Localization of a ferricyanide-reactive site of cytochrome b–c_1 complex, possibly of cytochrome b or ubisemiquinone, at the outer face of submitochondrial particles

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When succinate oxidation by submitochondrial particles is blocked by antimycin, NoHOQnO or funiculosin, addition of ferricyanide restores oxygen uptake coupled to membrane potential generation. The effect of ferricyanide is abolished by mucidin or myxothiazol, as well as by KCN. The data strongly favor a cyclic redox loop mechanism in site 2 and show that either heme of the ferrous cytochrome b or ubisemiquinone formed in the QH_2-oxidizing center of complex b–c_1 is accessible to ferricyanide at the outer (M) side of the submitochondrial particle membrane.

Respiratory chain *Q-cycle* *Cytochrome* b *Membrane potential* *Cytochrome* b–c_1 *site inhibitor* *Redox center topography*

1. INTRODUCTION

Ferricyanide has been widely used as a non-penetrating oxidant in studies of membrane-bound redox chain topography [1,2]. In intact mitochondria, electrons entering the respiratory chain via the internally localized dehydrogenases are accessible to ferricyanide only via the externally localized cytochromes c and c_1, so that reduction of $Fe(CN)_6^{3-}$ by succinate or NAD-dependent substrates is highly sensitive to antimycin [3–6].

In inverted submitochondrial particles (SMP), succinate dehydrogenase and NADH dehydrogenase are exposed at the outer (M) side of the membrane and react readily with ferricyanide [1,2,7].

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Abbreviations: NoHOQnO, 2-(n-nonyl)-4-hydroxyquinoline N-oxide; PCB^-, phenyldicarbaundecaborane anion; SMP, submitochondrial particles; $\Delta\psi$, transmembrane electric potential difference

Scattered evidence for reaction sites other than the dehydrogenases can be found in [8–12] but the identity of these sites remains obscure. The situation has been complicated by the presence of uninverted and ruptured particles in the conventional preparations of SMP in which ferricyanide could be reduced via cytochromes c and c_1 with low K_m.

It was found in [13–16] that addition of ferricyanide to SMP in the presence of succinate and antimycin restored membrane potential generation. This effect of ferricyanide was not observed under anaerobic conditions and was inhibited and prevented by cyanide. As only the coupled inside-out vesicles are seen by the PCB^- uptake method [17,18] used in [13,14], the observation pointed to the presence of a ferricyanide-reactive site of cytochrome b–c_1 complex, possibly of cytochrome b, on the M-side of the membrane.

Here we give further evidence that ferricyanide can drain electrons from the respiratory chain of SMP via some component(s) of cytochrome b–c_1 complex exposed at the outer face of the mem-

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brane. This withdrawal of electrons results in release of the antimycin-inhibited electron transfer through site 2. The data imply that either ferrocytochrome(s) *b* or ubisemiquinone generated in center *o* of the Q-cycle [19] is accessible to ferricyanide from the M-side of the membrane.

2. MATERIALS AND METHODS

Antimycin and NoHOQnO were from Serva. Mucidin [20] was kindly donated by Dr V. Musilek (Institute of Microbiology, Acad. Sci. CSSR, Prague). Myxothiazol [21,22] was a gift from Dr Trowizsch (Gesellschaft für Biotechnologische Forschung, Braunschweig). Funiculosin [23,24] was obtained from Dr P. Bollinger (Sandoz, Basel). The data given in the figures were obtained with $K_3Fe(CN)_6$ from Reachim (chemically pure grade, re-crystallized once) and have been subsequently reproduced with analytical grade ferricyanide samples from Reanal, Apolda, Fluka and Merck. Other reagents were largely from Sigma and Serva. Sonic Mg, Mn, succinate, ATP–SMP were prepared from heavy beef heart mitochondria essentially as in [25]. The succinate oxidase activity of SMP was ~10% sensitive to protamine and was stimulated by added cytochrome *c* by 10–15%, which indicates that most of the particles in the preparation are closed inside-out vesicles. Membrane potential generation was assayed by a penetrating ion method [17,18] as modified in [26] using PCB^- as a probe. Oxygen consumption was measured in a 1 ml cell with a Clark-type electrode fed into a PL-7e polarograph (CSSR) connected with a Servograph REC-80 recorder.

3. RESULTS

Fig.1 shows typical recordings of $\Delta\psi$ generation in beef heart SMP. Addition of succinate results in uptake of PCB^- which is reversed by antimycin (fig.1a,b). Subsequently, ferricyanide brings about generation of $\Delta\psi$ in accordance with [13,14]. The effect of ferricyanide is fully inhibited by KCN (trace a) or by ascorbate which rapidly reduces $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ (trace b). Ferrous iron-hexacyanide added instead of the ferric form was without effect on $\Delta\psi$ generation (not shown). Accordingly, the ferricyanide-induced PCB^- uptake by antimycin-inhibited SMP decayed upon reduction of the oxidant by excess succinate; the effect could be reproduced repeatedly under these condi-

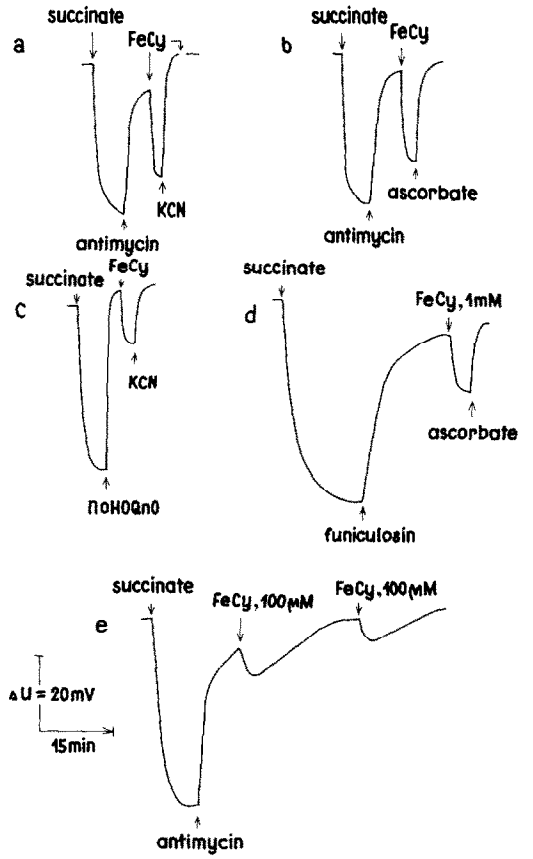

Fig.1. Ferricyanide-induced generation of membrane potential by submitochondrial particles in the presence of succinate and antimycin-type inhibitors. Beef heart SMP (about 0.5 mg protein/ml) in a medium containing 0.25 M sucrose, 5 mM fumarate, 5 mM $MgSO_4$, 30 mM sodium–Hepes (pH = 7.5, t = 23°C). Additions: succinate (20 mM); ferricyanide (FeCy) (2.5 mM) unless indicated otherwise; ascorbate (5 mM); KCN (2 mM); antimycin (0.6 μg/ml); funiculosin (0.3 μg/ml); NoHOQnO (0.8 μg/ml). The traces show energy-dependent PCB^- uptake monitored electrometrically [17,18] with a phospholipid-impregnated Teflon filter as the PCB^--selective permeable membrane [26]. The downward deflection of the traces corresponds to decreased PCB^- concentration in the medium, resulting in a change of electric potential difference (U) across the PCB^--selective membrane according to the Nernst equation, so that ΔU of 20 mV corresponds to approx. 2-fold change in $[PCB^-]$. Initial $[PCB^-]$ was 1 μM.

tions by adding more $Fe(CN)_6^{3-}$ (trace e). The same results have been obtained with SMP inhibited by NoHOQnO (trace c) or funiculosin (trace d). At the same time, ferricyanide failed to restore membrane potential generation when the respiratory chain was blocked by mucidin or myxothiazol (fig.2a,b). Moreover, mucidin and myxothiazol completely inhibited PCB^- uptake stimulated by ferricyanide in the presence of succinate and antimycin (fig.2c,d), NoHOQnO or funiculosin (not shown).

The sensitivity of the $Fe(CN)_6^{3-}$-supported generation of $\Delta\psi$ in antimycin-inhibited SMP to cyanide and anaerobiosis ([13–16] and here) pointed out that electron transfer through cytochrome oxidase was in some way involved in the effect. Therefore, we investigated whether ferricyanide could diminish the effectiveness of the antimycin block. As shown in fig.3, this has indeed been found to be the case. When ferricyanide is added to SMP supplemented with succinate and inhibited either by antimycin (trace a), NoHOQnO (trace b) or funiculosin (trace c), a substantial stimulation of oxygen uptake occurs. This ferricyanide-induced succinate oxidase activity is inhibited by myxothiazol (trace a), mucidin (trace c) and KCN (trace b); accordingly, ferricyanide does not stimulate electron transfer from succinate to oxygen in SMP inhibited by myxothiazol (trace d) or mucidin (not shown).

Addition of ferrous iron-hexacyanide to antimycin-inhibited SMP brought about a negligible increase of the oxygen uptake rate as compared to the effect of ferricyanide (trace e). Also, we found that ascorbate largely eliminated the O_2 con-

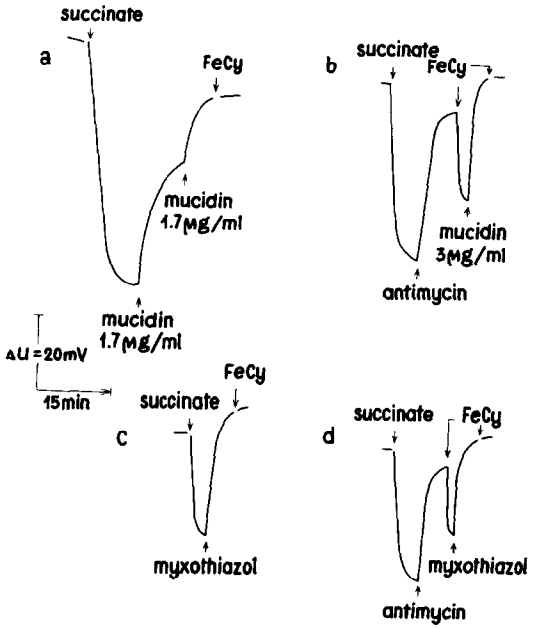

Fig.2. Inhibitory effect of myxothiazol and mucidin on the ferricyanide-induced $\Delta\psi$ generation in submitochondrial particles in the presence of succinate, antimycin and ferricyanide. Basic conditions as in fig.1. Additions: succinate (20 mM); ferricyanide (5 mM) (a), (2.5 mM) (b,c) or (1 mM) (c,d); antimycin (0.4 μg/ml); myxothiazol (0.5 μg/ml); mucidin (as indicated in the fig.).

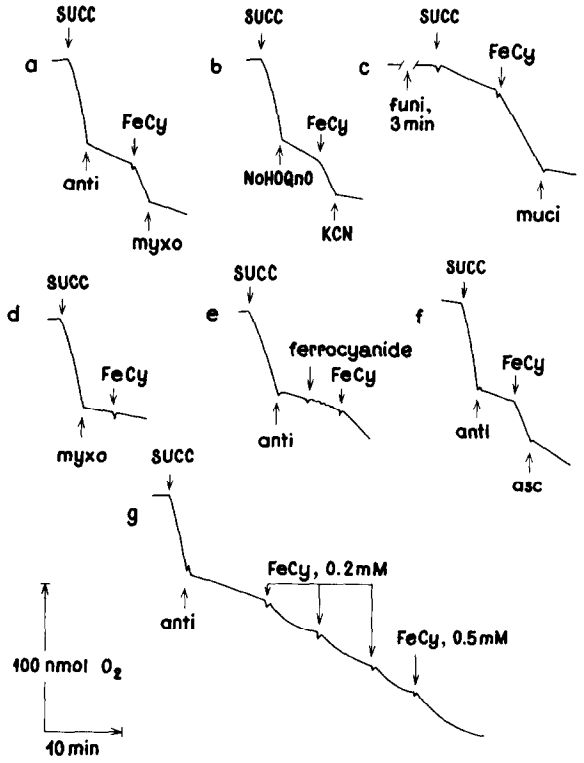

Fig.3. Effect of ferricyanide on the oxygen uptake by submitochondrial particles in the presence of b–c_1 site inhibitors. Beef heart SMP (1.3 mg protein/ml) in a medium containing 0.3 M sucrose, 20 mM sodium–Hepes (pH = 7.5), 5 mM $MgSO_4$, 3 μM rotenone and 1 μM carbonyl cyanide m-chlorophenylhydrazone (t = 25°C). Additions: succinate (20 mM); ferricyanide (1 mM unless indicated otherwise); antimycin (1 μM); NoHOQnO (10 μM); funiculosin (4 μM); mucidin (6 μM); myxothiazol (1 μM); ferrocyanide (1 mM); ascorbate (2 mM).

sumption induced by ferricyanide in the presence of succinate and antimycin (tracc f). Therefore, it is clear that the observed stimulation of respiration is due to reaction of SMP with ferric iron-hexacyanide rather than to sluggish oxidation of the accumulating ferrocyanide. It can also be ruled out that ferricyanide causes irreversible destruction of either antimycin or the antimycin-binding site. As illustrated in fig.3g, repetitive small additions of $Fe(CN)_6^{3-}$ to antimycin-inhibited SMP bring about transient bursts of O_2 uptake which decay as the oxidant is exhausted in the presence of excess succinate (cf. fig.1e).

4. DISCUSSION

As shown here, there is good correlation between the ability of ferricyanide to restore $\Delta\psi$ generation and oxygen consumption in antimycin-inhibited SMP supplemented with succinate. It is clear from the present and previous [13–16] data, that electron transfer through cytochrome oxidase is necessary for (or at least is operative during) membrane energization under these conditions. Therefore, a minimal hypothesis as to the mechanism of the paradoxical $\Delta\psi$ generation in SMP in the presence of succinate, antimycin, O_2 and $Fe(CN)_6^{3-}$ would be that the membrane is energized by cytochrome oxidase. Nevertheless, electron transfer through site 2 can itself be coupled to generation of membrane potential in these conditions as suggested in [13] (fig.4). This interesting possibility is presently under investigation.

There is little doubt that the reduction of ferricyanide involved in the observed effects occurs at the M-side of the membrane. Firstly, measuring PCB^- uptake one monitors only reactions in the inside-out coupled vesicles [5,17,18], so that a possible contribution of the unsealed or mitochondrially oriented particles is completely eliminated. Secondly, ferricyanide does not release the antimycin inhibition in mitochondria [1–6], hence the effect observed in SMP could not have been due to the reaction of the oxidant at the C-side of the membrane, even if it did penetrate the membrane.

What is the nature of the ferricyanide-reactive site? The 5 specific inhibitors of electron transfer 'between b and c_1 cytochromes' used in this work

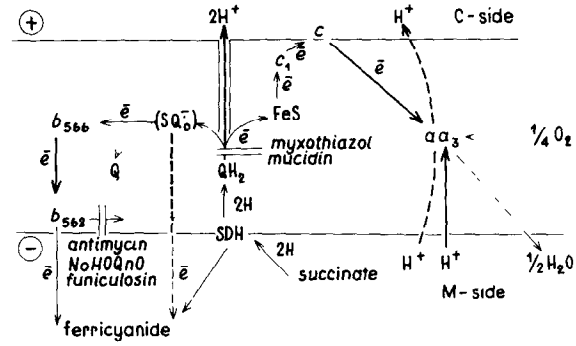

Fig.4. Possible mechanism of the ferricyanide-supported energy-coupled electron flow through the antimycin-inhibited respiratory chain of submitochondrial particles. The electron transfer diagram is based on the Q-cycle model [19]. SDH, succinate dehydrogenase. Energy-conserving vectorial H^+ and e^- transfer reactions are depicted by thick arrows and migration of oxidized substrates (Q and O_2) by dotted lines. The ferricyanide-induced release of electron transfer in the presence of the antimycin-type inhibitors is ascribed to the reaction of the oxidant with cytochrome(s) b, although interaction with ubisemiquinone (– – –) cannot be ruled out. Since some of the electrons donated by succinate to the cytochrome chain via CoQ return to the M-phase, iron-hexacyanide serving as the final sink, the answer to the question whether electron flow through site 2 in the antimycin-inhibited state is itself coupled to generation of $\Delta\psi$ will depend critically on the fate of protons liberated from QH_2 upon oxidation by FeS_{Rieske}. If both protons are released to the C-side (into the lumen of the particles) as shown, then charge separation across the membrane will inevitably occur, regardless of the specific molecular mechanism of the b–c_1 site. Note that the release of one of the QH_2-bound protons to the C-phase is charge compensated by electron transfer to cytochrome c.

belong to two different classes. Antimycin, NoHOQnO and funiculosin are believed to block electron transfer from cytochromes b to ubiquinone in center i of the Q-cycle [19,27–30] whereas mucidin and myxothiazol suppress cooperative oxidation of ubiquinol in center o [30–32] (fig.4). This grouping of the inhibitors persisted through the present experiments and it looks likely that ferricyanide can release the inhibition of center i but not of center o.

As illustrated by fig.4, electrons could be accepted by $Fe(CN)_6^{3-}$ either from b cytochromes or directly from ubisemiquinone generated in center

o. According to the Q-cycle, in both cases oxidation of ubiquinol via center *o* would be uncoupled from the antimycin-blocked *b*-562 ⟶ Q reaction in center *i* and restoration of the succinate oxidase activity will occur. It is not yet possible to discriminate between these two possibilities.

Localization of the cytochrome *b*-562 heme group at the M-side of the membrane has been postulated [19,33] and is indirectly corroborated by studies on the redox equilibrium between cytochromes *b* and succinate/fumarate couple in energized SMP [34,35]. On the other hand, it was reported [36] that the hemes of ferric cytochromes *b*-566 and *b*-562 are located within 10 Å from the C-aqueous phase. In contrast, we have shown [37,38] that the cytochrome *b*-562 redox center is accessible to a membrane-impermeable electron donor $Ru(NH_3)_6^{2+}$ in SMP but not in mitochondria. Therefore, it is reasonable to suggest that cytochrome *b*-562 can donate electrons to ferricyanide at the outer surface of SMP.

As to the interaction of ferricyanide with the center *o*-generated ubisemiquinone, such a reaction may seem incompatible with the presumed Q-cycle topography of redox centers in coupling site 2 [19]. Indeed, a Q-cycle with center *o* exposed at the matrix side of the mitochondrial membrane would probably have diverged somewhat too far from the original model [39]. Nevertheless, one cannot exclude the possibility that under certain conditions ubisemiquinone formed in center *o* in the depth of cytochrome b–c_1 complex can migrate to some ubisemiquinone-binding site(s) at the M-side of the membrane where it becomes accessible to ferricyanide. The presence of a ubisemiquinone species readily accessible to $Fe(CN)_6^{3-}$ in SMP but not in mitochondria has been described [12,40].

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