ISOLATION OF A PHOSPHORYLATED INTERMEDIATE INVOLVED IN THE ADP-ATP EXCHANGE REACTION

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A nucleoside diphosphate kinase highly specific for the ADP-ATP exchange reaction has been isolated from beef heart mitochondria. This report will deal with the isolation of a phosphorylated intermediate of the exchange enzyme capable of reacting with ADP to form ATP. Kinetic experiments are also presented ; their results strongly suggest that the phosphorylated enzyme is a reactive intermediate in the ADP-ATP exchange reaction.

The procedure for purification of the exchange enzyme is summarized in Table I. All operations are performed at 0°-3°C. Beef heart mitochondria are extracted overnight with 0.025 M Tris-HCl buffer (pH 10) (Step I). The supernatant fluid is brought to pH 9 and applied to a column of DEAE cellulose (0.85 mEq/g., medium) equilibrated with 0.025 M Tris-HCl (pH 9). The enzyme is rapidly eluted with the same buffer (Step II). The enzyme solution is dialyzed against distilled water, concentrated by freeze-drying and brought to pH 5. It is then transferred to a CM cellulose column (0.6 mEq/g., medium) equilibrated with 0.025 M phosphate buffer (pH 5). Some inactive material is removed by washing the CM cellulose with the same buffer. The active fraction is eluted using 0.01 M phosphate buffer (pH 7) (Step III), dialyzed and concentrated as in Step II. The solution from Step III is then poured on a small column of neutral aluminum oxide (Merck) previously washed with 0.025 M Tris-HCl buffer (pH 8.5 The enzyme is directly eluted by addition of the same buffer (Step IV). The last step (IV) conveniently eliminates any traces of adenylate kinase without excessive loss of exchange activity.

Table I - Purification of the ADP-ATP exchange enzyme

Step	Volume (ml)	Protein (mg)	Total ADP-ATP exchange activity (units)	Total adenylate kinase activity (units)	Specific ADP-ATP exchange activity (units/mg)
I	42	176	20.11	34.03	0.11
II	115	12	6.77	2.45	0.56
III	22	1.1	3.06	0.02	2.78
IV	4.5	0.12	0.80	<0.00001	6.66

The exchange activity was measured with the following medium : 0.25 µmole of [^{32}P]ADP (70,000 cpm), 6 µmoles of ATP, 12 µmoles of $MgCl_2$, 100 µmoles of triethanolamine-HCl buffer (pH 7.4), enzyme in a final volume of 1 ml. After 5 minutes of incubation at 28° the reaction was stopped by 0.1 ml of 30 % TCA. The adenine nucleotides separation was achieved by paper chromatography (KREBS and HEMS 1953). The areas corresponding to ADP and ATP were located under UV light and their respective sections cut out from the paper strips. The radioactivity was determined with a Geiger-Muller counter. The amount of incorporation was calculated as described by BOYER et al. (1959). Adenylate kinase activity was estimated spectrophotometrically with hexokinase and glucose 6-phosphate dehydrogenase. Protein was estimated by a modified Folin Lowry reaction.
One unit of enzyme is defined as that amount which catalyzes the exchange of one µmole of [^{32}P]ADP per minute (exchange enzyme) or the conversion of one µmole of ADP into ATP (adenylate kinase).

The relative exchange activity (Conditions in Table I) between [^{32}P]ADP and the following nucleoside triphosphates : ATP, UTP, CTP, ITP and GTP (used at the final conc. of 6.10^{-3}M) is 100 %, 45 %, 27 %, 12 % and 3 % respectively.

As shown in Fig. 1, the labeling of the exchange enzyme by ^{32}P is achieved by incubation of the enzyme with [γ-^{32}P]ATP and $MgCl_2$. The radioactive protein is easily separated from ADP and the non reacted [γ-^{32}P]ATP by column chromatography on DEAE cellulose. The good correlation between the exchange activity and the radioactivity of the main protein fraction eluted at pH 8.5 suggests a phosphorylation of the exchange enzyme during the incubation with [γ-^{32}P]ATP. The small adjacent peak of ^{32}P and exchange activity is probably due to a slight retention of protein on the column.

In order to prove the phosphorylation of the enzyme by ATP and to exclude the hypothesis for an adsorption of the nucleotide on the protein, a second set of experiments was carried out with an incubation mixture containing [^{14}C,γ-^{32}P]ATP instead of [γ-^{32}P]ATP. As shown in Fig. 2 the initial radioactivity ratio $^{32}P/^{14}C$ of the [^{14}C,γ-^{32}P]ATP is 0.85. After incubation and separation as

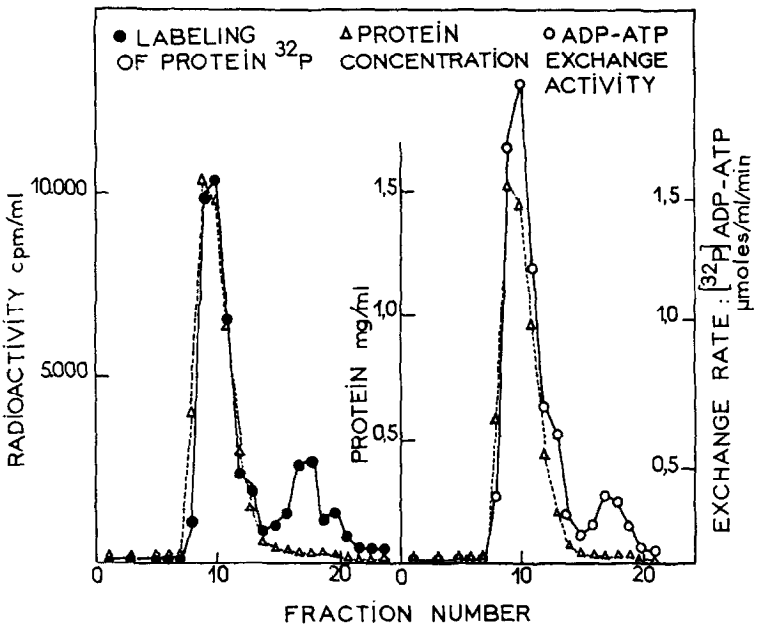

Fig. 1 - Correlation between ^{32}P labeling of the exchange enzyme and ADP-ATP exchange activity.

The labeling of the exchange enzyme (3.5 mg) was carried out in a medium containing 7.9 µmoles of [γ-^{32}P]ATP (379 cpm/mµmole), prepared according to GLYNN and CHAPPELL (1964), 9 µmoles of $MgCl_2$, 50 µmoles of Tris-HCl (pH 7.3) final volume 2.5 ml). After 5 min. of incubation at 28°, the reaction mixture was chilled, adjusted to pH 8.5 with diluted NaOH and transferred to a DEAE cellulose column equilibrated with 0.025 M Tris-HCl buffer (pH 8.5). The labeled protein was eluted with the same buffer. The exchange activity was measured as described in Table I.

Table II - Transfer of $^{32}P_i$ from the phosphorylated exchange enzyme to ADP

^{32}P incorporated in ATP (cpm)	γ-$^{32}P/\beta$-^{32}P in ATP	^{32}P released as P_i (cpm)
19,075	153	2,030

The ^{32}P exchange enzyme (27,200 cpm) was incubated with 0.6 µmole ADP, 3 µmoles $MgCl_2$, 20 µmoles Tris-HCl buffer pH 7.3 in a final volume of 1 ml, for 5 min. at 28°. The reaction was stopped with 0.05 ml of 30 % TCA. After centrifugation the ADP and ATP present in the supernatant fluid were separated by bidimensional paper chromatography : formic acid and isopropyl ether for the separation of $^{32}P_i$, isobutyric acid/ammonia for the descending step (KREBS and HEMS, 1953). The distribution of the radioactivity between the γ-P and the β-P of ATP was assayed according to LATURAZE and VIGNAIS (1964).

described in Fig. 1, the exchange enzyme is selectively labeled by ^{32}P as the radioactivity ratio $^{32}P/^{14}C$ of the emerging protein peak is now 12.1. These data clearly show that the exchange enzyme is phosphorylated by ATP. When the phosphorylated exchange enzyme is incubated with ADP, $[\gamma\text{-}^{32}P]ATP$ is formed. The radioactivity is located in the terminal phosphate of ATP (Table II).

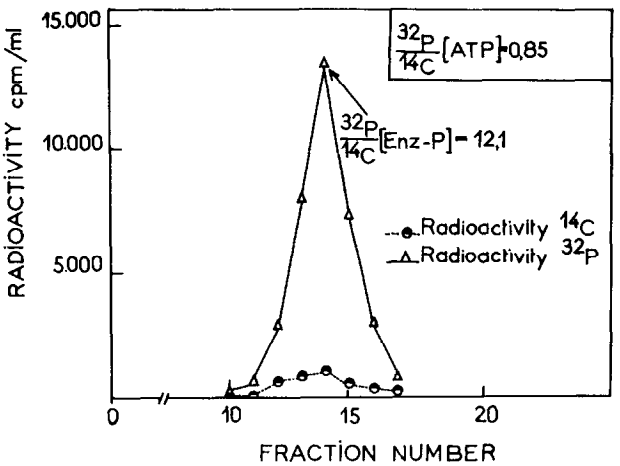

Fig. 2 - Evidence for a phosphorylated form of the ADP-ATP exchange enzyme. The exchange enzyme (4.9 mg) was incubated for 5 minutes at 28° with 4.5 µmoles $MgCl_2$, 4.1 µmoles $[^{14}C,\gamma\text{-}^{32}P]ATP$ (36,000 cpm/µmole $[\gamma\text{-}^{32}P]ATP$ and 42,000 cpm/µmole $[^{14}C]ATP$), 30 µmoles Tris-HCl pH 7.3 in a volume of 1.5 ml. The separation of the labeled enzyme was carried out as described under Fig. 1. The enzyme fractions were tested for ^{14}C and ^{32}P activities with a liquid scintillation spectrometer (Packard Co. Tri-Carb).

Preliminary studies point out a marked lability of the phosphorylated form of the exchange enzyme. For instance, a 30 min. incubation at 60°, pH 7, of the phosphorylated enzyme leads to a complete release of radioactivity as ^{32}P-orthophosphate (Fig. 3). When incubated at 0°, the ^{32}P-enzyme showed greater stability at pH 3.3 than at pH 10. However, the ^{32}P-enzyme is extensively dephosphorylated when exposed at 60°, pH 3.3, for 30 min.. The lability of the phosphate link of the ^{32}P-exchange enzyme does not fit with that of phosphohistidine or phosphoserine, but it resembles that of the phosphorylated form of the Na^+, K^+ activated ATPase (NAGANO et al. 1965).

The kinetic data we present now are consistent with the formation of a phosphorylated enzymic intermediate during the ADP-ATP exchange reaction.

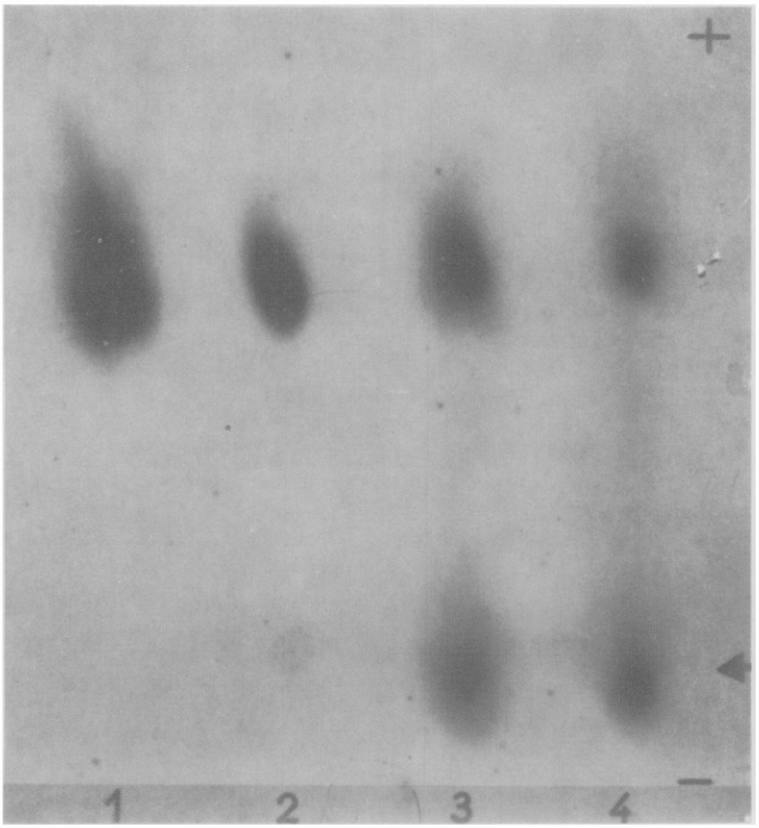

Fig. 3 - Lability of the ^{32}P-intermediate.
The ^{32}P-labeled enzyme fractions eluted from the DEAE cellulose column were dialyzed then concentrated against polyvinylpyrrolidone and dialyzed again. The samples were incubated for 30 minutes at pH 3.3, pH 7 and pH 10. High voltage electrophoresis (50 V/cm) was then carried out using 0.05 M citrate buffer (pH3.8) and Whatman n°3MM paper. The paper strips were dried and autoradiographied. On this document the figures refer respectively to : 1) $^{32}P_i$ (reference), 2) fraction incubated at pH 7 at 60°, 3) fraction incubated at pH 10 at 0°, 4) fraction incubated at pH 3.3 at 0°.

The rate of exchange is relevant to several parameters (Mg^{++}, ATP and ADP concentrations) which will be briefly discussed here. Orthophosphate has no effect on the ATP-ADP exchange reaction. Furthermore the ATP-ADP exchange enzyme does not catalyze any ATP-P_i exchange either in the presence or in the absence of ADP. These results show a sharp difference with the enzyme isolated by CHIGA and PLAUT (1959).

The exchange is inhibited by high concentrations of ADP. In the non-inhibitory range of concentration of ADP an optimal Mg^{++}/ATP ratio of 2 is needed for a maximal rate of exchange. The exchange has been measured at

different concentrations of ADP and at fixed concentrations of ATP, the Mg^{++}/ATP ratio being kept at the fixed value of 2. As shown in Fig. 4, the plot of $1/v$ versus 1/ADP at various fixed concentrations of ATP gives a family of parallels which is characteristic of a "ping-pong" mechanism

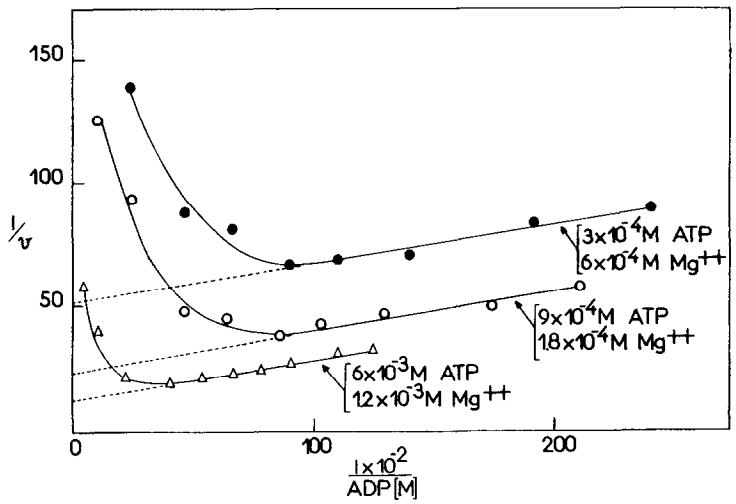

Fig. 4 - Plot of the reciprocal velocity against reciprocal ADP concentration. The medium used was the following : [^{32}P]ADP (70,000 cpm), ATP and $MgCl_2$ as indicated ; 100 µmoles triethanolamine-HCl buffer (pH 7.4), final volume 1 ml. The reaction was initiated with the enzyme and stopped after 5 min. by 0.1 ml of 30 % TCA. Rates are expressed in µmoles of ADP exchanged per minute.

according to the nomenclature given by CLELAND (1963). MOURAD and PARKS (1965) have recently shown a similar mechanism for a non specific NDP kinase isolated from human erythrocytes. This mechanism, when applied to the ADP-ATP exchange reaction, assumes the formation of a phosphorylated enzyme intermediate in a first stage of reaction of the enzyme with ATP, and assumes also the dephosphorylation of this intermediate in a second stage of reaction with ADP. These data indicate that the phosphorylated form of the exchange enzyme described in the first part of this report is a reactive intermediate in the ADP-ATP exchange reaction.

WADKINS and LEHNINGER (1963) have purified an ADP-ATP exchange enzyme from beef liver mitochondria ; their studies were essentially concerned with the involvement of this enzyme in oxidative phosphorylation. A correlation between results reported in this paper and the role of the ADP-ATP

exchange reactions in oxidative phosphorylation, either branched on the coupling reactions (ZALKIN et al., 1965) or directly included in them (WADKINS and LEHNINGER, 1960, 1963) is under investigation.

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