BINDING OF ADENINE NUCLEOTIDES BY MITOCHONDRIA DURING ACTIVE UPTAKE OF CA^{++}

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The active accumulation of Ca^{++} by mitochondria may be supported by the energy-conserving mechanisms of electron transport (Rossi and Lehninger, 1963; Brierley et al., 1963). However, accumulation of Ca^{++} under these circumstances also requires the presence of ATP in the system (DeLuca and Engstrom, 1961; Vasington and Murphy, 1962; Rossi and Lehninger, 1963, 1964; Brierley et al., 1963). It has been suggested (Vasington, 1963; Brierley et al., 1963) that the ATP is necessary for the maintenance of the structural state of the mitochondria in the presence of Ca^{++}, a powerful swelling agent. This explanation, however, does not account for the fact that the supporting action of ATP is the same in the presence or in the absence of oligomycin, a reagent which completely suppresses the ATP-induced contraction of swollen mitochondria (Neubert and Lehninger, 1962). Furthermore, ATP has also been shown to be necessary for respiration-supported accumulation of Sr^{++} by rat liver mitochondria despite the fact that Sr^{++} has no damaging effects on the mitochondrial structure (Carafoli, Weiland and Lehninger, 1964).

A possible clue to this specific role of ATP in Ca^{++} accumulation is the finding made by Lehninger et al.,(1963 a, b) that significant amounts

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of easily-hydrolyzed phosphate (labile to 1 N HCl at 100° for 7 minutes) accumulate in rat liver mitochondria during accumulation of Ca^{++} and inorganic phosphate. This observation has been pursued further; in this communication it is shown that large amounts of adenine nucleotides are accumulated by rat liver mitochondria from the suspending medium during uptake of Ca^{++} supported by electron transport. The uptake of nucleotides is Ca^{++}-dependent, has the same requirements as Ca^{++} uptake, and its sensitivity to inhibitors is similar.

EXPERIMENTAL

The general conditions for measuring the uptake of Ca^{++} were those of Rossi and Lehninger (1963). For measuring binding of adenine nucleotides, ATP-8-C^{14} (1000-1500 c.p.m.), was incubated with the mitochondria; at the end of the incubation period, the tubes were quickly cooled to 0° and centrifuged in the cold at 20,000 x g for 5 minutes. The pellets were washed twice with 0.25 M sucrose, dissolved in 85% formic acid, plated, and counted at infinite thinness in a low background gas-flow apparatus. When determination of Ca^{++} was carried out, the washed pellets were resuspended in 0.1 M succinate buffer pH 4.6, and processed according to the method of Walser (1960). Protein was determined on the mitochondrial suspensions by a biuret reaction.

RESULTS

Fig. 1-A compares the rate of binding of labeled adenine nucleotides to the mitochondria with the rate of accumulation of Ca^{++}. The adenine nucleotides are not bound instantaneously, but rather at a rate which indicates that the binding accompanies the accumulation of Ca^{++}. About 4-5% of the labeled ATP added was bound in the 20 minute period. However, since the Ca^{++}-stimulated ATPase activity hydrolyzes most of the added ATP in such experiments, it appeared possible that the binding of labeled nucleotide was limited by the amount of ATP present. That this is probably true is shown by the experiment in Fig. 1-B. The binding of labeled nucleotide increases with its concentration and appears to reach saturation only above 6.0 mM ATP.

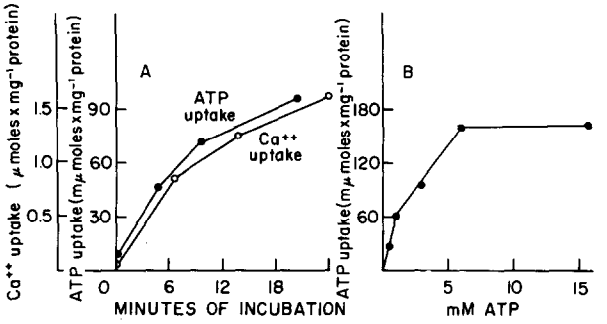

Figure 1. A = Ca^{++} and ATP uptake by mitochondria. Test system contained 10 mM Tris-HCl, pH 7.0, 80 mM NaCl, 10 mM $MgCl_2$, 4.0 mM NaK-phosphate, 10 mM Na-succinate, 3.0 mM ATP-Na (+1500 c.p.m. ATP-8-C^{14}), 4.0 mM $CaCl_2$ and rat liver mitochondria (15 mg protein) in a total volume of 10.0. Temperature. 30°. B = ATP uptake by mitochondria as a function of the added ATP concentration. Test system as in Fig. 1A, with variable amounts of ATP. Time, 20 minutes, temperature, 30°.

Typical data in Table 1 show that the labeled ATP is bound maximally in a system containing respiratory substrate, Mg^{++}, Ca^{++} and P_i. Omission of any

Table 1

Requirements for binding of C^{14}-adenine nucleotides by rat liver mitochondria

The test system contained 10 mM Tris HCl pH 7.0 , 80 mM NaCl, 10 mM $MgCl_2$, 4.0 mM NaK-phosphate, 10 mM Na succinate, 3.0 mM ATP-Na (+1500 c.p.m. ATP-8-C^{14}), 4.0 mM $CaCl_2$, and rat liver mitochondria (15 mg protein) in a total volume of 10.0 ml. Incubated 20 min. at 30°.

System	Nucleotide uptake mμmoles x mg^{-1} protein
Complete	88.1
Mg^{++} omitted	47.7
Succinate omitted	45.0
P_i omitted	52.0
Ca^{++} omitted	16.6
Complete	91.5
Ca^{++} omitted	19.1
Complete + 1 μg oligomycin x mg^{-1} protein	91.2
Complete + 0.2 mM 2,4-dinitrophenol	35.0

component greatly limits the ATP binding. These requirements are identical with those required for maximum accumulation of Ca^{++} (Vasington and Murphy, 1962), (Brierley et al., 1963), (Rossi and Lehninger, 1963). The typical data in Table 1 also show that the aerobic binding of adenine nucleotides is not inhibited by oligomycin but is inhibited strongly by 2,4-dinitrophenol, in agreement with the action of these agents on Ca^{++} accumulation in liver mitochondria (Vasington and Murphy, 1962). There appears to be a significant base-line ATP accumulation which is not dependent on added Ca^{++}; this amounts to some 15-20% of the maximum nucleotide uptake.

The maximum amounts of adenine nucleotide bound at saturation (i.e. high concentrations of ATP) in experiments such as that in Fig. 1B were about 160 mμmoles adenine nucleotide per mg mitochondrial protein, or about 10 times the normal content in freshly isolated mitochondria. This may be compared with the much smaller amounts of ATP and ADP (i.e. ~1 mμmole per mg protein) bound by mitochondria in the atractyloside-sensitive reaction recently described by Bruni, Luciani and Contessa (1964).

In several experiments in which uptake of both Ca^{++} and labeled adenine nucleotide were measured, under conditions of widely varying Ca^{++} concentrations and at different time intervals, between 60 and 90 mμmoles of adenine nucleotide were bound per μmole of Ca^{++} accumulated. This ratio does not suggest a simple stoichiometry between Ca^{++} and adenine nucleotide binding but does indicate that the binding of adenine nucleotides accompanying Ca^{++} accumulation is a relatively conspicuous process.

It appears most likely that the form of the labeled nucleotide bound still contains one or both of the acid-labile phosphate groups, since the earlier experiments of Lehninger et al., (1963 a, b) showed that the amounts of easily-hydrolyzed P (7 min. P_i) accumulated with Ca^{++} are of the order of 0.05-0.10 μmole per μmole Ca^{++} accumulated. In addition, Bruni et al., (1964) have demonstrated that both P^{32}-labeled ATP and ADP are bound to rat liver mitochondria.

It appears possible that the nucleotide is bound at or near the sites of Ca^{++} binding, and that bound nucleotide aids in forming and stabilizing calcium phosphate deposits in the mitochondria, in such a manner that the Ca^{++} does not "leak" out again (Greenawalt et al., 1964; Carafoli et al., 1964; Rossi and Lehninger, 1964). The significance of the nucleotide-binding reaction in active Ca^{++} uptake, oxidative phosphorylation, and in the sites of action of oligomycin and atractyloside is under further investigation.

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BIBLIOGRAPHY

Brierley, G. P., Murer, E. and Green, D. E., Science, 140, 60 (1963).
Bruni, A., Luciani, S. and Contessa, A. R., Nature, 201, 1219 (1964).
Carafoli, E., Weiland, S. and Lehninger, A. L., Biochim. Biophys. Acta, Submitted for publication.
Carafoli, E., Rossi, C. S. and Lehninger, A. L., J. Biol. Chem., In press.
DeLuca, H. F. and Engstrom, G. W., Proc. Nat. Acad. Sci. U.S., 47, 1744 (1961).
Greenawalt, J. W., Rossi, C. S. and Lehninger, A. L., J. Cell Biol., In press.
Lehninger, A. L., Rossi, C. S. and Greenawalt, J. W., Biochem. Biophys. Research Comm., 10, 444 (1963a).
Lehninger, A. L., Rossi, C. S. and Greenawalt, J. W., Fed. Proc., 22, 526 (1963b).
Neubert, D. and Lehninger, A. L., Biochim. Biophys. Acta, 62, 556 (1962).
Rossi, C. S. and Lehninger, A. L., Biochem. Zeit., 338, 698 (1963).
Rossi, C. S. and Lehninger, A. L., J. Biol. Chem., Submitted for publication.
Schneider, W. C. in Manometric Techniques, W. W. Umbreit, R. Burris and J. E. Stauffer (eds.), Burgess Press, Minneapolis, 1956, p. 188.
Vasington, F. D., J. Biol. Chem., 238, 1941 (1963).
Walser, M., Anal. Chem., 32, 771 (1960).