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CYTOCHROME *c* REACTIVITY IN ITS COMPLEXES WITH MAMMALIAN CYTOCHROME *c* OXIDASE AND YEAST PEROXIDASE

EUGENE MOCHAN[a,*] AND PETER NICHOLLS[b,**]

[a] *Department of Biochemistry, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.) and* [b] *Laboratory of Biochemistry, University of Amsterdam, Plantage Muidergracht 12, Amsterdam C (The Netherlands)*

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SUMMARY

1. The ascorbate reducibility of cytochrome *c* (beef or horse heart) in its complexes with cytochrome *c* oxidase (beef heart) and cytochrome *c* peroxidase (yeast) has been studied.

2. The rate of cytochrome *c* reduction in both cases is diminished by about 90 % in media of low ionic strength (5 mM phosphate, pH 7.0–7.4), the second order velocity constant decreasing from approximately 100 $M^{-1} \cdot s^{-1}$ to between 9 and 12 $M^{-1} \cdot s^{-1}$.

3. The observed decrease in ascorbate reducibility is consistent with the formation of 1:1 complexes between cytochrome *c* and cytochrome aa_3 (*i.e.* 1 cytochrome *c*: 2 heme *a*), and between cytochrome *c* and cytochrome *c* peroxidase (1 cytochrome *c*:1 cytochrome *c* peroxidase) , with dissociation constants between 10^{-6} and 10^{-7} M in 5 mM phosphate.

4. The rate of the cyanide reaction with ferric cytochrome *c*, and the rate of spontaneous release of cyanide from cyanferrocytochrome *c*, are not appreciably affected by the binding of cytochrome *c* in a complex with cytochrome *c* oxidase.

5. An analogy is drawn between the decrease in the rate of cytochrome *c* reduction in the complexes and the decrease occurring at high ionic strength in free solution; it is concluded that the result does not militate against the view that the cytochrome *c*–oxidase complex is the physiologically active form of cytochrome *c* in the mitochondrial membrane.

6. From the rate of ascorbate reduction of membrane-bound ("endogenous") cytochrome *c* it is calculated that between 60 and 80 % may be bound in the form of the oxidase complex.

INTRODUCTION

The reactivity of bound cytochrome *c* has been the subject of disagreement. Tsou[1] reported that the endogenous cytochrome *c* of submitochondrial particles

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine; cytochrome aa_3, the presumed minimal functional unit of cytochrome *c* oxidase, containing two heme *a* equivalents (one of cytochrome *a* and the other of cytochrome a_3).

* To whom correspondence should be addressed.

** Present address: Sidney Sussex College, Cambridge, Great Britain.

failed to react with cyanide, Slater[2] that such cytochrome *c* was only slowly reduced by ascorbate. Chance and co-workers[3,4] have found that the bound ferrous form reacts with ferricyanide and with the cytochrome *c* peroxidase–peroxide system, but at reduced rates compared to soluble cytochrome *c* and with evidence of heterogeneity. A possible decline in the redox potential of cytochrome *c* on binding in the mitochondrial membrane[5] would require that either the rate of oxidation or the rate of reduction (or both) must differ from that in solution.

Various authors have described the formation of cytochrome *c*–cytochrome oxidase complexes[6–9]. We have advanced the view that membrane-linked cytochrome *c* is largely in the form of such a complex [10]. We have also claimed[11] that such "endogenous" cytochrome *c* reacts with reducing agents and with cyanide as does free cytochrome *c*. Similarly we have shown that oxidase bound cytochrome *c* is still catalytically active[8] and that a similar situation appears to prevail with cytochrome *c* peroxidase of yeast[12,13]. Some such reactivity must of course be retained if the bound cytochrome *c* is to act as a catalytically effective electron transfer agent. The alternative possibility would be a certain mobility of cytochrome *c* from the oxidase-bound form to a reductase-bound[14] form, *via* a flexible lipid link[3,15] rather than in a "locked" electrostatic complex with the oxidase.

We now wish to report experiments on enzyme-bound cytochrome *c*, describing its ascorbate reducibility and reactivity with cyanide. At low ionic strength we find that the rate of the ascorbate reaction is diminished on binding, thus persuading us to modify our earlier views[8] concerning the reduction of bound cytochrome *c*. However, we intend to show that this may still be consistent with a functional role for the cytochrome *c*–oxidase complex (and possibly the cytochrome *c*–yeast peroxidase complex) in electron transfer in the mitochondrial membrane.

MATERIALS AND METHODS

Cytochrome *c* oxidase, prepared from beef heart by a modification of the method of Fowler *et al.*[16], was the generous gift of Mr K. J. H. van Buuren of the Jansen Institute. Cytochrome oxidase concentrations are expressed in molarity of heme *a* ($\Delta\varepsilon_{mM}$(605–630 nm) reduced–oxidized = 14.0). The cytochrome aa_3 concentration is exactly one half of this value. Cytochrome *c* peroxidase and apo-cytochrome *c* peroxidase were prepared from yeast by the methods of Yonetani and Ray[17] and Yonetani[18], respectively. Beef heart cytochrome *c* was kindly provided by Dr B. F. van Gelder of the Jansen Institute. Horse heart cytochrome *c* was Sigma type III or type VI (Sigma Chemical Co.).

Spectrophotometry was carried out with Unicam SP1800 (cytochrome *c* peroxidase) and Cary 14 (cytochrome oxidase) spectrophotometers. The $\Delta\varepsilon_{mM}$ (reduced–oxidized) for cytochrome *c* 550 nm was taken as 19.7.

Sodium ascorbate was obtained from Sigma Chemical Co. or from British Drug Houses. Analar KCN was dissolved in glass distilled water and neutralized with HCl before use. Sodium dithionite was stored in dark air-tight containers in the cold. Reactions involving cytochrome oxidase were carried out at 25 °C in 5 mM potassium sodium phosphate buffer pH 7.4, containing 0.5 % Tween-80. The oxidase before dilution was stored as an approximately millimolar solution (heme *a*) in 50 mM Tris buffer pH 7.8 containing 0.5 % Tween-80. Reactions involving cytochrome *c* peroxidase were performed at 25 °C in 5 mM potassium phosphate buffer pH 7.0.

RESULTS

Fig. 1 illustrates typical time courses for cytochrome *c* reduction by ascorbate in the presence and absence of cytochrome oxidase, in phosphate buffer containing sufficient cyanide to inhibit the catalytic oxidation of cytochrome *c* by the oxidase. When ascorbate is added to a system containing oxidase, cytochrome *c* and cyanide (Fig. 1B), the reduction rate of cytochrome *c* is dramatically diminished (*cf.* Fig. 1A). Since the reaction of cytochrome oxidase with cyanide is slow in the absence of substrate[19], the reduction is not monotonically exponential as it is in the absence of the oxidase. After a few seconds, however, the oxidase becomes inhibited and the reduction of cytochrome *c* proceeds at a maximal rate about 10 % of that in the absence of the oxidase. Under these conditions, where cyanide was added immediately before the ascorbate, the reaction of cyanide with cytochrome *c* itself is too slow ($t_{0.5}$ approx. 1 h, see below) to influence the course of reduction. Subsequent addition of a 10-fold greater quantity of ascorbate (Fig. 1C) gives a rate of reduction equivalent to that produced by the original concentration of ascorbate added to cytochrome *c* free in solution.

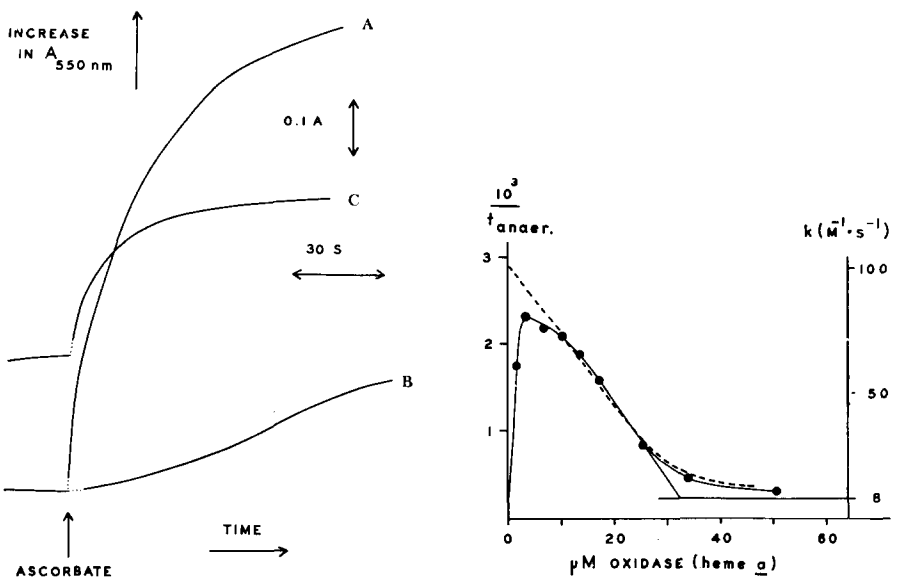

Fig. 1. Reduction of cytochrome *c* by ascorbate in the presence and absence of cytochrome oxidase. Ascorbate added as indicated to 39 μM cytochrome *c* in 5 mM phosphate, 25 °C, pH 6.8 with 1.8 mM cyanide. A, control (cytochrome *c* alone), + 0.85 mM ascorbate. B, *plus* 66 μM (heme *a*) oxidase, + 0.85 mM ascorbate. C, system from B allowed to reach equilibrium inhibition (after approx. 5 min), + 8.5 mM ascorbate.

Fig. 2. Effect of increasing oxidase concentration on anaerobiosis time in presence of fixed cytochrome *c* and ascorbate concentrations. $t_{anaer.}$ = anaerobiosis time in s. ●—●, experimental observations; – – – –, theoretical curve for decline in rate of ascorbate reduction ($k_{free\ c}$ = 100 $M^{-1}\cdot s^{-1}$; $k_{bound\ c}$ = 8 $M^{-1}\cdot s^{-1}$; and $[cyt.]_{free}[oxidase]_{free}/[cyt.]_{bound} = K_d$ = 0.33 μM; 17 μM cytochrome binding sites contained in 31 μM oxidase), where $1/t_{anaer.}$ $\alpha v = (k_{free}\ [cyt.]_{free} + k_{bound}\ [cyt.]_{bound})$ [ascorbate]. 2 mM ascorbate, 17 μM cytochrome *c*, oxidase as indicated. 5 mM phosphate, pH 7.4, 25 °C. Left hand ordinate: $10^3/t_{anaer.}$ = rate of respiration (μequiv·s^{-1}). Right hand ordinate: effective 2nd order velocity constant for reduction of cyt. *c* by ascorbate ($M^{-1}\cdot s^{-1}$).

It is difficult to compare quantitatively rates of cytochrome *c* reduction measured directly in the presence and absence of oxidase; even under anaerobic conditions eletrons reaching cytochrome *c* are immediately equilibrated into cytochromes *a* and a_3 and possibly the oxidase copper atoms. To check the semi-quantitative result of Fig. 1, we therefore measured the anaerobiosis times for the oxidation of ascorbate by cytochrome *c*–cytochrome oxidase mixtures at a fixed concentration of cytochrome *c* and progressively increasing concentrations of the oxidase. The results of such a series of experiments are summarized in Fig. 2.

The anaerobiosis time in absence of either cytochrome *c* or oxidase (due to ascorbate autoxidation) is too long to be measured satisfactorily in an open cuvette (even with a covering layer of paraffin)*. At low concentrations of oxidase, as previously reported[20], a rate of reaction (proportional to the reciprocal of the anaerobiosis time) is found which increases with the oxidase concentration. Above 5 μM oxidase this trend is lost. The steady state reduction level of cytochrome *c* is now almost imperceptible and the reaction rate is solely determined by the rate of electron transfer from ascorbate to cytochrome *c*. This latter part of the curve may be approximately fitted by an equation assuming that the maximum rate of reduction of cytochrome *c* by ascorbate is governed by an apparent velocity constant of 100 $M^{-1} \cdot s^{-1}$, and the minimum rate (when all cytochrome *c* is bound) by a constant of 8 $M^{-1} \cdot s^{-1}$. The number of cytochrome *c* binding sites (high affinity) is half the heme *a* concentration (or equal to the concentration of 'cytochrome aa_3'), and the apparent dissociation constant between 10^{-6} and 10^{-7} M; the theoretical curve in the figure is drawn with $K_d = 0.33$ μM, and the dependence of K_m on cation concentration[8] indicates a probable binding constant of 0.5-2.0 μM in 5 mM phosphate**. Although this is slightly higher than the value obtained directly by the method of Fig. 2, it should be noted that secondary binding sites associated with cytochrome *a* can introduce an effect interpreted as a rather higher affinity than the primary site actually shows[21]. The problem of the binding constant and number of binding sites does not alter the conclusion concerning the reactivity of cytochrome *c*, that the rate of reduction by ascorbate is decreased to about 10 % of that in free solution.

In 5 mM phospahte at pH 7.3 a rate constant of 120 $M^{-1} \cdot s^{-1}$ was obtained for the reduction of 38 μM cytochrome *c* by ascorbate (concentrations from 0.3–4 mM). Addition of 66 μM (heme *a*) oxidase reduced this rate constant to 30 $M^{-1} \cdot s^{-1}$ (4 mM ascorbate). The apparent rate of reduction of cytochrome *c* in such a system, measured during the transition to anaerobiosis was about 0.04 s^{-1}. The apparent rate of reduction of cytochrome *a* (also measured at anaerobiosis using the absorption at 605 nm) was 0.02 s^{-1}. As two electrons are added per equiv of cytochrome *a* or a_3 reduced[22] and as cytochrome *c* is delivering electrons to the bound oxidase concurrently with its own reduction (Eqn 1),

$$\text{Ascorbate} \xrightarrow[\text{slow}]{e^-} \text{cytochrome } c \overset{e^-}{\underset{\text{rapid}}{<}} \begin{cases} \text{cytochrome } a \\ \text{cytochrome } a_3 \\ 2\,Cu^{2+} \end{cases} \qquad (1)$$

* Polarographic experiments suggest that the observed autoxidation rate of 0.2 μM $O_2 \cdot min^{-1} \cdot (mM\ ascorbate)^{-1}$ in air would be proportional to oxygen concentration, giving rise to the removal of half the dissolved oxygen in about 10 h.

** Direct measurement of the K_m under catalytic conditions gives a value of 1.0 μM (P. Nicholls, unpublished experiments).

an approximate value of the rate constant for cytochrome *c* reduction will be given by Eqn 2:

$$k[\text{ascorbate}] \simeq 0.4\left(66 \cdot 2 \cdot \frac{0.02}{0.04} + 39\right)\Big/39 = 1.1\,\text{s}^{-1} \tag{2}$$

At 4 mM ascorbate the second order constant is therefore in close agreement with the value of 30 $M^{-1} \cdot s^{-1}$ estimated from the duration of the catalytic steady state.

If the ratio of reduction rates (between free and bound cytochrome *c*) is approximately 0.1, then the apparent velocity constant (k_{obs}) in any system is a measure of the proportion of bound and free cytochrome *c*, according to Eqn 3:

$$k_{obs} = \left(\frac{[\text{cyt. } c]_{free} + 0.1\,[\text{cyt. } c]_{bound}}{[\text{cyt. } c]_{total}}\right) k_{free} \tag{3a}$$

or

$$k_{obs} = (1 - 0.9\alpha)(k_{free}) \tag{3b}$$

where α = proportion of bound cytochrome *c*.Thus for a system containing 39 μM cytochrome *c* and 66 μM oxidase (heme *a*), $k_{obs}/k_{free} = 0.25$ and $\alpha = 0.83$; 32 μM cytochrome *c* is bound, leaving 7 μM cytochrome *c* and 1–2 μM binding sites unoccupied with K_d between 0.2 and 0.5 μM.

Similar, but more direct experiments, may be carried out with cytochrome *c* peroxidase of yeast. In this case, there is no problem of electron transfer from reduced cytochrome *c* to enzyme in the absence of the acceptor, H_2O_2. Previous samples of cytochrome *c* peroxidase[13] had, however, contained sufficient adventitious catalyts to induce autoxidative H_2O_2 from ascorbate and oxygen. With a new preparation of enzyme cytochrome *c* reduction by ascorbate (H_2O_2 free) can be followed in the presence of cytochrome *c* peroxidase (Fig. 3). The half time for cytochrome *c* reduction by 133 μM ascorbate increases from 50 s (k approx. 100 $M^{-1} \cdot s^{-1}$) to over 300 s ($k \leqslant 15\ M^{-1} \cdot s^{-1}$) in presence of a 14-fold excess of cytochrome *c* peroxidase. The inability of fluoride (3.3 mM) to have any appreciable effect on this inhibition, showed that cytochrome *c* peroxidase was not functioning catalytically under these conditions. Apo-cytochrome *c* peroxidase, which also forms a reversible sephadex-detectable complex with ferricytochrome *c* (E. Mochan, unpublished experiments), exhibits a similar inhibition, while horse radish peroxidase (60 μM) which does not readily

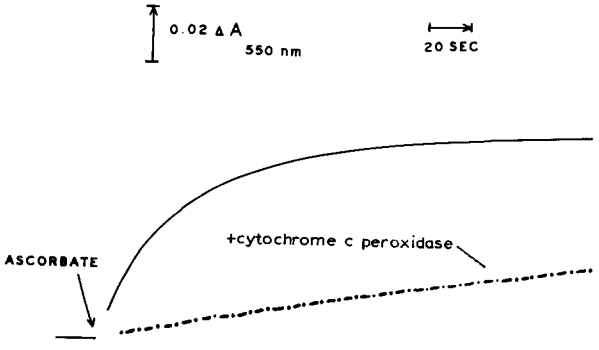

Fig. 3. Reduction of cytochrome *c* by ascorbate in presence and absence of cytochrome *c* peroxidase. 133 μM ascorbate added as indicated to 4.0 μM cytochrome *c* in the presence and absence of 14.7 μM cytochrome *c* peroxidase in 5 mM phosphate, pH 7.0, 25 °C.

form such complexes with ferricytochrome c[23], has no effect. The inhibitory effect of cytochrome c peroxidase thus appears to be related to its ability to form a reversible complex with ferricytochrome c[23,24]. Fig. 4 shows that a sharp decline in reduction velocity occurs on titrating a fixed cytochrome c concentration with increasing amounts of cytochrome c peroxidase. As with cytochrome c oxidase, the rate of reduction of fully complexed cytochrome c is about 10% of the rate in solution. The affinity of cytochrome c peroxidase for cytochrome c measured in this way (K_d of about 0.5 μM in 5 mM phosphate) may be compared with the values obtained by kinetic measurements (K_m approx. 2 μM, ref. 13) or estimated from chromatographic studies (K_d approx. 1 μM, refs 12 and 23). The lower value of Fig. 4 may represent a true difference between static and kinetic measurements, or may reflect the occurrence

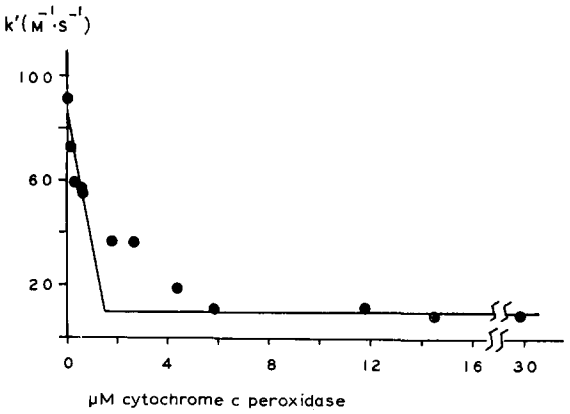

Fig. 4. Effect of cytochrome c peroxidase on ascorbate reduction rate of cytochrome c. Effective 2nd order velocity constant for reduction of cytochrome c by ascorbate ($M^{-1} \cdot s^{-1}$) is plotted against cytochrome c peroxidase concentration in 5 mM phosphate, pH 7.0, 25 °C.

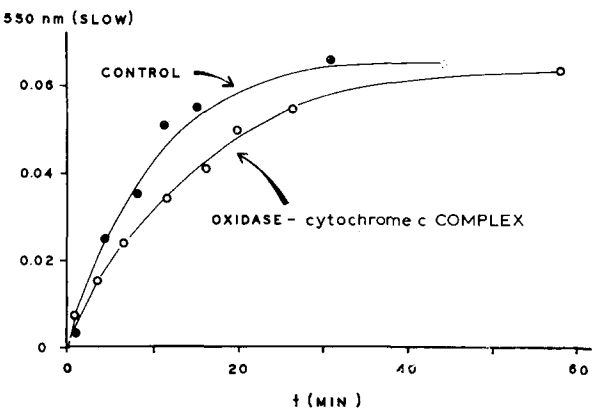

Fig. 5. Binding of cyanide by cytochrome c in presence and absence of cytochrome oxidase. ○—○, 60 μM cytochrome c incubated with 150 μM cytochrome oxidase in 4 mM phosphate–8 mM Tris, 0.1% Tween-80, pH 7.4, *plus* 10 mM cyanide (neutralized). ●—●, 60 μM cytochrome c without oxidase (control). Ordinate represents the proportion of slow absorption change at 550 nm on addition of dithionite (*i.e.* the amount of cyancytochrome c, see refs 23 and 24) to samples of the incubation mixtures diluted out at the indicated times (0.2-ml sample added to 2.5 ml 5 mM phosphate).

of secondary binding sites on cytochrome *c* peroxidase as have been postulated for the oxidase[21].

In contrast to the decline in reactivity measured with ascorbate, the rate of reaction of bound cytochrome *c* with cyanide seems identical with the rate in solution. Fig. 5 shows the formation of cyanferricytochrome *c* during incubation with cyanide, as measured by the decline in dithionite reducibility at 550 nm, (although complete reduction is given by dithionite, the product in the case of cyanferricytochrome *c* is ferrocytochrome *c* cyanide[25], with absorption maximum at 555 nm). A barely significant decrease in the rate constant, from 0.18 $M^{-1} \cdot s^{-1}$ to 0.11 $M^{-1} \cdot s^{-1}$ occurs with the enzyme-bound cytochrome *c* (*cf.* Kimelberg and Lee[26]). In addition the rate of crevice closure and elimination of cyanide from the ferrous form may be measured by the slow appearance of absorption at 550 nm (Fig. 6a) or by the disappearance of the cyanferrocytochrome *c* peak at 555 nm (Fig. 6b), following the addition of dithionite. In this reaction (Eqn 4) also, the binding of cytochrome *c* to the enzyme does not influence the rate.

$$\left[-Fe^{2+} \quad CN^- \; X-\right] \longrightarrow \left[-Fe^{2+} \quad X-\right] + CN^- \tag{4}$$

The rate constant for the reaction of Eqn 4 obtained under the present conditions (approx. $6 \cdot 10^{-3}$ s^{-1}) in 5 mM phosphate with beef heart cytochrome *c* may be compared with that obtained previously ($4 \cdot 10^{-3}$ s^{-1}) with horse heart cytochrome

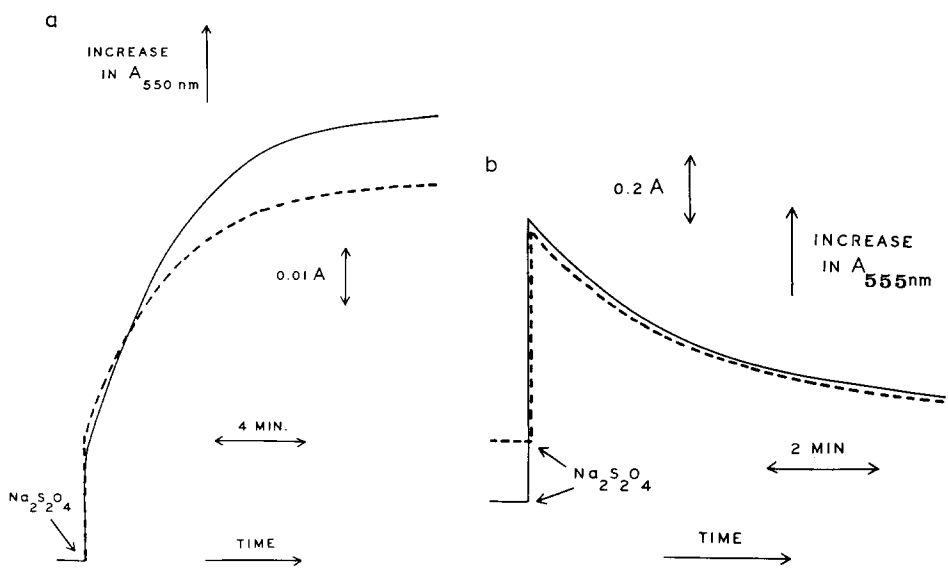

Fig. 6. Rate of dissociation of cyanide from cyanferrocytochrome *c* in presence and absence of cytochrome oxidase, (a) Change at 550 nm on addition of dithionite to 4.4 μM cytochrome *c* in 5 mM phosphate, pH 7.4, previously incubated for 60 min with cyanide (10 mM) in the presence (– – – –) and absence (——) of cytochrome oxidase. Final concentrations: CN^-, 0.75 mM; oxidase, 11 μM (*cf.* Fig. 5). Appearance of reduced cytochrome *c* with *k* approx. $5 \cdot 10^{-3}$ s^{-1} in each case. (b) Change at 555 nm on addition of dithionite to 60 μM cytochrome *c* in 4 mM phosphate–8 mM Tris incubated for 45 min with 10 mM cyanide in the presence (– – – –) and absence (——) of 150 μM oxidase (without dilution). Disappearance of cyanferrocytochrome *c* with *k* approx. $6.3 \cdot 10^{-3}$ s^{-1} in each case.

c in 100 mM phosphate. Binding to the oxidase does not have the accelerating effect that can be induced, for example, by copper ions in this reaction[27].

Is the ascorbate reaction typical of the behavior of redox agents? Preliminary experiments[11] with the ascorbate–TMPD system suggest that the rate of reduction of cytochrome *c* by TMPD may be little affected by complex formation with cytochrome oxidase. This is also indicated by the catalytic activity of systems containing bound cytochrome *c* towards ascorbate–TMPD as substrate. Ferricyanide oxidized reduced cytochrome *c* in the presence of the oxidase, but the normal rate of this reaction is so high (approx. $10^6\ M^{-1}\cdot s^{-1}$) that a 10-fold decrease would go undetected in our system.

The most interesting case is represented by the enzymatic reductase sytems of the inner mitochondrial membrane. An antimycin-sensitive NADH–cytochrome *c* reductase system of high specific activity* was therefore used in conjunction with the cytochrome oxidase preparation; and at high oxidase concentrations an increased anaerobiosis time was observed with NADH and the reductase. Unfortunately, the reductase is strongly inhibited by Tween-80 (>90 % at 0.1 %), and although the oxidase preparation employed was fairly stable when diluted out in 5 mM phosphate alone, in the absence of Tween-80 a co-precipitation between the reductase and oxidase took place. This latter phenomenon, therefore, and not the sequestering of cytochrome *c*, may account for the increased anaerobiosis time at high oxidase concentrations. Similar experiments with a succinate cytochrome *c* reductase preparation have also given inconclusive results.

Another question that may be asked of the cytochrome *c*–cytochrome oxidase complex is whether, in addition to effects on bimolecular rate constants, there are changes in monomolecular rates (reductant or oxidant independent rates of electron transfer to or from cytochrome *c* iron). We have no evidence, from our studies, of any such limiting rates, either in free cytochrome *c* or in the enzyme–cytochrome *c* complex. However, the maximal first order reduction rates measured were no greater than approximately 0.1 s^{-1} with ascorbate, and 5 s^{-1} with ascorbate–TMPD[11]. We have so far not studied the enzyme–cytochrome *c* complexes under conditions where the rate of cytochrome *c* reduction approaches that observed *in situ* in the mitochondrial membrane (approx. 100 s^{-1}).

DISCUSSION

The reaction between ascorbate and cytochrome *c* is highly sensitive to pH and ionic strength[7,28,29]. Decreasing the pH to below 5.5 causes a decrease in reduction rate to below 2 $M^{-1}\cdot s^{-1}$. It should be noted that the velocity constants used here are apparent velocity constants, approximately twice the value of the true constant for the reaction of Eqn 5a. As the rate constant for the reaction of Eqn 5b is much greater than that of Eqn 5a (approx. 10^3-fold, ref. 29),

$$AH_2 + \text{cytochrome } c\ Fe^{3+} \rightarrow AH\cdot + \text{cytochrome } c\ Fe^{2+} + H^+ \quad (5a)$$

$$AH\cdot + \text{cytochrome } c\ Fe^{3+} \rightarrow A + \text{cytochrome } c\ Fe^{2+} + H^+ \quad (5b)$$

The magnitude of the apparent overall constant is always determined by that for Eqn 5a. Table I lists the values of the overall constant that have been reported

* Generously provided by Mr S. Albracht of the Jansen Institute, University of Amsterdam.

under various conditions, in high ionic strength and in combination with the enzymes and with submitochondrial particles, (*i.e.* "endogenous" cytochrome *c*).

The decrease in reduction rate in the complexes at pH 7.4 is of the same order of magnitude as the decrease produced by phosphate buffer ions, and rather less than the decrease produced by low pH. An anionic binding site near the active center of cytochrome *c* is described by Dickerson *et al.*[30], and Margoliash[31] has speculated on the role of such binding. The data[29,30] suggest a binding of phosphate to ferricytochrome *c* with K_d of approximately 10^{-2} M and a decline of 80–90 % in the rate constant. Thus the cytochrome *c*–phosphate complex remains reducible. It is possible to speculate that the binding of the oxidase to cytochrome *c* may resemble, or involve the same sites as, the binding of phosphate, and that the decline in reducibility has a common origin in the change in ionic environment of the heme group. It is not necessary to

TABLE I

RATES OF CYTOCHROME *c* REDUCTION BY ASCORBATE

Experiments at 25 °C; $k_{app} = v$ (cytochrome *c* reduction)/[ascorbate] [cytochrome c^{3+}].

Reference	*pH*	*Phosphate concentration (mM)*	*Cytochrome c*	k_{app} $(M^{-1} \cdot s^{-1})$
	7.4	5	Free	100–150
This paper	7.4	5	Oxidase-bound	8
28	7.0	5	Cytochrome *c* peroxidase-bound	10
11,28	7.4	50	Free	50
	7.4	50	Endogenous *	15
28,29	7.4	80–200	Free	30
	6.4	80–200	Free	12

* Of Keilin–Hartree submitochondrial particles.

TABLE II

BINDING OF CYTOCHROME *c* TO OXIDASE AND PEROXIDASE

Values for dissociation constant obtained at pH 7.0 to 7.4, 25 °C, and phosphate concentration $\leqslant$ 10 mM.

Reference	*Method*	*Dissociation constant (K_d)*	
		Cytochrome oxidase (μM)	*Cytochrome c peroxidase (μM)*
8, 20, 22	Kinetic	0.5–1.0	2
7, 8, 13	Chromatographic	0.1–4.0 *	1–2
This paper	Ascorbate reducibility	0.3–0.5	0.5 **

* High values from ref. 8 (which assumed 2 cytochrome *c*: cytochrome aa_3 complex), lower values estimated from ref. 7.

** Lower value than obtained by other methods (see text).

assume that the reaction with the anionic ascorbate involves the same site, however, because in each case the anion-bound cytochrome *c* molecule remains directly reducible.

Table II compares the binding constants, observed at low ionic strengths, for the cytochrome *c*–enzyme complexes, as measured by three different methods (kinetics of cytochrome *c* oxidation, change in cytochrome *c* reducibility, and direct chromatography).

The relationship between the proportion of bound cytochrome *c* and the overall rate constant for ascorbate reduction (Eqn 3) gives a method for estimating the amount of endogenous cytochrome *c* bound to the oxidase. A decline of 70 % in its reduction rate (Table I) is consistent with the presence of 80 % in the oxidase-bound state and 20 % in the free state (of the ascorbate accessible cytochrome *c*). If some 25 % of the endogenous cytochrome *c* may be ascorbate inaccessible[11], perhaps in a lipid milieu resembling that of the phospholipid vesicles[26], we may estimate the total distribution of cytochrome *c* in Keilin–Hartree particles as: 60 % oxidase-bound, 15 % free (aqueous), and 25 % lipid-bound.

Kuboyama *et al.*[32] have reported that a complex between cytochrome *c* and cytochrome oxidase is effective in reconstituting succinate oxidase activity when mixed with succinate cytochrome *c* reductase. The low K_m for cytochrome *c* shown by the succinate reductase and the low maximal turnover of this system (approx. 3 electrons$\cdot s^{-1}\cdot$(cytochrome c_1)$^{-1}$) suggest that the observed activity may have been due to free cytochrome *c* at the ionic strength used (25 mM phosphate + 40 mM succinate, pH 7.4). Moreover, the complex which they obtained[9] contained 1 mole of cytochrome *c* per mole of heme *a* (*i.e.* $2c$:aa_3) while in the present system (Fig. 2) only 0.5 mole cytochrome *c*/1 mole heme *a* is tightly bound (secondary binding sites may exist of lower affinity[21]). It is clear, however, that the bound cytochrome *c* of the particle is catalytically active[21]. If, as suggested above, it is in the form of the oxidase complex, the latter must be active. And this activity depends on the monomolecuar rate constant, for which we have been unable to give a limiting value, and not any bimolecular rate constant.

Finally, it is worth noting that the reactions involving cyanide are almost unaffected by the state of cytochrome *c* binding, despite the probability that both binding of cyanide to the ferric form and its dissociation from the ferrous form involve conformational changes[25]. Again, the enzymes and the particles[11] are similar in this respect. The complexes formed do not impose any great stress on the cytochrome *c* molecule, either in its stable structural conformation, or in its ability to change from one conformation to another.

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