NEW ATP INCORPORATING ACTIVITIES FROM RAT LIVER MITOCHONDRIA: THE ATTACHMENT OF A PORTION OF ATP TO A PRONASE-SENSITIVE RECEPTOR*

David P. Philipp and Peter Parsons
Department of Biochemistry, University of Massachusetts
Amherst, Massachusetts 01002

Received October 23,1973

SUMMARY: Studies using a Brij 58 detergent extract of rat liver mitochondria reveal that these organelles can catalyze the time-dependent incorporation of a portion of [^{3}H]ATP into an acid-insoluble product. The activities studied using 8 mM Mn^{++} or 15 mM Mg^{++} are stimulated by dithiothreitol and by CTP, GTP or UTP, while that studied using 2 mM Mg^{++} is not. The incorporated tritium remains bound after incubation in the presence of excess unlabeled ATP and chromatography on Sephadex G-25. The labeled product is insensitive to ribonuclease A and snake venom phosphodiesterase, but is sensitive to pronase. The attached portion of the ATP molecule released upon treatment of the product has been tentatively identified as adenosine for the activities studied using 2 mM Mg^{++} or 8 mM Mn^{++} and as AMP (80%) and adenosine (20%) for the reaction studied using 15 mM Mg^{++}.

During our investigation of RNA polymerase from rat liver mitochondria, we observed that while intact mitochondria incorporated all four ribonucleotides to similar extents, a Brij 58 detergent extract of these organelles incorporated ATP to a much greater extent than CTP, GTP or UTP into acid-insoluble material. It is evident that this ATP incorporation is not the result of RNA polymerase (1,2) nor poly (A) synthetase (3) activities, but instead, reflects the enzymatic attachment of a portion of the ATP molecule to a pronase-sensitive substrate, an activity not previously reported from mitochondria. After some preliminary studies we have defined reaction conditions which suggest the presence of more than one activity of this type. This report describes some of the characteristics of these activities and presents preliminary data on the nature of the synthesized products.

METHODS: Preparation of the Enzyme. Mitochondria from the livers of male Sprague-Dawley rats were isolated in 0.25 M sucrose-1 mM EDTA, pH 7.5, according to the method of Reid and Parsons (1) and suspended in 10 mM Tris-1 mM EDTA, pH 8.5 at approximately 60 mg/ml, as determined by the Lowry protein assay (4). Addition of an aqueous 10% Brij 58 (Sigma) detergent solution to a final concentration of 0.05% resulted in the

*This work was supported in part by a Biomedical Sciences Support Grant to the University of Massachusetts, FR-07048.

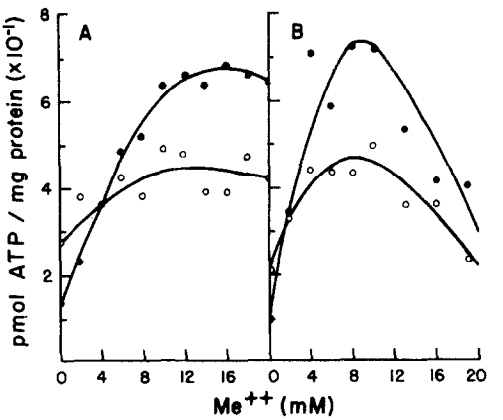

Fig. 1. Dependence of ATP incorporation on divalent cation concentration in the presence (-●-) or absence (-o-) of 5mM DTT. In a final volume of 0.2 ml, 50 λ of enzyme preparation was incubated with 25mM Tris-HCl, pH 8.5, 30mM KCl, 0.225mM malate, 3.75mM pyruvate, 1mM CTP, 1mM GTP, 1mM UTP, 0.1mM ATP, 1.5 μCi [2,8-^{3}H]ATP (32.7 Ci/mmole), and increasing amounts of $MgCl_2$(A) or $MnCl_2$(B). The incubations were performed at 37°C for 30 minutes.

solubilization of the activities to be described. After centrifugation at 170,000 x g for 1 hour, the resulting supernatant was passed through Sephadex G-25 equilibrated with 10 mM Tris-1 mM EDTA, pH 8.5. The protein fractions of the void volume were combined and constitute the enzyme preparation.

ATP Incorporation Assays. Individual experimental reaction conditions are defined in Results. The amount of incorporation of tritiated nucleotide into acid-insoluble material was measured by the cellulose filter disc method described by Niles and Westhead (5), unless otherwise specified.

RESULTS: The response of the ATP incorporating activities to varying Mg^{++}, Mn^{++} and dithiothreitol (DTT) concentrations are given in Figs. 1 and 2. Fig. 1A shows that 5 mM DTT only stimulates ATP incorporation at Mg^{++} concentrations greater than 4 mM. When Mg^{++} is replaced by Mn^{++}, the reaction reaches a maximum at 8-10 mM Mn^{++} and is stimulated by 5 mM DTT (Fig. 1B). Fig. 2 shows that the reaction using 8 mM Mn^{++} or 15 mM Mg^{++} is considerably enhanced by the presence of DTT concentrations above 4 mM, whereas the activity observed using 2 mM Mg^{++} is not affected by the presence of DTT. The distinct difference in response to DTT in the presence of low versus high Mg^{++} concentrations suggests the presence of more than one activity.

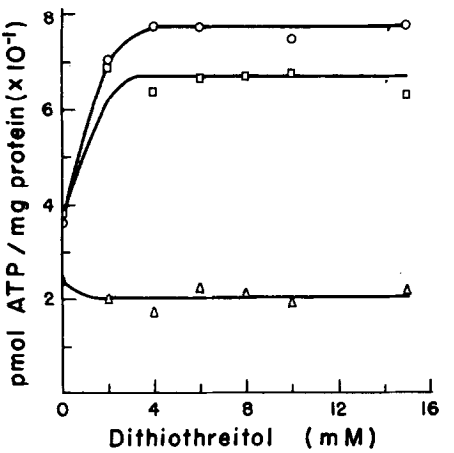

Fig. 2. Dependence of ATP incorporation on DTT concentration. Incubations were carried out as described in Fig. 1 at divalent cation concentrations of 8mM Mn^{++} (-o-), 2mM Mg^{++} (-Δ-), or 15mM Mg^{++} (-□-), with increasing amounts of DTT.

The ability of the enzyme preparation to utilize each of the four nucleoside triphosphates as substrate is shown in Table 1. It is clear that under each of the conditions described, ATP is utilized to a much greater extent than CTP, GTP or UTP. The low activity seen with the other three nucleotides suggests that virtually none of the product obtained using ATP as substrate is a result of RNA polymerase activity.

The results of Table 1 prompted an investigation of the effects of CTP, GTP and UTP on the ATP incorporating activities. In the presence of 2 mM Mg^{++}, the reaction was unaffected by either CTP, GTP, or UTP at a concentration of 1 mM. Each of these nucleotides, however, when present at 2 mM, inhibited the incorporation of ATP 12-30%. Furthermore, when CTP, GTP and UTP were all present at 1 mM each, inhibition was increased to 50%, suggesting that this inhibition may be due simply to the total nucleotide concentration present. On the other hand, when the reactions were carried out in the presence of 8 mM Mn^{++} or 15 mM Mg^{++}, CTP, GTP and UTP, whether present individually at either 1 or 2 mM or together at 1 mM each, enhanced the activities up to 100% in some cases. The presence of 5 mM DTT did not alter the responses to the other nucleotides under any of the cation con-

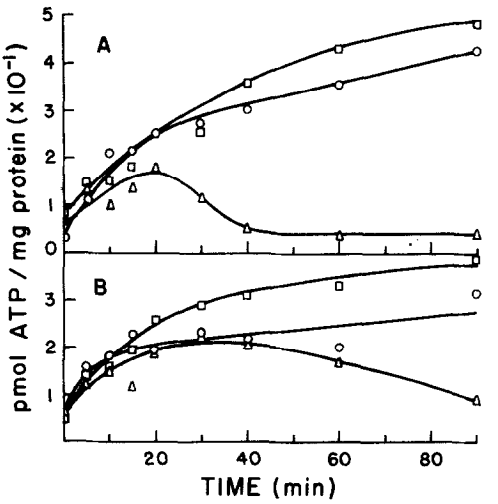

Fig. 3. Kinetics of ATP incorporation in the presence (A) or absence (B) of 5mM DTT. In a final volume of 1.0 ml, 0.20 ml of enzyme preparation was incubated as described in Fig. 1 using 8mM Mn^{++} (-o-), 15mM Mg^{++} (-□-) or 2mM Mg^{++} (-Δ-) with the following exceptions: 0.01mM ATP and 5 μCi [2,8-^{3}H] ATP (32.7 Ci/mmole) were used and CTP, GTP and UTP were omitted from the 2mM Mg^{++} incubations. At various times, 0.10 ml aliquots were assessed for acid-insoluble tritium.

ditions studied. Since the presence of CTP, GTP and UTP greatly inhibited the activity studied at 2 mM Mg^{++}, they were omitted from subsequent studies at this cation concentration.

Fig. 3 shows the kinetics of the ATP incorporating activities carried out in the presence and absence of 5 mM DTT. The kinetics obtained using 2 mM Mg^{++} are clearly different from those obtained at the other cation concentrations. In this case after 30 minutes degradation of the product exceeds its synthesis. The presence of 5 mM DTT enhances the rate of this net decrease of product. These results are consistent with the conclusion that the enzyme preparation contains more than one activity.

Additional studies revealed that under each of the conditions studied the ATP incorporating activities were linearly dependent on the amount of enzyme preparation present between 3 and 9 mg/ml protein. The enzymatic nature of these activities is further demonstrated by the inability of the enzyme preparation to catalyze product formation after it has been pre-

Table 1. Relative Incorporation of the Four Nucleoside Triphosphates into Acid Insoluble Material

Reaction Conditions	pmoles Nucleotide Incorporated/mg Protein/30 Min. ATP	CTP	GTP	UTP
8 mM Mn^{++}	52.2	4.57	1.39	1.32
2 mM Mg^{++}	21.4	7.10	2.82	2.04
15 mM Mg^{++}	47.8	3.57	1.49	1.61

Incubations were carried out as described in Fig. 1 except that the labeled nucleotide was present at 0.05 mM. The specific activities of the original [2,8-^{3}H]ATP, [5'-^{3}H]CTP, [8-^{3}H]GTP and [5-^{3}H]UTP in Ci/mmole were 32.7, 28.4, 5.7 and 25.5, respectively. The 8 mM Mn^{++} and 15 mM Mg^{++} incubations contained 5 mM DTT, whereas the 2 mM Mg^{++} incubation contained no DTT.

heated for 30 minutes at 50°. Furthermore, three facts support the conclusion that this ATP incorporation is not the result of non-specific association between ATP and some macromolecule. First, there is no significant loss of tritium from the products synthesized for 30 minutes using 8 mM Mn^{++} or 15 mM Mg^{++} as described in Fig. 3A, or using 2 mM Mg^{++} as described in Fig. 3B, when they are incubated for 2 hours at 37° in the presence of a 150-fold excess of unlabeled ATP. Secondly, these products remain intact upon chromatography on Sephadex G-25. Finally, these products are stable to incubation at pH 3.0 for 3 hours at 25° as well as cold 10% trichloroacetic acid.

In order to characterize the nature of the products of the ATP incorporating activities, they were subjected to the action of three degradative enzymes. Synthesis of tritium labeled product was carried out under the three cation conditions described above and terminated after 30 minutes by the addition of a 150-fold excess of unlabeled ATP. The three product preparations were then incubated for 1 hour at 37° after the addition of one of the following: 100 µg/ml pronase (Calbiochem, grade B, autodigested at 2 mg/ml for 1 hour at 37°), 50 µg/ml RNase A (Worthington), or 150 µg/ml snake venom phosphodiesterase (Sigma,

Crotalus adamanteus, type VI). The products synthesized using 8 mM Mn^{++}, 15 mM Mg^{++} and 2 mM Mg^{++}, as described above, were 74%, 73%, and 96% sensitive to pronase, respectively. Furthermore, the products obtained using 8 mM Mn^{++} and 2 mM Mg^{++} were completely insensitive to RNase A and phosphodiesterase, while the product synthesized using 15 mM Mg^{++} was 13.6% and 8.0% sensitive to RNase A and phosphodiesterase, respectively. These results strongly suggest that the activities observed result not in the incorporation of ATP into nucleic acid but rather in the enzymatic attachment of a portion of the ATP molecule to a pronase-sensitive receptor.

Further information on the identity of the product was obtained by studying the chemical nature of the tritiated compound released from each product after removal of [^{3}H]ATP precursor by Sephadex G-25 and treatment for 3 hr at 50°, pH 7.0, which results in the release of 70% of the tritiated components. The released tritiated compounds were identified by cellulose thin layer chromatography utilizing an ammonium acetate, pH 7.5 : 95% ethanol (30:70) solvent system. The results identify this component as adenosine for the products synthesized using 8 mM Mn^{++} or 2 mM Mg^{++}, and as a mixture of adenosine (20%) and AMP (80%) for the product synthesized using 15 mM Mg^{++}.

The heterogeneity of the tritiated components released from the product made using 15 mM Mg^{++} may be a reflection of more than one activity. The fact that no [^{3}H]ATP was recovered from the treated products further suggests that these reactions involve enzymatic activities catalyzing the covalent linkage of a portion of ATP to a macromolecular receptor. Also, the fact that the only component released from the products synthesized using 2 mM Mg^{++} or 8 mM Mn^{++} was adenosine is a further indication that the activities observed at least under these two conditions do not represent the formation of nucleic acid. We wish to stress that the results of this experiment must be considered tenta-

tive since it is conceivable that there may be enzymes in the preparation which alter the structure of the ATP component either before or after its release from the product.

DISCUSSION: While several cases of modification of proteins by reaction with ATP have been described, no such activity has been reported from mitochondria. The data presented here describe mitochondrial activities studied at 2 mM Mg^{++}, 15 mM Mg^{++} and 8 mM Mn^{++} which apparently utilize ATP as substrate to form acid-insoluble products. The insensitivity of the synthesized products to both RNase A and snake venom phosphodiesterase and their sensitivity to pronase strongly suggest that the macromolecular receptor of this ATP substrate is a protein. The fact that the activities studied are not observed using intact mitochondria suggests that they are not the result of contaminating extramitochondrial enzymes. Furthermore, since the products of these activities are stable to acid conditions and do not undergo exchange of tritium when incubated in the presence of excess unlabeled ATP, it is clear that they do not reflect the reaction between ATP and tRNA ligases involved in amino acid activation (6).

We have also observed the formation of a non-RNA, pronase sensitive, acid insoluble product by an enzyme preparation from sonicated mitochondria which uses UTP and CTP as nucleotide precursors (7). The existence of such activities in these organelles may help explain why we were not able to obtain complete sensitivity to Actinomycin D or Rifamycin during our studies on mitochondrial RNA polymerase using UTP as substrate (1). In addition, such activities must be taken into account when studying possible poly (A) synthetase activities, which are often characterized by methods which do not rule out the type of product formation reported here.

Studies now in progress, involving not only the isolation and characterization of the ATP incorporating activities, but also the synthesized products will hopefully provide answers to many of the questions concerning the nature of these reactions.

REFERENCES

1. Reid, B. D. and Parsons, P., Proc. Natl. Acad. Sci., USA, 68, 2830 (1971).

2. Gallerani, R., Saccone, C., Cantators, P., and Gadaleta, M.N., FEBS Letters, 27, 37 (1972).

3. Jacob, S. T. and Schindler, D. G., Biochem. Biophys. Res. Commun., 48, 126 (1972).

4. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

5. Niles, E. G. and Westhead, E. W., Biochemistry, 12, 1723 (1973).

6. Allende, C. C., Chaimovich, H., Catica, M. and Allende, J. E., J. Biol. Chem., 245, 93 (1970).

7. Dubin, J. and Parsons, P., Unpublished results.