DISRUPTION OF MITOCHONDRIA AND SOLUBILIZATION OF CYTOCHROME OXIDASE BY Na_4EDTA AND BY TRIS-BUFFER AT HIGH pH VALUES

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We recently reported zeolite solubilization of cytochrome oxidase and cytochrome b from yeast and beef heart mitochondria (Person and Zipper, 1964). Because the synthetic zeolite used (Zeolite 3A, Linde Co., Tonawanda, N.Y.) produces a high pH (10-10.5) in aqueous systems, and notwithstanding the well-known denaturation and inactivation of cytochrome oxidase by NaOH or KOH above pH 9 (Keilin, 1925; Lemberg and Pilger, 1964), we investigated the possible role of high pH as a means of solubilizing cytochrome oxidase and cytochrome b. We found eventually, that simple extraction of isolated yeast and beef heart mitochondria with Na_4EDTA or with Tris-buffer at high pH values will put cytochrome oxidase and cytochrome b into the aqueous phase. The resultant aqueous extracts are enzymatically active, optically clear and homogeneous systems, colored deep red-green to pale yellow-green according to concentration and reagent used. No bile salts, detergents, organic solvents or sonic oscillations are used.

EXPERIMENTAL: Glass redistilled water was used. 0.05M Na_4EDTA (Na_4 ethylenediamine tetra-acetate) and 0.05M Tris-(2amino 2-hydroxymethyl 1,3-propanediol) buffer were prepared at respective pH values of 10.5 and 9.0 (20-25°C). Subsequent procedures were performed at 0°-4°C. Frozen-thawed Candida utilis (Lake States Yeast and Chemical Co., Rhinelander, Wisconsin) and fresh beef heart muscle were starting materials. Preparation of mitochondria, cytochrome oxidase assay and protein determination method are given in Person and Zipper,

(1964). Isolated yeast and heart mitochondria were washed three times with water and following each wash were recovered by centrifugation at 144,000 x g, for 30 minutes. The recovered mitochondrial pellets (approx. 1 ml.vol. containing 150-200 mg. prot.) were dispersed in 15-25 ml extracting medium and the pH checked. Additional medium was added (if required) to maintain the respective pH values. The various systems were then centrifuged for one hour at 144,000 x g (yeast) and 25,000 x g (heart), and the supernatant optically clear and homogeneous extracts recovered for anaylsis. Although details will not be given now, we wish to report that successful extractions were also made with 2-amino 2-methyl 1-propanol, N-methyl glucamine and with NH_4OH. Extractions with NaOH and KOH at pH's between 8-10.5 gave negative results.

RESULTS: Both Na_4EDTA (pH 10.5) and Tris (pH 9.0) solubilize cytochrome oxidase and cytochrome b from both yeast and heart mitochondria (above pH values uncorrected to 0°-$4^{\circ}C$.) The action of Na_4EDTA with yeast mitochondria is immediate; all other combinations require more prolonged extractions (3-16 hours). The extractions work only with freshly prepared or frozen mitochondria, but not for lyophilized mitochondria. Even prolonged extraction of lyophilized (yeast or heart) mitochondria with any of the media mentioned above (24 hours) dissolves out only cytochrome c (see Fig. 1-curve b). Fig. 1 curve a, shows a typical absorption spectrum of an Na_4EDTA extract of heart muscle mitochondria. The absorption maxima are: α peaks - 603-604 mμ (cytochrome oxidase), 560-550 mμ (combined b and c components); β peaks 530-520 mμ region (combined b and c components); γ peaks - 443 mμ (cytochrome oxidase); 432 mμ (b components). A reduced absorption for cytochrome c at 415 mμ is not seen and may be masked by the other γ absorbancies. Similar spectra were obtained in recordings from Na_4EDTA extracts of yeast, and also from Tris extracts of yeast and heart mitochondria. Of all extracting mediums used, Na_4EDTA is most rapid and complete in its action (with the yeast mitochondrial system). Results with heart mitochondria are more variable.

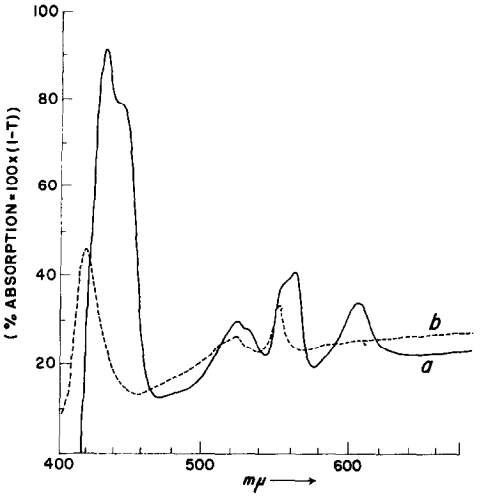

Fig. 1 Direct tracings of recorded ratio spectra of a) pH 10.5, Na_4EDTA extract of fresh heart mitochondria, and b) pH 9.0, 0.05 M Tris extract of lyophilized yeast mitochondria. Dithionite-reduced extract in sample cuvette; non-dithionite-reduced extract in reference cuvette. Ordinate scale is in % Absorption i.e.100x(1-T) where T=I/Io.

The specific activities of 0.02-0.1 ml aliquots of extracts have varied between 0.116-1.62 μM cyt. c oxid/min/mg.prot. The latter value was for a 16 hour Tris extract of freshly prepared yeast mitochondria.

DISCUSSION: The above procedures provide new means for disruption of mitochondria and solubilization of mitochondrial components without use of bile salts, detergents, organic solvents or sonic oscillations; and with surprisingly little inactivation or denaturation of cytochrome oxidase, considering the high pH values used. Undoubtedly alkyl amine groups, and NH_3 itself, are involved in protecting the oxidase at high pH, as well as in disruption of mitochondria and solubilization of the oxidase. Mechanisms of action now being studied in our laboratory appear to be manifold, as follows: a) at the pH values used, lipid and lipoprotein dispersal and solubilization will occur via emulsification, saponification and ammonolysis (Schulman and Montagne, 1960; Becher, 1957; Eckey and Miller, 1954). b) The

alkaline earth cations Ca^{++} and Mg^{++} will form complexes with NH_3 and amine groups. Especially with Na_4EDTA at pH 10.5 will Ca^{++} and Mg^{++} be chelated very strongly (Martell and Calvin, 1952). The possibility that other cationic components are also being complexed remains open. c) Mitochondrial structural protein is also solubilized, and can be precipitated from the extracts by addition of $(NH_4)_2SO_4$ to 12.3-12.5% concentration at neutral pH (unpublished observations). As shown by Criddle et al (1963) structural protein is soluble at high pH, and is precipitated at 12.3% $(NH_4)_2SO_4$ concentration at neutral pH.

Further consideration of mechanisms brings us again to the role of water. As in our zeolite 3A solubilization of cytochrome oxidase (Person and Zipper, 1964), so in the case of the present experiments, negative results are obtained when lyophilized mitochondria are used. Evidently, then, water molecules are present not only as bulk water, but also as important structural and structure maintaining components of the mitochondrion. Such water must also be involved (directly or indirectly) in electron transport catalysis, because lyophilization of a variety of mitochondrial and cytochrome oxidase preparations almost always causes 30-50% losses in activity. This lyophilization-induced loss in activity is restored in variable degree by incubation in aqueous media (our unpublished experiments; McGuiness and Wainio, 1962). In view of the almost obligatory structural ordering of water molecules at lipid-water interfaces and hydrocarbon-water interfaces (see Grassl, 1963 for identificattion of lipid-soluble hydrocarbon component of heme a), it is understandable that lyophilization should produce serious structural and catalytic alterations in mitochondrial components. In addition, and apart from phase considerations of water-lipid systems, the fundamental role of water in maintenance of protein structure as advocated in the theoretical and experimental studies of Klotz (1962) is very pertinent to our studies, and must be considered. Also in connection with water involvement, the inability of zeolite 3A and the agents mentioned in this study to solubilize lyophilized mitochondrial components stands in important contrast with the

action of deoxycholate and other detergents, which do disrupt lyophilized mitochondria and which do solubilize cytochrome oxidase and other lyophilized mitochondrial components.

In summary, on the basis of present results and our previous zeolite experiments (Person and Zipper, 1964), we consider 1) that water and the alkaline earth cations Ca^{++} and Mg^{++} are not only chemical species of the mitochondrial environment, but also that they are integral and inter-related components of the mitochondrion, of great importance both for its structure and catalytic activity, and 2) that with the aid of the outlined methods of mitochondrial disruption, new approaches to mitochondrial dissection and recombination are made available.

A report giving fuller details of structural and catalytic inter-relationships between water, the alkaline earth cations and lipid components of mitochondria, (as revealed by the procedures we have described) is now in preparation.

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REFERENCES

Becher, P., Emulsions: Theory and Practice, Chap. 6. Reinhold Publ. Corp., New York (1957)

Criddle, R. S., Bock, R. M., Green, D. E. and Tisdale, H., Biochem., 1, 827 (1962)

Eckey, E. W. and Miller, L. P., Vegetable Fats and Oils, p. 144, Reinhold Publ. Corp., New York (1954).

Grassl, M., Coy, U., Seyffert, R., and Lynen, F., Biochm. Zeit., 338, 771 (1963)

Keilin, D., Proc. Roy. Soc. Lond. B., 98,312 (1925)

Klotz, I., in M. Kasha and B. Pullman (Eds.) Horizons in Biochemistry, Albert Szent-Gyorgyi Dedicatory Volume, Academic Press, Inc., New York p. 523, (1962)

Lemberg, R., and Pilger, T.B.G., Proc. Roy. Soc. B, 159, 436 (1964)

McGuiness, E. T., and Wainio, W. W., J. Biol. Chem., 237, 3273 (1962)

Martell, A. E., and Calvin, M., Chemistry of Metal Chelate Compounds, p. 41, Prentice-Hall, Inc., Englewood Cliffs, N.J., (1952)

Person, P., and Zipper, H., Biochem. Biophys. Res. Comm., 17, 225 (1964)

Schulman, J. H., and Montagne, J. B., Ann. N. Y. Acad. Sci., 92, 366 (1961)