Mitochondrial Phosphate Transport Protein. Reversions of Inhibitory Conservative Mutations Identify Four Helices and a Nonhelix Protein Segment with Transmembrane Interactions and Asp39, Glu137, and Ser158 as Nonessential for Transport†

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ABSTRACT: The mitochondrial phosphate transport protein (PTP) has six (A−F) transmembrane (TM) helices per subunit of functional homodimer with all mutations referring to the subunit of the homodimer. In earlier studies, conservative replacements of several residues located either at the matrix end (Asp39/helix A, Glu137/helix C, Asp236/helix E) or at the membrane center (His32/helix A, Glu136/helix C) of TM helices yielded inactive single mutation PTPs. Some of these residues were suggested to act as phosphate ligands or as part of the proton cotransport path. We now show that the mutation Ser158Thr, not part of a TM helix but located near the center of the matrix loop (Ile141−Ser171) between TM helices C and D, inactivates PTP and is thus also functionally relevant. On the other side of the membrane, the single mutation Glu192Asp at the intermembrane space end of TM helix D yields a PTP with 33% wild-type activity. We constructed double mutants by adding this mutation to the six transport-inactivating mutations. Transport was detected only in those with Asp39Asn, Glu137Gln, or Ser158Thr. We conclude that TM helix D can interact with TM helices A and C and matrix loop Ile141−Ser171 and that Asp39, Glu137, and Ser158 are not essential for phosphate transport. Since our results are consistent with residues present in all 12 functionally identified members of the mitochondrial transport protein (MTP) family, they lead to a general rule that specifies MTP residue types at 7 separate locations. The conformations of all the double mutation PTPs (except that with the matrix loop Ser158Thr) are significantly different from those of the single mutation PTPs, as indicated by their very low liposome incorporation efficiency and their requirement for less detergent (Triton X-100) to stay in solution. These dramatic conformational differences also suggest an interaction between TM helices D and E. The results are discussed in terms of TM helix movements and changes in the PTP monomer/dimer ratio.

Steady-state oxidative phosphorylation requires the transport of inorganic phosphate from the cytosol into the mitochondrial matrix. This ion transport is highly substrate-specific and regulated to preserve the electrochemical membrane potential that is essential for oxidative phosphorylation.

The mitochondrial PTP[1] catalyzes this transport of inorganic phosphate. The transport is electroneutral and is postulated to be a phosphate/proton symport (*1*). PTP belongs to a family of mitochondrial transport proteins (MTPs) (*2*) that appear to function as homodimers[2] and that have subunits [mass of about 30 kDa; see review (*2*) for exceptions] consisting of three similar tandem repeats (TRs). Some experimental evidence supports a structural model in which these proteins possess six transmembrane (TM) helices per subunit or two per TR (*3*). Structural information obtained from protease treatment and labeling studies (*4*−*8*) suggests that the N- and C-terminals of PTP and other MTP family members are located on the cytosolic (intermembrane space) side of the inner membrane.

In earlier studies, we have used a site-directed residue replacement approach to identify residues in the PTP that are important for transport and that are at structure-sensitive locations. Two such residues have been located near the

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[1] Abbreviations: PTP, phosphate transport protein; MTP, mitochondrial transport protein; TR, tandem repeat; HCA, human carbonic anhydrase; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; LIE, liposome incorporation efficiency; PVDF, poly(vinylidene difluoride); ORF, open reading frame.

[2] All mutations refer to the PTP subunit. A single mutation PTP refers to the homodimeric PTP with the same single mutation in each subunit.

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membrane center and three near the matrix ends of three TM helices (*9*−*15*); i.e., transport is inactivated by replacing them with a similar residue. We describe now an additional transport-inactive mutant that demonstrates that the matrix loop, which connects TM helices C and D, is functionally significant. We have constructed double mutation PTPs by incorporating Glu192Asp, located on the other side of the membrane (intermembrane space end of TM helix D), with each one of the six transport-inactivating mutations. Three of these double mutation PTPs catalyze transport. In addition, all the double mutation PTPs, except the one with a mutation in the matrix loop Ile141−Ser171, differ dramatically in their conformation from those of the single mutation PTPs. We discuss the results in terms of transmembrane interactions between different regions of PTP, changes in monomer/dimer PTP ratio, and transport-inactivating mutations, some of which are expected to affect all members of the functionally identified MTP family and some that are specific for PTP.

MATERIALS AND METHODS

Preparation of Mutant Plasmids. Table 1 shows the codons used in the construction of replacement mutants of Ser158 with Met, Ile, Val, Cys, Ala, Thr, Gly, and Pro. The mutations were generated by PCR, and the PCR fragments (after trimming their ends with restriction enzymes) either were ligated into the yeast shuttle vector pAP-W3 (*9*) and then recloned into the bacterial expression vector pNYns, using the unique restriction sites *Sal*I and *Kpn*I, or were ligated directly into pNYns. pNYns was constructed from pNYHM131 (*10, 16*) by removing a second *Sal*I site (379 bp beyond the *Nde*I site) by site-directed mutagenesis using the following mutagenic primers: 5′AAGGGAGAGCGTTGACCGATGCCCTTG3′ and 3′TTCCCTCTCGCAACTGGCTACGGGAAC5′. Underlined bases are the mutated *Sal*I restriction site. More recently, mutant plasmids were constructed with the Stratagene QuikChange Mutagenesis Kit using PCR primers purified by gel electrophoresis. The entire mutant gene in each vector construct was sequenced (ABI373A automated DNA sequencer equipped with Stretch upgrade) after analyzing the *Sal*I/*Kpn*I restriction digest of the final mutant PTP vectors.

Transformation of S. cerevisiae and E. coli. The *S. cerevisiae* PTP null mutant (CG379mir::URA3) (*17*) was transformed with the yeast shuttle vector construct and growth on glycerol (YPG: 5 g of yeast extract, 10 g of peptone, 10 g of agar, 50 mL of 30% glycerol, made to 500 mL with H_2O) and glucose (YPD: 5 g of yeast extract, 10 g of peptone, 10 g of agar, 50 mL of 20% glucose, made to 500 mL with H_2O) plates was determined as described (*9*).

Selection of Second Site Revertants and Transfer of Revertant Yeast Shuttle Vector to E. coli. The *S. cerevisiae* PTP null mutant was transformed with pAP-W3 containing the PTP gene with the transport-inactivating mutation. Colonies that did grow on the YPG plates were replated onto fresh YPG plates to confirm the growth.

For characterization of the plasmid of a revertant, it was transferred to *E. coli* DH5α. The *smash and grab* method was used. Yeast cells were broken with glass beads, and the plasmid was extracted according to a published procedure (*18*). This plasmid extract was then electroporated into DH5α cells (*19*).

Expression and Purification of PTP. E. coli BL21(DE3) was transformed with the wild-type and mutant PTP genes in pNYns as described (*10*). Expression of the protein was induced by IPTG in the presence of ampicillin. The protein was purified from the inclusion bodies as described (*10*).

Reconstitution of PTP and Transport Assays. The wild-type and the mutant PTPs were incorporated into liposomes that had been prepared as described (*10*). The frozen reconstitution mix was thawed for 10 min at room temperature, vortexed at a low speed for 6 s, and centrifuged in a Beckman TL100 tabletop ultracentrifuge in a TLA100.2 rotor at 10^4g (1.7×10^4 rpm) for 15 min at 4 °C. Then 150 μL of the supernatant was transferred to a 3.5 mL tube (Sarstedt 55.484) on ice, 18 μL of C_iDTT (10 mM Tris, 15 mM dithiothreitol, 10 mM PIPES, adjusted to pH 8.0 with KOH) was added, and the mixture was incubated for 25 min on ice under a high-purity argon atmosphere. These are the stock proteoliposomes. Transport assays were started immediately, and each transport assay was stopped with freshly prepared sodium mersalyl, which was prepared by adding just enough NaOH to a mersalylic acid suspension to yield a clear solution after extensive vortexing (*10*). The pH gradient-dependent net phosphate uptake by the proteoliposomes was determined exactly as published (*10*).

Proteinase K Digestion and PTP/Peptide Extraction from Proteoliposomes. Fifty microliters of stock proteoliposomes was digested with Proteinase K (Boehringer Mannheim) and analyzed by SDS−PAGE in the presence or absence of the inhibitor PMSF.

Digestion of the stock proteoliposomes, i.e., PTP protected by incorporation into the liposomal membrane and PMSF addition before exposure to SDS for SDS−PAGE, was carried out as follows: 0.5 μL of Proteinase K diluted with C_i to 40 or 10 μg/mL was added to 50 μL of stock proteoliposomes. This solution was kept at room temperature (21 °C) for 24 h; then 0.5 μL of PMSF (100 mM in 2-propanol) was added, and the mixture was kept 10 min at 21 °C, diluted with a 500 μL of C_i plus 5 μL of PMSF mixture, and centrifuged at 6×10^4g for 30 min (4 °C). The pellet was taken up in 42.5 μL of water. This suspension was added to a mix of 134 μL of chloroform and 67 μL of methanol in a 1.5 mL Eppendorf cup and vigorously vortexed for 30 s and centrifuged for 5 min to separate the phases. The upper phase was dried in a SpeedVac for 45 min at 22 °C. The resulting pellet was analyzed by SDS−PAGE.

Digestion of the PTP, partly exposed by SDS solubilization of the proteoliposomes in the absence of PMSF, was carried out with the stock proteoliposome and Proteinase K mixture (see above) at 0 °C for 10 min and then diluted with 500 μL of C_i at 4 °C and centrifuged at 6×10^4g for 30 min (4 °C). The pellet was taken up in 42.5 μL of water. Chloroform/methanol extraction, followed by SDS−PAGE, was carried out as desribed above.

Preparation of Yeast Mitochondria. The yeast was grown in synthetic galactose medium to select for transformants with the plasmid. Mitochondria were prepared as described (*20*).

Western Blot. After SDS−PAGE, the protein in the gel was blotted onto a PVDF membrane (Millipore) and probed with a polyclonal rabbit antibody raised against yeast PTP (*21*). Antibody binding was detected with the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

Protein Quantitation. PTP (before liposome incorporation) was analyzed by SDS−PAGE followed by Coomassie Blue (Serva Blau R, C.I. 42660) staining and destained for 24 h with gentle shaking. HCA (Sigma 4396) was used as a protein standard. The dried gel was scanned with an HP ScanJet 6100C, and the results were analyzed with Scion Image software.

PTP in the stock proteoliposomes was quantitated as follows. Immediately after the transport assay, 30 μL of the stock proteoliposomes was 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 $3 \times 10^5 g$ (9×10^4 rpm) at 4 °C. To the pellet was added 22.5 μL of the SDS gel electrophoresis sample buffer (*22*), 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 its staining intensity 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 (*10*). The silver-stained gels were scanned and evaluated like the Coomassie Blue-stained gels.

Determination of PTP Solubility. PTP was solubilized and purified from inclusion bodies according to the standard procedure (*10*). The phosphatidylcholine addition was omitted. BioBeads SM2 with suspension water were transferred to preweighed 1.5 mL Eppendorf cups. After repeatedly adding and removing water with a micropipet to definitively establish the correct weight of the aliquot of BioBeads SM2 without micropipet-accessible water, soluble PTP was added and the mixture rocked at 4 °C in a Labquake (Dubuque, IA) for 45 min. The mix was centrifuged for 1 min at 4 °C in an Eppendorf tabletop centrifuge; the supernatant was transferred to a new Eppendorf cup and kept on ice overnight. The supernatant was centrifuged at $10^4 g$ (1.7×10^4 rpm) for 15 min at 4 °C, and the resulting supernatant was assayed spectrophotometrically for Triton X-100 and by SDS−PAGE for PTP.

RESULTS

Transformation of Yeast with pAP-W3 and Mutant PTP Plasmids. The PTP null mutant yeast [CG379mir::URA3 (*17*)] was transformed with pAP-W3 (*9*) and mutant PTP plasmids. The transformants were grown on YPG medium to determine their physiological competence. Yeast cells transformed with single mutation PTP plasmids (Ser158Met, Ser158Cys, Ser158Ala, Ser158Gly) grow like the wild type (pAP-W3-transformed). However, yeast transformed with the Ser158Thr mutant PTP plasmid show no growth. Since the side chain H of Gly and the methyl of Ala are sterically smaller than the OH-methyl of Ser, one indeed might not expect these mutations to block a phosphate transport path. The Met and Thr results are somewhat unexpected. Mutations in PTP can block yeast growth on glycerol plates in several ways: decreased PTP biosynthesis and thus fewer mutant PTPs incorporated into the membrane, decreased transport activity, or improper incorporation of PTP into the membrane. Western blots demonstrate (data not shown) that the Ser158Thr mutant PTP is present in mitochondria of yeast grown on galactose at about the same concentration as wild-type PTP. Thus, biosynthesis is not inhibited. Below, we present transport activity and conformation data of the Ser158Thr mutant PTP.

Table 1: Transport and Liposome Incorporation of Ser158 and Related Mutant PTPs

mutant	codon	*V* (at 0.66 mM P_i) [μmol of P_i min^{-1} (mg of PTP)$^{-1}$]	LIE (%)	PTP in reconstitution mix (μg)
wild type	TCC	201.6	16.8	0.140
		220.8	11.9	0.076
Ser158Met	ATG	216.6	16.3	0.083
Ser158Ile	ATC	127.2	25.2	0.061
Ser158Val	GTC	166.8	19.6	0.078
Ser158Cys	TGC	225.0	15.2	0.067
Ser158Ala	GCC	148.2	16.7	0.135
Ser158Thr	ACC	3.6	15.1	0.074
Ser158Gly	GGC	214.2	15.7	0.113
Ser158Pro	CCC	243.0	1.6	0.135
		166.8	1.5	0.148

Phosphate Transport Catalyzed by the Reconstituted PTPs. Table 1 lists the *V*s (based on initial 10 s P_i uptake at 0.66 mM extraliposomal P_i, no intraliposomal P_i). Purification and reconstitution of each mutant with our standard method yields 10−15 separate aliquots that are kept at −70 °C. Six mutants, using one frozen aliquot each, are assayed at the same time in those experiments where only *V* and PTP protein quantitation were determined. Transport was determined first, followed immediately by PTP protein quantitation in proteoliposomes by SDS−PAGE.

Table 1 shows that the *V*s catalyzed by the various PTPs are not too different (except that catalyzed by the Ser158Thr mutant PTP) from the wild type. Decreasing the amount of wild-type protein in the reconstitution mix yields less protein in the stock proteoliposomes (rows 1 and 2). The difference between *V*s catalyzed by the two Ser158Pro mutation PTPs is due to the difficulty of quantitating the small amount of protein in these proteoliposomes. Ser158Thr mutation PTP transport activity is detectable, and this may explain why the Ser158Thr mutation PTP yeast yield microcolonies on YPG plates, while those without plasmid show absolutely no colonies.

Liposome Incorporation Efficiency of Ser158 Mutant PTPs. We demonstrated earlier (*23*) that centrifugation ($10^4 g$ for 15 min) of PTP proteoliposomes prepared by the freeze−thaw procedure has only a minor effect on transport activity per microliters remaining in the supernatant. At the same time, there is a significant loss of protein and lipid from the supernatant. We have quantitated the PTP in the supernatant (stock proteoliposomes) to be able to express the transport activity per unit of PTP protein and to be able to calculate the LIE for each mutant PTP, i.e., the percent of PTP in the reconstitution mix that is present in the stock proteoliposomes. Table 1 shows the LIE for various PTP mutants. It shows that this LIE is very much decreased when Ser158 is replaced with Pro but not when it is replaced with Thr. We have varied the amount of BioBeads SM2 used for the detergent-minimizing step and followed this by an assay of PTP protein in the stock proteoliposomes and their transport activity. The transition from transport-inactive (too much Triton X-100) to transport-active stock proteoliposomes occurred at about the same BioBeads SM2 concentration for wild-type, Ser158Thr, and Ser158Pro PTPs.

Probing the Conformation of Ser158Thr Mutant PTP. It is possible that the low transport activity of the Ser158Thr PTP is due to a conformation dramatically different from that of the wild type, and yet not made apparent by its LIE (Table 1). We looked for differences between protease-generated peptide maps of the wild-type and Ser158Thr PTPs. The C-terminal of yeast PTP, contrary to that of the beef heart PTP, is not accessible to carboxypeptidases A and Y (*24*). A hydropathy plot of the yeast PTP (*24*) suggests that the N-terminal is also buried in the protein or in the membrane. Since the protein is thus expected to be largely protected from proteases by membrane lipids and since it is not clear which regions of the protein are accessible to proteases, we chose Proteinase K to probe the PTP conformation. Proteinase K is sensitive to PMSF inhibition, and its cut sites are not very residue-specific.

Stock proteoliposomes were digested for 24 h (21 °C), and the digestion was stopped with PMSF before solubilization (see Materials and Methods). The digestion pattern reflects the accessibility of the protease to the liposome-embedded PTP. Sufficient PMSF was added to the proteoliposome and Proteinase K mix 10 min before the centrifugation step and also to the dilution medium to prevent digestion of BSA (present in some samples as a protease sensor) added to the water-resuspended proteoliposome pellet and taken through the steps that lead to the SDS−PAGE analysis. Under these conditions, only very little digestion of the PTPs occurred; i.e., only a single peptide band of less than 26 kDa can be detected in addition to the undigested PTP. The wild-type and Ser158Thr PTPs are similarly protected by the lipids of the liposomes from the Proteinase K.

The very closely spaced PTP bands (around 30 kDa) in the PMSF-inhibited sample most likely are due to the reaction of PMSF with the undigested PTP. It is a series of well-defined bands with very similar mobilities. The highest mobility band is the same as the undigested PTP not exposed to PMSF or Proteinase K. This interpretation is quite reasonable since transport catalyzed by PTP is sensitive to PMSF (results not shown). PMSF reacts with Ser residues, some of which may be important for the PTP-catalyzed transport.

In the second type of digestion, stock proteoliposomes were exposed to a very low concentration of Proteinase K (remaining after washing the proteoliposomes and extracting the lipids) and also to SDS to increase its susceptibility to protease digestion. After a 10 min incubation at 0 °C, the proteoliposome and Proteinase K mixture (same mixture as in the first type of digestion) was diluted with 10 volumes of medium C_i, the proteoliposomes were pelleted by centrifugation, and the lipids were extracted with chloroform/methanol. The resulting protein was added to the SDS−PAGE sample buffer, and the peptides were separated by electrophoresis. This exposure of PTP to the SDS−PAGE sample buffer and to residual Proteinase K does generate a number of protein bands that are the same in size and concentration for the wild-type and Ser158Thr PTPs.

Selection of a Second Site Revertant. The yeast with the double mutation Asp39Asn Glu192Asp PTP was identified through its ability to grow on YPG plates. This double mutation PTP gene was subcloned into the expression vector pNYns and expressed in *E. coli* BL21(DE3). The resulting protein was purified and reconstituted. Table 2 shows its transport activity as well as its LIE.

Table 2: Effect of the Glu192Asp Mutation on Transport and Liposome Incorporation of Single Mutation PTPs

mutant	*V* (at 0.66 mM P_i) [μmol of P_i min^{-1} (mg of PTP)$^{-1}$]	LIE (%)	PTP in reconstitution mix (μg)
wild type	221.4	13.8	0.151
Glu192Asp	48.0	7.1	0.115
	70.2	6.0	0.115
His32Ala	0.0	9.9	0.128
His32AlaGlu192Asp	1.2	0.9	0.115
	1.2	2.0	0.282
Asp39Asn	1.2	21.9	0.128
Asp39AsnGlu192Asp	45.0	2.9	0.128
Glu126Gln	0.0	17.9	0.141
Glu126GlnGlu192Asp	2.4	2.4	0.102
Glu137Gln	0.6	12.4	0.102
Glu137GlnGlu192Asp	19.2	1.2	0.179
	15.0	1.4	0.282
	15.6	1.5	0.397
Ser158Thr	3.6	15.1	0.074
Ser158ThrGlu192Asp	158.7	13.8	0.143
Asp236Asn	0.0	17.9	0.077
Asp236AsnGlu192Asp	2.4	1.2	0.064
	0.6	7.3	0.192

Construction of Double Mutant PTPs with Glu192Asp. We constructed double mutants (Figure 1) by adding the Glu192Asp mutation to the single Ser158Thr mutation PTP gene as well as to each of the other five conservative mutations that are located within or at the matrix ends of TM helices. These mutant PTP genes were expressed, and the resulting proteins were purified and reconstituted. Table 2 shows the transport activities and the LIE of these mutant proteins.

Conformation Differences among Mutant PTP Proteins Are Detectable by Protein Solubility. Dramatic differences in the LIE of mutant PTPs must be sufficiently basic to the PTP structure to be detectable in the absence of the liposome lipids. When PTP is solubilized from the bacterial inclusion bodies, first with Sarkosyl and then with Triton X-100 (*10*), it will remain in solution when centrifuged at $2.3 \times 10^5 g$ for 50 min (4 °C). When the Triton X-100 concentration is decreased by exposing the protein preparations to BioBeads SM2, PTP is readily pelleted by centrifugation. At higher concentrations of BioBeads SM2, the PTP protein is adsorbed to the beads.

We chose the His32Ala Glu192Asp PTP (Table 2) as an example of a low LIE (0.9%) mutant and the Ser84Ala PTP (*25*) as an example of a mutant with a high LIE (36.0%). Figure 2 shows wild-type PTP. As the Triton X-100 concentration is decreased, the PTP (0.25 mg/mL) stays in solution until the detergent concentration is lowered to about 0.03%. Interestingly, a higher PTP concentration (about 0.74 mg/mL) requires a proportionately higher detergent concentration (about 0.09%) to stay in solution. Carrying out the same experiments with the two mutant PTPs (Figure 3) shows that the solubility properties of these two mutants are significantly different. Less Triton X-100 is required to keep the low LIE His32Ala Glu192Asp PTP in solution than the high LIE Ser84Ala PTP.

DISCUSSION

Metabolite membrane transport proteins belong to a protein family that has not yet been crystallized, and thus their high-

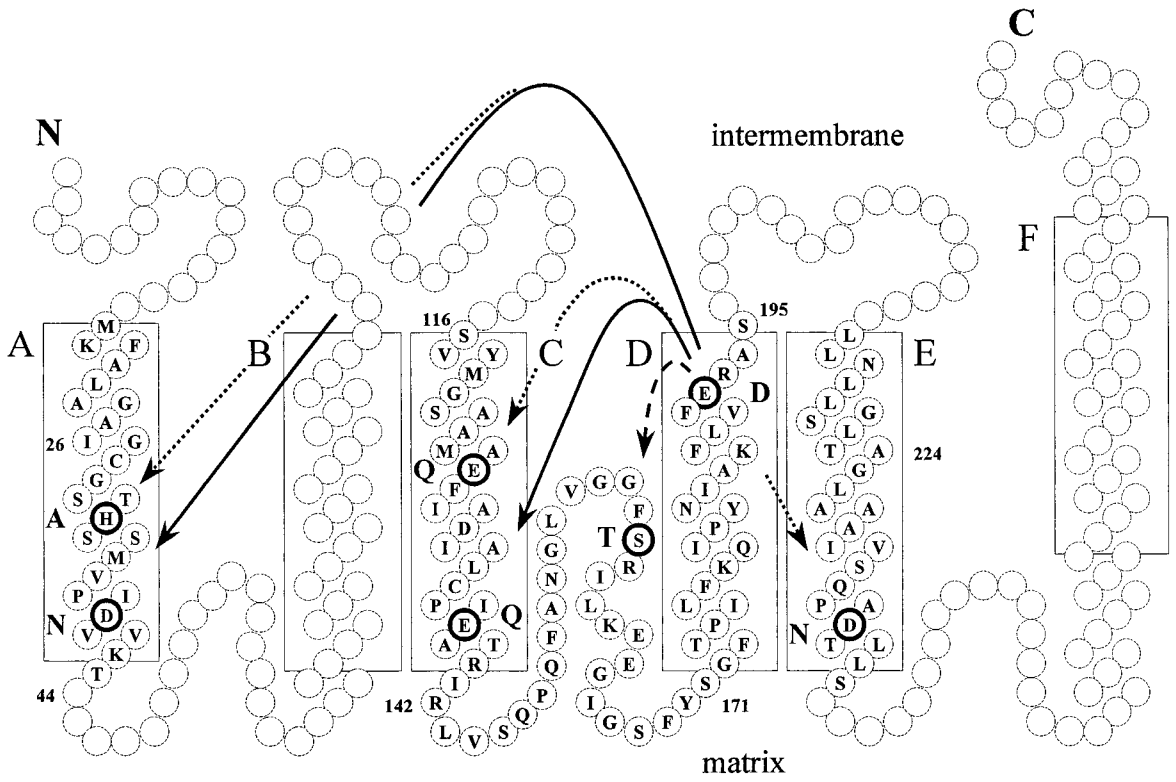

FIGURE 1: Residues (boldface circles) used in the construction of the double mutant PTPs. Arrows indicate double mutants with reversed inhibition and decreased LIE (—), reversed inhibition but no decrease in LIE (– –), or no reversed inhibition but decreased LIE (···). The boldface letter next to the wild-type residue is the residue in mutant PTP.

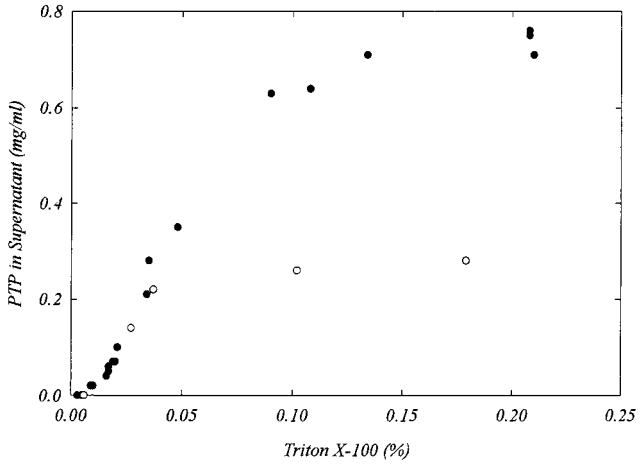

FIGURE 2: Solubility of wt PTP as a function of Triton X-100. Triton X-100 was removed with increasing amounts of BioBeads SM2. Titrations were started with 0.25 mg of PTP/mL (○) and 0.74 mg of PTP/mL (●).

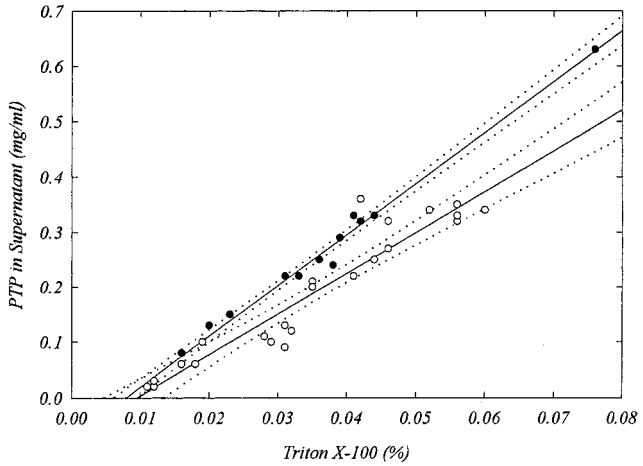

FIGURE 3: Determination by first-order linear regression of Triton X-100 required to keep low LIE His32AlaGlu192Asp PTP (●) and high LIE Ser84Ala PTP (○) in solution. The dotted lines indicate the 95% confidence interval.

resolution structures are not yet available. These proteins appear to have highly flexible structures that make it difficult to purify them as uniformly unique structures. This flexibility can be inferred from some of our results which demonstrate that a transport-inactivating movement of a protein domain on one side of the membrane can be overcome by the movement of another domain on the other side of the membrane.

We used site-directed mutagenesis to identify residues in the PTP that are associated with functionally and structurally significant domains (*20*). These residues are located on TM helices and may have a role in aligning TM helices, in providing ligands for the P_i transport path, or in providing residues for the cotransport of protons. It is most likely that the TM helices are aligned or interconnected in ways that permit their ends to undergo movements in the plane of the membrane surface as protons are cotransported with P_i ions.

The TM helices are connected via non-TM-helix protein segments that have a poorly characterized role, which may in fact be limited to a passive TM helix ends-connecting loop. Interestingly, the matrix loop that connects TM helices C and D consists of 18−48 residues (32 residues within yeast PTP) within the 12 functionally identified members of the MTP family (*2*). Our identification of the Ser158Thr mutation, which is located near the center of this matrix loop, as transport-inactivating is thus of special interest (Table 1). Ser158Thr is also physiologically incompetent since it is expressed at normal wild-type PTP protein concentration in

Table 3: Residues at Equivalent Locations in PTP and in the Other Functionally Identified Members of the Mitochondrial Transport Protein Family (*2*) (http://genome-www.stanford.edu/Saccharomyces/)

gene	ORF	helix D	helix A	helix A	helix C	helix C	helix E	Ile141 to Ser171[a]
MIR1 (PTP)	YJR077C	E192	H32	D39	E126	E137	D236	GFS158RI
AAC1	YMR056C	D203	K28	E35	G129	D140	D240	VYK169KT
AAC2	YBL030C	D212	K38	E45	G138	D149	D249	VYK178KT
AAC3	YBR085W	D201	K27	E34	G127	D138	D238	VYK167KT
ARG11	YOR130C	E191	K28	D35	G118	E129	D235	TIK157AI
CAC	YOR100C	E216	V50	D57	A152	E163	D261	AAK182TI
CTP1	YBR291C	N194	A27	E34	L120	E131	D236	NYS160SL
ACR1	YJR095W	S197	A25	D32	I122	E133	D236	AAY163TI
OAC1	YKL120W	D195	V37	E44	G139	F150	D251	YSE161FI
DIC1	YLR348C	D190	T28	D35	V129	E140	D229	VYK156IY
FLX1	YIL134W	T206	T24	D31	G136	W147	Q248	GVQ172QL
−	YMR241W	R194	T35	E42	T128	E139	E236	VFK160NI

[a] The equivalent Ser158 location within the other proteins was assumed to be the same number of residues away from the helix D consensus sequence (*2*).

the yeast mitochondrial membrane but cannot rescue the yeast PTP null mutant on glycerol plates.

If this Ser158 resides in the P_i transport path, replacing it with Thr will most likely block the transport and yet it will not significantly perturb the structure of PTP; i.e., the Thr hydroxyl group simply penetrates somewhat into the P_i transport path. On the other hand, if this Ser158 hydroxyl group forms a hydrogen bond with a peptide bond carbonyl oxygen or a carboxyl group oxygen of a residue on an adjacent TM helix or other protein structure, which in turn lines the phosphate transport path, one might expect a more significant structural change in PTP as Ser158 is replaced with the transport-inactivating Thr158.

To help us differentiate between these two possibilities, conformation changes induced in PTP by the Ser158Thr mutation were investigated in two ways: LIE and peptide patterns generated by Proteinase K digestion. Table 1 shows that the LIE is not altered. PTP was exposed to Proteinase K after incorporation into proteoliposomes where most of the protein is protected by membrane lipids from digestion. Indeed, even after exposure to Proteinase K for 24 h at 21 °C, almost no digestion has taken place. On the other hand, when washing most of the Proteinase K away, removing most of the lipid by chloroform/methanol extraction, and exposing the protein to SDS, a large number of peptides are generated. The overall peptide patterns are the same for the two proteins. This method eliminates obvious situations where transport inactivation of the Ser158Thr mutation PTP occurs because the protein is only partly, compared to the wild-type PTP, embedded in the membrane. The results point to the Thr158 hydroxyl group extending into the P_i transport path and thus blocking P_i transport.

Replacing Ser158 with a sterically larger, yet more hydrophobic residue (Table 1), the transport is not inhibited. This could be because the hydrophobic nature of this side chain induces it to move from the polar transport path into the adjacent hydrophobic region, avoiding interference in the transport process. None of the more nonpolar residues blocks transport (Table 1). Proline, the most polar of the replacement residues, also does not block transport but does perturb the protein structure sufficiently to decrease its LIE (Table 1).

We used this novel mutation and five transport-inactivating conservative point mutations to probe their structural relationship to the mechanism of transport. A single second site mutation was identified that suppresses the transport inactivation of the Asp39Asn mutation (Table 2). This mutation, Glu192Asp, is located on the other side of the membrane. Glu192 is a residue that is present, at an equivalent location (*2*), in some of the other functionally identified mitochondrial transport proteins (Table 3) and thus is not unique to PTP. As single mutation PTPs, Asp39Asn PTP and Glu192Asp PTP have a wild-type LIE (Table 2). The double mutant PTP, however, shows a very low LIE (Table 2). This implies that the Asp39Asn mutation induces the C-terminal of helix A to move away from the N-terminal of helix D and this results in blocked transport. However, if the other ends of helices A and D can be moved closer to each other, e.g., with Glu192Asp, to relieve some of the strain generated by the Asp39Asn mutation, transport can function again.

We constructed double mutation PTPs (Figure 1), pairing each of the other five transport-inactivating single mutations with Glu192Asp. Each double mutation (except that with Ser158Thr) PTP has a very low LIE (Table 2). This suggests that the double mutations have significantly altered the orientations of TM helices. Going back to the earlier suggestion that Thr158 blocks transport because its hydroxyl interferes sterically with the passage of P_i, the argument can now be made that by moving the C-terminal end of helix D with the Glu192Asp mutation, the N-terminal moves further from the Thr158 hydroxyl group and thus reopens the phosphate transport pathway (Table 2). Since the Glu192Asp mutation induces only helix D to move and since this has no effect on the LIE, one would expect Ser158Thr Glu192Asp to also have the same LIE. This is indeed the case (Table 2).

These mutation-induced helix movements are inherent to the protein and should not depend on protein/liposome membrane interactions. To assess these liposome membrane-independent structure changes, the solubility of two PTP mutants was monitored as a function of the detergent Triton X-100 concentration. Figure 2 shows that the minimum Triton X-100 concentration required to keep the wild-type PTP protein in solution is proportional to the amount of protein. The quantitation of the BioBeads makes it technically difficult to obtain these results. Water was added and removed from the wetted BioBeads at least 10 times to establish their weight with all micropipet-accessible water removed. Several double mutants of Table 2 have some of the lowest LIEs. A comparison was made between His32Ala Glu192Asp PTP and Ser84Ala PTP, which has a very high

LIE (*25*). The slopes of the two lines [mg of PTP in solution mL^{-1} (% of Triton X-100)$^{-1}$] in Figure 3 were determined with a first-order linear regression analysis (Sigma Plot) and give values of 9.22 (correlation coefficient = 0.995) for the lower (0.9%) LIE and 7.40 (correlation coefficient = 0.949) for the higher (36%) LIE mutant PTP. These values, as expected, are higher and lower than the 8.86 of the wild-type PTP in Figure 2. Thus, the low LIE mutant PTP requires the least detergent to stay in solution. This is logical since we expect the low LIE mutants to have more exposed polar surface, i.e., a relatively more open subunit/subunit interface. This should result in less effective interaction with lipid membranes and thus lower LIE. This also suggests that low LIE PTPs may have fewer dimeric PTPs. Dimeric PTPs are expected to incorporate more readily into liposomes.

The mutation Glu192Asp can suppress the inhibitory effect of the single mutations Asp39Asn, Glu137Gln, and Ser158Thr, but not His32Ala, Glu126Gln, and Asp236Asn (Tables 1 and 2). This implies that Asp39, Glu137, and Ser158 are not essential for PTP function. This also implies that helix D can interact with helix A, helix C, and the matrix loop Ile141−Ser171. In addition, LIE results (Table 2) demonstrate that helix D can interact with helix E. These helix interactions must be interpreted of course also with respect to the homodimeric nature of PTP. Thus, a helix D−helix A interaction can also be a helix D−helix A′ interaction where helix A′ belongs to the other PTP subunit.

These PTP mutation results are completely consistent with the types of residues present at equivalent locations within the other 11 functionally identified members of the mitochondrial transport protein family (*2*). Table 3 shows that Asp39 has been replaced in six proteins with a Glu and, as expected, none of these six proteins has a Glu192. Since the Asp39Glu PTP is inactive (*20*), these results suggest that Asp39, possibly via a salt bridge, retains a critical distance to a neighboring residue. Increasing this distance with a Glu or Asn (increased distance by eliminating a salt-bridge constraint) blocks transport which can be overcome by moving the C-terminal of helix D by an Asp192, Asn192, or Arg192, but not by a Glu192. Glu192 can be replaced by other residues in the presence of Asp39, as indicated by the observation that both Glu192Asp PTP and Glu192 Gln PTP are active (*20*) (Table 2). The Glu137Asp PTP is active (*20*). The Glu137Gln mutation inactivates PTP unless Glu192 is replaced with another residue (Table 2). Thus, the two proteins that have a Phe or Trp substitution at the Glu137 position also have an Asp or Thr at the Glu192 position (Table 3), respectively. Finally, Glu192Asp cannot suppress the Asp236Asn inhibition (Table 2). Thus, Asp236 is present in all proteins, except two where we find a Gln or Glu. These proteins also do not retain the Glu192 but have it substituted with Thr or Arg at this position. None of the proteins have a His32, Glu126, or Ser158 (CTP1 has a Ser158 equivalent Ser160). These three residues must thus be unique to PTP and its function.

The mutation/activity data and the sequences of the 12 function-identified mitochondrial transport proteins (*2*) establish a first general rule pertaining to the general transport mechanism catalyzed by mitochondrial transport proteins: Glu192 requires the presence of Asp39, Glu137 (or Asp137), and Asp236. Retaining activity after replacing the residue at one of these three matrix-facing locations requires that Glu192 be replaced with Asp or another residue. For a Asp236 replacement, Asp192 may not be sufficient to restore activity.

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