were stored in liquid nitrogen. Low-temperature diffuse reflectance spectra (at 100 K) were obtained according to De Vries et al. [11] and EPR spectra according to Albracht et al. [8].

Kinetic experiments. Freeze-quench experiments were performed at 20°C as described previously [12]. Three types of reaction were carried out: (i) reduction of the enzyme with succinate, (ii) reduction of the enzyme with 2,3-dimethyl-1,4-naphthoquinol and (iii) oxidation of the reduced enzyme with fumarate. In all the experiments, the anaerobic solutions (flushed with N_2) used comprised 0.1 M Tris-HCl (pH 7.6)/glucose (80 mM)/glucose oxidase (0.4 g/ml)/catalase (40 mg/l) to remove oxygen (standard solution).

(i) Syringe A contained the enzyme (about 20 μM) in the standard solution. Syringe B contained 0.1 M sodium succinate in the same solution. The fully oxidized reference sample was obtained with syringe B containing no succinate. As the fully reduced reference, the corresponding reference sample obtained in the experiment under (iii) was used.

(ii) Syringe A contained the enzyme as described under (i). Syringe B contained 0.2 mM 2,3-dimethyl-1,4-naphthoquinol in the standard solution, membrane particles from *V. succinogenes* (0.2 g/l) and 10 mM formate. 2,3-dimethyl-1,4-naphthoquinol was formed on the reduction of 2,3-dimethyl-1,4-naphthoquinone by formate which is catalyzed by the particles. As fully oxidized and reduced samples, the references under (i) and (iii) were used respectively.

(iii) Syringe A contained the enzyme in the standard solution supplemented with 10 mM formate, 1 μM 2,3-dimethyl-1,4-naphthoquinone, 1 μM benzyl viologen and membrane particles from *V. succinogenes* (0.2 g/l). Under these conditions, the enzyme was maintained in the fully reduced state. Syringe B contained 0.1 M sodium fumarate in the standard solution. The syringes, the mixing chamber and all tubings were surrounded by a nitrogen atmosphere. The tubings were flushed with nitrogen before use. The reduced control sample was obtained without fumarate in syringe B. The complete absence of any $[4Fe\text{-}4S]^{3+}$ signal indicated total reduction of the enzyme. As the fully oxidized sample, the reference under (i) was used.

Results

Redox titration

When the titration was carried out with the mediator mixture I (see Methods), the results depicted in Fig. 1 were obtained for the Fe-S clusters. Both behave like one electron carriers with apparent midpoint potentials of −130 mV (4-Fe cluster) and −125 mV (2-Fe cluster). Earlier studies have led to the proposal that the Fe-S clusters transfer electrons between the high-potential cytochrome *b* and the fumarate/succinate couple (E_0' = +24 mV) [4,8,10]. It is, therefore, surprising to find the midpoint potentials of the Fe-S clusters more than 100 mV more negative than that of the cytochrome *b*. Redox titration with mediator mixture II (see Methods) in which the concentration of some dyes was considerably lower than in mixture I, gave values of −80 and −95 mV for the 4-Fe and the 2-Fe cluster, respectively.

In a titration of the enzyme with the fumarate/succinate couple under anaerobic conditions in the absence of mediators (Fig. 2), virtually complete reduction of the 4-Fe and the 2-Fe cluster was achieved with apparent midpoint potentials at −24 and −59 mV, respectively. The fumarate/succinate couple appears to be effective down to −140 mV, which is 100 mV lower than its midpoint potential under the conditions used (−41 mV at pH 8.1). It should be mentioned here, that under aerobic conditions, only partial reduction was obtained with excess of succinate [8].

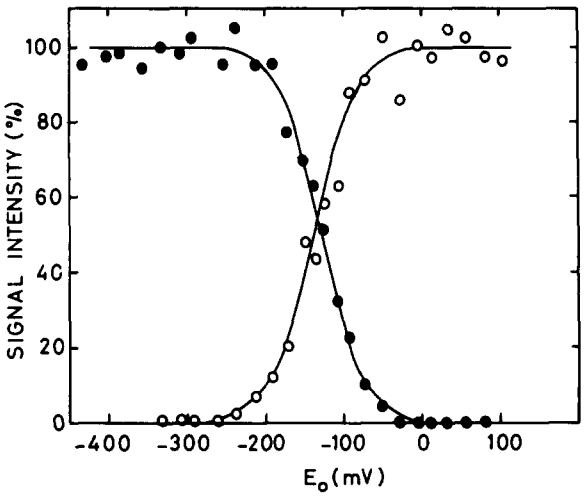


Fig. 1. The behaviour of the Fe-S clusters in menaquinol: fumarate oxidoreductase during potentiometric titration. The titration was carried out with mediator mixture I (see Methods), starting at −430 mV. The degree of reduction of the Fe-S clusters was determined from the amplitudes of the EPR signals. The signal for the $[4Fe\text{-}4S]^{3+}$ cluster (oxidized form) was measured at 8 K with a microwave power of 0.26 mW (○———○); the signal of the $[2Fe\text{-}2S]^{1+}$ cluster (reduced form) was measured at 50 K with a microwave power of 2.2 mW (●———●). The lines represent theoretical $n = 1$ Nernst curves with E_m values of −130 mV (○———○) and −125 mV (●———●). The normalized signal intensity of the 4-Fe cluster in the completely oxidized enzyme equals that of the 2-Fe cluster in the fully reduced enzyme and was taken as the 100% value.

The degree of reduction of the cytochromes *b* in the enzyme was determined from the absorbance changes at 560 nm in the low-temperature diffuse-reflectance spectra of the samples in the EPR tubes [11]. With the fumarate/succinate couple only one cytochrome *b* with a midpoint potential of −23 mV ($n = 1$) could be reduced (not shown). In the presence of the mediators, the optical measurements were perturbed by colour changes of the dyes below a potential of −300 mV and only approximate values could be obtained for the two *b* cytochromes (−250 mV and −55 to −70 mV). The data obtained from the titration experiments are summarized in Table I.

Also, the response of the flavin radical was quite different with both titration methods (not shown). In the presence of mediators, the radical signal appeared with a half-maximal amplitude at

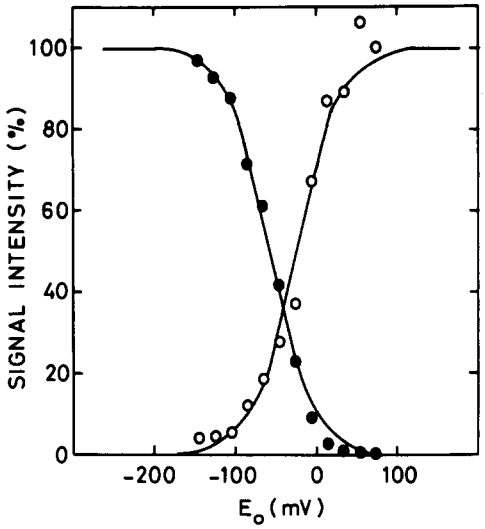


Fig. 2. Behaviour of the Fe-S clusters in a titration of the enzyme with the fumarate/succinate couple. The potential in each sample (see Methods) was calculated from the fumarate/succinate ratio and the pH used (8.1). The E'_0 (pH 7.0) for this couple was taken as 24 mV [32]. Other conditions were as in Fig. 1. The lines are theoretical $n = 1$ Nernst curves with E_m values of −24 mV (○———○, relative intensity of the signal of the $[4Fe\text{-}4S]^{3+}$ cluster) and −59 mV, (●———●, relative intensity of the signal of the $[2Fe\text{-}2S]^{1+}$ cluster). The 100% values for the signals of the $[4Fe\text{-}4S]^{3+}$ and the $[2Fe\text{-}2S]^{1+}$ clusters were obtained from samples which were oxidized with excess of $K_3Fe(CN)_6$ or reduced with excess of $Na_2S_2O_4$.

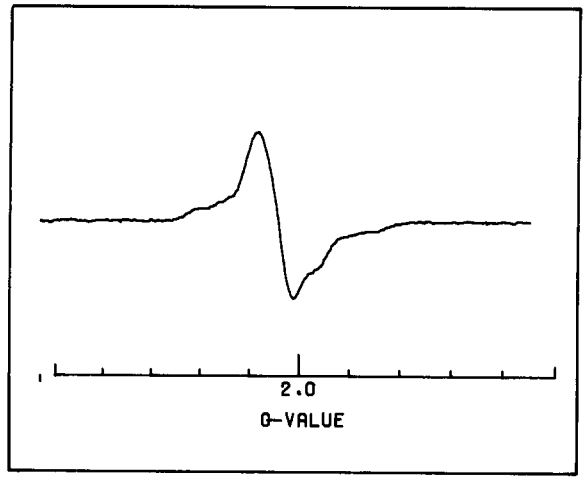


Fig. 3. EPR signal of the flavin radical of fumarate reductase complex reduced with excess of succinate (anaerobic conditions). The peak-to-peak width of the signal is 1.2 mT. EPR conditions: microwave frequency, 9121.0 MHz; temperature, 192 K; microwave power, 0.26 mW (nonsaturating); modulation amplitude, 0.5 mT. The g-value scale is in units of 0.01.

−120 mV and reached a plateau representing 19% of the enzyme concentration at −190 mV. Instead of the bell-shaped titration curve expected for the equilibria $FAD \rightleftharpoons FADH^{\cdot} \rightleftharpoons FADH_2$, the signal persisted and even increased at low potentials. The signal of the flavin radical could be distinguished from those of the mediators under all conditions, because it was much broader and showed characteristic shoulders at both sides of the main line. the signal (Fig. 3) has a peak-to-peak width of 1.2 mT which is characteristic for anionic 8α-substituted flavin radicals also observed in many other flavoproteins [13]. With the fumarate/succinate couple, the radical appeared with a half-maximal amplitude at −20 mV. The maximal amplitude

TABLE I

OXIDATION-REDUCTION POTENTIALS OF THE PROSTHETIC GROUPS OF FUMARATE REDUCTASE COMPLEX UNDER SEVERAL CONDITIONS

(A) Potentiometric titration in the presence of electron-mediating dyes (mixture I, see Methods). Experimental conditions as in Fig. 1. (B) The potential was adjusted by the fumarate/succinate couple. Experimental conditions as in Fig. 2. (C) The data were taken from Ref. 2 and had been measured by potentiometric titration at room temperature in the presence of mediators. h.p., high-potential; l.p., low-potential.

Condition	Observation temperature (K)	Half-maximal intensity of flavin radical	Midpoint potential (mV) of			
			[4Fe-4S]	[2Fe-2S]	Cytochrome *b*	
					h.p.	l.p.
A pH 7.5	≤ 200	−120 mV	−130	−125	−55	−250
B pH 8.1	≤ 200	−20 mV	−24	−59	−23	-
C pH 7.5	295	-	-	-	−20	−200

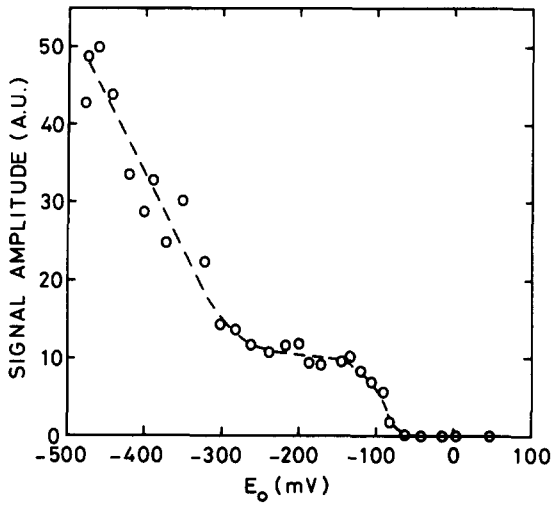


Fig. 4. Behaviour of the power-saturated EPR signal of the $[2Fe\text{-}2S]^{1+}$ cluster during a potentiometric titration. The mediator mixture II (see Methods) was used. The signal was measured under saturating conditions with a microwave power of 257 mW at 11 K. The amplitude of the g_x line is plotted in arbitrary units. Under nonsaturating conditions, the amplitude of the signal was constant between −500 and −150 mV, and a E_m value of −95 mV was found.

represented 41% of the enzyme concentration. Although the fumarate/succinate couple was really functional between −50 and −140 mV (Fig. 2), the radical signal was constant in this potential span, an observation that we do not understand.

Besides their effect on the apparent midpoint potentials, the mediators also caused an appreciable shift (1.5 mT) of the g_x line of the signal of the 2-Fe cluster to higher field. In addition, the width of the g_x line nearly doubled in the presence of the mediators on raising the potential from −200 to −100 mV, while it was constant between −200 and −400 mV. No changes in width or position of this g_x line were observed during the titration with the fumarate/succinate couple. With both methods, no differences or changes in width or position of the g_z and g_y lines of the signal of the 2-Fe cluster were found.

Previously, we have noticed that the spin-lattice relaxation rate of the 2-Fe cluster reduced by succinate increased when $Na_2S_2O_4$ was added [8]. We have now examined further this effect by measuring its EPR signal during the redox titration under greatly saturating conditions (Fig. 4). An apparent signal increase started below −250 mV and continued at lower potentials. When measured under nonsaturating conditions, there was no difference in signal amplitude between −450 and −150 mV, measured in the temperature region 7–60 K. Fig. 4 thus demonstrates that the spin relaxation of the [2Fe-2S] cluster at 11 K is continuously enhanced below −250 mV, rendering the signal to become less saturated and thus more intense.

Since it has become uncertain whether the $g = 2.02$ signal in the oxidized enzyme is due to a $[4\text{-}Fe\text{-}4S]^{3+}$ or a [3Fe-xS] cluster [14–20], attention was also paid to the $g = 4.3$ signal in the system. As one of the iron atoms of a 4-Fe cluster can sometimes easily leave this cluster under oxidizing conditions, whereby a $[3Fe\text{-}4S]^{1+}$ cluster might be formed [20,21], the lone iron ion might equilibrate with the mediators to give a $g = 4.3$ signal. In the fumarate reductase complex as isolated, where the $g = 2.02$ signal is maximal, only a very small $g = 4.3$ signal can be observed which does not respond to the addition of $K_3Fe(CN)_6$, succinate or $Na_2S_2O_4$. In the presence of the mediator mixture I, which contained 1 mM $FeCl_3$, a $g = 4.3$ signal was observed which titrated as a one-electron carrier with a midpoint potential of −40 mV. Its intensity, estimated from a spectrum at 50 K with a copper standard as a reference [22], accounted for about 7 μM Fe^{3+}, which was half the enzyme concentration used in this experiment. It is probably due to enzyme-bound iron originating from the added $FeCl_3$. Half-field signals, indicative for possible magnetic interactions were not observed under any of the conditions used.

Kinetic properties

The kinetics of the redox response of the components of the enzyme were measured using the freeze-quench technique. Three types of reaction were carried out: (i) reduction of the enzyme with succinate, (ii) reduction with 2,3-dimethyl-1,4-naphthoquinol and (iii) oxidation of the reduced enzyme with fumarate. In the experiment of Fig. 5, the enzyme in the oxidized state was reacted with succinate. The time-course of the reduction of the iron-sulphur clusters and cytochrome *b* within the first 100 ms could be approximated by pseudo-first order kinetics. The rate constants used in these

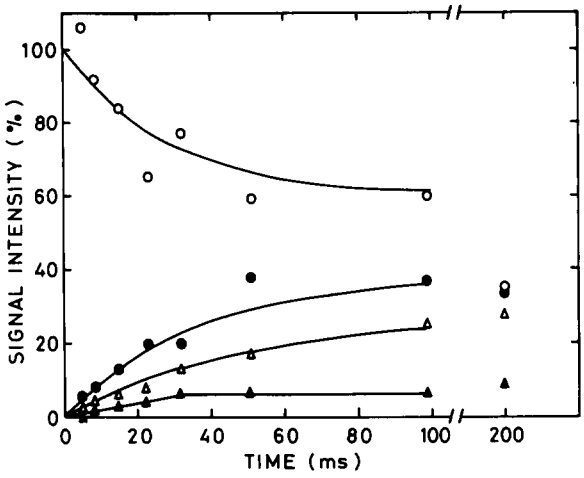


Fig. 5. Reduction of the enzyme (20 μM) with succinate (0.1 M) measured with the freeze-quench technique at 20°C. (See Methods for experimental conditions.) ○———○, EPR signal intensity of the $[4Fe\text{-}4S]^{3+}$ cluster (oxidized cluster); ●———●, EPR signal intensity for the $[2Fe\text{-}2S]^{1+}$ cluster (reduced cluster); △———△, amplitude of the 560 nm band in diffuse-reflectance spectra (reduced cytochrome *b*); ▲———▲, intensity of the EPR signal of the flavin radical measured as in Fig. 3. The redox state of the Fe-S clusters was measured as in Fig. 1.

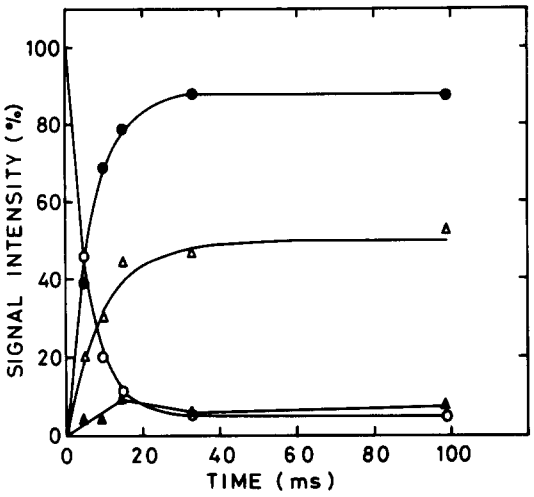


Fig. 6. Reduction of the enzyme (20 μM) with 2,3-dimethyl-1,4-naphthoquinol (0.2 mM) measured with the freeze-quench method at 20°C. See Methods for experimental conditions. The symbols are the same as in Fig. 5.

approximations ranged between 23 and 30 s^{-1}. Thus, the rate constants were about 2-times greater than the turnover number of the enzyme in suc-

TABLE II

PSEUDO-FIRST ORDER RATE CONSTANTS (k) OF THE REDUCTION AND THE OXIDATION OF THE COMPONENTS OF FUMARATE REDUCTASE COMPLEX (20°C)

The rate constants were obtained by a least-squares fit to the data in Figs. 5–7, plotted as $-\ln(I/I_0)$ against time (I is the signal intensity at time t, I_0 is the intensity at $t = 0$). The turnover number (TN) of the corresponding enzymic reactions are based on the FAD contents of the enzyme (5.4 μMol/g protein) and were measured in the presence of 0.1 mM 2,3-dimethyl-1,4-naphthoquinone (DMN) or 0.1 mM 2,3-dimethyl-1,4-naphthoquinol ($DMNH_2$) as described [4], n.r., not reduced.

Reaction	Component observed	k (s^{-1})	Corresponding enzymic reaction	TN (e^-/s)
Oxidized enzyme + succinate (Fig. 5)	$[2Fe\text{-}2S]^{1+}$	30	succinate: DMN oxidoreductase	13
	$[4Fe\text{-}4S]^{3+}$	24		
	cyt. *b* (−20 mV)	23		
	cyt. *b* (−200 mV)	n.r.		
Oxidized enzyme + $DMNH_2$ (Fig. 6)	$[2Fe\text{-}2S]^{1+}$	172	$DMNH_2$: fumarate oxidoreductase	133
	$[4Fe\text{-}4S]^{3+}$	143		
	cyt. *b* (−20 mV)	155		
	cyt. *b* (−200 mV)	n.r.		
Reduced enzyme + fumarate (Fig. 7)	$[2Fe\text{-}2S]^{1+}$	45	$DMNH_2$: fumarate oxidoreductase	133
	$[4Fe\text{-}4S]^{3+}$	50 [a]		
	cyt. *b* (−20 mV)	140		
	cyt *b* (−200 mV)			

[a] Rapid phase. The reaction is biphasic; the estimated rate for the slow phase is less than 14 s^{-1}

cinate oxidation by 2,3-dimethyl-1,4-naphthoquinone (13 s^{-1}) (Table II). The rate constant of the appearance of the flavin radical (2 s^{-1}) is much smaller than the turnover number. About 40% of the iron-sulphur clusters and of the high-potential cytochrome *b* (20% of the total cytochrome *b*) was reduced within the first 100 ms, while the intensity of the flavin radical increased to 6% of the enzyme concentration. After 200 ms, the degrees of reduction were nearly the same except for the 4-Fe cluster which was reduced a little further. The final degrees of reduction attained in this experiment were lower than in the titration experiments, where the Fe-S clusters (Fig. 2) and the high-potential cytochrome *b* were fully reduced by succinate. Full reduction of the components in the experiment of Fig. 5 was presumably prevented by the presence of trace amounts of oxygen which have been excluded from the titration and the other kinetic experiments.

The reduction of the enzyme by 2,3-dimethyl-1,4-naphthoquinol (Fig. 6) was faster than that by succinate. Complete reduction of the components was attained 30 ms after the addition of 2,3-dimethyl-1,4-naphthoquinol. The pseudo-first order rate constants of the reactions of the Fe-S clusters and the high-potential cytochrome *b* were slightly greater than the turnover number of the enzyme in fumarate reduction by 2,3-dimethyl-1,4-naphthoquinol (Table II).

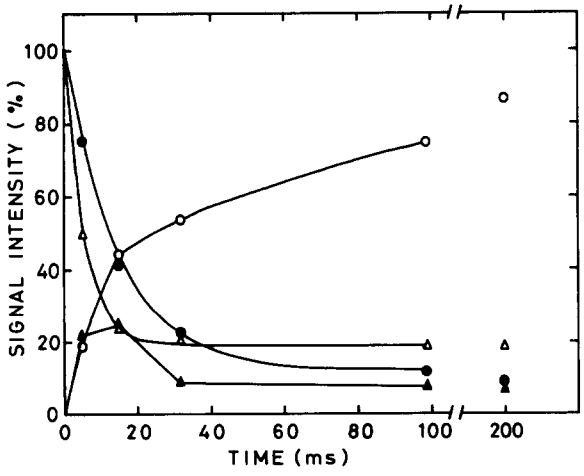


Fig. 7. Oxidation of the reduced enzyme (20 μM) with fumarate (0.1 M) as measured with the freeze-quench technique at 20°C. See Methods for experimental conditions. The symbols are used as in Fig. 5.

The oxidation of the completely reduced enzyme (including the low-potential cytochrome *b*) by fumarate proceeded in an unexpected way (Fig. 7). Nearly the total amount (81%) of the two cytochromes *b* was oxidized in an apparently homogeneous reaction. The reaction was characterized by a rate constant of 140 s^{-1} which is a little greater than the turnover number of the enzyme in fumarate reduction by 2,3-dimethyl-1,4-naphthoquinol under the conditions used (133 s^{-1}) (Table II). The oxidation rate of the other components was slower than the turnover number. The signal of the 4-Fe cluster appeared in a biphasic reaction. Approximately half of the total signal responded about one-third as fast as cytochrome *b*, while the residual part developed much slower and required more than 200 ms for maximal intensity. The oxidation of the 2-Fe cluster could be approximated by a first-order reaction with a rate constant (45 s^{-1}) which is about one-third the turnover number. The flavin radical appeared with the velocity of the initial phase of oxidation of the 4-Fe cluster and reached a maximum concentration (25% of the enzyme concentration), when the cytochromes *b* had reached their final oxidation level (15 ms after fumarate addition). From this point on, the radical concentration decreased until the steady-state concentration (8% of the enzyme concentration) was attained.

During all these reactions, no change in the very small $g = 4.3$ signal was observed, excluding any conclusions about a possible 4-Fe to 3-Fe cluster conversion. A change in the lineshape of the $g = 2.02$ signal, that might also point to such a transition, was not observed.

Discussion

The apparent midpoint potential of the prosthetic groups

The E_m value of the high-potential cytochrome *b* of the fumarate reductase complex was measured earlier as −20 mV at room temperature in the presence of mediators. This value agrees very well with that determined here (−23 mV) in frozen samples (173 K) using the fumarate/succinate couple. From this it is concluded that the effects of

freezing on the oxidation-reduction potential is negligible in the presence of fumarate/succinate. This is likely to be valid also with the other prosthetic groups of the enzymes. It is, therefore, proposed that the E_m values measured with fumarate/succinate reflect the actual potentials of the redox groups.

However, freezing of the sample in the presence of the mediators does affect the apparent E_m value of all the prosthetic groups (Table I). It is concluded that the mediators cause a modification of the enzyme during the freezing of the sample. Accordingly, the titration with lower concentrations of mediators (Mixture II) resulted in higher midpoint potentials. The modifying influence of the dyes is also demonstrated by the strange behaviour of the width and position of the g_x line of the signal of the [2Fe-2S] cluster.

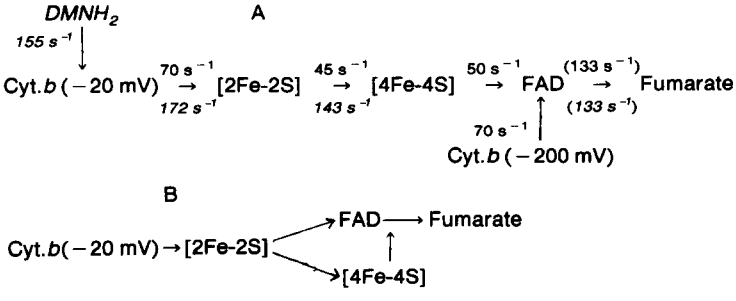
An effect of freezing on the redox potential, reminiscent to those reported here, has been noted by Porras and Palmer [23,24] in a potentiometric study on xanthine oxidase. These investigators found that the potential of the Fe-S clusters and of Mo in this enzyme, obtained by circular dichroism measurements at room temperature in the presence of mediators, was 20–110 mV higher than the potentials obtained by other workers [25,26] by way of EPR spectroscopy of frozen samples in a similar mixture.

The increase of the spin-lattice relaxation rate (at 11 K) of the [2Fe-2S] cluster at potentials below −250 mV (Fig. 4) is a phenomenon that we do not understand, but which confirms our earlier observations with the fumarate reductase complex reduced with succinate or with dithionite. It is known that succinate dehydrogenase responds in a similar way to low redox potentials [27–30]. In this case, however, the changes were interpreted by some investigators as the reduction of a second [2Fe-2S] cluster (see Ref. 31 for a review). The sulphide contents of the purest preparations of fumarate reductase complex [4] leave no room for more than one 4-Fe and one 2-Fe cluster in this enzyme. Since dithionite alone has the same effect on the relaxation of the 2-Fe cluster, we conclude that the effect (Fig. 4) is not merely an artifact due to the mediators.

Kinetics of the redox response

From cleavage and reconstitution experiments with the fumarate reductase complex, it is known [8,10] that FAD and the 4-Fe cluster are situated on the subunit carrying the fumarate site. The high-potential cytochrome *b* was found to react directly with 2,3-dimethyl-1,4-naphthoquinol. The 2-Fe cluster was recognized to be a necessary component of the electron transport from 2,3-dimethyl-1,4-naphthoquinol to the fumarate site.

The kinetic response of the components to the addition of 2,3-dimethyl-1,4-naphthoquinol (Fig. 6 and Table II) is consistent with the results obtained from the reconstitution experiments. The velocities of reduction of cytochrome *b* and the Fe-S clusters were about as fast or faster than the turnover number of the enzyme. Therefore, these components are kinetically competent as members of the catalytic process even in a linear sequence as depicted in Scheme IA. The response of the



Scheme I. Sequence of interaction of the components of the fumarate reductase complex. The rate constants (taken from Table II) refer to reduction of the complex by 2,3-dimethyl-1,4-naphthoquinol (italic numbers) or to its oxidation by fumarate (normal numbers). The bracketed numbers represent the turnover numbers of the enzyme. The arrows indicate the directions of electron flow.

components was probably limited by the reaction of the high-potential cytochrome *b* with 2,3-dimethyl-1,4-naphthoquinol, since the concentration of 2,3-dimethyl-1,4-naphthoquinol applied was below the K_m of the enzyme. This may explain why all the pseudo-first order rate constants of the components were close to the turnover number. Greater rate constants should result with 2,3-dimethyl-1,4-naphthoquinol at a greater concentration.

The rate constant of oxidation of the cytochromes *b* by fumarate (140 s^{-1}) is consistent with the turnover number of the enzyme in fumarate reduction by 2,3-dimethyl-1,4-naphthoquinol, while those of the Fe-S clusters are less than half this value (Fig. 7 and Table II). The rate constant evaluated for cytochrome *b* refers to both cytochromes, each of which is present in the enzyme in the same molar amount as each of the Fe-S clusters. On the basis that the two cytochromes are oxidized with identical velocities, this means that the individual turnover numbers are half the rate constant. Thus, each of the components is oxidized with a turnover number which is half of the turnover number of the enzyme or less. A tentative explanation is that the two cytohromes are oxidized by fumarate via different pathways. The high-potential cytochrome *b* should be oxidized via the 2-Fe cluster as concluded from the reconstitution experiments [10], while the low-potential cytochrome *b* might transfer its electrons to FAD either directly or mediated by the benzyl viologen present.

The low-potential cytochrome *b* was shown earlier not to be involved in fumarate reduction by 2,3-dimethyl-1,4-naphthoquinol [10], but to function in the reduction of menaquinone by formate [2,7,33]. It is, therefore regarded as a contaminant of the preparation of the fumarate reductase complex. Under the conditions of the experiment of Fig. 7, the low-potential cytochrome *b* was reduced by formate in the presence of benzyl viologen and the formate dehydrogenase of the membrane particles from *V. succinogenes*.

Both the reconstitution [10] and the kinetic experiments did not decide whether the 4-Fe cluster or the FAD reacts directly with the 2-Fe cluster. Therefore, an arrangement of the components as depicted in Scheme IB would also be consistent with the experimental results.

Possible consequences of a 3-Fe to 4-Fe cluster conversion

Very recently, experimental evidence has emerged indicating that the $g = 2.02$ signal in oxidized succinate dehydrogenase is exerted by a 3-Fe cluster [34]. Likewise, the EPR signal of the oxidized fumarate reductase complex could also be due to a [3Fe-4S] cluster. This type of cluster may be speculated to convert to a 4-Fe cluster on reduction (reaction a).

$$[3Fe\text{-}4S]^{1+} \overset{+e}{\rightarrow} [3Fe\text{-}4S] \overset{+Fe^{2+}}{\rightarrow} [4Fe\text{-}4S]^{2+} \quad (a)$$

Only the $[3Fe\text{-}4S]^{1+}$ cluster would be detected by EPR. On oxidation reaction (b) might occur.

$$[4Fe\text{-}4S]^{2+} \overset{-e}{\rightarrow} [4Fe\text{-}4S]^{3+} \overset{-Fe^{2-}}{\rightarrow} [3Fe\text{-}4S]^{1+} \quad (b)$$

In this case, both $[4Fe\text{-}4S]^{3+}$ and the $[3Fe\text{-}4S]^{1+}$ cluster would be detectable by EPR. The Fe^{2+} could be oxidized to Fe^{3+} which might give an EPR signal.

We have looked for signs that point to a possible 3-Fe to 4-Fe cluster interconversion. With regard to Fe^{3+}, we found no significant changes in the very small $g = 4.3$ signal during the kinetic experiments or the titration with the fumarate/succinate couple. Only in the presence of 1 mM $Fe^{2+/3+}$ we observed a $g = 4.3$ signal which titrated as a $n = 1$ component with a midpoint potential of -40 mV. Thus, the Fe^{3+} signal does not provide evidence for cluster interconversion.

Ackrell et al. [34] proposed that in succinate : Q oxidoreductase, the $[4Fe\text{-}4S]^{2+}$ cluster may be reduced to the $[4Fe\text{-}4S]^{1+}$ state. If the fumarate reductase reduced with succinate would contain a $[4Fe\text{-}4S]^{2+}$ cluster, it is feasible that at very low redox potentials, reduction to the $[4Fe\text{-}4S]^{1+}$ state occurs. In the case of fumarate reductase, such a cluster would be expected to have an extremely fast spin-lattice relaxation, since no additional EPR signals were observed even at 7 K. Magnetic interaction with such a cluster might provide an explanation for the enhanced relaxation of the $[2Fe\text{-}2S]^{1+}$ cluster.

If cluster conversion would take place, the EPR detectable $[4Fe\text{-}4S]^{3+}$ cluster could be so short-lived that it might not be detected in the redox

titration experiments. However, the cluster could be expected to contribute to the EPR spectra in the kinetic experiments like that of Fig. 7. One of the possible explanations of the biphasic response of the amplitude of the $g = 2.02$ signal (Fig. 7) is the rapid formation of a $[4Fe\text{-}4S]^{3+}$ cluster which is then converted to a $[3Fe\text{-}4S]^{1+}$ cluster (reaction b). The two signals are then expected to be rather similar in lineshape, but to differ in width. The 3-Fe signal should be narrower and, therefore, exhibit a greater amplitude. An apparent change in the width of the $g = 2.02$ signal during the oxidation reaction (Fig. 7) could, however, not be noticed. Such a change could easily be obscured by interference of the signals of the flavin radical and the 2-Fe cluster.

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