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KINETICS OF REDUCTION OF THE CYTOCHROME C_3 FROM DESULFOVIBRIO VULGARIS

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SUMMARY

The reduction of cytochrome c_3 from *Desulfovibrio vulgaris* by dithionite or carboxyl radicals follows biphasic kinetics. The data are consistent with lack of heme to heme electron exchange within a single protein molecule. The kinetics of reduction with hydrated electrons are also reported.

Cytochrome c_3 from the sulfate-reducing bacterium *Desulfovibrio vulgaris* is a small protein containing four heme *c* molecules (1-4) covalently attached through thioether bonds to a single polypeptide chain of 107 residues (5). Histidine groups bind to both axial coordination positions of each iron ion (6-9). Broadening of the heme proton resonances observed in the NMR experiments upon partial reduction of concentrated solutions of cytochrome c_3 from *D. vulgaris* or from the parent bacterium *Desulfovibrio gigas* (8-10) indicates a relatively fast rate for the electron exchange arising either between different protein molecules or within a single protein unit. The preferential mode of electron transfer is important with respect to the physiological role of the cytochrome c_3. We therefore entered upon an investigation of the oxidoreduction kinetics of this hemoprotein, using stopped-flow and pulse-radiolysis techniques which provide different time scales of observation.

METHODS

Cytochrome c_3 was purified from *D. vulgaris* as described previously (11).

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Its concentration was determined using $\varepsilon_{408} = 690\ mM^{-1}.cm^{-1}$ per mol of protein. The purity index was $(A^{red}_{553} - A^{red}_{570})/A^{ox}_{280} = 3.16$.

Dithionite solutions, obtained by addition of deaerated buffer to weighted amounts of $Na_2S_2O_4$ in an argon-flushed septum-stoppered flask, were calibrated by optical titration of FMN. Stopped-flow measurements were carried out using a Durrum-Instruments apparatus (mixing dead-time 2.5 ± 0.2 ms) operated under anaerobic conditions and thermostated at 20°C. The pulse-radiolysis equipment was a modified Febetron 707 (12) allowing irradiation of solutions contained in Suprasil quartz cells (optical path length 2.5 cm) with single pulses of electrons (16 ns total duration and ca. 1.8 Mev energy). The effect of the irradiation was detected optically and recorded photographically on a cathode ray oscilloscope. Saturated nitrous oxide in formate buffer (160 mM, pH 8.1) was used as a hydrated electron scavenger (13,14).

RESULTS AND DISCUSSION

Deaerated (99.995% argon) solutions of cytochrome c_3 (3.2 to 4.3 μM) in 25 mM borate buffer (pH 9.1) exhibit biphasic kinetics of reduction when mixed (stopped-flow) with dithionite (0.7 to 60 mM) in the same buffer. The extrapolation of the lines that fit the slow phase in the log plots, yields a single ordinate intercept corresponding to the value of absorbance expected from half-reduction (Fig.1). Consequently, the four protein-bound hemes are equivalent two by two with regard to their reduction rate. No intermediate is evidenced in the 440-610 nm range. The slopes for both the rapid and slow phases are proportional to the square root of the dithionite concentration. Therefore, each heme group reacts specifically with the one-electron donor SO_2^- resulting from the dissociation of the dithionite ion according to Lambeth and Palmer (15). Using the equilibrium and rate constants of dithionite monomerization calculated by these authors (15), namely $K_d = 1.4 \times 10^{-9}$ M and $k_d = 1.7\ s^{-1}$, it appears that the rate of dissociation of $S_2O_4^{2-}$ is nearly 20-fold as fast as the initial rate of reduction of the four heme groups of the cytochrome c_3. This is suffi-

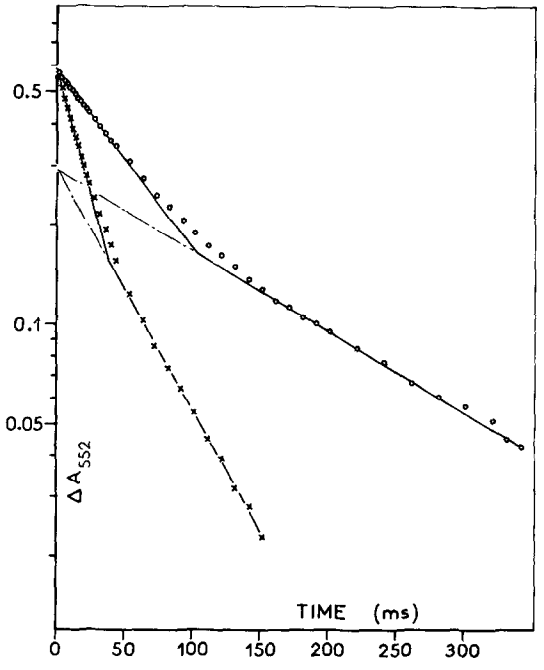

Figure 1. Log-plot for the reduction (stopped-flow) of cytochrome c_3 from *Desulfovibrio vulgaris* (4.3 μM) with 4.8 mM (o) and 56.5 mM (×) sodium dithionite.

cient to maintain a steady-state with respect to SO_2^- although its concentration is comparable to the total heme content. With k_1 and k_2 as the rate constants for the "rapid" and "slow" hemes, the whole initial rate of reduction is

$$v_i = 2\,(k_1+k_2)\,[C]\,K_d^{1/2}\,[S_2O_4^{2-}]^{1/2} = 4\,\alpha\,[C] \qquad (1)$$

where $[C]$ is the protein concentration and α the apparent rate constant of the initial reaction, i.e. the initial slope of the log plot, $d(\ln\Delta A)/dt$. If β is the corresponding slope for the second phase of the reaction, it comes

$$k_2\,K_d^{1/2}\,[S_2O_4^{2-}]^{1/2} = \beta \qquad (2)$$

which allows the calculation of k_1 and k_2 to 6.8 and 2.1×10^6 $M^{-1}.s^{-1}$, respectively, with $K_d = 1.4\times10^{-9}$ M (15). The α/β ratio is constant over the whole range of dithionite concentrations investigated. Electron transfer between "rapid" and "slow" hemes is thus negligible ($k_{exch} < 2\ s^{-1}$) on the time scale of these experiments in which the reaction is half-over in 17 to 160 ms, depending on the concentration of dithionite.

The reaction of cytochrome c_3 (2.5 μM) with the hydrated electrons (e^-_{aq}) formed from the pulse-radiolysis set up (5-12 krad) in deaerated formate buffer (160 mM), results in a direct reduction of the hemes without the formation of any detectable transient species in the 425-600 nm wavelength range. e^-_{aq} decays through both self-recombination and reaction with ferric hemes, so that about half of the heme sites only are reduced for doses of 5 krad (i.e. $[e^-_{aq}]_o$ = 15 μM). Therefore, absorbance changes have been measured in parallel at 550 and 600 nm ; from the known ε values, the concentrations of e^-_{aq} and reduced heme are calculated as a function of time (Fig.2). The initial rate of reduction leads to a $k_3.[Fe^{III}]_o$ value of 2×10^5 s^{-1} ; $[Fe^{III}]_o$can be adjusted to different values since one to four hemes can react simultaneously, i.e. $[Fe^{III}]_o \leq 4\times[\text{cytochrome}]$, but a single value of $[Fe^{III}]_o$ can fit the course of the whole reaction. Given the rate of the disappearance of e^-_{aq} without protein in the buffer used, the computer-simulation of the reaction indicates $[Fe^{III}]_o$ = 10 μM, i.e. the four heme groups are kinetically equivalent towards e^-_{aq} attack and k_3 is 2×10^{10} $M^{-1}.s^{-1}$, near the limit for a diffusion-controlled reaction. Carboxyl radicals are formed together with e^-_{aq} in these experiments but they react more slowly, so that they do not interfere noticeably with e^-_{aq} in the kinetics of reduction of ferric hemes.

The reduction of cytochrome c_3 by the carboxyl radical anion (COO^-) has been investigated in nitrous oxide-saturated formate (160 mM) buffer (13,14). The effect of COO^- upon ferric hemes is studied using 10 krad doses ($[COO^-]_o$ = 60 μM) corresponding to reduction of ca. 25% of the total heme content (10 μM) after completion of the reaction, with an initial rate $k_4.[Fe^{III}]_o$ of 1075 s^{-1} (Fig.2). Given the rate of the self-recombination of COO^- ($2.k = 1.8\times10^9$ $M^{-1}.s^{-1}$) in the same buffer, a computer-simulation of the reaction kinetics gives a $[Fe^{III}]_o$ value equal to half the concentration of protein-bound hemes, and k_4 is 2.1×10^8 $M^{-1}.s^{-1}$. Therefore, two heme groups react more rapidly with COO^- than the other two hemes, as also observed with SO^-_2 .

Although the four hemes are undistinguishable with regard to the rate of

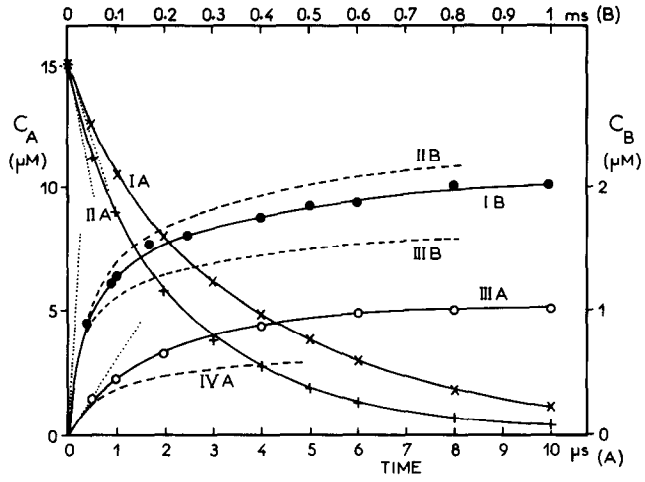

Figure 2. Reduction of cytochrome c_3 (2.5 μM) by pulse-radiolysis at pH 8.1. *(A)*. Reaction with 15 μM e^-_{aq} (left and bottom scales) : e^-_{aq} decay without (*IA*) and with (*IIA*) cytochrome c_3 and reduced heme formation (*IIIA*) ; curves are calculated with $[Fe^{III}]_o$ = 10 μM (full lines) or with $[Fe^{III}]_o$ = 5 μM (dashed line, curve *IVA*). *(B)*. Reaction with COO^- (60 μM) (right and top scales) : reduced heme formation (*IB*) ; curves are calculated with $[Fe^{III}]_o$ = 5 μM (full line, *IB*), 10 μM (dashed line, curve *IIB*) or 2.5 μM (dashed line, curve *IIIB*).

e^-_{aq} attack, the kinetics of reduction of dilute solutions of cytochrome c_3 by SO_2^- agree with a slow rate ($< 2\ s^{-1}$) of intramolecular electron transfer. Interaction between different cytochrome molecules is thus necessary to explain the rate of the heme-heme electron exchange evidenced from NMR studies in concentrated solutions (8-10). Consequently, any tight and stereospecific association of the cytochrome c_3 with its physiological partners would limit the turnover of the corresponding electron transfer chain. On the other hand, the inequivalence of the heme groups towards reduction at equilibrium (4,8-10) suggests a slightly different redox potential for each active site. However, the biphasic course of reduction is more obvious with SO_2^- (Fig.1) or COO^-. Differences in sterical hindrance rather than in net electrostatic charge around the hemes may account for this behavior. Actually, we found that a 0.5 M increase of ionic strength (using KCl) results in a 24% decrease of the whole reduction rate but leaves the k_1/k_2 ratio unchanged in experiments with SO_2^- .

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