Single Replacement Constructs of All Hydroxyl, Basic, and Acidic Amino Acids Identify New Function and Structure-Sensitive Regions of the Mitochondrial Phosphate Transport Protein[†]

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ABSTRACT: The phosphate transport protein (PTP) catalyzes the proton cotransport of phosphate into the mitochondrial matrix. It functions as a homodimer, and thus residues of the phosphate and proton pores are somewhat scattered throughout the primary sequence. With 71 new single mutation per subunit PTPs, all its hydroxyl, basic, and acidic residues have now been replaced to identify these essential residues. We assayed the initial rate of pH gradient-dependent unidirectional phosphate transport activity and the liposome incorporation efficiency (LIE) of these mutants. Single mutations of Thr79, Tyr83, Lys90, Tyr94, and Lys98 inactivate transport. The spacings between these residues imply that they are located along the same face of transmembrane (TM) helix B, requiring an extension of its current model C-terminal domain by 10 residues. This extension superposes very well onto the shorter bovine PTP helix B, leaving a 15-residue hydrophobic extension of the yeast helix B N-terminus. This is similar to the helix D and F regions of the yeast PTP. Only one transport-inhibiting mutation is located within loops: Ser158Thr in the matrix loop between helices C and D. All other transport-inhibiting mutations are located within the TM helices. Mutations that yield LIEs of <6% are all, except for four, within helices. The four exceptions are Tyr12Ala near the PTP N-terminus and Arg159Ala, Glu163Gln, and Glu164Gln in the loop between helices C and D. The PTP C-terminal segment beyond Thr214 at the N-terminus of helix E has 11 mutations with LIEs >20% and none with LIE <6%. Mutations with LIEs >20% are located near the ends of all the TM helices except TM helix D. Only a few mutations alter PTP structure (LIE) and also affect PTP transport activity. A novel observation is that Ser4Ala blocks the formation of PTP bacterial inclusion bodies.

The PTP[1] belongs to a family of 35 mitochondrial transport proteins (*1*). These proteins have significant sequence similarities, yet the substrates that the 16 function-identified members of this MTP family transport are of different structures. One of the challenges is to be able to predict which transport protein catalyzes the transport of which substrate. With this challenge in mind, it will be important to understand the mechanism of transport of at least one of the transporters quite well in order to facilitate an understanding of the mechanism of catalysis and of the basis of substrate specificity of the others.

We report now the construction of 71 new single amino acid replacement per subunit mutants of PTP. The rationale for preparing these constructs is that, contrary to multimeric channels that are built on a "barrel-stave" plan with the ion-conducting pore formed by repetition of a transmembrane subunit around an axis of 4-, 5-, or 6-fold symmetry (*2*), the homodimeric structure of PTP (*3*) requires the amino acids that line the phosphate and proton pores to be somewhat scattered throughout the primary sequence and their locations to be not quite so predictable. Our new constructs complete the single amino acid replacements of all hydroxyl, basic, and acidic residues of PTP. This is the first time that this has been done to any member of the mitochondrial transport protein family (*1*). We chose to replace these particular types of residues since a high-resolution structure of the bacterial periplasmic phosphate binding protein (PBP) demonstrated that hydroxyl and acidic amino acids are its ligands of inorganic phosphate (*4*) and acidic groups have been demonstrated to undergo protonation/deprotonation cycles during proton transport catalyzed by bacteriorhodopsin (*5*). Also basic residues can act as ligands for inorganic phosphate or as proton donors/acceptors in the proton transport pathway. Basic residues can also be part of structure/function-essential salt bridges (*6, 7*).

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[1] Abbreviations: PTP, phosphate transport protein; MTP, mitochondrial transport protein; LIE, liposome incorporation efficiency; TM, transmembrane; PBP, phosphate binding protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, DL-dithiothreitol; IPTG, isopropyl 1-thio-β-D-galactoside; psi, pounds per square inch; ABB, After-BioBeads PTP.

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Our results show that helix B should be extended by 10 residues toward the C-terminus of PTP, that 19 of 20 function-sensitive residues are located within the transmembrane helices, that the replaced C-terminal residues (residues between the N-terminus of helix E and the PTP C-terminus) show only normal (6%−20%, 18 residues) or high (>20%, 11 residues) LIE, and that the mutation Ser4Ala prevents the formation of bacterial PTP inclusion bodies.

MATERIALS AND METHODS

Preparation of Mutant PTP Gene Plasmids. Mutant yeast PTP genes were constructed with the Stratagene QuikChange mutagenesis kit using PCR primers purified by PAGE. The entire mutant gene in each plasmid was sequenced (ABI373A automated DNA sequencer equipped with Stretch upgrade) to confirm the presence of only the single mutation and no other mutations in the wild-type PTP gene. Since we obtained no inclusion bodies with the PTP mutant Ser4Ala, the PTP gene fragment between *Nde*I and *Kpn*I, which includes the Ser4Ala mutation, was subcloned into the wild-type PTP pNYns plasmid to eliminate possible random mutations introduced during the mutagenesis step and that might have been responsible for the inhibition of the protein expression.

Replacement Residues in Mutant PTPs. Most amino acid replacements were made with Ala. However, the following Glu were replaced with Gln: Glu48, Glu67, Glu95, Glu126, Glu137, Glu163, Glu164, Glu192, Glu196, and Glu305. The following Asp were replaced with Asn: Asp39, Asp108, Asp130, and Asp236. Ser158 was replaced (Figures 1 and 3) with Thr. Arg44 was not identified in Figures 1−4 since the Arg44Ala mutant PTP bacterial inclusion bodies did not solubilize under our standard sarkosyl procedure (*8*). This mutant most likely should be in the figures since the yeast (*Saccharomyces cerevisiae*) PTP null mutant was not able to grow on glycerol plates (1% yeast extract, 2% peptone, 2% agar, 3% glycerol) after transformation with the mutant Arg44Ala gene in the pAP-W3 yeast shuttle vector (*8*).

Expression of Mutant PTPs. The yeast PTP gene (*9*) in the plasmid pNYns (*10*) was expressed in *Escherichia coli* BL21(DE3) cells (Novagen). The transformed *E. coli* cells were grown to an OD (600 nm) of 0.8, IPTG was added to 1.0 mM, and the cells were grown for another 3 h. The cells were harvested and the pellets stored at −20 °C. The cell pellet from a 250 mL culture was thawed in TE medium (10 mM Tris·HCl, 0.1 mM EDTA, 1 mM DTT, pH 7.0), and the suspension was passed twice through a French press at 16000 psi (4 °C). The mix was centrifuged, and the pellet was dispersed in TE and separated into four aliquots. These were centrifuged at 1100*g* for 2.5 min. Each supernatant was centrifuged at 12000*g* for 2.5 min, and the pellets were stored at −70 °C.

Reconstitution of Mutant PTPs. Purified lipids (Avanti Polar Lipids) in chloroform were stored at −70 °C. Soybean L-α-phosphatidylcholine (4 mg) was dried in a glass ampule under a stream of argon and placed under a high vacuum for 2 h. C_i medium (250 μL) (10 mM Tris base, 10 mM PIPES, adjusted with KOH to pH 8.0) was added and the lipid dispersed by vortexing. The mix was transferred to a small polystyrene test tube, sonicated until optically clear, and then stored on ice for later use. Soybean L-α-phosphatidylethanolamine (6.3 mg), 5.0 mg of soybean L-α-phosphatidylcholine, and 0.2 mg of egg calcium phosphatidate were mixed, dried, sonicated in 250 μL of C_i medium, and stored under argon at 4 °C.

TE (264 μL) was added to an inclusion body mutant PTP pellet and vortexed until well dispersed. Then 66 μL of sodium *N*-lauroylsarcosinate (Fluka) (17 mg/mL) was added and the mixture vortexed thoroughly. Then 586 μL of water was added and the mixture centrifuged for 2.5 min at 12000*g* (4 °C). To the pellet was added 90 μL of TE, and the pellet was thoroughly dispersed by vortexing. Then 210 μL of sodium *N*-lauroylsarcosinate was added and the mixture vortexed. Water (532 μL) was added and the mixture centrifuged for 50 min (4 °C) at 180000*g*. The supernatant was saved and stored on ice. A desalting column (4 cm × 4 mm diameter) (Bio-Gel P-6DG, Bio-Rad) was equilibrated with 2 mL of C_iXD medium [C_i medium, 0.48% Triton X-100 (Anatrace) (v/v), 5 mM DTT]. The supernatant (200 μL) was passed though the column and collected. To 200 μL of the pass-through was added 2 μL of the phosphatidylcholine dispersion, and the mix was added to 42 mg of washed Bio-Beads SM2 that had been wetted with C_iD (C_i medium, 5 mM DTT). The mixture was shaken for 45 min in a labquake at 4 °C. The mix (ABB) was separated from the Bio-Beads by gravity and the supernatant stored overnight on ice.

Reconstitution was completed the next day by mixing 110 μL of the optically clear phospholipid mixture with 81 μL of C_iD medium and 4 μL of ABB. This mixture, under argon, was frozen in liquid nitrogen during vigorous agitation and then stored at −70 °C as a frozen reconstitution mixture.

Transport Assays of Reconstituted Mutant PTPs. A frozen reconstitution mixture was thawed at room temperature for 10 min and then gently vortexed for 6 s. The sample was then centrifuged for 15 min at 10000*g* (4 °C). The supernatant (150 μL) was transferred to a test tube, and 18 μL of C_i (15 mM DTT) was added. This final mix (proteoliposomes) was stored on ice for 25 min. The anion-exchange column [11.5 cm × 8 mm diameter, AG 1-X8 (50−100 mesh, formate form, Bio-Rad)] was at 4 °C. To 20 mL of C_e (0.66) transport medium (10 mM Tris base, 10 mM PIPES, 0.66 mM sodium phosphate, pH 6.8) was added 20 μL (0.2 mCi) of carrier-free [^{32}P]P_i (Amersham), and the mixture was stored overnight at room temperature to decrease the amount of [^{32}P]P_i label that does not bind to the anion-exchange column. The transport-blocking mersalyl solution is prepared fresh immediately before use by adding 64 mg of mersalylic acid (Aldrich) to 1 mL of water, and then 10 N NaOH (about 13.5 μL) is added for almost all mersalylic acid to go into solution. The GNN medium consists of 5% (v/v) glycerol and 0.1 mM sodium azide. A typical transport experiment is as follows: 500 μL of C_e (0.66) transport medium with [^{32}P]P_i is added to the column, followed by 1 mL of GNN and then 3 mL of GNN. The 3 mL of medium leaving the column, while the 3 mL of GNN is entering the column, is collected in a scintillation vial. It reflects the amount of [^{32}P]P_i not retained by the column in the absence of proteoliposomes. Then 5 μL of the transport-blocking mersalyl solution is added to 500 μL of C_e (0.66) transport medium with [^{32}P]P_i, followed by 25 μL of the proteoliposomes. About 10 s after having added the proteoliposomes, the mix is vortexed to simulate addition of mersalyl for

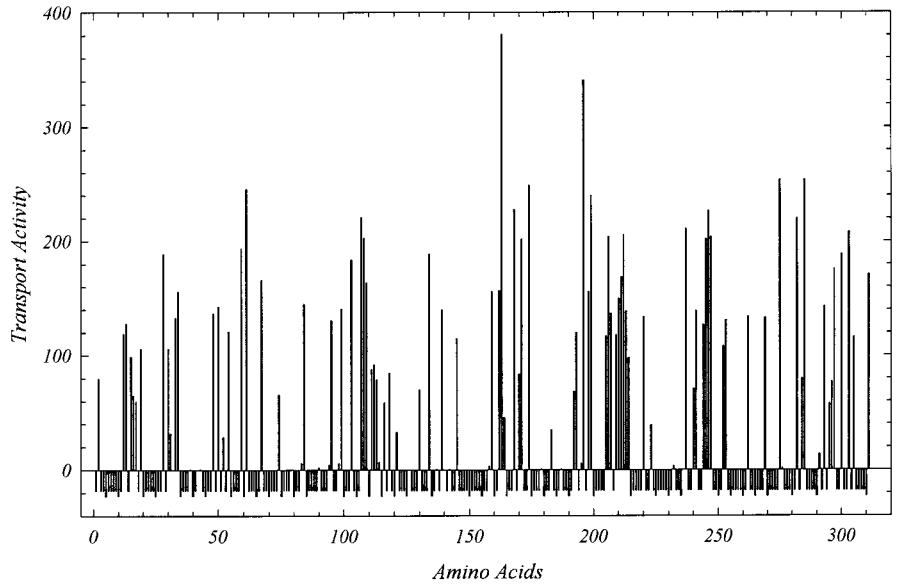

FIGURE 1: Phosphate transport catalyzed by single amino acid replacement mutants of yeast PTP. Negative vertical bars indicate the position of residues which were not used to construct mutants. The amino acids axis reflects the primary sequence of the yeast PTP from the N-terminal Met1 to the C-terminal His311. Transport activity is the initial rate in μmol of P_i min^{-1} (mg of PTP)$^{-1}$ of pH gradient-dependent net phosphate uptake by the proteoliposomes (see Materials and Methods).

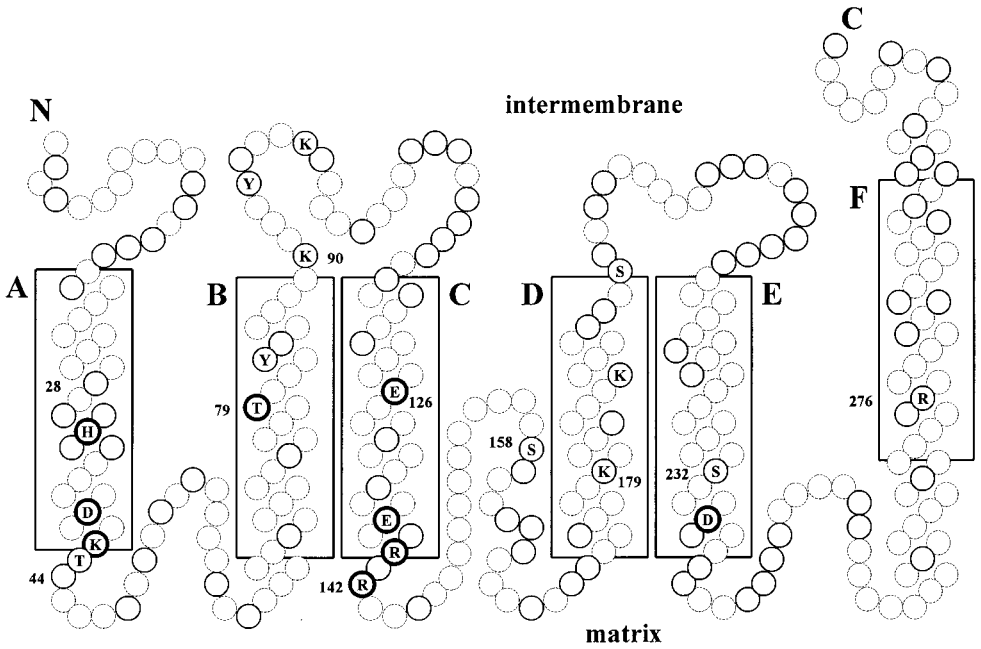

FIGURE 2: Diagram of yeast PTP showing amino acids whose replacement yields single mutation PTPs with blocked or severely inhibited transport activity. Single letter code is used for amino acids. Heavy circles identify residues whose replacement yields PTP transport activity with less than 0.6 μmol of P_i min^{-1} (mg of PTP)$^{-1}$; light circles with residues indicate mutants with transport activity of >0.6 and <6.0 μmol of P_i min^{-1} (mg of PTP)$^{-1}$. Light solid circles without amino acid labels indicate other single amino acid replacement mutants that were constructed and assayed.

transport stop at 10 s. The mix is added to the anion-exchange column, and again the 3 mL eluate is collected. This sample reflects the traces of [^{32}P]P_i taken up by the proteoliposomes when PTP is blocked by mersalyl. This mersalyl-before-proteoliposome experiment is repeated, followed by two experiments when mersalyl is added 10 s after the proteoliposome addition. Finally, the mersalyl-before-proteoliposome experiment is repeated; i.e., the blocked P_i uptake before and after the actual P_i uptake experiments should be closely the same.

LIE Determination of the PTP Mutants. LIE is the percentage of PTP protein in the reconstitution mix that is present in the proteoliposome fraction used to assay transport. The PTP protein (ABB), before addition to the reconstitution mix, was quantitated, using human carbonic anhydrase (Sigma) as standard, in Coomassie Blue-stained polyacrylamide gels after SDS−PAGE. The PTP protein in the proteoliposomes was quantitated as follows: 30 μL of the proteoliposomes, immediately after the transport assays, was added to 500 μL of C_e (0.66) transport medium (without [^{32}P]P_i) at room temperature, and the mixture was centrifuged at 240000*g* for 30 min (4 °C). To each pellet was added 22.5 μL of SDS−PAGE sample buffer, and the sample was submitted to SDS−PAGE. The gel was Coomassie Blue-

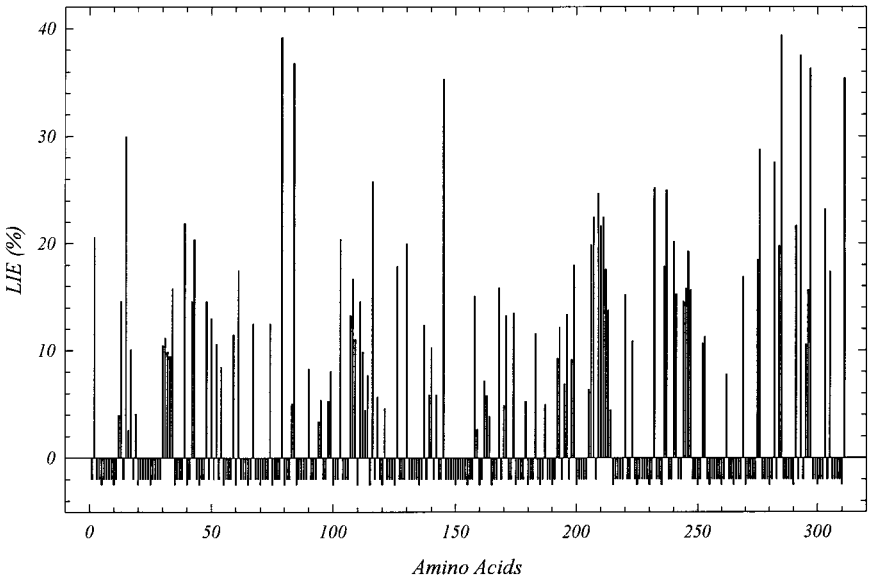

FIGURE 3: LIE of PTP mutants with single amino acid replacements. The amino acids axis identifies the location of the replaced residue with respect to the Met1 to His311 primary sequence of yeast PTP. Amino acids that were not used to construct mutants are indicated by negative vertical bars.

and then silver-stained and the protein quantitated by optical scanning using human carbonic anhydrase as a standard.

RESULTS

Transport Activity of Mutant PTPs. Figure 1 shows the results of the transport assays of all the new mutants as well as relevant mutants from earlier studies (*8*, *10*, *11*). All of the data reflect the results of at least two initial rate transport assays using the same PTP proteoliposome preparation. In many cases they also reflect the results from several independently prepared PTP proteoliposome preparations. These initial rate transport activities vary from 0.0 to about 380 μmol of P_i taken up into the proteoliposomes per minute per milligram of PTP protein. The wild-type yeast PTP catalyzes an initial uptake rate of about 180 μmol of P_i min^{-1} (mg of PTP)$^{-1}$ in this proteoliposome assay system.

Figure 2 shows a diagram of the most recent PTP model (*10*). It indicates those residues that upon replacement catalyze P_i uptake at <0.6 μmol of P_i min^{-1} (mg of PTP)$^{-1}$ (heavy circles with single letter code amino acid) and those with uptake rates of >0.6 but <6.0 μmol of P_i min^{-1} (mg of PTP)$^{-1}$ (light circles with single letter code amino acid). These transport activity ranges were chosen to keep the number of function-significant residues to a minimum. All the transport blocking mutations [<6.0 μmol of P_i min^{-1} (mg of PTP)$^{-1}$] fall into sequence regions associated with TM helices, except Ser158Thr. The spacing between Tyr94Ala and Lys98Ala along the PTP sequence suggests that they, together with Thr79Ala, Tyr83Ala, and Lys90Ala, are on the same face of a helix, which in this case is helix B.

Liposome Incorporation Efficiency of Mutant PTPs. We observed some time ago that in our reconstitution process the fraction of mutant PTP molecules that incorporates into the proteoliposomes depends clearly on the location within the PTP sequence of the replaced amino acid. While we observed that PTP mutants with high LIE required about 30% more Triton X-100 to stay in solution than those with low LIE (*10*), we have acquired some evidence that those mutants with high LIE have a dimeric structure in which the subunits have a greater affinity for each other. Thus we expect the LIE results to yield information on the subunit/subunit interface of the homodimeric PTP.

Figure 3 shows the LIE of the single replacement per subunit PTP mutants. The PTP protein was quantitated in the proteoliposomes that were used in the transport studies. The LIE values range from about 2% to 40%. Figure 4 identifies residues that upon replacement yield PTPs with very low LIE ($<6\%$) with a heavy circle and single letter code amino acid. Those residues that upon replacement yield PTPs with high LIE ($>20\%$) are shown with single letter code and within a light circle. The result that stands out is that none of the residues in the most C-terminal third of PTP yield, upon replacement, a PTP with very low LIE.

Lack of Inclusion Bodies with the Ser4Ala Mutant PTP. Expression of the Ser4Ala mutant PTP resulted in no inclusion bodies. We did reclone the mutant gene into the wild-type plasmid (see Materials and Methods) to make sure that the lack of inclusion bodies was not due to a random mutation generated in the expression plasmid during the preparation of the mutation in the PTP gene. The resulting construct also yielded no inclusion bodies.

DISCUSSION

One of the physiologically essential members of the MTP family is the PTP, which catalyzes the transport of inorganic phosphate into the mitochondrial matrix where the phosphate is used primarily in the oxidative phosphorylation of ADP to ATP. In this investigation we report the characteristics of single residue replacement per subunit mutants of all hydroxyl, basic, and acidic residues to identify the regions of the PTP that are essential for the transport function. We conclude that there are 20 residues that upon replacement by an alanine or a residue similar to the replaced residue (conservative replacement) yield PTPs with completely blocked or severely inhibited transport activity (Figure 1).

A more detailed analysis of the results points to the effect of Tyr94Ala and Lys98Ala. Comparing the location of these two residues with the other three residues of helix B that

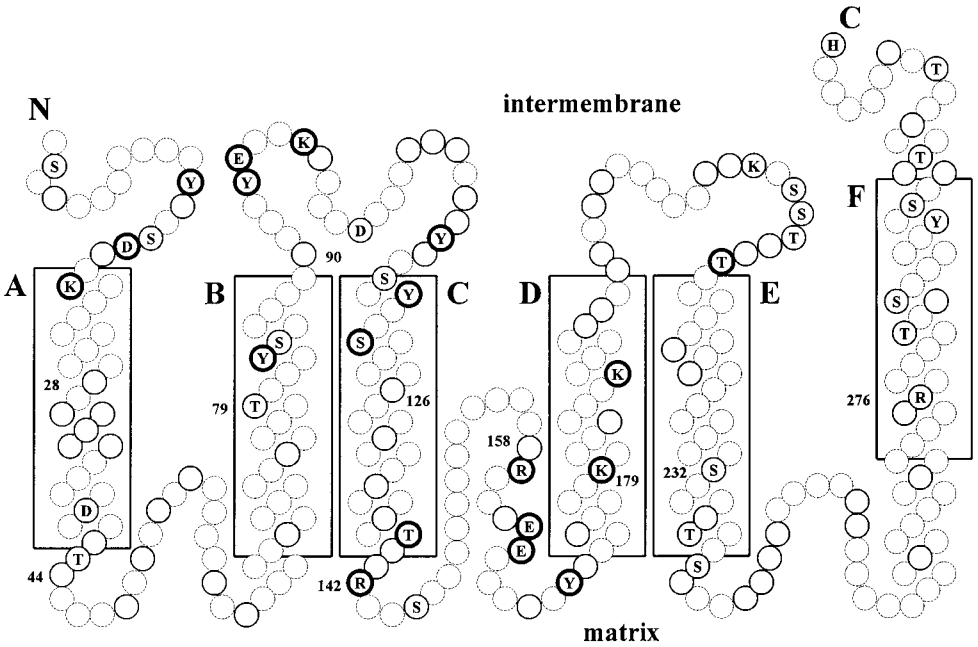

FIGURE 4: Diagram of yeast PTP showing amino acids that upon replacement yield single mutation PTPs with LIE dramatically different from wild-type PTP LIE. Single letter amino acid code is used. Heavy circles indicate residues whose single mutation PTP has a LIE of <6%. Residues with light circles yield PTP mutants with a LIE of >20%. Light solid circles without amino acid labels refer to other single site mutants that have been constructed and assayed.

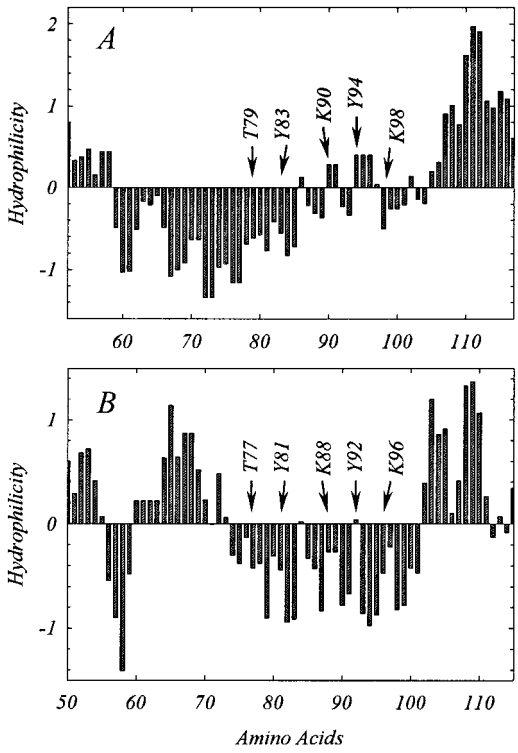

FIGURE 5: Hydrophilicity plots of the TM helix B region for (A) yeast PTP (*9*) and (B) bovine PTP (*12*). Amino acid numbers are with respect to the N-terminal residue of the PTP primary sequences. The two sequences were aligned on the basis of the function-significant residues of Figure 2, i.e., Thr79, Tyr83, Lys90, Tyr94, and Lys98 for yeast PTP and Thr77, Tyr81, Lys88, Tyr92, and Lys96 for bovine PTP.

block transport (Figure 2), it becomes clear that all five are located on the same face of helix B and thus could readily line the phosphate transport path. Helix B's hydrophilicity plot (Figure 5) is not typical for a TM helix. However, when we line up these five residues of helix B of the beef heart PTP (Thr77, Tyr81, Lys88, Tyr92, Lys96) (*9*) with those of helix B of yeast PTP (Figure 5), we find that the beef heart PTP residues are located quite nicely in a hydrophobic region appropriate for a TM helix, i.e., 28 overwhelmingly hydrophobic locations bordered at both ends by hydrophilic locations. The five residues of the yeast PTP are located within a much more mixed hydrophilic/hydrophobic region bordered, as expected, on the most likely helix B C-terminal end by a very hydrophilic region. However, near the N-terminal end, where the beef heart PTP sequence shows high hydrophilicity, the yeast PTP region is still highly hydrophobic. On the basis of this comparison, Tyr94 and Lys98 fit well into a yeast PTP TM helix B structure. We are left with the question of why helix B of yeast has an additional 15 residues of high hydrophobicity at its N-terminus. This kind of hydrophobic N-terminal extension is also present in the yeast TM helices F (Figure 6) and D (Figure 5 in ref *9*). Since PTP [like all members of the MTP family (*1*)] does have a triplicate repeat of about 100 residues each, it is not too surprising that helices B, D, and F show similar hydrophilicity profiles. It should also be noted that all members of the MTP family do have hydrophobic regions for helices A, C, and E that are much better defined than those for helices B, D, and F (*1*).

Our results demonstrate (Figure 2) that function-sensitive regions are almost exclusively restricted to the TM helices. Ser158Thr is located in a loop and does inhibit transport. However, Ser158 is not essential since Ser158Ala does not inhibit transport (*10*).

We are most interested in identifying those function-significant residues that are unique to PTP. They are the ones that line the phosphate and the proton transport pathways and that are responsible for coupling these two pathways. We have therefore aligned the 20 function-significant residues of PTP and their immediately adjacent residues with

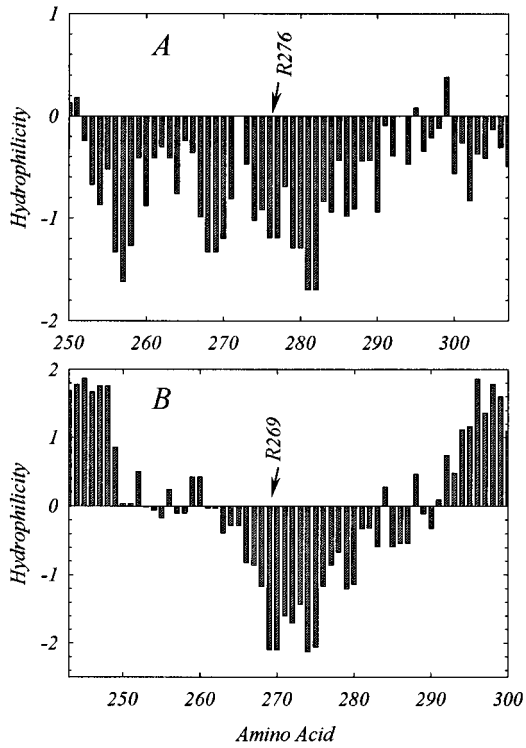

FIGURE 6: Hydrophilicity plots of the TM helix F region for (A) yeast PTP (*9*) and (B) bovine PTP (*12*). Amino acid numbers are with respect to the N-terminal residue of the primary PTP protein sequences. The two sequences were aligned on the basis of the function-significant residue of Figure 2, i.e., Arg276 of yeast PTP and Arg269 of bovine PTP.

the similar regions of the other 15 function-identified members of the MTP family (Figure 7).

We should point out that the 20 function-significant residues of the yeast PTP are also present, with only minor conservative replacements, in both the beef heart (*12*) and the rat liver (*13*) PTP sequences. Both of these mammalian sequences have a Cys instead of Thr43; they both have a Lys instead of Arg140; both have an Arg instead of Lys179; both have a Thr (instead of Ser195) shifted by one residue toward the N-terminus to retain its location on the same side of helix D as the two other function-significant residues (Lys179, Lys187). Thus the 20 function-significant yeast PTP residues are evolutionarily conserved and as such indeed functionally important.

His32 and Glu126 are two residues unique to PTP (Figure 7) and probably intimately involved in proton cotransport (*11*). Another residue nearly as unique to PTP is Thr43 (on the same face of helix A as PTP's His32 and Asp39). While AAC2 and four other transport proteins do have a basic residue (Lys or Arg) and an acidic residue (Glu or Asp) at locations similar to the His32 and Asp39 of PTP, none of them have a residue similar to Thr at a location similar to PTP's Thr43 (Figure 7). From among the His32 and Asp39 equivalent residues of these five proteins, only Lys38 and Glu45 of AAC2 have been replaced to characterize their function significance. Lys38, but not Glu45, was found to be essential for AAC2 transport (*14*, *15*). Interestingly, Asp39 is also not essential for PTP since we have shown earlier that the inactive Asp39Asn PTP mutant can be reactivated by the second site Glu192Asp mutation (*10*). Nevertheless, the set of residues (His32, Asp39, Thr43) on the same face of helix A is unique to PTP (Figure 7). The combination of Thr79, Tyr83, Lys90, Tyr94, and Lys98 (on the same face of helix B) and the combination of residues on the same face of helix D (Lys179, Lys187, Ser195) are also unique to PTP (Figure 7). Two proteins that transport tricarboxylates (CTP1, Yhm2p) (Figure 7) do have basic residues at locations equivalent to PTP's Lys179 and Lys187. These two Arg's (Arg181, Arg189) of CTP1 have been replaced and found to be essential for transport (*16*). CTP1 has a Lys197 at PTP's Ser195 location (Figure 7). The function significance of this residue has, however, not yet been determined.

It is also quite interesting that there are a number of residues that are common to all members of the MTP family. Their presence suggests common mechanics in transport as catalyzed by the MTPs. Thus we recently documented five residues that are perfectly conserved among function-identified members of the MTP family [*1*; the functions of four other members have recently been identified (*17*−*19*)]. These absolutely conserved residues are a Pro and a Lys in TM helix A, a Pro in TM helix E, and two Gly in TM helix F (*1*). These residues must be critically important to have been retained through evolution. The Lys is indeed functionally essential for PTP (Lys42) (*8*) (Figures 1 and 2) and for AAC2 (Lys48) (Figure 7) (*14*). We found that in PTP there are three residues (Glu192 at the intermembrane space end of helix D; Asp39 at the matrix end of helix A; Glu137 at the matrix end of helix C) with a very close functional relationship (*10*). These three residue locations are also entities of a general MTP family transport mechanism since there appears to be a rule that restricts the types of residues at the Asp39 and Glu137 locations once a residue is chosen for the Glu192 site in a functional transport protein (*10*).

Figure 7 suggests that other members of the MTP family may have function-significant residues at locations similar to those of PTP. However, such function significance has only been established in a few cases. Lys48 (*14*), Asp149 (*15*), Arg152 (*14*), and Asp249 (*20*, *21*) (equivalent to Lys42, Glu137, Arg140, and Asp236 of PTP, respectively) of AAC2 have been shown to be function-significant.

Figure 7 suggests possible interhelix contacts: i.e., Lys187 (PTP) has been replaced by Tyr in the ADP/ATP transporters (AAC1, AAC2, AAC3), and for Tyr94 (PTP) there is Lys in the ADP/ATP transporters. This could imply that helices B and D are next to each other and that their position is stabilized with a noncovalent interaction between Lys187 and Tyr94. If this interaction is purely structural, these two residues should be exchangeable to Tyr187 and Lys94 without affecting transport activity.

The yeast protein coded for by the gene YER053C is highly sequence-similar to PTP (*1*). However, when expressed as bacterial inclusion bodies and reconstituted, it shows no phosphate transport activity (H. Wohlrab and A. Tzagoloff, unpublished observations). A comparison of the sequences of these two proteins now shows that YER053C lacks the function-significant residue Ser195, and other residues in the loop neighboring this residue are missing or differ significantly from those in PTP. Thus our newly identified function-significant PTP residues provide a reasonable explanation for the lack of P_i transport function of the YER053C-encoded protein.

protein	gene	helix A		helix B	
PTP	YJR077C	T**H**$_{032}$S	I**D**$_{039}$VV**K**$_{042}$**T**$_{043}$R	P**T**$_{079}$LLG**Y**$_{083}$S	F**K**$_{090}$FGG**Y**$_{094}$EVF**K**$_{098}$K
AAC1	YMR056C	A**K**$_{028}$T	I**E**$_{035}$R**VK**$_{038}$L$_{039}$L	AN$_{083}$VLR**Y**$_{087}$F	LN$_{094}$FAF**K**$_{098}$DKI**K**$_{102}$S
AAC2	YBL030C	A**K**$_{038}$T	I**E**$_{045}$R**VK**$_{048}$L$_{049}$L	AN$_{093}$VIR**Y**$_{097}$F	LN$_{104}$FAF**K**$_{108}$DKI**K**$_{112}$A
AAC3	YBR085W	A**K**$_{027}$T	I**E**$_{034}$R**VK**$_{037}$I$_{038}$L	AN$_{082}$VIR**Y**$_{086}$F	LN$_{093}$FAF**K**$_{097}$DKI**K**$_{101}$L
Ant1p	YPR128C	AN$_{018}$I	L**D**$_{025}$LS**K**$_{028}$**T**$_{029}$I	V**T**$_{080}$TVA**T**$_{084}$F	VY$_{091}$FF**W**$_{095}$TFI**R**$_{099}$K
CTP1	YBR291C	E**A**$_{027}$C	F**E**$_{034}$FA**K**$_{037}$**T**$_{038}$R	AF$_{076}$IIGN$_{080}$T	I**R**$_{087}$FLGF$_{091}$DTI**K**$_{095}$D
Yhm2p	YMR241W	VT$_{035}$T	L**E**$_{042}$VV**K**$_{045}$**T**$_{046}$T	ST$_{086}$KGAV$_{090}$L	AE$_{097}$AEYR$_{101}$FKSL$_{105}$G
Sfc1p	YJR095W	EA$_{025}$L	L**D**$_{032}$TI**K**$_{035}$V$_{036}$R	AV$_{078}$VIGI$_{082}$I	I**R**$_{089}$FSS**Y**$_{093}$EFY**R**$_{097}$T
Dic1p	YLR348C	AT$_{028}$M	L**D**$_{035}$LA**K**$_{038}$V$_{039}$R	AA$_{074}$VLRQ$_{078}$C	V**R**$_{085}$FGA**Y**$_{089}$DLL**K**$_{093}$E
Oac1p	YKL120W	AV$_{037}$T	I**E**$_{044}$LI**K**$_{047}$I$_{048}$R	AA$_{090}$YIYQ$_{094}$I	S**R**$_{101}$LGF**Y**$_{105}$EPI**R**$_{109}$S
Odc1p	YPL134C	EL$_{026}$L	L**D**$_{033}$VV**K**$_{036}$**T**$_{037}$R	SP$_{087}$ILME$_{091}$A	I**K**$_{098}$FSGN$_{102}$DTFQ$_{106}$T
Odc2p	YOR222W	EL$_{027}$T	L**D**$_{034}$VV**K**$_{037}$**T**$_{038}$R	SP$_{085}$MLME$_{089}$A	T**K**$_{096}$FACN$_{100}$DQYQ$_{104}$K
CaGluAsp	YNL083W	S**R**$_{242}$T	F**D**$_{249}$RL**K**$_{252}$V$_{253}$F	LN$_{311}$VIKV$_{315}$F	I**K**$_{322}$FGSF$_{326}$EVT**K**$_{330}$K
Crc1p	YOR100C	AV$_{050}$F	F**D**$_{057}$LI**K**$_{060}$V$_{061}$R	PP$_{105}$LLGV$_{109}$T	VS$_{116}$FW**GY**$_{120}$DVG**K**$_{124}$K
ARG11	YOR130C	G**K**$_{028}$V	F**D**$_{035}$TV**K**$_{038}$V$_{039}$R	A**S**$_{075}$PLVG$_{079}$A	AT$_{086}$L**FVS**$_{090}$YNQC$_{094}$S
Flx1p	YIL134W	TT$_{024}$L	L**D**$_{031}$LL**K**$_{034}$V$_{035}$R	IN$_{080}$LFGN$_{084}$A	VY$_{091}$FGL**Y**$_{095}$GVT**K**$_{099}$E

gene	helix C	helix D			helix E	helix F
YJR077C	A**E**$_{126}$F	L**E**$_{137}$AT**R**$_{140}$I**R**$_{142}$L	F**K**$_{179}$Q	A**K**$_{187}$F	A**S**$_{195}$E	V**S**$_{232}$QPA**D**$_{236}$T	T**R**$_{276}$L
YMR056C	AG$_{129}$G	L**D**$_{140}$YA**R**$_{143}$T**R**$_{145}$L	LG$_{190}$I	LY$_{198}$F	FK$_{206}$P	A**S**$_{236}$YPL**D**$_{240}$T	AN$_{282}$I
YBL030C	AG$_{138}$A	L**D**$_{149}$YA**R**$_{152}$T**R**$_{154}$L	VG$_{199}$I	LY$_{207}$F	LK$_{215}$P	C**S**$_{245}$YPL**D**$_{249}$T	AN$_{291}$I
YBR085W	AG$_{127}$A	L**D**$_{138}$FA**R**$_{141}$T**R**$_{143}$L	VG$_{188}$I	LY$_{196}$F	LK$_{204}$P	C**S**$_{234}$YPL**D**$_{238}$T	AN$_{280}$I
YPR128C	AA$_{135}$S	MA$_{146}$VVA$_{149}$T**R**$_{151}$Q	AL$_{191}$T	TY$_{199}$A	KE$_{207}$V	V**T**$_{240}$QPLI$_{244}$V	PQ$_{283}$L
YBR291C	GL$_{120}$L	F**E**$_{131}$AI**K**$_{134}$TA$_{136}$L	M**R**$_{181}$Q	V**R**$_{189}$L	IK$_{197}$T	S**T**$_{232}$MPL**D**$_{236}$T	P**R**$_{276}$L
YMR241W	LT$_{128}$M	V**E**$_{139}$IT**R**$_{142}$H**K**$_{144}$S	I**R**$_{181}$Q	S**R**$_{189}$F	VE$_{197}$D	WN$_{232}$QPI**E**$_{236}$V	P**R**$_{280}$I
YJR095W	GI$_{122}$T	M**E**$_{133}$VV**K**$_{136}$I**R**$_{138}$L	A**R**$_{184}$Q	AN$_{192}$F	LK$_{200}$E	SN$_{232}$APL**D**$_{236}$T	P**R**$_{282}$V
YLR348C	VV$_{129}$N	L**E**$_{140}$AA**K**$_{143}$**R**R$_{145}$N	V**R**$_{177}$G	SQ$_{185}$V	FK$_{193}$N	VC$_{225}$SPA**D**$_{229}$V	PS$_{268}$F
YKL120W	SG$_{139}$I	LF$_{150}$LV**K**$_{153}$T**R**$_{155}$L	IF$_{182}$K	GL$_{190}$F	AI$_{198}$L	VM$_{247}$NPW**D**$_{251}$V	AQ$_{291}$V
YPL134C	AG$_{133}$A	F**E**$_{144}$LV**K**$_{147}$I**R**$_{149}$L	W**R**$_{186}$H	GY$_{194}$F	IR$_{202}$K	LN$_{233}$TPF**D**$_{237}$V	P**K**$_{279}$V
YOR222W	AG$_{129}$M	F**E**$_{140}$LI**K**$_{143}$I**R**$_{145}$M	W**R**$_{182}$N	GY$_{190}$F	VR$_{198}$N	LN$_{229}$TPF**D**$_{233}$V	P**K**$_{278}$V
YNL083W	AG$_{358}$M	ID$_{369}$TL**K**$_{372}$F**R**$_{374}$V	VG$_{416}$I	LD$_{424}$L	LK$_{432}$K	VV$_{472}$YPIN$_{476}$L	PT$_{520}$L
YOR100C	SA$_{152}$I	T**E**$_{163}$R**VK**$_{166}$VV$_{168}$L	A**R**$_{203}$D	LY$_{211}$F	SK$_{219}$N	AV$_{257}$FPI**D**$_{261}$T	PA$_{300}$L
YOR130C	AG$_{118}$S	V**E**$_{129}$LV**K**$_{132}$C**K**$_{134}$L	I**R**$_{178}$E	AW$_{186}$F	VK$_{194}$K	SI$_{231}$FPA**D**$_{235}$T	IT$_{271}$L
YIL134W	SG$_{136}$L	IW$_{147}$VI**K**$_{150}$T**R**$_{152}$I	FG$_{193}$V	YF$_{201}$A	KQ$_{209}$R	LV$_{244}$YPFQ$_{248}$L	AN$_{289}$L

FIGURE 7: Function-significant amino acids (bold, single letter amino acid code) of PTP within the other 15 function-identified members of the MTP family (*1*, *17*−*19*). The sequences were aligned with DNAStar (version 3.02) (*1*), and the amino acids at the same location of all 16 proteins are shown. The subscript number refers to the residue position with respect to the N-terminal residue of the protein. The yeast gene reference number is shown as well as the abbreviated name of the encoded protein. Primary substrates transported by the proteins: phosphate (PTP); ADP/ATP (AAC1, AAC2, AAC3); ADP/ATP/AMP (Ant1p, peroxisomal); tricarboxylate (CTP1, Yhm2p); dicarboxylate (Sfc1p, Dic1p); oxodicarboxylates (Oac1p, Odc1p, Odc2p); glutamate/aspartate (CaGluAsp, Ca^{2+}-regulated); carnitine (Crc1p); ornithine (ARG11); flavin (Flx1p).

The LIE's of the mutants do differ dramatically (Figure 3). We have not as yet unequivocally identified the reason for these differences. While we do have evidence that the subunits of those mutants with high LIE show a higher affinity for each other within the homodimeric PTP, additional investigations are required. There is as yet no obvious correlation between LIE and transport activity. A comparison of Figures 2 and 4 shows that replacing Asp39 or Thr79 yields PTP mutants with no transport activity yet high LIE. On the other hand, when Arg142 is replaced, the resulting PTP mutant also shows no transport activity yet its LIE is low. It is clear that we do not yet understand why the replacement of only some function-significant residues affects the interaction of PTP with the lipid bilayer. If the subunit−subunit interface of the homodimeric PTP plays a critical role in transport and in LIE, then our results should become helpful in mapping the subunit−subunit contact face.

Finally, our observation that the Ser4Ala mutation completely prevents the accumulation of bacterial PTP inclusion bodies may be due to, among other possibilities, a redirection of the transport protein to the bacterial membrane. Our observation should call attention to the possibility that factors other than a low concentration of bacterial tRNAs for eukaryotic codons may prevent the formation of bacterial inclusion bodies.

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