Biochemistry

 Volume 33, Number 32 August 16, 1994

Accelerated Publications

Yeast Mitochondrial Phosphate Transport Protein Expressed in *Escherichia coli*. Site-Directed Mutations at Threonine-43 and at a Similar Location in the Second Tandem Repeat (Isoleucine-141)†

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Received May 10, 1994; Revised Manuscript Received June 15, 1994®

ABSTRACT: Yeast mitochondrial phosphate transport activity has been reconstituted from the import receptor (MIR) expressed as inclusion bodies in *Escherichia coli*. This result undermines the suggestion [Murakami, H., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3358–3362] that the MIR has been misidentified as the phosphate transport protein (PTP). PTP was solubilized with *N*-lauroylsarcosinate and Triton X-100 and purified with a yield of about 2 mg/L of induced bacterial culture. This PTP, reconstituted in liposomes, catalyzes phosphate uptake with a V_{max} [24.5 °C, net (zero trans), pH_i = 8.0, pH_e = 6.8] of 0.61 mmol of phosphate min^{-1} (mg of PTP)$^{-1}$ and a K_m of 1.30 mM. This V_{max} is higher and the K_m about the same as that obtained with PTP purified from mitochondria. Replacement of Thr43 and Ile141 by other amino acids results in three types of PTP: (a) 2.5–5.0% V_{max} of wild-type PTP (PTP_{wt}) (Thr43Cys; Thr43Ser; Ile141Cys), (b) <0.1% V_{max} (detection limit of assay) of PTP_{wt} (Thr43Ala; Thr43Asp), and (c) proton transport uncoupled from phosphate transport (Ile141Cys). K_m changes are not significant. Activity of Thr43Cys confirms results obtained with mitochondrially expressed protein. Thus, yeast PTP requires Thr43 and mammalian PTP the similarly located Cys42 for high transport activity. Thr43 and Ile141 are each situated between two basic residues (LysThrArg vs ArgIleArg). Cys substitutions in either of these positions confer the same high *N*-ethylmaleimide sensitivity to the yeast PTP_{wt} as displayed by the mammalian PTP. On the basis of single substitutions and a bacteriorhodopsin-like pattern of Glu126, His32, and Glu137, helices I and III (second tandem repeat) have been suggested to facilitate proton transport. Thus it is interesting that replacing Ile141, which is located near the matrix end of transmembrane helix III (similar to Thr43 and helix I), with a polar Cys perturbs helix III and its local environment sufficiently to yield uncoupled proton transport.

The mitochondrial phosphate transport protein (PTP)[1] is responsible for transporting inorganic phosphate into the mitochondrial matrix (Wohlrab, 1986; Krämer & Palmieri, 1992; Ferreira & Pedersen, 1993). This phosphate is utilized in the matrix for the oxidative phosphorylation of ADP to ATP. PTP was first identified in mitochondria from beef heart on the basis of its *N*-ethylmaleimide reactivity and its ability to transport phosphate in a reconstituted system (Wohlrab, 1980). Its gene was identified in and cloned from the yeast *Saccharomyces cerevisiae* (Phelps & Wohlrab, 1991) in order to carry out site-directed mutagenesis and to have an assay for its physiological competence. Thus, yeast with mutant PTPs that possess low or no transport activity will not be able to grow on medium with glycerol as the sole carbon

† Supported by a grant from the NIH (GM33357).
® Abstract published in *Advance ACS Abstracts*, July 15, 1994.
[1] Abbreviations: PTP, phosphate transport protein; PTP_{wt}, wild-type PTP; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol. Thr43Asp denotes PTP with Thr43 replaced with Asp.

0006-2960/94/0433-9371$04.50/0

Table 1: Oligonucleotides for Mutant Constructs[a]

name	(anti)sense[b]	sequence
YpNYM	S	5′-CGAAATTAATACGACTCA-3′
YPH27	AS	3′-CGGGTGGTTGGTAACTTTAAC-5′
Thr43	S	5′-GAT GTC GTT AAG ACG AGA ATC CAA C-3′
Thr43Cys	S	5′-GAT GTC GTT AAG TGT AGA ATC CAA C-3′
	AS	3′-CTA CAG CAA TTC ACA TCT TAG GTT G-5′
Thr43Ala	S	5′-GTC GTT AAG GCG AGA ATC CA-3′
	AS	3′-CAG CAA TTC CGC TCT TAG GT-5′
Thr43Asp	S	5′-GAT GTC GTT AAG GAT AGA ATC CAA C-3′
	AS	3′-CTA CAG CAA TTC CTA TCT TAG GTT G-5′
Thr43Ser	S	5′-GTC GTT AAG TCG AGA ATC CA-3′
	AS	3′-CAG CAA TTC AGC TCT TAG GT-5′
Ile141	S	5′-GAA GCC ACT AGA ATC AGA TTG GT-3′
Ile141Cys	S	5′-GAA GCC ACT AGA TGC AGA TTG GT-3′
	AS	3′-CTT CGG TGA TCT ACG TCT AAC CAG AG-5′

[a] Oligonucleotides were used in the PCR-based site-directed mutagenesis procedure as published (Phelps & Wohlrab, 1991). The underlined codon represents the original or the new mutant amino acid. [b] S is the sense and AS is the antisense oligonucleotide.

source. On this basis a series of mutant PTPs with insufficient phosphate transport activity have been identified (Phelps & Wohlrab, 1993; Wohlrab & Phelps, 1993; Briggs and Wohlrab, unpublished results).

The yeast PTP has recently been expressed in *Escherichia coli*, and some of the activities that one might expect a mitochondrial import receptor to possess have been identified (Murakami et al., 1993). We have now solubilized this protein from the bacterial inclusion bodies and have reconstituted its phosphate transport activity. Indications are that this bacterially expressed PTP has the functional characteristics of the protein expressed in yeast mitochondria. Advantages in expressing this intrinsic membrane protein in bacteria are (1) preparation of PTP is facilitated dramatically and (2) relatively large amounts of PTP and its mutants can readily be prepared, independent, for example, of whether the mutated protein has the sequence motifs required for physiological incorporation into the mitochondrial membrane. We have utilized this system to express several new mutant PTPs. Some mutants have a dramatically lower V_{max} and others no (or extremely low) transport activity. Two have a cysteine at a new location that highly sensitizes the transport towards *N*-ethylmaleimide. One of these also shows a dramatically higher proton permeability in the absence of phosphate, and this suggests that proton transport has been uncoupled from phosphate transport. Some of these results have been communicated as an abstract (Wohlrab & Briggs, 1994).

MATERIALS AND METHODS

Preparation of Mutant PTP Plasmids. Mutant PTPs were prepared exactly as described (Phelps & Wohlrab, 1991). However, in the present experiments plasmid pNYHM131 (Figure 1) was used with oligonucleotide primers as indicated in Table 1. Manipulations of the plasmid were carried out with *E. coli* strain DH5α, which showed a much higher transformation efficiency than the expression strain BL21 (DE3). The PCR construct as well as its subcloning sites was completely sequenced in the double-stranded vector. A novel method was used to eliminate false stops in the DNA sequencing reactions by including Klenow fragment (Briggs, unpublished observation). The protocol for Sequenase version 2.0 (U.S. Biochemicals) was followed. Reagents were obtained in a nucleotide kit for sequencing with Sequenase T7 DNA polymerase and 7-deaza-dGTP (U.S. Biochemicals). After extension reactions were stopped by incubation with ddNTPs (3-μL total volume), 0.5 μL of Klenow cocktail (1 mM dNTPs, 0.6 unit/μL Klenow fragment) was mixed with each reaction.

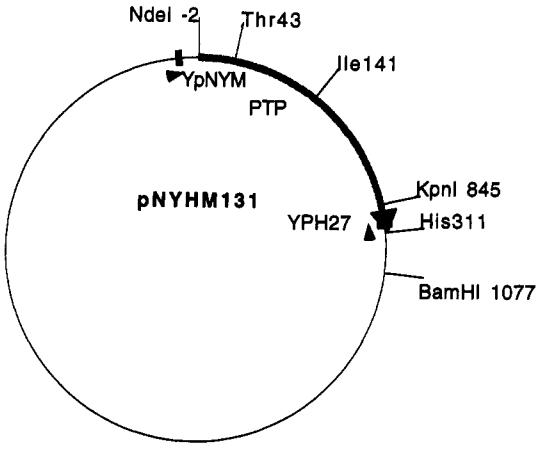

FIGURE 1: Map of plasmid pNYHM131 (Murakami et al., 1993). Restriction sites (numbers refer to nucleotides from the first codon of the coding region of PTP) used in the construction of mutants, locations (amino acid number from the N-terminus of PTP) of the two mutated amino acids as well as the C-terminal amino acid, and the location and direction of the two nonmutant oligonucleotides (see Table 1) are indicated. The coding region of the yeast PTP is shown.

Samples were incubated for 20–30 min at 37 °C, loading buffer was added, and samples were heated in a 65 °C water bath for 3 min, placed on ice for 1 min, and loaded onto the sequencing gel. The expression strain BL21 (DE3) was transformed with the final construct.

Expression and Purification of E. coli-Expressed PTP. A total of 1 L of 2 × YT medium (plus 100 mg of ampicillin) was inoculated with a fresh overnight colony of transformed BL21 (DE3) and grown to an OD_{600} of 0.6 (about 5 h) under vigorous shaking at 37 °C. Expression of PTP was initiated by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM plus 100 mg of ampicillin. Growth was continued for 3 h, and the cells were harvested and stored at −20 °C.

The cell pellet from 250 mL of the cell culture with the expressed PTP was suspended in TE (10 mM Tris base, 0.1 mM EDTA, 1 mM DTT, adjusted to pH 7.0 with NaOH) (4 °C) and passed twice through a French pressure cell; this was followed by centrifugation at 12100*g* for 10 min (4 °C). All the following steps were carried out at 0–4 °C. The pellet was homogenized with 10.1 mL of TE and centrifuged 1100*g* for 2.5 min; 8.8 mL of the supernatant was centrifuged 12100*g* for 2.5 min. The pellet at this stage may be stored at −20 °C. The pellet was dispersed with 330 μL of TE/SA [3.4 mg of sodium *N*-lauroylsarcosinate (Fluka)/mL of TE], and 586 μL of H_2O was added. The resulting suspension was centrifuged at 12100*g* for 10 min. The pellet was dispersed

in 300 μL of TE/SB [11.9 mg of sodium *N*-lauroylsarcosinate (Fluka)/mL of TE); then 532 μL H_2O was added, followed with centrifugation at 226000*g* for 50 min. The resulting supernatant was passed through a gel filtration column (Bio-Gel P-6DG, Bio-Rad) that had been equilibrated with C_i/DTT/X-100 [10 mM Tris base, 10 mM PIPES, 0.5% Triton X-100 (106 mg of Triton X-100/20 mL of final solution), 5 mM DTT; pH adjusted to 8.0 with KOH]. To 200 μL of the resulting PTP was added 22 μL of plant phosphatidylcholine (Avanti) (16 mg/mL of C_i). C_i is 10 mM Tris base and 10 mM PIPES, with the pH adjusted to 8.0 with KOH. This mix was added to 46 mg of washed Bio-Beads SM-2 (Bio-Rad) and stirred for 45 min. The suspension (PTP–ABB) was removed from the Bio-Beads SM-2 and used in the reconstitution after storage for about 12 h at 0 °C under high-purity argon.

Reconstitution of PTP. PTP–ABB was characterized by SDS–PAGE. Its concentration was determined from comparison to human carbonic anhydrase B (Sigma) in the Coomassie Blue-stained SDS gel. The same volume of PTP–ABB was used in all the reconstitution experiments, i.e., 4 μL (120 μg PTP/mL). Specific transport rates were recalculated for small differences in final PTP–ABB protein concentration in the reconstitution medium. The reconstitution medium consisted of 0.09 mg of calcium phosphatidate (egg), 2.20 mg of phosphatidylcholine (plant), and 2.77 mg of phosphatidylethanolamine (plant) in 191 μL of C_i made to 0.077% glycerol and 1.5 mM DTT. The mixture was flushed with high-purity argon and frozen in liquid nitrogen during rapid agitation. The frozen samples were stored at –80 °C.

Transport Assays. Phosphate transport was assayed as previously described (Phelps & Wohlrab, 1991). Kinetic experiments were carried out at four different extraproteoliposomal phosphate concentrations: 0.66, 1.0, 1.5, and 5.0 mM. Time points were 0, 5, 10, 15, and 20 s. Transport was stopped with 1.26 mM sodium mersalyl (final concentration during transport assay), which was prepared fresh immediately before each experiment by suspending the acid in water and adding just sufficient NaOH to completely dissolve it.

Reagents. Lipids were obtained from Avanti and stored at –80 °C. Calcium phosphatidate was prepared essentially as described (Wohlrab et al., 1984).

RESULTS

Purification of PTP Expressed in E. coli. The inclusion bodies were purified by differential centrifugation and PTP purified as described in Materials and Methods. Figure 2 shows a preparation of *E. coli*-expressed and purified PTP and also a preparation from an identically treated host cell culture [BL21 (DE3)] that had not been transformed with pNYHM131. All the mutants yielded a similar amount of purified PTP, except Ile141Cys, which had a 75% lower yield. The reason for this is not clear. PTP is expressed to a much higher concentration than the 2 mg/L of culture obtained after purification.

Some Transport Characteristics of Mutant PTPs. The reconstituted PTP preparations were thawed, gently vortexed for 6 s, and centrifuged at 17 K for 15 min in a TLA 100.2 rotor in a TL100 (Beckman) centrifuge to minimize mersalyl-insensitive phosphate uptake. This centrifugation step leads to a loss of about 50% of the protein (Wohlrab et al., 1984) and is especially critical since all the mutants reported here catalyze low transport activities. Table 2 shows the V_{max} and K_m of the phosphate transport (net uptake, zero trans, with pH_e = 6.8 and pH_i = 8.0) catalyzed by the various mutant

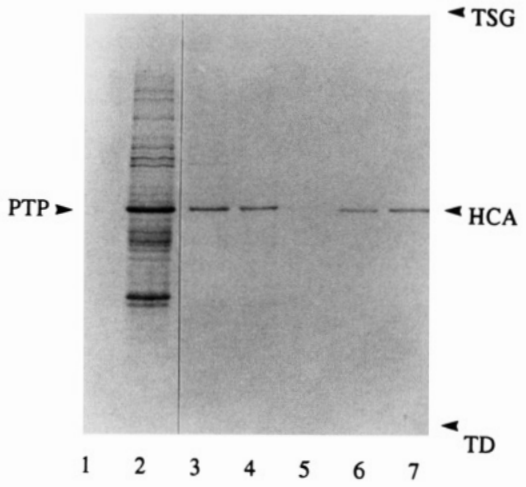

FIGURE 2: SDS–PAGE of bacterially expressed PTP. Lanes 1 and 2 are samples from just before the gel filtration column, from host cells grown without vector (essentially no protein since inclusion bodies are absent), and host cells grown with the expressed vector of mutant Thr43Asp, respectively. Lanes 3 and 4 are samples after Bio-Beads SM-2 treatment of Thr43Ser and Thr43Asp, respectively. No sample was applied to lane 5. Lanes 6 and 7 are human carbonic anhydrase B (HCA) taken up in 20% (v/v) sample buffer (Kolbe et al., 1984) at 0.0125 mg/mL with 5 and 10 μL applied to the gel, respectively. *N*-Ethylmaleimide (2 μL, 100 mM) was added to the other samples (20 μL), and after 10min on ice, 5.5 μL of 100% sample buffer was added. A total of 4 μL was applied to lanes 1 and 2 and 4 μL to lanes 3 and 4 after the samples were diluted with three volumes of 20% (v/v) sample buffer. TSG is the top of the separating gel; TD is the location of the tracking dye.

Table 2: Kinetic Characteristics of Mutant PTPs

mutant	K_m (mM P_i)	V_{max} [μmol of P_i min^{-1} (mg of PTP)$^{-1}$]	temp (°C)
wild type	1.30	606	24.5
	0.93	478	23.0
Thr43Cys	1.39	30.7	24.0
Thr43Ser	1.20	18.4	23.0
	1.05	14.2	22.0
Thr43Ala		<0.5	20.5
Thr43Asp		<0.5	21.5
Ile141Cys	0.95	27.2	22.5

PTPs. The K_m of transport catalyzed by PTP_{wt} is like that reported in earlier publications; the V_{max} is somewhat higher (Guérin et al., 1990). Thr43Cys shows a much lower V_{max}. This confirms the published results on PTP (Thr43Cys) purified from yeast mitochondria and reconstituted (Phelps & Wohlrab, 1991). Thr43Ser catalyzes a similarly low V_{max}. Thr43Ala and Thr43Asp catalyze transport at a rate below our detection limit. Even Ile141Cys shows a 95% decrease in V_{max} compared to PTP_{wt}.

N-Ethylmaleimide Sensitivity of Ile141 Mutant PTP. It has been shown that Cys42 of beef heart PTP is the amino acid from among eight cysteines of the protein that is responsible for the very high *N*-ethylmaleimide sensitivity (Kolbe & Wohlrab, 1985). Yeast PTP does not show this high sensitivity (Guérin et al., 1990) because it has a Thr43 at a similar location between two basic residues. Replacing this Thr43 with Cys confers high *N*-ethylmaleimide sensitivity to the yeast PTP (Phelps & Wohlrab, 1991).

A topological arrangement of the yeast PTP (Figure 3) shows that Ile141 is at a location similar to that of Thr43 except it is within the second tandem repeat. We prepared the mutant Ile141Cys. This mutant shows the same high sensitivity to *N*-ethylmaleimide (Figure 4) as the Thr43Cys mutant.

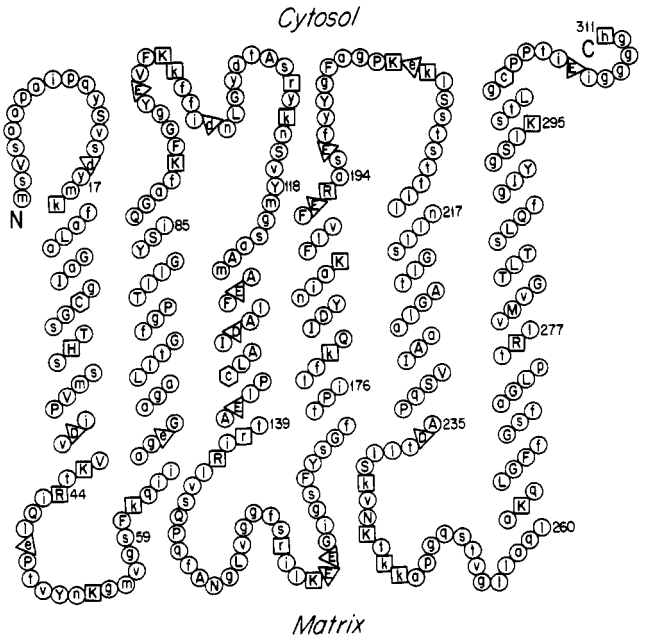

FIGURE 3: Topographical representation of PTP. Upper case letters represent amino acids at same location in the beef heart and yeast sequences. Symbols: acidic amino acid (△), basic amino acid (□), and cysteine (○).

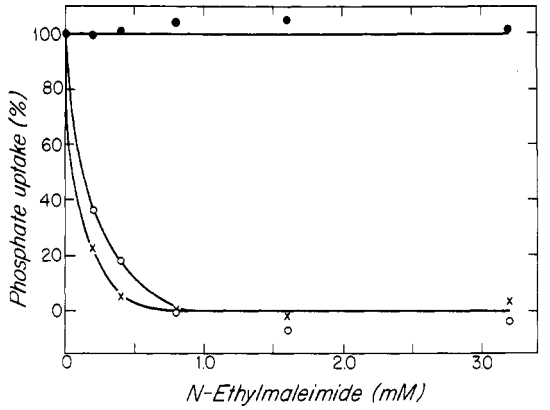

FIGURE 4: *N*-Ethylmaleimide titration of phosphate transport catalyzed by PTP in liposomes. *N*-Ethylmaleimide was added to the medium just before transport was started by addition of the proteoliposomes. Symbols: PTP_{wt} (●), Thr43Cys (○), and Ile141Cys (×).

Stability of the Trans-Proteoliposomal pH Gradient. The phosphate transport assay is started by the addition of the proteoliposomes to [^{32}P]P_iC_e (10 mM Tris base, 10 mM PIPES, various concentrations of P_i, adjusted to pH 6.8 with KOH). The inside of the proteoliposomes is pH 8.0. When proteoliposomes are added to C_e in the absence of [^{32}P]-inorganic phosphate, phosphate transport is started 10 or 20 s later by the addition of [^{32}P]inorganic phosphate. Initial transport rates are essentially independent of this delay with PTP_{wt}, Thr43Ser, or Thr43Cys (Figure 5). However, phosphate uptake drops to 0 within 10 s after Ile141Cys proteoliposomes are exposed to the pH gradient (Figure 5).

DISCUSSION

PTP is a very interesting transport protein for several reasons. It is present in all cells that possess mitochondria. It is a homodimer (Phelps & Wohlrab, 1993) with a subunit size of 32.8 kDa (cDNA) (Phelps et al., 1991). The subunit consists of three sequence-similar tandem repeats of about 100 amino acids each (Runswick et al., 1987; Phelps et al., 1991). Residues that determine the high substrate specificity

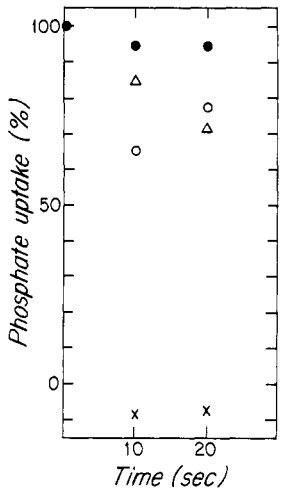

FIGURE 5: Phosphate transport (phosphate taken up in 10 s) initiated after the reconstituted proteoliposomes were exposed to the pH gradient for various times in the absence of phosphate. Symbols: PTP_{wt} (●), Thr43Cys (△), Thr43Ser (○), and Ile141Cys (×).

of PTP may be similar to the ligands that interact with inorganic phosphate in the bacterial periplasmic binding protein (Luecke & Quiocho, 1990). Phosphate transport is electroneutral [for process review, see Wohlrab (1986)]. PTP is most likely a proton/phosphate cotransporter since the phosphoric acid concentration in the cytosol is very low and the K_m for transport is about 1 mM. Additional support for this kind of transport mechanism comes from residues Glu126, His32, and Glu137 (see Figure 3), which are essential (Asp130 is not) for yeast growth on glycerol (Wohlrab & Phelps, 1993) and which are similar in type and location to the major proton-transfer residues Asp96, Lys216 (Schiff base), and Asp85 of bacteriorhodopsin [for review, see Khorana (1993)].

Recent kinetic studies (Stappen & Krämer, 1994), however, suggest that PTP acts as a OH^-/P_i^- antiporter. Their mechanism may not apply to the physiological situation of high-matrix pH as used in our experiments.

The suggestion that a mitochondrial import receptor (MIR) (Murakami et al., 1990; Pain et al., 1990) has been misidentified as a PTP (Murakami et al., 1993) is undermined by the present experiments as well as by published experiments on the PTP mutant Thr43Cys purified from yeast mitochondria and reconstituted (Phelps & Wohlrab, 1991). Should PTP, however, also act physiologically as an import receptor, then its molecular mechanism is more complex than that of a typical cation/proton cotransporter.

The bacterial expression system increases the number of systems available for investigating PTP. The yeast PTP gene was cloned in order to permit site-directed mutagenesis and to establish an assay for the physiological competence of PTP. Thus, when the transport activity of a mutant PTP is less than 10% of PTP_{wt}, the growth of yeast on glycerol media is blocked (Phelps & Wohlrab, 1991). This has also recently been shown for the mitochondrial ADP/ATP translocase (Klingenberg & Nelson, 1994). The bacterial expression system cannot be used for such a physiological transport assay. However, mutant PTPs can easily be prepared in large quantities and be purified and reconstituted to characterize catalytic activities.

We have somewhat modified the MIR purification procedure of Murakami et al. (1993) and incorporated some features of the method used to reconstitute transport activity of the mitochondrial oxoglutarate carrier expressed as bacterial inclusion bodies (Fiermonte et al., 1993). This bacterially expressed, purified, and reconstituted yeast PTP_{wt} catalyzes a higher V_{max} than the protein purified from yeast mitochon-

dria. In addition, the bacterially expressed Thr43Cys shows the same low transport activity (Table 2) as the protein purified from yeast mitochondria (Phelps & Wohlrab, 1991). These results legitimize this expression system as a tool for studying mutant PTPs.

Cys43 is the residue responsible for the high sensitivity of PTP to *N*-ethylmaleimide (Kolbe & Wohlrab, 1985; Phelps & Wohlrab, 1991). This must be due to the location of Cys in or near the proton and/or phosphate transport paths and because the two neighboring basic residues lower the pK_a and make it highly *N*-ethylmaleimide-reactive. We have replaced Ile141 with Cys and find a PTP with an *N*-ethylmaleimide sensitivity and V_{max} like that of Thr43Cys. This result fits with transport activities of PTPs with other substitutions in this loop between helices III and IV (A. Phelps and H. Wohlrab, unpublished results). There are three other motifs of two basic residues separated by a single nonbasic residue, i.e., Tyr113, Glu206, and Thr245. Thr43, Ile141, and Thr245 face the mitochondrial matrix. Tyr113 and Glu206 face the cytosol. Interestingly, helix V has in yeast Glu206 near its cytosolic end and Thr245 near its matrix end; in mammalian PTP (human PTP, Dolce et al., 1991; rat PTP, Ferreira et al., 1989; bovine PTP, Runswick et al., 1987) it has Pro206 near its cytosolic end and Glu245 near its matrix end. Have other residue replacements compensated this shift of a Glu from matrix to cytosol? Will a Cys between these basic residues, especially those facing the cytosol, affect the V_{max} and the *N*-ethylmaleimide sensitivity of PTP_{wt}?

Ile141Cys is able to transport protons in the absence of phosphate (Figure 5). One might speculate that the low pK_a of Cys141 helps the proton bypass the P_i coupling site and makes the matrix water more accessible. Another possibility is that Cys141 disturbs the interface between the homodimeric subunits and thus leads to uncoupled phosphate transport. Reacting Cys141 with *N*-ethylmaleimide blocks phosphate transport (Figure 4) and may also block the uncoupled proton flux.

We have investigated more thoroughly the Thr43 site. Why does replacing Thr with the smaller Cys result in such a low V_{max} (Table 2)? Mammalian PTPs have a Cys at a similar position. From other substitutions we already know how critical helix I is to transport. Thus, Cys28, which inhibits transport by forming a disulfide with Cys28 of the second subunit (Phelps & Wohlrab, 1993; Wohlrab et al., 1984), faces the same side of helix I as His32, Asp39, and Thr43. By selectively replacing residues not conserved between yeast and mammalian PTP, we should be able to increase the V_{max} of Thr43Cys and obtain new insights into structural elements important for this proton-compensated phosphate transport.

Thr43Ser shows the same low transport activity as Thr43Cys. Ser is a generally accepted replacement of Cys, and therefore the results are not surprising. Thr43Ala and Thr43Asp show no activity, i.e., activity at the detection limit of our current assay method. At this stage of our investigations we have not identified enough of the transport-critical residues to interpret these results more extensively.

It is clear that single residue replacements have dramatic effects on phosphate transport. This new bacterial expression system permits the rapid preparation and characterization of additional amino acid replacements and thus will help us identify amino acids important for this proton/phosphate cotransport process.

ACKNOWLEDGMENT

We thank Dr. Debkumar Pain for making plasmid pNYHM131 available to us, Dr. Anne Phelps for discussions and critical comments, and Dr. Saroj Joshi for comments and suggestions on the bacterial expression of proteins.

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