PURIFICATION AND RECONSTITUTION OF THE PHOSPHATE TRANSPORTER FROM BOVINE HEART MITOCHONDRIA*

Ranajit K. Banerjee, Howard G. Shertzer+, Baruch I. Kanner‡ and Efraim Racker

Section of Biochemistry, Molecular and Cell Biology
Cornell University, Ithaca, New York 14853

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SUMMARY: Phosphate transporter of bovine heart mitochondria was purified by solubilization of submitochondrial particles with octylglucoside and fractionation of the extract with ammonium sulfate. After reconstitution into liposomes the purified protein catalyzed phosphate transport which was sensitive to mersalyl and other SH reagents. Transport measured either as P_i/OH or P_i/P_i exchange was proportional to protein concentration and time. The P_i/OH but not the P_i/P_i exchange was stimulated several fold by valinomycin plus nigericin in the presence of K^+. The reconstituted system provides a suitable assay during purification of the mitochondrial phosphate transporter.

The transport of inorganic phosphate is catalyzed by two different carrier systems present in the inner membrane of mitochondria. One of them (P_i carrier) is influenced by the pH gradient and is believed to catalyze a P_i/OH exchange (1-3); the other (P_i-dicarboxylic acid carrier) mediates the exchange of P_i with certain dicarboxylic acids (4,5). Only the P_i/OH exchange carrier was shown to be sensitive to N-ethylmaleimide, whereas both of them are sensitive to mercurials like mersalyl (6). The P_i/OH carrier is regarded as the major contributor to phosphate transport during oxidative phosphorylation (7,8,9). The present paper deals with the solubilization of this phosphate carrier from bovine heart mitochondria and its functional reconstitution into the phospholipid vesicles.

*Supported by Grant CA-08964 from the National Cancer Institute, DHEW.
+Present address: Department of Biology, Texas A&M University, College Station, Texas 77843
‡Present address: Department of Biochemistry, Hadassah Medical School, Hebrew University, Jerusalem, Israel
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, ethylenediaminetetra-acetate; DTT, dithiothreitol; NEM, N-ethylmaleimide.

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MATERIALS AND METHODS

A-particles were prepared from bovine heart mitochondria (10). Octylglucoside was synthesized as described (11). N-ethylmaleimide, p-hydroxymercuribenzoic acid, valinomycin and dithiothreitol were products of Sigma. Nigericin was a gift from Dr. R. Hosley of Eli Lilly. N-cyclohexylmaleimide was obtained from Nutritional Biochemicals Corporation, sodium mersalyl from K & K Laboratories, crude soybean phospholipids (asolectin) from Associated Concentrates, Dowex-1X8 chloride, 50-100 mesh and Biobeads SM-2 from Bio-Rad Laboratories and Dowex-1X8 chloride, 20-50 mesh from J.T. Baker. [^{32}P] was purchased from ICN Pharmaceuticals, Inc. and hydrolyzed with 2 N HCl for 2 hours at 100°C. All other chemicals were of reagent grade purity.

Reconstitution and assay of phosphate transport - Thirty µmoles of partially purified soybean phospholipids (12) in chloroform were dried with a stream of N_2, dissolved in a small amount of ether and dried again. After addition of 2 ml of a solution containing 10 mM tricine KOH (pH 7), 5% glycerol, 2 mM DTT, 20 mM EDTA and about 30 to 200 µg protein, the mixture was sonicated under N_2 in a bath type sonicator at 0-10° for 7-8 min. An aliquot (0.3 ml) was assayed for P_i/OH exchange at room temperature with stirring in the presence or absence of 0.5 mM mersalyl or 0.12 mM $HgCl_2$. After addition of 10 µl of 1 M KP_i and 2 µl (2 µg) each of valinomycin and nigericin, 50 µl of 0.2 M [^{32}P] KP_i (7×10^6 cpm) were added. The reaction was stopped by the addition of 0.5 mM mersalyl or 0.12 mM $HgCl_2$. The vesicles were immediately passed through a column of 3 ml of Dowex-Cl 1X8, 50-100 mesh over 1 ml of Dowex-Cl, 1X8, 20-50 mesh equilibrated with 5% glycerol and 0.1 mM NaN_3. The vesicles were eluted with 3 ml of equilibrating solution at a slow flow rate and counted with 12 ml of ACS scintillation fluid in a Beckman LS-230 Liquid Scintillation Counter. For measurements of the P_i/P_i exchange the procedure was the same except that the vesicles were prepared with 33 mM KP_i (pH 7) and the reaction was started by the addition of 50 µl of [^{32}P] KP_i without ionophores.

RESULTS AND DISCUSSION

Purification of phosphate transporter - A variety of detergents were tested for the extraction of the P_i transporter from submitochondrial particles. Cholate and deoxycholate in the presence of salts extracted the transporter from the membrane, but the preparations were labile and the results variable. With either Triton X-100 or octylglucoside, reproducible and stable preparations were obtained. Octylglucoside was chosen for the preparation of extracts for further purification because it extracted almost all the transport activity from submitochondrial particles and because the active protein remained in solution following subsequent steps of purification.

All operations were carried out at 0-4°C. The extraction medium contained in a final volume of 40 ml, 90 mg A-particles, 5% glycerol, 10 mM EDTA, 2 mM DTT, and 50 mM KP_i buffer (pH 8.0). Octylglucoside was added slowly to the

Table I

Partial Purification of Phosphate Transporter from A-particles

The purification procedure and the assay of P_i/OH exchange in the presence of valinomycin and nigericin were as described under "Materials and Methods." The activity was corrected for the mersalyl-insensitive rates of about 20 nmoles/min/mg protein.

	Protein mg/ml	Total Protein mg	Specific Activity nmole/min/mg	Total Activity nmole/min
A-particles	45	90	17	1530
Supernatant after Biobead treatment	0.68	25.5	59	1504
Precipitate I (0-50% ammonium sulfate saturation)	5	5	102	510

medium during stirring to a final concentration of 30 mM. The mixture was stirred for 30 min and centrifuged at 160,000 x g for one hour. The supernatant was treated with 12 g of washed Biobeads SM-2 (13) for 2 hours and centrifuged at 12,000 x g for 5 min. The supernatant was collected and passed through a layer of glass wool. An equal volume of saturated ammonium sulfate was added during stirring and the mixture was kept at 4° for 10 min. After centrifugation at 27,000 x g for 15 min the floating precipitate was collected and dissolved with 1 ml of a solution containing 10 mM tricine KOH (pH 7.5), 0.5 mM EDTA, 1 mM $MgCl_2$, 0.05 mM DTT and 5% glycerol. The protein fraction (0-50p) was stored at -70° in small aliquots. Repeated freezing and thawing was avoided. Protein was determined according to Lowry et al. (14).

The activities of fractions obtained during the purification of the transporter with octylglucoside are shown in Table I. Preparations of P_i transporter from A-particles contained rutamycin-sensitive ATPase. With ASUA-particles as starting source, preparations free of ATPase, with specific activity of 800, have been obtained; however, the procedure is more laborious and the yield has been low.

Table II

Effect of Valinomycin and Nigericin on Phosphate Transport

The assay for P_i transport was performed with vesicles reconstituted with 65 µg of Precipitate I (see Table I) as described under "Materials and Methods." The data were corrected for $HgCl_2$-insensitive rate of 19 nmoles/min/mg protein.

	P_i Transport	
	P_i/OH	P_i/P_i
	nmoles/min/mg	
Complete system	32	87
" + valinomycin	64	
" + nigericin	58	
" + valinomycin + nigericin	117	99

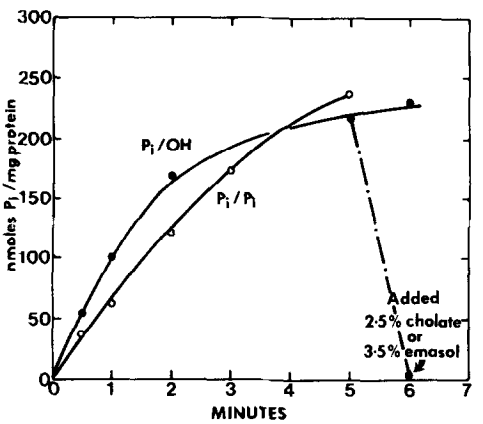
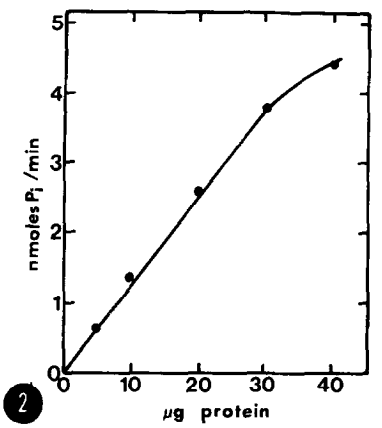


Fig. 1. Time course of P_i/OH and P_i/P_i exchange. Reconstitution with 100 µg Precipitate I (see Table I) and assays were performed as described under "Materials and Methods." Corrections were applied (see Table I).

Properties of reconstituted P_i transport - It was shown previously that the ATP-ADP exchange (but not the ATP/ATP exchange) catalyzed by the nucleotide transporter activity was stimulated by KP_i (15). In the accompanying paper (16) we show that this stimulation required a protein fraction which can be separated from the nucleotide transporter. The elimination of the phosphate effect by N-ethylmaleimide and the lack of stimulation of the ATP/ATP exchange (15) sugges-



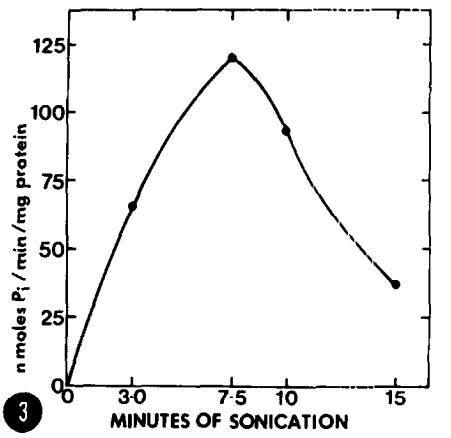


Fig. 2. Effect of protein concentration on P_i/OH exchanges. Reconstitution was performed with varying amounts of protein (32 to 340 μg) as described under "Materials and Methods." Aliquots containing the amounts of protein shown in the figure were added in the assay. Corrections were applied (see Table I).

Fig. 3. Effect of sonication time. Reconstitutions were performed as described under "Materials and Methods" with 100 μg of Precipitate I (Table I) and varying times of sonication. Corrections were applied (see Table I).

ted that the stimulation was caused by a P_i transport mechanism which is electrogenic and which eliminates the membrane potential caused by the charge difference between ADP and ATP.

In view of these considerations we examined the effect of K^+-valinomycin, which collapses the membrane potential, on P_i transport of reconstituted vesicles. As shown in Table II the P_i/OH exchange was stimulated two-fold in the presence of K^+ by valinomycin or nigericin alone and over three-fold when both were added. P_i/P_i exchange was not stimulated under the same conditions. The stimulation by valinomycin of P_i/OH and not of P_i/P_i exchange, suggests that like ADP/ATP transport, P_i transport is electrogenic. The stimulatory effect of nigericin can be explained in terms of the need to collapse the proton gradient and to regenerate K^+ inside the vesicles.

Mersalyl and $HgCl_2$ at 0.05 mM inhibited P_i transport in the reconstituted systems. When compared to values obtained with liposomes prepared without protein,

about 80% of P_i transport was sensitive to the SH-reagents. The residual rates in the presence of the mercurials of about 20 nmoles/min/mg protein were corrected for in all experiments. The observed K_i values for $HgCl_2$ and mersalyl were 6.5 μM and 25 μM respectively. At 50 μM concentration N-ethylmaleimide, N-cyclohexylmaleimide, N-benzylmaleimide and p-hydroxymercuribenzoate inhibited about 60 to 70%.

Figure 1 shows the time course of the P_i/OH and P_i/P_i exchanges. In both cases, transport was linear for about 2 min. The initial rate of P_i/OH exchange in the presence of valinomycin and nigericin was slightly higher than that of P_i/P_i exchange. As shown in Fig. 1, the radioactivity was completely released when the vesicles were treated with 2.5% cholate or 3.5% emasol after 5 min of incubation. Fig. 2 shows that the initial rate of P_i/OH exchange is linear with protein concentration up to 200 μg protein reconstituted with 30 μmoles of phospholipids. This proportionality makes the procedure suitable for the assay of P_i transport activity during purification of the transporter. Fig. 3 shows that the transport activity was dependent on sonication and that optimum rates were obtained after 7 to 8 min of sonication. Activity decreased rapidly with prolonged sonication.

The observations that sonication of the phospholipid is essential for transport, that radioactivity is released on addition of detergents and that transport is stimulated several fold by valinomycin plus nigericin, indicates that we are dealing with a transmembranous process.

The orientation of the reconstituted transporter appears to be the same as an intact mitochondria since both N-ethylmaleimide and N-benzylmaleimide inhibited equally well. This is in contrast with the P_i transport in submitochondrial particles (17) which was inhibited by the hydrophobic (N-benzylmaleimide) but not by the hydrophilic maleimide (N-ethylmaleimide).

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