Characterization of purified and unidirectionally reconstituted Pho84 phosphate permease of *Saccharomyces cerevisiae*

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Abstract Hydropathy analysis of the amino acid sequence of the Pho84 phosphate permease of *Saccharomyces cerevisiae* suggests that the protein consists of 12 transmembrane domains connected by hydrophilic loops. The Pho84 protein has been modified by a gene fusion approach, yielding two different N-terminal His-tagged chimeras which can be expressed in *Escherichia coli*, purified and functionally reconstituted into defined proteoliposomes. The continuous epitopes in the N- and C-terminal sequences of the Pho84 chimeras were shown to be accessible in proteoliposomes containing the purified active Pho84 proteins. Site-specific proteolysis of the immunoreactive N-terminal sequence in the reconstituted protein suggests a unidirectional insertion into liposomes.

Key words: Phosphate transporter; Pho84; *PHO* gene; Plasma membrane; Reconstitution

1. Introduction

The Pho84 phosphate permease of *Saccharomyces cerevisiae*, encoded by the *PHO84* gene [1], belongs to a family of phosphate:H^+ symporters and is a member of the major facilitator superfamily [2]. This hydrophobic integral membrane protein consisting of 587 amino acid residues catalyzes the coupled transport (symport) of phosphate and H^+ across the yeast plasma membrane by conversion of the energy stored in an electrochemical H^+ gradient into energy for translocation of phosphate into the cell (see [3–5] for reviews). The synthesis and activity of this transporter are regulated at the transcriptional level by the *PHO* regulatory pathway [6,7]. Synthesis and activation of this high-affinity transporter is strictly regulated and is favored at external phosphate concentrations lower than 100 μM. Activation of the Pho84 phosphate transport system thus allows the cells to scavenge phosphate from the environment under conditions of phosphate limitation. However, upon exhaustion of external phosphate to concentrations below 50 μM, de novo synthesis of the Pho84 protein is halted and high-affinity phosphate transport across the plasma membrane is abolished by a degradative rerouting of the protein to the vacuole ([7], Petersson and Persson, unpublished information). Although recent evidence has been presented that additional gene products such as Pho86 [8], Pho87 [9], Pho88 [10] and Gtr1, a putative GTP-binding protein [11], act together with the Pho84 permease in a regulatory unit, strong evidence that the Pho84 protein contains the phosphate translocation pathway has been obtained in intact cells by gene disruption analyses [1,12], in inverted plasma membrane vesicles [13] and, unequivocally, by *Escherichia coli* expression of a histidine-tagged Pho84 protein which was shown to catalyze an uncoupler-sensitive accumulation of phosphate after purification and reconstitution [14]. Thus, proteoliposomes reconstituted with this polypeptide catalyze the high-affinity phosphate transport observed in intact cells and inverted plasma membrane vesicles. Accordingly, in the presence of a proton electrochemical gradient (Δp), hydrogen ions move down their electrochemical gradient, driving up-hill translocation of phosphate. Based on hydropathy analysis, a secondary structure model has been proposed (see [5]) in which the transporter contains 12 transmembrane segments connected by hydrophilic loops and expanded N- and C-termini at the same membrane face. In order to characterize the topology and kinetics of the purified transporter, an unidirectional reconstitution of the protein into liposomes is desirable. In the present study, we show that two purified Pho84 fusion proteins with highly polar His-tagged peptides of 23 and 33 amino acid residues, respectively, attached to the N-terminus of Pho84 can be unidirectionally reconstituted into catalytically active proteoliposomes with their N- and C-termini exposed on the exterior face of the vesicles. Furthermore, interactions between the termini of the reconstituted Pho84 protein and antibodies directed against these perturb Pho84-catalyzed phosphate transport.

2. Materials and methods

2.1. Materials

[^{32}P]orthophosphate (carrier free), anti-rabbit Ig donkey antibody-conjugated horseradish peroxidase (HRP) and an enhanced chemiluminescence detection kit were obtained from Amersham Pharmacia Biotech (Sweden). Rabbit antisera against synthesized peptides corresponding to partial sequences of the N- and C-termini of the Pho84 protein were raised and purified as described previously [13]. AntiXpress (Axp) antibodies were obtained from Invitrogen (The Netherlands). Alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse secondary antibodies and CDP-Star chemiluminescent substrate were purchased from Perkin-Elmer (Sweden). Chromatographically pure phosphatidylcholine (egg), phosphatidylethanolamine (egg), lysophosphatidylcholine (egg) and phosphatidylserine (bovine spinal cord) were purchased from Lipid Products (UK). The endoproteinase enterokinase (EK) (bovine, light chain) was purchased from Calbiochem (USA). Bio-Beads (SM-2) were obtained from Bio-Rad (Sweden).

2.2. Cloning and expression of Pho84 in E. coli

The Pho84 protein was expressed as a histidine-tagged protein in *E. coli* BL21(DE3)pLysS cells, carrying the expression plasmid pET16b (Novagen). The N-terminal His_{10}-tagged fusion protein was detergent-solubilized and purified by immobilized Ni^{2+} affinity chromatography

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Δp, proton electrochemical gradient; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)

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in the presence of 0.1% Triton X-100 as described previously [14]. In order to clone and express a histidine-tagged Pho84 protein containing a unique EK protease cleavage site and an additional antibody recognition epitope in the sequence connecting the fused domains, the *PHO84* gene was PCR-amplified from the PET16b construct and was cloned into a pTrcHisB plasmid (Invitrogen, The Netherlands). The novel fusion construct encodes a sequence of six consecutive histidine residues and the amino acid sequence GMASMTGGNNMGR-DLYDDDDKD harboring an AXp epitope (DLYDDDDK) and an EK recognition site (DDDDK) at the 5′-end of *PHO84*, yielding plasmid pTrcHisB/His_6-AXp/EK-*PHO84* (Fig. 1). The resulting construct was verified by DNA sequencing of double-stranded plasmid DNA using an ABI 377 automated DNA sequencer and the Thermo-Sequenase dye terminator cycle sequencing kit (Amersham Pharmacia). *E. coli* TOP10 cells (Invitrogen) harboring pTrcHisB/His_6-AXp/EK-*PHO84* were grown aerobically in TB medium [15] at 30°C in the presence of 100 μg/ml ampicillin essentially as described for BL21(DE3)pLysS harboring PET16b/His_{10}-*PHO84* [14]. Pho84 expression was in both cases initiated by addition of 1 mM isopropyl thio-β-D-galactoside to cell cultures which had reached an A_{600} of 0.6. After further growth for 4 h in the case of PET16b/His_{10}-*PHO84* and for 24 h in the case of pTrcHisB/His_6-AXp/EK-*PHO84*, cells were harvested by centrifugation as described [14].

2.3. Purification and reconstitution of Pho84 chimeras

Harvested cells were resuspended at a concentration of 10 g wet weight per 100 ml in 20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 2 mM imidazole and 2% Triton X-100 and subjected to lysis in the presence of lysozyme (about 1 mg/g cells) and 1 mM phenylmethylsulfonyl-fluoride followed by pulsed microtip sonic treatment (Branson Sonifier 250) at a 40% output efficiency until the lysate was homogeneous and no longer viscous. Both pET16b-expressed His_{10}-Pho84 and pTrcHisB-expressed His_6-AXp/EK-Pho84 were solubilized from the cytoplasmic membrane of their expression hosts in the presence of Triton X-100 essentially as described [14]. Detergent-solubilized protein samples were filtered through a 0.45 μm filter and applied onto an immobilized Ni^{2+} affinity resin, pre-equilibrated with 20 mM sodium phosphate, pH 8.0, alternatively 20 mM Tris-HCl, pH 8.0, in the presence of 0.5 M NaCl, 2 mM imidazole and 0.2% Triton X-100, followed by removal of non-bound and loosely bound proteins by washing with eight bed volumes of equilibration buffer and by eight bed volumes of equilibration buffer containing 60 mM imidazole, respectively. Fractions eluted in the presence of 120 mM imidazole were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein detection. Pho84-containing fractions were pooled and subjected to buffer exchange by use of a HiPrep desalting (Amersham Pharmacia) column. Collected fractions containing purified Pho84 in 20 mM Tris-HCl, pH 8.0, 0.2% Triton X-100, 0.2 M NaCl and 10% glycerol were pooled and reconstituted.

2.4. Reconstitution of Pho84 chimeras

Reconstitution of the purified His_6-AXp/EK-Pho84 and His_{10}-Pho84 chimeras 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 [14]. The appropriate amounts of lipids were dried under N_2 (g), re-dissolved in diethyl ether, dried, resuspended to a final concentration of 20 mg/ml in 25 mM K^+-*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 6.6, and sonicated to near clearness with a microtip sonicator (Branson Sonifier 250) at a 20% output efficiency. The purified His_6-AXp/EK-Pho84 and His_{10}-Pho84 contained in elution buffer (20 mM Tris-HCl, pH 8.0, 0.2% Triton X-100, 0.2 M NaCl and 10% glycerol) was mixed with the liposomes at a protein concentration of 0.15 mg/ml. The protein-lipid mixture was incubated for 30 min at 4°C, after which the detergent was removed by a sequential Bio-Beads treatment essentially according to the method of Knol et al. [16]. Fresh Bio-Beads (80 mg), extensively washed with methanol, ethanol and finally rinsed in water, were added per ml of liposome mixture and incubated for 2 h at 4°C under slow agitation. After removal of the Bio-Beads by centrifugation, fresh Bio-Beads (150 mg/ml) were added. After an additional 2 h, the beads were removed and the procedure was repeated with a 12 h treatment. Following removal of the Bio-Beads, the proteoliposomes were collected by centrifugation at 133 500 × *g* for 1 h and resuspended in 25 mM K^+-HEPES, pH 6.6, at a lipid concentration of 20 mg/ml, frozen in liquid N_2 and stored at −80°C. Preparation of liposomes devoid of protein was accomplished essentially as described above except that protein and Triton X-100 additions were excluded.

2.5. Transport measurements with proteoliposomes

Phosphate uptake was measured in proteoliposomes containing His_{10}-Pho84 and His_6-AXp/EK-Pho84. Proteoliposomes (1 μl) pre-loaded with 25 mM K^+-HEPES, pH 6.6, were diluted 200-fold in 25 mM Tris-succinate, pH 4.5, containing 0.11 mM [^{32}P]orthophosphate (0.18 Ci/μmol; 1 mCi = 37 MBq) to generate a Δp (interior alkaline). The suspension was immediately blended in a vortex mixer and incubated at 25°C. Transport assays were terminated at a given time by quenching of the reaction with 2 ml ice-cold dilution buffer (25 mM Tris-succinate, pH 4.5) containing 150 mM LiCl and immediate filtration using Supor-200 filters (0.2 mm pore size, Pall-Gelman Sciences). Filters were washed with an additional 2 ml of the quench buffer and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

2.6. Proteolytic cleavage

Purified, detergent-solubilized protein (10 μg) and proteoliposomes containing the reconstituted His_6-AXp/EK-Pho84 chimera (10 μg) were incubated with 0.2 U EK for 5 h at 37°C followed by 16 h at room temperature, in the presence of 50 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. The liposomes were washed two times with 1 ml 25 mM K^+-HEPES, pH 6.6, pelleted by centrifugation at 247 000 × *g* for 30 min and resuspended in 50 μl of the same buffer. Subsequently, liposomes were solubilized in 1.3% SDS and immediately subjected to SDS-PAGE followed by Western blotting. Immunodetection was accomplished with AXp antibody.

2.7. Analytical methods

SDS-PAGE was performed using a 12% polyacrylamide and bispolyacrylamide gel system [17]. Gels were stained with GelCode Blue Stain Reagent (Pierce). Specific detection of detergent-solubilized and reconstituted Pho84 chimeras was achieved by Western blot analysis using purified Pho84 anti-(C-terminal peptide) and anti-(N-terminal peptide) antibody and anti-rabbit Ig donkey antibody-conjugated HRP or AXp antibody and goat-anti mouse secondary antibody conjugated to alkaline phosphatase. After a short incubation with chemiluminescent substrate, the blot was exposed to film for 1–2 min. The molecular mass of separated proteins was determined by the relative mobilities of the pre-stained marker proteins (Bio-Rad). Protein determination was performed by use of the commercially available Bio-Rad *DC* Protein Assay kit (Bio-Rad). Bovine serum albumin was used as standard.

3. Results

3.1. Properties of constructed Pho84 chimeras

We have previously shown that a constructed histidine-tagged version of the H^+-coupled Pho84 phosphate permease of *S. cerevisiae* can be expressed in the cytoplasmic membrane of *E. coli* and that immobilized Ni^{2+} affinity chromatography of the Triton X-100-solubilized His_{10}-Pho84 chimera allows for an efficient purification of the protein [14]. Although the purified His_{10}-Pho84 catalyzes a high-affinity uncoupler-sensitive uptake of phosphate when reconstituted in proteoliposomes with a defined phospholipid composition [14], it was desirable to construct an alternative histidine-tagged Pho84 protein in order to facilitate immunological studies of the reconstituted chimera. The novel His_6-Pho84 protein contains an AXp antibody epitope and a partially overlapping EK recognition site in the sequence connecting the His_6 sequence to the N-terminus of the Pho84 protein (Fig. 1). The His_6-AXp/EK-Pho84 protein can, like the His_{10}-Pho84 protein, be expressed in the cytoplasmic membrane of *E. coli*, solubilized with Triton X-100, purified by immobilized Ni^{2+} affinity chro-

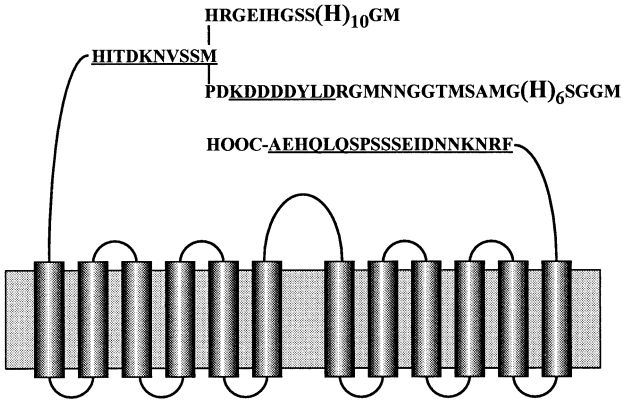

Fig. 1. Schematic secondary structure model of the Pho84 chimeras. The model is based on hydropathy analysis of the deduced Pho84 amino acid sequence [1]. Putative transmembrane segments are shown as boxes. The N- and C-terminal regions of the transporter corresponding to the epitopes recognized by the anti-(N-terminal peptide) antibody and anti-(C-terminal peptide) antibody, the Axp antibody recognition sites in the histidine-Pho84 chimera are indicated by underlined one-letter amino acid codes.

matography and reconstituted into proteoliposomes. As shown in Fig. 2, the purified protein in the detergent-solubilized state and in proteoliposomes could be detected as a single band with an apparent molecular mass of about 75 kDa by staining and by immunodetection of the epitopes contained within the Pho84 protein by use of anti-(N-terminal peptide) and anti-(C-terminal peptide) antibodies and by the AXp epitope in the His_6-AXp/EK-Pho84 protein.

3.2. Activity of Pho84 in proteoliposomes

The Triton X-100-solubilized, purified His_6-AXp/EK-Pho84 protein was reconstituted into defined proteoliposomes composed of PC/PE/PS/LPC. The H^+-coupled symport activity of reconstituted His_6-AXp/EK-Pho84 protein was investigated by measuring ^{32}P accumulation in proteoliposomes loaded with 25 mM K^+-HEPES, pH 6.6. A Δp (interior alkaline and negative) was imposed across the proteoliposomal membrane by a 200-fold dilution of the proteoliposomes into a buffer with pH 4.5. In Fig. 3, it is shown that Δp-driven uptake of phosphate occurs at an initial rate of 0.3 μmol/min/mg protein. In the presence of the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP), phosphate accumulation was almost completely inhibited after an initial rapid rate of uptake of 0.3 μmol phosphate/mg during the first min of the reaction. As the His_6-AXp/EK-Pho84 fusion protein is functionally competent when reconstituted into proteoliposomes, it is clear that the N-terminal sequence attachment does not lead to inactivation of the transport function. However, in an experiment where the anti-(N-terminal peptide) and anti-(C-terminal peptide) antibodies were incubated with proteoliposomes harboring the purified His_{10}-Pho84 protein, the rate of Δp-driven phosphate accumulation was inhibited by 50% in the presence of the anti-(N-terminal peptide) antibody and by 75% in the presence of anti-(C-terminal peptide) antibodies (Fig. 4). In contrast, treatment of the proteoliposomes with an antibody directed against the PMA1 H^+-ATPase of *S. cerevisiae* did not affect the transport activity of the Pho84 protein.

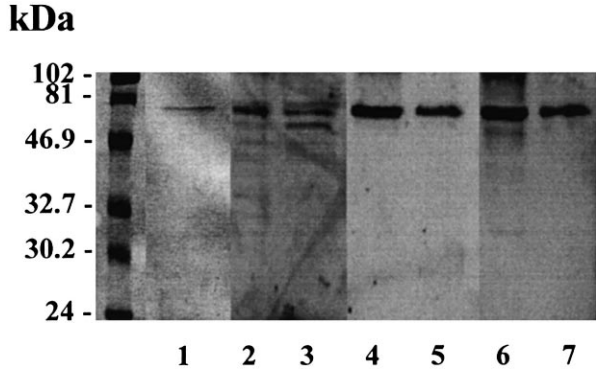

Fig. 2. SDS-PAGE of purified and reconstituted His_6-AXp/EK-Pho84 in proteoliposomes. The presence of purified His_6-AXp/EK-Pho84 protein (2 μg) in proteoliposomes was visualized by 12% PAGE followed by protein staining (lane 1). The immunoreactivity of purified Triton X-100-solubilized (1.5 μg) and reconstituted (4 μg) His_6-AXp/EK-Pho84 protein was analyzed by Western blot analysis with anti-(N-terminal peptide) antibody (lane 2 and 3, respectively), anti-(C-terminal peptide) antibody (lane 4 and 5, respectively) and AXp antibody (lane 6 and 7, respectively). The protein in proteoliposomes was solubilized with 1.3% SDS and subjected to SDS-PAGE.

3.3. Orientation of Pho84 in proteoliposomes

In order to investigate whether the degree of inhibition of Δp-driven phosphate accumulation observed when the proteoliposomes harboring the Pho84 protein were treated with anti-

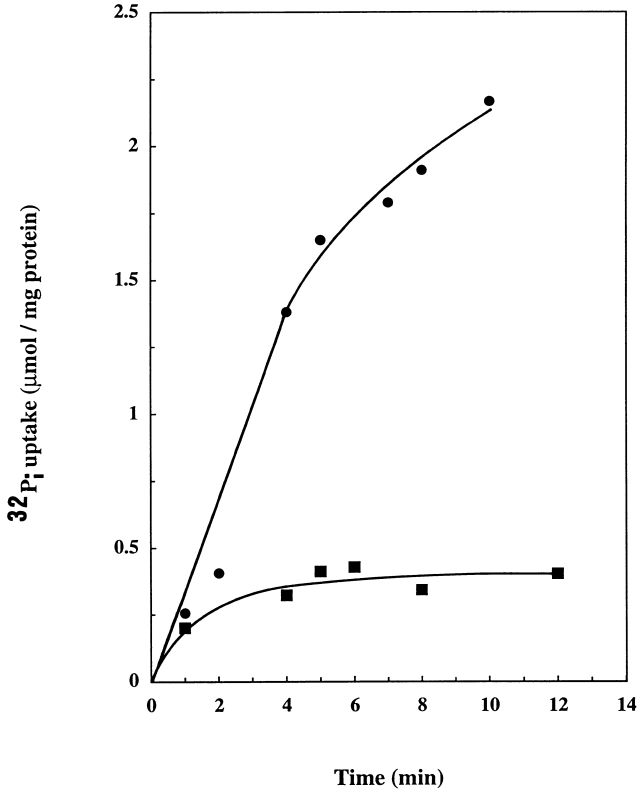

Fig. 3. Uncoupler-sensitive phosphate transport catalyzed by His_6-AXp/EK-Pho84 in proteoliposomes. Proteoliposomes containing 0.1 mg purified His_6-AXp/EK-Pho84/ml were assayed for phosphate uptake as described in Section 2 in the absence (l) and in the presence (n) of 20 μM CCCP. At given times, transport was stopped and the samples were assayed by rapid filtration and liquid scintillation spectrometry.

(N-terminal peptide) and anti-(C-terminal peptide) antibodies was due to a mixed orientation of the reconstituted protein, the N-terminal region of the His_6-AXp/EK-Pho84 was selected for determination of the orientation of the reconstituted protein in proteoliposomes. In this analysis, the partially overlapping AXp epitope and EK recognition site were used for site-specific proteolysis and immunodetection. Proteoliposomes harboring the purified His_6-AXp/EK-Pho84 subjected to treatment with EK were analyzed by SDS-PAGE and the AXp/EK epitope was detected by Axp antibody. As shown in Fig. 5, where the accessibility of the AXp epitope was monitored after proteolytic treatment, the proteolytic site was highly accessible as the enzyme cleaved reconstituted His_6-AXp/EK-Pho84 to the same extent as the detergent-solubilized non-reconstituted protein. The obtained results clearly indicate a unidirectional orientation of the Triton X-100-reconstituted protein in the PC/PE/PS/LPC proteoliposomes where the N- and C-termini are outwardly exposed.

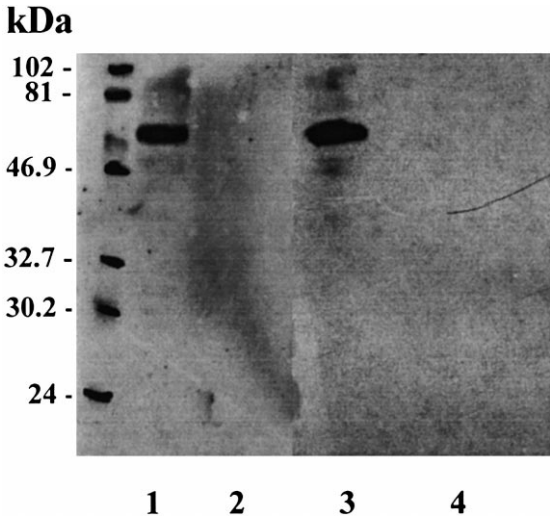

Fig. 5. Proteolytic cleavage of Triton X-100-solubilized and reconstituted His_6-AXp/EK-Pho84. Aliquots (30 μl) of detergent-solubilized His_6-AXp/EK-Pho84 protein (lanes 1 and 2, 1 μg) and proteoliposomes containing reconstituted His_6-AXp/EK-Pho84 protein (lanes 3 and 4, 2 μg) were incubated in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of EK as described in Section 2 and were analyzed by SDS-PAGE followed by Western blot analysis.

4. Discussion

Support for a secondary structure model of the polytopic Pho84 transporter in which the 12 transmembrane segments traverse the membrane in a zigzag fashion has been obtained by hydropathy profile analysis of the amino acid sequence (see [5]). In this paper, we describe experimental evidence for the topological arrangement of this transporter by applying a gene fusion approach together with analysis of the transport activity, accessibility of immunoreactive domains and site-specific proteolysis. The results obtained describe the oriented functional reconstitution of two Pho84 chimeras, His_{10}-Pho84 and His_6-AXp/EK-Pho84, into proteoliposomes with a defined lipid composition. Both chimeras were expressed in *E. coli*, purified by immobilized Ni^{2+} affinity chromatography in the presence of Triton X-100. As judged by a comparison between the two Pho84 chimeras, the attachment of 23 or 33 additional residues harboring the His_{10} and the His_6-AXp/EK sequences to the N-terminus, both constructions yielded a functional Pho84 protein, suggesting that at least the N-terminus of the protein can be modified without a major influence on the activity. However, immunodecoration of N-terminal or C-terminal sequences in the reconstituted protein results in a partial loss of phosphate transport activity. The differences in transport activities observed with the anti-(N-terminal peptide) and anti-(C-terminal peptide) antibodies cannot be attributed to an effect of transporter orientation. Rather, the lowered transport rate observed with the anti-(C-terminal peptide) antibody-treated Pho84 protein as compared to that of the anti-(N-terminal peptide) antibody might be explained by a previously proposed weaker antigenicity of the N-terminal domain in the fusion protein (see Fig. 1) as compared to the wild-type Pho84 protein [14] and/or a higher sensitivity in the protein towards a modification of the C-terminal.

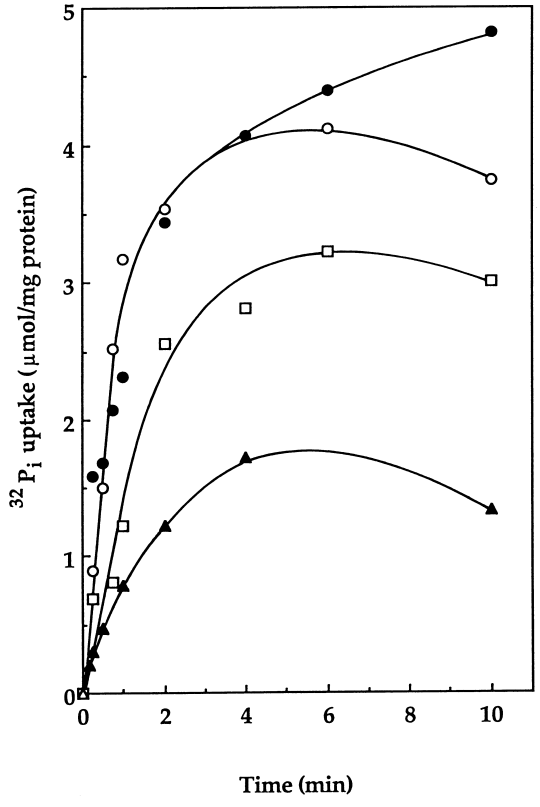

Fig. 4. Antibody-specific inhibition of phosphate uptake in proteoliposomes containing purified His_{10}-Pho84 protein. Proteoliposomes containing purified reconstituted His_{10}-Pho84 protein at a protein and lipid concentration of 0.1 and 11 mg/ml, respectively, were incubated in the absence (m) or in the presence of affinity-purified anti-(N-terminal peptide) antibody (o), in the presence of affinity-purified anti-(C-terminal peptide) antibody (s) and in the presence of an antibody directed against the PMA H^+-ATPase of *S. cerevisiae* (l) for 10 min at 25°C at a molar ratio of 1:1 with respect to the Pho84 protein. Aliquots (1 μl) of the incubated proteoliposomes were assayed for phosphate uptake as described in Section 2.

As the Pho84 protein previously has been shown to catalyze phosphate uptake in tightly sealed isolated inverted plasma membrane vesicles of *S. cerevisiae* cells expressing the Pho84 protein [13], the direction of phosphate transport catalyzed by the Pho84 protein seems to be determined by the direction of the driving force, as for many other secondary transport sys-

tems which operates bidirectionally, rather than by the orientation of the protein in the membrane.

The partial inactivation of the Pho84 transporter does not reflect a bidirectional topological arrangement of the reconstituted protein as proteolytic cleavage of the N-terminus of the His6-AXp/EK-Pho84 chimera resulted in no detectable Pho84 signal when EK-treated proteoliposomes were solubilized and analyzed by SDS-PAGE/Western blot analysis. The unidirectional liposomal insertion of membrane proteins such as the Pho84 permease, of which the inner and outer surface differ in hydrophilicity, is possible due to the lower tendency of large hydrophilic domains to traverse the lipid bilayer once closed liposomal structures have been formed as a consequence of detergent-binding to the polystyrene Bio-Beads. The obtained results suggest that the Pho84 permease, in analogy with LacS lactose permease of *Streptococcus thermophilus* [18] and the PutP proline transporter of *E. coli* [19] reconstituted in the presence of Triton X-100 followed by detergent removal by Bio-Beads, is unidirectionally incorporated into the liposomes with its N- and C-termini and large central hydrophilic loop protruding towards the exterior.

Taken together, the results shown in this work with purified histidine-tagged Pho84 chimeras provide the first experimental evidence for a functional unidirectional orientation of the Pho84 transporter reconstituted into proteoliposomes with its termini protruding towards the exterior. Although the protein is able to catalyze a bidirectional transport of phosphate, the establishment of the orientation of the reconstituted transporter in proteoliposomes is critical as it can be expected that the kinetics of transport differ depending on the direction of transport.

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References

[1] Bun-ya, M., Nishimura, M., Harashima, S. and Oshima, Y. (1991) Mol. Cell. Biol. 1, 3229–3238.
[2] Pao, S.S., Paulsen, I.T. and Saier Jr., M.H. (1998) Microbiol. Mol. Biol. Rev. 62, 1–34.
[3] Borst-Pauwels, G.W.F.H. (1981) Biochim. Biophys. Acta 650, 88–127.
[4] Persson, B.L., Berhe, A., Fristedt, U., Martinez, P., Pattison, J., Petersson, J. and Weinander, R. (1998) Biochim. Biophys. Acta 1365, 23–30.
[5] Persson, B.L., Petersson, J., Fristedt, U., Weinander, R., Berhe, A. and Pattison, J. (1999) Biochim. Biophys. Acta, pp. 1–18 (in press).
[6] Oshima, Y. (1997) Genes Genet. Syst. 72, 323–334.
[7] Martinez, P., Zvyagilskaya, R., Allard, P. and Persson, B.L. (1998) J. Bacteriol. 180, 2253–2256.
[8] Yompakdee, C., Bun-ya, M., Shikata, K., Ogawa, N., Harashima, S. and Oshima, Y. (1996) Gene 171, 41–47.
[9] Bun-ya, M., Shikata, K., Nakade, S., Yompakdee, C., Harashima, S. and Oshima, Y. (1996) Curr. Genet. 29, 344–351.
[10] Yompakdee, C., Ogawa, N., Harashima, S. and Oshima, Y. (1996) Mol. Gen. Genet. 251, 580–590.
[11] Bun-ya, M., Harashima, S. and Oshima, Y. (1992) Mol. Cell. Biol. 12, 2958–2966.
[12] Martinez, P. and Persson, B.L. (1998) Mol. Gen. Genet. 258, 628–638.
[13] Fristedt, U., Berhe, A., Ensler, K., Norling, K. and Persson, B.L. (1996) Arch. Biochem. Biophys. 330, 133–141.
[14] Berhe, A., Fristedt, U. and Persson, B.L. (1995) Eur. J. Biochem. 227, 566–572.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Knol, J., Venhoff, L., Liang, W.-J., Henderson, P.J.F., Leblanc, G. and Poolman, B. (1996) J. Biol. Chem. 271, 15358–15366.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Knol, J., Sjollema, K. and Poolman, B. (1998) Biochemistry 37, 16410–16415.
[19] Jung, H., Tebbe, S., Schmid, R. and Jung, K. (1998) Biochemistry 37, 11083–11088.