Biochimica et Biophysica Acta, 408 (1975) 170–179

BBA 46997

A SPECTRAL SHIFT IN CYTOCHROME *a* INDUCED BY CALCIUM IONS

MÅRTEN WIKSTRÖM* and HERKKO SAARI

*Department of Medical Chemistry***, *University of Helsinki, Helsinki (Finland)*

(Received May 15th, 1975)

SUMMARY

Ca^{2+} induces a red shift in the absorption spectrum of ferrocytochrome *a* when added to uncoupled mitochondria, sub-mitochondrial particles or isolated cytochrome aa_3. The shift is identical within experimental error to the previously reported energy-linked shift in intact mitochondria (Wikström, M. K. F. (1972), Biochim. Biophys. Acta 283, 385-390). One mol of calcium produces the shift in one mol of cytochrome *a*, the K_D being approx. 20–30 μM. The calcium-induced shift is readily reversed by chelating agents such as EDTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*'-tetra-acetic acid (EGTA) and ATP and is insensitive to uncoupling agents and inhibitors of calcium transport (La^{3+} and ruthenium red). It is shown that the binding site for calcium that is responsible for the spectral shift is located on the outside of the permeability barrier of the mitochondrial cristae membrane.

It is proposed that calcium simulates the energy-linked shift in cytochrome *a* by binding to a site of cytochrome aa_3 that is occupied by protons in energized mitochondria and that is located at the external surface of the mitochondrial membrane.

INTRODUCTION

We have previously reported [1–3] that energization of the mitochondrial membrane with ATP induces a red shift in the absorption spectrum of ferrocytochrome aa_3. This was the first direct evidence for energy-linked alteration of the physicochemical properties of respiratory carriers in the mitochondrial membrane. Subsequently, an energy-linked spectral change has also been reported for ferricytochrome aa_3 of intact mitochondria [4, 5] that is consistent with a high to low spin shift in heme iron, suggesting a considerable conformational change. The energy-linked changes in cytochrome aa_3 are dependent on the phosphate potential (a function of the ratio $[ATP]/[ADP][P_i]$) rather than on the concentration of ATP [3–6] and are prevented and reversed by oligomycin and uncouplers of oxidative phosphorylation [1–6]. As shown by Wikström and Saari [5], the spectral changes in ferrous and ferric aa_3 may also be produced by coupled electron transport and by an

* Person to whom correspondence should be addressed at the Department of Biophysics and Physical Biochemistry, University of Pennsylvania, Philadelphia, Pa. 19174, U.S.A.

** Postal address: Siltavuorenpenger 10A, 00170 Helsinki 17, Finland.

electrical diffusion potential across the mitochondrial membrane (positive polarity outside). In both these cases the spectral changes are insensitive to oligomycin.

From these findings it appears clear that the spectral changes in cytochrome aa_3 are intimately linked to the energy state of the mitochondrial cristae membrane and are useful intrinsic probes of this energy state (see ref. 5). Whether these spectral changes do reflect the formation of true primary intermediates in mitochondrial energy conservation and oxidative phosphorylation [4, 6] has been questioned recently [3, 5], but thorough study of these changes may elucidate the energy conservation process and the mechanism of respiratory control in mitochondria [5]. In this respect it was of interest that the spectral shift in ferrocytochrome aa_3 was found to be produced by Ca^{2+} in uncoupled mitochondria [3]. Thus there may be a chance to study the molecular shifts inherent in energization of the mitochondrial membrane by simulating them in fully de-energized and possibly in isolated systems.

In this paper we report on the properties of the energy-independent, calcium-induced shift in the spectrum of ferrocytochrome aa_3. It is shown that this shift occurs in cytochrome *a*, that the binding site for calcium appears to be located on the outside of the inner mitochondrial membrane, and that the shift can be demonstrated in isolated cytochrome oxidase.

RESULTS

Fig. 1 shows the effect of ATP added to anaerobic mitochondria supplemented with phenazine methosulphate to abolish energy-dependent oxidation of cytochrome aa_3 (see ref. 2). The effect of ATP is studied with the dual wavelength technique at a wavelength couple maximally sensitive to the energy-linked red shift in cytochrome *a* (see refs 2 and 3). In the upper trace it is shown that under these experimental conditions ATP does not cause any net change in absorption. However, the addition of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) after the ATP produces an upward deflection of the trace (in the upper trace of Fig. 1 this is followed by a downward deflection due to the calcium added before FCCP; see below). Addition of uncoupler without previously added ATP has no effect. The inability of ATP to produce a net change in absorption under these conditions is explained by the experiment shown in the lower trace of Fig. 1. The addition of ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetaacetic acid (EGTA) (or EDTA) after anaerobiosis produces an increase in the absorption difference. This effect is insensitive to uncoupling agents. ATP added after the EGTA now causes a decrease in absorption, and this effect of ATP is reversed by the uncoupler FCCP. Subsequent addition of calcium at higher concentrations than those of EGTA, again decreases the absorption to the initial level. As will be shown below, all these deflections (i.e. those induced by ATP, FCCP, EGTA and calcium) are due to generation (ATP, calcium) or abolition (FCCP and EGTA) of the typical spectral red shift in ferrocytochrome *a* (see Figs 2 and 4).

From these data it is clear that ATP without previous addition of EGTA (Fig. 1, upper trace) produces a dual effect, an increase in absorption due to chelation of calcium (this effect can be directly demonstrated in the presence of FCCP, not shown), and a decrease in the absorption due to the energization of the mitochondrial membrane (Fig. 1, lower trace). The two effects are equal in magnitude but of opposite direction, resulting in the observed zero net effect (Fig. 1, upper trace). From these

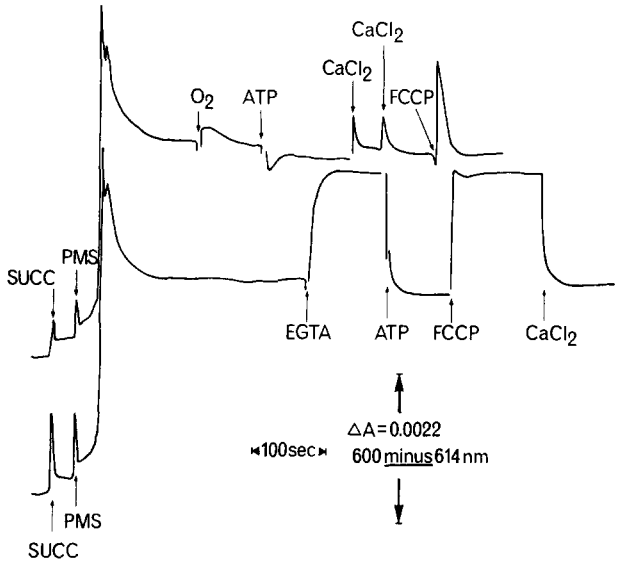

Fig. 1. Spectral shift in ferrocytochrome aa_3 induced by ATP and Ca^{2+}. Standard reaction medium supplemented with 4 μM rotenone, 2 mM $MgCl_2$ and mitochondria (approx. 2.5 mg protein/ml). 6.7 mM succinate (succ.) and 20 μM phenazine methosulphate (PMS) were added after which anaerobiosis is observed as a biphasic deflection at the wavelength couple 600–614 nm due to the presence of two components in the 605 nm peak of cytochrome aa_3 (see refs 2 and 10). Upper curve: O_2 addition by stirring followed by anaerobiosis, 0.67 mM ATP, two additions of 40 μM $CaCl_2$ and 1 μM FCCP. Lower curve: 80 μM EGTA, 0.67 mM ATP, 1 μM FCCP and 160 μM $CaCl_2$.

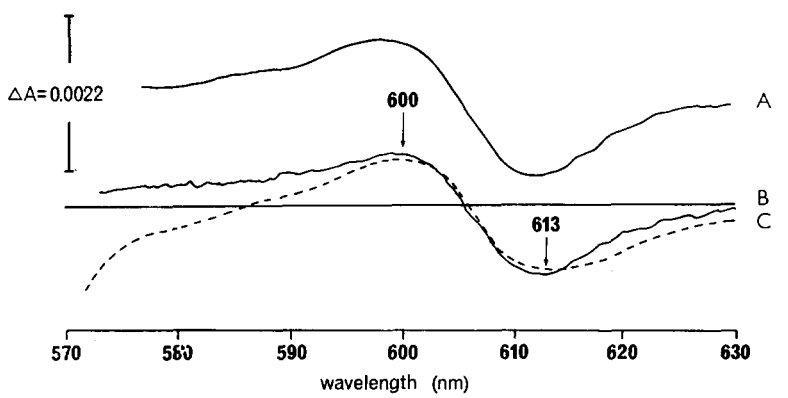

Fig. 2. Wavelength dependence of spectral shift in ferrocytochrome aa_3. Standard reaction medium supplemented with 4 μM rotenone, 6.7 mM succinate, 5 μM phenazine methosulphate and mitochondria (approx. 2.5 mg protein/ml). Curve A: 1 μM FCCP and 20 μM $CaCl_2$ were further added. After anaerobiosis the suspension was divided into two cuvettes and the baseline recorded (not shown). Then 80 μM EGTA was added to the sample cuvette and the difference (sample minus reference) recorded. B: Further addition of 100 μM EGTA. After anaerobiosis and recording the baseline, 0.67 mM ATP was added to the reference cuvette and 1 μM FCCP to the sample. C: 1 mM KCN, 20 mM $CaCl_2$ and 1 μM FCCP were further present and the mixture remained aerobic. After recording the baseline 80 μM EGTA was added to the sample cuvette. An upward deflection denotes higher absorption in the sample cuvette relative to the reference.

data then it appears that Ca^{2+} may be able to produce a spectral shift in ferrocytochrome aa_3 similar to the energy-linked shift both in magnitude and wavelength dependence (see below), but under fully non-energized conditions (see also ref. 3).

Fig. 2 shows the wavelength dependence of the shifts caused by energization of the mitochondrial membrane with ATP, and the shift produced under non-energized conditions by calcium in the absence and presence of cyanide. The spectra are very similar, if not identical, both in extent and in wavelength dependence. Thus energization of the mitochondrial membrane and binding of Ca^{2+} under non-energized conditions both appear to cause the same or a similar molecular change in the ferrocytochrome aa_3 complex.

The difference spectrum in the Soret region induced by calcium (Fig. 3) suggests, as indicated by the inserted model, that we are dealing here with red shifts in two separate absorption peaks. The difference spectrum at 77 °K (Fig. 4) confirms this interpretation, clearly showing two separate shift spectra. Fig. 5 shows that although very little difference between the shift spectra in the presence and absence of cyanide was observed in the α-region, a slight difference becomes apparent in the Soret. The finding of a shift in two peaks in the Soret region (Figs 3 and 4) and the relative insensitivity of the shift to cyanide (in the presence of cyanide and air cytochrome a_3 is mainly in the ferri form complexed with cyanide, while it is in the ferro form in the absence of the ligand under the anaerobic conditions employed) are strong evidence in favour of our previous proposal [2, 3] that the red shift in reduced cytochrome *c* oxidase is in cytochrome *a*. Ferrocytochrome *a* has been shown to have a unique split absorption band in the Soret region [7] which agrees excellently with the observed

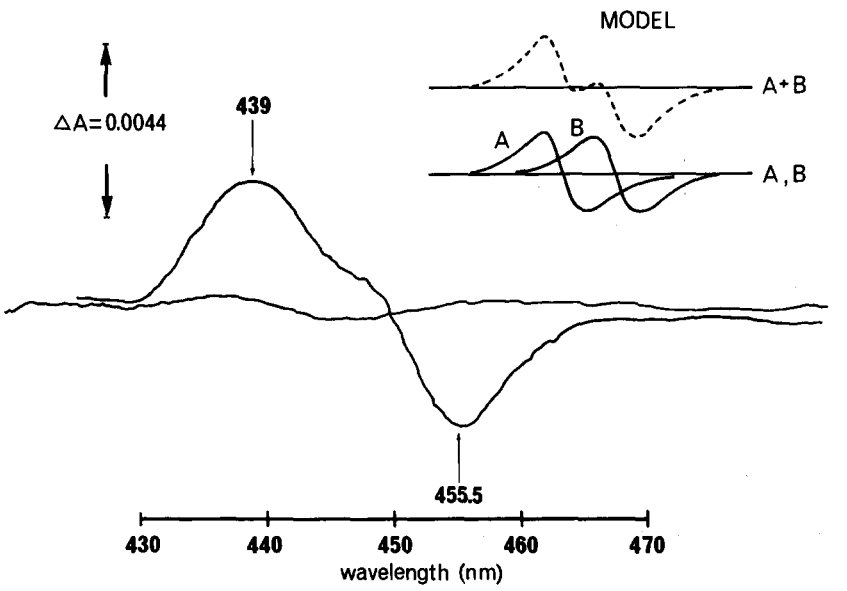

Fig. 3. Calcium-dependent spectral shift in cytochrome aa_3. Experimental conditions as described in the legend to Fig. 2A. The inset shows a model of a shift in two separate absorption peaks (A and B) and how these may add up to a difference spectrum (A+B) similar to that observed experimentally. The spectra show the difference EGTA (sample) minus Ca^{2+} (reference).

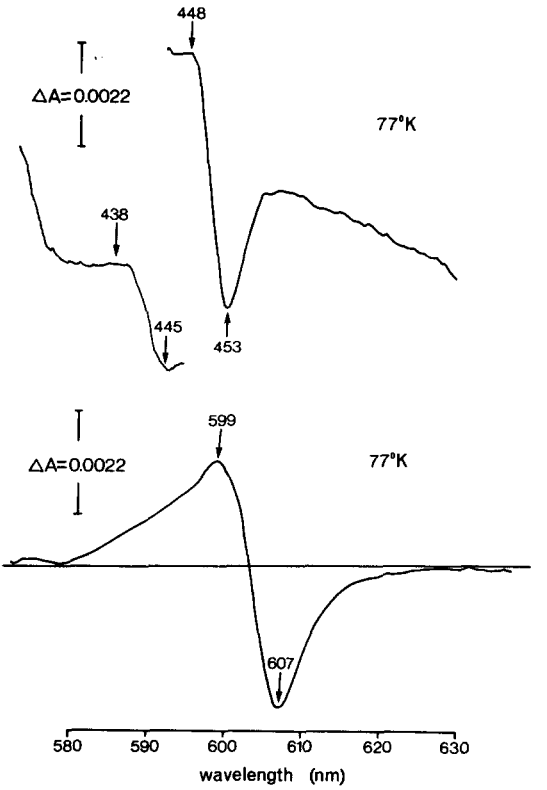

Fig. 4. Calcium-induced spectral shift in cytochrome aa_3 at 77 °K. Experimental conditions as described in the legend to Fig. 2A, except that the difference spectra were recorded at 77 °K in cuvettes of 2 mm light path. The spectra show the difference EGTA (sample) minus Ca^{2+} (reference).

shift in two absorption peaks.

Titration of the calcium-induced red shift in uncoupled mitochondria reveals a K_D of approx. 20–30 μM for calcium and a 1 : 1 stoichiometry between the spectrally shifted cytochrome and calcium (Fig. 6). Neither the kinetics nor the extent of the calcium-induced spectral shift is affected by lanthanum ions or by ruthenium red, the well known inhibitors of calcium translocation by mitochondria [8, 9] suggesting that calcium need not be translocated across the mitochondrial membrane to reach the appropriate binding site (see also below for further evidence supporting this notion). Fig. 7 shows the transition of rat liver mitochondria from the aerobic to the anaerobic state as measured at the wavelength couple maximally sensitive to the red shift. In experiment A all calcium is complexed by EGTA and upon anaerobiosis only reduction of cytochromes a and a_3 is seen. At the wavelength couple employed, reduction of the two hemes in cytochrome oxidase causes increase in absorption at wavelengths slightly above and below 606 nm, respectively (see refs 2 and 10) which results in sequential upward and downward deflections at the wavelength couple used. When calcium is present during the aerobic phase (curve C), it is taken up by the mitochondria and released upon anaerobiosis. The release of calcium is seen as a gradual red shift in the spectrum corresponding to the slow final downward deflection of

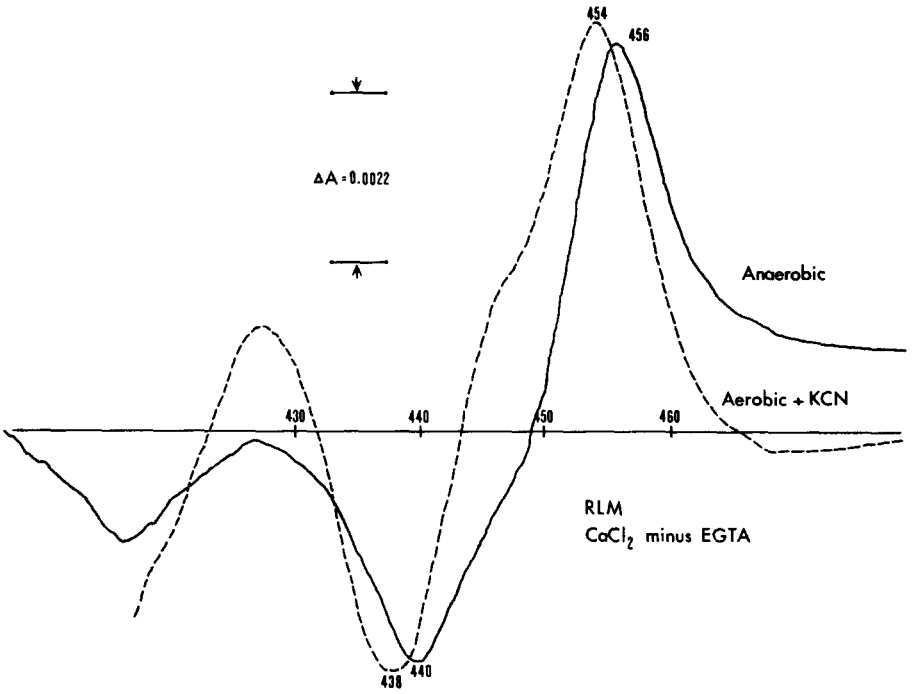

Fig. 5. The effect of cyanide on the calcium-induced shift. Experimental conditions as described in the legend to Fig. 2A and C, except that the EGTA was added to the reference cuvette.

Curve C. Complexation of the released calcium by EGTA reverses this shift (see also Fig. 1). When La^{3+} is present aerobically together with calcium, uptake of the latter is largely inhibited and most of the calcium remains in the extramitochondrial space. Thus, as seen in trace B, the spectrum of ferrocytochrome *a* is largely red-shifted immediately upon anaerobiosis (compare trace B with traces A and C).

These experiments demonstrate that the calcium-reduced red shift is apparent only when calcium is present in the extramitochondrial space, supporting the view that the calcium binding site responsible for the shift in cytochrome aa_3 is located outside the permeability barrier of the membrane.

We reported in a previous communication [3] that manganese was also able to produce the red shift in ferrocytochrome *a*. This conclusion was made from preliminary experiments with rat liver mitochondria to which EGTA was added first to get rid of endogenous calcium. Subsequent titration with manganese produced the red shift, but this was apparently due to binding of manganese to the EGTA, liberating the calcium. We reached this conclusion from experiments with isolated cytochrome *c* oxidase preparations [11] with very low calcium content to which the ions could be added without previous addition of chelating agent. Fig. 8 shows that calcium shifts the absorption spectrum of isolated ferrocytochrome aa_3 in the same way as in intact mitochondria*. Similar results have been obtained with submitochondrial particles

* This has recently been confirmed by Dr Peter Nicholls (Biochim. Biophys. Acta (1975), in the press).

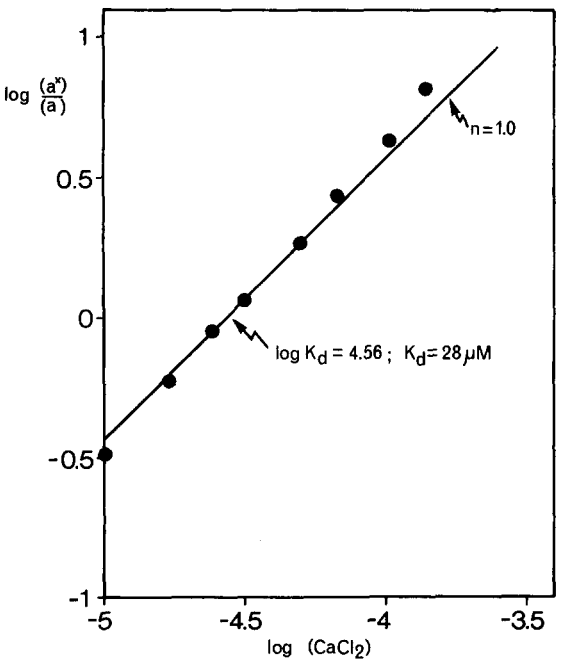

Fig. 6. Titration of the spectral shift with calcium. Standard reaction medium was supplemented with 4 μM rotenone, 1 μM FCCP, 6.7 mM succinate, 10 μM phenazine methosulphate and mitochondria (approx. 2.5 mg protein/ml). After anaerobiosis 40 μM $CaCl_2$ was added and the titration carried out by adding aliquots of EGTA and measuring at 600 minus 612 nm. The concentration of free calcium was plotted logarithmically against the ratio of the two forms of cytochrome *a* assuming that the cytochrome is entirely in the *a** form in the presence of excess Ca^{2+} and in the *a* form with excess EGTA.

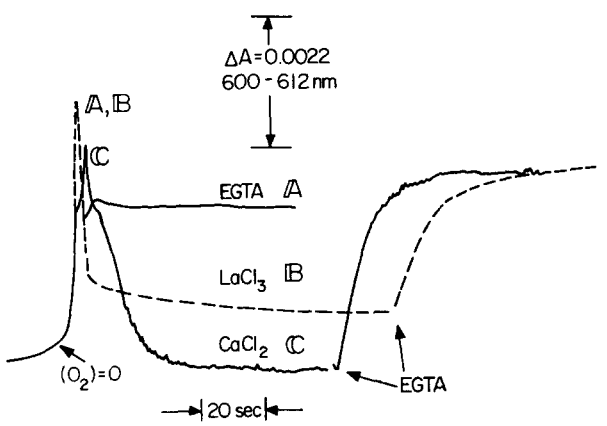

Fig. 7. Kinetics of the calcium-induced shift on anaerobiosis. Mitochondria were suspended at approx. 2.5 mg protein/ml in the standard reaction medium to which the following additions were made: 4 μM rotenone, 30 μM $CaCl_2$ and 6.7 mM succinate. The figure shows the spectrophotometric recording at 600 minus 612 nm starting from the point of anaerobiosis of the suspension (O_2 = 0). The deflections on anaerobiosis are partly due to sequential reduction of cytochromes *a* and a_3 and partly to the spectral shift in ferrous cytochrome *a*. In A (EGTA), 100 μM EGTA was added initially. In B ($LaCl_3$) 10 μM of lanthanum was added initially. Additions of 100 μM EGTA were made as indicated.

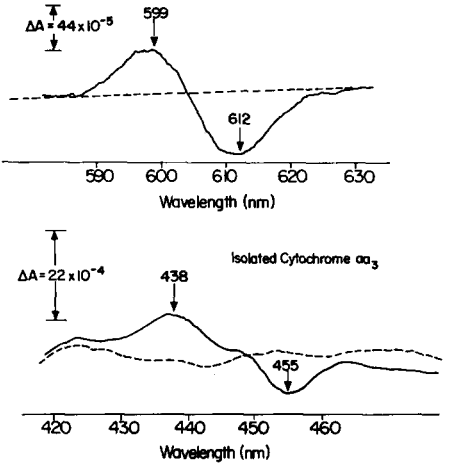

Fig. 8. Calcium-induced shift in isolated cytochrome oxidase. Isolated cytochrome *c* oxidase was suspended in 20 mM HEPES/KOH buffer (pH 7.0) at a concentration of 0.5 μM aa_3. 25 μM cytochrome *c* and 6.7 mM ascorbate were added. After anaerobiosis 100 μM $CaCl_2$ was added and the suspension was divided into two cuvettes. After recording the baseline (dotted lines) 0.33 mM EGTA was added to the sample cuvette and the difference spectrum (sample minus reference) recorded at room temperature. An upward deflection denotes higher absorption in the sample cuvette relative to the reference.

and also with phospholipid vesicles inlaid with cytochrome aa_3. The K_D for calcium was the same within experimental error in the isolated cytochrome aa_3 and intact mitochondria. With isolated cytochrome oxidase it was found that the shift is highly specific for calcium. Thus, no shift was produced by either lanthanum ($< 100\ \mu$M), manganese (< 1 mM) magnesium (< 20 mM), potassium (< 50 mM) or sodium (< 50 mM). This specificity is clearly different from that of the calcium-accumulating system of intact mitochondria [12].

DISCUSSION

In this paper we have demonstrated for the first time that an energy-linked change in the spectral properties of a respiratory carrier may be simulated in isolated and purified preparations of the respiratory carrier. This may be of importance for the future elucidation of the molecular basis for the structural effects in respiratory carriers that accompany energization of the mitochondrial membrane.

Ca^{2+} produce a spectral shift by binding to reduced cytochrome aa_3 that is indistinguishable within experimental error from the shift produced by energization of the mitochondrial membrane. Thus the question arises whether the energy-linked shift could be due to binding of calcium to the mitochondrial membrane. This, however, appears very unlikely from the data presented here. First, the energy-linked shift is unaffected by very high concentrations of calcium chelators. Secondly, both the energy-linked and the calcium-induced shifts are unaffected by lanthanum and ruthenium red, known inhibitors of energy-linked calcium binding to the mitochondrial membrane [8, 9]. Thirdly, the ion specificity of the two processes is different, and

finally, it was clearly shown (Fig. 7) that the calcium-induced shift becomes apparent only after "de-energization" and efflux of calcium from the mitochondria. We therefore conclude that the energy-linked shift is not due to calcium binding.

The question of how closely calcium simulates the energy-linked conformational change cannot be answered at present. However, the similarity of the extent and wavelength dependence and the insensitivity towards cyanide of the Ca^{2+}-induced and the energy-linked shifts suggest that the underlying molecular changes may be closely similar.

The data discussed above also strongly suggest that the binding site for calcium responsible for the spectral shift is located external to the permeability barrier of the membrane. On the basis of these results and the observed calcium-induced shift in purified ferrocytochrome aa_3 (Fig. 8), we suggest that the binding site for calcium is located in a region of the cytochrome aa_3 molecule that extends to the external surface of the cristae membrane. This is in accordance with the proposals [13–17] that part of cytochrome *c* oxidase extends to the outside of the inner mitochondrial membrane.

It is clear that we cannot estimate the extent of conformational change in the ferrocytochrome aa_3 molecule upon energization or calcium binding on the basis of the data reported here. Very small geometrical changes in the heme could be the basis for the observed spectral shift. However, it is of significance that the binding of calcium to an externally exposed region of the cytochrome aa_3 complex* produces a change that is felt by the heme (cf. allosteric interactions). Thus it may be proposed that interphase events at the mitochondrial membrane, such as the binding of ions to respiratory chain complexes may be reflected to the redox centers and may therefore regulate respiratory chain activity. We have recently obtained evidence for this type of allosteric regulation of cytochrome *c* oxidase conformation by electrical field-coupled binding of a proton to the ferric aa_3 complex [5].

Due to the relatively high K_D of calcium in causing the spectral shift, it appears improbable that the binding site would normally be controlled by this cation. We have suggested [3] that the red shift in the ferrocytochrome *a* spectrum upon energization of the mitochondrial membrane may be due to binding of protons to this site and this notion has recently gained experimental support [5].

MATERIALS AND METHODS

Rat liver mitochondria were isolated essentially as described by Schneider [18] in a medium consisting of 0.25 M sucrose and 1 mM EDTA, pH 6.9. The mitochondria were subsequently washed twice in the same medium, but omitting the EDTA, and finally suspended in 0.25 M sucrose buffered with a small amount of Tris · HCl to pH 6.9. The mitochondrial preparations were used immediately after isolation. Mitochondrial protein concentrations were determined by the Folin procedure [19] with bovine serum albumin as standard. The isolated mitochondria routinely showed respiratory control ratios with succinate as substrate (rotenone present) in excess of 6 as determined polarographically.

* We cannot fully exclude binding of calcium to phospholipid molecules in close association to the protein because the isolated cytochrome *c* oxidase still contains lipid.

Dual wavelength and split beam spectrophotometry was performed with the Aminco DW-2 spectrometer using standard cuvettes with 1 cm light path at 25 °C, if not stated otherwise.

The standard reaction mixture consisted of 0.2 M sucrose/0.02 M KCl/0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer, pH 7.2 (standard reaction medium). Further constituents and additions are specified in the figure legends.

The uncoupling agent carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr. P. G. Heytler. All other other reagents were purchased commercially at highest available purity.

REFERENCES

1 Wikström, M. K. F. and Saris, N. E. L. (1970) in Electron Transport and Energy Conservation, (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 77–88, Adriatica Editrice, Bari
2 Wikström, M. K. F. (1972) Biochim. Biophys. Acta 283, 385–390
3 Wikström, M. K. F. (1974) Ann. N.Y. Acad. Sci. 227, 146–158
4 Erecińska, M., Wilson, D. F., Sato, N. and Nicholls, P. (1972) Arch. Biochem. Biophys. 151, 188–193
5 Wikström, M. K. F. and Saari, H. T. (1975) Mol. Cell. Biochem., in press
6 Wilson, D. F. and Brocklehurst, E. S. (1973) Arch. Biochem. Biophys. 158, 200–212
7 Gilmour, M. V., Wilson, D. F. and Lemberg, R. (1967) Biochim. Biophys. Acta 143, 487–499
8 Mela, L. (1968) Arch. Biochem. Biophys. 123, 286–293
9 Moore, C. (1971) Biochem. Biophys. Res. Commun. 42, 298–305
10 Wilson, D. F., Lindsay, J. G. and Brocklehurst, E. S. (1972) Biochim. Biophys. Acta 256, 277–000
11 Kuboyama, M., Yong, F. C. and King, T. E. (1972), J. Biol. Chem. 247, 6375–6383
12 Chance, B. (1965), J. Biol. Chem. 240, 2729–2748
13 Racker, E., Brunstein, C., Lanter, A. and Christiansen, R. O. (1970) in Electron Transport and Energy Conservation (Tager, J. M. Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 235–252, Adriatica Editrice, Bari
14 Capaldi, R. A. (1973) Biochim. Biophys. Acta 303, 237–241
15 Mitchell, P. and Moyle, J. (1970) in Electron Transport and Energy Conservation, (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds) pp. 575–587, Adriatica Editrice, Bari
16 Vail, W. J. and Riley, R. K. (1974) FEBS Lett. 40, 269–273
17 Klingenberg, M. and von Jagow, G. (1970) in Electron Transport and Energy Conservation, (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds) pp. 281–290, Adriatica Editrice, Bari
18 Schneider, W. C. (1948) J. Biol. Chem. 176, 259–266
19 Lowry, O. H., Rosenbrough, O. H., Farr, N. J. and Randall, R. (1951) J. Biol. Chem. 193, 265–275