Replacements of Basic and Hydroxyl Amino Acids Identify Structurally and Functionally Sensitive Regions of the Mitochondrial Phosphate Transport Protein[†]

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ABSTRACT: The mitochondrial phosphate transport protein (PTP) from the yeast *Saccharomyces cerevisiae* has been expressed in *Escherichia coli*, purified, and reconstituted. Basic and hydroxyl residues were replaced to identify structurally and functionally important regions in the protein. Physiologically relevant unidirectional transport from extraliposomal (cytosol) pH 6.8 to intraliposomal (matrix) pH 8.0 was assayed. Replacements that affect transport most dramatically are at Lys42 (matrix end of helix A), Thr79 (helix B), Lys90 (cytosol end of helix B), Arg140 and Arg142 (matrix end of helix C), Lys179 and Lys187 (helix D), Ser232 (helix E), and Arg276 (helix F). The deleterious nature of these mutations was confirmed by the observation that the yeast PTP null mutant transformed with any one of these mutant genes cannot grow or has difficulties growing with glycerol as the primary carbon source. More than 90% of transport activity can be blocked by various mutations without affecting growth on glycerol. Alterations in the structure of the transport protein caused by the mutations were characterized by determining the fraction of PTP incorporated into liposomes during reconstitution. The incorporation of all PTPs (wild type and mutant) into liposomes is 15.5 ± 8.4 ng of PTP/25 μL and fairly independent of the amount of PTP in the initial reconstitution mix (49−212 ng of PTP/25 μL). Arg159Ala and Lys295Gln show the smallest incorporation of 2.3 ± 1.6 ng of PTP/25 μL and 2.6 ± 0.2 ng of PTP/25 μL, respectively. Ser145Ala shows the largest incorporation of 37.0 ng of PTP/25 μL. These three mutants show near wild-type reconstituted transport activity. Two of these three mutations are located in the loop connecting the matrix ends of helices C and D, Ser145 at its N-terminal (the matrix end of helix C) and Arg159 near its center. Lys295 is located at the C-terminal of PTP beyond helix F. These results, together with those from other mutations, suggest that like helix A, the protein segment consisting of the loop connecting helices C and D and helix D as well as the C-terminal of PTP beyond helix F faces the subunit interface of this homodimer. The role of the replacement-sensitive residues in the phosphate or in the coupled proton transport path is discussed.

Mitochondria are responsible for providing the bulk of ATP to the cell. The phosphorylation of ADP for this purpose occurs within mitochondria. The P_i for this phosphorylation is transported via the PTP (PHT, PHC, PIC, PIT, MIR) (*1*) as phosphate/proton cotransport from the cytosol into the mitochondrial matrix, following a pH gradient of about 1.2 pH units with a cytosol pH 6.8 and a matrix pH 8.0.

We are interested in understanding the function of this protein at the molecular level and have thus replaced several of its basic and hydroxyl residues. Mutant PTPs from yeast mitochondria have already been constructed and characterized in which the Cys (*2*, *3*), the acidic and His residues (*4*), Ile141 (*5*), and other residues (*6*, *7*) have been replaced. Several of these residues have been suggested (a) to be at the interface between the subunits of the homodimer (*2*), (b) to be primary members of a proton cotransport path (*4*), or (c) to be at a location where residue replacement uncouples the proton and phosphate transport paths (*5*). We report now that replacements of basic and hydroxyl residues point toward additional regions in PTP that are structurally and functionally significant. We have determined the physiologically relevant transport mode of the mutants and the fraction of the purified PTP in the reconstitution mixture that is incorporated into the transport-active proteoliposomes. We discuss the implications of these findings.

MATERIALS AND METHODS

Preparation of Mutant PTP Plasmids. The yeast shuttle vector pAP-W3 (6) was used to express the mutant PTPs in *Saccharomyces cerevisiae* (*6*). The ability of the mutant PTPs to rescue the *S. cerevisiae* PTP null mutant on glycerol was tested on YPG plates (*6*). PTP was expressed in *Escherichia coli* as inclusion bodies with the vector pNYHM131 (*5*, *8*). Mutant PTP genes were prepared according to a PCR protocol (*6*). Codons of the wild-type and replacement residues are shown in Table 1.

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[1] Abbreviations: M, matrix; C, cytosol; PTP, phosphate transport protein; PHT, phosphate transporter; PHC, phosphate carrier; PIC, phosphate carrier; PIT, phosphate transporter; MIR, mitochondrial import receptor; LIE, liposome incorporation efficiency; P_i, inorganic phosphate; X, any amino acid; HCA, human carbonic anhydrase.

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

PTP[a] (mutations)	codons (wild type/mutant)	PTP in reconstitution mixture (ng)[b]	PTP in fractionated and transport-active proteoliposomes (ng)[c]	incorporation efficiency of PTP into liposomes (%)
wild type		140.0	23.0 ± 3.0	16.4
LYS42ala	AAG/GCG	155.9	22.8	14.6
LYS42arg	AAG/AGG	114.5	16.8 ± 0.3	14.7
ARG44ala[d]	AGA/GCA			
ARG44lys	AGA/AAA	133.9	18.2	13.6
THR79ala	ACT/GCT	58.1	22.8 ± 4.6	39.2
SER84ala	TCT/GCT	47.6	17.5 ± 2.3	36.8
SER84ala(C)[e]		111.0	31.9	28.7
LYS90arg	AAG/AGG	92.5	18.2 ± 2.8	19.7
SER116ala	TCT/GCT	83.7	21.6 ± 1.6	25.8
arg140ala	AGA/GCA	149.8	15.4	10.3
arg140lys	AGA/AAA	146.2	23.9	16.3
arg140gln	AGA/CAA	143.6	18.8	13.1
ARG142ala	AGA/GCA	96.0	5.7	5.9
ARG142lys	AGA/AAA	94.3	11.4	12.1
ser145ala	TCC/GCC	104.8	37.0	35.3
arg159ala	AGA/GCA	148.9	2.3 ± 1.6	1.5
arg159ala (D)[f]		69.6	2.8	4.0
arg159lys	AGA/AAA	125.1	10.3	8.2
lys179ala	AAG/AGG	106.6	5.7	5.3
lys179arg	AAG/AGG	119.8	9.7	8.1
LYS187ala	AAA/GCA	163.9	8.2 ± 0.8	5.0
LYS187arg	AAA/AGA	104.0	7.4	7.1
SER232ala	TCC/GCC	83.7	21.1	25.2
thr237ala	ACT/GCT	66.1	16.5	25.0
lys262ala	AAA/GCA	211.4	16.5	7.8
ARG276ala	CGT/GCT	89.0	25.6	28.8
ARG276gln	CGT/CAA	91.6	9.1 ± 0.4	9.9
ARG276lys	CGT/AAA	73.1	11.7 ± 0.3	16.0
LYS295ala	AAG/GCG	104.8	11.1 ± 3.7	10.6
LYS295gln	AAG/CAG	49.3	2.6 ± 0.2	5.3

[a] Capitalized residues are the same in rat liver and bovine heart PTP (see also Table 2). [b] Values are averages of two independently obtained results. [c] Values with standard deviation are based on three or more independent results; others are averages of two independent experiments. [d] Inclusion bodies of this mutant remained insoluble during our standard purification/reconstitution procedure. [e] Purification and reconstitution was carried out with twice the initial inclusion bodies protein concentration. [f] Purification and reconstitution was carried out with half the normal amount of inclusion bodies protein.

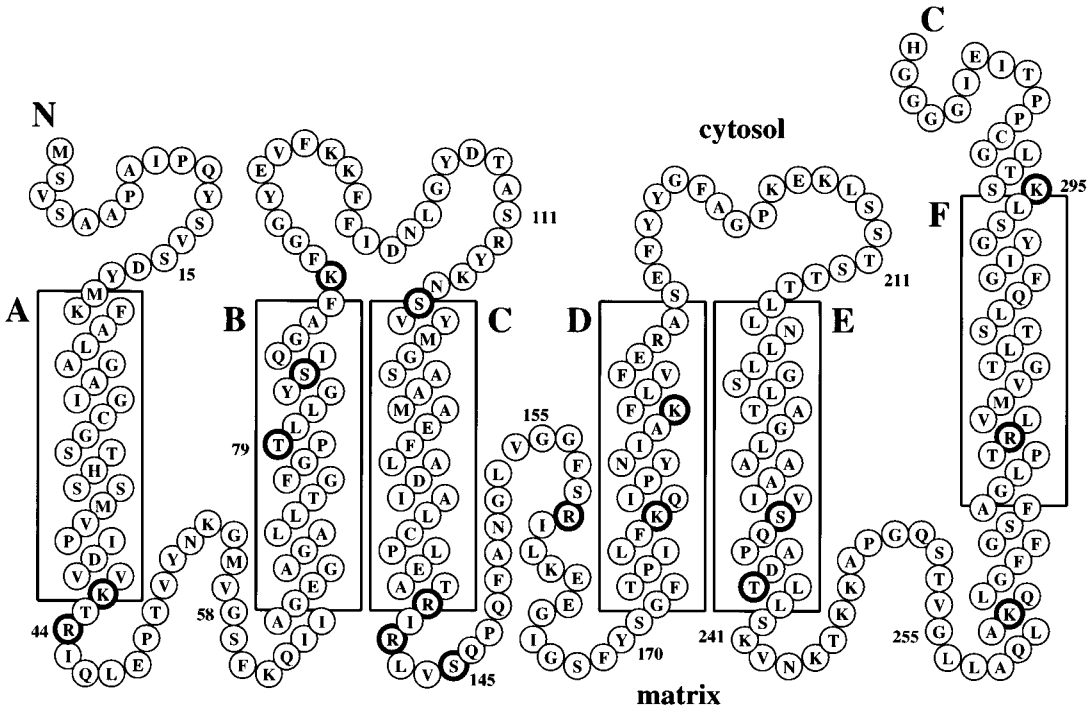

FIGURE 1: Transmembrane topography of yeast PTP. Residues with heavy circles and letters have been mutated in experiments presented in this report.

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 the subcloning sites. Often the whole PTP gene was sequenced.

Sequencing was carried out manually and also with an ABI373A automated DNA sequencer equipped with Stretch upgrade.

Reconstitution of PTP and Transport Assays. PTP was reconstituted after purification from the *E. coli* inclusion bodies and its transport activity assayed as described (*5*). Transport-active proteoliposomes were obtained from the supernatant of the "freeze−thaw vortex" reconstitution mix after centrifugation at (10^4)*g* for 15 min at 4 °C. They were incubated on ice for 25 min with 15 mM dithiothreitol immediately before the transport assays.

Protein Determination. The concentration of PTP in the reconstitution mix was determined from Coomassie Blue-stained (Serva Blau R, CI 42660) SDS−polyacrylamide gels (destained for about 6 h with gentle shaking) of PTP from the fraction used to prepare the proteoliposomes by mixing with the liposomes. Human carbonic anhydrase (Sigma 4396) was used as protein standard. The protein was quantitated after scanning it with a Hewlett-Packard ScanJet 6100C flat bed scanner, and the images were integrated with the Scion Image for Windows software.

PTP in the proteoliposomes was determined as follows by SDS−polyacrylamide gel electrophoresis/silver stain (a) to avoid a lipid extraction step which adds another processing step and thus provides another opportunity for protein loss (*9*) or (b) to avoid a Western blot-type assay (*10*) which may possibly not detect PTP due to a modified antigenic site or due to the masking of the antigenic site by lipids. Transport-active proteoliposomes were prepared as described above. Immediately after the transport assay, 30 μL of 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 (3×10^5)*g* (90 000 rpm) at 4 °C. To the pellet was added 22.5 μL of the SDS gel electrophoresis sample buffer (*11*), 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 addition to the reconstitution mixture (*5*). 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 dilute HCl. This [^{32}P]P_i was diluted with 1000 volumes of transport medium (C_e) and kept at room temperature for at least 24 h to decrease radioactive counts that do not exchange to the anion-exchange column.

RESULTS

PTP Incorporation into Liposomes. Mutant PTPs (see Figure 1 for location of mutations) were expressed in *E. coli* as inclusion bodies. They were then purified from the inclusion bodies and reconstituted (*5*). We quantitated the PTP in the fractionated proteoliposomes used for the transport assays. Table 1 shows the efficiency of transport protein incorporation into the liposomes (ratio of PTP in liposomes to PTP in reconstitution mixture) for the wild-type PTP as well as the mutants. This efficiency is about 16% for the wild type and ranges from a low of 1.5% for Arg159Ala to 39.2% for Thr79Ala.

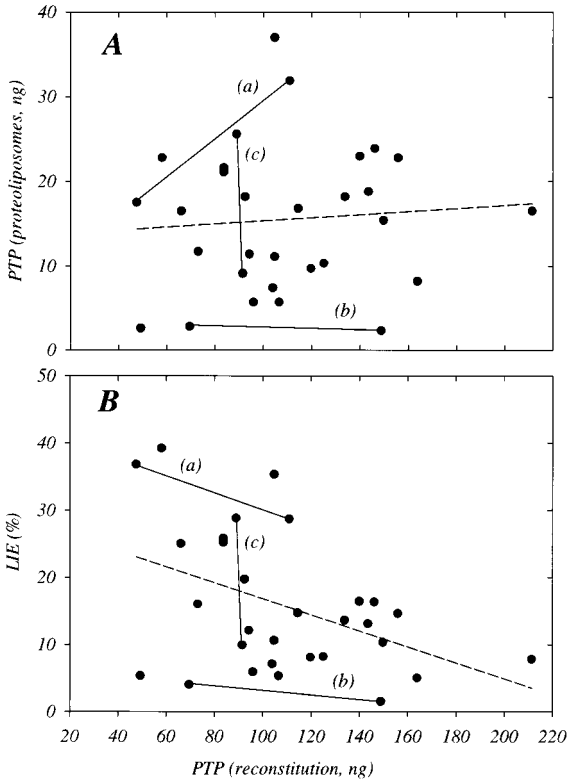

FIGURE 2: Incorporation of PTP wild type and PTP mutants into proteoliposomes (results from Table 1). (A) PTP protein in the proteoliposomes used for the transport experiments (the proteoliposomes obtained after freeze−thaw vortexing of the reconstitution mixture and centrifugation) as a function of PTP protein in the reconstitution mixture. (B) Liposome incorporation efficiency of the PTP protein (percent of PTP protein of the reconstitution mixture that is present in the proteoliposomes used for the transport experiments) as a function of PTP protein in the reconstitution mixture. The slashed lines are the linear regression lines of all the points within a plot (SigmaPlot 4.0 for Windows NT 4.0). Experiments with different amounts of PTP protein but the same PTP mutant in the reconstitution mixture are connected with (a) [Ser84Ala, Ser84Ala(C)] and (b) [Arg159Ala, Arg159Ala(D)]. Points of experiments of two different PTP mutants with the same amount of PTP protein in the reconstitution mixture [Arg276Gln, Arg276Ala] are connected by (c).

A closer look at PTP incorporation into the transport-active proteoliposomes shows (Figure 2B), that the incorporation efficiency decreases as the amount of protein in the reconstitution mix increases. This result at the same time shows that the amount of protein incorporated, over the range investigated (Figure 2), is independent of the amount of PTP in the reconstitution mix (Figure 2A). Incorporation with some mutants dramatically rises above or falls below the linear regression line (Figure 2A). Thus only 2.8 ng of Arg159Ala(D) (see Table 1 for nomenclature) is incorporated with a starting protein of 69.6 ng, while 22.8 ng of Thr79Ala gets incorporated with 58.1 ng of PTP in the reconstitution mix. This is about a 10-fold difference with nearly the same amount of PTP in the reconstitution mix.

The detergents (Sarkosyl, Triton X-100) are important for the reconstitution process. We investigated the role of the detergent-to-protein ratio for a mutant with a high liposome incorporation efficiency (Ser84Ala) and a mutant with a low liposome incorporation efficiency (Arg159Ala). The purification steps were carried out with twice the amount of inclusion bodies (Table 1, Ser84Ala(C)) or half the amount of the

Table 2. Transport Characteristics of Transport Proteins

location of mutation within PTP	PTP[a] (mutation)	same site amino acid in bovine heart/rat liver PTP[a]	V^b [mmol (min)$^{-1}$ (g of PTP)$^{-1}$][c]	temperature (°C)	growth with glycerol[d]
	wild type		153.6 ± 29.0	21.5 to 24.0	+
M-end helix A	LYS42ala[e]	LYS/LYS	0.1	22.5	-
	LYS42arg		1.2	24.0	±
	ARG44ala	ARG/ARG	nd[f]		-
	ARG44lys		60.0	22.5	+
helix B	THR79ala	THR/THR	0.5	21.0	±
	SER84ala	SER/SER	145.2 ± 7.2	21.5	+
	SER84ala(C)[g]		126.0	21.5	
C-end helix B	LYS90arg	LYS/LYS	4.8	21.5	-
C-end helix C	SER116ala	SER/SER	58.8	24.5	+
M-end helix C	arg140ala[e]	lys/lys	0.2	24.5	-
	arg140lys		15.6	24.5	+
	arg140gln		4.2	24.5	-
	ARG142ala[e]	ARG/ARG	0.5	24.5	±
	ARG142lys		42.0	24.5	+
M-loop helices C/D	ser145ala	thr/thr	115.2	22.0	+
	arg159ala	lys/lys	156.0 ± 10.8	21.5 to 24.0	+
	arg159ala(D)[h]		120.0	21.5	
	arg159lys		208.2	23.5	nd
helix D	lys179ala	arg/arg	1.2	24.0	±
	lys179arg		45.6	24.0	+
	LYS187ala	LYS/LYS	1.2	21.5	-
	LYS187arg		8.4	24.0	-
helix E	SER232ala	SER/SER	3.6	22.0	±
	thr237ala	ser/ser	211.2	21.5	+
M-loop helices E/F	lys262ala	lys/gln	134.4	22.5	+
helix F	ARG276ala	ARG/ARG	1.8	22.0	±
	ARG276gln		1.8 ± 1.2	24.5	±
	ARG276lys		8.4	24.5	+
C-end helix F	LYS295ala	LYS/LYS	58.2	21.5	+
	LYS295gln		45.0	21.5	+

[a] Residues in capital letters are the same in yeast (*15*), bovine heart (*16*), and rat liver (*17*) PTP. [b] Transport rate was determined from the initial 10s of the phosphate uptake per gram of liposome-incorporated PTP and reflects initial uptake rate (*4*). [c] Standard deviation from six independent experiments; other values are averages of two independent results. [d] The *Saccharomyces cerevisiae* PTP null mutant was transformed with the mutant PTP in the yeast shuttle vector pAPW3 (*6*) and plated onto YPG plates: (+) normal growth, (−) no growth, (±) poor growth. [e] These mutants show mersalyl-sensitive P_i uptake of less than that of the ARG44ala mutant, which showed no detectable protein in the reconstitution mix and the proteoliposomes. This phosphate transport was less than 0.15% of that of the wild-type PTP. [f] Not determined; in the case of the ARG44ala mutant, its inclusion bodies remained insoluble under our standard purification/reconstitution conditions. [g] Purification and reconstitution was carried out with twice the initial inclusion bodies protein concentration. [h] Purification and reconstitution was carried out with half the normal amount of inclusion bodies protein.

inclusion bodies (Table 1, Arg159Ala(D)) to determine whether this affects the incorporation; that is, does an increased (decreased) detergent-to-protein ratio cause increased (decreased) denaturation or monomerization of the protein and thus a decreased (increased) incorporation of PTP into the liposomes. This may partly be the case for Ser84Ala, that is, the liposome incorporation of PTP increases as detergent-to-protein ratio decreases, but of course at the same time the PTP to liposome ratio increases which results in more PTP partitioning into the liposome. This effect is also more pronounced with Ser84Ala than with all the other mutants within the pool of all mutants as indicated by the linear regression line of Figure 2A. It is not observed with Arg159Ala which may be much more sensitive to the detergents. These aspects are being investigated further.

Transport by Basic Residue Mutant PTPs. pH gradient-dependent net phosphate transport was determined with the reconstituted proteoliposomes. The four basic residues (Lys42, Arg44, Arg140, Arg142) at the ***matrix end of helices*** A and C (Figure 1 and Table 2) are very sensitive to replacement by a charge-neutral residue. The very low mersalyl-sensitive transport rate that these mutants catalyze is not significant since it is less than that of the Arg44Ala mutant with no detectable protein in the reconstitution mix or in the proteoliposomes (see Table 2); that is, [^{32}P]P_i transport without any protein is 0.15% of that of the wild-type PTP. Lys42 with an Arg replacement also yields transport of less than 1% of the wild-type PTP transport. The other three residues can be replaced with Lys (Arg44, Arg140, Arg142) and retain most of the wild-type transport activity.

Three basic residues (Lys179, Lys187, Arg276) are located ***within helices*** (Figure 1). Their replacement may affect transport (a) because a salt bridge essential for preserving structure-sensitive function (*5*, *12*, *13*) is eliminated, or (b) because as a member of the proton cotransport path (*4*) or (c) because as a P_i ligand of the phosphate transport path (*14*), the transport is inactivated. Lys179 and Lys187 are located within helix D, and transport tolerates their replacement by Arg but not by a charge-neutral residue (Table 2). Lys187Arg, however, cannot support growth on glycerol. Similarly, Arg276 within helix F can be replaced by Lys, but replacements with Ala or Gln practically eliminate transport. It should be noted that Lys187 and Arg276 are more sensitive to replacement by Arg and Lys, respectively, than Lys179.

Two basic residues at the ***cytosol end of helices*** B (Lys90) and F (Lys295) (Figure 1) are also sensitive to substitutions (Table 2). Replacement of Lys90 by Arg decreases transport as dramatically as Lys187Arg and Arg276Lys, and like Lys187Arg, Lys90Arg cannot support growth on glycerol.

Table 3. Sequence Similarities of Members of the Mitochondrial Transport Protein Family Bordering Mutated Residues of the Phosphate Transport Protein[a]

name[b]	(Lys-42, Arg-44)	(Thr-79, Ser-84, Lys-90)	(Ser-116)	(Arg-140, Arg-142, Ser-145)	(Arg-159)
PTP	PIDVV**K**T**R**IQL$_{47}$	GFGP**T**LLGY**S**IQGAF**K**FGG$_{93}$	YKN**S**VYM$_{119}$	PLEAT**R**I**R**LV**S**QP$_{147}$	GFS**R**ILK$_{162}$
ANT	PIERV**K**LLIQN	GNTANVIRYFPTQALNFA-	FAGNLAS	SLDYA**R**T**R**LAADS	VYK**K**TLK
UCP	PLDTA**K**V**R**LQI	GLPAGIQRQISFASL**R**IGL	TLGNRIS	PTEVV**K**V**R**LQAQS	AYRIIAT
OTP	PLDLV**K**N**R**MQL	GLSAGLLRQATYTTT**R**LGI	GFLLKAV	PAEVALI**R**MTADG	LFRIVQ-
CTP	PTEYV**K**TQLQL	GLSSLLYGSIPKAAV**R**FGM	DSRRGLL	PMETV**K**V**K**FIHDQ	VREIVR-
DTP	PLDLA**K**V**R**LQ-	GLSAAVLRQCTYTTV**R**FGA	AYLLPCS	FADVVNI**R**MQNDS	VYKIYRY
ORT	PFDTV**K**V**R**LQT	GIASPLVGACLENATLFVS	G--QILI	PVELV**K**C**K**LQVAN	IKAIIT-
CAT	PLDTV**K**V**R**LQT	GMAAPIIGV**T**PMFAVCF--	TYPQLFT	PGERI**K**CLLQI--	AKKLYQ-
FTP	PLDLL**K**V**R**LQL	GLSINLFGNAIAWGVYFGL	NSLIYLS	PIWVI**K**T**R**IM**S**TS	VQQLLRT
	* * * *				

name	(Lys-179, Lys-187)	(Ser-232, Thr-237)	(Lys-262)	(Arg-276)	(Lys-295)
PTP	GFTP--ILF**K**QIPYNIA**K**FLV$_{190}$	AIV**S**Q-PAD**T**LLSK$_{241}$	QLA**K**QLG$_{265}$	GLPT**R**LVM$_{279}$	GSL------**K**STL$_{298}$
ANT	GFLPSVVGIVVYRGLYFGMYD	ASTCSYPLD**T**VRRR	KIVAAEG	GCGANILR	DQLQMILFG**K**KFK
UCP	GTTPNLLRNVIINCVELVTYD	TFLAS-PADVVKTR	TMLTKEG	GFVPSFLR	EQL------**K**KEL
OTP	GCIPTMARAVVVNAAQLASYS	TAA**S**M-PVDIVKTR	KVVRYEG	GFTPYYAR	EQMN-----**K**AYK
CTP	GLTATVLKQGSNQAIRFFVMT	VFGNT-PLDVIKTR	QIL**K**NEG	GTVP**R**LGR	DEVV-----**K**LLN
DTP	GWKPNMVRGILMTASQVVTYD	TTVCS-PADVMKTR	DAVRKEG	GWLPSFTR	EQL------**K**KHR
ORT	GQSGTFIRESFGGVAWFATYE	NASIF-PAD**T**VKSV	KIFGKFG	GLGITLFR	ETLSAL
CAT	GTALTLMRDVPASGMYFMTYE	WVVAI-PPDVLKSR	ELIREEG	GFNAVMIR	EIPM-----**K**ILN
FTP	GLVPALF-GVSQGALYFAVYD	SVTLVYPFQLLKSN	LIIANDG	GLSANLVR	ENL------**K**HRL
	*	*	*	*	

[a] Residues marked with * are identical among all members of the mitochondrial transport protein superfamily with known transport function. Location similarities for Lys at Lys-295 depend somewhat more on the alignment algorithm, and for ORT it is beyond the C-terminal of the protein. Alignments of a large number of members of this gene family have been published (*18*, *19*). [b] PTP, phosphate transport protein, *Saccharomyces cerevisiae* (yeast), Locus 127277 (*15*); ANT, adenine nucleotide translocase 2, *Saccharomyces cerevisiae* (yeast), Locus 584738 (*20*); UCP, uncoupling protein 1, *Mesocricetus auratus* (golden hamster), Locus 1351354 (*21*); OTP, 2-oxoglutarate/malate transport protein, *Bos taurus* (bovine), Locus 126664 (*22*); CTP, citrate transport protein, *Rattus norvegicus* (rat), Locus 418141 (*23*); DTP, dicarboxylate transport protein, *Saccharomyces cerevisiae* (yeast), Locus 1077385 (*24*, *25*); ORT, ornithine transporter, *Saccharomyces cerevisiae* (yeast), Locus 2497988 (*26*); CAT, carnitine/acylcarnitine transporter, *Rattus norvegicus* (rat), Locus 2497984 (*27*); FTP, FAD transport protein, *Saccharomyces cerevisiae* (yeast), Locus 731875 (*28*).

Replacing Lys295 by electroneutral residues (Gln, Ala) has minimal effects on transport.

Basic residues in ***matrix-side helix-connecting loops***, that is, Arg159 (helix C/D) and Lys262 (helix E/F) (Figure 1), yield transport-active PTP upon substitution by a charge-neutral (Arg159 or Lys262) or basic residue (Arg159) (Table 2).

P_i Transport by Hydroxyl Residue Mutant PTPs. Two hydroxyl residues near the ***matrix end of helices*** C (Ser145) and E (Thr237) (Figure 1) were replaced by Ala, and transport was not affected (Table 2). Three ***intrahelix*** residues (Thr79 and Ser84 within helix B; Ser232 within helix E) were replaced by Ala. Thr79Ala catalyzed transport barely above background. Ser232Ala affected transport almost as severely as the Thr79Ala mutation. However, Ser84Ala showed normal transport activity. Ser116 at the ***cytosol end of helix*** C has been replaced with Ala, and transport is not affected.

DISCUSSION

Basic amino acids and hydroxyl amino acids have been shown to be present in the highly specific P_i binding site of

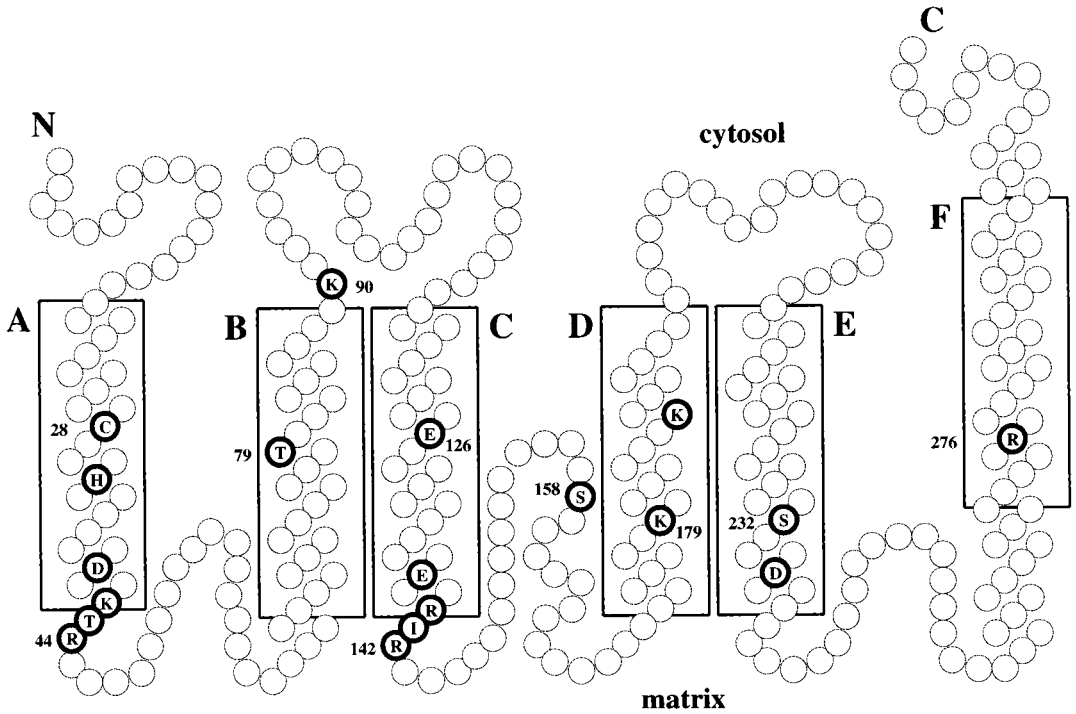

FIGURE 3: Residues in the yeast PTP important for function or at functionally critical location (*2*, *4*−*7*).

the bacterial phosphate binding protein (*14*). We have now replaced several of these types of amino acids in PTP in order to help us identify residues and regions in the PTP that may be functionally and structurally important.

Helix A (Figure 1) has residues near its C-terminal (matrix end) that are very sensitive to substitution by charged or charge-neutral residues. Thus Asp39 cannot effectively be replaced by Glu or Asn without eliminating 99.5% of transport activity (*4*). Replacing Thr43 with Cys lowers the transport rate dramatically (*6*), and transport is blocked completely after this Cys is reacted with *N*-ethylmaleimide (*6*); that is, sterically this location cannot tolerate the *N*-ethylmaleimide group. Now we find that Lys42 cannot be replaced by Ala and a replacement by Arg is barely tolerated (Table 2). Lys42 also is the only basic residue among the four basic residues at similar locations (Lys42, Arg44, Arg140, Arg142) that is strictly conserved as a Lys among all of the sequenced and functionally identified members of the mitochondrial transport protein superfamily (Table 3). However, the neighboring Arg44 can be replaced by Lys, while replacing it with Ala creates dramatic structural changes that prevent it from being solubilized by our standard concentrations of Sarkosyl and from having its transport activity readily assayed. That, indeed, there is something also physiologically wrong with Arg44Ala is indicated by its inability to rescue the yeast PTP null mutant on glycerol plates (Table 2). Whether this is due to improper or low incorporation of Arg44Ala into the mitochondrial membrane or due to lack of functional transport activity or both is being investigated. Arg44 and Asp39 may be structurally linked since only the adenine nucleotide transporter and the citrate transport protein do not have a basic residue at the location of Arg44 (Table 3). Instead they have a Leu (adenine nucleotide transporter) and Gln (citrate transport protein), and it is also *only* these two transporters that have a Glu instead of Asp at the equivalent PTP Asp39 site (Table 3). Transport by PTP may be highly sensitive to substitutions within helix A because of helix A's suggested role in the proton cotransport path (*4*) and because helix A forms such a functionally critical link between the subunits of the PTP homodimer that the disulfide between the two Cys28s blocks transport (*2*).

Residues at the C-terminal (matrix end) of helix C tolerate substitutions somewhat more readily. Glu137Asp, but not Glu137Gln, is transport-active (*4*). Arg140Ala and Arg142Ala show only background transport activity (Table 2). Substituting either of these two residues with Lys yields PTP mutants with physiologically reasonable transport activity. This location in PTP is of interest since replacing Ile141 with Cys uncouples proton transport from phosphate transport (*5*). Ile141 is on the same side of the helix C surface as Glu126 and Glu137, which have been suggested to be part of the proton cotransport path (*4*). From a structural standpoint, it is important to note that Arg142Ala has a much more dramatic effect on the structure of PTP, as indicated by its low liposome incorporation, than Arg140Ala. This is analogous to the relationship between Arg44Ala and Lys42Ala. The effect of Arg142Ala on PTP structure is less severe than that of Arg44Ala, possibly reflecting the overall lower sensitivity of the matrix end of helix C residues to replacement than those of helix A.

Two basic residues at the C-terminals (cytosol end) of helices B (Lys90) and F (Lys295) have been replaced. Lys90Arg reduces dramatically the transport activity and also does not permit growth on glycerol. Lys295Ala and Lys295Gln are transport-active. However Lys295Gln has a very low liposome incorporation efficiency. Cys300Ser, which has normal transport activity (*2*, *3*), also shows dramatically lower liposome incorporation (Wohlrab, personal communication). The structure of PTP appears to be especially sensitive to replacement mutations in its C-terminal beyond helix F.

The sensitivity of PTP function to Lys90 replacements suggests that other residues in helix B (Figure 1) may be of

interest. The replacement of Thr79 with Ala blocks transport just about completely, while no such effect is observed with Ser84. Thr79 and Ser84 are five residues apart and are thus located within opposite faces of helix B and thus may be exposed to rather different spatial and functional restrictions in their immediate environments. Interestingly Lys90 is separated from Thr79 by 10 residues, placing both on the same side of the helix B surface.

Two Ser residues (Ser116, Ser145) at either end of helix C are not sensitive to substitution. Ser116Ala and Ser145Ala behave like wild-type PTP. Ser232, located just one turn of helix E away from Asp236 and the aqueous matrix space, is very sensitive to substitution by Ala. This is not unexpected since Asp236 is even more sensitive to replacements with Asn or Glu than Asp39 (*4*). Thr237Ala, which like Ser145 is near the matrix end of a helix and which is about 100° away from Asp236 on the helix E surface, yields a somewhat higher transport rate than wild-type PTP (Table 2). Such increases in transport were already observed with Glu163Gln and Glu196Gln (*4*) at the matrix and cytosol ends of helix D, respectively.

Two basic residues within helix D (Lys179, Lys187) tolerate replacement by Arg. While the activity of the Lys187Arg mutant is inhibited to about 5% of wild-type PTP transport activity, this relatively high activity is nevertheless insufficient for growth on glycerol and suggests that coupling between proton and phosphate transport may not be functioning properly in this mutant. The replacement of either residue by Ala reduces transport to just above the background, that is, 0.15% of wild-type PTP. Also, these four mutants incorporate poorly into liposomes. Arg276 within helix F tolerates Ala and Gln substitutions poorly, but a Lys substitution yields P_i transport that supports growth on glycerol. Interestingly, the transport rate of this mutant is the same as that of the Lys187Arg and Lys90Arg mutants. Yet neither of these latter two mutants can support growth of the yeast on glycerol. Lys90 and Lys187 are in similar locations of the triplicate repeat sequence of PTP, that is, in helix B (Gly75 Phe76 X Pro78 X_{11} Lys90) and in helix D (Gly172 Phe173 X Pro175 X_{11} Lys187).

Lys262 (matrix loop connecting helices E and F) is completely insensitive to Ala substitutions. The Arg159Ala mutant (matrix loop connecting helices C and D) shows wild-type transport activity but dramatically reduced incorporation into proteoliposomes. Also within this matrix loop, Glu163Gln and Glu164Gln are both transport-active (*4*), yet they also show dramatically lower liposome incorporation (*4*). Also Ser158Thr shows very low liposome incorporation, yet this mutant is almost completely transport-inactive (*7*). Residue replacements in this matrix loop connecting helices C and D generate a PTP structure with dramatically lower LIEs.

We have now demonstrated function-sensitive residues in all six helices of PTP (Figure 3). It remains to be demonstrated which of these are P_i ligands of the phosphate transport path or participate directly in the coupled proton transport path. The dramatic structural effects of mutations in (a) the region consisting of helix D plus the matrix loop connecting helices C and D and (b) the C-terminal segment of PTP beyond helix F are likely due to interactions at the interface between the subunits of the homodimeric PTP. An increased exposure of polar residues in these regions to the aqueous medium could make the homodimer more susceptible to dissociation into monomers. Since the PTP monomers have their intersubunit-specific contact sites mainly within the membrane, they are expected to have a much lower tendency to be incorporated into liposomes by themselves than as homodimeric PTP.

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