Mitochondrial Phosphate Transport Protein. Replacements of Glutamic, Aspartic, and Histidine Residues Affect Transport and Protein Conformation and Point to a Coupled Proton Transport Path†

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ABSTRACT: The homodimeric mitochondrial phosphate transport protein (PTP), which has six transmembrane helices per subunit, catalyzes inorganic phosphate transport in an electroneutral and pH gradient-dependent manner across the inner membrane. We have replaced the Glu, Asp, and His residues of the yeast PTP to assess their role in the transport mechanism. Mutants with physiologically relevant transport activity were identified by their ability to rescue the PTP null mutant yeast from glycerol medium. Five residues appear critical for transport: His-32 in helix A, Glu-126 and -137 in helix C, and Asp-39 and -236 at the matrix ends of helices A and E. These mutant PTPs are expressed at near normal levels in yeast. This yeast PTP and the mutants were expressed in *Escherichia coli* as inclusion bodies, solubilized, purified, and reconstituted. Their transport activities correlate well with the physiological assays. None of the transport inactivating mutations appear to be due to major protein conformation changes as assayed by the efficiency of PTP incorporation into liposomes. Only the Glu95Gln (cytosolic helices B and C-connecting segment), Glu163Gln and Glu164Gln (matrix helices C and D-connecting segment), and Glu126Asp (helix C) show a near 70% decrease in liposome incorporation efficiency. In addition, mutations at either end of helix D increase phosphate transport 2-fold. We would like to suggest that Glu-126, His-32, and Glu-137 (similar to Asp-96, Lys-216, and Asp-85 of bacteriorhodopsin) form a proton cotransport pathway that is coupled in an as yet undefined manner (possibly via His-32) to a phosphate transport pathway, which may include helix D.

Inorganic phosphate must be transported into the mitochondrial matrix where it is used for the phosphorylation of ADP by oxidative phosphorylation. The resulting ATP is transported by the ADP/ATP translocase out of the matrix into the cytosol in exchange for ADP. The return of inorganic phosphate back into the mitochondrial matrix is catalyzed by the PTP.[1] This transport protein was first partially purified and reconstitution of its transport activity demonstrated to be feasable by Racker and co-workers (Banerjee et al., 1977).

PTP has been purified as a single protein and incorporated into liposomes. Phosphate transport activity has been demonstrated in these proteoliposomes. Since purification of membrane proteins is difficult and the purified protein is hardly ever completely without other contaminating proteins, the identification of a protein sequence with transport function at this stage remained in doubt. The recent expression of the yeast mitochondrial PTP from a yeast shuttle vector in a yeast PTP null mutant, together with a single amino acid replacement that changed its inhibitor sensitivity, has provided considerable confidence in the correlation of function and sequence (Phelps & Wohlrab, 1991). More recently, the yeast PTP has been expressed in *Escherichia coli* as inclusion bodies. Its purification from these inclusion bodies and reconstitution of transport activity, as well as a modification of transport inhibitor sensitivity by amino acid replacements, represent now the most definitive evidence identifying amino acid sequence with transport function (Wohlrab & Briggs, 1994).

The inorganic phosphate transport is electroneutral. Since only traces of phosphoric acid are present in the cell at near neutral pH, this transport occurs probably as a phosphate/proton cotransport. Amino acid residues, that most likely are part of such a proton cotransport pathway, are acidic like Glu and Asp and also His. We have now replaced most of these relevant residues with other residues and conclude that PTP may utilize a proton pathway similar to that of bacteriorhodopsin. Some of these results have been communicated as abstracts (Wohlrab & Phelps, 1993; Wohlrab et al., 1995).

MATERIALS AND METHODS

Preparation of Mutant PTP Plasmids. The yeast shuttle vector pAP-W3 (Phelps et al., 1991) was used to express the mutant PTPs in *Saccharomyces cerevisiae* (Phelps & Wohlrab, 1991). For expression of PTP in *E. coli*, the

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[1] Abbreviations: PTP, phosphate transport protein; Glu126Gln, replacement of Glu-126 with Gln; PCR, polymerase chain reaction; MIR, mitochondrial import receptor (same as PTP); SDS, sodium dodecyl sulfate; MTP family, mitochondrial transport protein family; HCA, human carbonic anhydrase.

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Table 1: Mutant Codons and Incorporation of Transport Protein into Liposomes

PTP	codons (wild type/ mutant)	PTP in reconstitution mixture (μg)	PTP in fractionated and transport-active proteoliposomes (μg)	incorporation efficiency of PTP into liposomes (%)
wild type		1.270	0.214	16.8
		0.688	0.082	11.9
His32ala[a]	CAC/GCC	0.969	0.096	9.9
His32gln	CAC/CAA	0.900	0.115	12.8
His32asn	CAC/AAC	0.900	0.116	12.9
His32arg	CAC/CGC	0.880	0.085	9.6
His32lys	CAC/AAA	0.968	0.126	13.1
his311	CAT			
glu48gln	GAA/CAA	1.104	0.161	14.6
glu67gln	GAA/CAA	0.984	0.123	12.5
Glu95gln	GAA/CAA	0.604	0.032	5.4
Glu126gln	GAA/CAA	1.100	0.197	17.9
Glu126asp	GAA/GAT	1.208	0.046	3.8
Glu137gln	GAA/CAA	0.760	0.094	12.4
Glu137asp	GAA/GAT	1.080	0.097	9.0
Glu163gln	GAA/CAA	0.692	0.040	5.8
Glu164gln	GAA/CAA	0.716	0.028	3.9
Glu192gln	GAA/CAA	0.776	0.072	9.3
Glu196gln	GAG/CAG	0.668	0.090	13.4
glu206	GAA			
Glu305gln	GAA/CAA	1.384	0.240	17.4
asp16	GAC			
Asp39glu	GAT/GAA	1.144	0.192	16.8
Asp39asn	GAT/AAT	0.992	0.217	21.9
asp103	GAC			
asp108asn	GAT/AAT	0.816	0.136	16.7
Asp130asn	GAC/AAC	0.568	0.114	20.0
Asp236glu	GAC/GAA	0.804	0.152	18.9
Asp236asn	GAC/AAC	0.604	0.108	17.9

[a] Capitalized residues are conserved between beef and yeast PTP.

pNYHM131 vector (Murakami et al., 1993) was used. Amino acid replacements were carried out according to a PCR protocol (Phelps & Wohlrab, 1991). Codons of the new residues as well as of the wild type are shown in Table 1.

The final plasmid construct was characterized by restriction mapping with endonuclease cuts at the subcloning sites and by sequencing of the subcloned PCR fragment and subcloning sites. Often the whole PTP gene was sequenced.

Transformation of S. cerevisiae and E. coli. The PTP null mutant (mir) of *S. cerevisiae* (CG379mir::URA3) (Murakami et al., 1990) was transformed with the yeast shuttle vector constructs (Phelps & Wohlrab, 1991). Growth on glycerol and glucose plates was determined as described (Phelps & Wohlrab, 1991). *E. coli* BL21 (D3) was transformed, and expression of the mutant PTPs was initiated, according to published procedures (Wohlrab & Briggs, 1994).

Preparation of Yeast Mitochondria. Yeast were grown in galactose medium (1.5 L of medium in a 6 L flask, 30 °C). Galactose medium (1.5 L) was prepared with 10.0 g of Yeast Nitrogen Base w/o Amino Acids (dehydrated, Difco Laboratories), 30 g of D-(+)-galactose (Sigma G-0625), 45 mg of L-leucine (Sigma L-800), 30 mg of L-histidine (Sigma H-8125), and 30 mg of adenine (hemisulfate salt, Sigma A-3159). The yield of yeast was about 3 g wet weight/L of medium.

Mitochondria were prepared from glass bead-broken cells. To 18 g of wet yeast pellet, add 150 mL of SE medium [436 g of D-sorbitol (Sigma S-6021) and 14.9 g of Na_2-EDTA·$2H_2O$, make to 4 L with water and adjust pH with NaOH to 6.5]. Carry out all steps at 0−4 °C. Disperse the yeast pellet and centrifuge (four 50 mL tubes) at 1100*g*, 2.5 min. To each pellet add 5 mL of SE and pool the four suspended pellets. Add 40 mL of glass beads (0.5 mm, acid cleaned, and washed with SE) in an 80 mL blending bottle; top off with SE and blend for 60 s; pour off broken cell mix and rinse beads 2× with fresh SE. Pool the blended cells and rinses and spin at 1100*g*, 2.5 min, saving the supernatant. Repeat blending/rinsing cycle with blended cells two times (total of 3 cycles). Spin pooled supernatant 8700*g*, 7.5 min. Discard supernatant. Save pellet (mito pellet I). Take yeast cell debris pellet, blend 60 s, and rinse with SE two times. Spin 270*g*, 2.5 min. This should yield a homogeneous white pellet with no dark residue (mitochondria) on top of pellet. Spin supernatant at 8700*g*, 7.5 min. Save pellet (mito pellet II). Suspend mito pellets I and II with glass/Teflon homogenizer in SE and pool. Spin at 270*g*, 2.5 min. Take supernatant and spin at 8700*g*, 7.5 min. Suspend pellet in small volume of SE, rapid freeze in liquid nitrogen, and store, at −70 °C.

Immunoblots. Various mitochondrial preparations were separated on SDS gels and blotted onto nitrocellulose membranes. PTP was visualized after complexing with PTP specific antibodies (Pain et al., 1990), with peroxidase-conjugated F(ab′)$_2$ fragment of goat anti-rabbit IgG, and incubation with *o*-dianisidine and H_2O_2 (Joshi & Burrows, 1990).

Reconstitution of PTP and Transport Assays. Reconstitution and transport assays were carried out as described (Wohlrab & Briggs, 1994). Transport-active proteoliposomes were obtained by centrifuging the freezed−thawed−vortexed reconstitution mix at (10^4)*g* for 15 min at 4 °C.

The supernatant was incubated at 0−4 °C for 25 min with 15 mM dithiothreitol before being used in the transport assay.

Protein Determination. The concentration of PTP in the reconstitution mix was determined from Coomassie Blue-stained (Serva Blau R, C.I. 42660) SDS polyacrylamide gels (destained for 24 h with gentle shaking) of the PTP fraction just before mixing it with liposomes, using human carbonic anhydrase (Sigma 4396) as a protein standard. The gel was scanned using a CCD video camera module with a Sony TV Zoom Lens hooked up to a computer running "NIH Image 1.53" software.

PTP in the proteoliposomes was determined as follows. Transport-active proteoliposomes were prepared as described above. Immediately after the transport assay, 30 μL of the stock proteoliposomes were added to 500 μL of C_e (10 mM Tris, 10 mM PIPES, pH adjusted to 6.8 with KOH) at room temperature and centrifuged in a Beckman TL100 ultracentrifuge for 30 min at $(3 \times 10^5)g$ (90 000 rpm) at 4 °C. To the pellet was added 22.5 μL of the SDS gel electrophoresis sample buffer (Kolbe et al., 1984), and duplicate 10 μL samples were applied to the gel. After electrophoresis, the gel was stained with Coomassie Blue, destained, and then silver-stained. HCA served as reference and had been calibrated in a separate experiment with standard dilutions of freshly prepared PTP at the purification stage just before its addition to a reconstitution mix (Wohlrab & Briggs, 1994). The silver-stained gels were scanned like the Coomassie Blue-stained gels.

Reagents. [^{32}P]P_i was obtained from Amersham in a carrier free form in diluted HCl. This [^{32}P]P_i was diluted into transport medium and kept at room temperature for at least 24 h to decrease counts that do not exchange to the anion exchange column.

RESULTS

Physiological Competence of Mutant PTPs. Growth on plates with glycerol as primary carbon source requires oxidative phosphorylation. Figure 1 shows glycerol plates of yeast with wild type PTP that clearly is able to support growth. On the other hand, the PTP null mutant (mir), Asp39Asn, and Glu137Gln are not able to support yeast growth on glycerol, while Glu126Gln and Asp236Asn do support some growth, which however is much less than that with the wild type (Figure 1).

Expression of Mutant PTPs in Yeast. Mutant PTPs, that cannot support growth on glycerol, may lack ability to transport P_i, not only because a functionally essential residue has been replaced, but also because PTP is not incorporated into the membrane. Figures 2 and 3 show SDS gels of mutant PTPs that cannot, or only poorly, support yeast growth on glycerol. The proteins in the gel were blotted and probed with PTP antibodies. Figures 2 (His-32 mutants) and 3 (Asp-39, Glu-126, Glu-137, Asp-236 mutants) show that these mutant PTPs are expressed at near normal levels in the membrane.

Efficiency of PTP Incorporation into Liposomes. Mutant PTPs were expressed in *E. coli* as inclusion bodies. They were then purified from the inclusion bodies and reconstituted. We determined directly the total amount of PTP in the fractionated proteoliposomes used for the transport assays. Table 1 shows the efficiency of transport protein incorporation into the liposomes for the wild type PTP as well as the mutants. This efficiency is about 15% for the wild type and ranges from a low of 3.8% for Glu126Asp to a high of 21.9% for Asp39Asn.

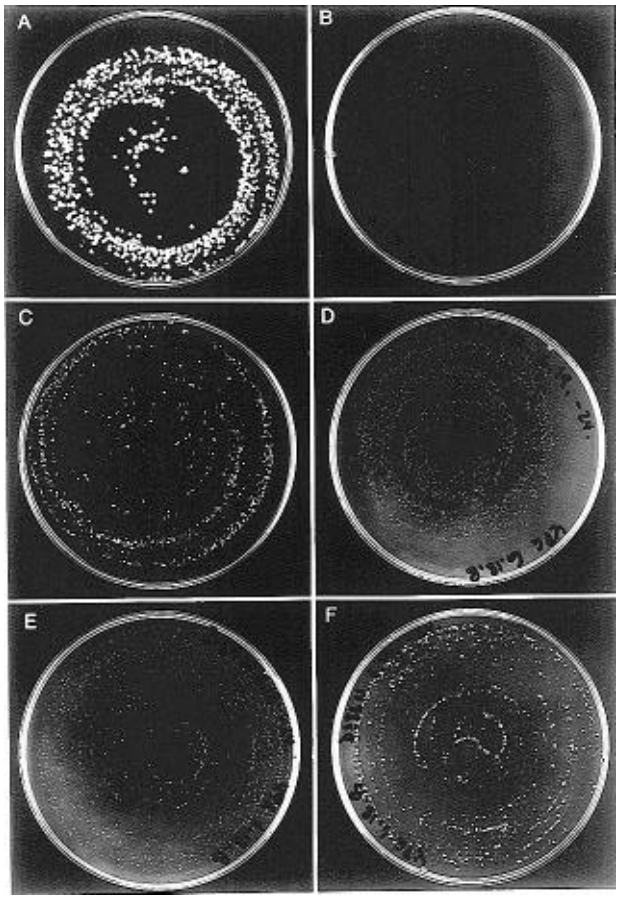

FIGURE 1: Growth of yeast on YPG plates. Yeast cells were spread on YPG plates and incubated for 5 days at 30 °C. All plates show CG379 mir::URA3 transformed with: (A) pAP-W3; (B) nothing; (C) pAP-W3 (Glu126Gln); (D) pAP-W3 (Glu137Gln); (E) pAP-W3 (Asp39Asn); (F) pAP-W3 (Asp236Asn). Codon replacements in the PTP gene of pAP-W3 as indicated in Table 1.

P_i Transport by Mutant PTPs. pH gradient-dependent net phosphate transport was determined with the reconstituted proteoliposomes. Table 2 presents the results for all the constructed and assayed mutants. Most obvious are the results that mutant PTPs, with His-32 replaced with several different residues, are not able to transport P_i. Replacement of this His-32 with basic residues Arg and Lys permits sufficient transport to permit kinetic analyses. However, replacement with Ala, Gln, or Asn yields no or barely detectable transport, i.e., less than 0.5% of the wild type. This is true also when transport is assayed with 5 mM P_i; i.e., an increased K_m does not explain the lack of transport at 0.66 mM P_i.

Replacing Glu-126, Glu-137, Asp-39, and Asp-236 also blocks transport completely as detected in our assay. Glu-126 and Glu-137 can be replaced with Asp, and the resulting transport activity is sufficient to support growth on glycerol. Replacement of either of these two residues with Gln inactivates transport. Replacement of Asp-39 or Asp-236 with Glu or Asn blocks transport (Table 2) and normal growth by yeast on glycerol (Figure 1).

Table 2 shows several entries with K_ms without a corresponding V_{max}. The V_{max} and K_m values were determined in our initial set of investigations. At that time the method for

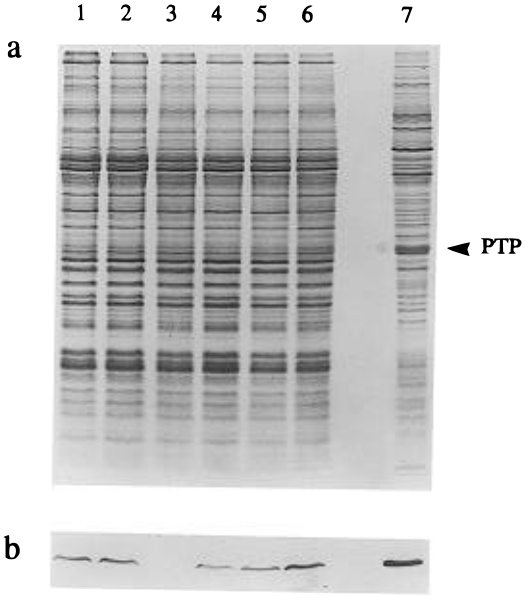

FIGURE 2: (a) Coomassie Blue-stained SDS gel of mitochondria from yeast CG379 mir::URA3 transformed with pAP-W3 with codon replacements in the PTP gene as indicated in Table 1 and grown in dextrose medium. (b) Immunoblot of the SDS gels probed with PTP (MIR) antiserum (see Materials and Methods). Lanes: (1) His32Gln; (2) His32Arg; (3) mir (null); (4) His32Asn; (5) His32Ala; (6) pAP-W3; (7) pAP-W3 (lactate medium).

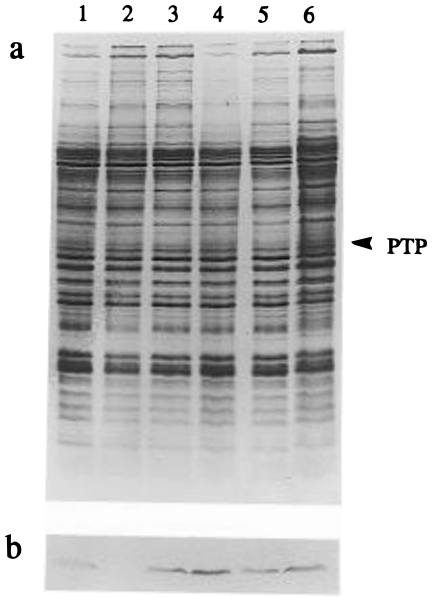

FIGURE 3: (a) Coomassie Blue-stained SDS gel of mitochondria from yeast CG379 mir::URA3 transformed with pAP-W3 with codon replacements in the PTP gene as indicated in Table 1 and grown in dextrose medium. Lanes: (1) His32Asn; (2) mir (null); (3) Asp39Asn; (4) Glu126Gln; (5) Glu137Gln; (6) Asp236Asn. (b) Immunoblot of the SDS gels probed with PTP (MIR) antiserum (see Materials and Methods).

determining PTP in proteoliposomes had not yet been established. Thus, in the absence of PTP concentrations, only the K_m, which does not depend on the protein concentration, is shown.

DISCUSSION

The mitochondrial phosphate transport protein is well suited for a detailed investigation into the molecular mechanism of transport. Primary reasons are the following: (1) the transported substrates (phosphate and proton) are simple;

Table 2: Transport Characteristics of Reconstituted Transport Proteins

PTP	V (at 0.66 mM P_i)[a] [(μmol of P_i) (10 s)$^{-1}$ (mg of PTP)$^{-1}$]	V_{max} [(μmol of P_i) min^{-1} (mg of PTP)$^{-1}$]	K_m (mM)	temp[b] (°C)
wild type	33.6	649	1.1	22.0
	36.8	911	1.2	24.5
His32ala[c]	0.0			24.0
His32gln	0.2			24.0
His32asn	0.1			24.5
His32arg	0.5		1.2	23.5
His32lys	0.6		1.0	23.5
his311				
glu48gln	22.8	288	0.7	23.5
glu67gln	27.6	454	0.9	24.0
Glu95gln	21.9		1.3	23.5
Glu126gln	0.0			22.0
Glu126asp	9.4		3.2	22.5
Glu137gln	0.1			22.0
Glu137asp	3.5		1.4	24.0
Glu163gln	63.5		1.2	24.0
Glu164gln	7.7		1.5	24.0
Glu192gln	11.4		1.0	22.0
Glu196gln	56.8		1.2	25.0
glu206				
Glu305gln	19.3	385	0.8	23.5
asp16				
Asp39glu	0.2		2.8	22.0
Asp39asn	0.2		1.3	23.0
asp103				
asp108asn	33.8		0.8	23.0
Asp130asn	11.7		1.0	23.5
Asp236glu	0.1			23.5
Asp236asn	0.0			23.5

[a] V experiments were conducted at 23.5 °C. [b] Temperature at which V_{max} and K_m were determined. [c] Capitalized residues are conserved between beef and yeast PTP.

(2) an example for a well-defined transport pathway for protons is bacteriorhodopsin (Khorana, 1993; Lanyi, 1993); the high resolution X-ray structures of the wild type periplasmic bacterial phosphate binding protein (Luecke & Quiocho, 1990) and two mutants (Wang et al., 1994; Yao et al., 1996) provide an example of amino acid ligands in a binding site highly specific for P_i. Other reasons are as follows: (1) PTP from mammalian sources as well as from yeast has been purified and sequenced; (2) there is only a single PTP gene in yeast and its absence produces a strong phenotype; (3) the characterization of mutant PTPs has been enormously facilitated by the successful development of a method for the expression of the yeast PTP in *E. coli*, and its reconstitution after purification from the inclusion bodies (Wohlrab & Briggs, 1994).

The residues that we have investigated by replacements (Table 1 and Figure 4) have a subgroup that consists of those that are conserved between yeast and mammalian PTP. All those that do not fall into this subgroup (Glu-48, Glu-67, Asp-108) can be replaced with Gln or Asn without affecting transport or liposome incorporation efficiency (Tables 1 and 2). Among those residues that are strictly conserved, only one (Asp-236) is strictly conserved also among all members of the MTP family (Table 3). Replacement of this residue with Asn or Glu blocks transport but has no effect on liposome incorporation efficiency. Our interpretation is that inhibition is due to the local residue replacement effect rather than a global conformation change due to misfolding of the protein. Among the other four residues that are strictly

Table 3: Sequence Similarities of Members of the Mitochondrial Transport Protein Family (and Bacteriorhodopsin) Bordering Phosphate Transport Important Residues[a]

name	(His-32, Asp-39)	(Glu-126, Glu-137)	(Glu-163, Glu-164)	(Glu-192, Glu-196)	(Asp-236)
PTP[b]	IGCGST**H**SSMVPI**D**VVK$_{42}$	MA**E**FLADIALCPL**E**ATR$_{140}$	SRILK**EE**GIG$_{167}$	VF**E**RAS**E**F$_{197}$	SQPA**D**TLL$_{239}$
AAC[c]	VAAAIS**K**TAVAPI**E**RVK$_{32}$	AAGATSLCFVYPL**D**FAR$_{137}$	TKIFKS**D**GLR$_{170}$	VY**D**TAKGM$_{200}$	SYPF**D**TVR$_{234}$
UCP[d]	VAACLA**D**IITFPL**D**TAK$_{37}$	MTGGVAVLIGQPT**E**VVK$_{137}$	RIIATT**E**SFS$_{170}$	TY**D**LMKGA$_{200}$	ASPA**D**VVK$_{236}$
OGC[e]	LAGMGATVFVQPL**D**LVK$_{46}$	TAGATGAFVGTPA**E**VAL$_{144}$	FRIVQ**EE**GVP$_{178}$	SYSQSKQF$_{208}$	SMPV**D**IVK$_{244}$
CTP[f]	LAGGI**E**ICITFPT**E**YVK$_{37}$	AGVA**E**AVVVVCPM**E**TVK$_{134}$	REIVR**E**QGLK$_{165}$	VMTSLRNW$_{195}$	NTPL**D**VIK$_{232}$
BR[g]	ILGFGV**K**ASV**D**LVMFLL$_{206}$	AL**D**LLLLPTTFLW**D**AYR$_{82}$			

[a] The PTP important residues are bold and underlined (see also Table 1). [b] Phosphate transport protein from yeast (*S. cerevisiae*) (Phelps et al., 1991). [c] ADP/ATP translocase 1 from beef (Aquila et al., 1982). [d] Uncoupling protein from hamster (Aquila et al., 1985). [e] Oxoglutarate carrier from beef (Runswick et al., 1990). [f] Citrate transport protein from rat liver (Kaplan et al., 1993). [g] These bacteriorhodopsin sequences are shown from C- to N-terminal (Dunn et al., 1983).

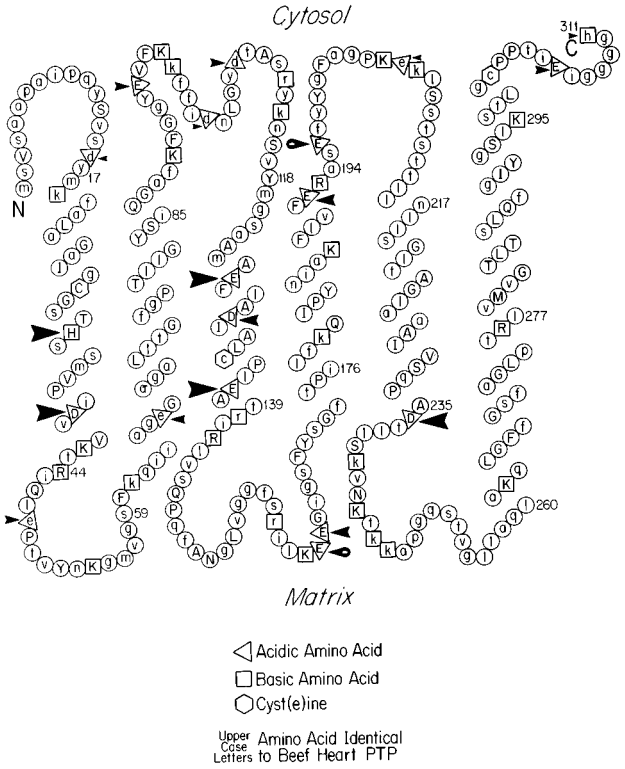

FIGURE 4: Transmembrane topography of yeast PTP. Arrowheads mark residues that have been replaced. Largest arrowhead indicates replacement with greatest inhibition of transport. Smallest arrowheads identify residues that have not yet been replaced. Arrowheads with loop indicate replacements that increase transport activity (see Table 2). Helix closest to N-terminal is A; that closest to C-terminal is F.

conserved among PTPs and whose sterically conservative replacement blocks transport, two (His-32, Glu-126) are unique to PTPs and are not present in other members of the MTP family, and two (Asp-39, Glu-137) are conserved only by residue type among other members of the MTP family (Table 3). Replacing Asp-39 with Glu or Asn yields the same results as replacing Asp-236 with Glu or Asn. This is also true for Glu-137, except that both replacements (Asp, Gln) yield a somewhat lower liposome incorporation efficiency. The Asp replacement does retain 10% of the wild type transport activity (*V* in Table 2). Replacing Glu-126, which is not present in other members of the MTP family, yields PTPs with properties similar to Glu-137 replacements. However, the Glu126Asp shows, surprisingly, a dramatic drop in liposome incorporation efficiency, yet retains more than 25% of the wild type *V* (Table 2). The Gln replacement, on the other hand, retains the wild type liposome incorporation efficiency with no transport activity. The replacements of His-32 (Tables 1 and 2) yield basically the same liposome incorporation efficiency as the wild type with traces of transport activity only with the basic residues Arg and Lys. It will be of interest to determine in what way, beyond a decrease in V_{max}, this residual transport differs from that of the wild type.

None of the replacements that block transport have a dramatic effect on liposome incorporation efficiency nor on incorporation into the mitochondrial membrane (Figures 2 and 3). Four other replacements show the most significant decrease in liposome incorporation efficiency. Among these, Glu95Gln (cytosolic side) shows a significantly lower transport activity, Glu163Gln (matrix side) doubles the wild type transport activity, and Glu164Gln (matrix side) has a significantly lower transport activity. Liposome incorporation efficiency is a novel new way to identify differences in membrane protein conformations. We have not yet identified protein characteristics that influence this process. A hydrodynamic or lipid/detergent binding comparison of wild type PTP, Glu126Gln, and Glu126Asp will be useful in further characterizing the structural differences.

Intramembrane acid residues have been implicated in salt bridges such as those that involve the quaternary ammonium of phosphatidylcholine (Iwata et al., 1995). King et al. (1991) showed that, in the lactose carrier of *E. coli*, the Lys358Thr replacement and also the Asp237Asn replacement generate a functional defect. Both mutations together, however, permit active transport. These results and others (Sahin-Toth et al., 1992; Dunten et al., 1993) suggest that Lys358 and Asp237 form an intramembrane salt bridge. Five residues yield transport-inactive PTPs upon replacement (Table 2). We have looked for second site revertants for these transport-inactive replacement PTPs and have found three (Ligeti et al., unpublished experiments). None of these suggest salt bridges.

The structural data on the bacterial periplasmic phosphate binding protein (Luecke & Quiocho, 1990; Wang et al., 1994; Yao et al., 1996) show that Asp-56 in this protein is a residue critically important for differentiating between sulfate and phosphate binding, i.e., it accepts the H from the dibasic or monobasic phosphate to form a hydrogen bond. Charge repulsion between this Asp-56 and sulfate prevents sulfate

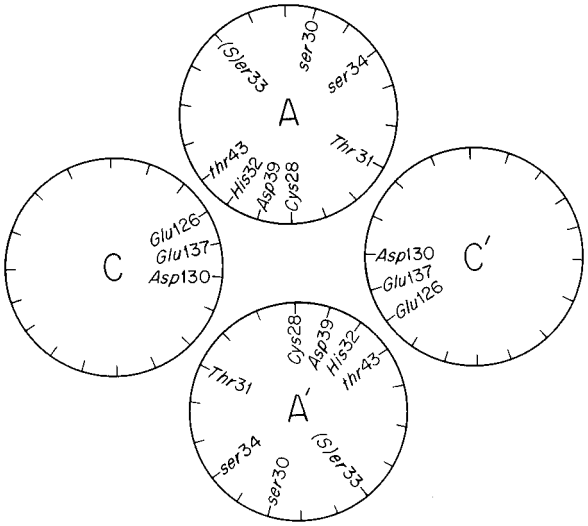

FIGURE 5: Helix wheel diagram of two helices of the two subunits of the homodimeric yeast PTP. Constraints in the diagram are the following: (a) Cys-28 of helices A and A′ (A′ is helix of other subunit of PTP homodimer; A and A′ are the most N-terminal helices) form, under oxidizing conditions, a transport-inhibiting intersubunit disulfide bond (Phelps & Wohlrab, 1993); and (b) Glu-126 and −137 are near to His-32 for an optimal proton transport pathway. Capitalized residues are conserved among PTPs from yeast, rat liver, and beef heart (Phelps et al., 1991). Those residues capitalized and in parentheses are Ser or Thr in rat liver and beef heart PTP.

from binding. In fact, replacing Thr-141 with an Asp eliminates dibasic phosphate binding since the second H of the monobasic phosphate is required for the interaction with the new Asp-141. Whether some of the acid residues of PTP (Glu-163, -164, -192, -196) play such a role in the generation of very high substrate specificity of PTP remains to be established.

Acid residues are an essential part of the proton transport path in bacteriorhodopsin (Mogi et al., 1988; Butt et al., 1989; Khorana, 1993; Lanyi, 1993). Replacing Asp-85 and Asp-96, two residues that undergo protonation/deprotonation during proton transport, with the larger Glu does not block transport, only decreases it. Replacing Asp-212, only indirectly involved in transport and located in the same helix as the Schiff base (Lys-216/retinal), with Glu decreases the transport to only 6% of the wild type. In PTP, Glu-126 and Glu-137 are separated by the same number of residues along helix C as Asp-85 and Asp-96 in bacteriorhodopsin (Table 3). His-32 would replace the Schiff base. The His-32 environment would be modulated by P_i instead of retinal and light. Asp-39 may function, similar to Asp-212 (Table 3), on the proton ejection side of PTP. The helix wheel diagram of Figure 5 suggests how placing helices A, A′, C, and C′ of the homodimeric PTP may satisfy steric requirements for (1) the participation of Glu-126, His-32, and Glu-137 in a proton cotransport path and (2) the formation of an intersubunit and transport-inhibitory disulfide bond (Phelps & Wohlrab, 1993). Additional investigations will elucidate the roles of these residues in PTP.

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