Reduction and Activity of Cytochrome *c* in the Cytochrome *c*–Cytochrome *aa*₃ Complex

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1. Uncharged reductants, such as *NNN'**N'*-tetramethyl-*p*-phenylenediamine and diaminodurene, reduce cytochrome *c* at both high and low ionic strength, unlike ascorbate, which is effective only at low ionic strength. 2. The 'tightly bound' cytochrome *c*–cytochrome *c* oxidase complex, with 1 equiv. of cytochrome *c* per cytochrome *aa*₃, can be prepared by simple mixing of the two component species. Its properties are not affected by co-sonication of the mixture. Bound cytochrome *c* is more rapidly reduced by *NNN'**N'*-tetramethyl-*p*-phenylenediamine and diaminodurene than is free cytochrome *c*. 3. At high ionic strength, when the complex is largely dissociated, addition of reductant under aerobic conditions in the presence of cyanide, or under anaerobic conditions, induces a rapid reduction of cytochrome *c* followed by the reduction of cytochrome *a*. At low ionic strength, addition of reductant induces a rapid reduction of cytochrome *a* while cytochrome *c* remains largely oxidized, the rate-limiting step now being the reduction of cytochrome *c*. 4. The results are interpreted in terms of direct reduction of cytochrome *c* in its tight complex with the oxidase, followed by rapid intramolecular electron transfer to both cytochrome *a* and the associated e.p.r.-detectable Cu atom.

The cytochrome *c*–cytochrome *c* oxidase complex prepared by simple mixing cannot readily be reduced by ascorbate (Mochan & Nicholls, 1972). In this respect cytochrome *c* in the complex behaves like cytochrome *c* at high ionic strengths (Minnaert, 1961; Yamazaki, 1962), and the two kinds of behaviour are explained by the common hypothesis that ascorbate reacts at an anion-binding site on the cytochrome *c* that can also be occupied by phosphate or by the cytochrome oxidase molecule itself.

Nevertheless, bound cytochrome *c* in the mitochondrion and submitochondrial particles seems to be catalytically competent (Nicholls, 1974), and Kuboyama *et al.* (1962*a*) have suggested that at least one form of the complex is more active in ascorbate oxidation than a mixture of components. Catalytic competence in the respiratory chain demands that the component concerned be capable of reacting with both an electron-donor system and an electron-acceptor system. Wilson *et al.* (1975) showed that electrons from cytochrome *c* can be accepted by at least two rapidly reacting sites on the

oxidase, and Nicholls & Hildebrandt (1978*b*) indicated that under near-equilibrium conditions both cytochrome *a* haem and the associated e.p.r.-detectable Cu atom could probably act as electron acceptors from either cytochrome *c* in solution or artificial reductants such as *NNN'**N'*-tetramethyl-*p*-phenylenediamine.

Whether such electron transfer can take place within the initially formed 'tight' complex between cytochrome *c* and cytochrome *c* oxidase seems uncertain. Ferguson-Miller *et al.* (1976) and Smith *et al.* (1979*a,b*) indicate rather low turnover rates for the 'tight' complex formed under kinetic conditions. van Gelder *et al.* (1979*a*) concluded that it was the low-affinity site, and not the 'tight' site, that was the primary electron-accepting site on the oxidase. The reactivity observed by Kuboyama *et al.* (1962*a,b*) is not necessarily in conflict with such a viewpoint, as the 'Kuboyama complex' contains more than 1 mol of cytochrome *c* per mol of cytochrome *aa*₃.

Mitochondria with no more than 1 mol of cytochrome *c* per mol of cytochrome *aa*₃ are capable of electron transfer from substrate to O₂. If mitochondrial cytochrome *c* is largely cytochrome oxidase-bound (Nicholls, 1974), this implies either

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that dissociation and reassociation is involved in reduction or that complex III is capable of reducing the bound cytochrome *c in situ* (Nicholls, 1976). Margoliash (1972) originally championed the latter viewpoint and Chance *et al.* (1970) the former one, but more recently Speck *et al.* (1979) have adopted the idea that reductase activity must involve at least a partial dissociation of the cytochrome *c*-cytochrome oxidase complex.

The present experiments were carried out to study further the effect of complex-formation on reducibility and the catalytic competence of the tightly associated complex of the molecule of cytochrome *c* and the 'molecule' of cytochrome *aa₃*. A preliminary account of part of the work was presented at the 11th International Congress of Biochemistry in Toronto (Hill, 1979).

Materials and Methods

Ox heart cytochrome *c* oxidase (cytochrome *aa₃*) was prepared by the method of Kuboyama *et al.* (1972) and stored at -75°C in 100 mM-sodium/potassium phosphate buffer (pH 7.4)/0.25% Tween 80 solution as described by Nicholls & Hildebrandt (1978a). Cytochrome *c* (horse heart) was type VI from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and the reagents Tween 80, sodium ascorbate, Tris base (Trizma) and Mops (4-morpholinepropane-sulphonic acid) were also from Sigma. NaN_3 , KCN and anthraquinonesulphonic acid were from BDH Chemicals (Poole, Dorset, U.K.). Diaminodurene was from Polysciences (Warrington, PA, U.S.A.) and *NNN'*-tetramethyl-*p*-phenylenediamine, as the dihydrochloride, from Pfaltz and Bauer (Stamford, CT, U.S.A.). Sephadex gels were from Pharmacia Fine Chemicals (Uppsala, Sweden). N_2 was Linde Prepurified grade (Union Carbide Canada, Toronto, Ont., Canada). All solutions were made up in glass-distilled water except for diaminodurene, a stock solution of which was made in ethanol before dilution with the appropriate buffered reaction medium.

Conventional spectrophotometry was carried out with an Aminco-Chance DW-2 instrument used in either split-beam or dual-wavelength mode. Stopped-flow spectrophotometry was performed either aerobically in the presence of cyanide or anaerobically in a Durrum-Gibson apparatus. Two instruments were used, one of which (at Brock University) comprised a Durrum D-110 stopped-flow unit linked to a Gilford single-beam monochromator-spectrophotometer and a Gould OS4000 storage oscilloscope. The signal was stored and fed into a standard 10 mV recorder via the Gould output unit 4001. The other instrument, located at the State University of New York at Buffalo, also contained the Durrum unit, but was modified for anaerobic

work with a stainless-steel flow compartment; the light-source was the Durrum monochromator and the signal was stored in a Tektronix oscilloscope interfaced with a computer system programmed for data accumulation and analysis [see Figs. 3–5 below containing print-outs of $\log(\text{absorbance change})$ versus time].

Anaerobic conditions were achieved with both systems by filling the storage syringes from a tonometer apparatus. Although such solutions can be made initially almost completely O_2 -free, leaks at the Teflon seals on the standard stopped-flow apparatus usually restore a few micromolar O_2 to the reaction syringes. The anaerobic experiments therefore required the use of the Teflon-free 'Buffalo' device.

Column chromatography of complexes was performed on $2\text{ cm} \times 25\text{ cm}$ Sephadex G-100 columns, equilibrated with the appropriate buffers at 4°C . Mixtures of cytochrome *c* and cytochrome *aa₃* were applied to the column and the spectrum of the leading fraction was taken after elution. In some cases the mixture was sonicated before being applied to the column. Sonication for periods of between 15 and 45 min with Ultrasonic model W-375 cell disrupter (micro-tip, power output 80 w) had no significant effect on complex-formation or stability (see the Results section).

Cytochrome *aa₃* concentrations were determined by using the ϵ_{mm} (reduced minus oxidized, 605–630 nm) value of $27\text{ mm}^{-1}\cdot\text{cm}^{-1}$ (Nicholls, 1978). Changes in the single-beam stopped-flow apparatus were measured at 605 and 445 nm (cytochrome *aa₃*) by using reduced-minus-oxidized absorption coefficients of 24.8 and $170\text{ mm}^{-1}\cdot\text{cm}^{-1}$ respectively, and at 550 nm (cytochrome *c*) by using an absorption coefficient of $19.7\text{ mm}^{-1}\cdot\text{cm}^{-1}$ (Margoliash & Frohwirt, 1959).

Results

Reduction of cytochrome *c* was first-order with respect to time and proportional to reductant concentration for all three systems employed: ascorbate, *NNN'*-tetramethyl-*p*-phenylenediamine (plus ascorbate) and diaminodurene, as observed by previous workers (Yamazaki, 1962; Minnaert, 1961). Thus the rate of reduction by ascorbate declined with increasing ionic strength from $1600\text{ M}^{-1}\cdot\text{s}^{-1}$ in 10 mM-Tris/Mops buffer to $300\text{ M}^{-1}\cdot\text{s}^{-1}$ in 10 mM-phosphate buffer to $60\text{ M}^{-1}\cdot\text{s}^{-1}$ in 67 mM-phosphate buffer, whereas the rate with *NNN'*-tetramethyl-*p*-phenylenediamine was slightly stimulated by low phosphate concentrations, changing from $4 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ in 10 mM-Tris/Mops buffer to $5.5 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ in 10 mM-phosphate buffer and declining slightly to $2 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$ in 67 mM-phosphate buffer (30°C ,

pH 7.4, 0.1% Tween). Table 1 summarizes the rates observed with the three reducing systems under the conditions used for stopped-flow experiments.

To study the influence of complex-formation with the oxidase on cytochrome *c* reduction, mixtures of the two were then made by dissolving crystals of cytochrome *c* in concentrated solutions of ox heart enzyme that had been dialysed against the appropriate buffer system. The initial cytochrome *c*/cytochrome *aa*₃ ratio varied from 2.5 to 5.5. At both high and low ionic strength two bands were seen, but at high ionic strength the leading band was green, whereas at low ionic strength it was more brown in hue because of the associated cytochrome *c*.

Table 2 summarizes the compositions of the chromatographic fractions obtained under several conditions. One molecule of cytochrome *c* is bound tightly to the enzyme at low ionic strength; a second molecule is bound more loosely. The complex can be obtained by simple mixing. Its formation is not promoted by co-sonication of its components. Reducing conditions do not affect its formation, and it is dissociated either by salts or by polycations. The catalytic activity of the complex, either in the 'mixed'

form or the 'co-sonicated' form, determined by the oxygen-electrode method, is the same as that of a mixture made directly in the polarographic chamber (not shown). It was concluded that at low ionic strength the behaviour of a 1:1 mixture of cytochrome *c* and enzyme would be that of the complex, whereas the behaviour at high ionic strength (0.3 M-phosphate buffer) would be that of a mixture permitting chemical interaction but in which both species existed largely in their dissociated forms.

Preliminary experiments showed that the complex was more reducible by diaminodurene than was a mixture of its components. Dual-wavelength experiments with 50 μ M-cyanide present under aerobic conditions showed that low diaminodurene concentrations that reduced cytochromes *c* (550–540 nm) and *a* (605–630 nm) over periods of several tens of seconds in 0.3 M-phosphate buffer reduced the mixture 'instantaneously' ($t_{\frac{1}{2}} < 0.5$ s) in 10 mM-Tris/Mops buffer.

With such a slowly reacting mixture, cytochromes *c* and *a* remain near equilibrium over the course of the reaction, with cytochrome *a* consistently slightly more reduced (E'_0 about 40 mV

Table 1. Rate constants for reduction of horse heart cytochrome *c* by three reducing systems at high and low ionic strengths

All reactions were carried out at pH 7.4 and 20°C in the presence of 0.1% Tween 80. Those performed at low ionic strength were performed in 10 mM-Tris/Mops buffer, and those at high ionic strength in 300 mM-sodium phosphate buffer. Cytochrome *c* reduction was monitored at 550 nm (single beam) or 550–540 nm (dual wavelength), with concentrations between 2 and 7 μ M and reductant concentrations giving reaction half-times varying from 0.1 to 50 s. Abbreviation: TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

Reductant	k ($\text{M}^{-1} \cdot \text{s}^{-1}$)	
	Low ionic strength	High ionic strength
Ascorbate	725	25
TMPD + ascorbate (18 μ M)	3.5×10^4	3×10^4
Diaminodurene	2.8×10^3	4.6×10^3

Table 2. Complex-formation between cytochrome *c* and cytochrome *aa*₃ demonstrated by Sephadex-gel chromatography. Chromatography was performed on 2 cm $\times$ 25 cm columns of Sephadex G-100 at high ionic strength [50 mM-sodium phosphate buffer (pH 7.4)/0.1% Tween-80] or at low ionic strength [10 mM-Tris/Mops buffer (pH 7.4)/0.1% Tween-80]. Initial cytochrome *c*/cytochrome *aa*₃ ratios varied from 2.5 to 6.0, with starting cytochrome *aa*₃ concentrations of approx. 50 μ M. Abbreviation: TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

Conditions	Cytochrome <i>c</i> /cytochrome <i>aa</i> ₃ ratio (leading fraction)	
	High ionic strength	Low ionic strength
No sonication	0	0.95–1.50
15 min sonication	0	0.95
45 min sonication	0	1.10
No sonication + ascorbate (2 mM) + TMPD (150 μ M)	0	1.35–1.50
No sonication + polylysine (30 mg/ml)	—	0.35

more positive than that of cytochrome *c*), according to eqn. (1):

where Cu_D is the e.p.r.-detectable Cu atom. The value of K_3 in the presence of both cytochromes is therefore obtained by dividing the total reduction rate by the product of ferric cytochrome *c* and reductant concentrations. Because there are two electron acceptors associated with cytochrome *a*, presumably haem and copper (Nicholls & Hildebrandt, 1978*b*; Wilson *et al.*, 1975), twice the reduction rate of cytochrome *a* must be added to that of cytochrome *c*. If this sum is equal to the rate

of cytochrome *c* reduction in free solution, we conclude that the presence of the oxidase has not affected the reduction process directly. Thus with $60\mu\text{M}$ -diaminodurene added to $7\mu\text{M}$ -cytochrome *c* and $7\mu\text{M}$ -cytochrome *aa*₃ in 300mM-phosphate buffer containing 0.1% Tween 80 and $50\mu\text{M}$ -KCN, the reduction rate of cytochrome *c* was 0.075 s^{-1} and that of cytochrome *a* 0.12 s^{-1} , giving a sum of 0.32 s^{-1} . In separate experiments the corresponding reduction rates were 0.3 s^{-1} and 0.06 s^{-1} , giving the essentially similar sum of 0.42 s^{-1} .

Fig. 1 shows the results of stopped-flow experiments with diaminodurene as reductant for cytochrome *c*/cytochrome *aa*₃ mixtures in the

Fig. 1. Stopped-flow time courses of reduction of cytochrome *c* and cytochrome oxidase in the presence of cyanide by diaminodurene

(a) Reaction at high ionic strength. The assay medium was 300mM-sodium phosphate buffer (pH 7.4)/0.1% Tween 80 at 20°C . One syringe contained $3.6\mu\text{M}$ -cytochrome oxidase, $3.0\mu\text{M}$ -cytochrome *c* and $80\mu\text{M}$ -KCN dissolved in buffer, and the other syringe contained 2mM-diaminodurene and 0.5% ethanol in buffer. Absorbance change was monitored aerobically in the 'Brock' stopped-flow apparatus attached to a Gilford single-beam spectrophotometer. The wavelengths employed are indicated on the Figure. (b) Reaction at low ionic strength. The assay medium was 10mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C . One syringe contained $5.10\mu\text{M}$ -cytochrome oxidase, $4.6\mu\text{M}$ -cytochrome *c* and $100\mu\text{M}$ -KCN in buffer. The other syringe contained 0.5mM-diaminodurene and 0.125% ethanol in buffer. Other conditions were as in (a).

presence of cyanide. Fig. 1(a) shows the reduction pattern at three wavelengths, 550, 605 and 445 nm, at high ionic strength. At 445 and 605 nm, an initial lag in reduction is seen, but the two traces show identical reduction half-times. Reduction at 550 nm occurs almost exponentially. Thus the reduction of cytochrome *c* precedes that of the oxidase. At higher diaminodurene concentrations the duration of the lag phase at 445 and 605 nm decreased, but at all reductant concentrations employed the reoxidation of cytochrome *c* was measurably slower than its reduction. In Fig. 1(b) the experiment is carried out at low ionic strength. A lag phase is again apparent, but in this case it is in the reduction of cytochrome *c*, and the reduction of cytochrome *a* (traces at 605 and 445 nm) is almost exponential in character. In the complex the net reduction of cytochrome *a* precedes that of cytochrome *c*; the latter's re-oxidation is faster than its reduction.

Unlike the situation in the conventional spectrophotometer, in the experiments of Fig. 1 the reaction is removed from equilibrium; thus the rate of reduction of cytochrome *c* in Fig. 1(a) is identical with its rate of reduction alone. The rate of electron entry into the complex (the sum of the rate of cytochrome *c* reduction plus twice the rate of

cytochrome *a* reduction) indicates a second-order reduction rate constant for reduction of bound cytochrome *c* by diaminodurene of $5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, over 10 times the rate in solution (Table 1).

Fig. 2 shows the results of similar experiments with ascorbate plus *NNN'*-tetramethyl-*p*-phenylenediamine as reductant system. At high ionic strengths (Fig. 2a) reduction of cytochrome *c* precedes that of cytochrome *a*; at low ionic strength (Fig. 2b) reduction of cytochrome *a* precedes that of cytochrome *c*. The general features are similar to those seen with diaminodurene, except that small initial rapid phases are seen at 550 and 605 nm, preceding the 'lag' phases. As these are not seen at 445 nm, they may be attributed to the transient formation of the oxidized cation radical, Wurster's Blue (Nicholls, 1976). The calculated rate constant for reduction of bound cytochrome *c* by *NNN'*-tetramethyl-*p*-phenylenediamine is now $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, a 5-fold increase over that seen with free cytochrome *c* (Table 1).

Ascorbate alone behaves quite differently from ascorbate plus *NNN'*-tetramethyl-*p*-phenylenediamine. At high ionic strength the mixture of cytochrome *c* and cytochrome *aa*₃ is reduced in a quasi-equilibrium fashion according to eqn. (1), but

Fig. 2. Stopped-flow time courses of reduction of cytochromes *c* and *aa*₃ by ascorbate plus *NNN'*-tetramethyl-*p*-phenylenediamine in media of high and low ionic strength

(a) The assay medium was 300 mM-sodium phosphate buffer (pH 7.4)/0.1% Tween 80 at 20°C. In one syringe was 3.6 μM -cytochrome *c*, 3.8 μM -cytochrome *aa*₃ and approx. 30 μM -KCN (partially degassed) and in the other syringe was degassed 1 mM-ascorbate and 150 μM -*NNN'*-tetramethyl-*p*-phenylenediamine in buffer. (b) The assay medium was 10 mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C. In one syringe was 5.8 μM -cytochrome *c*, 5.4 μM -cytochrome *aa*₃ and approx. 30 μM -KCN (partially degassed) and in the other syringe was degassed 2 mM-ascorbate and 150 μM -*NNN'*-tetramethyl-*p*-phenylenediamine in buffer. Absorbance changes were measured in the 'Brock' stopped-flow system described in the Materials and Methods section, at the wavelengths indicated on the Figure.

at low ionic strength cytochrome *c* in the complex is reduced very slowly, as reported by Mochan & Nicholls (1972). Cytochrome *a* is reduced to a greater extent than is cytochrome *c* at low ionic strengths, presumably via the small amount of free reduced cytochrome *c* in solution.

Results obtained in the fully anaerobic system were similar to those obtained in the presence of cyanide. It may be noted that isolated cytochrome *aa*₃ in the 'resting' ferric state is rather inactive until reduction and/or respiration have been initiated (Antonini *et al.*, 1977; Nicholls & Hildebrandt,

Fig. 3. Anaerobic reduction of cytochrome *c* in the cytochrome *c*-cytochrome *aa*₃ complex by ascorbate plus NNN'N'-tetramethyl-*p*-phenylenediamine

The assay medium was 10mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80, at 20°C. In one syringe was 5.8 μM -cytochrome *c* and 5.4 μM -cytochrome *aa*₃ in buffer. In the other syringe was 2mM-ascorbate and (a) 750 μM -NNN'N'-tetramethyl-*p*-phenylenediamine, (b) 75 μM -NNN'N'-tetramethyl-*p*-phenylenediamine or (c) 7.5 μM -NNN'N'-tetramethyl-*p*-phenylenediamine. Data are in the form of semi-logarithmic plots of absorbance change against time as printed out from the on-line computer of the 'Buffalo' stopped-flow system. The wavelength monitored was 550 nm.

1978a), and that therefore the initial reduction seen is that of cytochrome *a* only, at a rate differing by at least two orders of magnitude from that of cytochrome *a*₃ (Andréasson, 1975). Fig. 3 shows the

Fig. 4. Anaerobic reduction of cytochrome *a* in the cytochrome *c*-cytochrome *aa*₃ complex by ascorbate plus NNN'N'-tetramethyl-*p*-phenylenediamine

The assay medium was 10mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C. In one syringe was 5.8 μM -cytochrome *c* and 5.4 μM -cytochrome *aa*₃ in buffer. In the other syringe was 2mM-ascorbate and (a) 750 μM -NNN'N'-tetramethyl-*p*-phenylenediamine, (b) 75 μM -NNN'N'-tetramethyl-*p*-phenylenediamine or (c) 7.5 μM -NNN'N'-tetramethyl-*p*-phenylenediamine. Data are in the form of semi-logarithmic plots of absorbance change against time as printed out from the on-line computer of the 'Buffalo' stopped-flow system. The wavelength monitored was 605 nm.

reduction of cytochrome *c* in its complex with the cytochrome *aa*₃ in the absence of O₂, by several different *NNN'**N'*-tetramethyl-*p*-phenylenediamine concentrations. At 550 nm, a lag phase is seen, whose duration is inversely related to the concentration of *NNN'**N'*-tetramethyl-*p*-phenylenediamine. The reduction of cytochrome *a* at 605 nm (Fig. 4) shows no lag phase, and closely parallels the initial reduction at 445 nm (Fig. 5). However, in the anaerobic system a second phase was seen at the latter wavelength, contributing about half the total absorbance change. This slow reduction, not seen in the aerobic cyanide system (Fig. 1), was presumably due to the appearance of reduced cytochrome *a*₃ (Gibson *et al.*, 1965).

The calculated rate constant for reduction of bound cytochrome *c* under these conditions was $1.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, in essential agreement with the value obtained with the cyanide-inhibited complex

Fig. 5. Anaerobic reduction of cytochromes *a* and *a*₃ in the cytochrome *c*-cytochrome *aa*₃ complex by ascorbate. The assay medium was 10 mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C. In one syringe was 5.4 μM-cytochrome *aa*₃ and 5.8 μM-cytochrome *c* in buffer. In the other was 2 mM-ascorbate and (a) 75.0 μM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine or (b) 7.5 μM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine. Data are in the form of semi-logarithmic plots of absorbance change against time as printed out from the on-line computer of the 'Buffalo' stopped-flow system. The wavelength monitored was 445 nm.

(Fig. 2) and about 4-fold greater than the reduction rate in free solution.

An estimate of the degree to which the cytochrome *c*-cytochrome *a* system approximates to equilibrium during the transition to the reduced state may be made by calculating the mass-action product (Nicholls, 1976) according to eqn. (2):

$$\Gamma = [a^{2+}][c^{3+}]/[a^{3+}][c^{2+}] \quad (2)$$

where Γ may be computed for all times after the initial addition of reductant until $t = \infty$, at which $\Gamma = K_{\text{eq}}$. Fig. 6 shows the changes in the calculated value of Γ over time for the three concentrations of *NNN'**N'*-tetramethyl-*p*-phenylenediamine used in Figs. 3 and 4. (The values of cytochrome *a*²⁺ were determined at 605 nm uncorrected for cytochrome *a*₃ contribution, since we did not discern a slower phase of reduction at this wavelength. In addition, the Γ values behaved similarly at low *NNN'**N'*-tetramethyl-*p*-phenylenediamine concentrations with the cyanide-bound oxidase, where

Fig. 6. Electron transfer between cytochromes *c* and *a* as a function of time in the electrostatic cytochrome *c*-cytochrome *aa*₃ complex.

Γ values at discrete times are plotted against time for three different concentrations of *NNN'**N'*-tetramethyl-*p*-phenylenediamine indicated where Γ values are the apparent mass-action ratio values, $[a^{2+}][c^{3+}]/[a^{3+}][c^{2+}]$, during the course of the reaction (see the text). The assay medium was 10 mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C. All other conditions were the same as indicated in the legends to Figs. 3 and 4. (a) 375 μM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine; (b) 37.5 μM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine; (c) 3.75 μM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine.

Table 3. Rate constants (cf. eqn. 1) for cytochrome *c* reduction in low-ionic-strength medium in the absence and presence of cytochrome *c* oxidase

Rate constants were measured in 10mM-Tris/Mops buffer (pH 7.4)/0.1% Tween 80 at 20°C, in the presence or absence of equimolar concentrations of ox heart cytochrome *aa*₃. Cytochrome *c* concentrations between 2 and 9 μM were employed, and the reduction was monitored at 550nm. Abbreviation: TMPD, *NNN'**N'*-tetramethyl-*p*-phenylenediamine. The rate constant (*k*₃) is obtained from the slope of a plot of reductant concentration against rate.

Reductant	<i>k</i> ₃ (M ⁻¹ ·s ⁻¹)	
	Isolated cytochrome <i>c</i>	Bound cytochrome <i>c</i>
Ascorbate	~800	≤10
TMPD	3.5 × 10 ⁴	1.3 × 10 ⁵
Diaminodurene	2.8 × 10 ³	5.0 × 10 ⁴

cytochrome *a*₃ remains oxidized.) In all three cases the Γ value approaches 5 at longer time intervals. This must approximate the value of K_{eq} . At high *NNN'**N'*-tetramethyl-*p*-phenylenediamine concentrations and shorter times (trace *a*), $\Gamma < K_{eq}$, as expected for a reaction out of equilibrium. At the intermediate concentration (trace *b*), $\Gamma \approx K_{eq}$, throughout the reaction, as expected for a reaction proceeding under near-equilibrium conditions (eqn. 1). But at the lowest *NNN'**N'*-tetramethyl-*p*-phenylenediamine concentration (trace *c*) the initial reduction of cytochrome *a* so far exceeds that of cytochrome *c* that $\Gamma \gg K_{eq}$. Such behaviour, which requires an extension of the model in eqn. (1), is further discussed below.

Table 3 summarizes the rate constants obtained for the reduction of bound cytochrome *c*.

Discussion

Early transient-state kinetic studies of the reduction of cytochrome *aa*₃ by ferrocytochrome *c* (Gibson *et al.*, 1965) treated the process as a bimolecular reaction involving cytochrome *c* and the cytochrome *a* portion of the oxidase, and reported second-order rate constants of about 10⁷M⁻¹·s⁻¹. Later work focused on the number and type of rapid electron acceptors in the cytochrome oxidase molecule (Wilson *et al.*, 1975; van Buuren *et al.*, 1974; Greenwood *et al.*, 1976). From Andréasson (1975), as well as Wilson *et al.* (1975), it is possible to conclude that two electrons can be donated to the fully oxidized enzyme. The first electron is in rapid equilibrium between cytochrome *a* and the e.p.r.-detectable Cu atom, and the second completes the reduction of both, giving a 'half-reduced' form of the enzyme (cf. Nicholls & Hildebrandt, 1978*b*).

Early steady-state kinetic studies (Smith & Conrad, 1956; Minnaert, 1961) pointed out the possibility of both inhibitory and active complexes of cytochrome *c* and cytochrome *aa*₃. More recent steady-state experiments (Errede & Kamen, 1978; Ferguson-Miller *et al.*, 1976) indicate the presence of

a tight binding site for cytochrome *c* on the oxidase. van Gelder *et al.* (1979*a*) in stopped-flow experiments find that reduction of the enzyme takes place rapidly even when the tight site is occupied by an inert cytochrome *c* analogue such as porphyrin cytochrome *c*. But more recently they have observed a rapid partial reduction of cytochrome oxidase when mixing ferrocytochrome *c* with excess oxidase, and they conclude that this represents rapid electron transfer from cytochrome *c* bound at the tight site (van Gelder *et al.*, 1979*b*). This rapid step occurs with a half-time of about 2ms, almost identical with that observed by Chance (1967) for the oxidation of cytochrome *c* bound in intact mitochondria.

The 1:1 cytochrome *c*-cytochrome *aa*₃ complex prepared in the present experiments, tightly associated by chromatographic criteria, was reversible by salt addition. Unlike that of Kuboyama *et al.* (1962*a*), our complex was not affected by sonication, although we have found that cytochrome *aa*₃-containing vesicles may form a less readily reversible complex with added cytochrome *c* (P. Nicholls & V. Hildebrandt, unpublished work; cf. Nicholls, 1974). Reduced cytochrome *c* apparently binds to partially reduced oxidase (Table 2), in accordance with the kinetic prediction of Smith & Conrad (1956) and in contrast with the suggestion by Schroedl & Hartzell (1977) that oxidation of cytochrome *a* is needed for cytochrome *c* binding to occur.

Reduction of cytochrome oxidase in the complex presumably relies on the mediation of bound cytochrome *c* and, in agreement with previous workers (Wilson *et al.*, 1975), results in the reduction of the cytochrome *a* haem and probably another acceptor, which may be the e.p.r.-detectable Cu atom (Cu_D; eqn. 1). At low ionic strength, when the complex is the predominant species, the reductibility of bound cytochrome *c* and therefore the oxidase by anionic ascorbate is markedly depressed, whereas reactivity towards the neutral reductants *NNN'**N'*-tetramethyl-*p*-phenylenediamine and di-

aminodurene is enhanced (Table 3). Since cytochrome *c* dissociates very slowly from the complex, the neutral reductants must be able to reduce bound cytochrome *c*. Assuming that cytochrome *a* is reduced via cytochrome *c*, the intramolecular rate constant (k_2) may be derived from the kinetics at 605 nm (Fig. 4) according to eqn. (3):

$$v = k_2[c^{2+}a^{3+}]/2 \quad (3)$$

where the factor 2 appears because the reduction of the Cu atom is probably dynamically linked to the reduction of the haem (eqn. 1; Greenwood *et al.*, 1976). In Figs. 3–5, when $[c^{2+}a^{3+}]$ is 0.25 μ M (50% reduction of cytochrome *a*, 16% reduction of cytochrome *c*), v is 35 μ equiv. $\cdot$ s⁻¹; the value of k_2 is then obtained as 280 s⁻¹, well over the maximal catalytic-centre activity under similar conditions of approx. 100 s⁻¹.

Although it is possible that some free reduced cytochrome *c* contributes to the observed reduction, the maintenance of Γ below the equilibrium value (Fig. 6) over the first 30 ms supports the idea that the reaction observed is genuinely intramolecular. The role of such tightly bound cytochrome *c* in the functioning of the respiratory chain has been discussed by Nicholls (1965, 1974) and by Ferguson-Miller *et al.* (1976). This theory requires that the physiological reductase system (cytochrome *bc*₁ complex) be capable of reducing bound cytochrome *c* directly, like *NNN'*-tetramethyl-*p*-phenylenediamine or diaminodurene, and unlike ascorbate. Mutual incompatibility of reaction media has so far prevented direct checks of this hypothesis with isolated reductases and cytochrome *c* oxidase (Mochan & Nicholls, 1972; A. N. Malviya & P. Nicholls, unpublished work). Ascorbate reducibility of all cytochrome *a* under conditions where bound cytochrome *c* is poorly reactive, and *NNN'*-tetramethyl-*p*-phenylenediamine reducibility of all cytochrome *c* under conditions where bound cytochrome *c* is in the form of the non-reducible cyanide complex (B. C. Hill & P. Nicholls, unpublished work), do, however, show that the route via free cytochrome *c* and the secondary binding site is kinetically significant (cf. van Gelder *et al.*, 1979a).

Mochan & Nicholls (1972) suggest that the areas on the cytochrome *c* molecule for phosphate binding, ascorbate reduction and oxidase interaction, all overlap. We now propose that *NNN'*-tetramethyl-*p*-phenylenediamine and diaminodurene interact at a different site on the cytochrome *c* molecule, thus allowing for its reduction while bound in the cytochrome *c*-cytochrome *aa*₃ complex. This site is also available to attack by certain pteridine reagents when cytochrome *c* is bound to phospholipid vesicles (Cannon & Erman, 1978). It may also be the site by which electrons enter from cytochrome *c*₁ (Nicholls, 1976; Chiang & King,

1979). Although certain cytochrome *c* modifications affect its catalytic competence in both reductase and oxidase assays (Speck *et al.*, 1979), we do not believe that this finding demonstrates that the pathway of electron transfer must be similar in the two cases.

The entry of electrons into the cytochrome oxidase within the complex does not reflect a simple progress to equilibrium (Fig. 6). Both in the cyanide-inhibited system (Figs. 1 and 2) and in the initial stages in the anaerobic system (Figs. 3 and 4) cytochrome *a* always behaves as if it had a more positive redox potential than bound cytochrome *c* (+230 mV; Kimelberg & Lee, 1970). Quasi-equilibrium ($K_{eq} \sim 5$; Fig. 6) occurs at an apparent E'_0 value of about +270 mV. But the intermediate Γ value of 14 at 4 s after addition of low concentrations of *NNN'*-tetramethyl-*p*-phenylenediamine (Fig. 6, trace c) indicates the existence of an initially more positive potential that relaxes to the less positive state at longer time intervals. Nicholls & Petersen (1974) and Babcock *et al.* (1978) have proposed that the E'_0 of cytochrome *a* is a function of the redox state of other groups on the enzyme.

Although interpretation of intramolecular events may be complicated, some degree of catalytic competence for the 1:1 complex formed between cytochrome *c* and cytochrome *c* oxidase seems proven. The relative importance of, and differences in behaviour between, the two types of cytochrome *c* binding site remain an area of uncertainty (Ferguson-Miller *et al.*, 1978; Errede & Kamen, 1978; Smith *et al.*, 1979b).

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