INVOLVEMENT OF WATER IN ELECTRON TRANSPORT IN COMPLEXES III AND IV OF THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

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In this communication we present some preliminary findings on the "crossover points" which we observed when isolated electron transport complexes III and IV from beef heart mitochondria were studied in 90% glycerol. Three considerations led us to study electron transport in a medium of low water activity: 1) Such studies might allow the resolution of those segments of the electron transport chain which take place in a non-aqueous environment. 2) Any electron transfer where protons from the medium were reactants might be inhibited. 3) Any energy conserving transitions taking place within the isolated complexes and which would be very short lived in aqueous dispersion might be stabilized in a relatively non-aqueous medium. Further evidence relating to the involvement of protons was obtained by parallel studies where D_2O was used as the solvent.

We conclude from our findings that water is required for electrons to flow between cytochromes $\underline{b}$ and $\underline{c}_1$ in complex III and between cytochromes $\underline{a}$ and $\underline{a}_3$ in complex IV. We further conclude that this water requirement relates to a specific proton involvement in electron transport at these "crossover" points.

MATERIALS AND METHODS

Purified complex III (reduced CoQ*-cytochrome $\underline{c}$ reductase) was prepared according to the procedure of Rieske et al. (1) and assayed by a modification of

*The following abbreviations will be used: CoQ, coenzyme Q; CoQ_2H_2, reduced coenzyme Q_2; TMPD, N,N,N',N' tetramethyl-p-phenylenediamine; EPR, electron paramagnetic resonance spectroscopy.

the method of Hatefi et al. (2). Preparations of complex IV (cytochrome oxidase were made according to the procedures of Fowler et al. (3) and of Griffiths and Wharton (4). Cytochrome oxidase activity was assayed by the method of Griffiths and Wharton (4). Spectrophotometric measurements were made using the Beckman DK 2 spectrophotometer with a cooled cell compartment.

RESULTS AND DISCUSSION

Solutions of complex III (20 mg protein/ml in a Tris-sucrose-histidine buff could be mixed in all proportions with glycerol to give clear, stable dispersion When CoQ_2H_2 was added to these dispersions the resultant pattern of spectral changes in the region of the α bands of cytochromes b and c_1 was found to depend on a number of factors. These factors included the previous state of reduction of the complex; the amount, concentration and solvent of the CoQ_2H_2 solution add the temperature; and the final glycerol concentration of the medium. The most pronounced differences between the glycerol and aqueous control systems were obtained under the following conditions: (a) Complex III was first fully oxidized with ferricyanide; (b) a small amount of the reductant was added as a dilute suspension in glycerol, (c) the temperature was maintained at 0°-5°; and (d) the concentration of glycerol was 90 per cent or higher. Under these conditions there was an instantaneous, partial reduction of cytochrome b but a pronounced l: of some minutes before the α peak of reduced cytochrome c_1 became apparent (Fig. 1-A). When water was the solvent no such lag could be detected (Fig. 1-B). The time taken for the height of the 554 mμ peak (cytochrome c_1) to exceed that of the 562 mμ peak (cytochrome b) decreased with decreasing glycerol concentrations so that below 75% glycerol this lag was only transient. This finding presumably accounts for the failure of previous workers to observe any "crossover" points when electron transport was studied kinetically in 50% glycerol (5). When sucro: solutions of extremely high viscosity were substituted for glycerol, no effects analogous to those in 90% glycerol were observed. Viscosity alone was thus elim: ed as a factor responsible for the delayed reduction of cytochrome c_1.

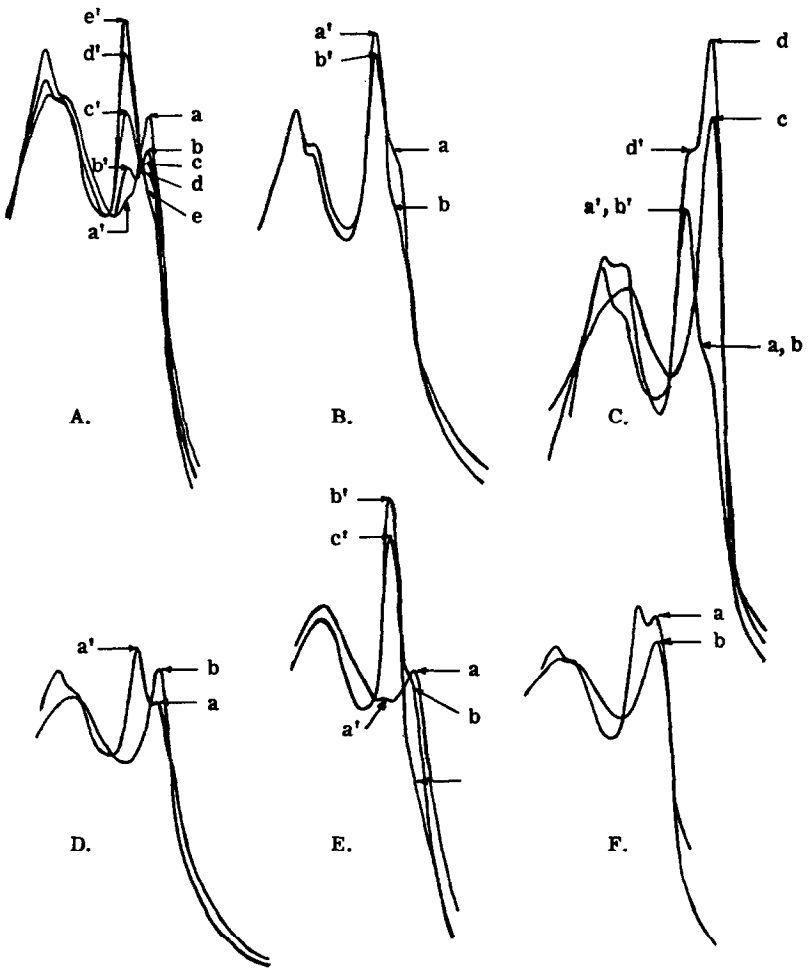

Fig. 1-A. Spectra of Complex III in 90 per cent glycerol; reduction of cytochromes b and c_1 by CoQ_2H_2. CoQ_2H_2 (ca. 0.02 ml glycerol solution containing 0.5 mg CoQ_2H_2 per ml.) was added and mixed with Complex III (2 mg. protein/ml. in 90% glycerol). Spectra were recorded at (a) 2 min., (b) 3.5 min., (c) 5.5 min., (d) 9 min., and (e) 14 min. after addition of CoQ_2H_2. Letter designations and their primed counterparts refer to the α bands of cytochrome b (562 mμ) and c_1 (554 mμ), respectively. The temperature was 4°.

Fig. 1-B. Spectra of Complex III in water; reduction of cytochromes b and c_1 by CoQ_2H_2. Other than water being the solvent medium the conditions were the same as those described for Fig. 1-A. Spectra were recorded in sequence (a) 30 sec. after the addition of CoQ_2H_2 and (b)immediately after the addition of ferricyanide (ca. 0.01 ml of 0.01 M K ferricyanide). Spectral designations are the same as those described in Fig. 1-A.

Fig. 1-C. Spectra of Complex III in water; effects of antimycin A on the reduction of cytochromes b and c_1 by CoQ_2H_2. Conditions were the same as those described for Fig. 1-B. Spectra were recorded immediately after the addition in sequence of (a) CoQ_2H_2, (b) antimycin A (ca. 0.01 ml of a 10 mM solution in ethanol), (c) ferricyanide (ca. 0.01 ml of 0.01 M K ferricyanide), and (d) $Na_2S_2O_4$. Spectral designations are the same as those described for Fig. 1-A.

Fig. 1-D. Spectra of Complex III in 90 per cent glycerol; effects of transient oxidation on the reduction of cytochrome b and c_1 by CoQ_2H_2. Conditions were the same as those described for Fig. 1-A. Spectra recorded at 7 min. after addition of CoQ_2H_2 (a) and immediately after the addition of ferricyanide (ca. 0.(ml. of 0.01 M K ferricyanide) (b) Spectral designations are the same as those described for Fig. 1-A.

Fig. 1-E. Spectra of Complex III in D_2O; reduction of cytochromes b and c_1 by CoQ_2H_2. Other than the solvent medium being D_2O the conditions were the same as those described for Fig. 1-A. Spectra were recorded at (a) 30 sec., (b) 2 min., and (c) 4 min. after addition of CoQ_2H_2. Spectral designations are the same as those described for Fig. 1-A.

Fig. 1-F. Spectra of Complex III in water; reduction of cytochromes b and c by $CoQH_2$ at pH = 10. Other than pH, the conditions were the same as those descril for Fig. 1-B. The pH was adjusted to 10 by addition of dilute KOH. Spectra were recorded immediately after addition in sequence of (a) CoQ_2H_2 and (b) ferricyanide (ca. 0.01 ml of 0.01 M K ferricyanide) to the sample.

The site of this inhibition was of interest because it corresponds with respect to location in the respiratory sequence to a "crossover" point in controlled respiration (6) as well as to the site of inhibition by antimycin A (7). One paradoxical feature of submitochondrial particles inhibited with antimycin A is that, in the presence of substrate, cytochrome b becomes more reduced when the degree of oxidation of the terminal cytochromes is increased by aeration (8,9) An analogous phenomenon was also observed by us with antimycin A-treated Complex III in water. Following reduction by CoQ_2H_2 the degree of reduction of cytochrome b was markedly increased when cytochrome c_1 was oxidized either by the addition of a trace of ferricyanide or by the addition of ferricytochrome c (Fig. 1-C). As can be seen in Fig. 1-D a similar though less pronounced effect was obtained in the absence of antimycin when 90% glycerol was the solvent. In contrast, the addition of ferricyanide to the CoQ_2H_2-reduced Complex III in water resulted in a preferential oxidation of cytochrome b (Fig. 1-B).

As with Complex III, solutions of Complex IV also formed clear stable suspensions when mixed with glycerol in all proportions. When substrate, (ascorbate-cytochrome c or ascorbate-TMPD), was added to suspensions of complex IV in 90% glycerol there was an immediate appearance of a peak at 605 mμ on the difference spectrum. This peak could only be slightly and temporarily decreased by vigorous aeration. The ratio of peak height of the Soret band at 444 mμ to that of the α

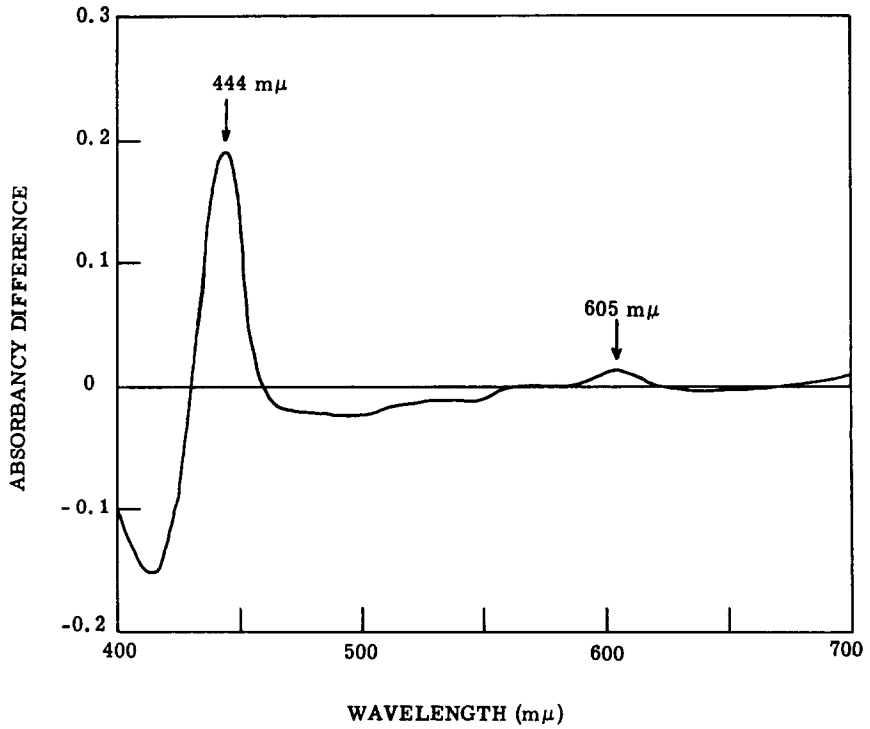

Fig. 2. Difference spectrum ($Na_2S_2O_4$ reduced minus ascorbate-TMPD reduced) of Complex IV in 90 per cent glycerol.

band at 605 mμ varied slightly from preparation to preparation of cytochrome oxidase but it was usually less than 4.0. In contrast, ratios of up to 7.0 were obtained when the preparations were reduced with dithionite plus dithiothreitol prior to mixing with glycerol. This suggested that in glycerol only cytochrome *a* was readily reduced by substrate. Figure 2 is a difference spectrum between the fully reduced complex and that reduced only by substrate (in both cases in 90% glycerol). This spectrum corresponds to that calculated for cytochrome a_3 in water (10).

Further confirmation of a block between cytochromes *a* and a_3 in glycerol was obtained by studying the effect of carbon monoxide on the spectrum of the substrate-reduced complex. On CO treatment, a difference spectrum, (substrate reduced + CO) minus (substrate reduced), was produced that was indistinguishable from the corresponding spectrum in an anaerobic aqueous system; however, in

the case of the glycerol system this spectral change occurred relatively slowly. On the other hand, the dithionite reduced complex in glycerol exhibited immediate spectral changes on treatment with CO. This was a further indication that cytochrome $\underline{a}_3$ was only very slowly reduced by substrate in glycerol.

EPR studies of complex IV in glycerol* showed that when cytochrome $\underline{a}$ (but not cytochrome $\underline{a}_3$) was reduced by substrate there was a corresponding decrease in the signal a g=2.0 of the oxidized copper usually thought to be associated with cytochrome $\underline{a}$ (10). Thus the block did not appear to be between cytochrome $\underline{a}$ and this associated copper atom.

The glycerol treatment did not appear to cause any significant denaturation of either complex III or complex IV during the time of the experiment. When glycerol dispersions of the complexes were diluted with water and assayed for catalytic activity it was found that, even after some hours in glycerol, up to 80% of the original activities were recoverable.

The effects of glycerol described above could be explained either in terms of an aqueous environment being required for electron transport within certain sectors of the complexes or, more specifically, in terms of the involvement of a proton or hydroxyl ion.

When complex III was studied in solution in D_2O it was found that, on addition of substrate, part of the cytochrome $\underline{b}$ was reduced significantly earlier than cytochrome $\underline{c}_1$ when compared with a control in water (Fig. 1-E). Moreover when the complex was studied in water at pH 10 a similar pattern of reductions was observed. Furthermore when cytochrome $\underline{c}_1$ was allowed to become partially reduced at pH 10 the addition of ferricyanide resulted in a preferential oxidation of this cytochrome, cytochrome $\underline{b}$ remaining reduced (Fig. 1-F). These observations appeared to implicate a proton in the electron transfer segment between cytochromes $\underline{b}$ and $\underline{c}_1$.

Further evidence for proton involvement was the finding that the catalytic activities of complexes III and IV were reduced by 40% and 60% respectively

*Orme-Johnson, W., Rieske, J. S. and Baum, H., unpublished observations.

when studied in 95% D_2O. In neither case was any irreversible loss of activity detected on dilution of D_2O solutions of the complexes with water.

The precise nature and site of the proton involvement in complex III is not clear. The pattern of spectral changes reported here is presumably the resultant of several kinetic processes. Similar patterns (each with slightly different characteristics, but always more pronounced at high pH) can be obtained in the presence of high concentrations of cyanide and azide. Moreover an apparent preferential reduction of cytochrome b can also be observed when electrons are fed in very slowly under carefully poised conditions. This can be achieved either by using reduced ubiquinones of longer chain length or succinate (in the case of preparations substantially free of succinic dehydrogenase or coenzyme Q). In either case however the pattern of reduction differs markedly from that in the glycerol or D_2O systems in that the appearance of the absorption peak corresponding to the reduction of cytochrome b is not rapid. In aerobic systems, a further factor which complicates the interpretation of spectral observations is that antimycin A increases the rate of autoxidation of cytochrome b, an effect that is greatly enhanced by the presence of cyanide.* Whatever the full significance of these patterns (which may reflect built in control features of electron transport) we believe that the involvement of protons in the reduction of at least one chain component is strongly indicated.

As an extension of these findings we have studied the respiration in D_2O of mitochondria and of submitochondrial particles in a number of metabolic states.** Our findings in general confirm and extend those reported by Laser and Slater (12). We have tentatively concluded that the pattern of inhibition of respiration in D_2O under various conditions primarily reflects the same proton involvement in electron transport as was demonstrated in this study.

In summary, the experiments in glycerol indicate that water is required for

*Baum, H. and Rieske, J. S., unpublished observations.

**Baum, H., Rieske, J. S., Lenaz, G. and Ozawa, T., unpublished observations.

electrons to flow between cytochromes b and c_1 in complex III and between cytochromes a and a_3 in complex IV. The experiments in D_2O and supportive evidence obtained at high pH demonstrate that this water requirement relates to a specific proton involvement. It is still an open question however as to whether protons from the medium have direct access to sites within the interior of each complex or whether electron transport is controlled by other processes taking place elsewhere in the complex, possibly at an aqueous interface. Certainly the pattern of spectral changes discussed above indicate a highly organized system; and it should also be borne in mind that there is evidence that reduction of complex III*** or treatment with antimycin A (13) each result in gross structural changes within the complex. The observations presented here are therefore considered significant both in regard to the detailed organization of the components involved in terminal electron transport, and to the mechanism of energy coupling in mitochondria. These problems are under active investigation.

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