Reactions of cytochrome c oxidase with sodium dithionite

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The reduction of cytochrome c oxidase (EC 1.9.3.1) by dithionite was investigated by stopped-flow spectrophotometry and flow–flash techniques in the presence of CO. Of the two haem groups present in the enzyme, that associated with cytochrome a is the first reduced. The second-order rate constants for reduction of a number of redox proteins (cytochrome c, stellacyanin and azurin) by the $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ anions are reported, and the values are compared with those determined for cytochrome c oxidase. These results are discussed in terms of the accessibility and charge distribution of the electron-entry site of cytochrome c oxidase.

Studies on the electron-transfer reactions between metalloproteins and small inorganic/organic molecules have been the subject of considerable interest in recent years (see, e.g., Cummins & Gray, 1977; Al-Ayash & Wilson, 1979; Saleem & Wilson, 1982). This interest stems from the fact that such studies yield valuable information about the nature of the metal site in protein and the mechanism by which electrons transfer into or out of the metalloprotein. The results of such investigations have, in general, been discussed in terms of the Marcus theory (Wherland & Gray, 1977) of outer-sphere electron transfer. Many of the reactions appear to conform to this mechanism once the effects of hydrophobic and electrostatic interactions have been adequately taken into consideration (Scott & Gray, 1980). There are, however, some small reductants, such as the chromous ion and the $SO_2^{\cdot-}$ anion, that are exceptions and appear to act also as inner-sphere reagents (Sutin & Yandell, 1972; Creutz & Sutin, 1973).

Most of the proteins examined possess a single redox site, and comparatively little information has been obtained on those that possess multiple metal centres. In particular, there is relatively little known about the reactions of cytochrome c oxidase with redox agents other than its natural substrates [see, however, Dawson et al. (1972), Scott & Gray (1980), Greenwood et al. (1977), Petersen & Cox (1980) and Halaka et al. (1981)].

In the present paper we report experiments on the reduction of cytochrome c oxidase (EC 1.9.3.1) by $Na_2S_2O_4$. Cytochrome c oxidase, the terminal electron acceptor of the mitochondrion, contains four metal centres: two haem a groups and two copper atoms. The haem a groups dominate the optical spectrum in the Soret and visible-light regions. Both haem groups make major contributions in the Soret region, and it is probable that the one associated with cytochrome a makes the largest contribution to the α-region around 600 nm (Wikström et al., 1981; Brunori et al., 1981). In order to distinguish the haem groups we have exploited the definitive property of ferrous cytochrome a_3 to bind CO. The iron–CO bond is photosensitive, and we have used this to determine the kinetics of cytochrome a_3 reduction by monitoring the onset of photosensitivity during reduction of cytochrome c oxidase by dithionite.

We conclude that cytochrome a is the first of the haem groups to be reduced and that cytochrome a_3 is reduced slowly. By studying the rate of cytochrome a reduction as a function of solution parameters and comparing this with rates for other metalloproteins, both haem- and copper-containing, we believe that the site of electron entry from either $SO_2^{\cdot-}$ or $S_2O_4^{2-}$ is less accessible than are those of the single-site proteins studied. At the present stage we cannot unambiguously decide whether the electrons enter oxidase from dithionite via cytochrome a or through the e.p.r.-detectable copper atom Cu_A.

Materials and methods

Bovine heart cytochrome c oxidase was prepared by a modification of the Yonetani (1960) method, and its concentration is expressed in terms of total

haem a ($\varepsilon_{605}^{red.} = 21\,000\,M^{-1}\cdot cm^{-1}$). *Rhus vernicifera* stellacyanin ($\varepsilon_{604}^{ox.} = 4100\,M^{-1}\cdot cm^{-1}$) was prepared by the method of Reinhammar (1970). Azurin ($\varepsilon_{625}^{ox.} = 3500\,M^{-1}\cdot cm^{-1}$) was purified from *Pseudomonas aeruginosa* as described by Parr et al. (1976). Cytochrome c type VI ($\varepsilon_{550}^{red.} = 27\,600\,M^{-1}\cdot cm^{-1}$) was obtained from Sigma Chemical Co. Solutions of dithionite at known concentration were prepared assuming a molecular weight of 174 and 92% purity as determined by redox titration against lumiflavin acetate (Lambeth & Palmer, 1973). Dithionite solutions were prepared under N_2 with degassed buffer and were used within a few hours of being prepared.

Absorption spectra were recorded with a Perkin–Elmer type 575 spectrophotometer. Kinetic experiments were performed with a Durrum–Gibson stopped-flow apparatus equipped with a 2 cm observation chamber (dead time $\simeq$ 3 ms).
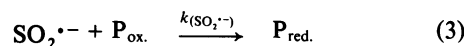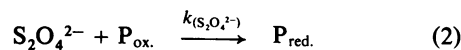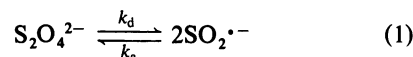
In some experiments a DataLab DL 901 transient recorder interfaced to a Commodore PET microcomputer was added to this system. Simulations and statistical analyses were also performed with this computer. Numerical integration of differential rate equations was performed with a PDP DEC 10 computer as previously described (Wilson et al., 1981).

Flow–flash experiments

A deoxygenated solution of oxidized (resting) cytochrome c oxidase was placed under an atmosphere (0.1 MPa) of CO in a 1 cm cuvette sealed with a rubber vaccine cap. The reduction was initiated by injecting a solution of dithionite through the seal to give the desired final reductant concentration. After rapid mixing by inversion, the cuvette was placed in the monitoring beam of a flash-photolysis apparatus. A series of flashes were given at timed intervals, and the amplitude of the absorbance change initiated by the actinic light was determined by recording the output of a photomultiplier, monitoring the observation beam on a storage oscilloscope (Tetronix 564). This absorption change increased with time as cytochrome a_3 became reduced by dithionite and thus produced the photolabile complex with CO. This method of following the reduction of cytochrome a_3 is a simplification of that described by Gibson et al. (1965).

Calculation of rate constants for reduction of metalloproteins by sodium dithionite: Schema 1

Following the work of others (Lambeth & Palmer, 1973; Sutin & Yandell, 1972), we have assumed that under our anaerobic conditions the solution chemistry of the reactions of $Na_2S_2O_4$ with metalloproteins can be totally described as follows:

$$S_2O_4^{2-} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} 2SO_2^{\cdot -} \tag{1}$$

$$S_2O_4^{2-} + P_{ox.} \xrightarrow{k_{(S_2O_4^{2-})}} P_{red.} \tag{2}$$

$$SO_2^{\cdot -} + P_{ox.} \xrightarrow{k_{(SO_2^{\cdot -})}} P_{red.} \tag{3}$$

where k_d is the rate constant for dissociation of $S_2O_4^{2-}$ and k_a the reverse dimerization rate constant, $k_{(SO_2^{\cdot -})}$ is the second-order rate constant for reduction by $SO_2^{\cdot -}$ and $k_{(S_2O_4^{2-})}$ is the corresponding constant for $S_2O_4^{2-}$; $P_{ox.}$ and $P_{red.}$ refer to the oxidized and reduced forms of the metalloprotein respectively.

Thus both the species $SO_2^{\cdot -}$ and $S_2O_4^{2-}$ may, in principle, act as reducing agents. The observed rate constants for reduction of metalloproteins may therefore have contributions from both species. Rate constants (together with their corresponding standard errors) for reduction were obtained by regression analysis of the experimental data. Both linear and curvilinear regressions were performed (Snedecor & Cochran, 1967), fitting the data to eqns. (4) and (5), which are modified from the work of Lambeth & Palmer (1973):

$$k_{obs.} = k_{(SO_2^{\cdot -})}\cdot [SO_2^{\cdot -}] + c \tag{4}$$

$$k_{obs.} = k_{(SO_2^{\cdot -})}\cdot [SO_2^{\cdot -}] + k_{(S_2O_4^{2-})}\cdot [SO_2^{\cdot -}]^2/K_{eq.} + c \tag{5}$$

$K_{eq.}$ is the equilibrium constant for reaction (1). A value of $0.85 \times 10^{-9}\,M$ was used throughout (Lambeth & Palmer, 1973). $[SO_2^{\cdot -}]^2/K_{eq.}$ is therefore assumed to be equivalent to $[S_2O_4^{2-}]$. c is the value of $k_{obs.}$ at zero total dithionite concentration.

Where only one reductant species is considered to make a significant contribution, we have chosen this to be the radical, as from earlier work this seems to be by far the more reactive species (Lambeth & Palmer, 1973).

The quality of fit of each set of experimental data to eqns. (4) and (5) was compared by using the F-test (Snedecor & Cochran, 1967). Where $P > 0.1$ [i.e. the probability of linearly distributed points fitting eqn. (5) by chance] we have discarded the quadratic form, since the error on $k_{(S_2O_4^{2-})}$ does not allow us confidently to distinguish the value of this rate constant from zero. The better equation to fit the data under these circumstances is thus the linear eqn. (4) to which eqn. (5) collapses.

The model implicit in this approach (eqns. 1–3), namely that both $S_2O_4^{2-}$ and $SO_2^{\cdot -}$ may act as reducing agents and that both are present at adequate concentrations, was tested by solving the appropriate differential equations by computer numerical methods described elsewhere (Wilson et al., 1981). The value of k_d was taken as $1.7\,s^{-1}$ and that of k_a as $2 \times 10^9\,M^{-1}\cdot s^{-1}$ (Lambeth &

Palmer, 1973). The values for $k_{(SO_2^{\cdot-})}$ and $k_{(S_2O_4^{2-})}$ for each metalloprotein were taken from the statistical procedure described above.
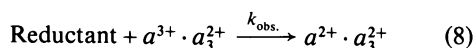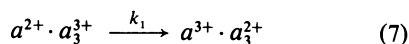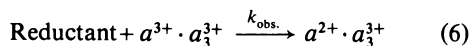
The computer generated progress curves that reproduced the experimentally observed first-order form. In addition, the concentration-dependence of $k_{obs.}$ on total dithionite concentration matched the experimentally determined values extremely closely. However, the value of $k_{obs.}$ at zero dithionite [c of eqns. (4) and (5)] could not be predicted. The physical significance of this value is unclear.

These simulations indicated that the concentrations of $SO_2^{\cdot-}$ and $S_2O_4^{2-}$ were slightly perturbed from equilibrium throughout the course of the reduction, and thus the assumption used for the statistical analysis (i.e. that $[SO_2^{\cdot-}]^2/K_{eq.}$ is equivalent to $[S_2O_4^{2-}]$) is not strictly valid. Despite this, the model fits the experimental data well.

The continuous lines in Figs. 4(a) and 4(b) were drawn by using the values of $k_{obs.}$ obtained from the model shown in eqns. (1)–(3), with the values of $k_{(SO_2^{\cdot-})}$, $k_{(S_2O_4^{2-})}$ and c obtained from the statistical procedure.

Simulation of the reduction of cytochrome c oxidase by sodium dithionite: Schema 2

Here we present a scheme specifically for the reduction of cytochrome c oxidase that enables the concentrations of the oxidation states of both cytochrome a and a_3 to be calculated during the time course of the reaction. The scheme is as follows:

$$\text{Reductant} + a^{3+} \cdot a_3^{3+} \xrightarrow{k_{obs.}} a^{2+} \cdot a_3^{3+} \quad (6)$$

$$a^{2+} \cdot a_3^{3+} \xrightarrow{k_1} a^{3+} \cdot a_3^{2+} \quad (7)$$

$$\text{Reductant} + a^{3+} \cdot a_3^{2+} \xrightarrow{k_{obs.}} a^{2+} \cdot a_3^{2+} \quad (8)$$

The reductant need not be specified here in terms of $SO_2^{\cdot-}$ or $S_2O_4^{2-}$, as $k_{obs.}$ is the observed pseudo-first-order rate constant for the rapid reduction of cytochrome a at a given total dithionite concentration (eqns. 6 and 8). k_1 is the internal electron-transfer rate constant of cytochrome c oxidase from cytochrome a to a_3 (eqn. 7).

Analytical solutions of the differential equations describing this scheme allow the concentration of each species of the oxidase to be expressed as a percentage of the total enzyme present for given values of $k_{obs.}$ and k_1. By taking the following differential absorption coefficients, $4000\,M^{-1}\cdot cm^{-1}$ for $(a_3^{2+} - a_3^{3+})$ and $17000\,M^{-1}\cdot cm^{-1}$ for $(a^{2+} - a^{3+})$, the overall time course at 605 nm could be satisfactorily simulated for a known cytochrome c oxidase concentration (see Fig. 1). The values for the differential absorption coefficients obtained by this simulation are in reasonable agreement with

those determined experimentally by Yonetani (1961). Fig. 5 was also calculated from this reaction scheme (see the Discussion section).

Results

On rapidly mixing $Na_2S_2O_4$ solutions with resting cytochrome c oxidase, complex progress curves were obtained at all wavelengths monitored. The forms of these curves were dependent on the monitoring wavelength, suggesting, in agreement with other workers (Halaka et al., 1981), that at least two distinct chromophores undergo redox changes. Fig. 1 shows representative progress curves obtained at 605 nm at two dithionite concentrations. Over the first 30 s of the reaction these optical data may be adequately fitted by the sum of two exponentials. However, a third, very slow, process, taking some minutes to complete, could also be discerned, but made only a very small contribution to the absorbance change at 605 nm under these conditions. The fastest of the processes made the major contribution to the absorbance change at 605 nm at all dithionite concentrations explored. The fitting procedure employed for Fig. 1 (see the Materials and methods section) used $\Delta\varepsilon$ of 17 000 and $4000\,M^{-1}\cdot cm^{-1}$ for the fast and slow processes respectively. The
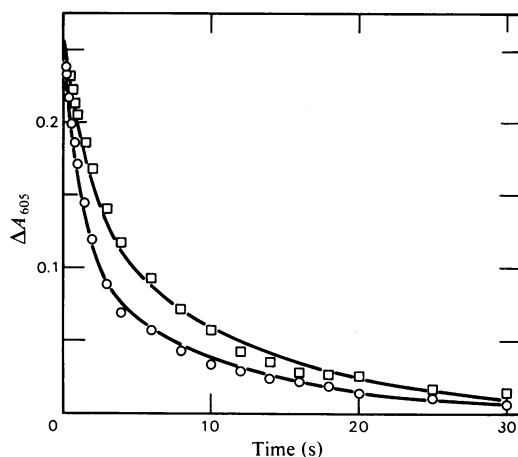

Fig. 1. *Time course for the reduction of cytochrome c oxidase by sodium dithionite*

The buffer was 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, containing 0.1% Tween 80, and the temperature was 25°C. The concentrations of reactants after mixing were 25 μM-cytochrome c oxidase and (O) 12.5 mM-dithionite or (□) 3.1 mM-dithionite. The continuous lines were drawn by using the model described in the Materials and methods section (Schema 2) with $k_{obs.} = 0.64\,s^{-1}$, $k_1 = 0.1\,s^{-1}$ (O), and $k_{obs.} = 0.37\,s^{-1}$, $k_1 = 0.1\,s^{-1}$ (□).

difference spectrum for the faster phase is reported in Fig. 2.

The values of the absorption coefficients for the absorbance change at 605 nm are similar to those reported by Petersen & Cox (1980) for the rapid reduction of cytochrome a and the slower reduction of cytochrome a_3. The overall spectral distribution (Fig. 2) is in agreement with that reported by Greenwood et al. (1977) for the reduction of cytochrome a. Thus these data indicate that reduction of cytochrome a precedes that of cytochrome a_3.
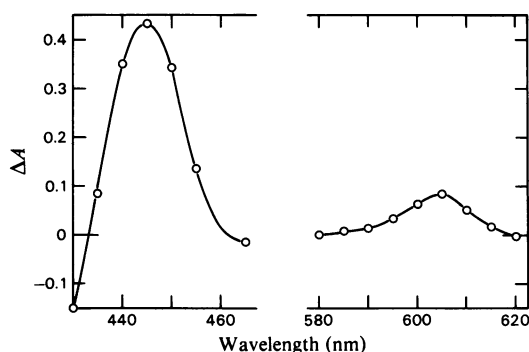

Fig. 2. *Kinetic difference spectrum for the fast phase of cytochrome c oxidase reduction by dithionite at 25 °C in 0.1 M-phosphate buffer, pH 7.4, containing 0.05% Tween 80*

Concentrations of reactants after mixing were: 5 μM-cytochrome c oxidase (total haem) and 5 mM-dithionite.

To test this hypothesis we undertook experiments in which we monitored the onset of photosensitivity, i.e. the reduction of cytochrome a_3, on mixing $Na_2S_2O_4$ with cytochrome c oxidase under an atmosphere of CO. After photodissociation of the cytochrome c oxidase·CO complex by an intense actinic flash, the size of the rapid absorbance change due to CO recombination with ferrous cytochrome a_3 increased with the time elapsing from the start of reduction. Figs. 3(a) and 3(b) report this type of experiment with a variety of reducing systems. It is clear that when dithionite is the sole reductant the onset of photosensitivity is slow and, to a first approximation, independent of dithionite concentration. The time course of appearance of photosensitivity was, however, heterogeneous, with a slower phase having a $t_{\frac{1}{2}}$ approx. 50 s. This heterogeneity of the reduction of cytochrome a_3 is similar to that reported by Halaka et al. (1981) with phenazine methosulphate as reductant.

Similar heterogeneity in the slow phase was also observed in our stop-flow experiments with dithionite, with a very slow phase having a small amplitude at 605 nm. Comparison of Fig. 3 with Fig. 1 shows that the reduction of cytochrome a_3 is much slower than the fast process observed at 605 nm. These experiments strongly support the conclusion that cytochrome a is the more rapidly reduced haem in cytochrome c oxidase.

Addition of cytochrome c enhanced the rate of reduction of cytochrome a_3 by dithionite (Fig. 3a). This rate is also increased over that by dithionite alone by using ascorbate and cytochrome c as the electron source. Under these circumstances the rate of reduction of cytochrome a_3 appears, however, to be rate-limited by the rate of reduction of cyto-
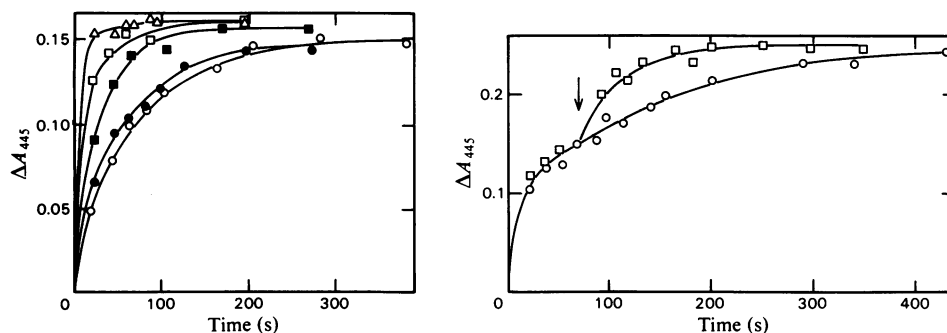

Fig. 3. *Onset of photosensitivity in cytochrome c oxidase*

The method is described in the Materials and methods section. (a) The enzyme concentration was 5 μM in Hepes buffer, pH 7.4, containing 0.1% Tween 80. The reductants used were O, 1 mM-dithionite; ●, 6.7 mM-dithionite; ■, 6.7 mM-dithionite, 2.5 μM-cytochrome c; □, 2.5 μM-cytochrome c, 10 mM-ascorbate; △, 2.5 μM-cytochrome c, 10 mM-ascorbate, 1.5 mM-tetramethyl-p-phenylenediamine. (b) The cytochrome c oxidase concentration was 7.5 μM in 0.1 M-phosphate buffer, pH 7.4, containing 0.1% Emasol. The reductant was 40 mM-dithionite in O and □. In □ 4 μM-cytochrome c, 7.7. mM-ascorbate and 0.8 mM-tetramethyl-p-phenylenediamine were added at 70 s (arrow).
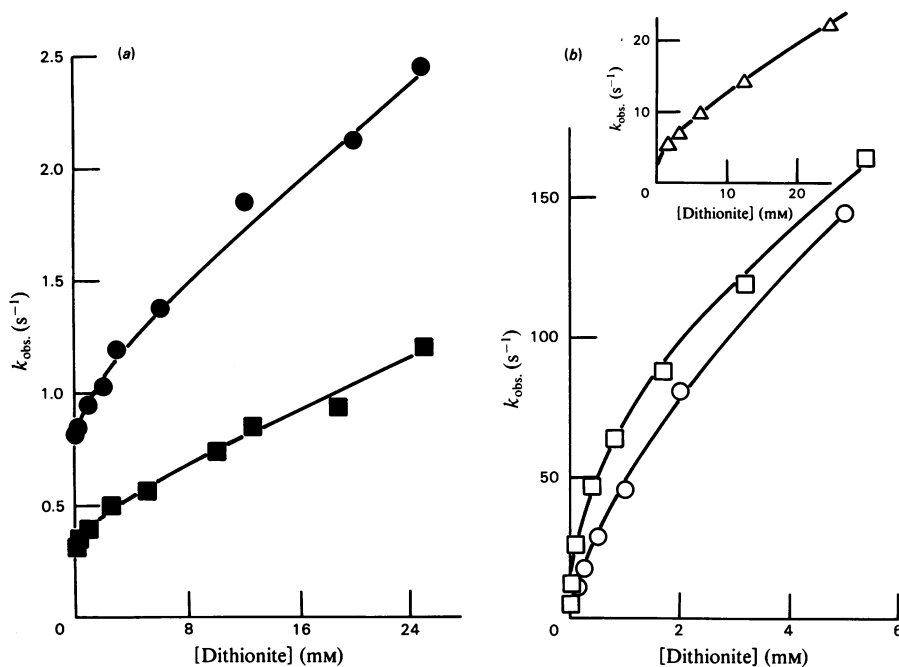
Fig. 4. *Concentration-dependence of dithionite reduction of selected metalloproteins*
The reactions were performed at 25 °C in 0.1 M-phosphate/0.8 M-NaCl buffer, pH 7.0, unless otherwise specified. All concentrations stated are after mixing. (*a*) ■, 5 µM-Cytochrome *c* oxidase in 0.1 M-phosphate buffer, pH 7.2, containing 0.05% Tween 80, at 605 nm; ●, 5 µM-cytochrome *c* oxidase in the presence of 0.1 M-NaSCN and 0.1% Tween 80, at 605 nm. (*b*) □, 8 µM-Cytochrome *c*, at 550 nm; ○, 22 µM-stellacyanin, at 604 nm; △ (insert), 25 µM-azurin, 20 °C, at 625 nm. The continuous lines were fitted as described in the Materials and methods section (Schema 1).

chrome *c* by ascorbate, as indicated by the enhancement on addition of *NNN'N'*-tetramethyl-*p*-phenylenediamine, which acts as a mediator between these components and which itself may donate electrons directly to cytochrome *a* (second-order rate constant = $1.3 \times 10^3 \, M^{-1} \cdot s^{-1}$; P. Sarti & M. T. Wilson, unpublished work).

It is apparent that dithionite reduction of cytochrome a_3 is slow under all the conditions explored, and, in order to ascertain whether there exists a specific inhibitory influence of dithionite in addition to its role as reducing agent, we undertook the experiments illustrated in Fig. 3(*b*). At high dithionite concentration (40 mM) we monitored the onset of photosensitivity firstly in the absence of other reductants and subsequently by injecting an ascorbate/tetramethyl-*p*-phenylenediamine/cytochrome *c* mixture during the course of dithionite reduction. The observed enhancement suggests the reductant systems act largely independently of each other, and reduction of cytochrome a_3 by the ascorbate/tetramethyl-*p*-phenylenediamine/cytochrome *c* system is not strongly inhibited by the presence of dithionite.

Having ascertained that cytochrome *a* is the first haem reduced, we conducted experiments to determine the dithionite-concentration-dependence of the rate of the reduction of this site, i.e. the pseudo-first-order rate ($k_{obs.}$) of the faster phase depicted in Fig. 1 (see Fig. 4*a*). For comparative purposes we have also conducted similar experiments with a number of other redox proteins, and these results are given in Fig. 4(*b*). From Fig. 4 we note that the rate of reduction is not strictly linear with respect to dithionite concentration, and, in agreement with the results obtained with other redox proteins by Lambeth & Palmer (1973), this supports the view that $SO_2^{\cdot-}$ as well as $S_2O_4^{2-}$ ions may act as reducing species. This view is also supported by our computer model, which is based on this premise and which simulates the experiments very satisfactorily (see the continuous lines in Fig. 4).

Taking the value of the equilibrium constant for the reaction $S_2O_4^{2-} \rightleftharpoons 2SO_2^{\cdot-}$ of $0.85 \times 10^{-9} M$, an analysis of the profiles reported in Fig. 4(*a*) for reduction in sodium phosphate buffer yields values for the rate constants for reduction by these species as reported in Table 1. A comparison of these values

Table 1. *Rate constants for the reduction of selected metalloproteins by dithionite at 25°C*
The data were calculated as described in the Materials and methods section.

| Protein | Conditions | Rate constants ($M^{-1} \cdot s^{-1}$) | | F | Degrees of freedom | P |
		$k_{(SO_2 \cdot^-)}$	$k_{(S_2O_4^{2-})}$			
Cytochrome c	0.1 M-Phosphate/0.8 M-NaCl, pH 7.0	$7.3 \times 10^7 \pm 0.2 \times 10^7$		1.3	1, 5	—
Cytochrome c	0.02 M-Phosphate/0.25 M-NaCl, pH 8.0*	4.59×10^7	1.5×10^4			
Plastocyanin	0.02 M-Phosphate/0.25 M-NaCl, pH 8.0*	3.48×10^7	1.45×10^5			
Stellacyanin	0.1 M-Phosphate/0.8 M-NaCl, pH 7.0	$5.3 \times 10^7 \pm 0.9 \times 10^7$	$8.8 \times 10^3 \pm 3.0 \times 10^3$	8.4	1, 3	0.2
Azurin	0.1 M-Phosphate/0.8 M-NaCl, pH 7.0, 20°C	$2.5 \times 10^6 \pm 0.4 \times 10^6$	$3.7 \times 10^3 \pm 0.6 \times 10^2$	37.5	1, 2	0.02
Cytochrome c oxidase						
Prep. 1	0.1 M-Phosphate/0.1% Tween 80, pH 7.0	$15.5 \times 10^4 \pm 1.9 \times 10^4$	—	4.8	1, 3	—
Prep. 1	0.1 M-Phosphate/0.8 M-NaCl/0.1% Tween 80, pH 7.0	$17.4 \times 10^4 \pm 1.9 \times 10^4$	—	2.6	1, 3	—
Prep. 1	0.1 M-Phosphate/0.8 M-NaCl/0.1 M-NaSCN/0.1% Tween 80, pH 7.0	$15.9 \times 10^4 \pm 0.6 \times 10^4$	—	0.03	1, 3	—
Prep. 2	0.1 M-Phosphate/0.05% Tween 80, pH 7.2	$8.4 \times 10^4 \pm 3.3 \times 10^4$	18.8 ± 5.8	10.5	1, 6	0.05
Prep. 2	0.1 M-Phosphate/0.8 M-NaCl/0.1 M-NaSCN/0.1% Tween 80, pH 7.0	$16.6 \times 10^4 \pm 2.9 \times 10^4$	35.3 ± 5.0	49.3	1, 6	0.005
Prep. 3	50 mM-Hepes/0.1% Tween 80, pH 7.4	$41.8 \times 10^4 \pm 3.3 \times 10^4$	–	0.85	4, 1	—

* Lambeth & Palmer (1973).

with those given for a number of redox proteins (Table 1) clearly shows that the rate constants for cytochrome c oxidase are approx. 10^3-fold lower than for cytochrome c, stellacyanin or plastocyanin. The rates for cytochrome c oxidase are, however, closer to the values obtained for azurin, but are nevertheless distinctly lower by 10–100-fold.

Table 1 also reports the rate constants for cytochrome c oxidase under a variety of conditions and shows the variation with preparation.

We quote values of the rate constants for reduction of cytochrome c oxidase by $S_2O_4^{2-}$ for only some experiments. By this, we do not imply that the mechanism changes with conditions, but imply only that we were not always able to distinguish this rate constant from zero with confidence. This lack of resolution is to be expected, given the very low value of the rate constant for $S_2O_4^{2-}$ reduction of cytochrome c oxidase. Where this constant is larger, e.g. with azurin, assignment is unambiguous.

Discussion

As described above, our results clearly indicate that, of the two haem groups in the enzyme, that associated with cytochrome a is the more rapidly

reduced by dithionite. Although this result is in keeping with the expectations based on experiments with the natural reductant, cytochrome c, which donates electrons rapidly to cytochrome a, the results of the experiments with dithionite may be interpreted with either cytochrome a or Cu_A as the initial entry site. Cu_A and cytochrome a are in rapid redox equilibrium ($1/\tau = 100 s^{-1}$; Greenwood et al., 1976) and thus an electron donated to the Cu_A site would be rapidly transferred to cytochrome a. In either case the results in Figs. 4(a) and 4(b) and Table 1 yield information on the nature of the site through which electrons enter cytochrome c oxidase from dithionite.

Of the proteins studied, cytochrome c, plastocyanin and stellacyanin have kinetically accessible electron-accepting sites. The low-spin hexa-co-ordinate ferric iron of cytochrome c is thought to be reduced by dithionite via electron entry either through the exposed haem edge or by close approach of the $SO_2 \cdot^-$ radical, which penetrates the haem crevice (Creutz & Sutin, 1973). Although cytochrome a is also considered to have a low-spin hexa-co-ordinate iron and to have a redox potential close to that of cytochrome c, it is clear that these two metalloproteins behave quite differently with
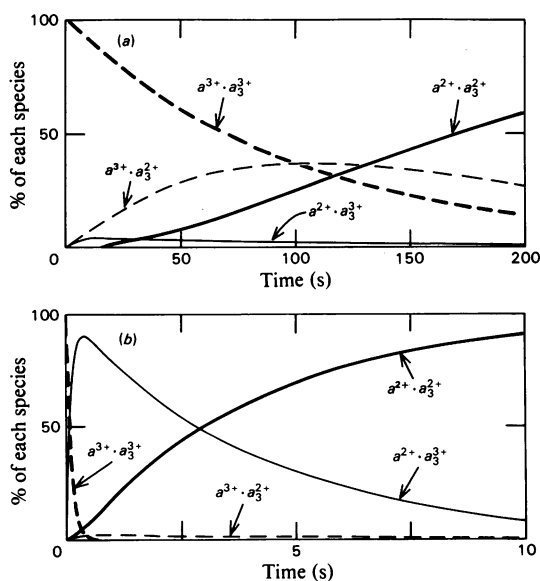
Fig. 5. *Computer simulation of the reduction of cytochrome c oxidase by sodium dithionite*
See the Materials and methods section (Schema 2). (a) Low dithionite concentration, approx. $50\,\mu M$ ($k_{obs.} = 0.01\,s^{-1}$, $k_1 = 0.25\,s^{-1}$); (b) high dithionite concentration, approx. $100\,\mu M$ ($k_{obs.} = 10\,s^{-1}$, $k_1 = 0.25\,s^{-1}$).

regard to dithionite reduction, cytochrome c being reduced approx. 10^3-fold more rapidly than cytochrome a. The data presented, however, do not at present allow us to distinguish between inner-sphere and outer-sphere mechanisms for the reduction of cytochrome a (or Cu_A) by the $SO_2^{\cdot-}$ ion.

Plastocyanin and stellacyanin also possess kinetically accessible sites, the Cu(II) atom being close to the surface with one of the histidine ligands exposed to the solvent. These proteins are rapidly reduced by dithionite.

The copper site of azurin, though similar in structure to that of plastocyanin and stellacyanin, except for a ligand change in the latter case, is appreciably more buried, as judged by the variability of self-exchange rates ($k_{11}^{corr.}$) (see Cummins & Gray, 1977).

The greater similarity of cytochrome c oxidase to azurin may well suggest that the initial electron-entry site in the oxidase enzyme is buried, and the relatively low rate reflects the kinetic inaccessibility of this site.

Electrostatic interactions between dithionite ions or the radical anion and the cytochrome c oxidase site are also relevant. The amino acid residues in the vicinity of the haem crevice of cytochrome c are largely positively charged and are considered by Koppenol et al. (1978) to direct negatively charged redox reactants to the haem edge. It is possible that a similar but reversed situation exists in cytochrome c oxidase, with negatively charged residues around the site repulsing the approach of anions. This suggestion agrees with the known strong electrostatic interactions between cytochrome c and its oxidase (van Buuren et al., 1974).

A negatively charged site on cytochrome c oxidase would also be in agreement with the relatively slight effect of added salt on the reduction rates (Table 1), and also with the very low rates of reduction by the doubly negatively charged $S_2O_4^{2-}$ species. The rates measured for this dianion are, under most conditions, very close to zero (see Table 1). However, arguments based solely on electrostatic considerations are only partially satisfactory. For example, in our hands (Table 1; cf. Lambeth & Palmer, 1973) the $S_2O_4^{2-}$ ion does not reduce cytochrome c (i.e. the measured rate constant is smaller than the s.e.m., as indicated by the F value), although, as pointed out, this protein has a positively charged site. However, this result is possibly due to the high salt concentration employed relative to that used by Lambeth & Palmer (1973).

It is possible that the low rate of $S_2O_4^{2-}$ reduction of cytochrome c oxidase is related both to charge repulsion and to the inaccessibility of the site to the larger reductant.

Our results concerning the order in which two haem groups are reduced by dithionite are in some conflict with the results reported by others (Halaka et al., 1981), but these discrepancies may well be due to the reductant concentrations employed. Under circumstances in which the internal electron transfer from cytochrome a to cytochrome a_3 is more rapid than the initial electron transfer to cytochrome a, it will appear that cytochrome a_3 is the first haem to be reduced. Such conditions pertain at low dithionite concentrations (approx. $50\,\mu M$), as employed by Halaka et al. (1981). To illustrate this point, we report in Fig. 5 computer simulations of the reaction at high and at low dithionite concentrations and demonstrate that the apparent order of haem reduction is a function of reductant concentration. For example, Fig. 5(a) shows that at low reductant concentrations the species $a^{3+} \cdot a_3^{2+}$ builds up while that of $a^{2+} \cdot a_3^{3+}$ remains close to zero. This is so even though the model is based on cytochrome a being the site reduced first. At higher dithionite concentrations (comparatively larger values of $k_{obs.}$; see Schema 2) the species $a^{2+} \cdot a_3^{3+}$ rapidly forms and cytochrome a_3 is reduced at a rate limited by internal electron transfer.

Finally, we point out that our analysis is only partial at this time and that a number of features remain unexplained. The time course of reduction of cytochrome c oxidase by a number of reducing

agents is heterogeneous, involving a very slow process with half-times $\geqslant 50$ s. This has been observed, not only for dithionite, but also for phenazine methosulphate (Halaka *et al.*, 1981), ruthenium hexammine (J. K. V. Reichardt & Q. H. Gibson, personal communication) and Cr^{2+} (Greenwood *et al.*, 1977). Whether or not this is related to the presence of multiple forms of resting cytochrome *c* oxidase, such as those observed by Brudvig *et al.* (1981), remains to be seen. However, analysis of the time course of reduction with these small molecules always yields one kinetic component with a rate constant $0.1–0.4$ s^{-1} [0.25 s^{-1} from our data; 0.19 s^{-1} from Halaka *et al.* (1981); 0.3 s^{-1} from Greenwood *et al.* (1977); $0.2–0.4$ s^{-1} from Scott & Gray (1980)]. These values stand in distinct contrast with the internal rate of reduction of cytochrome a_3 when resting cytochrome *c* oxidase is mixed with an excess of cytochrome *c*, the rate constant of this step being calculated as 5 s^{-1} (Wilson *et al.*, 1981). It is possible that the natural reductant cytochrome *c* and oxidant O_2 enhance this rate.

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