Cytochrome *b* reduction by hexaammineruthenium in mitochondria and submitochondrial particles

Evidence for heme b-562 localization at the M-side of the mitochondrial membrane

Wolfram S. Kunz and Alexander Konstantinov*

Institut für Physiologische Chemie, Medizinische Akademie Magdeburg, Leipziger Str. 44, Magdeburg, DDR and **A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR*

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In the presence of ascorbate, hexaammineruthenium mediates rapid reduction of cytochrome *b*-562 in submitochondrial particles but not in mitochondria. The reaction is obsreved in the combined presence of antimycin (or funiculosin) and myxothiazol, which implies direct interaction of $Ru(NH_3)_6^{2+}$ with *b* cytochrome(s). We assume that contrary to previous conclusions (Case and Leigh (1976) Biochem. J., 160, 769–783) redox centre of at least one of the oxidized cytochromes *b*, most probably of *b*-562, is exposed to the M-aqueous phase.

Respiratory chain *Redox center topography* *Cytochrome* b *Hexaammineruthenium* *Q-cycle*

1. INTRODUCTION

It is well established that mitochondrial cytochrome *b* apoprotein(s) is arranged transmembraneously [1–3], cytochrome *b*-566 moiety being accessible to p-diazobenzenesulfonate from the cytoplasmic (C-) side of the membrane [4,5]. Little is known, however, about the topography of the redox centres of cytochromes *b*-562 and *b*-566. According to [6], the iron porphyrin groups of the oxidized cytochromes *b* are localized within 10 Å from the C-side of the membrane.

In this paper we have compared reducibility of cytochrome *b*-562 in mitochondria and SMP by a membrane-impermeable redox mediator hexaammineruthenium (E_m = 50–100 mV [7,8]) which has been increasingly used in biochemical work in the recent years [9–12]. The results point to ferric cytochrome *b*-562 redox centre being localized at the inner face of mitochondrial membrane. In addition, evidence is obtained for centre *o* of the Q-cycle [13] being moderately accessible to $Ru(NH_3)_6^{2+}$ in mitochondria.

2. METHODS

$Ru(NH_3)_6Cl_3$ was obtained from Alfa Products (Denver). Myxothiazol [14] was a generous gift from Dr W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunschweig). Funiculosin [15] was kindly supplied by Dr P. Bollinger (Sandoz, Basel). Other reagents were commercial products of the highest purity available from Sigma, Serva, Fluka and Merck.

Mitochondria were isolated from pigeon heart as in [16]. Sonic SMP were prepared from heavy beef heart mitochondria essentially as described in [17] in the presence of Mg^{2+}, Mn^{2+}, ATP and succinate and were by ~90% closed inside-out vesicles as evidenced by the protamine sensitivity of their succinate oxidase activity. Spectrophotometrical

* To whom correspondence should be addressed

Abbreviations: SMP, submitochondrial particles; RuAm, hexaammineruthenium

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measurements were carried out in an Aminco DW2$^{a}_{TM}$ instrument in magnetically stirred 1 cm rectangular cells thermostatted at 25°C.

3. RESULTS

3.1. *Experiments with submitochondrial particles*

When 1 mM ascorbate is added to aerobic beef heart SMP inhibited by KCN and antimycin there occurs little or no reduction of cytochromes *b*. Subsequent addition of $Ru(NH_3)_6^{3+}$ which is rapidly reduced to $Ru(NH_3)_6^{2+}$ by the ascorbate brings about rapid increase in absorbance at 563 nm minus 575 nm (fig.1, trace a) due to partial reduction of cytochrome *b*-562 (spectra not included). As shown by trace b in fig.1, myxothiazol does not inhibit this reduction. Hence it is likely that $Ru(NH_3)_6^{2+}$ donates electrons to cytochromes *b* directly rather than via any of the CoQ-dependent pathways which are known to be eliminated by a combination of antimycin with myxothiazol ([18], unpublished observations of W.S. Kunz).

The extent of *b* cytochrome reduction by ascorbate + RuAm observed in the above experiments with the antimycin + KCN-inhibited SMP attained 30–35% of the dithionite-induced absorbance changes, which corresponds to about one-half of *b*-562 content. This incomplete reduction is most likely due to the fact that midpoint potentials of both ascorbate and RuAm are rather close to that of cytochrome *b*-562; besides, $Ru(NH_3)_6^{2+}$ is moderately autoxidizable [19], which further raises the actual steady-state redox potential of the mediator under aerobic conditions. However, one could suggest alternatively that only part of cytochrome *b*-562 is accessible to RuAm at the outer face of SMP. To resolve this problem, we have taken advantage of our recent finding that an antimycin-type inhibitor funiculosin [15] raises the E_m of cytochrome *b*-562 by >100 mV [20]. Indeed, having replaced antimycin with funiculosin, we were able to greatly promote the reduction of *b*-562 by ascorbate + RuAm.

As shown in fig.1c, addition of only 4 μM $Ru(NH_3)_6^{3+}$ to the KCN + funiculosin-inhibited SMP preincubated with ascorbate brings about rapid reduction of ~50% of the dithionite-reducible *b* cytochromes, which actually means complete reduction of *b*-562 as confirmed by appropriate spectral recordings (not included).

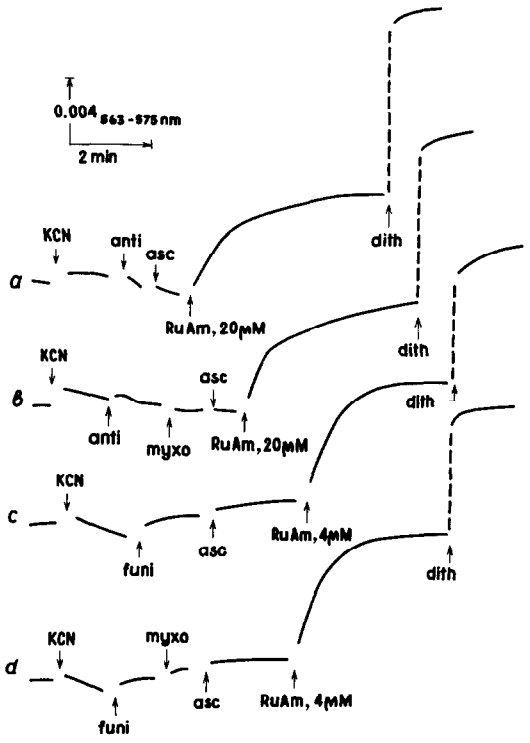

Fig.1. Hexaammineruthenium-mediated reduction of *b* cytochromes by ascorbate in submitochondrial particles. Beef heart SMP (1.2 mg protein/ml) in the medium containing 0.3 M sucrose, 20 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, pH 7.5, 1 mM EDTA, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone, 3 μM rotenone. Additions: KCN, 4 mM; antimycin (anti), 1 μg/mg protein; myxothiazol (myxo), 0.8 μg/mg protein; funiculosin (funi), 2 μg/mg protein; ascorbate (asc), 1 mM; RuAm, as indicated; dithionite (dith), a few crystals.

3.2. *Experiments with mitochondria*

The studies on RuAm interaction with cytochromes *b* in mitochondria encountered certain difficulties arising from the presence of endogenous substrates which, in the presence of antimycin and, especially, of funiculosin, tended to reduce cytochrome *b*-562 prior to any addition of ascorbate and RuAm. Eventually, we were able to obtain satisfactory results with mitochondria from pigeon heart.

As shown in fig.2a,b, antimycin addition to KCN-inhibited mitochondria results in a significant reduction of cytochrome *b*-562 (spectrum not included). This 'extra-reduction' [21,22] is not af-

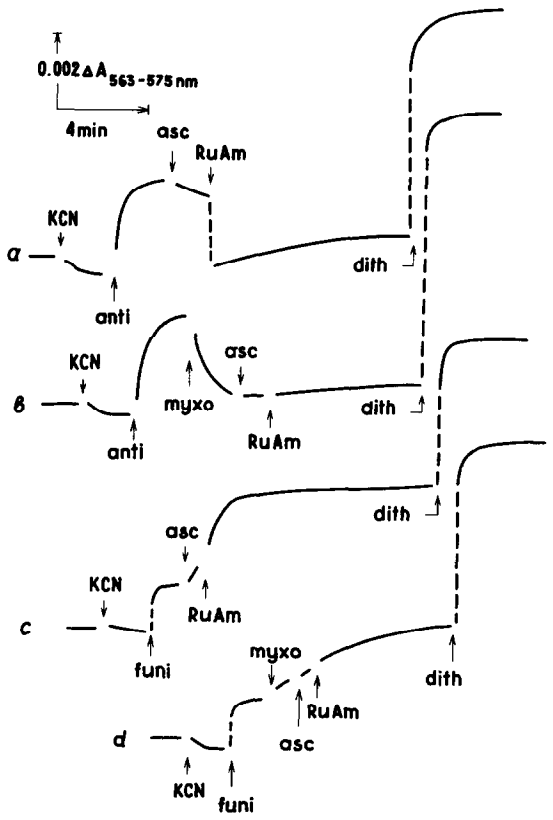

Fig.2. Effect of hexaammineruthenium on the reduction of cytochromes *b* in pigeon heart mitochondria. Pigeon heart mitochondria, 0.7 mg protein/ml. Incubation medium, additions and abbreviations as in fig.1, except that [RuAm] is 20 μM throughout.

fected significantly by ascorbate but relaxes instantaneously upon subsequent addition of RuAm*, which is followed by a very sluggish re-reduction of *b* cytochromes to not >10–15% of the dithionite level (cf. the rapid reaction in the corresponding experiment with SMP in fig.1a). Trace b in fig.2 shows that the antimycin-induced extra-reduction of cytochromes *b* can also be reversed by myxothiazol. In this case ascorbate + RuAm produce virtually no reduction of cytochrome *b* in contrast to what has been observed in SMP (fig.1b).

In the funiculosin-inhibited mitochondria significant endogenous reduction of *b*-562 could not be avoided which makes the results somewhat less distinct. Nevertheless it can be seen clearly enough from trace c in fig.2 that on a background of the funiculosin-induced partial reduction ascorbate promotes further absorbance increase which is markedly accelerated by RuAm. Importantly, this stimulating effect of the mediator is virtually absent in the presence of myxothiazol (trace d). Hence the RuAm-facilitated reduction of *b* cytochromes in mitochondria is quite different from the myxothiazol-insensitive reaction in SMP and probably occurs via centre *o* of the Q-cycle.

4. DISCUSSION

The results given in this paper show that a highly hydrophilic membrane-impermeable** redox-mediator $Ru(NH_3)_6^{2+}$ is a much more efficient electron donor to the middle segment of the respiratory chain than is ascorbate. Perhaps, it is net anionic charge of the membrane surface that discriminates between the reactions of cationic RuAm and anionic ascorbate with the membrane-bound redox carriers.

Indeed, as shown in our group by Dr E. Popova (results to be published elsewhere), cytochrome

* This effect belongs to reduced RuAm since it is not observed in the absence of ascorbate (data not shown); it is fully analogous to relaxation of extra-reduced state of *b* cytochromes by dichlorophenolindophenol and phenazine methosulfate described in [21,22] and interpreted as reduction of ubisemiquinone in the ubiquinol-oxidizing centre of complex b–c_1

** Since RuAm is a highly charged hydrophilic compound, one can expect it a priori to be membrane-impermeable. This is confirmed by the following experimental observations: (1) RuAm does not induce diffusion electric potential difference across a phospholipid bilayer membrane (the experiments kindly performed by Dr L.M. Tsofina); (2) RuAm serves as a good electron donor to cytochrome oxidase in mitochondria [9,10], but not in the inverted SMP; (3) RuAm does not reduce the photooxidized reaction centre bacteriochlorophyll in *Rhodospirillum rubrum* chromatophores but it reduces P-870$^+$ in milliseconds in proteoliposomes in which the pigment is exposed at the outer face of the membrane (experiments in collaboration with Drs L.A. Drachev, O.P. Kaminskaya, M. Mamedov and A.Yu. Semenov, to be published elsewhere)

b-562 in the funiculosin + myxothiazol-inhibited isolated complex b–c_1 can be readily reduced by ascorbate even in the absence of RuAm.

According to our data, there can be two different $Ru(NH_3)_6^{2+}$-reactive sites in complex b–c_1 located at the opposing sides of the coupling membrane.

First, in SMP, but not in mitochondria, $Ru(NH_3)_6^{2+}$ rapidly reduces cytochrome *b*-562 in the reaction which is insensitive to myxothiazol + antimycin (or funiculosin) and therefore is likely to involve direct electron donation to one of the *b* cytochromes. Although *b*-562 reduction by RuAm via *b*-566 cannot be entirely excluded, this possibility is much less likely than immediate interaction of the mediator with cytochrome *b*-562 redox centre because: (1) E_m of $b\text{-}566_{2+}$ is probably too low to allow for its rapid reduction by $Ru(NH_3)_6^{2+}$; (2) funiculosin which raises selectively E_m of *b*-562 [20] strongly accelerates the kinetics of the reaction; this were not to be expected in case of electron transfer to *b*-562 via *b*-566 since the energetically unfavourable reduction of the latter by RuAm would have been rate limiting; (3) there is certain evidence that of the two *b* cytochromes *b*-562 is nearer to the M-side of the membrane [5,13,22].

Thus, the data obtained provide strong evidence for localization of the ferric cytochrome *b*-562 heme at the M-side of the membrane as it had been suggested originally by Mitchell [13,23] and in agreement with the lack of energy-linked reduction of cytochrome *b*-562 in the succinate/fumarate-poised SMP [24,25] [26]. In contrast, Case and Leigh reported the hemes of both ferricytochromes *b*-566 and *b*-562 to be localized within 10 Å from the C-aqueous phase [6]. So far the reason for this discrepancy is not clear, but, in our opinion, the data on cytochromes *b* in [6] do not look so convincing as to allow for indisputable conclusions.

Second, experiments with mitochondria reveal some interaction of $Ru(NH_3)_6^{2+}$ with the b–c_1-locus of the respiratory chain at the C-side of the membrane. Thus, in the presence (but not in the absence) of ascorbate RuAm brings about relaxation of the antimycin-induced extra reduction of cytochromes *b* (fig.2a) and, in the funiculosin-inhibited mitochondria, the mediator facilitates myxothiazol-sensitive reduction of cytochrome *b*-562 by ascorbate (fig.2b). Both these activities are consistent with ubiquinol-oxidizing centre *o* of complex b–c_1 [13,25] being moderately accessible to $Ru(NH_3)_6^{2+}$ from the C-side of the membrane.

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