# Homodimeric Mitochondrial Phosphate Transport Protein. Transient Subunit/Subunit Contact Site between the Transport Relevant Transmembrane Helices A†

Anne Phelps and Hartmut Wohlrab*

*Boston Biomedical Research Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Watertown, Massachusetts 02472*

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ABSTRACT: The three Cys of the yeast (*Saccharomyces cerevisiae*) mitochondrial phosphate transport protein (PTP) subunit were replaced with Ser. The seven mutants (single, double, and complete Cys replacements) were expressed in yeast, and the homodimeric mutant PTPs were purified from the mitochondria and reconstituted. The pH gradient-dependent net phosphate (Pi) transport uptake rates (initial conditions: 1 mM [Pi]e, pHe 6.80; 0 mM [Pi]i, pHi 8.07) catalyzed by these reconstituted mutants are similar to those of the wild-type protein and range from 15 to 80 $\mu$mol Pi/min mg PTP protein. Aerobic media inhibit only the Pi uptake rates catalyzed by PTPs with the conserved (yeast and bovine) Cys28. This inhibition in the proteoliposomes is 84−95% and can be completely reversed by dithiothreitol. Transport by the wild type as well as by all mutant proteins with Cys28 is more than 90% inhibited by mersalyl. Transport catalyzed by mutant proteins with only Cys300 or only Cys134 is less sensitive, and that catalyzed by the no Cys mutant shows 40% inhibition by mersalyl. When dithiothreitol is removed from purified single Cys mutant proteins, only the mutant protein with Cys28 appears as a homodimer in a nonreducing SDS polyacrylamide gel. Thus, the function relevant transmembrane helix A, with Cys 28 about equidistant from the two inner membrane surfaces, is in close contact with parts of transmembrane helix A of the other subunit in the functional homodimeric PTP. The results identify for the first time not only a transmembrane helix contact site between the two subunits of a homodimeric mitochondrial transport protein but also a contact site that if locked into position blocks transport. The results are related to two available secondary transporter structures (lactose permease, glycerol-3-phosphate transporter) as well as to a low resolution projection structure and a high resolution structure of monomers of inhibitor ADP/ATP carrier complexes.

The mitochondrial PTP[1] is an integral membrane protein that is responsible for the transport of inorganic phosphate across the inner mitochondrial membrane (*1*). The inorganic phosphate is used in the mitochondrial matrix in the oxidative phosphorylation of ADP. We have purified PTP in a reconstitutively active form from the yeast *Saccharomyces cerevisiae* and cloned its gene (*2*, *3*). Although the transport mechanism of PTP is unknown at the molecular level, it has been shown to function as a homodimer (*4*) and as an electroneutral $H^+$-cotransporter (*5*). Much basic information on the molecular mechanism of this transport protein has also been obtained by the identification of amino acids essential for the transport (*6*). Thus, PTP thiols have been probed with inhibitors (*7*−*9*). Cys42 has specifically been identified as the only highly NEM reactive amino acid (*10*) of the bovine heart PTP and as the Cys responsible for the high NEM sensitivity of mammalian mitochondrial phosphate transport (*10*). Although the wild-type yeast protein shows only low sensitivity to NEM (*2*), the Thr43Cys mutant, equivalent to the bovine Cys42, has the same high NEM sensitivity as the bovine heart PTP (*11*).

The yeast PTP, like the mammalian PTP, is highly sensitive to the mercurial mersalyl and is inhibited by autoxidation in a DTT reversible manner (*12*). From among the eight Cys in the bovine protein and the three in yeast, only Cys28 (bovine Cys27) is conserved between them. Whether this residue is responsible for mersalyl inhibition and/or autoxidation inhibition of phosphate transport has been unclear. It is also not clear whether the inhibition by autoxidation is due to an intrasubunit or an intersubunit disulfide bond. The availability of the cloned PTP yeast gene in conjunction with a PTP null mutant makes an investigation of these issues feasible. We have used site-directed mutagenesis to systematically replace each of the Cys residues conservatively with Ser. The physiological competence of the novel mutant proteins was assessed. Each mutant protein was expressed and purified from yeast mitochondria and upon reconstitution was characterized with respect to catalytic transport activity, sensitivity to inhibitors, autoxidation, and dimer formation.

## MATERIALS AND METHODS

*Construction of Mutant PTP Genes.* The mutant PTP genes in which single Cys have been replaced by Ser were prepared by the following method (*11*, *13*). In each instance, oligo-

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* To whom correspondence should be addressed. Tel: 617-658-7775. Fax: 617-972-1753. E-mail: wohlrab@bbri.org.

[1] Abbreviations: PTP, phosphate transport protein; AAC, ADP/ATP carrier; nt, nucleotide; DTT, dithiothreitol; SAM, S-adenosylmethionine; thiamine PP, thiamine pyrophosphate; mDNA, mitochondrial DNA; NEM, N-ethylmaleimide.

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Table 1: Construction of Cys to Ser PTP Replacement Mutants

| mutant | Cys retained | codon change | DNA fragment subcloned | acceptor vector |
|---|---|---|---|---|
| C28S | C134, C300 | TGC to TCC | PvuII/EcoRI | pAP-W3 |
| C134S | C28, C300 | TGT to TCT | SalI/SpeI | pAP-W3 |
| C300S | C28, C134 | TGC to TCC | EcoRI/SpeI | pAP-W3 |
| C28S C134S | C300 | as above | EcoRI/SpeI[a] | pAP-W3(C28S) |
| C28S C300S | C134 | as above | EcoRI/SpeI[b] | pAP-W3(C28S) |
| C134S C300S | C28 | as above | KpnI/SpeI[c] | pAP-W3(C134S) |
| C28S C134S C300S | none | as above | EcoRI/SpeI[d] | pAP-W3(C28S) |

[a] EcoRI/SpeI fragment from C134S construct. [b] EcoRI/SpeI fragment from C300S construct. [c] KpnI/SpeI fragment from C134S C300S construct. [d] EcoRI/SpeI fragment from C134S C300S construct.

nucleotide primers complementary to the appropriate region of the PTP gene were synthesized with the correct point mutation (see Table 1) to direct the Cys to Ser substitutions. Following the fusion of the PCR-generated mutant fragments, the amplified product was digested with the appropriate restriction enzymes before being purified by the Geneclean II (Bio101) procedure. The restriction fragment was then ligated to the correct site in the pAP-W3 vector (*11*). The C28S mutant was constructed by ligating the PvuII-EcoRI (nt 394−959) fragment with the mutation to the pAP-W3 vector digested with the same restriction enzymes. The numbering of the restriction sites is based on the sequence of the original clone (*3*). C134S and C300S were constructed using the same strategy except that SalI-SpeI (nt 671−1655) and EcoRI-SpeI (nt 960−1655) were the ligated fragments carrying the C134S and C300S substitutions, respectively. The site-directed mutations were confirmed by sequencing (*14*). Regions of the plasmid that originated from the PCR fragments were also sequenced to avoid PCR-generated random mutations.

The C28S C134S mutant, which has only Cys300, was constructed by replacing the EcoRI-SpeI fragment of the C28S mutant with that from the C134S mutant. To generate the C28S C300S mutant, i.e., the mutant carrying only Cys134, the EcoRI-SpeI fragment of the C300S mutant replaced that from the C28S mutant. Ligating the KpnI-SpeI (nt 1429−1655) from the C300S mutant into the KpnI-SpeI site in the C134S mutant yields the construct C134S C300S, which has only Cys28. The C28S C134S C300S mutant, which has all of its Cys replaced by Ser, was constructed by ligating the EcoRI-SpeI fragment from the C134S C300S mutant into the EcoRI-SpeI-treated C28S mutant. The mutations were confirmed by sequencing.

*Preparation of Yeast Mitochondria and Purification and Reconstitution of PTP*. Plasmid-transformed yeast were grown in a semisynthetic medium with lactate as the sole carbon source (*11*). Mitochondria were prepared, PTP was purified, and its activity was reconstituted as previously described (*11*). The recorded transport rates reflect the difference in 32 Pi uptake between samples to which mersalyl is added to the medium before and 30 s after the proteoliposomes.

*Other Methods.* The yeast strain CG379 (*MAT*α *ade5 his7-2 leu2-3,112 trp1-189 ura3-52*) and CG379 *mir::URA3* (*MAT*α *ade5 his7-2 leu2-3,112 trp1-189 ura3-52 mir::URA3*) were obtained from Gunter Bobel (Rockefeller University). CNBr digestion of protein in SDS polyacrylamide gel slices was carried out for 40 min at 22 °C (*15*). SDS polyacrylamide gels were silver-stained according to Kolbe and coworkers (*15*).

## RESULTS

*Construction and Physiological Competence of PTP Mutants.* Mutants of the yeast PTP (Figure 1) were constructed by replacing Cys with Ser according to the methods described in the Materials and Methods section. Table 1 shows the codons used and identifies the mutants. Each of the novel plasmids expressed a physiologically competent PTP since the yeast PTP null mutant (CG379 *mir::URA3*) was readily rescued by them on YPG plates (data not shown). In contrast to the T43C mutant (*11*), each of the mutants directed the recovery immediately after plasmid transformation at approximately the same rate as the wild-type plasmid, indicating that replacement of each or all of the Cys residues does not markedly impair $Pi^-/H^+$-cotransport, or the coupling of Pi- and $H^+$-transport.

*Reconstituted Transport Activities of Mutant PTPs.* Table 2 shows the pH gradient-dependent net phosphate transport activity of the reconstituted mutants. To retain the sulfhydryl groups in a reduced state, the protein was purified in the presence of DTT. A gel filtration step was introduced to exchange the protein from purification medium to reconstitution medium (*2*). In these experiments, half of the purified protein was placed into reconstitution medium with DTT and the other half into medium without it. To achieve the conditions where protein-catalyzed transport rather than proteoliposome volume is rate limiting at the 30 s transport point, reconstitution was carried out at a low protein-to-lipid ratio. Except for the addition of mersalyl, which is freshly prepared prior to use, all samples were treated identically. It should be noted that the addition of mersalyl to the transport assay medium raises the pH by not more than 0.025 pH units. This pH change has a negligible effect on the pH gradient-dependent transport.

As already implied by the physiological competence of the mutant yeasts, all of the Cys can be replaced by Ser without significantly impairing the transport activity (Table 2). Although no obvious pattern for an activity decrease is apparent, the C300S mutant, the double Cys replacement mutants, and the mutant without Cys do catalyze somewhat lower transport activities than the wild type.

*Oxygen Sensitivity of Mutant PTPs.* The wild-type PTP reconstituted in the absence of DTT and under aerobic conditions catalyzes a dramatically inhibited phosphate transport activity (Table 2). Incubation of the proteoliposomes with the reducing agent for 20 min (shorter incubations were not carried out) before assaying transport led to a complete recovery of all transport activity. The reversible nature of this inhibition is indicative of a disulfide bond. Table 1 shows that this phenomenon is only exhibited in

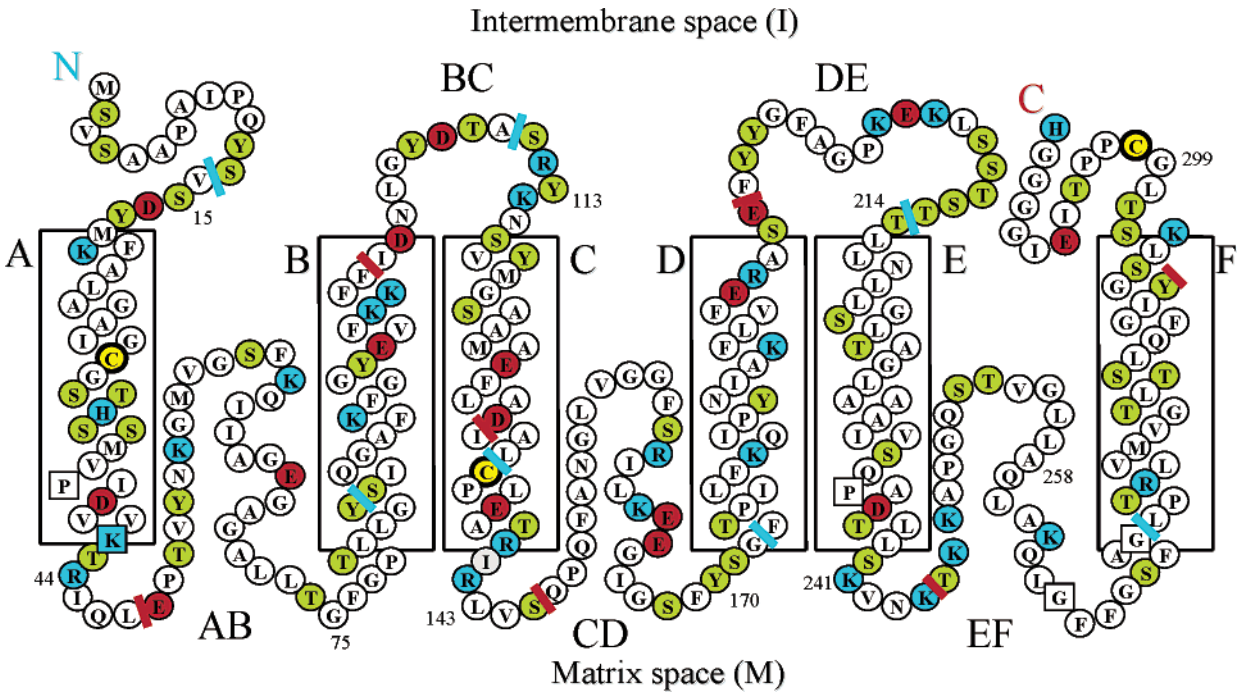


FIGURE 1: Topological model of the yeast PTP subunit (*6*). The three Cys are indicated in yellow. Other residue types: acidic (red), basic (blue), and hydroxyl (green). Rectangles (A−F) are suggested transmembrane helices. Sequences limited by blue to red bars indicate similar transmembrane helices of the bovine (T1 gene) AAC subunit (Ser1 to Val297) complexed with carboxyatractyloside structure (*35*). Alignments of AAC helix segments (in parentheses) were made using the absolutely or highly conserved Pro in helices A (Ala4 to Val37), C (Phe108 to Leu127, Tyr131 to Ala142), and E (Ile209 to Met239) as references. Alignments to helices B (Phe81 to Phe98), D (Phe176 to Gly199), and F (Ala273 to Asp291) were based on residues highly conserved among functionally identified members of the mitochondrial transport protein family (*36*). A single letter amino acid code was used. The five amino acids enclosed by squares are absolutely conserved among members of the mitochondrial transport protein family (*36*).

Table 2: Influence of DTT on Phosphate Transport Catalyzed by PTP Mutants[a]

| | | μmol Pi/min mg PTP | | | |
|---|---|---|---|---|---|
| | | | minus DTT[d] | | |
| mutant | μg PTP[b] | plus DTT[c] | minus DTT | plus DTT[f] | % inhibition[e] |
| wild type | 0.14 | 40 | 8 | 38 | 87 |
| C28S | 0.07 | 82 | 65 | 85 | |
| C134S | 0.05 | 62 | 9 | 56 | 94 |
| C300S | 0.11 | 24 | 4 | 28 | 84 |
| C28S C134S | 0.14 | 26 | 22 | 34 | |
| C28S C300S | 0.14 | 33 | 29 | 37 | |
| C134S C300S | 0.14 | 18 | 3 | 20 | 95 |
| C28S C134S C300S | 0.14 | 16 | 14 | 14 | |

[a] All experiments were carried out at least in duplicate, and duplicates differed by less than 10%. [b] Amount of PTP used for each Pi uptake experiment. [c] PTP was purified and reconstituted in the presence of DTT. [d] DTT was removed from purified PTP and reconstituted in the absence of DTT. [e] After subtracting mersalyl insensitive uptake. [f] Reversal of inhibition of the minus-DTT-prepared and reconstituted PTP by DTT (see Materials and Methods section).

those proteins carrying Cys28. In addition, because the Cys28 only mutant is clearly inhibited, an intermolecular disulfide must form between two protein molecules, i.e., the subunits of the homodimeric PTP.

This result is of interest for an additional reason. Because the hydrophilic DTT is not expected to be able to permeate the proteoliposomal membrane and because it is unlikely that the Cys28−Cys28 disulfide is accessible to DTT from both sides of the membrane, the data suggest that all of the transport active PTPs are oriented the same way in the proteoliposomal bilayer, i.e., with hydrophilic access from the outside of the proteoliposomes or by extrapolation from the intermembrane space of the mitochondria.

*Cys28 Is a Contact Site between the Two Subunits of the PTP Homodimer*. The inhibition of phosphate transport by the removal of DTT suggests that PTP forms a covalent dimer. Such a dimer should be detectable by SDS polyacrylamide gel electrophoresis (PAGE) carried out in the absence of a reducing agent. Figure 1 shows that from among the mutant PTPs with a single or no Cys only the C134S C300S mutant shows a protein band of approximately 59 kDa in the nonreducing SDS polyacrylamide gel. This band is not present when the gel is run under reducing conditions with mercaptoacetic acid (Figure 2). A CNBr digest of the 59 kDa protein band has the same peptides as the protein band at 32 kDa as well as the more diffuse protein band region on the lower mobility side of this 32 kDa band (Figure 3). We stimulated PTP autoxidation with o-phenanthroline and $CuSO_4$ (*16*). However, no significant additional shift of protein from the 32 kDa band to the 59 kDa protein was observed. This could be due to steric inaccessibility of the o-phenanthroline to the Cys28. It is also possible that the 20% SDS PAGE sample buffer increases the accessibility and/or reactivity of the Cys28−Cys28 disulfide toward residual DTT not removed by P6 gel filtration. The DTT could catalyze sulfhydryl-disulfide exchange and thus decrease the amount of disulfide-linked PTP dimer. To eliminate this possibility, after DTT was removed by P6 gel filtration, iodoacetate or iodoacetamide was added to a final concentration of 100 mM (*17*). The sample was incubated on ice (15 min) before and after (45 min) making it to 20% SDS PAGE sample buffer, before passing it through a gel

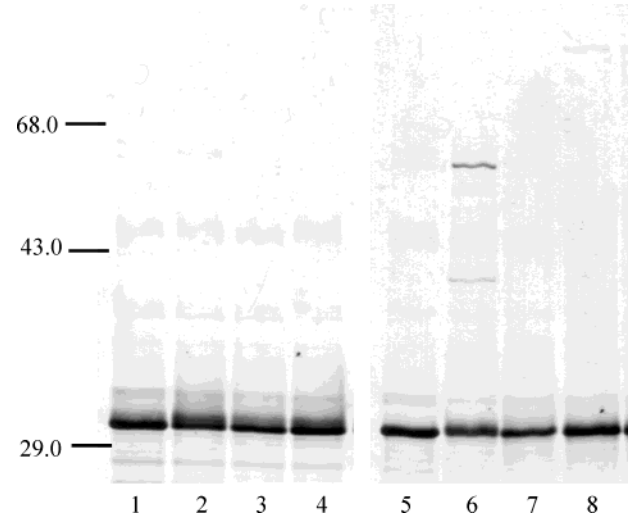


FIGURE 2: SDS PAGE of mutant PTPs in the presence (lanes 1−4) and absence (lanes 5−8) of mercaptoacetic acid. The purified mutant PTPs were used as follows: C28S C134S C300S (lanes 1 and 5), C134S C300S (lanes 2 and 6), C28S C300S (lanes 3 and 7), and C28S C134S (lanes 4 and 8). The standard protein markers are human carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), and bovine serum albumin (68.0 kDa). The gels were silver-stained.

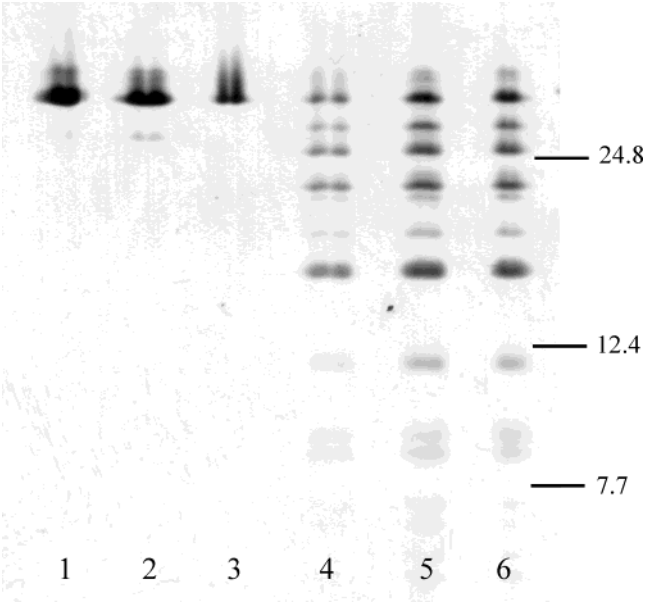


FIGURE 3: SDS PAGE of proteins in bands of mutant C134S C300S from the gel in Figure 1 (lane 6) digested without (lanes 1−3) and with (lanes 4−6) CNBr. Protein digests of the following bands are shown as follows: the lower mobility region of the 32 kDa protein band (lanes 1 and 6), the 32 kDa protein band (lanes 2 and 5), and the 59 kDa protein band (lanes 3 and 4). The gel was silver-stained. Protein standards are cytochrome *c* dimer (24.8 kDa), cytochrome *c* monomer (12.4 kDa), and the largest CNBr fragment (complete cleavage) of cytochrome *c* (7.7 kDa).

filtration column equilibrated in 20% SDS PAGE sample buffer (*15*). No significant enrichment of the dimeric form beyond the earlier observed amount was present. PTP was also purified in the complete absence of DTT. Again, about the same amount of dimer was detectable in the silver-stained gel. Because it appears that the Cys28−Cys28 disulfide is responsible for the inhibition of transport, it is most appropriate to characterize the state of PTP in the transport-inhibited proteoliposomes. Experiments along these lines are being carried out.

Table 3: Mersalyl Sensitivity of Transport Catalyzed by PTP Mutants[a]

| mutant | % inhibition | Cys present in mutant |
|---|---|---|
| C300S | 93 | 28, 134 |
| C134S | 93 | 28, 300 |
| wild type | 91 | 28, 134, 300 |
| C134S C300S | 90 | 28 |
| C28S | 84 | 134, 300 |
| C28S C134S | 84 | 300 |
| C28S C300S | 74 | 134 |
| C28S C134S C300S | 41 | none |

[a] All experiments were carried out at least in duplicate.

*Mersalyl Sensitivity of Mutant PTPs.* Another objective of these studies was the identification of Cys residue(s) that render PTP-catalyzed transport sensitive to the membrane impermeable mercurial mersalyl. Table 3 shows the effect of this reagent on the reconstituted transport activity of each of the PTP mutants. Those with Cys28 show the expected high sensitivity to mersalyl. However, the Cys300 and the Cys134 only mutants are also inhibited by this reagent, although to a lesser extent (84 and 74%, respectively). This result is surprising since each of these residues has been suggested to be located within the membrane: Cys134 according to topological predictions (*18*) and Cys300 from the lack of susceptibility of the yeast protein to carboxypeptidase A and Y (*3*). Furthermore, the Cys-less mutant is also inhibited by mersalyl. The amphipathic nature of mersalyl may permit non-Cys-related interactions with PTP to inhibit transport. A mersalyl-induced conformational change or a PTP conformation trapped by mersalyl rather than a Cys−mersalyl interaction may be responsible for much of the inhibition.

## DISCUSSION

Despite the important role that sulfhydryl reagents have historically played in the identification and characterization of mitochondrial transport proteins (*7*, *19*) and specifically of PTP (*8*), the three Cys of the yeast PTP are not essential for the catalytic activity of PTP (Table 2). However, some of these Cys are located at transport sensitive regions; that is, the DTT reversible autoxidation of Cys28 almost completely inhibits phosphate transport (Table 2). Cys28 is the residue that is essential for the conversion by $Hg^{++}$ of PTP to a rather substrate unspecific uniporter (*20*). Cys28 must be located at or near the phosphate and/or $H^+$-cotransport path(s). Additional evidence that helix A, within which Cys28 is located (Figure 5), has a direct role in transport comes from the observation that while the wild-type yeast PTP is essentially insensitive to NEM alkylation (*2*), the transport catalyzed by the T43C (near C-terminal of helix A) mutant PTP is extremely sensitive to alkylation by NEM (*11*); that is, steric hindrance of helix movements by the NEM blocks transport. This observation agrees with one made with the bovine PTP. Its transport is highly sensitive to NEM (*2*), and NEM reacts only with its Cys42 (similar to yeast PTP T43C) from among its eight Cys residues (partly due to its location between two basic residues) (*10*). Also, the replacement of the yeast helix A His32 by a number of residues always yields an inactive PTP (*21*). We have not been able to identify second site mutations able to suppress the H32A mutation (*22*).

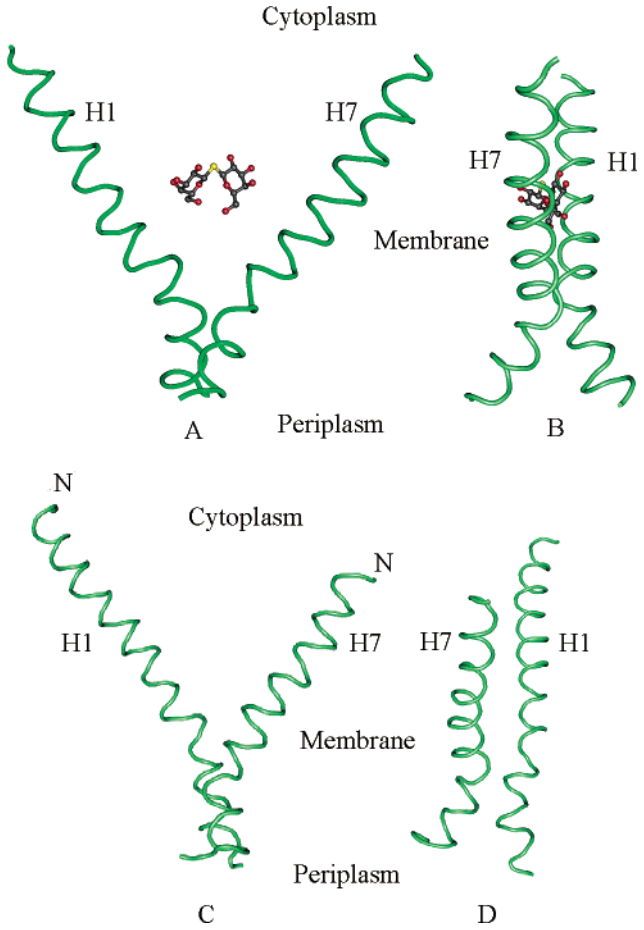


FIGURE 4: Helices H1 and H7 of the lactose permease Lac Y (A and B) (*31*) (pdb identifier 1PV7) and of the glycerol-3-phosphate transporter GlpT (C and D) (*32*) (pdb identifier 1PW4). Views B and D are from the right side of A and C, respectively. Lac Y shows the substrate homologue $\beta$-D-galactopyranosyl-1-thio-$\beta$-D-galactopyranoside. The N-terminals of the helices are indicated. The helical segments are for Lac Y (H1 N6 to H39; H7 Q219 to F251) and for GlpT (H1 E18 to E57; H7 N252 to H285). A single letter amino acid code is used. The diagrams were constructed with program Cn3D 4.1 at the ncbi.nlm.nih.gov web site.

Cys residue(s) are present within the transmembrane helix A region of a few other members of the mitochondrial transport protein family. However, from among the transporters of this family that have been identified with a specific function (Table 4), no other transporter has a Cys at the same location as PTP, i.e., same number of residues away from the absolutely conserved Pro (bold in Table 4). Eight of these 21 transporters do have a Cys in the helix A region. Among them, with a Cys only one position removed from that of PTP, is the oxaloacetate/sulfate transporter (Oac1p), which like PTP catalyzes proton cotransport. Human UCP2 (hUCP2) (Table 4) also catalyzes proton transport. Like all UCPs, it has no yeast transporter equivalent. Yet, like Oac1p, it has a Cys (Cys24) (*23*, *24*) at a site only one residue removed from that of the Cys28 of the yeast PTP. It has not yet been established whether transport by these two proteins is also sensitive to oxygen; that is, purification steps of hUCP2 include reducing agents (*25*) and it is not clear whether a reducing agent was used in the purification/reconstitution of Oac1p (*26*).

Direct evidence has been presented that two of the mitochondrial transport proteins function as homodimers.

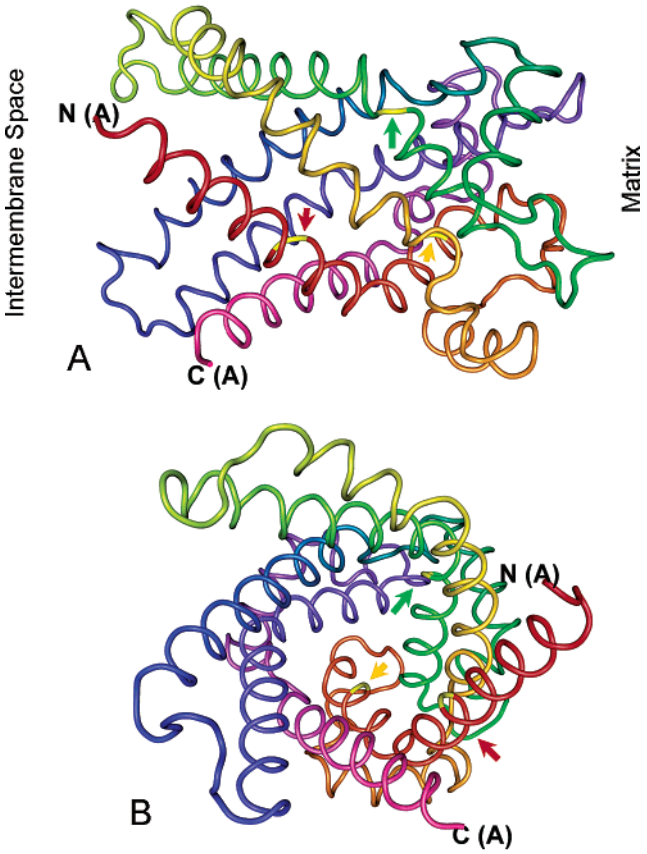


FIGURE 5: Tubular backbone diagram of the bovine (T1 gene) AAC subunit complexed with carboxyatractyloside (*35*). The red arrow points to location (yellow segment) equivalent to yeast PTP Cys28 (see Figure 1). The green arrow points to location (yellow segment) equivalent to Cys134 in yeast PTP. Yeast PTP Cys300 should be nine residues beyond bovine AAC Asp291 of aligned helix F (Figure 1). However, the C-terminal residue of bovine AAC is Val298. The orange arrow points to position (yellow segment) equivalent to Thr43 of yeast PTP. (A) View direction parallel to plane of inner membrane. (B) View perpendicular to plane of inner membrane from intermembrane space side of membrane.

When a homodimeric PTP is reconstituted with one NEM-inhibited subunit and another one that is not inhibited, the resulting dimeric PTP is transport inactive (*4*). In addition, one NEM per two bovine PTP subunits is sufficient to block phosphate transport (*27*). Evidence that the AAC functions as a dimer also comes from inhibitor studies (*28*). Function-related contact sites between the two subunits of such a homodimer have however not yet been identified. For yeast PTP, Cys28 must be located at the homodimeric subunit/subunit interface to generate a disulfide between the two Cys28 residues (Figure 1). The identification of a PTP dimer, with the subunits covalently linked only by a Cys28−Cys28 disulfide, is the first evidence for an intramembrane contact site between the two subunits of a mitochondrial transport protein. A contact site has been identified for UCP with a Cys304−Cys304 disulfide (*29*). This contact site is however structurally not well-defined since the C-terminal in that protein is hydrophilic, i.e., located outside of the membrane. This disulfide does not block UCP activity. The yeast PTP also has a Cys (Cys300) near its C-terminal. However, it is located within the membrane (*3*) and we have not been able to detect a covalent C28S C134S mutant PTP protein dimer (Figure 2). Contact between the two identical subunits along their transmembrane helix A (closest to PTP's N-terminal)

Table 4: Function-Identified Members of the Yeast Mitochondrial Transport Protein Family; Cys Residues in the Helix A Transmembrane Region

| protein/gene | gene | helix A | primary substrate | reference[a] |
|---|---|---|---|---|
| PTP | YJR077c | MKFALAGAIG**C**GSTH$_{32}$SSMV**P**IDVV**K**TRIQ | inorganic phosphate | |
| AAC1 | YMR056c | GVDFLMGGVSAAIAK$_{28}$TGAA**P**IERV**K**LLMQ | ADP, ATP | |
| AAC2 | YBL030c | LIDFLMGGVSAAVAK$_{38}$TAAS**P**IERV**K**LLIQ | ADP, ATP | |
| AAC3 | YBR085w | AINFLMGGVSAAIAK$_{27}$TAAS**P**IERV**K**ILIQ | ADP, ATP | |
| Ant1p | YPR128c | LESALTGAVASAMAN$_{18}$IAVY**P**LDLS**K**TIIQ | ATP (peroxisomal) | (*37, 38*) |
| CTP1 | YBR291c | LHSFLAGSLAGAAEA$_{27}$**C**ITY**P**FEFA**K**TRLQ | tricarboxylate | |
| Yhm2p | YMR241w | SNILLGA**C**LNLSEVT$_{35}$TLGQ**P**LEVV**K**TTMA | mDNA attachment | (*39*) |
| Sfc1p | YJR095w | AINLMAGGTAGLFEA$_{25}$L**CC**H**P**LDTI**K**VRMQ | succinate, fumarate | |
| Dic1p | YLR348c | KYPWWYGGAAGIFAT$_{28}$MVTH**P**LDLA**K**VRLQ | dicarboxylate | |
| Oac1p | YKL120w | FGSFVAGGLAA**C**IAV$_{37}$TVTN**P**IELI**K**IRMQ | oxaloacetate, sulfate | |
| Odc1p | YPL134c | IYQFTAGAIAGVSEL$_{26}$LVMY**P**LDVV**K**TRMQ | 2-oxoadipate | (*40*) |
| Odc2p | YOR222w | IYQFISGAVAGISEL$_{27}$TVMY**P**LDVV**K**TRFQ | 2-oxoglutarate | (*40*) |
| Crc1p | YOR100c | IKSFVAGGVGGV**C**AV$_{50}$FTGH**P**FDLI**K**VR**C**Q | carnitine | |
| ARG11 | YOR130c | ILDIINGSIAGA**C**GK$_{28}$VIEF**P**FDTV**K**VRLQ | ornithine | |
| Flx1p | YIL134w | QKEVISGLSAGSVTT$_{24}$LVVH**P**LDLL**K**VRLQ | FAD | |
| Leu5p | YHR002w | VRSGLAGGISGS**C**AK$_{48}$TLIA**P**LDRI**K**ILFQ | CoA (probably) | (*41*) |
| Tcp1p | YGR096w | WKTLLAGAVSGLLAR$_{31}$SITA**P**MDTI**K**IRLQ | thiamine PP | (*42*) |
| MRS3 | YJL133w | YHQLIAGAFAGIMEH$_{48}$SVMF**P**IDAL**K**TRIQ | iron | (*43*) |
| MRS4 | YKR052c | HSQLLAGAFAGIMEH$_{38}$SLMF**P**IDAL**K**TRVQ | iron | (*43*) |
| Sam5p | YNL003c | FLSLLSGAAAGTSTD$_{19}$LVFF**P**IDTI**K**TRLQ | SAM | (*44*) |
| MTM1[b] | YGR257c | KERMLSAGAGSVLTS$_{27}$LILT**P**MDVVRIRLQ | Mn into SOD2 | (*45*) |
| bovPTP[c] | | IL**C**GLGGIIS**C**GTTH$_{31}$TALV**P**LDLV**KC**RMQ | inorganic phosphate | (*10, 27, 46*) |
| hUCP2[d] | | TVKFLGAGTAA**C**IAD$_{28}$LITF**P**LDTA**K**VRLQ | proton | (*25*) |

[a] For references, see ref *36*. Most recent references are indicated. [b] This protein belongs to the group of six transporters that have one or two residue substitutions at three of the five residues that are absolutely conserved among transporters definitively identified with transporter function (*36*). Its $R_{037}$ is one of these residues that among the other transporters is conserved as a K. [c] Mature bovine PTP. [d] Human uncoupling protein 2 (UCP2).

would suggest that replacement mutants Ala21Cys and Met35Cys (both seven residues away from Cys28) should also generate, under oxidizing conditions, disulfide-linked and transport inactive PTP homodimers. Such expectations may however not be justified since the contact region(s) between the two A helices within a homodimer may be quite small, not including Ala21 and/or Met35, to keep the transport protein structure flexible. According to the ternary complex PTP model, on the basis of extensive kinetic data (*30*), the homodimeric PTP should have the same structure whether a binding site faces the matrix side or the intermembrane side of the membrane, i.e., always one subunit faces one side of the membrane while the other subunit faces the other side of the membrane. Thus, the helix A/helix A contact site is expected to be the same except during the actual transport step during which each substrate binding site changes from facing one side of the membrane to facing the other side.

The nature of the individual steps of the transport process determines in which conformation most PTP dimers are at any one time. Only about 10% of the purified C134S C300S mutant PTP preparation is present as a disulfide-linked homodimer (Figure 2). The size of this fraction is not unreasonable if one considers that typically only about 20% of PTPs of a purified wild-type PTP preparation is incorporated into liposomes in a transport functional form (*6*). A much smaller percentage of the PTPs of some purified PTP mutant preparations, especially of a double mutant as we have it here (C134S C300S), incorporate transport active into liposomes (low liposome incorporation efficiency) than of wild-type PTP (*6*, *22*). Such low liposome incorporation efficiency may be a reflection of only few purified protein molecules of the preparation being in a helix A/helix A contact site(s) conformation optimum for Cys28−Cys28 disulfide formation and transport. Also, our experimental conditions are most likely not optimum for maximizing the fraction of PTPs with helix A/helix A contact sites optimum for disulfide formation, i.e., the presence of Pi, a pH gradient, or a temperature of 22 °C instead of 4 °C.

Recently, the first higher resolution structures of two bacterial secondary transporters [lactose translocase (LacY) (*31*) and glycerol-3-phosphate transporter (GlpT) (*32*)] have been published. Their transport active unit also has 12 transmembrane helices; however, all are within a single protein. Interestingly, both also show a contact region between helices 1 and 7 (the two A helix equivalent of homodimeric PTP) (Figure 4). Because both of these proteins function with a ping-pong type or single binding site, alternating access mechanism (*32*, *33*), the contact site between the two helices clearly would be expected to be different when the protein's single binding site faces the cytosol than when it faces the periplasmic space.

Two structures of a mitochondrial AAC subunit complexed with an inhibitor have recently been published (*34*, *35*). The projection structure to 8 Å resolution of the atractyloside AAC3 monomer complex from yeast does not yet permit the identification of transmembrane helices with protein sequence (*34*). The high resolution structure (2.2 Å) of a bovine AAC (T1 gene) subunit complexed with carboxyatractyloside permits the identification of six transmembrane helices (Figure 5) (*35*). In this structure (mature protein), Ala18 is at a location equivalent to the yeast Cys28 with respect to the absolutely conserved Pro (Table 4). This Ala18 is located on the helix A side facing the center of the deep depression (ADP/ATP binding site) within the molecule (Figure 5). If this structure is similar to that of the yeast PTP, then such a location of Cys28 would imply significant interlinking between two subunits as would be expected from a tight coupling between the two subunits of a transport protein homodimer. Strong linking between the Cys28 location and the transporter's filter region (substrate specificity-generating region) located near the matrix side of the protein is suggested from purified bovine PTP experiments. We have shown that aerobic inactivation of the PTP protects the protein from inactivation by NEM (*27*). In the carboxyatractyloside ADP/ATP translocase subunit complex structure (*35*), the bovine PTP equivalent NEM reactive Cys42 (similar to yeast mutant T43C) is on the A helix face adjacent to the C-terminal end of helix C and the matrix interhelices loop AB in the filter region of the transporter. Thus, it is likely that changes in the subunit/subunit interaction in the bovine PTP A helix region of Cys27 (similar to yeast PTP Cys28), as they are expected to occur during coupled transport, will signal the filter region by helix A movement and thus contribute to the tight functional coupling between the two subunits of the homodimer.

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