AN ENERGY-DEPENDENT TRANSFORMATION OF A FERRICYTOCHROME OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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1. Introduction

The observation that the half-reduction potentials of cytochromes b_T and a_3 are dependent on the mitochondrial energy state [1–5] has provided direct evidence for a role of these cytochromes in the energy conservation reactions. One of the predictions arising from theoretical considerations related to the energy dependence of the half-reduction potentials is that these cytochromes have more than one oxidized or reduced species (or both), the interconversion of which is energy-dependent [1, 3]. It is expected that such conversions would give rise to measurable changes in the absorption spectra of the cytochrome but the size and nature of these spectral changes cannot be predicted.

We have undertaken a systematic search for these predicted spectral changes. In this paper evidence is presented for an energy (ATP)-dependent spectral change in an oxidized cytochrome of pigeon heart mitochondria.

2. Materials and methods

Pigeon heart mitochondria were isolated in 0.225 M mannitol – 0.075 M sucrose – 0.0002 M EDTA according to the method of Chance and Hagihara [6]. Spectral data were obtained using an Aminco–Chance dual wavelength spectrophotometer.

Oxidation–reduction potential measurements were performed in the apparatus designed by Dutton [7] using the oxidation reduction mediators indicated in the figure legend. Anaerobiosis was achieved by the addition of NADH which nonenzymatically reduces phenazine methosulfate. Potassium ferricyanide was used as oxidant and NADH as reductant.

3. Results and discussion

3.1. *Spectral changes induced by the addition of ATP to pigeon heart mitochondria*

The addition of ATP to a suspension of pigeon heart mitochondria in which the cytochromes are highly oxidized induces a spectral change characterized by maxima near 580 nm and 436 nm and minima at 416 nm, 480 nm and 655 nm (fig. 1 upper trace). This spectral change is prevented by the presence of either oligomycin or uncoupler. The sensitivity to these compounds indicates that the phenomenon is energy-dependent.

The oligomycin sensitivity of the ATP-dependent spectral shift as measured at 577 nm minus 540 nm is typical of that for oligomycin sensitive reactions in pigeon heart mitochondria. When limiting amounts of oligomycin are added the inhibition increases with time over a 2–3 minute time interval. In fig. 2 the oligomycin titer is given for the experimental conditions in which oligomycin is added before ATP and the absorbance change measured 1 min after the ATP addition. No inhibition is observed until the amount of oligomycin added reaches 0.3 μg/mg protein and then the absorbance change declines precipitously to near zero at 0.5 μg oligomycin/mg protein. These numerical values become somewhat smaller if longer equilibration times are used.

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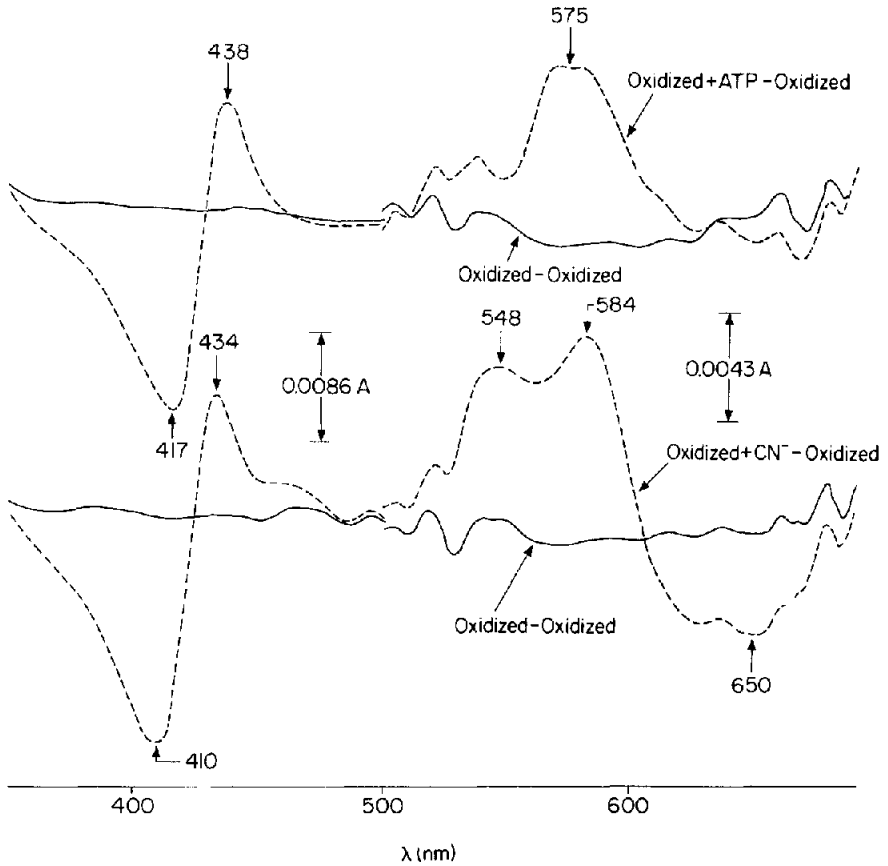

Fig. 1. The spectral changes induced on addition of ATP or cyanide to pigeon heart mitochondria in which the cytochromes are highly oxidized. The pigeon heart mitochondria were suspended in a medium containing 0.22 M mannitol, 0.075 M sucrose and 0.05 M morpholinopropane sulfonate, pH 7.2. A mitochondrial suspension of 1 mg protein/ml was treated with 670 μM potassium ferricyanide and used for the spectra of the wavelength range from 350 nm to 500 nm, while a suspension containing 2.5 mg protein/ml and 1.3 mM potassium ferricyanide was used for the wavelength range from 500 nm to 700 nm. The baselines were obtained using identical samples in each cuvette. The upper trace (+ATP) shows the spectral change induced by adding 2 mM ATP to the measure cuvette, and the lower trace (+CN) shows the spectral change induced by the addition of 250 μM KCN to the measure cuvette and allowing in the latter case 5 min for complete reaction. The spectra were obtained using an Aminco–Chance dual wavelength-split beam spectrophotometer and a measuring spectral bandwidth at half height of 4 nm.

The spectral change is a function of the phosphate potential. Preliminary measurements show that the change is half-maximal when the ratio of the concentration of ATP to the product of the concentrations of ADP and inorganic phosphate is approx. 10^3 M^{-1}

3.2. *The identification of the change as belonging to a ferricytochrome*

Although pigeon heart mitochondria have a very low content of endogenous substrate, it remains possible that the observed spectral changes are caused by an energy-dependent reduction of an as yet unknown component of the respiratory chain. Two lines of evidence argue against this possibility: (1) The ATP-induced spectral change is found to be undiminished in size when the mitochondria are suspended in a medium saturated with 100% oxygen gas and supplemented with 1.5 mM K-ferricyanide in order to maintain highly oxidizing conditions. As may be seen from the spectra, ATP causes only a negligible

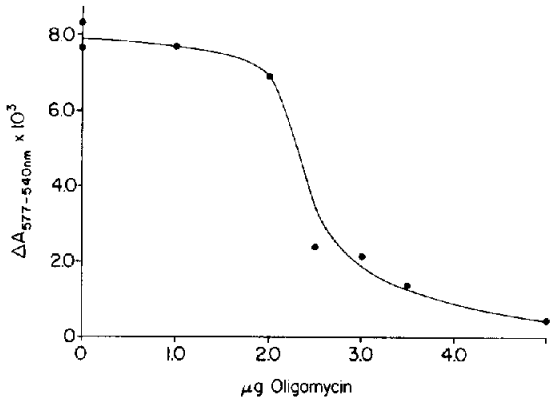

Fig. 2. The oligomycin sensitivity of the ATP induced absorbance change as measured at 577 nm minus 540 nm. Pigeon heart mitochondria were suspended at 2.7 mg protein/ml in a medium containing 0.22 M mannitol, 0.075 M sucrose and 0.05 M morpholinopropane sulfonate, pH 7.0. The final volume of the reaction mixture was 2.2 ml, and the light path was 1 cm. Ferricyanide (final concentration = 900 μM) and the amount of oligomycin indicated on the abscissa were added and the absorbance change initiated by the addition of 2 mM ATP. The absorbance change indicated on the ordinate was measured 1 min after the ATP addition.

reduction of any of the known components of the mitochondrial respiratory chain. (2) Separate experiments performed under anaerobic conditions (in the oxidation–reduction potential measuring apparatus of Dutton and co-workers [2, 7]) in which the absorbancy changes were measured at 577 nm–540 nm, demonstrate that the addition of ATP at an oxidation–reduction potential of +320 mV or greater causes an absorbancy change consistent with that of fig. 1 while the addition of ATP at oxidation–reduction potentials more negative than +210 mV causes less than 10% of the change observed at the more positive potential values. Furthermore, in the presence of ATP, the absorbance appears on addition of oxidant and disappears on the addition of reductant.

Attempts to determine the half-reduction potential of the component responsible for this absorbance change (using the wavelength pair 577 nm minus 540 nm) have given less than satisfactory results. The maximum ATP-induced absorbance change is approximately equivalent to that for a component having a concentration of 0.5 nmoles/mg protein and a millimolar extinction coefficient of 6 cm^{-1} at this wavelength pair. This is a small absorbance change. The measurements are further complicated because both the magnitude and the half-reduction potential are dependent on the phosphate potential.

3.3. *On the possible identification of the component undergoing the ATP-dependent spectral change*

The component was found to be associated with energy conservation site III by using the antibiotic antimycin A and cyanide. When an aerobic suspension of mitochondria is treated with 0.5 μg antimycin A/mg protein and an oxidizable substrate such as succinate plus glutamate, the *b* cytochromes are reduced while the cytochromes c, c_1, a and a_3 are oxidized. The addition of ATP to such a mitochondrial suspension elicits a small (10–20%) fraction of the absorbance change previously described. This apparent inhibition by antimycin A is the result of a small antimycin-insensitive electron flux, however, and if 1 mM potassium ferricyanide (which specifically accepts electrons on the oxygen side of the site of antimycin inhibition) is added, the absorbance change is increased to the control values.

The addition of 250 μM cyanide to a suspension of pigeon heart mitochondria in which the cytochromes are highly oxidized, induces the spectral change shown in fig. 1 (lower trace). Subsequent addition of ATP does not elicit a spectral change comparable to that obtained in the absence of cyanide. The cyanide sensitivity of the ATP-induced spectral change is good evidence that it is associated with energy conservation site III.

3.4. *On the origin of the energy dependent spectral change*

Cyanide is known to form low-spin derivatives of ferric hemoproteins [8, 9]. The cyanide-induced spectral change shown in fig. 1 (lower trace) is typical for the reaction of cyanide with a high-spin hemoprotein to form the low-spin cyanide compound. The similarities between the cyanide and the ATP-induced spectral changes provide additional evidence that the latter also represents a transition of a hemoprotein from a high-spin state to a low-spin state [10, 11].

3.5. *On the nature of the energy conservation reactions and the measurements of the half-reduction potentials in the presence of ATP*

The minimum equations necessary for describing the thermodynamics of energy conservation are:

1) $AH_2-I + B \longleftrightarrow A^* + I + BH_2$

2) $A^* + I + ADP + P_i \longleftrightarrow A-I + ATP$

3) $A-I + CH_2 \longleftrightarrow AH_2-I + C$

These equations are essentially the same as we presented earlier [1, 3] but they are in a general form in which the component A is designated as the energy-transducing element and the ligand effects are expressed as a binding or release of I. The oxidation–reduction reactions are written to indicate that the stoichiometry calls for 2 electrons to be transferred per ATP synthesized but this may occur as 2 sequential 1 electron steps. The essence of this mechanism is a directional electron flow with electrons being donated to A by a low potential donor (C) and accepted from A by a high potential acceptor (B). This directional electron flow serves to maintain the ratio of the activities of A* and I to A–I such that the free energy of interconversion of the former to the latter is equal to or greater than the energy required for ATP synthesis. This mechanism readily accounts for the net synthesis of ATP by a net electron flow from the low potential pool to the high potential pool, the energy-dependent reversed electron flow and the energy-dependent half-reduction potential of the energy transducing component.

It should be noted that theoretical considerations call for an additional reactant A^*H_2 and the appropriate reactions for its formation by reduction of A* and conversion of AH_2-I. Since thermodynamic evaluation of data taken at equilibrium deals with the state of the system and not the reaction pathways, for our purposes it is only necessary to consider the chemical species present in significant amounts at equilibrium. For example, the glyceraldehyde-3-phosphate dehydrogenase reaction may be analyzed as a 3 component system (1 reduced form, glyceraldehyde-3-phosphate; and 2 oxidized forms, 3-phosphoglycerate and 1,3-diphosphoglycerate) although a fourth component (a complex of glyceraldehyde-3-phosphate and inorganic phosphate [12]) is required to complete the formal equations for 2 oxidation–reduction couples.

No spectral evidence has been found for the additional species of cytochrome b_T as yet ([13–15]; see however [16–18]) but energy-dependent spectral changes in cytochrome oxidase have been observed by Wikstrom and Saris [19] and interpreted as either an oxidation of cytochrome a_3 or an energy-linked alteration of the spectrum of reduced cytochrome a_3. These observations have been confirmed (Lindsay and Wilson, unpublished results) and the latter interpretation proven to be correct. The energy-dependent spectral changes described in this communication may well be due to oxidized cytochrome a_3. If this can be unequivocally demonstrated, it will represent the evidence necessitating the expansion of the minimum required equations to include the additional species. The additional equations will make it much easier to explain some aspects of respiratory control and the energy-dependence of the oxidation–reduction state of cytochrome b_T during respiration [13, 20].

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References

[1] D.F. Wilson and P.L. Dutton, Biochim. Biophys. Res. Commun. 33 (1970) 53.
[2] D.F. Wilson and P.L. Dutton, Archiv. Biochem. Biophys. 136 (1970) 583.
[3] D.F. Wilson, P.L. Dutton and B. Chance, in: Energy Transduction in Respiration and Photosynthesis, Pugnochiuso 1970, eds. E. Quagliariello and S. Papa (Adriatica Ed., Bari, 1971) in press.
[4] B. Chance, D.F. Wilson, P.L. Dutton and M. Erecinska, Proc. Natl. Acad. Sci. U.S. 66 (1970) 1175.
[5] D.F. Wilson, J.S. Leigh, Jr., J.G. Lindsay and P.L. Dutton, in Oxidases and Related Redox Systems II, eds. T.E. King, H.S. Mason and M. Marrison, 1971, in press.
[6] B. Chance and B. Hagihara, in: Proc. Vth. Int. Congress Biochem., Moscow 1961, ed. A.N.M. Sissakian (Pergamon Press 5, 1963) p. 3.
[7] P.L. Dutton, Biochim. Biophys. Acta 226 (1971) 63.

[8] Y. Orii and K. Okunuki, J. Biochem. (Tokyo) 55 (1964) 37.
[9] K.J.H. vanBuuren, P. Nicholls and B.F. van Gelder, Biochim. Biophys. Acta, in press.
[10] R.J.P. Williams, Discussions Faraday Soc. 20 (1955) 231.
[11] T. Yonetani, D.F. Wilson and B. Seamonds, J. Biol. Chem. 241 (1966) 5347.
[12] P.D. Boyer, in: The Enzymes, Vol. 6, eds. P.D. Boyer, H. Lardy and K. Myrback (Acad. Press, New York, 1962) p. 45.
[13] M. Erecinska and B. Chance, in: Energy Transduction in Respiration and Photosynthesis, Pugnochiuso 1970, eds. E. Quagliariello and S. Papa (Adriatica Ed., Bari, 1971) in press.
[14] N. Sato, D.F. Wilson and B. Chance, FEBS Letters 15 (1971) 209.
[15] N. Sato, D.F. Wilson and B. Chance, Biochim. Biophys. Acta, 1971, in press.
[16] E.C. Slater, C.P. Lee, J.A. Berden and H.J. Wegdam, Nature 266 (1970) 1248.
[17] E.C. Slater, C.P. Lee, J.A. Berden and H.J. Wegdam, Biochim. Biophys. Acta 223 (1970) 354.
[18] A.J. Wegdam, J.A. Berden and E.C. Slater, Biochim. Biophys. Acta 223 (1970) 365.
[19] M.K.F. Wikstrom and N.E.L. Saris, in: Electron Transport and Energy Conservation, eds. E. Quagliariello and E.C. Slater (Adriatica Editrice, Bari, 1970) p. 77.
[20] D.F. Wilson, M. Koppelman, M. Erecinska and P.L. Dutton, Biochem. Biophys. Res. Commun. 44 (1971) 759.