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HIGH-ENERGY FORMS OF CYTOCHROME *b*

I. THE EFFECT OF ATP AND ANTIMYCIN ON CYTOCHROME *b* IN PHOSPHORYLATING SUB-MITOCHONDRIAL PARTICLES

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SUMMARY

1. As in non-phosphorylating particles, antimycin causes a shift of the wavelength of maximum absorption of ferrocytochrome *b* in phosphorylating particles to the red by 2 nm. The antimycin-effect curve is sigmoidal.

2. ATP also causes a red shift, less than that brought about by antimycin and additive to the effect of the latter.

3. In addition to a red shift ATP brings about an oxidation of cytochrome *b*.

4. The ATP-induced red shift also occurs in the presence of rotenone when NADH is electron donor and in the presence of malonate or 4,4,4-trifluoro-1-(2-thionyl)-1,3-butadione when succinate is the donor.

5. The ATP-induced oxidation of cytochrome *b* occurs in the presence of both rotenone and antimycin. The oxidant is probably ubiquinone.

6. It is proposed that mitochondria contain two species of cytochrome *b* in equal amounts, one (b_i) affected by antimycin and the other (b) not affected. Antimycin reacts preferentially with a form of b_i present in high concentrations in energized particles.

7. It is proposed that the ATP-induced red shift is due to the formation of $b^{2+} \sim X$, a low-potential cytochrome *b* ($b^{2+} \sim X \rightleftharpoons b^{3+} + X + e$).

8. It is proposed that $b_i^{3+} \sim X$ is a high-potential cytochrome *b* ($b_i^{2+} + X \rightleftharpoons b_i^{3+} \sim X + e$).

9. A mechanism of Site-II phosphorylation is proposed in which ATP synthesis is linked with intramolecular electron transfer within the dimer $b^{2+} \sim X \cdot b_i^{3+} \sim X$.

INTRODUCTION

One of the effects of antimycin on the Keilin and Hartree heart-muscle preparation (non-phosphorylating sub-mitochondrial particles) is to shift the α-band of ferrocytochrome *b* 1–2 nm towards the red[1–4]. The effect of antimycin concentration on

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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this red shift, like other effects of antimycin[3–5], is described by a sigmoidal curve[4]. BRYŁA *et al.*[4] have explained the sigmoidal antimycin-effect curves on the basis of the allosteric model of MONOD *et al.*[6]. They proposed that the antimycin-sensitive region of the respiratory chain exists in two conformation states in equilibrium with one another, and that antimycin combines preferentially with the conformation present in low concentration in this type of preparation. BONNER AND SLATER[7], SLATER[8] and WEGDAM *et al.*[9] have found a hyperbolic antimycin-effect curve with intact mitochondria in the presence of substrate and ATP, under anaerobic conditions, and a sigmoidal curve under otherwise identical conditions in the presence of uncoupler. This is to be expected according to the explanation of BRYŁA *et al.*[4] if the conformation binding antimycin more firmly is present in high concentration in intact mitochondria energized by substrate and ATP, and in low concentrations in uncoupled mitochondria.

In the present communication phosphorylating sub-mitochondrial particles prepared from beef heart have been used further to study the effect of ATP and antimycin on cytochrome *b*. Preliminary accounts of some of these results have appeared[8,10–12].

RESULTS

Three preparations of phosphorylating sub-mitochondrial particles were used: EDTA particles prepared by the method of LEE AND ERNSTER[13], A particles prepared by the method of FESSENDEN AND RACKER[14], and Mg–ATP particles prepared by the method of LÖW AND VALLIN[15]. For maximal catalytic activity in energy-coupled reactions, the EDTA and A particles require low concentrations of oligomycin in addition to oxidizable substrate or ATP[16]. Oligomycin is not necessary with ATP–Mg particles.

Fig. 1 shows the effect of antimycin on the spectra in the cytochrome region of EDTA particles reduced by NADH in the absence of oxygen. Antimycin shifts the peak

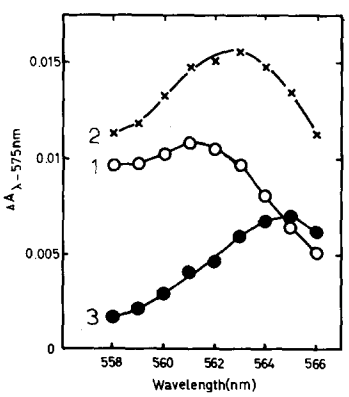

Fig. 1. Effect of antimycin on cytochrome *b* spectrum in EDTA particles. Particles (1 mg/ml) suspended in 0.2 M sucrose–0.05 M Tris–acetate (pH 7.4) containing 1.7 μg oligomycin per mg protein. NADH (1 mM) was added to the suspension and, after anaerobiosis was reached, 1.83 μg antimycin per mg protein were added. The reference wavelength was 575 nm, the test wavelength is shown on the abscissa. Separate runs were made at each wavelength. Curve 1, anaerobic *minus* aerobic; Curve 2, anaerobic + antimycin *minus* aerobic; Curve 3, anaerobic + antimycin *minus* anaerobic; (difference between Curves 1 and 2). Johnson Foundation dual-wavelength spectrophotometer used.

in the difference spectrum from 561 to 563 nm, with 575 nm as reference wavelength. The antimycin-effect spectrum has a maximum at 565 nm. These spectra are not affected by the addition of oligomycin or FCCP (not shown).

The spectra in Fig. 1 contain contributions from other cytochromes besides cytochrome *b*, particularly cytochrome c_1. In order to eliminate these contributions, the procedure of CHANCE[1], in which other cytochromes are first reduced by addition of ascorbate and cyanide (in some experiments, phenazine methosulphate was also present), was followed. The addition of succinate, NADH or $Na_2S_2O_4$ then specifically reduces cytochrome *b*. The wavelength pair, 566–560 nm, is isosbestic for ferri- and ferrocytochrome *b*, so that the addition of succinate, NADH or $Na_2S_2O_4$, after ascorbate and cyanide, has no effect on $\Delta A_{566-560\ nm}$. The subsequent addition of antimycin causes an increase in $\Delta A_{566-560\ nm}$ (*cf.* Fig. 1) that is a measure of the red shift uncomplicated by changes in the redox state of that fraction of the cytochrome *b* that is reducible in the absence of antimycin. Owing to some uncertainties in the wavelength setting of the Aminco–Chance dual-wavelength spectrophotometer, preliminary experiments were carried out with this instrument in order to determine the wavelength settings at which no change occurred when $Na_2S_2O_4$ was added subsequently to succinate, NADH or ascorbate + cyanide. $Na_2S_2O_4$ reduces as much cytochrome *b* in the absence of antimycin as NADH does in its presence[1,2].

Fig. 2 shows that, as with non-phosphorylating particles[4], the curve describing the effect of different concentrations of antimycin on the red shift is sigmoidal. ATP also causes a red shift, largely additive to that of antimycin. The effect of ATP is sensitive to oligomycin or uncoupler (FCCP).

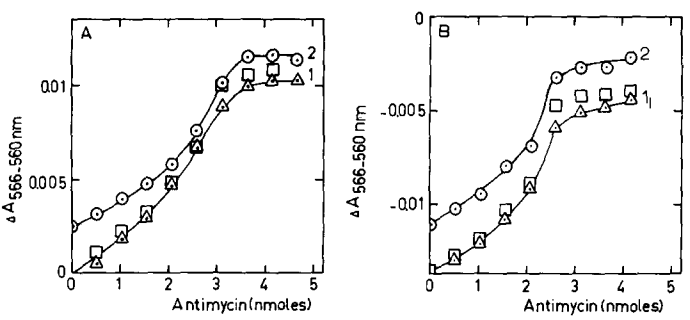

Fig. 2. Effect of concentration of antimycin on red shift of cytochrome *b* (measured at 566–560 nm) with Mg–ATP particles. To a suspension (3 mg/ml) of particles in 3.0 ml 0.2 M sucrose–0.05 M Tris–acetate (pH 7.4) were added successively 6.7 mM $MgSO_4$, 6.7 mM KCN and 8.3 mM ascorbate. A. Curve 1 (△——△). The reaction was started by the addition of 1 mM NADH and, when a steady state was reached, the indicated amounts of antimycin were added. The values plotted are the increase in $A_{566-560\ nm}$ due to the addition of antimycin. Curve 2 (⊙——⊙). The reaction was started by the addition of 1 mM NADH and, when a steady state was reached, 3 mM ATP was added, followed by the indicated amounts of antimycin. The values plotted are the increase in $A_{566-560\ nm}$ above the value reached after addition of NADH. The points □ refer to an experiment carried out in the same way as with Curve 2, except that oligomycin (1.1 μg/mg protein) was added before the ATP. B. Varying amounts of antimycin were added before the NADH. The reaction was started by the addition of 1 mM NADH and when a steady state was reached, 3 mM ATP was added, followed by 1.67 μM FCCP. Curve 1 (△—△), $A_{566-560\ nm}$ after NADH *minus* before NADH; Curve 2 (⊙——⊙), $A_{566-560\ nm}$ after ATP *minus* before NADH. Points □, after FCCP *minus* before NADH. Johnson Foundation dual-wavelength spectrophotometer used.

The effect of ATP, both alone and in the presence of antimycin, on the spectrum of A particles in the presence of oligomycin, with 560 nm as reference wavelength, is shown further in Fig. 3. In this experiment, succinate was used as hydrogen donor. At 566–560 nm, the effect of antimycin was the same whether added alone (Fig. 3B) or after the ATP (Fig. 3A). ATP also caused an increase at 566–560 nm, but a second effect of ATP, particularly noticeable in the presence of antimycin, is revealed at the higher wavelengths. Both effects of ATP are absent when $Na_2S_2O_4$ is the electron donor (Fig. 3C). As previously reported[4], the antimycin red shift is much less with $Na_2S_2O_4$ than with succinate as electron donor.

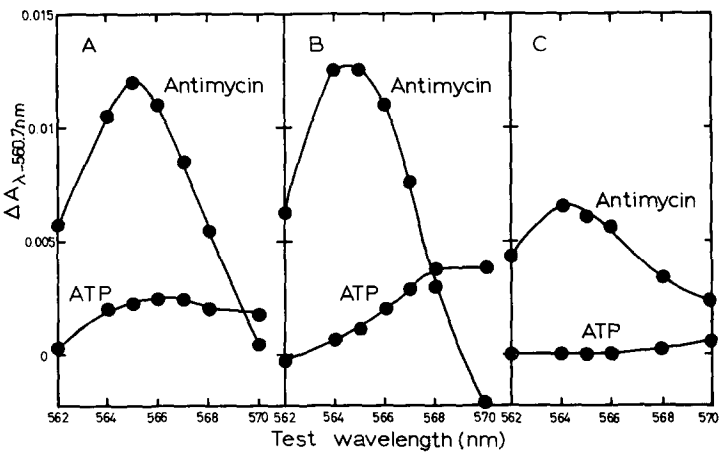

Fig. 3. Effect of ATP and antimycin on absorption spectrum of cytochrome *b* in A particles. The Aminco–Chance dual-wavelength spectrophotometer was used with the reference wavelength set at 560.7 nm (see text). The particles (2.5 mg/ml) were suspended in 0.17 M sucrose–0.042 M Tris–acetate (pH 7.4), containing 1 mM EDTA, 6.7 mM $MgSO_4$ and 0.2 μg oligomycin per mg protein. This was the amount of oligomycin found in preliminary experiments optimally to stimulate the ATP-driven reduction of NAD^+ by succinate catalysed by these particles. Cytochromes *c*, c_1 and aa_3 were reduced by addition of 1 μM phenazine methosulphate, 10 mM KCN and 6 mM ascorbate. When a steady state was reached, 16.7 mM succinate was added. When a steady state was reached, 4.2 mM ATP was added in A, followed, when a steady state was again reached, by 0.7 nmole antimycin per mg protein. The curve marked ATP gives the difference spectrum: after addition of ATP *minus* before addition of ATP. The curve marked antimycin is the difference spectrum: after addition of antimycin *minus* before addition of antimycin. In B, the order of addition of antimycin and ATP was reversed. In C, $Na_2S_2O_4$ was added before the antimycin, and ATP was the last addition.

That the second effect of ATP is due to oxidation of cytochrome *b* is made probable by the experiment shown in Fig. 4, that was carried out with Mg–ATP particles reduced with NADH in the presence of ascorbate and cyanide. The ATP effect, which is sensitive to FCCP, is maximal at 563 nm with reference wavelength at 575 nm. The wavelength pair 563–575 nm is suitable for studying the redox state of cytochrome *b*, and $A_{563-575\ nm}$ is relatively insensitive to the red shift.

In some experiments with A particles, the oxidation of cytochrome *b* by ATP was so much slower than the red shift that it was possible by extrapolation to plot the spectrum of the ATP-induced red shift separate from the oxidation. This is shown in Fig. 5. Both the antimycin- and the ATP-induced red shifts peak at 565 nm, at the wavelength settings used, and the two effects are additive.

The red shift brought about by ATP, with NADH as donor, both in the absence

(Figs. 6A and 6B) and presence (Figs. 6C and 6D) of antimycin, was also found in the presence of rotenone. The amount of rotenone used in the experiment shown in Fig. 6 was insufficient completely to inhibit reduction of cytochrome *b* by NADH, but slowed it considerably. Thus, oxidation of cytochrome *b* by NAD^+ cannot be involved in the red shift. The trace after the addition of antimycin shown in Fig. 6B indicates that cytochrome *b* is somewhat oxidizable in the presence of antimycin.

When succinate was substrate, the addition of malonate after the succinate also had no effect on the FCCP-sensitive red shift brought about by ATP (Fig. 7). In another experiment not shown 4,4,4-trifluoro-1-(2-thionyl)-1,3-butadione also had no effect.

Fig. 8 shows two parallel experiments, the upper trace illustrating the FCCP-sensitive red shift brought about by ATP, and the lower the FCCP-sensitive oxidation

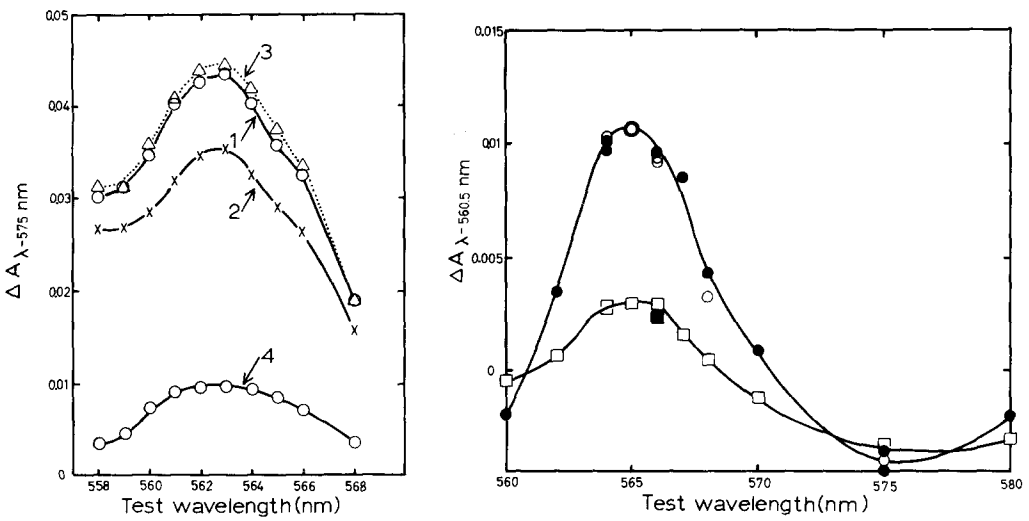

Fig. 4. Oxidation of cytochrome *b* in Mg–ATP particles by ATP in the presence of antimycin. The particles (3.0 mg/ml) were suspended in 0.20 M sucrose–0.05 M Tris–acetate (pH 7.4) containing 6.7 mM $MgSO_4$. Cytochromes *c*, c_1 and aa_3 were reduced by addition of 6.7 mM KCN and 8.3 mM ascorbate. Antimycin (0.4 nmole/mg protein) was added and the reaction to be studied started by the addition of 0.3 mM NADH. The NADH-effect spectrum, with 575 nm as reference wavelength, is given in Curve 1. When a steady state was reached, 3.0 mM ATP was added. The effect of NADH + ATP is given in Curve 2. When a steady state was again reached 1.0 μM FCCP was added. Curve 3 gives the effect of NADH + ATP + FCCP. Curve 4, the difference between Curves 2 and 3, represents the reverse of the FCCP-sensitive ATP effect. Johnson Foundation dual-wavelength spectrophotometer was used.

Fig. 5. Effect of ATP and antimycin on absorption spectrum of cytochrome *b* in A particles. The Aminco–Chance dual-wavelength spectrophotometer was used with the reference wavelength set at 560.5 nm. The particles (2.3 mg/ml) were suspended in 0.17 M sucrose–0.042 M Tris–acetate (pH 7.4), containing 1 mM EDTA, 6.7 mM $MgSO_4$ and 0.28 μg oligomycin per mg protein (optimal for ATP-driven reduction of NAD^+ by succinate). The reaction was started by the addition of 16.7 mM succinate. When anaerobiosis was reached, 0.85 nmole antimycin per mg protein (○) or 5 mM ATP (□) was added. The points represent the difference between after the addition and before. Points ● were obtained by adding antimycin after the ATP and represent the difference between after antimycin and before. The point ■ was obtained by adding ATP after the antimycin and represents the difference between after ATP addition and before. At the higher wavelengths a steady value was not reached when antimycin was added in the presence of ATP. The values plotted were obtained by extrapolation.

of cytochrome *b*, both followed in the presence of rotenone and antimycin. A control experiment showed that the extent of the oxidation of *b*, in the presence of antimycin, was the same in the presence and absence of rotenone. The rapid oxidation of cytochrome *b* by ATP in the presence of rotenone shows that NAD^+ is not the oxidant. Since the oxidant must lie between the rotenone-sensitive and antimycin-sensitive sites, it is most probably ubiquinone.

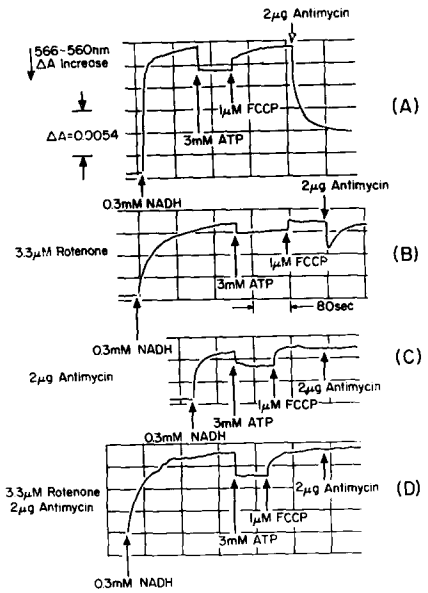

Fig. 6. Effect of rotenone and antimycin on FCCP-sensitive ATP-induced red shift in Mg–ATP particles, with NADH as donor. Conditions were the same as in Fig. 4. Other additions are as indicated.

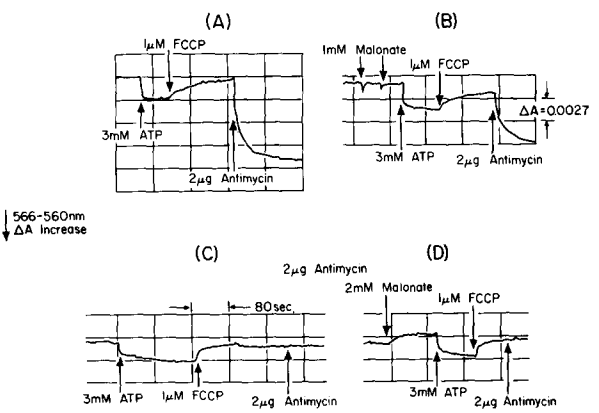

Fig. 7. Effect of malonate and antimycin on FCCP-sensitive ATP-induced red shift in Mg–ATP particles, with succinate as donor. Conditions were the same as in Fig. 4 except that the reaction mixture was supplemented with 5 mM succinate first before the trace was recorded. Other additions are as indicated.

DISCUSSION

It is necessary to separate two effects of antimycin and ATP on cytochrome *b* in phosphorylating sub-mitochondrial particles, namely oxidoreduction and a shift of the absorption peak to the red. With the reference wavelength at 560 nm, measurements at 566 nm are insensitive to oxidoreduction so that this wavelength pair is suitable for studying the red shift. The wavelength pair 563–575 nm is suitable for studying redox changes with minimal interference from the red shift.

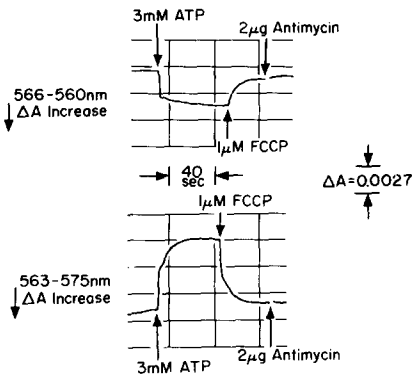

Fig. 8. The ATP-induced red shift (measured at 566–560 nm) and the ATP-induced oxidation of cytochrome *b* (measured at 563–575 nm) measured in the presence of rotenone and antimycin. Conditions were as in Fig. 4 except that the reaction mixture was supplemented with 3.3 μM rotenone, 1.3 μM antimycin and 0.3 mM NADH before the trace was recorded. Other additions are as indicated.

The stoichiometry of antimycin inhibition[17,4,18] shows that mitochondria and sub-mitochondrial particles contain two species of cytochrome *b* in equal amounts, one (b_i) affected by antimycin and the other (b) not affected. In the presence of electron donor, antimycin causes a red shift. As indicated in INTRODUCTION, a study of the antimycin-effect curves under different conditions has led to the conclusion that antimycin reacts preferentially with a form of cytochrome *b* present in high concentrations in energized particles, and in low concentrations in non-energized particles. Thus, the species in energized particles responsible for the red shift induced by antimycin may be written $(b_i^{2+} \sim X) \cdot$antimycin.

Both in the presence and absence of antimycin, ATP also brings about a red shift in phosphorylating sub-mitochondrial particles, with a maximum absorption at the same wavelength. The effects of ATP and antimycin are additive. We may write $b^{2+} \sim X$ for the spectroscopically observed species in the presence of ATP. In addition to the red shift ATP induces a slower oxidation of cytochrome *b*. The two effects may be described by the equations

$$ATP + b^{2+} + X \leftrightharpoons ADP + P_i + b^{2+} \sim X$$
$$b^{2+} \sim X + Q + H^+ \leftrightharpoons b^{3+} + X + QH\cdot$$

The redox couple

$$b^{2+} \sim X \leftrightharpoons b^{3+} + X + e$$

represents a species of cytochrome *b* with a redox potential lower than that of the couple

$$b^{2+} \leftrightharpoons b^{3+} + e$$

by 43 ΔG° mV, where $-\Delta G^\circ$ kcal is the free-energy change in the reaction

$$b^{2+} \sim X \leftrightharpoons b^{2+} + X$$

If $-\Delta G^\circ$ is 8 kcal, then $b^{2+} \sim X$ might be expected to have a potential 345 mV less than that of cytochrome *b*, which is 77 mV (ref. 19).

Cytochrome *b* with a potential as low as −270 mV has not been found in mitochondria. However, isolated cytochrome *b*, which reacts with CO, has been reported to have a potential of −340 mV (ref. 20).

WILSON AND DUTTON[21] have recently reported that, in the presence of ATP, mitochondria contain two species of cytochrome *b*, with redox potentials of 245 and 35 mV, respectively. In the presence of uncoupler, species with potentials of −55 and 35 mV, respectively, were identified. The species with potential of 35 mV is probably the same as that previously measured by HOLTON AND COLPA-BOONSTRA[19] ($E_0' = 77$ mV) in non-phosphorylating particles, *i.e.* that of conventional cytochrome *b*. We should like to suggest that the potentials of −55 and 245 mV, respectively, belong to b_i, and represent the equilibria

$$b_i^{2+} \leftrightharpoons b_i^{3+} + e \quad (E'_o = -55 \text{ mV})$$
$$b_i^{2+} + X \leftrightharpoons b_i^{3+} \sim X + e \quad (E'_o = 245 \text{ mV})$$

In agreement with this suggestion is the observation that ATP increases the degree of reduction of cytochrome *b* in intact mitochondria[9]. $b_i^{3+} \sim X$ is the species already proposed to explain the preferential binding of antimycin in energized particles.

Complex III of the respiratory chain contains 1 molecule of *b*, 1 molecule of b_i, 1 molecule of cytochrome c_1 and 1–2 molecules iron–protein per molecule of complex[17,18,22,23]. The evidence for the existence of two high-energy species, one a low-potential *b* and the other a high-potential b_i, suggests the following description of phosphorylation in Site II.

$$b^{3+} \cdot b_i^{3+} + e + X \leftrightharpoons b^{2+} \sim X \cdot b_i^{3+} \quad (1)$$
$$b^{2+} \sim X \cdot b_i^{3+} \leftrightharpoons b^{2+} \cdot b_i^{3+} \sim X \quad (2)$$
$$b^{2+} \cdot b_i^{3+} \sim X + e \leftrightharpoons b^{2+} \sim X \cdot b_i^{2+} \quad (3)$$
$$b^{2+} \sim X \cdot b_i^{2+} + X \leftrightharpoons b^{2+} \sim X \cdot b_i^{3+} \sim X + e \quad (4)$$
$$b^{2+} \sim X \cdot b_i^{3+} \sim X + ADP + P_i \leftrightharpoons b^{3+} \cdot b_i^{2+} + ATP + 2\,X \quad (5)$$
$$b^{3+} \cdot b_i^{2+} \leftrightharpoons b^{2+} \cdot b_i^{3+} \quad (6)$$
$$b^{2+} \cdot b_i^{3+} \leftrightharpoons b^{3+} \cdot b_i^{3+} + e \quad (7)$$

Sum: $ADP + P_i \overset{2e}{\leftrightharpoons} ATP$

According to the mechanism proposed, the catalyst for electron transfer and energy conservation is the dimer bb_i. An electron is fed into the low-potential *b* with formation of $b^{2+} \sim X$. The X is transferred to b_i^{3+}, with formation of the high-potential

$b_i^{3+} \sim X$ which can then take up a second electron, with simultaneous transfer of X back to b. The b_i delivers an electron to its acceptor with formation of $b_i^{3+} \sim X$. Electron transfer in the dimer from high-energy low-potential b to the high-energy high-potential b with elimination of X may be coupled with the synthesis of one molecule of ATP. After intramolecular transfer from b_i to b, the second electron is delivered into Site III. The source of the electrons for Reactions 1 and 3, and their destination in Reactions 4 and 7 will be the subject of another publication. $b^{2+} \sim X$ corresponds to $b^{2+} \sim I$ proposed by CHANCE AND WILLIAMS[24] and $b_i^{3+} \sim X$ to the $A \sim C$ (or $b^{3+} \sim I$, ref. 2) of SLATER[25]. The ATP-dependent species of ferrocytochrome b identified in this paper absorbs at 565 nm at room temperature, about 2 nm higher than classical ferrocytochrome b. Its relationship to the ATP-dependent species, absorbing at 555 nm at 77°K, reported by CHANCE AND SCHOENER[26,27] in pigeon-heart mitochondria and submitochondrial particles, is at present not clear. Since the latter species is reduced while other cytochromes are oxidized, it is presumably a low-potential species. However, a difference of 10 nm in the absorption maximum between room temperature and 77°K is unprecedented, and it seems unlikely that the species reported here and that by CHANCE AND SCHOENER[26,27] are identical.

According to the mechanism proposed, four different redox potentials of cytochrome b could be measured in mitochondria:

(i) $b^{2+} \leftrightharpoons b^{3+} + e$ (Eqn. 7)

(ii) $b^{2+} \sim X \leftrightharpoons b^{3+} + X + e$ (Eqn. 1)

(iii) $b_i^{2+} \leftrightharpoons b_i^{3+} + e$ (Eqn. 6)

(iv) $b_i^{2+} + X \leftrightharpoons b_i^{3+} \sim X + e$ (Eqn. 4)

As already explained, we suggest 77 mV (ref. 19) (or 35 mV (ref. 21)), −55 mV (ref. 21) and 245 mV (ref. 21) for the redox potentials i, iii and iv, respectively.

A phosphate potential of 17 kcal[28,29] requires a potential span of about 730 mV for a one-electron transfer reaction, and 365 mV for a two-electron transfer. If two electrons are fed into Site II at about zero potential, and are removed at a potential of about 395 mV (the potential of cytochrome a_3 according to WILSON AND DUTTON[30]) sufficient energy is available overall. However, the first reaction is thermodynamically unfavourable if $b^{2+} \sim X$ has a potential of about −270 mV, and the proposed mechanism implies a concerted mechanism in which the steady-state concentrations of $b^{2+} \sim X \cdot b_i^{3+} \sim X$, $b^{2+} \cdot b^{3+} \sim X$ and $b^{2+} \sim X \cdot b_i^{2+}$ would be low.

The ATP-induced red shift in sub-mitochondrial particles in the absence of antimycin may now be explained by Reactions 7–5 from right to left, followed by splitting of $b_i^{3+} \sim X$ which is apparently unstable in sub-mitochondrial particles. The ATP-induced oxidation of b may be explained by these reactions followed by Reaction 1 from right to left. This explanation is consistent with the finding (Fig. 3) that ATP cannot induce the red shift in the presence of $Na_2S_2O_4$ since, under these conditions, all the b will be in the form of $b^{2+} \cdot b_i^{2+}$, which cannot catalyse any of the reactions proposed.

Azzi *et al.*[31] have shown that 1-anilinonaphthalene-8-sulphonic acid is a sensitive extrinsic fluorochromic probe for the state of energization of the membrane in EDTA particles. The fluorescence increases when the membrane is energized by oxidation of

substrate, especially in the presence of oligomycin, or by the addition of ATP. Although both cytochrome *b* and the fluorochrome respond to ATP, they apparently do not detect the same conformation change, since it was found in the present studies that antimycin had no effect on the fluorescence of the probe under conditions in which it caused a shift in the absorption maximum of cytochrome *b* to the red. Moreover, the ATP-induced fluorescence change is much slower than the ATP-induced red shift[12].

According to the formulation used in this paper, the two high-energy forms of cytochrome *b* are distinguished by two species of *b* joined to the same ligand X. However, many other formulations differing in detail but not in principle, are possible. For example, different ligands may be involved (*cf.* ref. 21). It is also possible that $b^{2+} \sim X$ and $b_i^{3+} \sim X$ differ from b^{2+} and b_i^{3+}, respectively, in protein conformation rather than in ligand binding. However, the change in conformation must alter the environment of the iron atom sufficiently to cause the spectral shift.

METHODS

EDTA particles were prepared by the method of LEE AND ERNSTER[13], A particles by the method of FESSENDEN AND RACKER[14], and Mg–ATP particles by the method of LÖW AND VALLIN[15]. Antimycin was added in ethanolic solution, the concentration of which was determined spectrophotometrically using an absorption coefficient of 4.8 $mM^{-1} \cdot cm^{-1}$ (ref. 32).

The experimental procedures are described in the text and in the legends to the figures. In the experiments when cyanide and ascorbate were not present (Figs. 1 and 5), air was introduced with each addition of antimycin or ATP. This causes a momentary oxidation of cytochromes *c*, c_1 and aa_3, but control experiments, carried out at 550–540 nm (appropriate for cytochrome *c*), showed that these cytochromes are reduced after a few minutes, even when the respiration is maximally inhibited by antimycin. In this case, the oxygen is presumably consumed by the antimycin-resistant pathway[33]. With successive additions, of antimycin for example, the absorbance was allowed to reach a constant value after each addition before the subsequent addition.

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