COMPLEX FORMATION BY CYTOCHROME *c*: A CLUE TO THE STRUCTURE AND POLARITY OF THE INNER MITOCHONDRIAL MEMBRANE

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A reversible Michaelis complex between cytochrome *c* and cytochrome oxidase was postulated by Stotz [1] on kinetic grounds in 1938. As pointed out by Smith and Conrad [2], however, kinetic observations do not distinguish between an active ES complex and an inhibitory ES complex accompanied by an effective bimolecular reaction between free enzyme and free substrate. In the case of cytochrome oxidase this means that the complex of ferrocytochrome *c* with the oxidized form of the enzyme could be inert. Smith and Conrad [2] felt that the inhibition of the oxidase reaction by ferricytochrome *c* and by non-specific polycations favored the idea of an inert ES complex. Minnaert [3] derived various kinetic models, on the other hand, that incorporated a functional role for both active complexes with ferrocytochrome *c* and inhibitory complexes with ferricytochrome *c*. The physical existence of such complexes was demonstrated by Okunuki et al. [4] and by King and Takemori [5]. And Nicholls [6, 7] brought forward three kinds of argument for an active role of these complexes in electron transport, showing that (i) the effects of ionic strength on enzyme turnover and apparent affinity for cytochrome *c* are best explained by assuming intramolecular electron transfer within the complex, that (ii) the rate of electron transport in the intact respiratory chain was consistent with the existence of such a complex in situ in the membrane and that (iii) strong analogies existed between cytochrome oxidase and cytochrome *c* peroxidase, both of which form tight complexes with cytochrome *c* and oxidize it as a specific substrate, while non-specific peroxidases, which also catalyze cytochrome *c* oxidation, do not form ES complexes with it and therefore do not show the same kinetic pattern.

The complex between soluble cytochrome oxidase and cytochrome *c* has the following properties [4–6]:

a) approximately 1 mole of cytochrome *c* is bound per mole of heme *a* and the kinetics of cytochrome *c* oxidation indicate independence of binding sites;
b) both ferric and ferrous cytochrome *c* are bound, with approximately equal affinities [8];
c) the spectrum of both forms of the complex is that of an optical mixture of the components;
d) both forms of the complex are dissociated by cations, with *c* and cations engaging in direct competition; and
e) the affinity of enzyme for cytochrome *c* is largely unaffected by pH change in the region from 6.0 to 7.5, although above pH 8 some dissociation of the ferrous complex may occur [8].

Previous observations [7] indicated that these properties may also be found in the complex formed when cytochrome *c* binds to submitochondrial particles such as the *c*-deficient Keilin-Hartree particle. Two major differences emerge in this case. Firstly, the role of the complex is now not only the oxidation of cytochrome *c* in solution, but also, and probably more importantly, the transfer of electrons generated within the membrane (from succinate or NADH) to oxygen. This function may be subserved by only a proportion of the total number of sites that bind exogenous *c* and catalyze its oxidation [7]. Secondly, the occurrence of interchain electron transfer ("branching reactions") between the dehydrogenase level and

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cytochrome *c* permits the saturation of a single cytochrome *c*-oxidase complex in the membrane by a number of succinate-cytochrome *c* reductase chains. Therefore full restoration of succinoxidase activity in systems with a high maximal oxidase turnover may be achieved when only a few cytochrome *c* binding sites are occupied (i.e. only a few of the membrane-bound oxidase molecules are in the form of the complex).

Fig. 1 illustrates this behavior. The binding of a fixed amount of cytochrome *c* by progressively increasing quantities of Keilin-Hartree particles is monitored by measurement of succinate oxidase activity. This binding has the characteristics that:

(a) under the conditions studied (0.06 M phosphate pH 7.4), an apparent dissociation constant of 4 μM is obtained;

(b) the cytochrome *c* added is able to restore electron transfer to particles containing a tenfold molar excess of heme *a* over the *c* bound; and

(c) the bound cytochrome *c* turns over at approximately the same rate (40–50 sec^{-1}) as in the isolated cytochrome *c*-oxidase complex.

That the complex produced is identical with the oxidase complex is also indicated by its sensitivity to cations and other inhibitors [9]. Bound cytochrome *c* is reduced and oxidized *in situ* without dissociation, contrary to the view of Smith and Camerino [10]. This is shown by the facts that the turnover at maximal rates of succinate oxidation is some four times the rate of cytochrome *c* reduction in a cyanide-inhibited system, and the steady state reduction of exogenous cytochrome *c* (5%) is insufficient to maintain electron transfer at 20 sec^{-1} (electrons/sec/heme *a*). Endogenous cytochrome *c*, whether present *ab initio*, or added back to deficient particles, as in fig. 1, is between 20% and 25% reduced in the succinate-induced steady state (at 25°C in pH 7.4 60 mM phosphate).

In the intact mitochondrion, the binding of *c* has topological as well as chemical characteristics. Numerous studies have located the cytochrome system upon the inner mitochondrial membrane [11]. Unlike the other cytochromes, cytochrome *c* is readily dissociated from the mitochondrion by successive hypotonic and hypertonic treatment [12]. This suggests that this component is not deeply embedded in the lipid matrix. The question may therefore be asked:

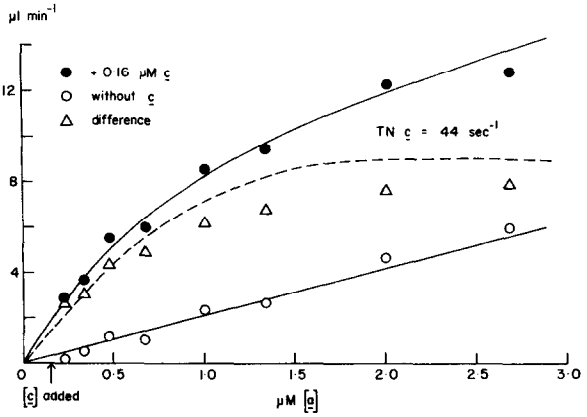

Fig. 1. Binding of cytochrome *c* to non-phosphorylating submitochondrial particles. Oxidation of 27 mM succinate measured manometrically at 30°C in 67 mM phosphate pH 7.4. Final volume was 3.0 ml per flask. Beef heart Keilin-Hartree particles used containing approximately 1.2 μmoles heme *a* (using ΔE mM 605–630 mμ reduced-oxidized = 14.0) per g protein (biuret reaction). Oxygen uptake in μl O_2 (N.T.P.) per min in presence and absence of 0.16 μM cytochrome *c*.

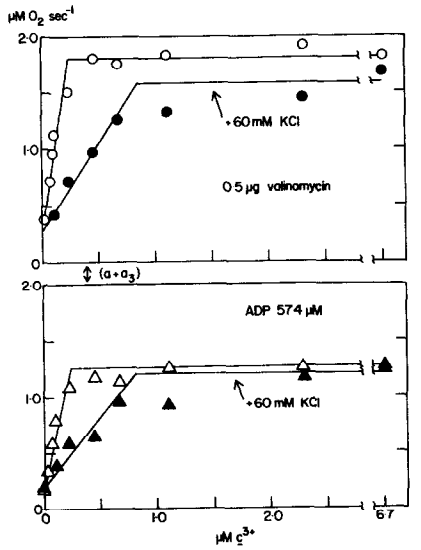

Fig. 2. Binding of cytochrome *c* to rat liver mitochondria in presence of ADP or valinomycin. Cytochrome *c*-deficient mitochondria, 0.4 μM heme *a*, 0.9 mg protein ml^{-1}. 6.7 mM succinate + 2.5 mM glutamate used as substrate with 2.1 μM rotenone in 1.5 ml final volume. Oxygen uptake measured polarographically. Reaction medium: 180 mM mannitol, 60 mM sucrose, 0.08 mM EDTA, 16 mM Tris-HCl, 10 mM potassium phosphate, 60 mM KCl where indicated, 0.2 mM $MgCl_2$, pH 7.35, 25°C. ($a + a_3$) indicates the cytochrome *c* concentration equal to the mitochondrial heme *a* concentration present.

Is it on the outside or inside of the inner mitochondrial membrane? The binding of cytochrome *c* to *c*-deficient rat liver mitochondria is characterized by an affinity and a dependence upon ionic strength similar to that shown by soluble oxidases. The procedure for obtaining such cytochrome *c*-deficient mitochondria [12] involves an initial hypotonic treatment, similar to that used by Parsons et al. [11] to remove the outer membrane, followed by the classical hypertonic treatment [13] used to extract cytochrome *c* from submitochondrial particles, in which the cytochrome *c* binding site is accessible to the reaction medium. This suggests that the binding site may be on the outside of the inner mitochondrial membrane. The experiment whose results are plotted in fig. 2 supports this conclusion.

In the lower part of this figure is seen the result of titrating *c*-deficient mitochondria, respiring in state 3, with increasing levels of cytochrome *c*. Although the results in 10 mM K^+ may be interpreted in terms of titration of a binding site [12], the shift in apparent titration point produced at higher K^+ concentrations is more easily envisaged as indicating a reversible binding with an affinity constant controlled by ionic strength. The apparent titration curve at low K^+ concentration may then be attributed to the saturation of the respiratory system when only 10 to 20% of available binding sites are occupied, as with the non-phosphorylating submitochondrial particles used in the experiment of fig. 1.

Valinomycin also induces respiration by coupling it to K^+ ion accumulation [14]. The top part of fig. 2 thus shows the restoration of electron transfer to *c*-deficient mitochondria in the "ion-pumping" state by cytochrome *c* addition. It may be noted that the apparent affinity for cytochrome *c* remains closely tied to the external KCl concentration and at each concentration is the same as for state 3 respiration. If cytochrome *c* were being bound inside the inner membrane, it would be anticipated that (a) no close dependence on external K^+ would be shown, and (b) the addition of valinomycin would markedly reduce the apparent affinity. We conclude that the binding of *c* is responding to the external ion concentration only.

These results may be compared with those of Palmieri and Klingenberg [14] who showed that energy-linked accumulation of azide by mitochondria was accompanied by an increase in the inhibition of cytochrome oxidase, suggesting that the azide binding site is inside. The energy released in this phase of electron transfer must also be communicated in some way to the ATPase located on the inside of the inner membrane [11]. One phosphorylation is coupled to the oxidation of two molecules of cytochrome *c*. If cytochrome a_3 (the ferric form of which is presumed to bind azide) is inside, while the cytochrome *c* binding site is outside, we have a situation in which an enzyme-substrate complex (presumably a protein-protein complex) is "plugged through" a lipoprotein membrane, somewhat as envisaged by Benson [15]. In addition, anisotropic electron transfer may now occur at this site, as envisaged in the theory of Mitchell [16], although it should be noted that the present evidence does not tell us whether this is coupled with proton transfer, or whether such proton transfer is associated with energy storage – the two other key concepts of the theory.

An alternative view of the binding of cytochrome *c* has been presented by Machinist et al. [17]. They believe that the binding of cytochrome *c* involves the membrane phospholipids. It is true that phospholipid depletion appears to alter both the binding of cytochrome *c* and the electron flux within the complex. However, the occurrence of electron transfer within the complex, and hence within the membrane, suggests that the formation of the complex involves protein-protein interaction rather than interaction with phospholipids. A conclusion of this type is supported by the analogy between mammalian cytochrome *c* oxidase and yeast cytochrome *c* peroxidase. The latter enzyme is soluble, loosely associated with the mitochondrion and has no lipid content [18]. A 1:1 complex has been demonstrated between yeast peroxidase and cytochrome *c* [19] in which heme-heme electron transfer occurs at a rate of up to 1300 sec^{-1} with horse cytochrome *c* and 7000 sec^{-1} with yeast cytochrome *c*. The smallest dissociation constants observed for this complex are of the same order of magnitude as with the cytochrome oxidase complex, though the affinities found at higher ionic strengths are slightly higher. As with cytochrome oxidase, both ferric and ferrous cytochrome *c* are bound with approximately equal affinity. The kinetic similarities between the two enzymes [6] are thus due to similarities in binding cytochrome *c*. These similarities extend to include

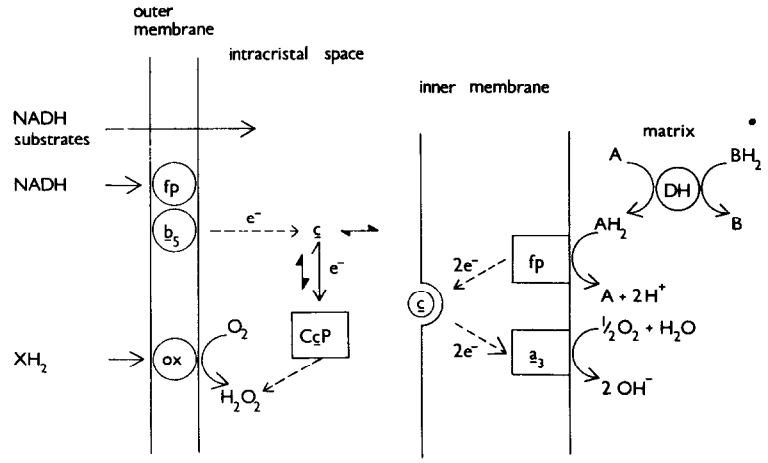

Fig. 3. Schematic picture of cytochrome *c* interactions and electron transport in the mitochondrion.

reactions with chemically modified cytochrome *c* and with polycations [20]. As well as providing an enzymic "model" free of the membrane and lipid dependent complications of the oxidase reaction, cytochrome *c* peroxidase reaction, cytochrome *c* peroxidase must itself be found a role in the economy of the mitochondrion [18]. It may be significant that in yeast the ratio of cytochrome *c*: cytochrome *a* is greater than in mammalian mitochondria, permitting the simultaneous existence of complexes of cytochrome *c* with oxidase and peroxidase.

Fig. 3 illustrates the roles in mitochondrial metabolism now attributed to cytochrome *c*. The mitochondrion represented here is a composite of the mammalian and yeast organelles, with yeast cytochrome *c* peroxidase, known to be "loosely" associated with the mitochondrion [18], arbitrarily assigned to the intracristal, or intermembrane, space. Cytochrome *c* may thus act as a mobile carrier, not between single assemblies on the inner membrane, for which dissociation and association are not required, but between the inner and the outer membranes, linking the oxidase and reductase systems present in each.

Acknowledgements

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