Studies of Cytochrome *c* Oxidase-Driven H^+-Coupled Phosphate Transport Catalyzed by the *Saccharomyces cerevisiae* Pho84 Permease in Coreconstituted Vesicles†

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ABSTRACT: The proton-coupled Pho84 phosphate permease of *Saccharomyces cerevisiae*, overexpressed as a histidine-tagged chimera in *Escherichia coli*, was detergent-solubilized, purified, and reconstituted into proteoliposomes. Proteoliposomes containing the Pho84 protein were fused with proteoliposomes containing purified cytochrome *c* oxidase from beef heart mitochondria. Both components of the coreconstituted system were functionally incorporated in tightly sealed membrane vesicles in which the cytochrome *c* oxidase-generated electrochemical proton gradient could drive phosphate transport via the proton-coupled Pho84 permease. The metal dependency of transport indicates that a metal−phosphate complex is the translocated substrate.

The H^+/phosphate cotransporter (Pho84p) of *Saccharomyces cerevisiae* is a polytopic membrane-spanning protein of the plasma membrane that catalyzes the coupled translocation of P_i and H^+ according to a symport mechanism (see refs *1*−*3* for reviews). This high-affinity transporter is encoded by the *PHO84* gene, which has been cloned and sequenced (*4*), shown to be derepressible by phosphate starvation, and regulated at the transcriptional level by the *PHO* regulatory pathway (*5*, *6*). Its synthesis is repressed by P_i concentrations of >100 μM (*6*). On the basis of hydropathy profile analysis of the primary amino acid sequence, a secondary structure model is proposed in which 12 transmembrane domains traverse the membrane in a zigzag fashion connected by hydrophilic loops, and with both N- and C-termini on the cytoplasmic side of the membrane (*2*). As in the case of several HXT sugar transporters (see refs *7* and *8* for recent reviews), the Pho84p exhibits a significant number of conserved residues between each of the two halves of the transporter, separated by the large central loop harboring 75 amino acids, suggesting that the protein has arisen from a duplication of an ancestral gene (*2*, *3*). The protein has been proposed to belong to the phosphate:H^+ symporter (PHS) family of functionally uniform members (*9*). Construction of a His-tagged version of the Pho84p has allowed for stable expression of the protein in the cytoplasmic membrane of *Escherichia coli*. The protein, solubilized from the membrane, purified, and reconstituted into liposomes, was shown to catalyze an uncoupler-sensitive transport of phosphate with a K_m typical of the high-affinity transporter (*10*). Thus, the proteoliposomes reconstituted with this polypeptide catalyze the high-affinity P_i transport activity observed in intact cells and in inside-out plasma membrane vesicles (*11*). Accordingly, in the presence of a Δp,[1] hydrogen ions move down their electrochemical gradient, driving the uphill translocation of P_i. As in other secondary transport systems which operate bidirectionally (*12*), the direction of P_i transport catalyzed by the Pho84p is determined by the direction of the driving force rather than by the orientation of the protein in the membrane. The dependence of P_i transport kinetics on membrane potential ($\Delta\psi$) and on the H^+ gradient (ΔpH) in proteoliposomes containing the purified protein is not known. Evidence for the presence of Δp-driven P_i uptake is lacking due to the presence of at least three different P_i symport systems in *S. cerevisiae* cells (see refs *2* and *3*), and the difficulty of varying the Δp without modifying other cellular parameters.

The study presented here describes the preparation and properties of highly active Pho84p/cytochrome *c* oxidase proteoliposomes, which are superior to previous reconstituted preparations of Pho84p (*10*). Thus, cytochrome *c* oxidase-generated Δp, which drives P_i transport into the liposomes,

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[1] Abbreviations: Δp, proton electrochemical gradient; $\Delta\psi$, membrane potential; ΔpH, transmembrane pH gradient; $DISC_3(5)$, 3,3′-dipropylthiodicarbocyanide iodide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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offers a suitable model system for studies of the interaction between the P_i transporter and the components of the driving force.

MATERIALS AND METHODS

Materials. ^{32}P (orthophosphate carrier free), anti-rabbit Ig donkey antibody-conjugated horseradish peroxidase (HRP), and the enhanced chemiluminescence detection kit were obtained from Amersham. His-Bind resin was from Novagen, and Sephadex G-25 was from Pharmacia. Chromatographically pure phosphatidylcholine (egg), phosphatidylethanolamine (egg), lysophosphatidylcholine (egg), and phosphatidylserine (bovine spinal cord) were purchased from Lipid Products. Biobeads SM2 were obtained from Bio-Rad. Pyranine and $DISC_3(5)$ were from Molecular Probes, Inc. All other materials were reagent grade and were obtained from commercial sources.

Purification and Reconstitution of Pho84p. The Pho84p was expressed as a His-tagged protein in *E. coli* BL21(DE3)-pLysS cells, carrying the expression plasmid pET16b. The protein was detergent-solubilized and purified on a His-Bind column in the presence of 0.1% Triton X-100 as described previously (*10*). Reconstitution of the purified protein into liposomes composed of 42.5% phosphatidylcholine (PC), 42.5% phosphatidylethanolamine (PE), 10% lysophosphatidylcholine (LPC), and 5% phosphatidylserine (PS) (all mass per volume) was performed essentially as described previously (*10*), with the modification that removal of Triton X-100 by rapid dilution was replaced by adsorption of the detergent onto polystyrene beads (Biobeads SM2). The appropriate amounts of lipids were dried under N_2(g), redissolved in diethyl ether, washed with ethanol, dried, resuspended to a final concentration of 20 mg/mL in 25 mM K^+/Hepes (pH 7.0) and 0.1% Triton X-100, sonicated to clarity, and mixed with purified Pho84 protein at a concentration of 0.14 mg/mL. The protein/lipid mixture was incubated for 30 min at 4 °C, after which the detergent was removed by a sequential Biobeads treatment essentially according to the method of Knol et al. (*13*). Fresh Biobeads (80 mg/mL of liposome mixture), extensively washed with methanol and ethanol and finally rinsed in water, were added and incubated at 4 °C under slow agitation. After 2 h, the Biobeads were removed by centrifugation and fresh Biobeads (150 mg/mL) were added. After an additional 2 h, the beads were removed and the procedure was repeated with a 12 h treatment. After removal of the Biobeads, the proteoliposomes were collected by centrifugation at 247000*g* for 10 min. Proteoliposomes were resuspended in 25 mM K^+/Hepes (pH 7.0) at protein and lipid concentrations of 0.32 and 32 mg/mL, respectively, and stored in liquid nitrogen.

Purification and Reconstitution of Beef Heart Cytochrome c Oxidase. Cytochome *c* oxidase from beef heart mitochondria was purified as previously described (*14*) and reconstituted into liposomes composed of 42.5% PC, 42.5% PE, 10% LPC, and 5% PS as described above. The appropriate amounts of lipids were dried under N_2(g), redissolved in diethyl ether, washed with ethanol, dried, resuspended to a final concentration of 20 mg/mL in 25 mM K^+/Hepes (pH 7.0) and 30 mM octyl β-D-glucoside, and sonicated to clarity. Purified cytochrome *c* oxidase at a concentration of 0.23 μmol/mL was added to 2 mL of the lipid/detergent mixture so the final concentration of cytochrome *c* oxidase became 2.2 nmol/mL. The protein/lipid/detergent mixture was allowed to equilibrate during dialysis at 4 °C twice for 1 h and once for 12 h against 500 volumes of 25 mM K^+/Hepes (pH 7.0). The dialyzed preparation was aliquoted and stored in liquid nitrogen.

Fusion of Pho84p and Cytochrome c Oxidase-Containing Liposomes. Fusion of the individually reconstituted proteoliposomes was accomplished by use of a freeze/thaw/sonication method (*15*). Thawing of the proteoliposomes was carried out at 25 °C. The sonication step consisted of two pulses each for 2 s at an output efficiency of 13 W. One hundred microliters of Pho84p-containing liposomes and 50 μL of cytochrome *c* oxidase-containing liposomes were mixed, and the mixture was added to 10 mM KCl, resulting in a final Pho84p/cytochrome *c* oxidase molar ratio of 63. Freshly prepared fused proteoliposomes were used in transport assay experiments.

Estimation of Membrane Potential ($\Delta\psi$) and pH Gradient (ΔpH). Fifteen microliters of fused proteoliposomes was diluted into 1 mL of oxygen-saturated 25 mM K^+/Hepes (pH 7.0) and $DISC_3(5)$ probe to a final concentration of 2.94 μM. After addition of 200 μM TMPD and 10 mM potassium ascorbate, the reaction was started by adding 10 μM cytochrome *c*. The decrease in fluorescence was monitored at an emission wavelength of 666 nm; the excitation wavelength was 643 nm (*16*). Where indicated, nigericin and valinomycin were added to final concentrations of 200 nM.

The prevailing pH gradient across the liposomal membrane was monitored by use of the fluorescent pH indicator pyranine (*17*). Following liposome entrapment of the probe during the freeze/thaw/sonication step as described previously (*16*), the external probe was removed by Sephadex G-25 gel filtration chromatography. Pyranine-containing proteoliposomes were diluted into oxygen-saturated buffer composed of 25 mM K^+/Hepes (pH 7.0) and 5 mM $MgSO_4$ to a final concentration of 46 nM with respect to cytochrome *c* oxidase, and the ΔpH was estimated from the changes in pyranine fluorescence monitored at an emission wavelength of 512 nm. The excitation wavelength was 454 nm.

Phosphate Transport Assay. Phosphate uptake was assayed in coreconstituted proteoliposomes energized by the Δp (inside negative and alkaline) generated by cytochrome *c* oxidase. To 15 μL of fused proteoliposomes, diluted into 975 μL of 25 mM K^+/Hepes (pH 7.0), were added 200 μM TMPD and 10 μM cytochrome *c* with 10 mM potassium ascorbate. After 30 s of water-saturated aeration, the reaction was started by addition of ^{32}P at a final phosphate concentration of 0.11 mM. At chosen time points, 50 μL of the sample was withdrawn, and the reaction was quenched in 2 mL of ice-cold 0.1 M LiCl and the mixture immediately filtered. The filter (type Supor-200, 0.2 μm, Gelman Sciences) was washed once with the same cold solution. Radioactivity retained on the filter was quantitated by liquid scintillation spectrometry. Prior to use, all glassware was washed in concentrated sulfuric acid and thoroughly rinsed in bidistilled water.

Protein Determination. Protein was assayed by use of the commercially available Bio-Rad *DC* Protein Assay kit. Bovine serum albumin was used as a standard.

RESULTS

Functional Coreconstitution of Pho84p and Cytochrome c Oxidase. Reconstitution of purified, detergent-solubilized Pho84p into liposomes was accomplished using the following lipid composition: 42.5% PC, 42.5% PE, 10% LPC, and 5% PS. Reconstitution of active H^+-pumping cytochrome *c* oxidase purified from beef heart mitochondria in liposomes composed of acetone/ether-washed *E. coli* lipids and L-α-phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w) has previously been shown (*15*). The same lipid composition was also used in the functional unidirectional reconstitution of the purified lactose transport system (LacS) of *Streptococcus thermophilus* (*13*). Although the purified Pho84p could be integrated into the liposomes composed of *E. coli* lipids and L-α-phosphatidylcholine, the protein was not active in this lipid environment (not shown). Also, fusion of liposomes containing purified Pho84 in the PC/PE/LPC/PS mixture with liposomes containing purified cytochrome *c* oxidase reconstituted in the *E. coli* lipids/L-α-phosphatidylcholine mixture abolished the Pho84p transport activity (not shown). As the purified cytochrome *c* oxidase maintained its H^+-pumping activity when reconstituted in liposomes composed of PC/PE/LPC/PS, this lipid composition was used for individual reconstitutions of Pho84p and cytochrome *c* oxidase. Although both proteins, Pho84p and cytochrome *c* oxidase, could be reconstituted into liposomes containing an identical lipid composition, the strategies of reconstitution were different. Fusion of the two proteoliposomal preparations was accomplished by a freeze/thaw/sonication technique, as described in Materials and Methods, and freshly prepared fused proteoliposomes were used in the experiments described.

Measurements of the Transmembrane Electric Potential and pH Gradient. After coreconstitution of cytochrome *c* oxidase with Pho84p, both $\Delta\psi$ and ΔpH were generated by cytochrome *c* oxidase in the presence of reduced cytochrome *c* (Figures 1 and 2). The use of the membrane-impermeable electron donor cytochrome *c* only permits activity of those oxidase molecules with the cytochrome *c* binding site exposed to the outside of the liposomes. This guarantees the formation of a Δp with the right polarity, that is, $\Delta\psi$, inside negative, and ΔpH, inside alkaline (*18*). Cytochrome *c* oxidase-generated $\Delta\psi$ across the membrane of the fused proteoliposomes was monitored with the fluorescent dye $DISC_3(5)$. Addition of ascorbate, cytochrome *c*, and the electron mediator TMPD resulted in a rapid generation of $\Delta\psi$ (Figure 1). Addition of the K^+/H^+ ionophore nigericin increased the transmembrane $\Delta\psi$ as anticipated, and this $\Delta\psi$ was stably maintained for at least 20 min (not shown). The $DISC_3(5)$ fluorescence was fully recovered upon addition of valinomycin, reflecting dissipation of the $\Delta\psi$ across the liposomal membrane (Figure 1A−E). Additions of bivalent cations such as Mn^{2+} or Mg^{2+} (Figure 1C) or EDTA (Figure 1E) did not perturb the generated $\Delta\psi$ across the membrane.

The magnitude of ΔpH was measured with the fluorescent pH indicator pyranine entrapped by the liposomes during the freeze/thaw/sonication step. After the addition of ascorbate, cytochrome *c*, and TMPD, the internal pH increased rapidly in the presence of valinomycin (Figure 2), reaching a steady-state ΔpH of 1.5 pH units. Calculation of the pH was achieved by titration of the fluorescent signal with base

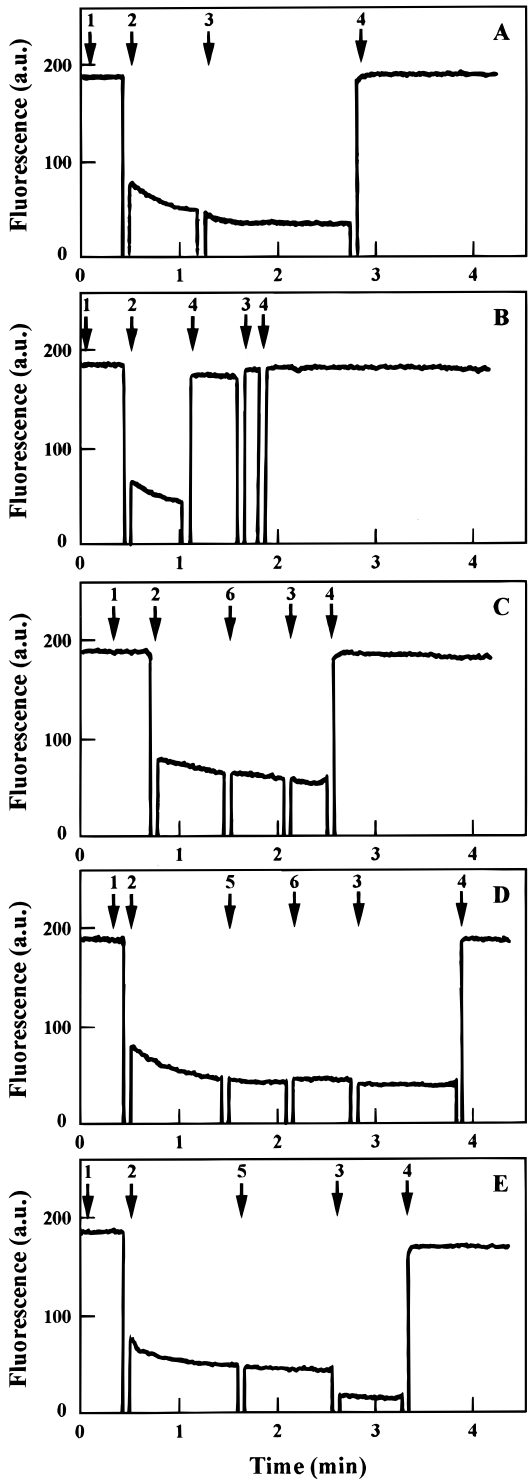

FIGURE 1: Generation of $\Delta\psi$ across proteoliposomal membranes containing Pho84p and cytochrome *c* oxidase. Fifteen microliters of fused proteoliposomes, diluted into 2 mL of oxygen-saturated 25 mM K^+/Hepes (pH 7.0), was equilibrated with $DISC_3(5)$, potassium ascorbate, and TMPD at final concentrations of 2.94, 10, and 200 μM, respectively, for 15−30 s (arrow 1) for panels A−D. The $\Delta\psi$ was generated by the addition of 10 μM cytochrome *c* (arrow 2). The decrease in fluorescence was enhanced by addition of 200 nM nigericin (arrow 3) and was fully abolished by addition of 200 nM valinomycin (arrow 4). Neither addition of bivalent cations Mn^{2+}, Mg^{2+}, and Co^{2+} at 2 mM (arrow 5) nor addition of 0.5 mM EDTA (arrow 6) affected the generated $\Delta\psi$. Shown in panel C and E are results from experiments in which the divalent cations and chelator, respectively, were included in the calibration solution (arrow 1).

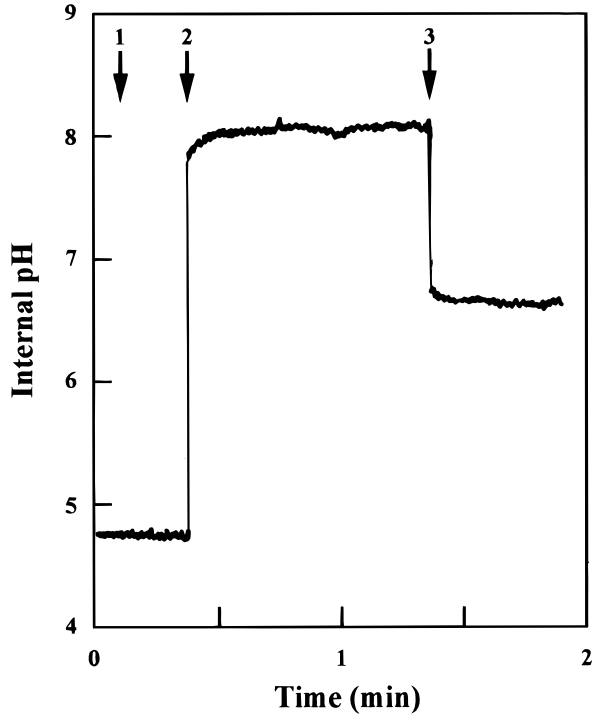

FIGURE 2: Internal pH change induced by the oxidation of reduced cytochrome *c* by proteoliposomes containing Pho84p and cytochrome *c* oxidase. Internal pH changes were measured by following the fluorescence of pyranine entrapped in the fused membranes as described in Materials and Methods. The reaction mixture contained potassium ascorbate, TMPD, and valinomycin at final concentrations of 10 mM, 200 μM, and 200 nM, respectively, in 25 mM K^+/Hepes (pH 7.0) (arrow 1). The reaction was initiated by addition of 10 μM cytochrome *c* (arrow 2). Nigericin (200 nM) was added as indicated (arrow 3). Fluorescence changes were calibrated by titration with NaOH and phosphoric acid in the presence of nigericin.

and measuring the pH values. Phosphate uptake and the magnitude of ΔpH and $\Delta\psi$ were measured under the same experimental conditions.

Assessment of Phosphate Transport. The Pho84 transporter has previously been shown to exhibit a pH optimum of 4.5 in catalysis of P_i transport (*19*, *20*). However, due to a pronounced decrease in cytochrome *c* oxidase activity at acidic pH values (*21*), the experiments described here were performed at pH 7.0, a pH value at which the Pho84 transporter retains about 50% of its transport activity. As shown in Figure 3A, the fused proteoliposomes were able to catalyze accumulation of ^{32}P upon energization. The level of ^{32}P accumulation was dependent on the Δp generated by the cytochrome *c* oxidase since uptake was abolished in the absence of cytochrome *c* (Figure 3A), and in the presence of valinomycin and nigericin (Figure 3B). The role of each component of the Δp in driving phosphate uptake was investigated in more detail by manipulating ΔpH and $\Delta\psi$ with nigericin and valinomycin. In the presence of nigericin, when Δp consists only of a membrane potential, the inital rate of phosphate uptake was lowered about 3-fold. Addition of valinomycin reduced the initial rate about 2-fold. However, the phosphate accumulation reached similar levels when measured for prolonged periods of time, irrespective of whether valinomycin or nigericin was present. In the presence of both ionophores, the initial rate of uptake measured during the first minute was similar to the rate measured in the

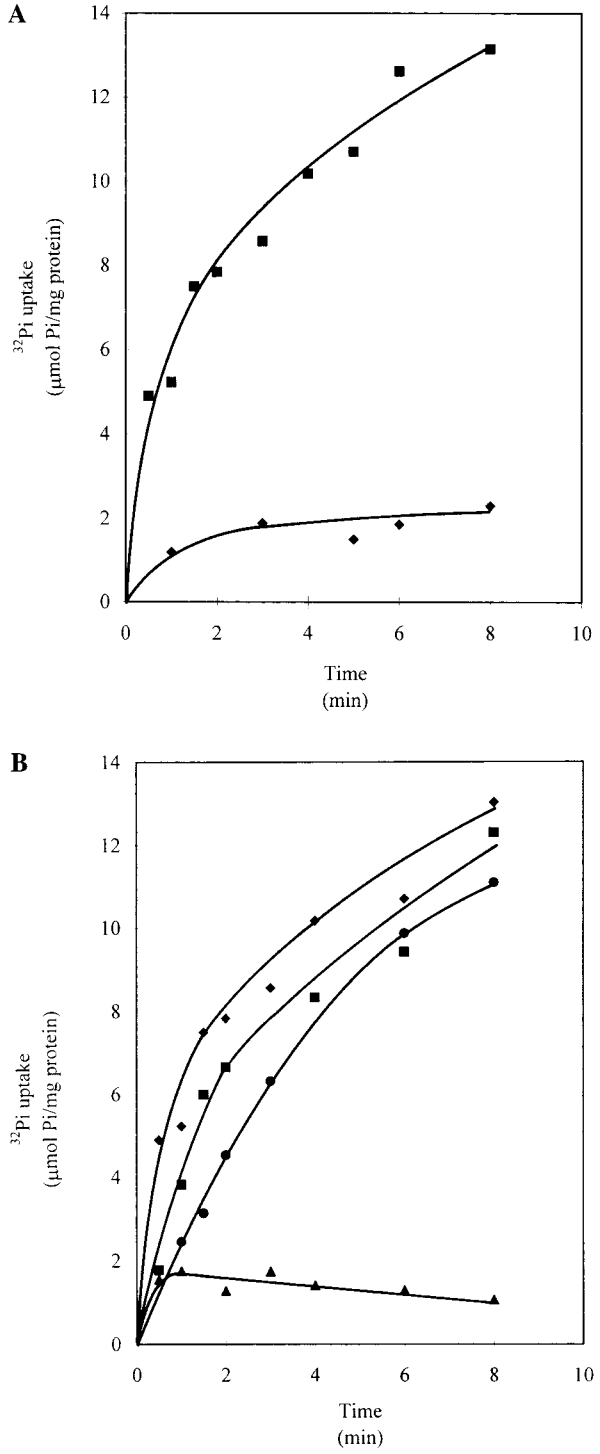

FIGURE 3: Δp-driven P_i accumulation by proteoliposomes reconstituted with purified cytochome *c* oxidase and purified Pho84 protein. Proteoliposomes containing purified Pho84 transporter and cytochome *c* oxidase at a molar ratio of 63 were prepared as described in Materials and Methods. (A) ^{32}P transport was assayed by use of 0.11 mM P_i in the presence of ascorbate and TMPD at final concentrations of 10 and 200 μM, respectively, in the presence (■) and absence (◆) of 10 μM cytochrome *c*. (B) Experimental details are the same as for panel A. P_i transport was assayed in the presence of ascorbate, TMPD, and cytochrome *c* in the presence of 200 nM valinomycin (■), in the presence of 200 nM nigericin (●), and in the absence (◆) and presence (▲) of both ionophores at 200 nM.

presence of ΔpH or $\Delta\psi$. The results show (Figure 3B) that both components of the Δp are competent and interchangeable in driving phosphate uptake.

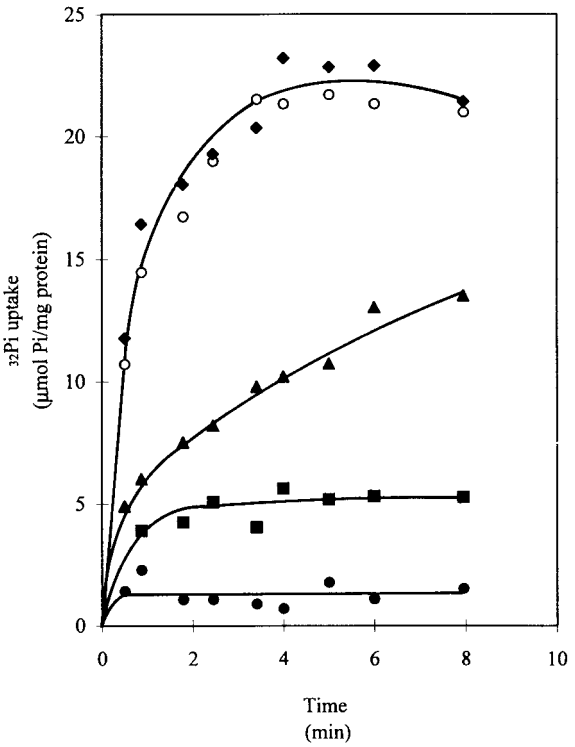

FIGURE 4: Δ*p*-driven P_i accumulation by proteoliposomes reconstituted with cytochrome *c* oxidase and purified Pho84. Experimental details are the same as described in the legend of Figure 3A. Transport was assessed in the absence (▲) and in the presence of 2 mM Mn^{2+} (◆), 2 mM Co^{2+} (○), 2 mM Mg^{2+} (■), and 0.5 mM EDTA (●).

Effect of Metal Ions on Phosphate Transport. Pho84-mediated phosphate uptake driven by an imposed Δ*p* was nearly completely inhibited in the presence of 0.5 mM EDTA (Figure 4). As shown in Figure 1, the generation or maintenance of the $\Delta\psi$ across the membrane was not affected by the chelating agent (Figure 1E). Addition of Mn^{2+} and Co^{2+} resulted in an approximately 3-fold stimulation of the Δ*p*-driven phosphate uptake rate, that is, from 7 to 20 μmol min^{-1} (mg of protein)$^{-1}$ (Figure 4). Phosphate accumulation was limited to about 5 μmol/mg after an initial rapid uptake during the first minute of uptake in the presence of Mg^{2+} which is still higher than that observed in the presence of EDTA (Figure 4). Addition of the divalent cations did not affect the generation or maintenance of the $\Delta\psi$ across the membrane as judged from the measurements with the fluorescent dye $DISC_3(5)$ (Figure 1C). The divalent cations Mg^{2+}, Mn^{2+}, and Co^{2+} form soluble, electroneutral metal−phosphate complexes ($MeHPO_4$). At the experimental P_i and Mg^{2+}, Mn^{2+}, and Co^{2+} concentrations, 36% (Mg^{2+}), 87% (Mn^{2+}), and 70% (Co^{2+}) of the P_i is at pH 7 present as metal−P_i complexes in the incubation mixture (*22*−*24*). These observations indicate that P_i is transported as $MeHPO_4$ in symport with H^+, in agreement with the phosphate uptake system in *Acinetobacter johnsonii* (*25*). When EDTA was added to energized proteoliposomes prior to addition of the divalent cations, phosphate uptake was essentially abolished as in the presence of EDTA alone (not shown).

DISCUSSION

The study presented here describes the successful reconstitution, from purified components, of a Δ*p*-driven Pho84 phosphate permease. In this study, phosphate transport was analyzed in defined proteoliposomes obtained by fusion of proteoliposomes containing *E. coli*-expressed, purified, and reconstituted Pho84 phosphate permease of *S. cerevisiae* and proteoliposomes containing purified and reconstituted cytochrome *c* oxidase from beef heart mitochondria. The Pho84 protein was expressed as a stable His-tagged chimera in *E. coli*, extracted from the bacterial cytoplasmic membrane by Triton X-100, and purified by Ni^{2+} chelation chromatography according to a previously published method (*10*). Although previous characterization of phosphate transport activity of the purified protein was accomplished by reconstitution into liposomes with the same lipid composition as used in this work, the driving force for Pho84-catalyzed phosphate accumulation was provided by an artificial pH gradient (interior alkaline). Reconstitution of Pho84 into liposomes composed of *E. coli* phospholipids and egg phosphatidylcholine as previously described for reconstitution of lactose transporter of *St. thermophilus* (*13*) and prepared according to the procedure described by Berhe et al. (*10*) resulted in an inactive membrane-bound Pho84 permease. In the study presented here, we have shown that purified cytochome *c* oxidase from beef heart can be functionally reconstituted into artificial lipid membranes with the lipid composition previously successfully employed for coreconstitution of purified nicotinamide nucleotide transhydrogenase and ATPase from beef heart mitochondria (*26*), purified beef heart nicotinamide nucleotide transhydrogenase and bacteriorhodopsin from *Halobacterium halobium* (*27*), and the Pho84 phosphate permease of *S. cerevisiae* (*10*). The incorporation of primary proton pumps in proteoliposomes thus constitutes an attractive possibility in studies of Δ*p*-dependent processes in isolated membrane vesicles with proteins from both bacterial or eukaryotic origin. Upon energization, the Pho84/cytochrome *c* oxidase proteoliposomes were able to actively transport phosphate in a process that is consistent with solute/H^+ symport (Figure 3). In agreement with previous findings, where the activity of cytochrome *c* oxidase was shown to be dramatically decreased at pH <5.5 (*21*), cytochrome *c* oxidase at the pH optimum for the Pho84 permease, that is, at pH 4.5, failed to generate a Δ*p*. Although Pho84-mediated phosphate transport is less efficient when assessed at pH 7, the transporter maintains approximately 50% of its catalytic power at that pH (not shown). The attractive feature of using cytochrome *c* oxidase as a Δ*p*-generating system for the Pho84p phosphate accumulation process is that it provides a well-defined model system in which a right-side-out oriented Δ*p* (interior negative and alkaline) can be generated. The rate of uptake of phosphate and the magnitude of ΔpH and $\Delta\psi$ were measured under the same experimental conditions. The role of each component of the Δ*p* in driving phosphate uptake was investigated in more detail by manipulating ΔpH and $\Delta\psi$. These studies with the ionophores nigericin and valinomycin (Figures 1, 2, and 3B) revealed that Pho84-mediated transport could be driven by either one of the components.

The observation that bivalent cations such as Mn^{2+} and Co^{2+} stimulate the phosphate uptake process in the proteoliposomes, while addition of a chelator such as EDTA eliminates phosphate transport, suggests that the Pho84p permease transport activity is dependent on the presence of bivalent cations. As in the case of the phosphate transport

system of *A. johnsonii 210A* (*25*), the presence of Mg^{2+} exerted an inhibitory effect on phosphate accumulation in reconstituted proteoliposomes as compared to the effects of Mn^{2+} and Co^{2+} (Figure 4). When the predominance of the $MeHPO_4$ complex under the experimental conditions used in this study and the influence on the Pho84p transport activity are considered, the obtained results suggest that P_i is most likely transported as a $MeHPO_4$ complex.

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