STIMULATION OF ADENINE NUCLEOTIDE TRANSLOCATION IN RECONSTITUTED VESICLES BY PHOSPHATE AND THE PHOSPHATE TRANSPORTER*

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SUMMARY: Highly purified adenine nucleotide transporter from bovine heart mitochondria was reconstituted with phospholipids to form vesicles which catalyzed atractyloside-sensitive adenine nucleotide translocation. When internal ATP was exchanged with external ADP, this reaction was enhanced by agents capable of collapsing a membrane potential, but not by inorganic phosphate. When the purified nucleotide transporter was reconstituted together with a second protein fraction, nucleotide transport was stimulated by inorganic phosphate. The stimulated rate was eliminated by mersalyl or other SH reagents. The second protein fraction could be replaced by preparations of purified phosphate transporter.

It was shown recently (1) that the exchange of internal ATP for external ADP in reconstituted vesicles, was stimulated by various agents that collapse the membrane potential. This stimulation was observed only when these agents caused either electrogenic movements of a negative charge inwards or of a positive charge outwards. Agents with reverse actions stimulated exchange of internal ADP for external ATP. One of the agents that stimulated the exchange of internal ATP for external ADP was P_i. This stimulation was eliminated by N-ethylmaleimide or mersalyl. In the present communication we report that vesicles reconstituted with highly purified adenine nucleotide transporter were no longer stimulated by P_i. The P_i effect can be restored, however, by reconstitution of the nucleotide transporter together with a second crude protein fraction, which catalyzes P_i transport in reconstituted liposomes. This crude protein fraction can be replaced by purified P_i transporter.

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Abbreviations: A-particles, depleted submitochondrial particles derived from bovine heart mitochondria by sonic oscillation in presence of ammonia; ASUA-particles, A-particles passed through sephadex G-25 column, followed by treatment with 2 M urea and then sonication in alkaline pH; EDTA, ethylenediamine-tetra-acetate; DTT, dithiothreitol.

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MATERIALS AND METHODS

ASUA particles were prepared by submitting ASU particles (2) to sonication at an alkaline pH (3). Highly purified adenine nucleotide transporter (36-44p fraction) was isolated from ASUA particles as described (1,4) except that the protein concentration during extraction was 20 mg/ml. The cholate insoluble residue was used as a crude source of P_i transporter. In some experiments it was used without further purification by suspension in a solution containing 50 mM sucrose, 0.5 mM EDTA, 1 mM $MgSO_4$, 0.5 mM dithiothreitol, and 10 mM Tris sulfate (pH 7.5). In other experiments it was partially purified by extraction of the cholate insoluble residue with 2% cholate, 0.5% deoxycholate in the presence of 10% saturated ammonium sulfate followed by centrifugation and fractionation of the supernatant with saturated ammonium sulfate. The pellet (or floating layer) obtained between 30 to 40% ammonium sulfate saturation contained P_i transporter activity, but, in contrast to the cholate insoluble residue, was rather unstable. Purified and stable P_i transporter was prepared as described in the accompanying paper (5).

Reconstitution was achieved as follows: Thirty μmoles of asolectin (6) in chloroform were dried under vacuum, then sonicated in a bath-type sonicator at 0°-10° in the presence of 50 mM ATP, 100 mM glycerol, 2 mM dithiothreitol, 10 mM Tricine-KOH (pH 7.0) and protein (amounts indicated in the legends to the figures and tables) in a final volume of 2 ml. The sonication was continued until the suspension was almost clear (10 to 15 min). With more extensive sonication and clarification, the activity was markedly reduced. The mixture was warmed to room temperature and was then passed over a 1 cm x 30 cm column of Dowex-1-acetate (7) which had been equilibrated with 136 mM glycerol containing 0.1 mM sodium azide. The vesicles were eluted and assayed with radioactive ADP as described previously (1). The reaction was stopped at 3 sec (unless otherwise indicated) with 25 μM atractyloside. Other additions were made just prior to ADP, except for sodium mersalyl (75 μM final concentration) which was added 5 min prior to ADP addition. The radioactive samples were counted with 12 ml Aqueous Counting Scintillant (Amersham/Searle) in a Beckman LS-230 Liquid Scintillation Counter.

[8-^{14}C] ADP (50 mCi/millimole) was obtained from New England Nuclear, Boston, Mass.; cholic and deoxycholic acids from Schwarz/Mann, Orangeburg, N.Y., were recrystallized from 70% ethanol (6); ADP, ATP, atractyloside from Sigma Chemical Co., St. Louis, Mo.; sodium mersalyl from K & K Laboratories, Plainview, N.Y.; crude soybean phospholipids (asolectin) from Associated Concentrates, Woodside, L.I., N.Y.; Dowex 1-X-8 chloride, 20 to 50 mesh, from either Bio-Rad Laboratories, Richmond, Calif., or J.T. Baker Chemical Co., Phillipsburg, N.J.

Protein was determined by the method of Lowry et al. (8).

RESULTS AND DISCUSSION

As shown in Table I, when vesicles were reconstituted with highly purified adenine nucleotide transporter, the exchange rate of internal ATP for external ADP was not stimulated by 20 mM KP_i. However, when the cholate insoluble residue obtained from ASUA particles during purification of the transporter was added during reconstitution, a nearly two-fold stimulation by P_i was observed. The rate without P_i was not significantly affected by the cholate

Table I

Stimulation ATP/ADP Exchange by P_i

Vesicles were reconstituted with internal ATP as described under "Materials and Methods" with 84 µg of the 36-44p fraction of nucleotide transporter and with 270 µg of the cholate-insoluble residue. The exchange was measured (1) in the presence and absence of 20 mM KP_i.

Preparation Used in Reconstitution	Adenine Nucleotide Exchange	
	$-KP_i$	$+KP_i$
	nmoles/min/mg of adenine nucleotide transporter	
Nucleotide transporter (34-44p fraction)	248	239
Cholate insoluble fraction	0	0
Both	277	486

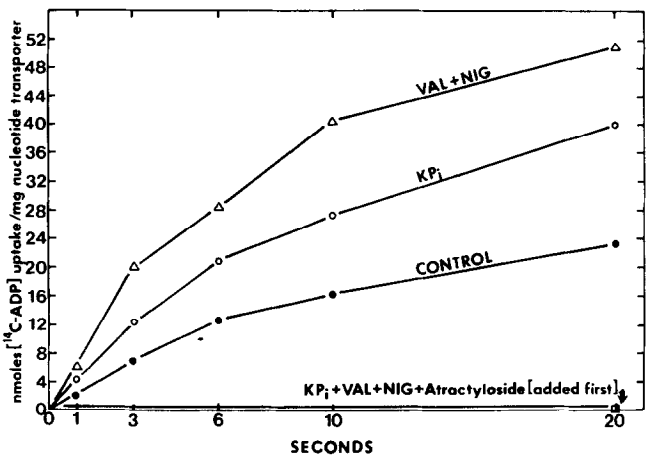

Fig. 1. Effect of phosphate, valinomycin and nigericin on the exchange of internal ATP with external ADP. Reconstituted vesicles containing each 54 µg of the 36-44p fraction of adenine nucleotide transporter plus 120 µg of the factor purified from the cholate insoluble residue were assayed as described under "Materials and Methods." The concentrations used were 25 µM atractyloside, 20 mM KP_i, 13 µg/ml valinomycin and 3 µg/ml nigericin.

insoluble residue, in line with the absence of detectable nucleotide transport activity in this fraction. This finding emphasizes the effectiveness of the cholate extraction procedure with regard to the adenine nucleotide transporter. The stimulation of nucleotide transport was used as an assay during the fractionation of the cholate insoluble residue (see Materials and Methods).

Table II

Effect of Sodium Mersalyl on Internal ATP for External ADP Exchange

Vesicles were reconstituted as described in "Materials and Methods" with 90 µg of the 36-44p fraction of the nucleotide transporter (1) together with 600 µg of cholate insoluble residue. Mersalyl, 75 µM, was added 5 min prior to the initiation of the reaction with [^{14}C] ADP.

Additions	Adenine Nucleotide Exchange	
	-mersalyl	+mersalyl
	nmoles/min/mg of adenine nucleotide transporter	
None	285	303
KP_i (20 mM)	544	345
KP_i + valinomycin (13 µg/ml) + nigericin (3 µg/ml)	758	840

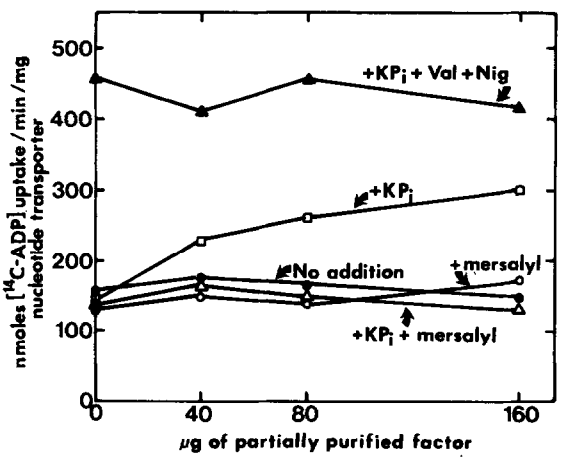

Fig. 2. Effect of increasing amounts of factor on the exchange of internal ATP with external ADP. Vesicles containing each 90 µg of the 36-44p fraction of the adenine nucleotide transporter and the amounts of factor indicated in the Figure. Mersalyl (75 µM) was added 5 min prior to the initiation of the reactions by [^{14}C] ADP.

The stimulation by P_i mediated by the second protein fraction was evident during the entire time course of the electrogenic exchange (Fig. 1). Also shown in this figure is the stimulation by valinomycin plus nigericin. This stimulated rate was equivalent to that of the ADP/ADP exchange (not shown). The stimulation by P_i was abolished by mersalyl, whereas the stimulation in

Table III

Effect of Phosphate Transporter on ATP/ADP Exchange by Nucleotide Transporter

For reconstitution of nucleotide transporter 180 µg of the 36-44p fraction of nucleotide transporter (1) and 130 µg of 0-50p fraction of the P_i transporter (5) were used. The mixture was sonicated for 8 min in all three experiments. Exchange activity was assayed as described under "Materials and Methods" in the presence of 20 mM KP_i.

Preparations used in Reconstitution	ATP/ADP Exchange*			
	-val -nig		+val +nig	
	nmoles/min/mg of adenine nucleotide transporter			
Nucleotide Transporter	102		210	
P_i Transporter	0		<20	
Both	233		237	

*The data represent the average of three experiments

the presence of valinomycin and nigericin was not (Table II). It is known that mersalyl is a potent inhibitor of P_i transport in mitochondria (9) and in submitochondrial particles (10). The elimination of the stimulation by P_i by mersalyl occurred at the same low concentration (75 µM) which inhibits P_i transport in mitochondria. Adenine nucleotide exchange was not affected at these concentrations either in the absence or presence of valinomycin plus nigericin under the conditions of the assay. However, prolonged exposure to 75 µM mersalyl gave rise to inhibition of nucleotide transport also (data not shown).

The stimulation by P_i increased with the amount of the factor purified from the cholate insoluble residue (Fig. 2), but there was not a strict linear relationship, possibly because of residual amounts of detergents carried over with factor. Only the P_i stimulated rate was accelerated with increasing amounts of the factor and the stimulation was always abolished by 75 µM mersalyl.

These findings clearly pointed to the P_i transporter of mitochondria as the responsible agent for the stimulation. Assays showed that the factor purified from the cholate insoluble residue catalyzed P_i transport after reconstitution

into liposomes. However, the data were erratic because of the great lability of the preparation. With the availability of a stable, purified P_i transporter (5), the question could be answered more decisively. It can be seen from Table III that the purified P_i transporter had no adenine nucleotide exchange activity when tested alone. In the presence of nucleotide transporter and P_i it more than doubled the rate of adenine nucleotide exchange. The stimulated rate was similar to that observed in the presence of valinomycin plus nigericin. As expected there was no further stimulation of nucleotide exchange when P_i transporter as well as the ionophores were present.

These experiments leave little doubt that the stimulatory factor in the cholate insoluble residue is in fact P_i transporter.

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