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Energy-linked change in the redox state and absorption spectrum of cytochrome *a in situ*

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SUMMARY

In the transition from the aerobic controlled state to anaerobiosis in rat liver mitochondria with succinate as substrate, cytochrome a_3 is reduced prior to cytochrome a, the two components being well separated both in time and in spectral properties. Addition of ATP to anaerobic rat liver mitochondria supplemented with succinate results in 70–80% oxidation of cytochrome a and 10–15% oxidation of cytochrome $c+c_1$ without any measurable change in cytochrome a_3. ATP also causes a red shift of 1–1.5 nm in the absorption spectrum of ferrocytochrome a. The shift, which is abolished by uncoupling agents and by oligomycin but is unaffected by cyanide and dithionite, may reflect a molecular event in the energy-conserving mechanism of the terminal "coupling site".

Wikström and Saris[1] attempted to determine the effect of ATP on cytochromes a and a_3 separately under conditions of "reversed electron flow" in mitochondria (see refs 2–4). Their data (see also ref. 5), which were based on the generally accepted assumption[6] that the spectral contribution of cytochrome a at 605 *minus* 630 nm is much larger than of cytochrome a_3, suggested that ATP causes a much larger oxidation of cytochrome a_3 than of cytochrome a when added to anaerobic mitochondria in the presence of succinate. We also demonstrated for the first time an ATP-linked shift in the absorption spectrum of reduced cytochrome c oxidase, which, on the basis of the above assumption, was interpreted[1,5] as an effect on ferrocytochrome a_3. The recent demonstration by Wilson

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulphate.

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et al.[7] and Muijsers *et al.*[8] that cytochromes *a* and a_3 contribute about equally to the absorption peak, both in the Soret and the α region, in anaerobic mitochondria[7] and in isolated oxidase[8], in the absence of inhibitors, necessitated a re-examination of this conclusion.

Fig. 1 shows the transition from the controlled State 4 to the anaerobic State 5 (for nomenclature, see ref. 9 but note that our State 4 is different from that defined by Chance and Williams[9] in that no ADP or P_i was added during the course of the experiment). The transition is measured at 605 *minus* 630 nm in rat liver mitochondria respiring on succinate. After anaerobiosis, reduction occurs in two distinct phases[1] that are best separated at a low respiratory activity (*e.g.* low pH), each comprising about 50% of the total redox change. The same effect is found in the Soret region[1]. Wikström and Saris[1] showed that distinction between the two phases was not possible in the presence of oligomycin or uncoupling agents. Thus the separation is due to a high phosphate potential at the point of anaerobiosis and the second phase of reduction is almost entirely caused by the spontaneously decreasing phosphate potential (*cf.* addition of ATP *plus* O_2 in Fig. 1). At a lower phosphate potential (O_2 addition after an anaerobic period, Fig. 1) the rate of reduction on anaerobiosis becomes faster. The effects of ATP, phenazine methosulphate (PMS) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) are further dealt with below. Fig. 2A shows the wavelength dependence of the two phases. The reason for the biphasic kinetics is clearly consecutive reduction of two components absorbing at approx. 603.5 and 607.5 nm, respectively, and with similar contribution to the total redox change. This is in good agreement with the finding[7,8] that the 605-nm band is composed of equal contributions from cytochromes *a* and a_3 in the absence of ligands to the latter. Wilson *et al.*[7] identified the short-wavelength component with cytochrome a_3 (E'_0 = 380 mV at pH 7.2) and the long-wavelength component with cytochrome *a* (E'_0 = 220 mV

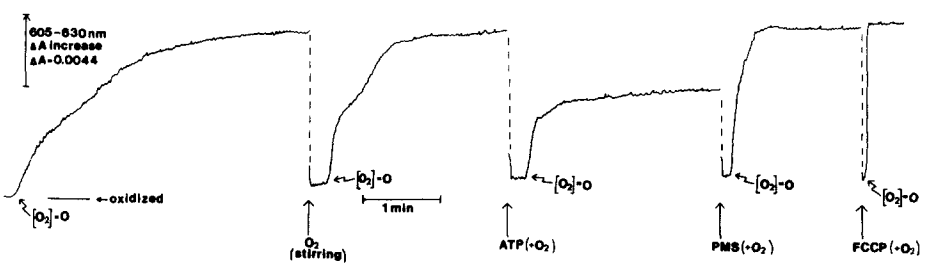

Fig. 1. State 4–State 5 transition and the effects of ATP and PMS in State 5. Rat liver mitochondria (2.3 mg protein/ml) were suspended in a medium consisting of 0.2 M mannitol–0.05 M sucrose–0.03 M morpholinopropane sulphonate (pH 6.8), in the presence of 3 μM rotenone. 6.7 mM sodium succinate was then added and the suspension became anaerobic after approx. 12 min. After anaerobiosis the following additions were made as indicated: O_2 by stirring, 4.8 mM ATP (with O_2), 20 μM PMS (with O_2) and 1 μM FCCP (with O_2). All measurements (Figs 1–3) were performed with the Aminco–Chance dual-wavelength spectrophotometer at a temperature of 21–22 °C using cuvettes of 1-cm light path, and a slit width of 0.2 mm. The line marked "oxidized" indicates the redox level before the addition of succinate.

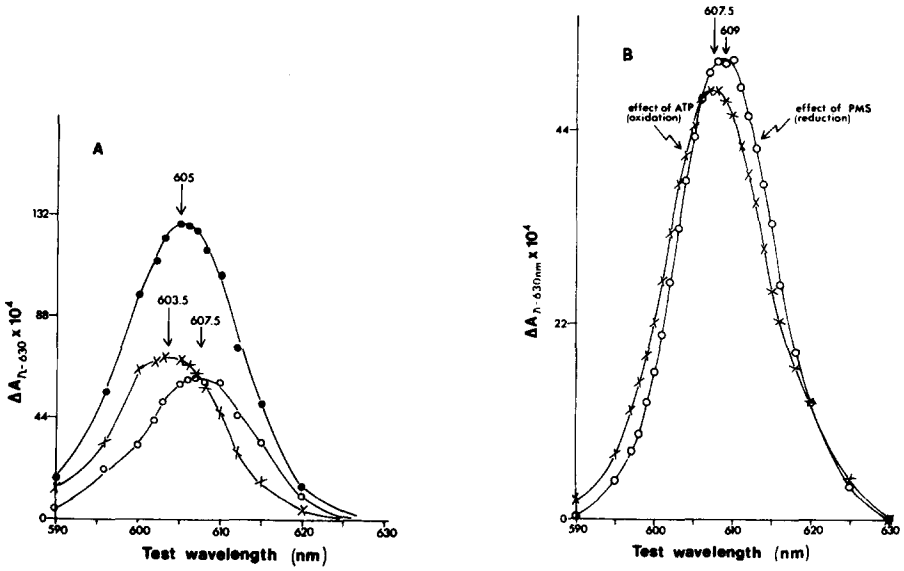

Fig. 2. The wavelength dependence of the State 4–State 5 transition and the effects of ATP and PMS. Rat liver mitochondria (3.6 mg protein/ml) were suspended in the medium described in the legend to Fig. 1, but at pH 7.2, and supplemented with 3 μM rotenone). 6.7 mM sodium succinate was then added and in A the State 4–State 5 transition was followed at different measuring wavelengths between 590 and 630 nm with a constant reference at 630 nm. A separate incubation was performed for each point. The approximate extent of the first (X——X) and second (O——O) phase of reduction and the total change on anaerobiosis (●——●) are plotted in the figure. In B an absolute spectrum with reference to the absorption at 630 nm was taken in State 5, after addition of 5 mM ATP to State 5, and after addition of 20 μM PMS to State 5 + ATP, by varying the measuring wavelength between 590 and 630 nm. The two spectra show the difference State 5 *minus* State 5 + ATP (X——X) and State 5 + ATP + PMS *minus* State 5 + ATP (O——O).

at pH 7.2) in pigeon heart mitochondria. A similar identification of the short-wavelength species with that of high midpoint potential and of the long-wavelength species with that of low midpoint potential has also been made in mitochondria from rat liver and beef heart (unpublished observations) and in isolated cytochrome aa_3 (ref. 10). Thus it seems evident from Figs 1 and 2A that cytochrome a_3 is reduced prior to cytochrome a in the State 4–State 5 transition.

Fig. 1 also shows that addition of ATP to anaerobic rat liver mitochondria in the presence of succinate results in 35–40% oxidation of cytochrome aa_3. This is accompanied by 10–15% oxidation of cytochrome $c+c_1$ measured at 550 *minus* 540 nm (not shown). Both effects are abolished by the further addition of PMS (see Fig. 1) and also by uncoupling agents and by oligomycin. From Fig. 2B it is seen that the component that is oxidized upon the addition of ATP absorbs maximally at 607 nm. The component that is re-reduced on subsequent addition of PMS (Figs 1 and 2B) absorbs at 608–609 nm (see below). Thus cytochrome a is oxidized to the extent of approx. 70–80% on addition of ATP while cytochrome a_3 remains largely reduced. This is in complete contrast to the

earlier conclusions[1,5], based on erroneous relative contributions of cytochromes *a* and a_3 to the α band, that a_3 is the component that is mainly oxidized under these conditions. Only in systems of considerably higher redox potential have we observed an ATP-linked oxidation of cytochrome a_3 (not shown, see refs 11 and 12).

If cytochromes $c+c_1$ (E'_0 = 230 mV at pH 7.2, ref. 13) and *a* (E'_0 = 190–220 mV at pH 7.2, refs 7, 13–15) are assumed to be in equilibrium in the presence of ATP under the above conditions the difference in apparent midpoint potentials is approx. 75 mV, cytochrome $c+c_1$ having the more positive midpoint potential. Since in the uncoupled state the difference in midpoint potentials between cytochromes $c+c_1$ and *a* equals 10–40 mV (see above and refs 7, 13–15), it may be concluded that ATP has lowered the apparent midpoint potential of cytochrome *a* by 35–65 mV with respect to cytochrome $c+c_1$. This is in excellent agreement with the observation by Hinkle and Mitchell[16] that ATP decreases the apparent midpoint potential of cytochrome *a* (defined as the component that does not react with CO) by about 40 mV in mitochondria where the redox potential of cytochrome $c+c_1$ is fixed with ferri-/ferrocyanide. The data of Fig. 2B also suggest that the apparent midpoint potential of cytochrome a_3 was not lowered below 220 mV in the presence of ATP since more than 20% oxidation of this component would have been detected.

These data are in contrast to the conclusion of Wilson and co-workers[14,15] that the midpoint potential of cytochrome a_3 is lowered below 200 mV by ATP and that the midpoint potential of cytochrome *a* remains unchanged. Recently Wilson[17] reported that ATP increases the midpoint potential of cytochrome *a* by 40 mV. The finding (Fig. 1) that cytochrome a_3 is reduced before cytochrome *a* in the State 4–State 5 transition is also not in agreement with a midpoint potential of cytochrome a_3 similar or lower than that of cytochrome *a* in the "energized" state[15]. Since the data reported by Wilson and co-workers[14,15,17] were not accompanied by measurements of spectra they could *per se* equally well be interpreted as an ATP-induced decrease in the apparent midpoint potential of cytochrome a_3 from 380 mV to approx. 250 mV (ascribed by Wilson[17] to the increased midpoint potential of cytochrome *a*) and of cytochrome *a* from 220 mV to about 160 mV (previously ascribed[15] to the decreased potential of a_3). If this were true, the data of Wilson *et al.*[14,15,17] would be in excellent agreement with those of Hinkle and Mitchell[16] as well as with those reported here.

As shown in Fig. 1, PMS abolishes the ATP-linked oxidation of cytochrome *a* (and of cytochrome $c+c_1$) in the presence of succinate. This is presumably due to a lowered redox potential at cytochrome $c+c_1$ caused by a PMS-induced transfer of reducing equivalents from the flavoprotein region to cytochrome $c+c_1$ making "reversed electron transfer" from cytochrome *a* to $c+c_1$ unfavourable. In the presence of ATP and PMS, ferrocytochrome *a* absorbs maximally at a longer wavelength (Fig. 2B). This is due to the presence of ATP since, as shown in Fig. 3, the addition of an uncoupler (or oligomycin[1], not shown) reverses the shift in the ferrocytochrome *a* spectrum and consequently causes a typical "shift spectrum" with extrema at approx. 600 and 613 nm (*cf.* ref. 1). This was previously interpreted[1,5] as a shift in the spectrum of ferrocytochrome a_3. Addition of ATP in the absence of uncoupler under conditions otherwise as in Fig. 3 produces a "shift

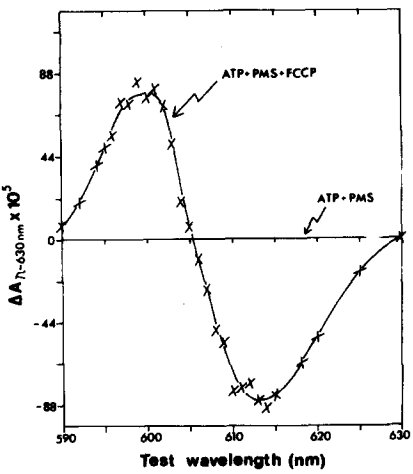

Fig. 3. Reversal of the ATP-induced spectral shift by FCCP. Experimental conditions as described in the legend to Fig. 2. After anaerobiosis 5 mM ATP and 20 μM PMS were added and the absolute spectrum (with reference to A at 630 nm) was taken. 1 μM FCCP was then added and the procedure repeated. The spectrum is the difference State 5 + ATP + PMS + FCCP *minus* State 5 + ATP + PMS.

spectrum" that is the opposite to that shown in Fig. 3, *viz.* a decrease in absorption at 600 nm and an increase at 613 nm (compare also the two spectra in Fig. 2B). The shift is unaffected by dithionite and can therefore hardly be attributed to an oxidation and a reduction effect of different origin. Under conditions where the shift is observed there is also no oxidation of copper detectable by EPR spectroscopy.

Thus Figs 2B and 3 suggest that ATP causes a red shift of 1–1.5 nm in the absorption spectrum of ferrocytochrome *a* and a slight (6%) increase in absorbance coefficient. This interpretation is strengthened by the finding (not shown) that the shift is unaffected aerobically by cyanide since under these conditions the spectral position of cytochrome a_3 is drastically changed[6,18,19] while that of cytochrome *a* is essentially unaffected. The effect of inhibitors of cytochrome *c* oxidase on the energy-linked shift in ferrocytochrome *a* absorption spectrum will be described in more detail in a subsequent paper.

Preliminary data have shown that the spectrum of ferrocytochrome *a* does not shift continuously with energy, but rather that energy affects an equilibrium between two electronically different forms of the cytochrome. Thus energy increases the number of cytochrome *a* molecules undergoing the same shift. Therefore the shift is not a direct physical response to a change in the heme environment (electric field, polarity *etc.*). It seems possible then that it reflects a molecular event in the energy-conserving mechanism of the terminal "coupling site", possibly a conformational change involving the heme iron. A secondary response towards "energization", not directly related to the energy-conservation mechanism cannot, however, be excluded at present.

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