ATP SYNTHETASE: A MIXTURE OF FACTOR A AND AVIDIN SENSITIVE ATP-P_i EXCHANGE ACTIVITY[1]

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SUMMARY: The ATP-P_i exchange activity of highly purified preparations of 'ATP synthetase' was inhibited by F_1-antiserum, Pullman inhibitor, azide and also by avidin (See You and Hatefi, 1973). The inhibition produced by the first three was relieved in the presence of ADP, and the avidin sensitivity was lost on pretreatment on the avidin with biotin. It is concluded that the ATP-P_i exchange resulted from the combined action of Factor A and a contaminating avidin-sensitive enzyme. The ADP necessary for the exchange reaction catalyzed by the latter was generated by the ATPase activity of Factor A.

INTRODUCTION: Two observations led us to suspect that the ATP-P_i exchange reaction observed in 'ATP synthetase' preparations (1,2) could be due partly to a contaminating protein. You and Hatefi (3) found that the ATP-P_i exchange activity observed in the partially purified 80 mM and 150 mM phosphate DEAE cellulose fractions was sensitive to avidin and demonstrated propionyl-CoA carboxylase activity in the preparation[3]. Also, recently we were able to separate the ATP-P_i exchange activity from the recoupling activity (Factor A) by filtration through Sepharose 4B. We had reported earlier that the ATP-P_i exchange activity of the 150 mM fraction of 'ATP synthetase' was uncoupler sensitive, but had recognized the presence of a trace of membrane contamination in the preparation which could be responsible for the uncoupler sensitivity (2). The present work and the observations of You and Hatefi (3) favor the

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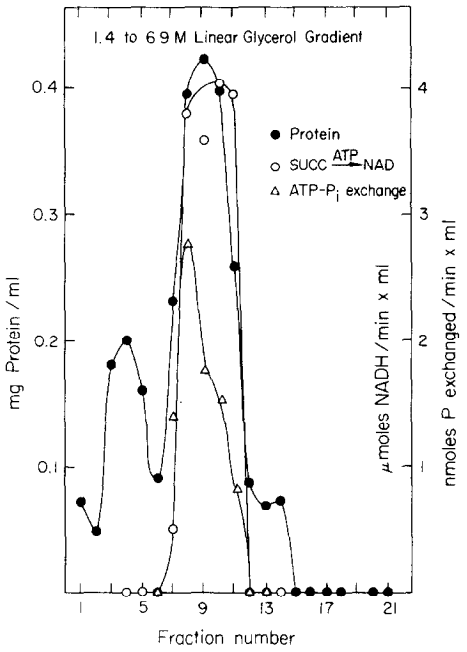

Figure 1: Second Density Gradient Fractionation. The active fraction from a first gradient is concentrated and 800 μg are placed on 5 ml gradients of 1.4 M to 6.9 M glycerol and centrifuged 15 hours at 37,000 RPM at 2° in a Beckman 500 50L rotor. The ATP-P_i exchange activity and activity in the ATP-dependent NAD reduction by succinate are measured as follows. 5 μl of the density gradient fraction was preincubated for 2 min at 38° with 0.5 mg urea particle. The protein was then diluted to 2.9 ml in a medium containing 6 μmoles ATP, 20 μmoles succinate, 10 μmoles $MgCl_2$, 150 μmoles Tris-SO_4 pH 7.8, 5 μmoles DTT, 3 μmoles NAD and 2 mg bovine serum albumin. This was incubated another 2 min at 38° and the reaction was started by adding 0.1 ml of 30 mM KCN.

latter explanation.

MATERIALS AND METHODS: A revised procedure for the preparation of the ATP-P_i exchange activity was used. Ten g of beef heart mitochondria were subjected to ultrasonication (full power, Bronson Sonifier) for 2 minutes at 0° in 500 ml of media containing 10 mM Tris-SO_4 pH 7.5, 2 mM EDTA and 10% glycerol. The pH of the crude extract was adjusted to 6.4 with 1 M KH_2PO_4 and centrifuged for 30 minutes at 35,000 RPM in a Beckman #35 rotor. The supernatant fluid was adjusted to pH 7.5 with 0.5 N NaOH, then applied directly to a 5 cm x 6 cm DEAE column (Schleicher and Schuell) and eluted sequentially with 10 mM P_i, 50 mM P_i and 125 mM P_i pH 7.5 buffer containing 10% glycerol and 0.1 mM DTT.

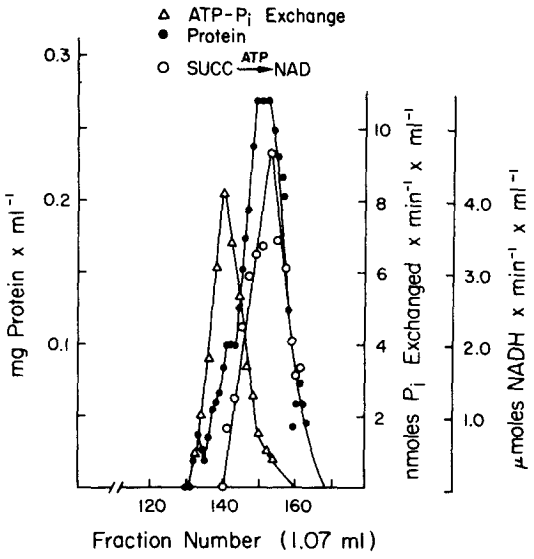

Figure 2: Sepharose 4B Chromatography. Assays are described in Table I and Figure 1. 10 mg of protein was applied to a 2 x 100 cm Sepharose 4B column. The ATPase activity in fractions 130 to 140 was sufficiently high to generate the ADP necessary for the exchange. For example, it was 3.5 μmoles/min x ml in Fraction 135.

The activity was recovered in the 125 mM fraction in 4 to 5 times the yeild obtained by the earlier procedure (5). This material was subjected to $(NH_4)_2SO_4$ fractionation; the precipitate appearing between 40 and 55% $(NH_4)_2SO_4$ saturation was dissolved at a concentration of 10 mg protein/ml in 10% glycerol, 0.5 mM EDTA, 0.1 mM DTT, 25 mM Tris-SO_4 pH 7.5 and subjected to two successive high speed centrifugations (30 minutes at 50,000 RPM in Beckman #65 rotor). Two milligrams of this material (S_3) was placed on a 5 ml linear glycerol gradient (1.4 M to 6.9 M) containing 25 mM Tris-SO_4 pH 7.5, 0.1 mM DTT and 0.5 mM EDTA. Alternatively, 10 mg of S_3 was placed on a 2 cm x 100 cm Sepharose 4B column and eluted with a buffer containing 10% glycerol, 25 mM Tris-SO_4 pH 7.5, 10 mM P_i, 0.1 mM DTT and 0.5 mM EDTA.

The ATP-P_i exchange activity was measured in a mixture containing 25-50 μg protein, 25 mM Tris-SO_4 pH 8.0, 10 mM P_i (containing 1.9×10^4 CPM $^{32}P_i$/μmole P_i), 1 mM DTT, 10 mM ATP and 15 mM $MgSO_4$ or where indicated 5 mM ADP, 5 mM ATP and 15 mM $MgSO_4$ all in 0.5 ml. The reactions were ususally started with

the addition of nucleotide and incubated 30 minutes at 38°. The reactions were terminated by the addition of 0.25 ml of 20% trichloroacetic acid. The orthophosphate was extracted and the organic phosphate determined as previously described (1).

The ATP dependent NAD reduction by succinate was assayed as previously described (4) using the urea depleted particle and the ammonia-EDTA depleted particle (AE particle).

RESULTS: The active fraction from the first gradient is concentrated and reapplied to a second density gradient. Essentially one major peak is observed with the activity for recoupling reversed electron flow coincident with the protein peak, but the ATP-P_i exchange activity is found on the heavy side of the protein peak (Fig. 1). If instead of fractionating as above by density gradient, the S_3 sample is filtered through Sepharose 4B, the peak for the ATP-P_i exchange activity is separated from the major protein peak with activity in recoupling of reversed electron flow as shown in Fig. 2. Since the ATP-P_i exchange activity was somehow dependent on the Factor A, as indicated by sensitivity to azide, ATPase inhibitor (6) and antiserum to F_1, we suspected that the Factor A could be generating the ADP necessary for the exchange by ATP hydrolysis. It should be noted that in our earlier work, ADP was used sometimes in the assay (ref. 1, Materials and Methods), but was routinely omitted since it did not affect the activity under the standard assay conditions. To test the above possibility, we assayed the ATP-P_i exchange activity with excess inhibitors and then determined if the inhibition would be reversed by ADP. The results (Table I) show that all the inhibitions are reversed by ADP. The inhibition by antiserum to F_1 (kindly provided by E. Racker) is only partially relieved by ADP, but this could be due to multiple antibodies in the antiserum. In confirmation of Hatefi's results, we find that avidin extensively inhibits the ATP-P_i exchange activity, and the inhibition may be reversed by pretreating the avidin with biotin (Table II).

TABLE I

Reversal of inhibition of ATP-P_i exchange by ADP

	nmoles/min x mg protein
Control	44.7
+ ADP	52.2
+ Anti-F_1 Serum (3.0 mg)	2.7
" " " + ADP	12.5
+ ATPase Inhibitor (13.3 µg)	9.4
" " + ADP	41.8
+ Azide (10^{-3} M)	3.9
" + ADP	42.7

The reaction mixtures contained 25 mM Tris-SO_4 pH 8.0, 10 mM phosphate pH 8.0, 1 mM DTT, 1.9 x 10^4 CPM $^{32}P_i$ per µmole P_i, 25 µg protein in a total volume of 0.45 ml. The reaction mixtures were preincubated 10 minutes on ice with the inhibitors before starting the exchange reaction with 0.05 ml of 0.1 M ATP plus 0.15 M $MgSO_4$. In the experiments with added ADP, the reactions were started with 0.05 ml of 0.05 M ADP plus 0.05 M ATP plus 0.15 M $MgSO_4$. The incubation was 30 minutes at 38°. Normal serum had no effect on the exchange activity.

TABLE II

Inhibition of ATP-P_i Exchange Reaction by Avidin

	S.A.
Control	46.4
+ 150 µg avidin	8.0
+ 150 µg biotin treated avidin	42.9

Assays were as described for Table I. The enzyme was pretreated with avidin for 10 minutes before assay. The avidin was pretreated with 5 µg of biotin.

From these results, it would appear that the ATP-P_i exchange is catalyzed by the contaminating avidin-sensitive enzyme, and the ADP necessary for the exchange is generated from ATP by Factor A. This would explain the sensitivity to inhibitors specific for the coupling reaction as well as to avidin.

The ATP-P_i exchange activity of the 150 mM fraction prepared by You and Hatefi (3) was insensitive to uncouplers. The preparations obtained by the

revised procedure described here lost uncoupler sensitivity after the glycerol gradient centrifugation step (unpublished data) and became phospholipase resistant (7). We now feel that the uncoupler sensitivity was probably associated with the traces of contaminating unusually small (300 Å) vesicles in our 150 mM fraction. The contamination would also explain the sensitivity to anti-F_1 in Table I, ref. 1.

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