ENERGY CONSERVATION IN DETERGENT-TREATED MITOCHONDRIA AND PURIFIED SUCCINATE-CYTOCHROME c REDUCTASE.*

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SUMMARY

The oxidation-reduction midpoint potential of mitochondrial cytochrome b_T becomes more positive when ATP is added or electron transport is activated. In the presence of antimycin A the electron transport dependent midpoint potential shift is stabilized. This energy dependent transformation is used to demonstrate competent energy conservation reaction in detergent-treated mitochondria and in purified succinate-cytochrome c reductase.

The study of mitochondrial electron transport and energy conservation has proven very difficult, partly because the electron transport chain is membrane bound and has resisted efforts for fractionation and purification with retention of a measurable coupling activity. Recent work which demonstrates an energy dependent change in the oxidation-reduction midpoint potential of cytochromes b_T and a_3 offers an experimental test for energy coupling that can be used in the absence of ATP interactions (1,2); moreover the literature contains two reports that raise the possibility of demonstrating the existence of energy coupling in purified preparations. Pumphrey (3) reported an anomalous reduction of cytochrome b when oxygen was added to an anaerobic suspension of detergent-clarified beef heart mitochondria which had been treated with antimycin A. More recently Rieske (4) reported an apparent change in the midpoint potential of cytochrome b in an antimycin A-treated cytochrome b-c_1 complex.

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In the present communication we will present evidence leading to two related conclusions. First, that antimycin A stabilizes an electron transport-generated "high energy" intermediate and second, that detergent-treated mitochondria and purified succinate-cytochrome c reductase retain a competent primary energy conservation reaction.

METHODS

Chicken heart mitochondria prepared by the method of Low and Vallin (5) for beef heart mitochondria were stored frozen at 60 mg protein/ml and thawed when needed. The thawed mitochondria were used after one of three treatments: A. after treatment of a 20 mg/ml suspension in 0.1 M phosphate buffer at pH 7.4 with 0.6% cholate, ammonium sulfate fractionation was used and the fraction between 0.25 and 0.4 saturation collected. This fraction was resuspended in 0.1 M phosphate to give an optically clear solution containing no cytochrome c but unchanged ratios of the other cytochromes. B. a succinate-cytochrome c reductase prepared by the method of King and Takemori (6) using cholate. C. a preparation of succinate-cytochrome c reductase prepared by using triton X-100 (7).

The apparatus used was that described by Dutton and associates (1,8). The optical measurements were made in stirred samples maintained strictly anaerobic by an atmosphere of ultra pure argon (less than 1 ppM of O_2). Four micromolar phenazine ethosulfate was added to permit the potentiometric measurement of the oxidation-reduction potential in the required range.

RESULTS

When a suspension of chicken heart mitochondria, clarified with cholate (preparation A, Methods), is supplemented with 1.2 mM succinate and the changes of the b cytochromes monitored at 561 minus 575 nm (Figure 1); the cytochromes b can be observed to rapidly attain a steady state level of reduction. Subsequent addition of cytochrome c activates oxygen consumption through cytochrome oxidase and, as a consequence, lowers the steady state reduction of the b cytochromes. When the oxygen in the suspending medium is consumed, a considerable

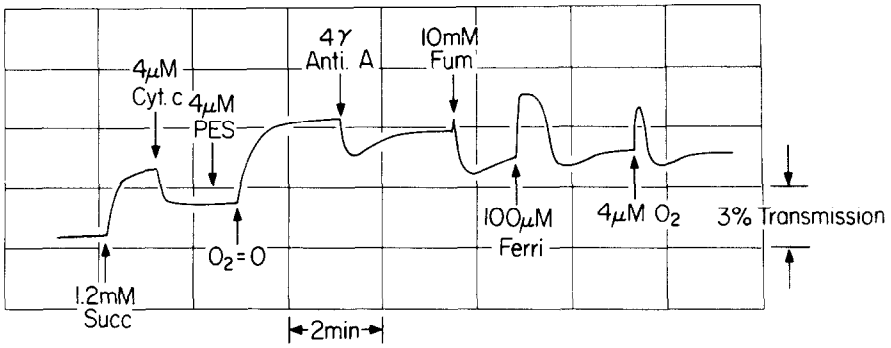

Figure 1. Energy conservation in detergent-treated chicken heart mitochondria. The cholate-clarified mitochondria (Methods Preparation A) were suspended at approximately 1.5 μM cytochrome b in a medium containing 50 mM morpholinopropane sulfonate as a buffer at pH 7.2 placed in a sealed cuvette with an argon atmosphere, and the indicated additions made. Abbreviations used are succ (succinate), cyt. c (cytochrome c), PES (phenazine ethosulfate), Fum (fumarate) and ferri (ferricyanide). The final volume was 5.0 ml.

increase in reduction occurs. The addition of 0.5 μg of antimycin A per mg protein and 10 mM fumarate establishes a more positive oxidation-reduction potential that results in reoxidation of some of the b cytochromes. The addition of ferricyanide causes an abrupt increase in absorbance nearly to the level recorded after anaerobiosis, an indication that the addition of the oxidizing agent evokes a reduction of a b cytochrome. The b cytochrome remains reduced until the ferricyanide is completely converted to ferrocyanide by electrons from succinate (largely through the phenazine ethosulfate [PES] bypass of the antimycin-inhibited site). The ferricyanide effect can be duplicated by oxygen in the presence of cytochrome c and an active cytochrome c oxidase. The PES may be omitted without influencing the b cytochrome reduction. Similar results are obtained with intact mitochondria or submitochondrial particles using oxygen, cytochrome c peroxidase complex ES or ferricyanide to induce the b cytochrome reduction (9).

The electron transport dependent reduction of the b cytochrome was next followed in purified succinate-cytochrome c reductase. The purified succinate-cytochrome c reductase preparation contains succinate dehydrogenase, the b

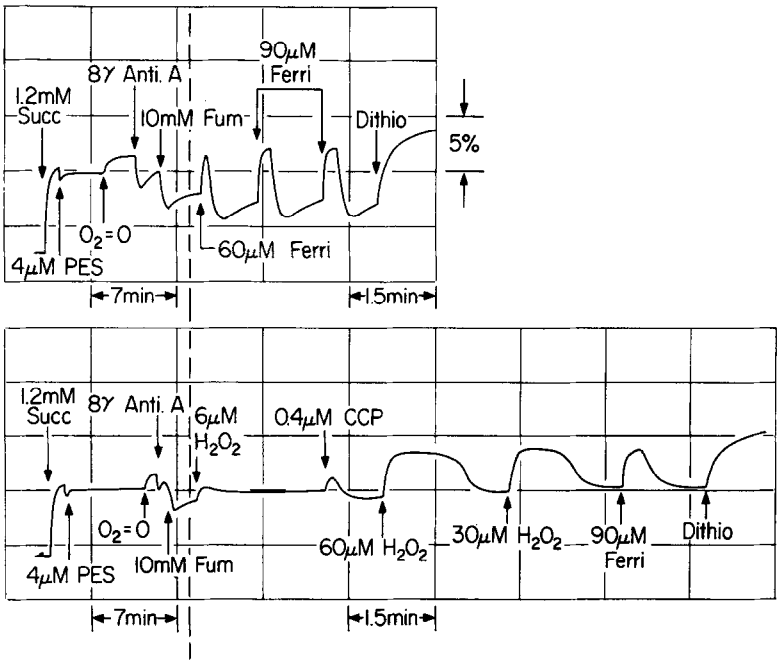

Figure 2. Energy conservation in the purified succinate-cytochrome c reductase. A succinate-cytochrome c reductase (Methods Preparation C) was suspended in a medium with 50 mM morpholinopropane sulfonate as a buffer at pH 7.2. The final cytochrome b concentration was approximately 2.5 μM. The dotted line indicates a change in recorder speed. The remaining conditions are the same as for Figure 1. Trace A differs from trace B in that 4 μM cytochrome c was added to the reaction medium of the latter before the trace was started. Additional abbreviations: CCP (cytochrome c peroxidase.)

cytochromes and cytochrome c_1 but is devoid of cytochrome oxidase and cytochrome c. In Figure 2 traces are presented from a dual wavelength spectrophotometer using the purified reductase. The addition of succinate and phenazine ethosulfate allows a significant oxygen consumption rate which exhausts the oxygen in the medium in about 5 minutes. Subsequent additions of antimycin A and fumarate establish a new more oxidized level of reduction of the b cytochromes. Ferricyanide causes a large reduction of the b cytochrome. As shown in Trace A, when a series of three additions is made, the duration of the reduction depends on the amount of oxidant added. Simultaneous measurements of the oxidation-reduction potential (the PES permits an approximate measurement) gives values of 65 mV before ferricyanide addition and greater than +300 mV in the presence of ferricyanide.

Because these preparations do not contain significant cytochrome oxidase activity, no effect of oxygen is observed. However, in the presence of cytochrome c and cytochrome c peroxidase (Trace B) the addition of H_2O_2 causes a reduction of the b cytochromes directly comparable to that caused by ferricyanide. The H_2O_2 effect is not observed if either cytochrome c or cytochrome c peroxidase is omitted from the reaction mixture. Since the electron transfer through the antimycin A-inhibited site is very slow, the duration of the reduction can be extended to several minutes by appropriate adjustment of the succinate and fumarate concentrations.

The spectrum of the ferricyanide induced absorbance change measured using the purified succinate-cytochrome c reductase (Figure 3) clearly shows a reduction of a b cytochrome having an alpha maximum at 565 nm and a shoulder near 560 nm, and an oxidation of cytochrome c_1. The spectrum of the b cytochrome is characteristic of cytochrome b_T (10,11).

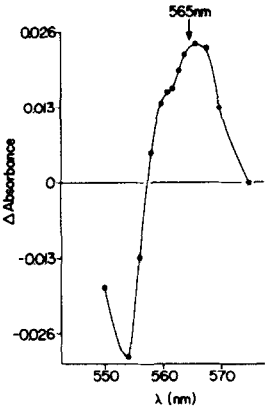

Figure 3. The spectrum of the ferricyanide-induced spectral change in antimycin A-treated succinate-cytochrome c reductase. The conditions used were the same as for Figure 2, except that the reference wave length was left at 575 nm and the measure wavelength moved to the values shown by the points in the Figure. The plotted absorbance changes are for 90 μM additions of ferricyanide.

A complete potentiometric titration of the oxidation-reduction potential dependence of the reduction of the b cytochromes in the succinate cytochrome c reductase preparation at pH 7.2 reveals the presence of three b cytochromes:

(1) an inactive fraction with a midpoint potential more negative than -100 mV, (2) cytochrome $\underline{b}_T$ with a midpoint potential of -30 mV and, (3) a more positive cytochrome(s) $\underline{b}$ with a midpoint potential of approximately 80 mV (these fractions contribute approximately 20%, 40% and 40%, respectively, to the absorbance change at 561 minus 575 nm). The presence of antimycin A in the anaerobic titration had no effect on the midpoint potentials of any of the three fractions of $\underline{b}$ cytochrome.

DISCUSSION

The direction of a chemical reaction is controlled by the change in Gibbs free energy in the overall process while the kinetic parameters define only the rate at which the reaction approaches equilibrium. The complete reduction of the cytochrome $\underline{b}_T$ (E_m = -30 mV) by the succinate-fumarate couple at +65 mV is an energy requiring process. This energy may be used either to chemically modify the cytochrome or to raise the electrons to a more negative potential required to reduce cytochrome $\underline{b}_T$ without chemical modification. The energy required is equivalent to at least 7 kilocalories (130 mV, n = 2.0). In mitochondria, ATP induces a change in the midpoint potential of cytochrome $\underline{b}_T$ from -30 mV to +245 mV (1,2,12). The reduction of cytochrome $\underline{b}_T$ by the activation of electron transport shows that the same energy dependent change is induced by electron transport through the second energy conserving site. It thus meets these two important criteria for the primary "high energy" intermediate at the second site.

It is most important to show that the observed electron transport-induced reduction of cytochrome $\underline{b}_T$ is a real expression of energy conservation rather than an artifact of the measuring technique. The reduction might possibly occur as the result of a transient generation of a low potential donor by an interaction of ferricyanide with a two electron donor to give a half-reduced form (i.e., reduced quinone + $Fe(CN_6)^{-3} \longrightarrow$ semiquinone + $Fe(CN)_6^{-4}$) with a lower oxidation-reduction potential. This possibility has been excluded by activating electron transfer by three different techniques: 1)

ferricyanide, 2) cytochrome c, cytochrome oxidase and 0_2 and 3) cytochrome c, cytochrome c peroxidase and H_20_2. Thus, the effect is not dependent on the species of oxidant used as long as the electrons are preferentially accepted from cytochrome c_1. Moreover, the last two oxidants can give completely stable cytochrome b_T reduction.

In the absence of a low potential donor generated by the added reagents, the cytochrome b_T reduction must be an inherent property of electron transfer through the second energy conservation site of the respiratory chain. Since these changes are observed in the purified succinate-cytochrome c reductase, this preparation must contain a competent primary energy conservation reaction whether the cytochrome b_T reduction is considered to be an expression of reversed electron transport or a chemical conversion of cytochrome b_T from a low potential to a high potential form. Relevant discussions of possible phosphorylation mechanisms have been presented (2,12-15).

Antimycin A appears to stabilize the electron transport-driven "high energy" intermediate at site II and to prevent the formation of this intermediate by added adenosine triphosphate (Wilson, Dutton and Erecinska, unpublished results).

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