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CYTOCHROME *c*-CYTOCHROME OXIDASE INTERACTION AT SUBZERO TEMPERATURES

B. CHANCE, C. SARONIO *, A. WARING and J.S. LEIGH Jr.

Johnson Research Foundation, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104 (U.S.A.)

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Summary

Cytochrome oxidase forms two distinctive compounds with oxygen at —105 and —90°C, one appears to be oxycytochrome oxidase (Compound A) and the other peroxycytochrome oxidase (Compound B). The functional role of compound B in the oxidation of cytochrome *c* has been examined in a variety of mitochondrial preparations. The rate and the extent of the reaction have been found to be dependent upon the presence of a fluid phase in the vicinity of the site of the reaction of cytochrome *c* and cytochrome oxidase. The kinetics of cytochrome *c* oxidation and of the slowly reacting component of cytochrome oxidase are found to be linked to one another even in cytochrome *c* depleted preparations, but under appropriate conditions, especially low temperatures, the oxidation of cytochrome *c* precedes that of this component of cytochrome oxidase. Based upon the identification of the slowly reacting components of cytochrome oxidase with cytochrome *c*, various mechanisms are considered which allow cytochrome *c* to be oxidized without the intervention of cytochrome *a* at very low temperatures, and tunneling seems an appropriate mechanism.

Introduction

The functional role of intermediate compounds of cytochrome oxidase and oxygen may best be assayed by their ability to oxidize ferrocytochrome *c*. This paper reports the kinetics and extent of cytochrome *c* oxidation by two identifiable intermediates of oxygen reduction, Compounds A and B [1—4].

The low temperature inhibition of electron transfer between cytochrome *c* and cytochrome oxidase was studied in 1960 [5]. Maintenance of mixed oxidation-reduction states of cytochrome *c* and cytochrome oxidase at 77 K

* Deceased, April 1975.

was observed over a period of 24 h. The oxidation-reduction state of cytochrome *c* remained unaltered in this experiment, under conditions where electron flow from NADH to cytochrome *c* was diminished by at least a factor of 10^7 with respect to the room temperature value [6]. In this respect the reaction of cytochrome *c* with cytochrome oxidase requires further study and has been provided by a previous paper [4] which indicates that cytochrome *c* oxidation is detectable at −70°C and reaches its maximum extent at much higher temperatures, approx. −30°C. An apparent energy of activation for this process can be measured, and the reaction appears to follow an Arrhenius law, with simple bimolecular reaction kinetics. In actuality, the situation is much more complicated, because the electron carriers of the respiratory chain act as autonomous assemblies within which electron transfer is rapid and between which electron transfer is slow [5]. However, at low temperatures the probability of electron transfer may be very non-uniform in different assemblies, due to the "freezing" of donor and acceptor in different orientations and mobilities due, for example, to eutectic formation in the freezing process. Decreasing the temperature may not only discontinually affect the kinetics of cytochrome *c* but also bring different components of cytochrome oxidase in to function as electron acceptors [4].

In this respect, the low temperature behavior of the mitochondria may be contrasted with that at room temperature, where electron transfer between cytochrome *a* and a_3 is more rapid than that between cytochrome *c* and *a*, so that there is a sequence of increasing reaction rates from cytochrome c_1 through cytochromes *c*, *a* and a_3 to oxygen [7]. The reason for the vast change of relative rates at low temperatures is not clear. One effect of "freezing" could be a restriction of the "mobility" of cytochrome *c*. However, freezing is a complicated phenomenon in the roughly iso-osmotic suspension of mitochondria, and eutectic formation is likely to occur. Those portions of the cytochrome oxidase molecule lying within the eutectics may show kinetic differences from those elsewhere.

At room temperature, flash photolysis activation of CO-bound cytochrome oxidase in the presence of oxygen allows formation of oxygen intermediates and the acceptance of electrons by oxygen from cytochrome a_3 in a time short compared to the half-time for cytochrome *c* oxidation, which is approx. 7 ms at 23°C [7]. The reaction kinetics appear to be purely exponential and are relatively easily fitted to a bimolecular mechanism with roughly equal forward and reverse velocity constants of approx. 300 s^{-1} at 23°C [7], which is consistent with the equilibrium constant for the cytochrome *c*-cytochrome *a* reaction, as determined in redox titrations [8]. The rate of electron transfer in the cytochrome chain is limited to values lower than the diffusion or electron transfer-limited rate; for example, in the reaction of cytochrome *c* with cytochrome *c* peroxidase, a value of 10^4 s^{-1} has been obtained [9,10]. The steps which may be considered to affect the reaction of cytochrome *c*, and which may be selectively altered at low temperatures, are (1) the lateral diffusion of cytochrome *c* on the cytochrome oxidase molecule and the lipid membrane [11,12]; (2) the rotational diffusion of the cytochrome *c* molecule [13,14]; (3) the "binding" of cytochrome *c* to one or more sites on the surface of cytochrome oxidase [15]; and (4) electron transfer by electron tunneling

[16—18] (equivalent to outer sphere electron transfer under these conditions).

The introduction of low temperature techniques for studying cytochrome *c* oxidation in the liquid and solid states of the mitochondrial membrane [19,20] affords a new approach to identify which of these factors are important in controlling the rate of this reaction. Since the reaction of cytochrome *c* with cytochrome oxidase serves as a model, and perhaps the best known model, for biological electron transfer, we present here some detailed studies using this new method. This paper is focussed on kinetic results, spectral data have been presented previously [4].

Methods

Preparations. Beef heart mitochondria were used in most of the experiments reported here, while mitochondria from pigeon heart, rat liver or flight muscle were employed in a few cases.

Beef heart mitochondria were prepared according to the method of Löw and Vallin [21] and are maintained in the frozen state at −30°C. For use, the mitochondria are suspended at approx. 30 mg of protein per ml in 30% ethyleneglycol (freezing point, −22°C), 225 mM mannitol, 75 mM sucrose and 5 mM Tris/sulfate buffer, pH 7.4 at 23°C in all experiments. The addition of 30% ethyleneglycol inhibits electron transfer from succinate to oxygen by about 20%, and energy coupling by about 50%, as assayed at 0°C *. On occasion, the mannitol/Tris may be replaced by 125 mM KCl/Tris. On other occasions, the water is replaced by ^2H_2O, by resuspending the centrifuged mitochondria in ^2H_2O/mannitol/Tris as above. Reduction of the cytochrome components is accomplished by the addition of 5 mM glutamate and 5 mM succinate. The mitochondria are then saturated with CO, and maintained at 0 or −20°C, if desired. At the temperature of these studies (−30°C and below), electron transfer from succinate is inhibited, and the reaction is essentially a single turnover of the cytochrome components.

Pigeon heart mitochondria were prepared by the current method [22]; a proteinase concentration of approx. 1 mg per pigeon heart was used.

Rat liver mitochondria were prepared as described previously [23,24].

Blowfly mitochondria were prepared according to the method of Chappel and Hansford [25], modified by Hansford [26]. The substrate employed was 20 mM DL-glycerol phosphate.

Mitoplasts were derived from isolated mitochondria by removal of the outer membrane either by an osmotic shock treatment [27] or by a controlled digitonin treatment [28].

Cytochrome *c*-depleted mitochondria were prepared according to the modified procedure [29] of Jacobs and Sanadi [30]. Repletion of cytochrome *c* was carried out by addition of soluble cytochrome *c* to the depleted mitochondria [29].

The change of pH with temperature has been recently shown by Bray and coworkers [31] to be largely abolished by the presence of large concentrations of serum albumin (≈40 mg/ml). Similar effects were observed with mitochon-

* Chance and Itsak, in preparation

dria; the system equilibrated with phenol red at 0°C showed no large shift of indicator on cooling to −30°C even with only 3.6 mg of mitochondrial protein per ml. Presumably the higher protein concentrations (5—25 mg/ml) used in these studies avoid significant pH shifts with temperature [32].

Physical techniques. The optical approach has been fully described in a previous publication [20] (see also Figs. 1, 2, 3 and 8). The selection of the wavelength pairs is based upon the earlier studies of Chance and Williams [24] as modified by recent work on the oxygen reduction intermediates of cytochrome oxidase [1—4]. In recording the later phases of the reaction, however, interferences due to prior steps may be observed. For example, considering the traces of Fig. 2, the fast initial reactions are due to a sequence composed of the photolysis of the CO compound, traces a, b and c, the formation of the oxy compound A, and its disappearance to form the peroxy compound B, trace a, and the formation of Compound B (traces b, c). Thus, three reactions have occurred within the initial phases of the charts presented in this paper. The start-point of the traces for the slowly reacting components of cytochrome oxidase are displaced by the pre-photolysis level by the absorption of Compound B. Similarly, the trace e labelled "cytochrome *b*" (566—540 nm) shows a fast response due to the β-bands of the CO compound of cytochrome a_3 and of Compounds A and B. The wavelengths chosen for recording cytochrome *c* and c_1 (550—540 nm (d) and 554—540 nm (f), respectively) do indeed show considerable overlap of the two compounds; their kinetics appear to be indistinguishable under these experiments. Observations of the formation of Compounds A and B at temperatures below those at which cytochrome *c* and c_1 are oxidized show negligible interference. However, in the presence of large amounts of added cytochrome *c*, or in cytochrome *c*-depleted preparations, or at lower temperatures, there is a small and rapid initial response due to the β-bands of Compounds A and B. In the infrared region, trace g at 830—930 nm identifies mainly the copper components, and the rapid phase of that trace is due to the fast oxidation of copper associated with heme a_3; on a slower time scale, the copper is that associated with heme *a* [3].

Results

The general characteristics of low temperature electron transport in intact mitochondria are illustrated in Fig. 1. At −26°C, the preparation is in the liquid state and the processes of oxygenation, flash photolysis, and rapid chemical reaction can be displaced without interruption. Time proceeds from left to right, and 1 s after the traces start, the aerobic, CO-inhibited mitochondrial suspension is vigorously stirred for approx. 3 s, as indicated by the blurring of the traces. A slight displacement of traces c, d and f occurs due to prior inhomogeneities of the suspension. Thereafter, however, the traces exhibit a slight slope due to photolysis of the CO compound by the measuring light, with the subsequent slow oxidation of cytochrome *c*.

Upon flash photolysis (designated "Flash"), all the traces except trace e show a regular progression of absorbance decreases. The significant differences between traces a, b and c, representing cytochrome oxidase kinetics, are due to

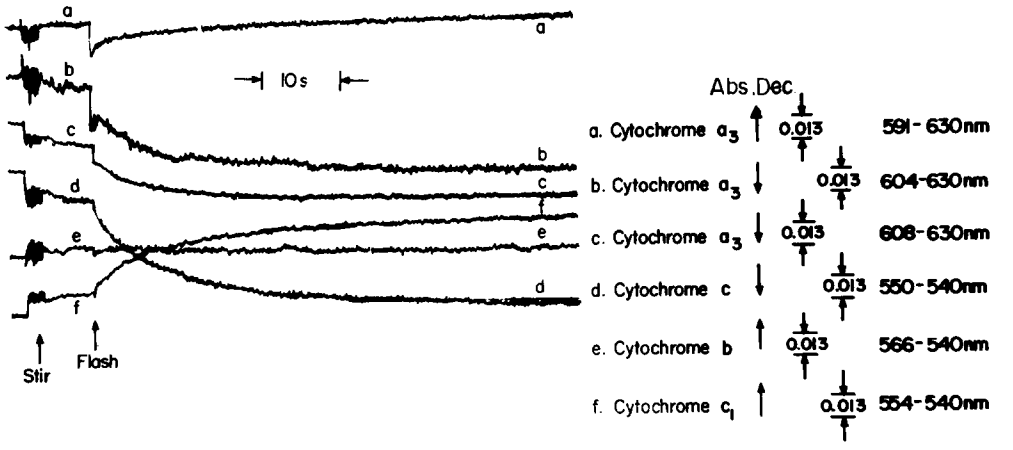


Fig. 1. Kinetics of cytochromes *c* and c_1 and of the slowly responding portion of cytochrome oxidase, measured above the freezing point. The chart indicates the complete process of oxygenation, flash photolysis, reaction with oxygen, and oxidation of cytochrome *c* at −26°C. Time proceeds from left to right. Pigeon heart mitochondria, 15 mg of protein per ml, 40% ethyleneglycol, 60% mannitol/sucrose/EDTA (MSE: 225 mM mannitol, 75 mM sucrose, 1 μM EDTA) in the presence of 10 mM succinate, 1.2 mM CO, and 50 μM oxygen.

the fast initial phases of the reaction with oxygen and indicate the presence of prior intermediates; the monotonic behavior of the "product" curves of the kinetics of cytochromes *c* and c_1 (traces d and f) suggests their absence. A further important feature of the kinetics is the parallelism between the slowly reacting portion of the cytochrome oxidase kinetics (trace b) and the kinetics of cytochromes *c* and c_1. Lastly, cytochrome *b* (trace e) does not appear to be measurably oxidized in the same temperature range as cytochromes *c* and c_1. Thus, the reaction is a "single turnover" of the components from cytochrome *c* to oxygen.

Some features of the kinetics of cytochrome oxidase and its intermediates are of interest. As described in Methods, traces a, b and c show three distinct reactions that precede the apparently simple kinetics shown here. Thus, the initial point of the kinetics of trace a, just following flash photolysis, represents fully formed Compound B, which subsequently decomposes. Similarly, traces b and c consist of an upward deflection due to photolysis (at −120°C), and as the reaction proceeds, a downward deflection due to the formation of Compounds A (at −100°C) and B (at −90°C), followed finally by the slow reaction shown here. The latter step is discussed in another paper [3,4] and has been associated with the oxidation of cytochrome *a*. A comparison of the amplitude of the initial downward deflection of trace a and the total change in trace b verifies that the formation and disappearance of Compound B at 591 nm represents a small part of the total redox change at 604 nm, in this case, only one-third. In traces b and c, the corresponding values are one-third and one-quarter, respectively. Thus, the absorbance changes due to the oxygen compounds of cytochrome oxidase as measured at 604 and 608 nm are between one-quarter and one-third of the total absorbance change at these wavelengths, supporting the view that these compounds are associated with

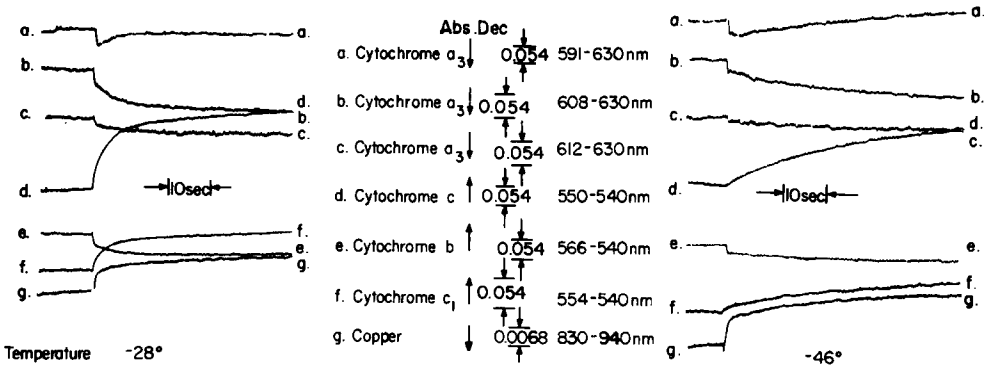


Fig. 2. The kinetics of oxidation of cytochromes *c* and c_1, and of the slowly responding portion and the infrared absorbance changes of the copper component of cytochrome oxidase, measured in a freeze-trapped sample at −28° C (left) and −46° C (right). The sample had been oxygenated prior to freezing, and the freeze-trapped sample was equilibrated at the two temperatures. Pigeon heart mitochondria, 30 mg of protein per ml, 20% ethyleneglycol, 80% MSE (see Fig. 1) in the presence of 10 mM succinate, 1.2 mM CO, and ≈50 μM oxygen.

cytochrome a_3, the minor contributor at these wavelengths, and that the slowly responding component is cytochrome *a*, the major contributor to absorbance at these two wavelengths.

Thus very similar kinetics are observed for cytochromes *a* and c_1, as if they remain in equilibrium at −26° C. This slowly reacting portion of cytochrome oxidase behaves quite differently at −26° C than at room temperature; its kinetics at −26° C are here attributed to cytochrome *a* oxidation, and in this experiment, the half-time of the slow phase is distinguishable from that for cytochrome *c* oxidation.

In Fig. 2, the reaction starts after oxygenation of the CO-blocked mitochondrial suspension at −20° C and freeze-trapping at −28° C, about 10° C below the freezing point. Under these conditions, flash photolysis produces the same large exponential response of cytochromes *c* and c_1 (traces d and f), together with fast and slow phases of the cytochrome oxidase signals (traces a, b and c). Again, the kinetics of the slowly responding portion of cytochrome oxidase closely approximate the kinetics of cytochromes *c* and c_1. At 591 nm, the disappearance of Compound B (upward deflection of trace a) seems to have a slightly shorter half-time than that at 608 nm (trace b). Cytochrome *b* (trace e) becomes reduced, following a rapid phase due to the interaction with the cytochrome oxidase kinetics. Finally, the infrared absorbance change attributed to copper (trace g) shows a phase consistent with the formation of the oxygen intermediates, followed by a smaller slow phase which parallels the oxidation of cytochrome *c*.

At −46° C, a number of changes have occurred in the kinetics of cytochrome oxidase (traces a, b and c), cytochromes *c* and c_1 (traces d and f), cytochrome *b* (trace e), and copper (trace g). The reactions of cytochromes *c* and c_1 are greatly slowed, and appear somewhat biphasic, as indeed is confirmed by appropriate kinetic plots. There remains, as before, a large component of the absorption at 608 nm that closely follows the kinetics of cytochromes *c* and

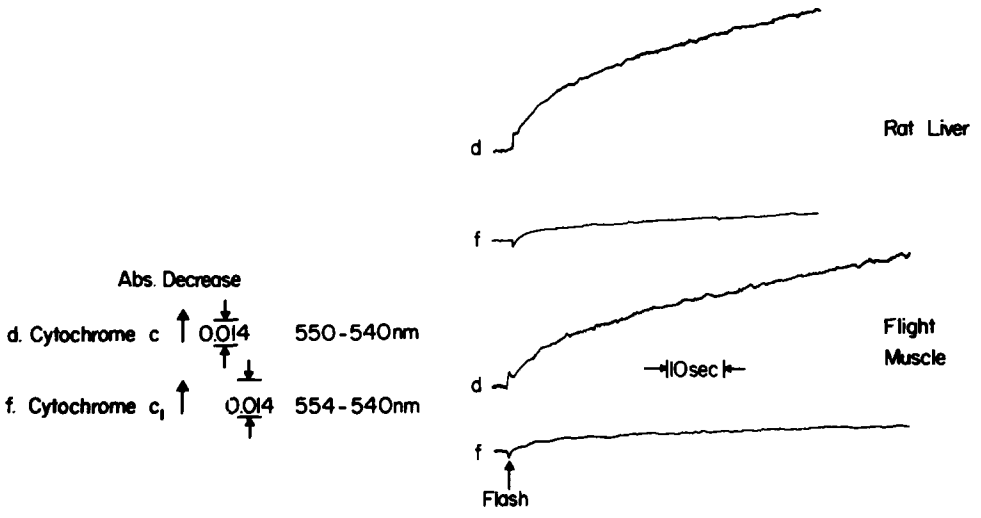


Fig. 3. Comparison of the kinetics of oxidation of cytochromes *c* and c_1 in mitochondria prepared from rat liver and from insect flight muscle. The conditions are identical to those of Fig. 2, except that the temperature in both experiments here is −37° C.

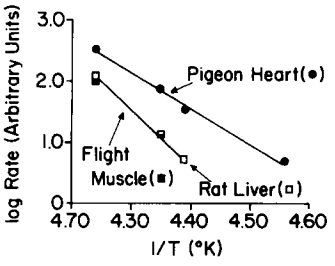
c_1. In trace a, the formation and disappearance of Compound A (not shown) is followed by the formation of Compound B and by a plateau prior to the disappearance of Compound B.

Two phases of trace g, representing the absorption of copper in the infrared region are well-defined. The rapid portion corresponds mainly to the component absorpting at 790 nm, and the second, slower phase to the component absorbing at 830 nm, identified with the copper atom associated with heme a_3 and heme *a*, respectively [3]. The kinetics of the slow phase parallel those of the slowly reacting portion of cytochrome oxidase measured at 604 and 608 nm. Heme *a* and its associated copper seem to be in equilibrium in the slow reactions at −46° C.

Kinetics of oxidation of cytochrome c in mitochondria from various sources

In order to establish the validity of using beef heart and pigeon heart mitochondria, Fig. 3 records the kinetics of cytochrome *c* oxidation in mitochondria prepared from rat liver and insect flight muscle at −37° C. The experiments are carried out under nearly identical conditions of protein concentration, temperature, optical sensitivity, time scale, etc. The traces show the initial phases of the oxidation of cytochromes *c* and c_1 (traces d and f), and the similarity of the kinetics and extent of the reaction in these and the preceding charts is apparent.

Some divergence is observed in the several preparations as the temperature decreases, as indicated by the Arrhenius plot of Fig. 4. In accordance with the data of Fig. 3, mitochondria from flight muscle and rat liver show identical kinetics over the observed temperature range. However, as the temperature is decreased, the rate of cytochrome oxidation in pigeon heart remains faster. No explanation for the smaller temperature dependence of cytochrome *c* oxidation in pigeon heart mitochondria is available.



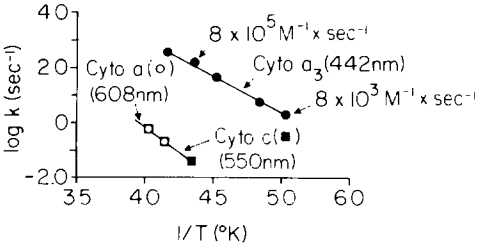


Fig. 4. Temperature dependence of the rate of reaction in pigeon heart, rat liver, and flight muscle mitochondria as measured at 550—540 nm as in previous figures.

Fig. 5. Temperature dependence of the reaction kinetics at 442, 550—540, and 608—630 nm. Conditions as in Figs. 1, 2 and 3.

The response of cytochrome *c* in baker's yeast is found to follow kinetics which are generally similar to those of the other preparations, but with a very large ratio of cytochrome *a* responses to those of cytochrome *c*; for example, the absorbance changes due to the cytochrome oxidase reaction at 608—630 nm and to the cytochrome *c* oxidation at 550—540 nm stand in a ratio of 9 : 1 at —31°C and 7 : 1 at —43°C, while this value is less than 2 : 1 in Figs. 2 and 3.

Comparison of cytochrome kinetics in intact mitochondria and in "mitoplasts"

The possibility that the motional properties of cytochrome *c* might be affected by contacts between the inner and the outer membrane has been put to a direct experimental test where the kinetics of oxidation of cytochrome *c* in rat liver mitochondria have been compared with that in mitoplasts prepared from rat liver mitochondria. The functionality of cytochrome *c* oxidation in both preparations has been demonstrated by the near identity of the half-times for cytochrome *c* oxidation in the two cases.

In summary, the overall properties of cytochrome *c* oxidation appear to be similar in a variety of mitochondria, and in preparations with and without the outer membrane present.

Relationship between the kinetics of cytochrome c and cytochrome oxidase

Fig. 5 quantitates the differences between the kinetics of cytochrome a_3 at 442 nm, those of cytochrome *c* at 550 nm, and those of the slowly reacting component of cytochrome oxidase at 608 nm. At 50 μM oxygen, the kinetics of cytochrome a_3, observed by the disappearance of the ferrous form, are very much faster and have a lower energy of activation than do the kinetics of the slowly reacting component at 608 nm (open circles) and of cytochrome *c* (black squares). It should be noted that the kinetics of the slowly reacting form of cytochrome oxidase can be observed only at higher temperatures; the extent of its reaction is too small to measure at lower temperatures.

Effect of energy coupling

Fig. 6 compares the kinetics of oxidation of cytochromes *c* and c_1 in coupled

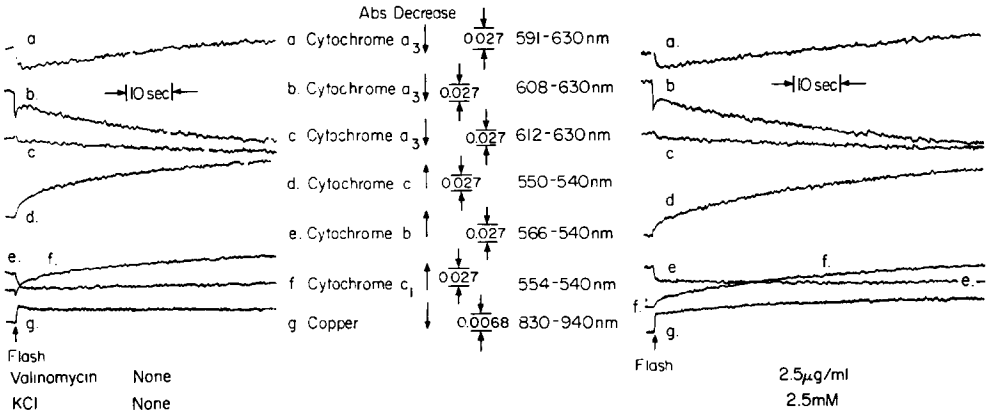


Fig. 6. The effect of energization and de-energization upon the kinetics of cytochromes *c* and c_1 and the slowly responding component and the copper component of cytochrome oxidase. Pigeon heart mitochondria under conditions similar to those of Fig. 2; temperature, −38° C. Additions of valinomycin and KCl as indicated.

(left) and uncoupled (right) pigeon heart mitochondria at −38° C. At 20% ethyleneglycol, as used here, 50% of respiratory control is retained (see footnote 1), and preliminary results with voltage sensitive probes have suggested that energy coupling can be observed at −28° C [33]. Thus, one would expect to find indications of the effect of coupling upon cytochrome *c* kinetics at −38° C. Following an initial rapid phase, there is a regular progression of cytochrome *c* oxidation from 10 s onwards. The slow reaction proceeds 50% more slowly in the uncoupled state than in the coupled state. The slowly reacting portion of cytochrome oxidase and cytochrome *c* appear to react at rates that are indistinguishable under the two conditions. The copper component responds equally rapidly in both cases, and cytochrome *b* shows no measurable response in either case. The more pronounced biphasicity of traces d and f might be due to "coupled" and "uncoupled" assemblies of respiratory carriers.

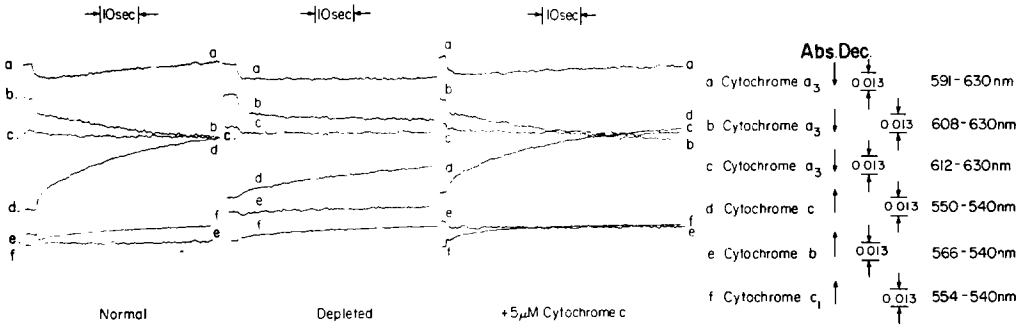


Fig. 7. The effect of depletion and repletion of cytochromes *c* upon the kinetics of oxidation of these cytochromes and of the slowly reacting component of cytochrome oxidase. The conditions are identical to those of Fig. 1, except that ≈100 μM oxygen is present and the temperature is −38° C. Panel A represents native mitochondria; Panel B, mitochondria depleted as indicated in Methods; Panel C, mitochondria repleted with 5 μM cytochrome *c*.

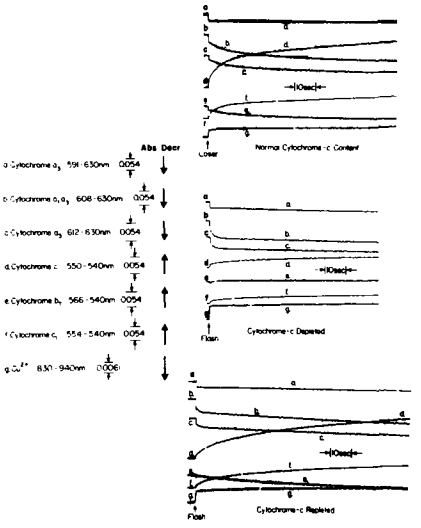


Fig. 8. Depletion and repletion of cytochrome *c* in pigeon heart mitochondria. Other conditions as in Fig. 7.

Depletion and repletion of mitochondrial cytochrome c

Depletion of approximately half of the cytochrome *c* of beef heart mitochondria slows the responses of cytochrome *c* and cytochrome oxidase by a factor of two. If, however, the depletion is more complete (about 80%) then a considerable change takes place, as shown in Fig. 7. While the time course of cytochrome *c* oxidation at −38°C is comparable to that shown in Fig. 3 in the normal preparation, the rate decreases 4-fold in the cytochrome *c* depleted preparation, and the slope for the slowly reacting component of cytochrome oxidase decreases 10-fold, suggesting that the latter component is closely linked to cytochrome *c* oxidation.

Repletion with 5 μM cytochrome *c* reactivates the rate and extent of cytochrome *c* oxidation, and increases the rate of the slow phase of cytochrome oxidase. The remarkable observation of a component of cytochrome oxidase that responds more slowly in the absence of cytochrome *c* and more rapidly in its presence seems inconsistent with a role for cytochrome *a* in electron transfer between cytochrome *c* and Compound B.

In order to explore this question more fully, Fig. 8 repeats the experiment of Fig. 7, using pigeon heart instead of beef heart mitochondria. The depletion and repletion procedures are carried out similarly, and the results indeed confirm and extend those of Fig. 7. In normal pigeon heart mitochondria, the kinetics of the slowly responding portion of cytochrome oxidase are comparable to those of cytochrome *c* in the initial 5 s. The ratio of the rates of absorbence change is 2.4 : 1. If these rates are converted to molar concentrations by the usual differential extinction coefficients ($\approx$19 $mM^{-1} \cdot cm^{-1}$ [34]) the ratio is 2 : 1, demonstrating that at this temperature the slowly responding portion of cytochrome oxidase reacts more rapidly than cytochrome *c*, as it is observed to do at room temperature.

The absorption change attributed to copper in the infrared region of the spectrum (trace g) has a slope one-fifth as steep as that of the slowly responding portion of cytochrome oxidase which, in view of the roughly 2-fold ratio of extinction coefficients, suggests that the copper of cytochrome oxidase is responding somewhat more rapidly. In addition, at this temperature, cytochrome *b* (trace e) shows a significant reduction, as is often characteristic of the initial phase of cytochrome *b* kinetics.

Depletion of cytochrome *c* to approx. 15% of normal reduces the rate of cytochrome *c* oxidation relative to the control by a factor of eight. The slowly responding portion of cytochrome oxidase has also slowed by a factor of eight. The infrared trace, however, indicates that the slowly responding copper component continues to respond at approximately the same rate as in the presence of normal amounts of cytochrome *c* (to within 10%). Cytochrome *b* (trace e) no longer becomes reduced at a measurable rate.

Repletion with 5 μM cytochrome *c* restores the rate of cytochrome *c* oxidation to 60% of the control value. But under these conditions, the reactivation of the slowly reacting portion of cytochrome oxidase does not occur to the extent observed with beef heart mitochondria; the rate is only 10% of that in normal mitochondria. The rate of absorption change in the infrared region appears to be unaltered from the control, and the reduction of cytochrome *b* has been reactivated.

These experiments confirm and extend the dependence of the response of the slow phase of the cytochrome oxidase kinetics upon the presence of cytochrome *c* and further show that reconstitution of cytochrome *c* oxidation may not reconstitute the response of this slow phase. These observations raise questions about the identity of the slowly reacting component of cytochrome oxidase, and of the essentiality of cytochrome *a* in cytochrome *c* oxidation.

The effect of pH

Further evidence for parallels in the rate and extend of oxidation of cytochrome *c* and of the slowly reacting portion of cytochrome oxidase in pigeon

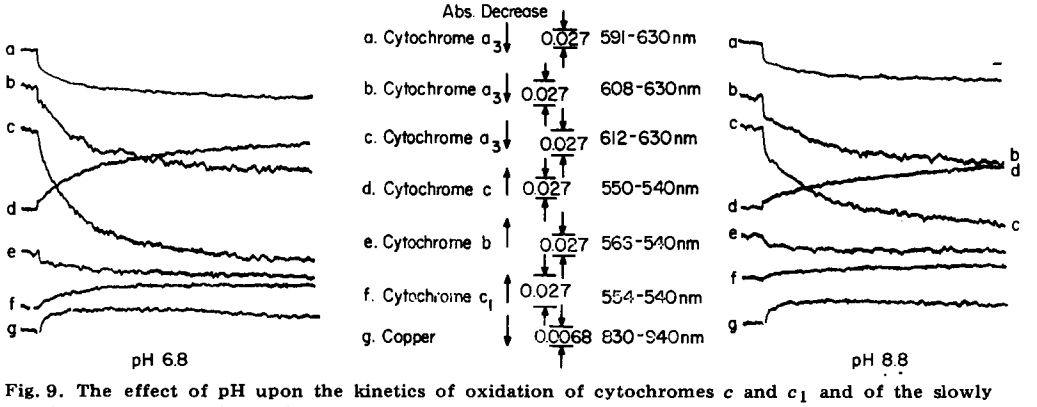


Fig. 9. The effect of pH upon the kinetics of oxidation of cytochromes *c* and c_1 and of the slowly reacting component of cytochrome oxidase, measured at pH 6.8 (left panel) and pH 8.8 (right panel). Conditions similar to those of Fig. 1, except 25 mg of mitochondrial protein per ml. The copper component of cytochrome oxidase is recorded as well (trace g).

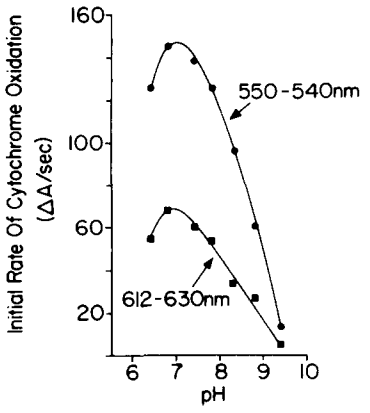


Fig. 10. The effect of pH upon the rate of oxidation of cytochrome *c* and c_1 and of the slowly reacting component of cytochrome oxidase. The rates are taken from the initial slope 10 s after photolysis, at 550—540 and 612—630 nm. Other conditions as in Fig. 9.

heart mitochondria is afforded by varying the pH as measured at 23°C in the range from 8.6 to 6.4, as illustrated in Fig. 9. The reaction rate at −27°C is measured from the slope approximately 10 s after initiation of the reaction, and shows a 3-fold decrease in rate for both components. The amount of cytochrome oxidase present as Compound B and the extent of copper oxidation are monitored and found to remain constant over the pH range studied.

The graph of Fig. 10 plots the variation of the slopes (at 10 s) for the oxidation of cytochrome *c* and of the slowly responding component of cytochrome oxidase of beef heart mitochondria. The two segments of the curve can readily be resolved into p*K* values of approx. 8 and 6. Of greatest interest is the contrast of these results with the usual pH profile for cytochrome oxidase activity [34] which shows a 2-fold increase per unit pH decreased from pH 8 to 6. Apparently, the rate-limiting steps differ from those at room temperature, thus allowing insight on phases of the reaction not otherwise observable.

Effect of the composition of the supporting electrolyte

Eutectics may form during the freezing process and contain varying concentrations of solutes. Mannitol has been used in the experiments described so far, and the properties in the presence of KCl seem worthy of investigation. Fig. 11 illustrates the kinetics of cytochrome *c* oxidation in the presence of appropriate concentrations of mannitol or KCl at −39°C.

The kinetics of oxidation of cytochromes *c* and c_1 are identical both in extent and rate under the two conditions, to within the experimental error. Thus the solute composition of the eutectics does not have a great impact upon the electron transfer reaction between cytochrome *c* and cytochrome oxidase. However, this is not the case with the slowly reacting portion of cytochrome oxidase (see traces a, b and c) where the amplitude and rate of the absorbance changes are smaller and slower in the presence of KCl than in the presence of mannitol; for example, the rise of the 591 nm trace is distinctly more rapid in the presence of mannitol. However, the copper component (trace g) shows no significant change. In summary, electron transfer between

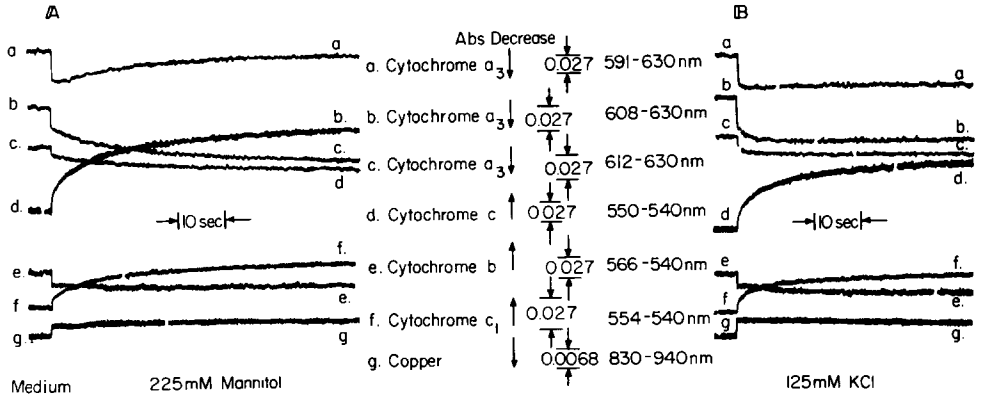


Fig. 11. The effect of changing from mannitol to KCl as the supporting electrolyte upon the kinetics of oxidation of cytochromes *c* and c_1 and of the slowly responding portion of cytochrome oxidase.

cytochrome oxidase and cytochrome *c* is unaffected by the composition (mannitol or KCl) of the eutectics, while the response of the slow component of cytochrome oxidase, is definitely altered.

The meaning of freezing

The freezing of the solvent could have significant effects upon the kinetics of cytochrome oxidation. The experiment is difficult since large changes of light scattering occur at the freezing point. An approach that takes advantage of super-cooling to study a sample in the super-cooled liquid state (Douzou, P., personal communication) seems attractive but has been unsuccessful, since nucleation occurs efficiently in the mitochondrial suspension and the range of super-cooling is too small to be useful. Instead, we have recorded the kinetics of cytochrome *c* oxidation above and below the freezing point in prior work (see ref. 20); the kinetics are slowed by a factor of approximately two by the 9° C difference of temperature, and their nature is clearly demonstrated to be identical. In the liquid state at −31° C and in the solid state at −40° C, the usual correlations between the kinetics of cytochromes *c* and c_1 and the slowly reacting portion of cytochrome oxidase are observed. In addition, the response of copper is similar and the lack of a cytochrome *b* response is characteristic. It is apparent that the liquid-solid transition of the solvent causes little change of the kinetics of cytochromes *c* and c_1 and of the slowly reacting form of cytochrome oxidase.

Proton nuclear magnetic resonance studies of the freezing phenomenon

Since the kinetic data do not appear to be greatly affected by the transition from the liquid to the solid phase, the question may be asked: What, indeed, occurs in the transition from liquid to solid? At what temperature does it occur? The hypothesis that the freezing could be detected by the decreasing amplitude of the NMR signal of the water protons has been explored in a 260 MHz instrument (Eastern Regional Facility). The profile for the decrease in amplitude of the integrated signal from the water protons is plotted on the abscissa as percent in the liquid phase in Fig. 12, trace a for a system

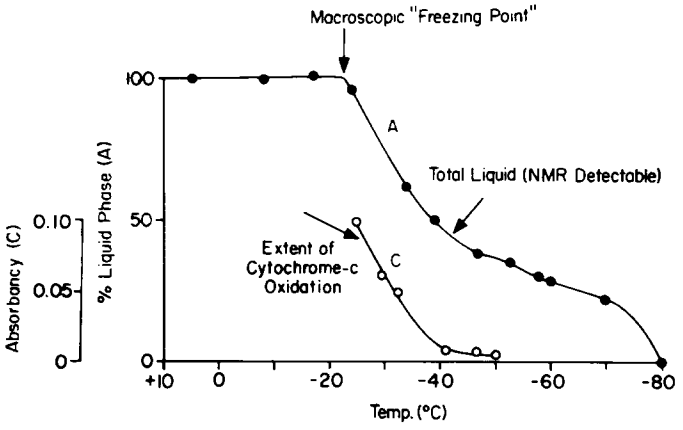


Fig. 12. A correlation of the percent of liquid water, as determined by nuclear magnetic resonance, with the extent of oxidation of cytochrome *c*, measured spectrophotometrically at the indicated temperatures.

containing 20% ethyleneglycol (mole fraction of 25%). Starting at room temperature, there is no detectable decrease of the percent in the liquid phase until the microscopic freezing point of −20° C is reached. Then a progressive decrease of the liquid phase reaches a level corresponding to the mole fraction of ethyleneglycol at a temperature of −60° C. Below −60° C, the liquid ethyleneglycol decreases, reaching zero at −80° C. Thus, not only are eutectics formed but also some of the ethyleneglycol is segregated in the eutectics with varying proportions of ethyleneglycol and water.

The reactivity of cytochrome *c* may be correlated with the magnitude of the liquid water phase (see trace c of Fig. 12). The extent of the cytochrome *c* reaction, recorded in a separate set of experiments, is maximal above −20° C but does not reach completion in this experiment because of the light scattering change that occurs at the melting point. From −20 to −50° C, the two traces run parallel. As interpreted in terms of a current model for cytochrome *c* oxidation [4], the cytochrome *c* molecule resides in an aqueous phase and is in contact with the membrane lipid and with the cytochrome oxidase model. The cytochrome *c* cytochrome oxidase reaction seems to require a fluid phase that allows the approach of cytochrome *c* to within electron tunneling distance.

Effect of ^2H_2O

The effect of substituting ^2H_2O for 90% of the H_2O, the remainder being ethyleneglycol in both cases, is indicated in Fig. 13, where the extent of cytochrome *c* oxidation at a particluar temperature serves as a measure of the fraction of the total cytochrome assemblies that are capable of electron transfer.

The results are plotted on a logarithmic scale as a function of reciprocal temperature. The data suggest that more of the assemblies of cytochrome oxidase and cytochrome *c* are able to react at a given temperature in the presence of ^2H_2O than in its absence. The curve further suggests that the difference is less at higher temperatures. Thus, there seems to be no measur-

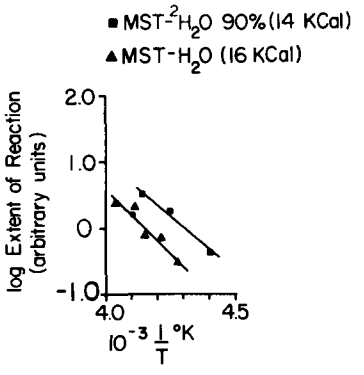


Fig. 13. Temperature profile for the extent of cytochrome *c* oxydation in the presence of H_2O and ^2H_2O. A comparison of the extent of oxidation of cytochrome *c* as a function of temperature in water (triangles) and in a 90% ^2H_2O medium (squares). The slopes of the two curves correspond to apparent energies of activation of 16 and 14 kCal, respectively. MST, 0.075 M mannitol/0.125 M sucrose/10 mM Tris buffer (pH 7.4).

able inhibitory effect of 80% ^2H_2O [35]; as was obtained at the higher ^2H_2O concentration and at higher temperatures.

Discussion

The activity of the intermediate compounds of cytochrome oxidase and oxygen in oxidizing cytochromes *c* and c_1 has been suggested in prior work [1—4]. Their function has been clarified and extended by the present study, and two aspects of the reaction of cytochrome *c* have been elucidated.

The first is the role of a fluid phase in the reaction, as suggested by the correspondence between the decrease of liquid water protons and the extent of cytochrome *c* as shown in Fig. 12. While it is generally agreed that motion is one of the most important parameters of reaction kinetics, the question arises here as to the scale of the motion; it is also very likely that nature will achieve the most reaction with the least motion. The scale of motions can extend from the smallest distance; i.e., nuclear motions involved tunneling $\approx$0.1 Å to motional properties of cytochrome *c* between the two relatively stationary donor and acceptor molecules in the membrane, cytochrome *c* reductase and cytochrome *c* oxidase possibly separated by 10 Å. A "branching" reaction studied previously [36] indicates that cytochrome *c* can mediate not only between reductase and oxidase that belong to a single "assembly" of respiratory enzymes, but that it can also mediate between different "assemblies", albiet at a considerably slower rate. This important experiment identifies different types of motion and different distances between assemblies and within an assembly, and in the direct reaction of cytochrome *c* with cytochrome oxidase proximal and distal orientations of donor and acceptor may occur in a particular assembly. In this discussion we wish to use lateral diffusion as a term which identifies the motion of cytochrome *c* in the plane of the membrane between assemblies or within a given assembly. Such motions can be of three types: (1) across the surface of the lipid bilayer between the macromolecular assemblies (see Fig. 14); (2) across

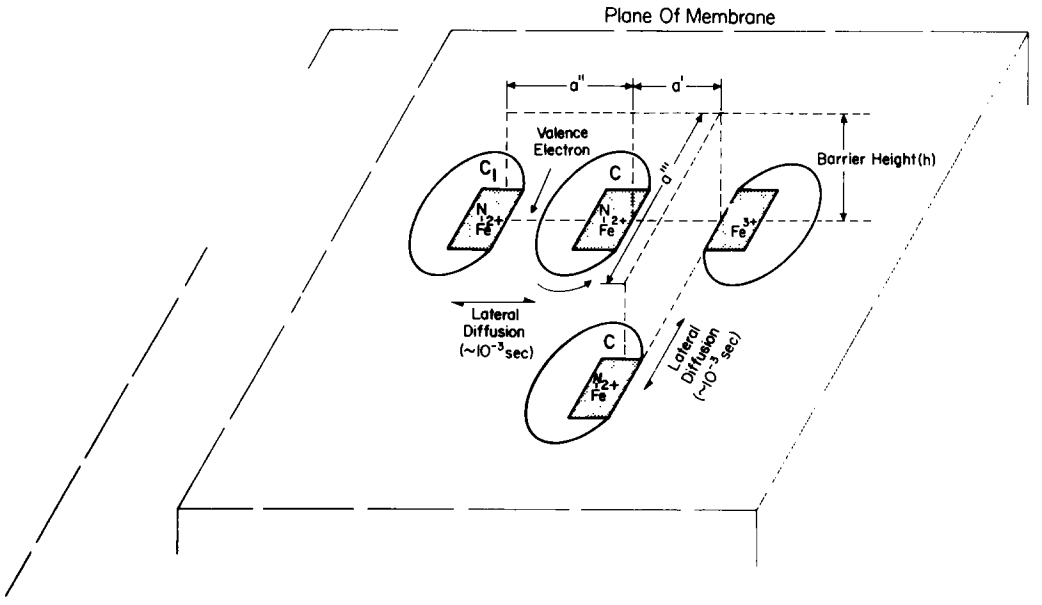


Fig. 14. Lateral diffusion and reaction of cytochrome *c* relative to immobilized cytochromes c_1 and *a*, with possible times for thermally assisted electron tunneling.

the surface of one macromolecule to another, possibly unfavorable energetically; and (3) a microscopic "vibration or translation" which would bring the heme of donor and acceptor into optimal orientation and minimal distance. These possibilities are discussed elsewhere [37—40]. In ref. 40, the enhanced association of cytochrome *c* with the total area of the oxidase molecule and the available phospholipid surface is considered, while here and elsewhere we consider only the translational and rotational possibilities (1—3 above) in the plane of the membrane [36—39]. The minimal motional requirement would be the nuclear motions involved in achieving configurations appropriate to rapid tunneling between donor and acceptor. Freezing of the solvent molecules is capable of altering any one of these motions. The schematic diagram (Fig. 14) illustrates the above possibility in which lateral diffusion in the plane of the membrane between the fixed donor and acceptor macromolecules occurs.

The model identifies a planar membrane with a diffusable cytochrome *c* molecule that can approach to within electron tunneling distance (a) of cytochrome oxidase, with a frequency that is equal to or greater than the characteristic turnover number of cytochrome *c* in establishing an equilibrium between cytochrome c_1 and cytochrome *a* (300 s^{-1} at 23°C). A liquid phase between the inner and the outer membrane, and even in the mitoplast (see Fig. 14) can be imagined, and this phase must be fluid for the cytochrome reaction. Movement of cytochrome *c* perpendicular to the plane of the membrane does not seem to be important, since removal of the outer membrane does not cause a loss of cytochrome oxidase activity under low temperature conditions.

The second aspect of the cytochrome *c*-cytochrome oxidase reaction concerns the function of the slowly reacting component of cytochrome oxidase. In Fig. 15 the amplitudes of the slowly reacting portion of cytochrome oxidase

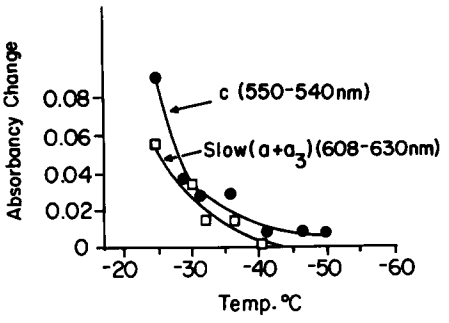


Fig. 15. Temperature profiles for cytochrome *c* (550—540 nm) and the slowly responding portion of cytochrome oxidase (608—630 nm).

are compared with those of cytochrome *c* in the inital phases of the reaction and they change as a function of temperature in the range from the freezing point down to the lowest temperature at which the reactions can be detected. The curves nearly reach their maximal amplitude at the freezing point (independent experiments with smaller concentrations of ethyleneglycol at higher temperatures confirm this observation; Waring, A., personal communication) and decrease in a parallel fashion to a temperature of approximately —40° C. At that point, the amplitude of the slow phase of cytochrome oxidase decreases more rapidly than that of cytochrome *c*. Although the system may not have reached equilibrium it appears from these data that the extent of the response of the slow phase of cytochrome oxidase differs from that of cytochrome *c* at temperatures below —40° C. This slow phase seems less likely to occur than at lower temperatures, than cytochrome *c* oxidation.

Depletion and repletion of cytochrome *c* shed considerable light upon the nature and function of the slowly reacting component of cytochrome oxidase. Depletion of cytochrome *c* reduces the rate of response of this component in proportion to the decrease of the rate of cytochrome *c* oxidation, just the opposite of what would be expected if the slowly reacting component were an intermediate in cytochrome *c* oxidation. In that case it would be re-oxidized more rapidly without the "electron transport load" of its supposed electron donor, cytochrome *c*. In beef heart mitchondria, repletion with cytochrome *c* causes the recovery of the slowly reacting component, but studies with pigeon heart show that this is not necessarily so; repletion of pigeon heart mitochondria in the experiment of Fig. 8 fails to reactivate the response of this portion of cytochrome oxidase, although the response in the infrared region due to the copper component appears to be unaffected.

The most likely assignment of this slowly reacting component of cytochrome oxidase is to cytochrome *a*, based upon observations at a variety of wavelengths which indicate that cytochrome a_3 has already reacted with oxygen at very low temperatures and has already appeared not only in the form of Compound B but also in the oxidized form (cytochrome a_3^{3+}) prior to the activation of the slowly reacting form of cytochrome oxidase. Further studies indicate that the copper component associated with heme a is similarly slowly reacting at low temperatures (Waring, A., unpublished data). At a temperature above —30° C and especially at room temperature, this portion of the 605 nm

absorption band responds very rapidly, in fact, even more rapidly than cytochrome *c*.

In a previous communication [3], explanations were offered for the anomalous behavior of cytochrome *a*, based upon the idea that steady state concentrations were maintained at a low value because of a temperature-dependent mid-potential. It is proposed that the low temperature E_m for Compound B is sufficiently positive that it is a good oxidant, and cytochrome *c* a good reductant.

Some shift of E_m with temperature is to be expected and Larroque et al. [41] find an increase of E_m of cytochrome *c* with decreasing temperature. Evidently, the increase of that of Compound B must be greater and that of cytochrome *a* must be much less or even change in the opposite direction so that cytochrome *a* responds even more slowly than cytochrome *c*, giving support to the idea that cytochrome *c* donates electrons directly to Compound B with cytochrome *a* in sluggish equilibrium. Nuclear controlled nonadiabatic tunneling from cytochrome *c* to cytochrome *a* at higher temperatures and to cytochrome a_3 (as Compound B) at low temperatures affords the most likely explanation [42].

The question of the rate-limiting step arises in these studies, and the answer may determine the mechanism. A maximal concentration of Compound B is found from −90 to −50°C. A minimal concentration of oxidized cytochrome *a* appears to be present in this temperature range. This situation holds true from the temperature range at which cytochrome *c* is initially observed to be oxidized (−70 to −60°C) to almost −40°C. Above −40°C, substantial concentrations of the slowly reacting form of cytochrome oxidase are becoming more rapidly oxidized than cytochromes *c* and c_1. Thus, at −23 to −30°C, donation of electrons from cytochrome *c* to cytochrome *a* would occur. At lower temperatures, cytochrome *c* appears to donate electrons directly to Compound B. Thus, an additional step is introduced at high temperatures and a break in the Arrhenius plot for cytochrome *c* oxidation observed [43] might indicate a change from that characteristic of electron donation to cytochrome *a* to that characteristic of electron donation to Compound B [4].

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