Purification of a Reconstitutively Active Mitochondrial Phosphate Transport Protein*

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The mitochondrial phosphate transport protein has been extracted from beef heart and insect flight muscle mitochondria with Triton X-100 and has been purified by taking advantage of its low affinity for hydroxylapatite. The purified fraction consists of the phosphate transport protein and the carboxyatractyloside-binding protein. The molecular weight of the phosphate transport protein has been revised from earlier estimates to about 34,000 by comparing its mobility on dodecyl sulfate-polyacrylamide gels to that of the γ subunit of beef heart mitochondrial F1-ATPase. The phosphate transport protein has been incorporated by the freeze-thaw sonication method into liposomes prepared from purified phosphatidylethanolamine:phosphatidylcholine:cardiolipin (4:1:0.25). The reconstituted system catalyzes phosphate-phosphate exchange, as determined by 32P efflux or uptake experiments, with an initial rate of 990 nmol of Pi/min × mg of protein. The exchange is sensitive to the phosphate transport inhibitors N-ethylmaleimide, mersalyl, p-hydroxymercuribenzoate, and HgCl2, but not to the dicarboxylate transport inhibitor n-butylmalonate or the adenine nucleotide transport inhibitor, carboxyatractyloside. Phosphate transport protein purified from N-[3H]ethylmaleimide-inhibited mitochondria contains most of the triutium label with only a trace in the carboxyatractyloside-binding protein and does not catalyze phosphate-phosphate exchange in the reconstituted liposomes.

The phosphorylation of ADP that is linked to respiration occurs in the mitochondrial matrix. Most ATP-utilizing reactions, however, happen outside the mitochondria. A high flux of ADP and P_i into the mitochondrial matrix must, therefore, accompany the efflux of ATP. The adenine nucleotide transporter is responsible for the ATP and ADP fluxes. It has been highly purified and demonstrated to be reconstitutively active (1). The identification and thus the purification of the protein responsible for the phosphate transport, on the other hand, have been hindered by the lack of a specific inhibitor. The phosphate transport can be inhibited by various sulfhydryl reagents (for a review, see Ref. 2). Attempts have been made to identify the protein responsible for this transport by using radioactively labeled sulfhydryl reagents (3-8) or by assaying for —SH reagent-sensitive phosphate binding sites of isolated membrane proteins (9, 10). Banerjee and co-workers (11, 12) have fractionated beef heart mitochondria and obtained a protein fraction that upon reconstitution was able to catalyze inhibitor-sensitive phosphate transport.

We have labeled intact mitochondria with only sufficient N-[3H]ethylmaleimide to completely inhibit the phosphate transport (19). Many proteins of the inner mitochondrial membrane react with N-[3H]ethylmaleimide and it was not clear which labeled protein was the phosphate transport protein. Mitochondria from the blowfly flight muscle are highly specialized and possess a simplified inner membrane (14, 15). We compared the N-[3H]ethylmaleimide-labeled inner membrane proteins from the flight muscle mitochondria to those of the sonic submitochondrial particles of rat heart (16) and the Lubrol-insoluble fraction of rat liver mitochondria (3, 13). We correlated the amount of N-[3H]ethylmaleimide that reacted with the various inner membrane proteins with the degree of phosphate transport inhibition (17) and identified those proteins whose alkylation could be prevented by mercurials (16). We were thus able to identify a protein on dodecyl sulfate-polyacrylamide gels with the phosphate transport protein (18).

We report now a simple and fast method for the purification of this phosphate transport protein. Furthermore, we demonstrate with reconstitution experiments that phosphate transport protein incorporated into liposomes catalyzes phosphate-phosphate exchange, which is sensitive to the various phosphate transport inhibitors. These experiments strongly support our earlier conclusion that this protein is the phosphate transport protein and is responsible for the primary mitochondrial phosphate transport system.

EXPERIMENTAL PROCEDURES

Purification of the Phosphate Transport Protein—Mitochondria were prepared from beef hearts according to published procedures (19) and stored in a −40°C freezer at a concentration of 8.4 nmol of cytochrome b/ml. The cytochrome b concentration of the mitochondrial suspension was determined with an Amino-Chance dual wavelength spectrophotometer (16). All of the following operations were carried out at 4°C unless noted otherwise. The thawed mitochondria were swollen in Medium S (10 mM sodium phosphate and 6.35 mM EDTA, pH 7.2) for 10 min and then centrifuged at 17,000 × g for 10 min. The pellet was suspended in Medium A (10 mM sodium phosphate, 0.1 mM EDTA, and 130 mM NaCl, pH 7.2) and centrifuged at 17,000 × g for 10 min. The pellet was washed once more with Medium A and taken up in Medium A at a cytochrome b concentration of more than 13 nmol/ml Triton X-100 (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1.4% and 13 nmol of cytochrome b/ml. The mixture was added to cold hydroxyapatite powder (Bio-Gel HTP, Bio-Rad, Richmond, CA) at 0.4 g/ml of solubilized mitochondria and blended on a Vortex mixer thoroughly. The resulting mixture was centrifuged at 27,000 × g for 15 min. The resulting clear supernatant is the purified phosphate transport protein.
Preparation of Liposomes—Phosphatidylethanolamine (8.4 mg), phosphatidylcholine (2.1 mg), and cardiolipin (0.5 mg) in the organic solvents of the commercial suppliers were mixed in a small, thin-walled glass test tube and the solvent evaporated under a stream of high purity argon while vortexing the tube. The lipids were suspended with a silicone rubber stopper. The lipids were clarified in a Heat Solvents of the commercial suppliers were mixed in a small, thin plastic test tube and sonicated until clear. The lipids were adjusted to pH 7.2 with NaOH and they were transferred to a small plastic test tube and sonicated until clear (2 min, 0-4°C).

Reconstitution of the Phosphate-Phosphate Exchange Activity—The medium of the purified phosphate transport protein was changed to one of lower anion concentration to permit 32P, retention on the anion exchange column and to have the same medium (except for dithiothreitol) inside and outside of the liposomes. The purified phosphate transport protein was passed through a small Bio-Gel P2 (100 to 200 mesh, 0.7 x 3 cm) column at a flow rate of 150 ml/min. The column had been equilibrated with Medium B (10 mM Tris-HCl, 2 mM MgCl2, 10 mM sodium phosphate, and 2 mM dithiothreitol, pH 7.2). The reconstitution was carried out by adding 40 μl of the purified phosphate transport protein (10 to 20 μg of protein) to 80 μl of the liposomes and 140 μl of Medium C with 32P. The mixture was blended on a Vortex mixer and frozen in a dry ice-ethanol bath (20) and thawed at room temperature. It was then sonicated in a small plastic test tube under argon at 0-4°C for 15 s. Various inhibitors in 3.75 μl of Medium C with 32P (or control media) were added to 25 μl of reconstituted liposomes, incubated for 3 min at 0-4°C, and added to 500 μl of Medium C at 22°C. The reaction was stopped by the addition of 10 μl of 250 mM mersalyl. The mixture was immediately placed on a Dowex AG1-X8 (chloride form, 50 to 100 mesh) anion exchange column (0.7 x 12 cm, 4-10°C) (21) which had been thoroughly washed with Medium D (5% glycerol and 0.1 mM sodium azide) and treated with 30 mg of bovine serum albumin and 150 μl of the initial liposome solution without phosphate transport protein. Fractions were collected into miniscintillation vials in a fraction collector. Liquisint (National Diagnostics, Somerville, NJ) was added and the samples counted in a Beckman scintillation counter.

Phosphate-phosphate exchange by 32P, uptake experiments was determined with 32P, present only in the 500 μl of Medium C. One anion exchange column was used for a series of up to 30 samples. Medium C with the proper dilution of 32P, was passed through the column at the beginning and the end of the experiment to make sure that the column still retained all of the extraliposomal 32P. Samples were run alternatively with and without N-ethylmaleimide and then all samples were repeated. The amount of 32P, recovered from the column varied less than 2% between repeated samples. The recovery of the first two samples from the column was generally lower than all of the following samples.

Polyacrylamide Gel Electrophoresis—Dodecyl sulfate-polyacrylamide slab gel electrophoresis was carried out as described (16). The mitochondrial were labeled with N-[3H]ethylmaleimide at 29.8 nmol/nmol of cytochrome b (16). The cylindrical gel of the N-[3H]ethylmaleimide-labeled phosphate transport protein was run, sliced, and counted as described (13).

Other Methods—Protein was determined in the presence of sodium dodecyl sulfate to eliminate the interference of Triton X-100 in the Lowry method (22). 32P, with a specific activity of 60 GBq/mmol was used for the 32P, efflux and uptake experiments.

Materials—N-[3H]ethylmaleimide was obtained from New England Nuclear (Boston, MA); the phospholipids were obtained from Supelco (Bellefonte, PA): phosphatidylethanolamine (plant), phosphatidylinoline (plant), cardiolipin (bovine); or from Applied Science Laboratories (State College, PA): cardiolipin (bovine); n-butylmalonate was obtained from Aldrich Chemical Co. (Milwaukee, WI) and carboxyatractyloside from Boehringer Mannheim (Indianapolis, IN).

RESULTS AND DISCUSSION

Molecular Weight Determination and N-[3H]Ethylmaleimide Labeling of the Phosphate Transport Protein—The dodecyl sulfate-polyacrylamide slab gel of Fig. 1 clearly shows that the phosphate transport protein has a smaller mobility and, therefore, a larger molecular weight than the 33,160 of the γ subunit of the mitochondrial F1-ATPase (23).

The protein was purified from N-[3H]ethylmaleimide-inhibited mitochondria and run on a cylindrical dodecyl sulfate-polyacrylamide gel. The optical density showed that the fraction contains significantly less phosphate transport protein than carboxyatractylloside-binding protein. Whether the co-purification of the phosphate transport protein and the carboxyatractylloside-binding protein implies that the two proteins occur as a complex in the inner mitochondrial membrane or whether it is just a reflection of a similar affinity for hydroxylapatite remains to be established. The cylindrical gel was sliced and the slices matched to the absorbance profile. The phosphate transport protein is the predominantly N-[3H]ethylmaleimide-labeled protein (Fig. 2). A trace of label,
Mitochondrial Phosphate Transport Protein

as expected (16), appears also in the carboxyatractyloside-binding protein.

Similar purification experiments have been carried out with mitochondria from the blowfly flight muscle (13) and here also the phosphate transport protein is extracted together with the carboxyatractyloside-binding protein.

We calculated from the amount of N-[3H]ethylmaleimide in the phosphate transport protein band in the dodecyl sulfate-polyacrylamide gel of the purified fraction and the labeled mitochondria that the phosphate transport protein was purified about 20-fold with a yield of about 35%.

Reconstitution of the Phosphate Transport Protein—The sonication time after the freeze-thaw step is optimum between 5 and 15 s; longer or shorter sonication times decrease the amount of 32P, exchange per unit of time. The highest exchange rates are obtained by keeping the sonication temperature 0-4°C. Fig. 3, a and b, clearly shows that the reconstituted vesicles catalyze phosphate-phosphate exchange. This exchange occurs with an intraliposomal dithiothreitol concentration of 310 μM and an extraliposomal concentration of 15 μM, the dithiothreitol carried over from the Bio-Gel P2 column medium change step. The phosphate transport protein can also be purified without dithiothreitol; however, we have not yet determined whether it is reconstitutively active.

Fig. 3a shows the phosphate-phosphate exchange as determined by 32P efflux, while Fig. 3b is the parallel 32P uptake experiment. The curves of the two figures look very much alike. It is possible to estimate a turnover number of our purified phosphate transport protein and to compare it to that of mitochondria. For various reasons, neither number is a good estimate. Coty and Pedersen (24) calculated a turnover number of 3500 min⁻¹ for the phosphate transport protein of rat liver mitochondria from phosphate-phosphate exchange experiments by assuming that (a) their major N-[3H]ethylmaleimide-labeled protein is the phosphate transport protein and that (b) none of this protein was solubilized by Lubrol. We obtained a maximum exchange rate of 990 nmol of phosphate/min X mg of protein. This rate is comparable to that obtained by Banerjee and Racker (11). It is, however, not possible to estimate a turnover number for their preparation since it is not clear which protein of their purified fraction is the phosphate transport protein. The absorbance trace of a Coomassie blue-stained polyacrylamide gel of our phosphate transport protein suggests that only 20% of the protein of the purified fraction is phosphate transport protein, while the other 80% is carboxyatractyloside-binding protein. This assumes that phosphate transport protein and carboxyatractyloside-binding protein stain equally well with Coomassie blue. If this factor is considered, we can calculate a turnover of 170 min⁻¹. We feel, however, that our system has by no means been optimized. Thus, it is not clear what fraction of the phosphate transport protein is actually incorporated into the liposomes. Hinkle and co-workers (25) find that only a small fraction of the erythrocyte glucose transporter gets incorporated into liposomes during the freeze-thaw procedure. It is also interesting to note that Kramer and Klingenberg (1) estimate that 5% of the adenine nucleotide transporters incorporated into liposomes are reconstituted with the right polarity and provide transport activity.

Preliminary experiments suggest that with the intraliposomal pH at 7.2, the same as the extraliposomal pH, the phosphate-phosphate exchange rate is significantly higher than the net phosphate efflux in the absence of uncouplers or ionophores. The Km for phosphate-phosphate exchange is

### Table I

| Inhibitor                        | % Inhibition |
|----------------------------------|--------------|
| Phosphate transport protein from beef heart mitochondria | None | 0 |
| p-Hydroxymercuribenzoate (1.35 mM) | 99 |
| Mersalyl (1.24 mM)                | 87 |
| HgCl₂ (1.00 mM)                   | 100 |
| N-Ethylmaleimide (0.50 mM)        | 3 |
| n-Butylmalonate (0.50 mM)         | 100 |
| Carboxyatractyloside (0.05 mM)    | 0 |
| Phosphate transport protein from beef heart mitochondria inhibited with 50 nmol of N-ethylmaleimide/nmol of cytochrome b | None | 90 |

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**Fig. 3.** Phosphate-phosphate exchange by the reconstituted liposomes. The exchange was determined by 32P efflux (a) and uptake (b). The inside and the outside phosphate concentration was 10 mM. The experiments were carried out as described under "Experimental Procedures." NEM is the inhibitor N-ethylmaleimide.
much smaller at pH 6.0 than at higher pH. This appears to be in agreement with the results of Fonoy and co-workers (26), who used intact rat liver mitochondria.

Inhibition of the Reconstituted Phosphate Transporter—One of the criteria for differentiating the phosphate transporter from the dicarboxylate transporter is the sensitivity of the former to N-ethylmaleimide, while both are sensitive to mercurials such as mersalyl. We have determined the sensitivity of the reconstituted phosphate-phosphate exchange to various inhibitors. Table I shows that only a small amount of N-ethylmaleimide beyond twice the concentration of dithiothreitol present is required to inhibit the transport. Mersalyl, p-hydroxymercuribenzoate, and HgCl₂ were kept above the amount of dithiothreitol present. It is not quite clear why Banerjee and co-workers (11, 12) were able to inhibit their reconstituted phosphate transport system with a concentration of inhibitor much below that of the 2 mM dithiothreitol present, unless the —SH group of the phosphate transport protein indeed has a much higher affinity for the various inhibitors. Our results with N-ethylmaleimide in Table I suggest that this is not true for our reconstituted system.

HgCl₂ behaved differently from the other inhibitors since at high concentrations it was less effective in inhibiting the exchange of 32P32P. It is possible that it perturbed the phosphate transport protein or carboxyatractyloside-binding protein to such an extent as to make them leaky to phosphate or to make the liposomes leaky. It is more likely that HgCl₂ was precipitated partly by phosphate and thus no longer available to react with —SH groups.

The reconstituted phosphate-phosphate exchange is insensitive to n-butylmalonate (Table I), which inhibits the dicarboxylate transporter in intact mitochondria (24, 27) and to carboxyatractyloside, which inhibits the adenine nucleotide transporter also in the reconstituted system (28).

We have also inhibited the mitochondria before solubilization with sufficient N-ethylmaleimide to block the phosphate transporter (50 nmol of N-ethylmaleimide/nmol of cytochrome b). We then purified the phosphate transport protein and used it in reconstitution experiments. The resulting reconstituted liposomes showed less than 10% of the N-ethylmaleimide-sensitive phosphate-phosphate exchange of the control reconstituted liposomes (see Table I).

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REFERENCES
1. Krämer, R., and Klingenberg, M. (1979) Biochemistry 18, 4209-4215
2. Fonoy, A. (1978) J. Bioenerget. Biomembr. 10, 171-194
3. Coty, W. A., and Pedersen, P. L. (1975) J. Biol. Chem. 250, 3515-3521
4. Briand, T., Touraille, S., Debise, R., and Durand, R. (1976) FEBS Lett. 65, 1-7
5. Hadvary, P., and Kadenbach, B. (1976) Eur. J. Biochem. 67, 573-581
6. Touraille, S., Briand, Y., and Durand, R. (1977) FEBS Lett. 84, 119-124
7. Alziari, S., Touraille, S., Briand, Y., and Durand, R. (1979) Biochimie 61, 891-903
8. Hofmann, H. D., and Kadenbach, B. (1979) Eur. J. Biochem. 102, 605-613
9. Kadenbach, B., and Hadvary, P. (1973) Eur. J. Biochem. 39, 21-264
10. Guerin, M., and Napias, C. (1978) Biochemistry 17, 2510-2516
11. Banerjee, R. K., and Racker, E. (1979) Membr. Biochem. 2, 203-225
12. Banerjee, R. K., Shertzer, H. G., Karnes, B. F., and Racker, E. (1977) Biochem. Biophys. Res. Commun. 72, 772-778
13. Wohlrab, H., and Greaney, J., Jr. (1978) Biochim. Biophys. Acta 503, 425-436
14. Van den Bergh, S. C., and Slater, E. C. (1962) Biochem. J. 82, 362-371
15. Wohlrab, H. (1976) J. Geront. 31, 257-263
16. Wohlrab, H. (1979) Biochemistry 18, 2098-2102
17. Wohlrab, H. (1978) Biochem. Biophys. Res. Commun. 83, 1430-1435
18. Wohlrab, H. (1979) in Function and Molecular Aspects of Membrane Transport (Quaglialietto, E., Klingenberg, M., Palmieri, F., and Papa, S., eds) pp. 387-390, Elsevier/North Holland Biomedical Press, Amsterdam
19. Joshi, S., and Sanadi, D. R. (1979) Methods Enzymol. 55F, 384-391
20. Kasahara, M., and Hinkle, P. C. (1972) J. Biol. Chem. 252, 7384-7390
21. Gasko, 0., Knowles, A., Shertzer, H., Suolinna, E. M., and Racker, E. (1976) Anal. Biochem. 3, 57-65
22. Kuosov, Y. Y., and Kalinchuk, N. A. (1976) Anal. Biochem. 88, 296-292
23. Knowles, A. F., and Penevsky, H. S. (1972) J. Biol. Chem. 247, 6624-6630
24. Coty, W. A., and Pedersen, P. L. (1974) J. Biol. Chem. 249, 2593-2598
25. Hinkle, P. C., Sogin, D. C., and Wheeler, T. J. (1980) in Second International Conference on Channels and Carriers in Biological Systems—Transport Proteins (Shamoo, A., Lazdunski, M., Racker, E., and Sachs, G., eds), New York Academy of Sciences, New York, in press
26. Fonoy, A., Palmieri, F., Ritvay, J., and Quaglialietto, E. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G. F., Klingenber, M., Quaglialietto, E., and Siliprandi, N., eds) pp. 283-286, Elsevier/North Holland Biomedical Press, Amsterdam and London
27. Chappell, J. B., and Crofts, A. R. (1966) in Regulations of Metabolic Processes in Mitochondria (Tagar, J. M., Papa, S., Quaglialietto, E., and Slater, E. C., eds) Vol. 7, pp. 293-314, BBA Library, American Elsevier Publishing Co., New York
28. Klingenberg, M., Riccio, P., and Aquila, H. (1978) Biochim. Biophys. Acta 503, 193-210