Mitochondrial Phosphate Transport

N-ETHYLMALEIMIDE INSENSITIVITY CORRELATES WITH ABSENCE OF BEEF HEART-LIKE Cys42 FROM THE SACCHAROMYCES CEREVISIAE PHOSPHATE TRANSPORT PROTEIN*

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The mitochondrial phosphate transport protein (PTP) has been purified in a reconstitutively active form from Saccharomyces cerevisiae and Candida parapsilosis. ADP/ATP carriers that copurify have been identified. The PTP from S. cerevisiae migrates as a single band (35 kDa) in sodium dodecyl sulfate gels with the same mobility as the N-ethylmaleimide-alkylated beef heart PTP. It does not cross-react with antisera against beef heart PTP. The CNBr peptide maps of the yeast and beef proteins are very different. The rate of unidirectional phosphate uptake into reconstituted beef heart PTP is stimulated about 2.5-fold by two basic residues (Lys-Cys4*-Arg) (5). These are located between two basic amino acids of this motif (Lys41-Cys42-Arg43), is reconstituted PTP catalyzes also net (zero-trans) Pi uptake and is sensitive to mersalyl and N-ethylmaleimide. The inhibitory N-ethylmaleimide reacts with only a single cysteine which is located between two basic residues (Lys-Cys4*-Arg) (6). These basic residues drop the pH of the cysteine and consequently make it more reactive than the other cysteines toward alkylating agents.

Mitochondria from the yeast Saccharomyces cerevisiae have been shown to contain the two systems for Pi transport (6). The phosphate transport can be inhibited by mersalyl (6, 7). Here we report now the isolation and reconstitution of PTP from yeast. Evidence is presented that the Cys42 of beef is replaced by a threonine in yeast PTP. This low sensitivity of yeast PTP' to N-ethylmaleimide and implies that Cys42, as has already been suggested from indirect evidence (4, 8, 9), is not essential for Pi transport as catalyzed by PTP. As an additional structural variant of PTP we report also some of the properties of this protein from the obligatory aerobic yeast Candida parapsilosis. A major protein, that copurifies with the PTP from S. cerevisiae and C. parapsilosis and has a similar mobility on SDS gels, has been identified from its amino acid sequence and immunologically as the ADP/ATP carrier (AAC). A novel method is described for removing this AAC from the S. cerevisiae PTP preparation.

EXPERIMENTAL PROCEDURES

Culture Conditions and Preparation of Mitochondria—Cells of the diploid wild strain S. cerevisiae (yeast foam) and of C. parapsilosis (CBS 7154) were grown aerobically at 28 °C in a complete medium: 1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, pH 4.5, supplemented with 2% glycerol. The cells were harvested in the logarithmic phase and the mitochondria isolated from protoplasts (10). Mitochondria were suspended at 40 mg of protein/ml (estimated by the biuret method) in 0.6 M mannitol, 2 mM EGTA, 10 mM Tris maleate, pH 6.8, and immediately frozen, i.e. with the help of a syringe, mitochondrial suspension droplets were put in contact with liquid nitrogen and stored at −70 °C.

Purification of an Active PTP Fraction—The method used to purify the phosphate transport protein from yeast mitochondria was adapted from that previously used for purifying beef heart mitochondrial PTP (11). All operations were carried out at 4 °C. Yeast mitochondria (17)}
mg of protein) were rapidly thawed and washed two times (10 min, 12,000 × g) with 7 ml of the following medium: 10 mM sodium phosphate, 0.1 mM EGTA, 130 mM NaCl, 5 mM DTT, pH 7.2. The pellet was then suspended in this medium and Triton X-100, diluted in the same medium, was added to obtain a final concentration of 1.55% (v/v) in a final suspension volume of 400 µl. The solubilized mitochondrial protein supernatant was centrifuged for 60,000 × g (for 4 h) and a second supernatant (370 µl) was then added onto a dry hydroxyapatite column (300 mg of hydroxyapatite powder (Chemical Dynamics Corporation, South Plainfield, NJ) in a glasswool-plugged Pasteur pipette) and eluted with 870 µl of the following buffer: 10 mM sodium phosphate, 0.1 mM EGTA, 130 mM NaCl, 5 mM DTT, 0.5% (v/v) Triton X-100, pH 7.2. The pass-through (450 µl) was collected and 0.4 µl of the pass-through protein (hydroxyapatite column was added (11) and stirred for 45 min with 200 µg of Bio-Beads SM2. 20% (v/v) glycerol was added to the Bio-Beads SM2-treated eluate, frozen in liquid nitrogen, and stored at −70 °C.

Proteoliposome Preparation—Liposomes for reconstitution were prepared according to Wohlrab et al. (4). The lipid composition (highly purified plant lipids, Avanti Polar Lipids) of each batch was 6.3 mg of phosphatidylethanolamine, 5.0 mg of phosphatidylcholine, and 0.9 mg of calcium phosphate in 250 µl of the reconstitution medium (10 mM Tris base, 10 mM 1,4-piperazinediethanesulfonic acid) with the pH, usually 8.0. 200 µl of the thawed protein fraction was passed through a Bio-Gel P-6 DG (Bio-Rad) column, eluted with the reconstitution medium containing 5 mM DTT as in (4). 60 µl of the pass-through was mixed with 100 µl of liposomes plus 25 µl of the reconstitution medium. All these manipulations were done under an argon atmosphere. The mixture was then frozen in liquid nitrogen, thawed, and vortexed at low speed at room temperature.

Phosphate Transport Assay—The transport experiments were carried out as in Ref. 12 at 22 °C. Proteoliposomes (25 µl) were added to a tube containing 500 µl of the reconstitution medium (usually at pH 6.8) supplemented with [32P]Pi. The reaction was stopped by mersalyl (0.62 mM) and the mixture passed through an anion-exchange column (AG 1-X8 chloride converted into the formate form, 50-100 mesh, Bio-Rad) to separate proteoliposomes from the [32P]Pi-containing medium (4). Nonspecific [32P]Pi uptake was estimated by adding mersalyl to the medium before proteoliposome addition.

Other Methods—A S. cerevisiae PTP preparation with only traces of AAC was obtained by adding N-ethylmaleimide (9.5 mM final concentration) to the high speed supernatant before adding it to the dry hydroxyapatite column. Standard and peptide SDS-polyacrylamide slab gel electrophoresis was carried out according to Ref. 13 with 16 and 20% acrylamide, respectively, both with an acrylamide/bisacrylamide ratio of 150/1. Samples were applied to the gel before addition of lipids. All samples had also been treated with 9.5 mM N-ethylmaleimide (20 min, 0 °C) to react with cysteines of the proteins and the DTT of the medium. Slab gels were silver-stained according to Refs. 5 and 14. CNBr digestion of protein in polyacrylamide gel slices was carried out according to (13). PTP content was determined on the SDS slab gels after silver-staining by spectrophotometrically scanning the stained protein band. Various concentrations of human carbonic anhydrase B (Sigma) were also electrophoresed on the same gel and stained. The integrated absorbance bands were used to generate an optical density versus protein concentration plot (4).

Electroblots were performed with a Janssen Semidry Electroblotter (Janssen Life Sciences Products) according to the method described by the manufacturer. The nitrocellulose sheets (0.45 µm, Schleicher & Schuell) were either stained with Amido Black (15) or incubated for 1 h at 37 °C in 20 mM Tris base, 0.9% (w/v) NaCl, 5% (w/v) bovine serum albumin, pH 8.2, to saturate the nonspecific binding sites of the sheets. The blotted transfer membranes were then incubated for 2 h at room temperature in 20 mM Tris base, 0.9% (w/v) NaCl, 0.1% (w/v) bovine serum albumin, 1% normal goat serum (Janssen Life Sciences Products), pH 8.2, containing the primary antiserum. The beef heart PTP antiserum (Rasmussen & Schuell) were either stained with Amido Black (15) or incubated for 1 h at 37 °C in 20 mM Tris base, 0.9% (w/v) NaCl, 5% (w/v) bovine serum albumin, pH 8.2, to saturate the nonspecific binding sites of the sheets. The blotted transfer membranes were then incubated for 2 h at room temperature in 20 mM Tris base, 0.9% (w/v) NaCl, 0.1% (w/v) bovine serum albumin, 1% normal goat serum (Janssen Life Sciences Products), pH 8.2, containing the primary antiserum. The beef heart PTP antiserum (Rasmussen & Schuell) were either stained with Amido Black (15) or incubated for 1 h at 37 °C in 20 mM Tris base, 0.9% (w/v) NaCl, 5% (w/v) bovine serum albumin, pH 8.2, to saturate the nonspecific binding sites of the sheets. The blotted transfer membranes were then incubated for 2 h at room temperature in 20 mM Tris base, 0.9% (w/v) NaCl, 0.1% (w/v) bovine serum albumin, 1% normal goat serum (Janssen Life Sciences Products), pH 8.2, containing the primary antiserum.
Yeast Mitochondrial Phosphate Transport Protein

FIG. 2. Purification of the yeast (C. parapsilosis) phosphate transport protein. Mitochondria (lanes 1 and 2) were solubilized, centrifuged, and the supernatant passed through a hydroxylapatite column. Lane 3, pass-through; lane 4, pass-through of the N-ethylmaleimide-treated supernatant; lane 5, pass-through of beef heart mitochondrial proteins. Amount of sample applied to gel (standard SDS-polyacrylamide gel) relative to starting mitochondrial suspension (40 mg/ml) (based on dilutions during preparative procedure and volume of sample applied to gel): lanes 1 (11 μg of mitochondrial protein was applied) (1/8), 2 (1/15), 3 (2/1), 4 (4/1), 5 (1/2). Abbreviations as in Fig. 1. cp, C. parapsilosis.

FIG. 3. Time dependence of net (zero-trans) phosphate uptake by proteoliposomes. ○, S. cerevisiae; ○, C. parapsilosis. Initial conditions were: pH, 6.8, pH, 8.0, [32P]Pi = 1.0 mM, (Pi) = 0.0 mM. Amount of phosphate transport protein was 0.25 and 0.16 μg, respectively, for each assay. Uptake reaction was stopped with 0.62 mM mersalyl. Zero time uptake was obtained by adding mersalyl to the incubation medium before proteoliposomes.

FIG. 4. Phosphate transport activity of S. cerevisiae PTP proteoliposomes: effect of the initial ΔpH. Proteoliposomes were prepared at pH (pH) 8.0 (○, □) or 6.8 (△, ○), respectively. External values of pH (pH) were 6.8 (○, △) or 8.0 (□, ○). In all cases the initial [32P]Pi = 1.0 mM and (Pi) = 0.0 mM. Phosphate transport protein was 0.21 μg/assay.

FIG. 5. Double-reciprocal plot of net (zero-trans) phosphate uptake catalyzed by proteoliposomes prepared with S. cerevisiae PTP. Initial conditions: pH, 6.8, pH, 8.0, [32P]Pi = 1.0 mM, (Pi) = 0.0 mM. Phosphate transport protein was 0.21 μg/assay. The initial phosphate uptake rates were determined from time-dependent phosphate uptake experiments with sample points at 0, 5, 10, and 15 s.

Unidirectional ΔpH-dependent Phosphate Uptake—Proteoliposomes prepared at pH 8.0 in the absence of phosphate were incubated in the reaction medium containing 1 mM [32P]Pi at pH 6.8. The reaction was stopped at different times by the addition of 0.62 mM mersalyl. Figure 3 shows that the hydroxylapatite pass-through fractions from either S. cerevisiae or C. parapsilosis catalyze net Pi uptake. The low incorporation of [32P]Pi, measured when mersalyl was added in the reaction medium before proteoliposomes (time 0 in Fig. 3), was identical to that observed with liposomes devoid of protein (not shown). It represented less than 10% of the incorporation observed after 60 s of proteoliposome incubation in the reaction medium. Different samples from the same batch of proteoliposomes yielded a reproducibility of Pi uptake values of ±10% at each incubation time.

To examine the role of ΔpH in Pi transport catalyzed by proteoliposomes, we tested different combinations of pH inside and outside the vesicles. From the results presented in Fig. 4, it is clear that the optimal conditions for Pi transport were obtained by establishing an initial alkaline inside transmembrane ΔpH. On the contrary, the worst conditions were an alkaline outside transmembrane ΔpH. The absence of a transmembrane ΔpH led to a low rate of Pi incorporation. These results are in accordance with the well established fact that Pi accumulation in mitochondria depends on the pH gradient across the inner mitochondrial membrane.

Dependence of the initial rate of unidirectional Pi transport in proteoliposomes as a function of external Pi concentration, between an external medium at pH 6.8 and an initial internal medium at pH 8.0, was measured (Fig. 5). The Kₐₜ and Vₐₜ values were determined from the double-reciprocal plot in Fig. 5.

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values are 2.2 mM and 170 nmol min⁻¹ µg⁻¹ protein, respectively. These values are very close to those (220 nmol min⁻¹ µg⁻¹) determined for beef heart mitochondrial PTP under the same experimental conditions (4).

It is known that Pᵢ uptake in mitochondria is mediated either by a Pᵢ:H⁺ symporter (PTP) or a dicarboxylate:Pᵢ antiporter (dicarboxylate carrier) (18). Yeast mitochondria are able to catalyze a succinate:Pᵢ exchange but not a fumarate:Pᵢ exchange (6). In order to characterize the purified phosphate transport protein of S. cerevisiae further, we tested the effect of dicarboxylates on the Pᵢ transport catalyzed by the proteoliposomes. Fig. 6 shows that neither succinate nor fumarate inhibit Pᵢ uptake, indicating that proteoliposomes are free of active dicarboxylate carrier. Arsenate, a competitive inhibitor of phosphate transport (19), decreased the rate of Pᵢ uptake into proteoliposomes. Fig. 6 shows also that acetate, expected to compete with phosphate for ΔνH⁺ utilization, decreases the rate of Pᵢ transport.

Effect of Thiol Reagents on the Reconstituted PTP—It is known that phosphate transport in yeast mitochondria, as in mammalian mitochondria (6), is inhibited by low concentrations of mersalyl. Fig. 7 shows that Pᵢ uptake is equally inhibited by mersalyl in proteoliposomes containing PTP from either S. cerevisiae or C. parapsilosis. The I₅₀ (60 µM) is similar to that determined for beef heart mitochondrial PTP (4). Pᵢ transport in S. cerevisiae mitochondria however is poorly inhibited by N-ethylmaleimide. Fig. 8 shows the effect of N-ethylmaleimide on mitochondrial swelling in an isosmotic ammonium phosphate medium. It has been observed that a preincubation, lasting 5 min, was necessary to get a maximum inhibition (Fig. 8A). Under these incubation conditions, 50% inhibition was obtained for a N-ethylmaleimide concentration of 0.75 mM (Fig. 8D). In an identical experiment, carried out with rat liver mitochondria, swelling was completely inhibited after 2 min incubation of mitochondria with 0.2 mM N-ethylmaleimide (20).

Fig. 6. Effect of different competitors of net Pᵢ transport or dicarboxylate/Pᵢ exchange on net (zero-trans) phosphate uptake catalyzed by proteoliposomes prepared with S. cerevisiae PTP. Initial conditions: pH 6.8, pH 8.0, [3pPLJ(P,J₈) = 1.0 mM, (Pᵢ) = 0.0 mM, 0.25 µg of PTP/assay; the incubation medium contained no additions (O) or the following additions (©): A, 2 mM arsenate; B, 2 mM fumarate; C, 2 mM succinate; D, 2 mM acetate. The mersalyl-insensitive Pᵢ uptake was subtracted.

Fig. 7. Mersalyl inhibition of net (zero-trans) phosphate uptake catalyzed by proteoliposomes prepared with PTP from S. cerevisiae (©) and C. parapsilosis (©). Initial conditions: pH 6.8, pH 8.0, [3pPLJ(P,J₈) = 1.0 mM, (Pᵢ) = 0.0 mM, 0.25 µg of PTP/assay. Different mounts of mersalyl were present in the incubation medium before addition of proteoliposomes. Mersalyl (0.62 mM) was added to all samples at 60 s. The mersalyl-insensitive phosphate uptake was subtracted.

Fig. 8. Effect of N-ethylmaleimide on swelling of S. cerevisiae mitochondria in isosmotic ammonium phosphate. Mitochondria were suspended in 0.45 M mannitol, 10 mM Tris maleate, pH 6.8, and preincubated in the presence or absence of N-ethylmaleimide at 22 °C. 100 µl of the suspension was put into 0.9 ml of 0.2 M ammonium phosphate, pH 6.8, and the swelling was monitored at 546 nm using an Eppendorf photometer. Mitochondria were incubated with 1.4 mM N-ethylmaleimide for different times (A) or for 5 min with different N-ethylmaleimide concentrations (B).

Fig. 9. N-Ethylmaleimide inhibition of net (zero-trans) phosphate uptake catalyzed by proteoliposomes prepared with PTP from S. cerevisiae (©) or C. parapsilosis (©). Initial conditions were as in Fig. 3. Different amounts of N-ethylmaleimide were present in the incubation medium before addition of proteoliposomes. Mersalyl (0.62 mM) was added to all samples at 60 s. The mersalyl-insensitive phosphate uptake was subtracted.

Fig. 9 shows that Pᵢ uptake, catalyzed by proteoliposomes made with the PTP from S. cerevisiae, has a low sensitivity to N-ethylmaleimide. 15 mM thiol reagent was required to inhibit the Pᵢ incorporation into proteoliposomes by 50%. The higher I₅₀ value found with proteoliposomes than with mitochondrial swelling experiments can be explained by the ab-
phoresed on a peptide gel (Fig. 1, lanes 6 and 7) was sequenced with an Applied Biosystems 477A gas-phase protein Sequencer with an Online phenylthiohydantoin derivative analyzer (Harvard Microchemistry Facility). The peptide was prepared from a CNBr digested yeast (S. cerevisiae) PTP (Fig. 1, lanes 6 and 7) About 40 pmol of digested protein was applied to the SDS-polyacrylamide gel (5) before blotting onto an Immobilon P membrane (21). pmol is the background subtracted yield. Underlined amino acids are the same for the yeast and beef protein.

 Partial Amino Acid Sequence—The Coomassie Blue-stained P1P protein band in the SDS-polyacrylamide gel (Fig. 1, lanes 2 and 3) was digested with CNBr and reelectrophoresed on a peptide gel (Fig. 1, lanes 6 and 7) (5). The separated peptides were blotted onto an Immobilon P membrane (21) and subjected to gas phase sequencing. A 26-kDa peptide (YPTP2) has an N-terminal sequence as shown in Table I. This sequence shows a dramatic similarity to the sequence of beef heart PTP (14). The N-terminal sequence (Met)-Ile-Leu-Phe-Gly. This sequence matches the shorter N-terminal sequence. The double band was submitted for sequencing and we obtained the sequence shown in Table II. This AAC sequence is highly homologous with AAC-9, yet its N terminus is 17 amino acids shorter than that of the immature AAC-2 (sequence of its gene). Since they most likely have the same C terminus (AAC-1 and AAC-2 have the same length C terminus (25)), the increased mobility of the C. parapsilosis AAC (Fig. 2) can most readily be explained by the shorter N-terminal sequence.

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DISCUSSION

The results reported in this paper clearly show that the ADP/ATP carrier from C. parapsilosis contains only two proteins: one is the ADP/ATP carrier, the other shows sequence homology with the mammalian PTP. The PTP migrates in SDS-polyacrylamide gel electrophoresis in the 35-kDa region both for S. cerevisiae and C. parapsilosis (Fig. 1, lane 3; Fig. 2, lanes 3 and 4). The S. cerevisiae ADP/ATP carrier, just like that from rat liver and rat heart (1, 24), appears to have a molecular mass larger than that of PTP. The ADP/ATP carrier from beef and C. parapsilosis mitochondria, on the other hand, have a smaller molecular mass. Indeed, it has been reported that in S. cerevisiae the ADP/ATP carrier has a lower mobility than the corresponding protein of other species (25, 27).

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A new method has been developed to remove the AAC from the purified S. cerevisiae PTP. The proteins are alkylated with N-ethylmaleimide before hydroxylapatite chromatography and the pass-through yields a PTP with only traces of terminal sequence. The double band was submitted for sequencing and we obtained the sequence shown in Table II. This AAC sequence is highly homologous with AAC-9, yet its N terminus is 17 amino acids shorter than that of the immature AAC-2 (sequence of its gene). Since they most likely have the same C terminus (AAC-1 and AAC-2 have the same length C terminus (25)), the increased mobility of the C. parapsilosis AAC (Fig. 2) can most readily be explained by the shorter N-terminal sequence.

The results reported in this paper clearly show that the phosphate transport catalyzed by the proteoliposomes is due to the phosphate transport protein. This conclusion is based on two pieces of evidence. First, the proteoliposomes catalyze a net phosphate uptake, which is pH-dependent and insensitive to succinate (the dicarboxylate carrier catalyzes Pi-Pi exchange). Second, the active fraction corresponds protein of other species (25, 27).
The CNBr digestion was carried out to make sure that the AAC or another protein did not for some unknown reason change its mobility to that of PTP. Fig. 1, lanes 6 and 7, clearly shows that the purified PTP has the same digestion pattern as the PTP copurified with the AAC. We have not yet determined the minimum N-ethylmaleimide concentration or the minimum reaction time required to increase the affinity of the ADP/ATP carrier for hydroxylapatite sufficiently to make a separation from the phosphate transport protein possible. This method should be able to yield an active phosphate transport protein preparation. The degree of purification with this procedure does depend on the commercial source of the hydroxylapatite and thus its chemical structure and composition. The concentration of Triton X-100 is also important for a successful increase in AAC affinity for hydroxylapatite. The method was not successful with the C. parapsilosis preparation (Fig. 2, lanes 3 and 4).

Since there are no extra cysteines in the N-terminal extension of AAC-2 (S. cerevisiae) (25) compared to that of AAC from C. parapsilosis (Table II), an internal cysteine must be responsible for making this conformational change possible.

The kinetic characteristics of the reconstituted PTP from S. cerevisiae are very similar to those from mammalian PTP. A Ka value of 2.3 nm has been reported for beef heart PTP (4). A turnover number of 6 x 10^3 min^-1 can be calculated for S. cerevisiae PTP by assuming a monomeric PTP of 35 kDa in agreement with the calculation previously reported for beef heart PTP (4). A striking difference however between yeast and mammalian PTP is the sensitivity to N-ethylmaleimide. Kolbe and Wohlrab (5) have shown that N-ethylmaleimide reacts only with a single amino acid residue in native PTP from heart and that this residue is a cysteine (Cys48), surrounded by the basic amino acids lysine (Lys31) and arginine (Arg29) (Ref. 5, see also Ref. 22). A similar structure has recently been reported also for rat liver mitochondrial PTP (23). Our results show that in S. cerevisiae PTP Cys48 is replaced by a Thr residue. Most likely, this substitution is responsible for the Cys48 sensitivity of the yeast PTP activity. It can be concluded that Cys48 has no essential role in the catalysis of P transport by the mitochondrial PTP. It thus also supports the conclusions drawn by Ligeti and Fonyo (8, 9) that the Cys48 has no essential role in phosphate binding. Their studies however were not able to determine whether the Cys48 is essential for phosphate transport. Studies reported by Wohlrab et al. (4) demonstrated that autooxidation of the purified beef heart PTP leads to reversible inactivation of transport function and protection from inhibition by N-ethylmaleimide. This suggests that Cys48 is sensitive to disulfide formation within a possible dimeric structure of the active transport protein. This may no longer be the case for the yeast protein, which lacks this Cys48.

Our observation that the yeast PTP retains normal sensitivity to mersalyl implies that another SH group in the protein must be reactive. Most likely this mersalyl sensitive SH group is at or near one of the active phosphate binding sites. The sequence of the yeast PTP and in vitro mutagenesis is expected to permit us to characterize much better the relationship of various SH groups to the function of the phosphate transport protein.

REFERENCES

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Mitochondrial phosphate transport. N-ethylmaleimide insensitivity correlates with absence of beef heart-like Cys42 from the Saccharomyces cerevisiae phosphate transport protein.

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