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The effect of the protonmotive force on the redox state of mitochondrial cytochromes

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Abstract

In the absence of kinetic limitations, as determined either by high substrate concentrations or by absence of respiratory chain inhibitors, we have observed that: (a) the relationship between the percentage reduction of the cytochromes and the protonmotive force is linear in the case of cytochrome *c* and biphasic in the case of cytochrome *b*, (b) the redox state of cytochrome *c* depends only on the membrane potential and not on the total proton motive force and (c) the alkalinization of the matrix enhances the extent of cytochrome *c* reduction because of the marked inhibitory effect on the cytochrome oxidase activity. Thus, although the redox states of the *b*, *c* and aa_3 mitochondrial cytochromes depend on the protonmotive force, the quantitative correlation between the two parameters and the relative effects of the electrical and chemical components of the force differ among the various cytochromes.

Key words: Mitochondrion; Cytochrome; Redox state; Membrane potential

1. Introduction

Following the observations of Chance and Williams [1,2], Muraoka and Slater [3] observed that during succinate oxidation in steady state rat liver mitochondria (RLM), the extent of reduction of the cytochromes *b*, *c* and aa_3 decreased from 40, 30 and 15% to 18, 15 and 10%, respectively, upon addition of ADP. While these observations indicate that the redox state of the cytochromes is under thermodynamic control, the relative roles of the kinetic and thermodynamic parameters, as well as those of the electrical and chemical components of pmf, respectively, in determining the % reduction of each cytochrome in steady state remain unknown. Furthermore, it remains also an open question the extent to which the reconstituted cytochrome proteoliposomes provide significant information on the kinetic behaviour of the cytochromes in intact mitochondria.

We have found that, during uncoupler titrations, the depression of the membrane potential is accompanied by a diminution of the % reduction of the cytochromes. The relationship is biphasic in the case of cytochrome *b* and proportional in the case of cytochrome *c*. However, the relationship between membrane potential and cytochrome reduction is also markedly affected by matrix alkalinization presumably as a consequence of the inhibitory effect of the high pH on the activity of cytochrome oxidase. As a consequence, divalent cation uptake leads to conditions where the large extents of cytochrome reduction are accompanied by marked diminutions of the membrane potential. Finally, we have found that in the case of cytochrome *c*, but not of cytochrome *b*, the variations of the percentage reduction correlate with the electrical component rather than with the total pmf.

2. Experimental procedures

Mitochondria were prepared as previously described [4,5]. The standard incubation medium used during the

Abbreviations: pmf, protonmotive force; BCECF, 2′,7′-bis (carboxyethyl)-5(6)carboxyfluorescein; cyt. *b*, cyt. *c*, cytochromes *b* and *c*; [^{14}C]DMO, 5,5-dimethyloxazolidine-2,4-dione; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; RLM, rat liver mitochondria; $TPMP^+$, triphenylmethylphosphonium ion.

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experiments contained: 0.2 M sucrose, 5 mM succinate, 30 mM Mops/Tris, 0.2 mM EGTA/Tris, 5 mM P_i/Tris, 5 μM rotenone, 1 μg/mg oligomycin, catalase, T 25°C (pH 7.4). Spectrophotometric studies were performed in a temperature-controlled, stirred cuvette using an SLM-Aminco DW-2000 dual-wavelength UV-VIS spectrophotometer connected to an IBM/PS2 computer system. Absorption spectra were obtained in the dual wavelength mode using a slit of 2 nm. On the basis of the correspondence between the spectral changes at 550–540 nm and at 421–407 nm: (a) induced by malonate, antimycin-A and myxothiazol and (b) occurring during the kinetic transitions in succinate-supplemented and in antimycin-inhibited mitochondria supplemented with sulfite [11], we have identified the species responsible for the cytochrome response in the Soret region at 421–407 nm as cytochrome *c*. Although most of the present experiments have been carried out at 421–407 nm, it has always been checked that the spectral changes at these wavelengths were proportional, although about 10-times larger, to those occurring at 550–540 nm. The electrical potential gradient, $\Delta\psi$, was evaluated by following the concentration of $TPMP^+$ in the incubation medium with a $TPMP^+$-sensitive electrode as described in Zoratti and Petronilli [6]. When necessary, the matrix volume was measured as previously described [7] and found to be equal to 1 μL/mg of protein. The transmembrane proton chemical gradient, ΔpH, was estimated by using two different techniques; first, by the distribution of the labelled weak acid [^{14}C]DMO (0.05 μCi/ml), as essentially described in Zoratti et al. [7]; second, by following the fluorescence change of the matrix pH indicator, BCECF, in BCECF-loaded mitochondria as described in Luvisetto et al. [8]. For the assay of calcium uptake under steady state conditions, different amounts of a stock solution of Ca^{2+}-NTA buffer were used. The amount of added calcium needed to achieve the free calcium concentrations in the range 1–50 μM was determined by using the computer program SPECS by Fabiato [22]. The final concentration of the NTA buffer in each measurement was 2 mM.

3. Results

Fig. 1A shows the effect of increasing FCCP concentrations on the relationship between $\Delta\psi$ and % reduction of cytochrome *c* at 421–407 nm. The 100% oxidation was taken as the absorbance after addition of malonate (5 mM), while the 100% reduction was assigned as the absorbance after exhaustion of the oxygen. There was a strict proportionality between the depressions of the transmembrane potential and of the % reduction of cytochrome *c*. Addition of increasing atractyloside concentrations to ADP-supplemented mitochondria caused, parallel to the inhibition of the rate of ATP synthesis, a proportional increase of $\Delta\psi$ and of the % reduction of cytochrome *c*. Finally, Fig. 1A shows also that addition of increasing FCCP concentrations to ADP-supplemented mitochondria caused a proportional decrease of $\Delta\psi$ and of % reduction of cytochrome *c*. These results demonstrate that the changes in % reduction of cytochrome *c* are proportional to the changes of $\Delta\psi$ and that the relationships between membrane potential and % reduction are independent of whether the titrations are carried out on mitochondria originally kept in state 4 or in phosphorylating conditions.

Fig. 1B shows the effect of increasing FCCP concen-

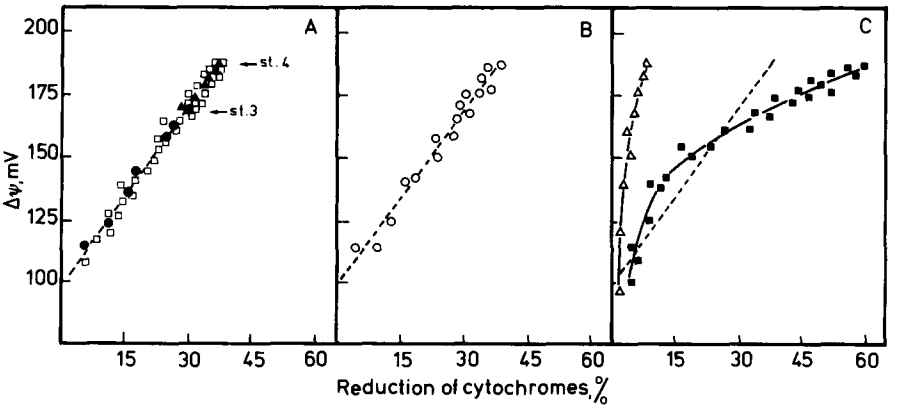

Fig. 1. Relationships between transmembrane electrical potentials and redox states of cytochromes *c*, *b*, aa_3. (A) RLM (2 mg/ml) were incubated in the standard incubation medium. After reaching anaerobiosis, the reaction was initiated with an excess of oxygen. After reaching state 4 equilibrium, increasing amounts of FCCP (0–100 pmol/mg) were added. Absorbance changes at 421–407 nm. Titrations with FCCP in the absence (□) or in the presence (●) of ADP (1.5 mM). Titrations with atractyloside (0–20 μM, ▲) in the presence of ADP (1.5 mM). Transmembrane electrical potentials were measured in parallel samples. (B) Titration with FCCP of the absorbance changes as recorded at the wavelength pairs 550–540 nm (○). (C) Titration with FCCP of the absorbance changes at the wavelength pairs 444–457 nm (△) and 565–575 nm (■), respectively. Dashed line in panels (B) and (C) represents the best-fit of the data in panel (A).

trations on the relationship between $\Delta\psi$ and % reduction of cytochromes *c* measured at 550–540 nm. The dashed line reported in Fig. 1B represents the best fit of the data of Fig. 1A.

Fig. 1C shows the effect of increasing FCCP concentrations on the relationship between membrane potential and % reduction of cytochromes *b* (measured at 565–575 nm) or cytochrome aa_3 (measured at 444–457 nm) [9,10]. For all the cytochromes the extent of reduction was calculated as percentage of the total extent of absorbance change between the absorbance level in anaerobiosis and the absorbance level after addition of malonate (5 mM). In state-4 mitochondria, the % reduction of the cytochromes decreased from 60% in the case of cytochrome *b*, to 40% in the case of cytochrome *c*, and to 10% in the case of cytochromes aa_3. While the changes of the % reduction of cytochrome *c* were proportional to those of the membrane potential this was not the case for either cytochrome aa_3 or *b*. In the case of cytochrome aa_3, due to the extensive oxidation of cytochrome aa_3 in state-4, the range of proportionality was very narrow. In the case of cytochrome *b* there was a biphasic behaviour. The first 40 mV of decrease of membrane potential were accompanied by a major variation (from 60% to 10%) of the % reduction of cytochrome *b*, while the further decreases of 40 mV were accompanied by the abolition of the remaining 10% reduction of cytochrome *b*.

To assess the differential effect of ΔpH and $\Delta\psi$ on the % reduction of cytochrome *c*, we have tested the effect of weak acids on state-4 mitochondria incubated in the absence of P_i. As shown in Table I addition of P_i resulted in an abolition of the ΔpH, in an increase of $\Delta\psi$ from 178 to 198 mV and in a parallel increase of reduction of cytochrome *c* from 33 to 42%. The increase of $\Delta\psi$ corresponded exactly to the depression of 0.3 unit of the ΔpH (about 20 mV), while the overall proton electrochemical gradient remained unchanged. Table I also shows that the behaviour of cytochrome *b* was different from that of cytochrome *c* in that addition of P_i resulted in a decreased, instead of an increased, reduction. The different behaviour of the two cytochromes was further confirmed by their responses after addition of nigericin (not shown).

Table I
P_i effects on the redox state of the cytochromes *b* and *c*

P_i (mM)	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)	cyt. *c* (%Red)	cyt. *b* (%Red)
/	178	20	198	33	65
5	198	0	198	42	60

Mitochondria (2 mg/ml) were incubated with standard incubation medium in the presence or absence of P_i (5 mM). After anaerobiosis, non-limiting amount of oxygen was added and, after reaching equilibrium, the transmembrane electrical potential and the % of reductions of cytochrome *c* were determined. The ΔpH was determined either by using isotopic distribution of [^{14}C]DMO or by following the fluorescence change of the matrix pH indicator, BCECF.

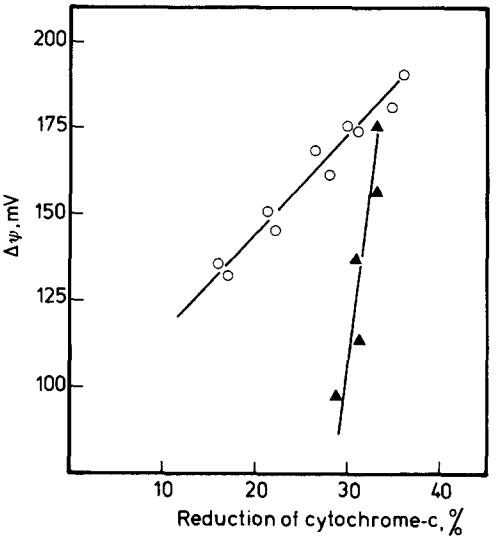

Fig. 2. Relationships between the transmembrane electrical potential and the redox state of cytochrome *c* during divalent cation uptake. RLM (2 mg/ml) were incubated in the absence (▲) or in the presence of P_i (5 mM) and A23187 (0.5 μg/mg) (○). After reaching anaerobiosis an excess of oxygen was added and, after reaching equilibrium, different amounts of Ca-NTA buffer solution were added. Free [Ca^{2+}] were varied between 0–50 μM. EGTA was omitted from the standard medium and, in the absence of P_i, mitochondria were supplemented with *N*-ethylmaleimide (20 μM). Absorbance changes were followed at 421–407 nm and transmembrane electrical potentials were measured in parallel samples.

In the early 1960's it was reported that divalent cation uptake resulted in a mitochondrial state characterized by a marked inhibition of the respiration and by a marked extent of cytochrome reduction. In view of the correlation between the respiratory inhibition and the high extent of reduction of the cytochromes, the divalent cation uptake was suggested to lead to a high energy mitochondrial condition, denoted as state 6. Fig. 2 shows the relationship between membrane potential and % reduction of cytochrome *c* during titrations with increasing concentrations of Ca^{2+}. The titrations were carried out under two sets of stationary state conditions. In the first (empty circle), mitochondria were supplemented with P_i and the ionophore A23187 in order to maintain a continuous cycling of the divalent cations and thus avoiding any increase of matrix pH. In the other (full triangle), both P_i and A23187 were omitted thus allowing for a limited divalent cation uptake and a large matrix alkalinization. Under these latter conditions, corresponding to those known as state 6, there was still a linear relationship between membrane potential and % reduction of cytochrome *c* but the extent of reduction of cytochrome *c* was, at each membrane potential, much higher. The experiment of Fig. 2 thus shows that the divalent cation

uptake leads to a condition where the high extent of reduction of cytochrome *c* is accompanied by a low and not by a high membrane potential. Similar results were obtained by titrating the response of cytochrome *b* instead of that of cytochrome *c*. On the other hand, when the titrations were repeated with the isolated proton pumps at site II, no effect of alkalinization was observed. This indicates that the effect of alkalinization requires the simultaneous operation of site II + III proton pumps. State 6 then corresponds to a kinetically inhibited state rather than to a high energy state.

4. Discussion

While the thermodynamic control of the redox state of the cytochromes in steady-state conditions is a long-standing concept, the quantitative relationship between % reduction and pmf has become a matter of interest only recently. Two problems have arisen. One is the assay of the quantitative effects of pmf. The other is a possible difference between the behaviour of the reconstituted proteoliposomes and of the intact mitochondria. In intact mitochondria a differential effect of pmf on the kinetics of the various pumps, and therefore of the kinetics of one over that of another proton pump, is likely to overlap with the direct effects of pmf over the kinetics of each pump.

This difference is exemplified by the behaviour of cytochrome *a*. In fact, while in proteoliposomes a large part of cytochrome *a* is reduced in the resting state and goes oxidized after stimulation of the respiration, in intact mitochondria most of cytochrome *a* is oxidized in the resting state and the small reduced part undergoes an oxidation which is difficult to correlate with the decline of the membrane potential [12–21].

In intact mitochondria the pattern of the relationships between % reduction of cytochrome *b* and *c* and protonmotive force is different from that of cytochrome *a* and also different between themselves. In the case of cytochrome *b* the relationship between membrane potential and % reduction is biphasic, with a slope which is very smooth (large decline in % reduction and slight decline in membrane potential) in the high potential range and very steep (small change in % reduction and large in membrane potential) in the low potential range. Whether this biphasicity is a consequence of the different potential dependence of the two cytochromes *b* contributing to the absorbance band at 565–575 nm remains to be clarified. A still different behaviour is obtained in the case of cytochrome *c*. During titrations with uncouplers in state-4 mitochondria, or with atractyloside in phosphorylating mitochondria, the relationship between redox state of cytochrome *c* and membrane potential is strictly proportional.

The proportionality between the % reduction of cytochrome *c* and the membrane potential raises, in turn, two other questions. The first is the dependence of the relationship on the matrix pH and the second is whether the % reduction of cytochrome *c* constitutes an internal probe of the pmf. As to the first question we find that the inhibition of the site III redox pump at alkaline pH causes a higher extent of reduction of all cytochromes. As a consequence, the slope of the relationship between membrane potential and % cytochrome reduction increases with the increase of matrix pH. As to the second question the data of Table I indicate that the % reduction of cytochrome *c* depends exclusively on the membrane potential rather than on the sum of the electrical and the chemical components of the pmf. This is consistent with the position of cytochrome *c* with respect to the topology of the respiratory chain and thus with the view that this cytochrome, at variance from the case of cytochromes *b* is not directly participating to the proton pumping processes.

In conclusion the redox state of cytochrome *c* is not affected by the pH while the alkalinization-induced inhibition of cytochrome oxidase introduces kinetic limitations in the activity of both cytochromes *c* and *b*. The present results emphasize that extrapolations of the kinetic data obtained on the reconstituted systems to the intact mitochondria are unwarranted.

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