pH Gradient-dependent Phosphate Transport Catalyzed by the Purified Mitochondrial Phosphate Transport Protein*

(Received for publication, October 5, 1981)

Hartmut Wohlrab and Nancy Flowers

From the Department of Cell Physiology, Boston Biomedical Research Institute, Boston, Massachusetts 02114 and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

The mitochondrion phosphate transport protein (Wohlrab, H. (1980) J. Biol. Chem. 255, 8170–8173), which co-purifies with the adenine nucleotide translocase, has been isolated with a modified procedure resulting in an increased yield and in a preparation that is stable to storage in liquid nitrogen. The phosphate transport protein was incorporated into liposomes (phosphatidylethanolamine/phosphatidylcholine/phosphatidic acid, 3.75:1:1), and phosphate/phosphate exchange rates were determined. With $pH_e$ (extraliposomal) = $pH_i$ (intraliposomal) = 7.2, we found a $K_m$ of 2.5 mm independent of $[Pi]$ and a $V_{max}$ of 12 pmol/min·mg of protein. Parallel phosphate transport experiments were carried out with liposomes containing phosphate transport protein isolated from mitochondria inhibited by N-ethylmaleimide. Phosphate transport unidirectionally [$pH_e$ = 6.8; ($pH_i$ = 1.0 mm, $P_i$ = 0 mm) reaches a maximum at 30 s and is 0.13 and 0.05 pmol/mg for active and inhibited protein, respectively. At $pH_e$ = 6.8 and $pH_i$ = 8.0, the respective amounts are 0.45 and 0.05. At $pH_i$ = 8.0, the uptake becomes the same with active and inhibited protein ($pH_i$ = 6.8, 0.15 and 0.14; $pH_i$ = 8.0, 0.25 and 0.20). At all the above $pH$ values, about the same uptake is observed with liposomes prepared without protein as those prepared with inhibited protein. The initial rate of protein catalyzed unidirectional flux ($V_{max}$ = 1.0 mm, $P_i$ = 0.0 mm, $pH_e$ = 8.0; $P_i$ = 0.0 mm, $pH_i$ = 8.0) is 2 pmol/min·mg of protein or 8 pmol/min·mg of phosphate transport protein estimated without the adenine nucleotide translocase.

Inorganic phosphate must be transported into the mitochondrial matrix for steady state oxidative phosphorylation of ADP to occur. We recently reported the purification of a protein from beef heart mitochondria which upon incorporation into liposomes catalyzed phosphate/phosphate exchange (1, 2). The exchange is sensitive to the known mitochondrial phosphate transport inhibitors but not to those of dicarboxylate or adenine nucleotide transport (2).

We show in this communication that the purified phosphate transport protein co-purified with the adenine nucleotide translocase catalyzes, after incorporation into liposomes, phosphate transport with characteristics similar to those of the protein in intact mitochondria (3–5). We specifically demonstrate that it can catalyze unidirectional phosphate transport, that the amount of phosphate accumulated depends on the intravesicular $pH$, and that the $K_m$ and $V_{max}$ are similar to those of mitochondria.

Experimental Procedures

Purification of the Phosphate Transport Protein—Mitochondria were prepared from beef hearts according to published procedures (6) and stored at a concentration of 13 nmol of cytochrome b/ml (−40 °C). The cytochrome b concentration was determined as described (7). All operations were carried out at 4 °C unless noted otherwise. Thawed mitochondria (6 ml) were centrifuged (17,000 g, 10 min), and the pellet was suspended in 80 ml of medium S (10 mM sodium phosphate, 0.33 mM EDTA, pH 7.2), kept on ice for 10 min, and centrifuged (17,000 g, 10 min). The pellet was suspended in 80 ml of medium A (10 mM sodium phosphate, 0.1 mM EDTA, 130 mM NaCl, 5 mM dithiothreitol, pH 7.2) and centrifuged (17,000 g, 10 min). The pellet was resuspended in medium A and centrifuged once more. The supernatant was completely removed and the pellet suspended in medium A to yield a final volume of 5 ml. 1 ml of a 16% Triton X-100 (Sigma) solution in medium A was added while vortexing the suspension. After centrifugation (165,000 g, 10 min, Beckman 50-Ti rotor), 1.6 g of washed SM-2 Bio-Beads (Bio-Rad) was added to the supernatant. The suspension was stirred for 30 min. The SM-2 Bio-Beads decrease the Triton X-100 concentration to 1.4%.

The supernatant, after the Bio-Beads had settled out, was put on a hydroxyapatite column (1 × 8.5 cm with 15 cm of tubing attached to the lower end to increase the flow rate) and fractions (about 1.2 ml) were collected at intervals of 1.5 min. The sample was eluted with medium A which contained 0.5% Triton X-100. The hydroxyapatite (hydroxylapatite, fast flow, dry powder, Calbiochem-Behring) had been suspended in and the column equilibrated with medium B (10 mM sodium phosphate, 0.1 mM EDTA, 130 mM sodium sulfate, pH 7.2). The fractions with high absorbance (275 nm) were pooled. 500 μl of a freshly prepared lipid dispersion (46 mg/ml) (see below) were added immediately, the mixture was vortexed, and the air was flushed out of the test tube with high purity argon. The test tubes (1 g/2-ml sample) were added and the mixture was stirred for 5 min. 200-μl fractions were pipetted into argon-flushed 1.5-ml cryotubes (Vanguard International, Neptune, NJ) and stored in a liquid nitrogen freezer.

The phosphate transport protein inhibited by N-ethylmaleimide was prepared from beef heart mitochondria with a modified purification procedure. The mitochondrial pellet from the first centrifugation was suspended in 30 ml of medium E (100 mM 4-morpholinopropane sulfonic acid, 0.2 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, 2 mM dithiothreitol, adjusted to pH 7.2 with NaOH) and centrifuged (17,000 g, 10 min). The pellet was suspended to 10 ml with medium E, and 500 μl of 100 mM N-ethylmaleimide in water was added. After 3 min on ice, the suspension was centrifuged (17,000 g, 10 min). The pellet was taken up in 80 ml of medium S and the procedure was finished as described above.

Preparation of Liposomes—11.5 mg (phosphatidylethanolamine (7.5 mg), phosphatidylcholine (2.0 mg), cardiolipin (2.0 mg)) or 9.5 mg (phosphatidylethanolamine (7.5 mg), phosphatidylcholine (2.0 mg)) of phospholipids in the organic solvents of the commercial supplier were transferred into sterile ampules (1 ml Pierce). The amount of phospholipid in each ampule was kept small to permit complete removal of solvents by blowing high purity argon into each ampule. To facilitate further solvent removal, 100 μl of diethyl ether were added to dissolve the lipids and the solvent was again removed by blowing argon into the ampules. This last step was repeated with 100 μl and then with 50 μl of diethyl ether to reduce the size of the lipid film to a minimum. The ampules were placed overnight into a high vacuum chamber (<1 μ) to remove remaining traces of solvent.

* This investigation was supported by National Institutes of Health Grant AG00100 and Grant PCM 8008916 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Commercially lyophilized phosphatidic acid (5 mg) was transferred into an empty ampule and 200 μl of medium C (10 mM Tris base, 2 mM MgCl₂, 10 mM 1,4-piperazine-N,N'-bis(2-ethanesulfonic acid), 2.5 mM carrier-free “PI (in deute HCl) was obtained from New England Bq eluted.

Commercial 32P₁ sample (1.7 Bq eluted) was added to the ampules with the sodium dodecyl sulfate and N-ethylmaleimide using the Lowry method (2). Since much of the purified protein was lost in the soybean phosphatidylcholine, bovine cardiolipin, egg phosphatidic acid, various amounts of phosphate for the intraliposomal concentration, pH was adjusted with 10 or 3 N KOH) was added. The phosphatidic acid was adjusted with 10 or 3 N KOH was added. The mixture was frozen, thawed (2, 9), and sonicated in small plastic test tubes under argon for 10 s at 13°C in the bath-type sonicator (2).

Phosphate Transport Assays—25 μl of liposomes into which phosphotransferase protein had been incorporated (30 °C) were added to 500 μl of reaction medium (medium C, 22 °C) to which carrier-free 32P₂ (1.7 x 10⁴ Bq) and various amounts of phosphate had been added. The pH of medium C was at the pH, (extraliposomal) before phosphate was added and was readjusted after the phosphate addition. The phosphate transport reaction was stopped with mersalyl (10 μl, 79 mM), which was prepared by adding mersalyl sodium to water followed with only enough NaOH to dissolve the mersalyl. Zero time points were obtained by adding the liposomes to the reaction medium plus mersalyl. 30 s after, the mixture was placed on an anion exchange column (see below) (4-10 °C) and eluted with 3 ml of medium D (5% glycerol, 0.1 mM sodium azide). The eluate (3.5 ml) was collected in one scintillation vial, 8 ml of scintillation mixture (Liquisint, National Diagnostics) were added and the vial was vortexed and counted. For the other time points, mersalyl was added 2 s after the liposomes; (30 – 5) s later, the sample was placed on the anion exchange column.

The anion exchange column (2) (AG 1-X, formate, 75-100 mesh, BioRad) was treated with 500 μl of bovine serum albumin (30 mg/ml), 100 μl of liposomes (46 mg/ml of medium C), 520 μl liposomes (1.77 mg/ml of medium C) and 500 μl of liposomes (1.77 mg/ml of medium C) before use. The affinity of the column for Pi was monitored by elution without liposomes through the column after second or third reaction mixture with liposomes. From a reaction mixture without liposomes and an acceptable commercial 32P₁, sample (1.7 x 10⁴ Bq) put through the column, less than 1.7 Bq eluted. With some unacceptable commercial 32P₂ samples, 34 Bq eluted.

Other Methods—Protein was determined in the presence of sodium dodecyl sulfate and N-ethylmaleimide using the Lowry method (10).

Materials—Phospholipids (soybean phosphatidylethanolamine, soybean phosphatidylcholine, bovine cardiolipin, egg phosphatidic acid) were obtained from Avanti Polar Lipids (Birmingham, AL); carrier-free 32P₁ (in deute HCl) was obtained from New England Nuclear or Amersham.

RESULTS AND DISCUSSION

Purification of the Phosphate Transport Protein—The mitochondrial phosphate transport protein was originally purified by centrifuging a mixture of hydroxyapatite and solubilized mitochondria. The pure protein together with the adenine nucleotide translocase was recovered from the supernatant (2). Since much of the purified protein was lost in the liquid trapped in the hydroxyapatite pellet, we increased the yield by passing the solubilized mitochondrial membrane through a hydroxyapatite column. This preparation also consists only of the phosphate transport protein and the adenine nucleotide translocase (11). This column procedure takes more time and we therefore developed conditions that protect the active preparation during storage in a liquid nitrogen freezer. Freezing the phosphate transport protein with or without added phospholipids results in an 80% loss of reconstituted transport activity. A 30- to 60-min exposure to SM-2 Bio-Beads after the addition of phospholipids and before freezing completely protected the transport activity from inhibition by freezing.

Phosphate/Phosphate Exchange Rates—We extended our phosphate/phosphate exchange studies (1, 2) in order (a) to increase the reconstituted activity and (b) to obtain kinetic properties similar to those reported for mitochondria (5). We modified our original assay system (2) by stopping the phosphate/exchange at various times with mersalyl. Mersalyl appears to be less competitive with phosphate than some other transport inhibitors and is thus more suitable for initial transport rate studies. We discovered that high mersalyl concentrations (4.4 mM) trap phosphate in liposomes prepared without protein; i.e. the activity of 32P₁, eluted from the anion exchange column is significantly higher for 4-s than 0-s points with liposomes prepared without transport protein or with transport protein inhibited by N-ethylmaleimide. The amount of 32P₁, trapped at 4 s and 36 s is about the same. At the low mersalyl concentrations that we use in our present experiments, the protein-independent mersalyl effect is almost completely eliminated. More recent experiments suggest that mersalyl at a concentration as low as 0.20 mM is sufficient to inhibit protein-catalyzed phosphate transport from the extraliposomal side at pH 6.8 or 8.0.

The transport assay was further modified in order to exclude mersalyl-insensitive phosphate leakage from the transport analysis. All the liposome samples were kept suspended in the reaction medium for 30 s before being placed on the anion exchange column. This experimental protocol eliminates the possibility that the smaller amount of 32P₁, recovered from the anion exchange column of a short time point sample is due to the earlier separation of the extraliposomal 32P₁, from the intraliposomal 32P₁, by the anion exchanger instead of due to the earlier addition of mersalyl. Investigating the maximum phosphate uptake as a function of time beyond 30 s, we carried out experiments in which the total time the liposomes spent in the reaction medium was increased to 1 min.

Fig. 1a shows the result of a series of experiments where medium C contained various amounts of sodium phosphate, the initial extraliposomal phosphate concentrations ([Pi]ᵢ). This phosphate concentration includes the increase or decrease caused by the 25 μl of liposomes added to the 500-μl reaction medium. We assumed for the purpose of this calculation that all the phosphate in the 25 μl of liposomes is extraliposomal. We determined 32P₁, uptake into the vesicles at 0, 4, 7.5, 15, and 30 s and estimated initial rates from the resulting graph. These initial rates are shown as double reciprocal plots in Fig. 1a. We have calculated an approximate Km for extraliposomal phosphate of 2.5 mM (pH 7.2) which appears to be independent of the intraliposomal phosphate concentration. It is very close to the value of 1.84 mM determined by Coty and Pedersen (5) using rat liver mitochondria at pH 7.4. It is not clear from their experiments what the intramitochondrial pH is and how a ΔpH across the membrane affects the Km. In Fig. 1b we used the results of Fig. 1a to determine a Vₘₐₓ with an extrapolated infinite internal phosphate concentration. This Vₘₐₓ is about 12 nmol of P/min of phosphate transport protein.

A preliminary amino acid analysis of the phosphate transport protein separated in the presence of sodium dodecyl sulfate from the adenine nucleotide translocase (11) shows a composition similar to that of the adenine nucleotide trans-
Rates of 30 Reconstituted Mitochondrial Phosphate Transport Activity

Since, however, liposomes prepared without any protein show the pH is raised to 8.0 by N-ethylmaleimide, we conclude that the liposomes are almost the same uptake pattern at the different external and internal pH values as the liposomes with the protein inhibited by N-ethylmaleimide. The initial extraliposomal pH was 6.8 and an internal pH of 8.0. Uptake in the presence of protein inhibited by N-ethylmaleimide is almost completely absent in both cases. When, however, the external pH is raised to 8.0 (Fig. 2, b and d), the phosphate uptake in the presence of protein inhibited by N-ethylmaleimide or active protein is the same. This suggests that either the liposomes have become more leaky and phosphate is trapped by mersalyl (as discussed earlier) or that a phosphate transport pathway insensitive to N-ethylmaleimide but sensitive to mersalyl is activated at the higher external pH in the protein.

While many more studies have to be carried out with this protein in its natural or synthetic membrane environment, we can conclude from the present set of results that the phosphate transport protein catalyzes unidirectional phosphate transport that is stimulated by a membrane pH gradient.

**Acknowledgments**—We thank Diane Costello for enthusiastic and expert help with the experiments and Louise Reed for her assistance.

![Fig. 1](https://example.com/image1.png)

**Fig. 1.** Phosphate/phosphate exchange catalyzed by the phosphate transport protein. Initial intraliposomal phosphate concentrations were 5.0 (●), 1.7 (○), 1.0 (△), and 0 mM (▲). Initial exchange rates have been used. The extrapolated maximum exchange rates of a are plotted in b to obtain a V_max at infinite internal phosphate concentration. Extraliposomal pH of 7.2 was the same as the intraliposomal pH.

![Fig. 2](https://example.com/image2.png)

**Fig. 2.** Unidirectional phosphate flux catalyzed by liposomes incorporated with phosphate transport protein (●) and phosphate transport protein inhibited by N-ethylmaleimide (○). The initial extraliposomal phosphate concentration was 1.0 mM and the initial intraliposomal phosphate concentration was 0 mM. The initial extraliposomal pH was 6.8 (a and c) and 8.0 (b and d) and the initial intraliposomal pH was 6.8 (a and b) and 8.0 (c and d).

![Fig. 3](https://example.com/image3.png)

**Fig. 3.** Unidirectional phosphate uptake catalyzed by the phosphate transport protein in the presence of a ∆pH gradient. The experiment was carried out as in Fig. 2c except that the transport was stopped after 10 s. The phosphate trapped by liposomes incorporated with phosphate transport protein inhibited by N-ethylmaleimide was subtracted.

preparing with protein inhibited by N-ethylmaleimide. The initial uptake rate is about 2 μmol of Pi/min·mg of protein or 8 μmol/min·mg of phosphate transport protein estimated without the adenine nucleotide translocase.

Acknowledgments—We thank Diane Costello for enthusiastic and expert help with the experiments and Louise Reed for her assistance.
Reconstituted Mitochondrial Phosphate Transport Activity

in preparing this manuscript. We also thank Dr. James Hughes and Vincent Sy for making beef heart mitochondria available.

REFERENCES

1. Wohlrab, H. (1980) in Proceedings of the 2nd International Conference on Carriers and Channels in Biological Systems—Transport Proteins (Shamoo, A., ed), pp. 364–367, New York Academy of Sciences, New York
2. Wohlrab, H. (1980) J. Biol. Chem. 255, 8170–8173
3. Palmieri, F., Quagliariello, E., and Klingenberg, M. (1970) Eur. J. Biochem. 17, 233–241
4. LaNoue, K. F., and Schoolwerth, A. C. (1979) Annu. Rev. Biochem. 48, 871–922
5. Coty, W. A., and Pedersen, P. L. (1974) J. Biol. Chem. 249, 2583–2588
6. Joshi, S., and Sanadi, D. R. (1979) Methods Enzymol. 55F, 384–391
7. Wohlrab, H. (1976) J. Gerontol. 31, 257–263
8. Holloway, P. W. (1973) Anal. Biochem. 53, 304–308
9. Kasahara, M., and Hinkle, P. C. (1977) J. Biol. Chem. 252, 7384–7390
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
11. Wohlrab, H., Brigida, M., Flowers, N., and Costello, D. (1981) in Calcium and Phosphate Transport across Biomembranes (Bronner, F., and Peterlik, M., eds) Academic Press, New York 99–102
pH gradient-dependent phosphate transport catalyzed by the purified mitochondrial phosphate transport protein.

H Wohlrab and N Flowers

J. Biol. Chem. 1982, 257:28-31.