Expression of the functional mature chloroplast triose phosphate translocator in yeast internal membranes and purification of the histidine-tagged protein by a single metal-affinity chromatography step

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ABSTRACT The mature part of the chloroplast triose phosphate–phosphate translocator was cloned into the yeast expression vector pEVP11. This construct was used to transform cells from both Saccharomyces cerevisiae and the fission yeast Schizosaccharomyces pombe. The chloroplast translocator protein was functionally expressed in the transformed yeast cells and represented about 1–2% of the cells. Histidine-tagged a sequence encoding a phosphate-phosphate translocator was cloned and/or homogeneity disproved by a single-step affinity chromatography using a Ni²⁺-nitrilotriacetic acid resin. Both the expressed triose phosphate translocator and the recombinant histidine-tagged protein possess substrate specificities identical to those of the authentic chloroplast protein, providing definitive evidence for its identity as the triose phosphate translocator and further disproving its assignment as the receptor for chloroplast protein import. The yeast expression system in combination with the Ni²⁺-nitrilotriacetic acid chromatography thus provides a valuable tool for the production of purified membrane proteins in a functional state.

During photosynthetic CO₂ fixation, the fixed carbon (in the form of triose phosphates and 3-phosphoglycerate) is exported from the chloroplasts into the cytosol, where it is converted into other substances such as sucrose and amino acids. This transport is mediated by the chloroplast triose phosphate–phosphate translocator (cTPT) with an apparent Mr of 29,000 (E29), which represents the most abundant polypeptide of the inner chloroplast envelope membrane, accounting for up to 10–15% of the total protein content of this membrane (for review, see ref. 1). The cDNA sequences of the translocator protein from spinach and pea chloroplasts have been determined (2, 3). Here we report the expression of the mature part of this hydrophobic plant translocator protein in its functional state, using fission yeast (Schizosaccharomyces pombe) and budding yeast (Saccharomyces cerevisiae) as a host. The level of expression is about 1% of the total yeast protein, and methods have therefore to be developed to facilitate efficient purification of the expressed membrane protein. To address this problem, a recombinant translocator protein with a C-terminal affinity tag of six histidine residues was engineered by the overlap extension method using the polymerase chain reaction (PCR) (4). The underlying idea is based on an elegant means of protein purification first reported by Hochuli et al. (5), who introduced an oligohistidine tag into mouse dihydrofolate reductase. This approach led to an efficient purification of the fusion protein by chromatography on Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA), albeit under denaturing conditions. Subsequently, Ni²⁺-NTA chromatography has also been used for the purification of soluble proteins in a native state (e.g., refs. 6 and 7). Here we demonstrate that a strongly hydrophobic membrane protein, cTPT, can be efficiently and rapidly purified in a functional state from transformed yeast cells by chromatography on Ni²⁺-NTA-agarose. Thus, the expression of the histidine-tagged TPT in yeast cells provides an abundant source of protein for structure determination and analysis of structure–function-relationships by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim or Pharmacia and were used according to the manufacturer's instructions. Radiochemicals were obtained from Amersham/Buchler. 4,4'-Diisothiocyanato[15N]stilbene-2,2'-disulfonate ([¹⁵N][H]DDDS) was a generous gift from H. Fasold (University of Frankfurt) and the yeast expression vector pEVP11 was kindly provided by N. Sauer (University of Regensburg). All other chemicals were of the highest purity available.

Cloning Procedures. The DNA encoding only the mature part of the spinach translocator protein was generated by PCR from the full-length cDNA clone encoding the entire precursor protein (2). As a sense primer, a synthetic oligonucleotide corresponding to the beginning of the mature part of the cTPT with an additional ATG codon was used (P1), and the second oligonucleotide was a (reverse) T7 primer. The resulting DNA fragment was first cloned into EcoRV-cut pBSC to yield clone pBSC-mPtra. This vector was digested with Sal I, the Sal I ends were filled in, and the Sal I-filled/BamHI fragment was then ligated into the Sac I-filled/BamHI-cut yeast expression vector pEVP11 containing the S. cerevisiae LEU2* gene (8) downstream of the alcohol dehydrogenase promoter of Sch. pombe. Cells from both Sch. pombe and S. cerevisiae were transformed with the resulting plasmid, pEVP-mPtra. Leu⁺ transformants were detected on agar minimal plates [1% (wt/vol) glucose/0.67% yeast nitrogen base without amino acids] and subsequently grown without leucine at 30°C.

A sequence encoding a C-terminal His₆ tag was inserted into clone pBSC-mPtra (coding for the mature cTPT) by the overlap extension method using the PCR (4). Two oligonucleotide primers (P2 and P3) oriented in opposite directions were synthesized; they were complementary to each other in
their 5' ends corresponding to the insertion sequence, whereas their 3' ends were complementary to the beginning of the noncoding region of the mPtra-cDNA or to the sequence upstream of the insertion point, respectively. In two independent PCRs, the T7 primer and a T3 primer were used as external primers, in combinations P2/T7 and P3/T3. The two PCR-generated DNA fragments containing the overlapping insertion sequences were subsequently fused by PCR amplification using the T3 and the T7 primer, respectively. The resulting fusion product containing the C-terminal His6 tag was then subcloned into pBSC and subsequently into the yeast expression vector pEVP11 as described above. This construct, pEVP11-mPtra(His)6, was used to transform Sch. pombe cells. Transformed cells were then disrupted and the 100,000 × g membrane fraction containing the expressed mature cTPT-His6 protein was used for further experiments.

Subcellular Fractionation. Transformed Sch. pombe (strain 1-32) and S. cerevisiae (strain RS 453) cells were grown for 1–2 days to an OD600 of 0.8–1.0 in minimal medium (1% glucose/0.67% yeast nitrogen base without amino acids) with shaking at 30°C. All the following procedures were performed at 4°C. The cells were collected by centrifugation at 3000 × g for 5 min and disrupted by agitation with glass beads in medium A (10 mM Tris–HCl, pH 7.6/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.1 mM leupeptin/0.1 mM chymostatin). After centrifugation at 700 × g for 10 min, the supernatant was centrifuged at 100,000 × g for 2 hr. The membrane pellet was suspended in medium A with 10% (wt/wt) sucrose and layered on top of a continuous sucrose gradient (20–60%, wt/wt) in medium A for isopycnic centrifugation at 160,000 × g for 14 hr. The cTPT was localized by functional reconstitution of each fraction into artificial membranes (see below). Marker enzyme activities were H+-ATPase (plasma membrane; ref. 9), NADPH-cytochrome c reductase (endoplasmic reticulum (ER) membrane; ref. 10), cytochrome c oxidase (mitochondrial membrane; ref. 11), and α-mannosidase (vacuolar membrane; ref. 12).

Purification of the Expressed cTPT-His6. All steps were performed at 4°C. Cell membranes from Sch. pombe cells containing the expressed cTPT-His6 protein were solubilized for 5 min in 100 mM sodium phosphate, pH 8.0/50 mM NaCl/10 mM imidazole/3% (wt/vol) n-dodecyl maltoside at a protein concentration of 10 mg/ml. Afterwards, the mixture was diluted 2-fold with the solubilization buffer (without detergent) and centrifuged for 5 min at 20,000 × g. The supernatant was then incubated with Ni²⁺-NTA-agarose (Diagen, Hilden, Germany) for 1 hr with continuous agitation (0.15 bed volume per ml of solubilized protein). The resin was subsequently packed into a column and washed with 20 bed volumes of 100 mM sodium phosphate, pH 8.0/10 mM imidazole/0.05% n-dodecyl maltoside (buffer A). The TPT-His6 protein was then eluted with 5 bed volumes of buffer A containing in addition 90 mM imidazole (100 mM imidazole total).

Reconstitution of Transport Activity. Liposomes were prepared from acetone-washed soybean phospholipids (100 mg/ml) by sonication for 10 min at 4°C in 100 mM Tricine-NaOH, pH 7.4/20 mM NaH2PO4 (unless stated otherwise)/30 mM potassium glutonate. Solubilized 100,000 × g membranes and fractions from the sucrose density gradient or protein eluted from the Ni²⁺-NTA column were incorporated into the liposomes, and reconstituted [32P]phosphate transport activities were measured as described (13).

RESULTS

Cloning and Expression of the Mature cTPT. The DNA for the mature part of the spinach cTPT (i.e., without its transit peptide) was amplified by PCR from the full-length cDNA clone encoding the entire precursor protein, pBS-prePtra. The PCR fragment containing an additional start codon was cloned into the yeast expression vector pEVP11 (8), and the resulting plasmid, pEVP11-mPtra, was used to transform cells from both S. cerevisiae and Sch. pombe. Control cells were transformed with pEVP11 only. Total RNA from Leu+ transformants of both S. cerevisiae (NSC-PT4) (Fig. 1A, lane 2) and Sch. pombe (NSP-PT7) (lane 4) gave strong signals on Northern blots indicating that cTPT mRNAs were efficiently transcribed, whereas mRNAs from control cells containing only the pEVP11 vector showed no signals (lanes 3 and 5). For unknown reasons, two transcripts differing in length were obtained in Sch. pombe. Cells from the Sch. pombe transformant NSP-PT7 were then disrupted by agitation with glass beads and fractionated into a soluble fraction and a 100,000 × g membrane pellet, and the expression of cTPT was analyzed by Western blot. Recombinant cTPT was completely absent from control cells and from the soluble fraction of the yeast transformant (Fig. 1B, lanes 7 and 10, respectively) and immunoreactive translocator protein could be detected only in the 100,000 × g pellet (lane 9). This indicates that the expressed cTPT was exclusively associated

![Fig. 1](image-url)
with the membrane fraction of NSP-PT7. The cTPT in this transformant was also detected by labeling of the cells with 5 μM [3H]DIDS, which is a strong inhibitor of chloroplast phosphate transport activity (15) and an almost selective labeling agent for the cTPT (refs. 15 and 16; Fig. 1C, lane 1). Although several yeast membrane proteins became labeled by this reagent, [3H]DIDS-labeled TPT (Mr, 29,000; E29) could be detected in membranes from transformed cells but not from control cells (Fig. 1C, lanes 2 and 3, respectively). This result further proves the identity of the TPT and [3H]DIDS-labeled E29 (ref. 16; Fig. 1C, lanes 1 and 2).

**Localization of the Expressed cTPT.** To determine the intracellular localization of the expressed cTPT in transformed *Sch. pombe* cells, the 100,000 × g membrane pellet (representing about 60% of the total yeast protein) was subjected to isopycnic sucrose gradient centrifugation to separate the different organelles on the basis of their equilibrium densities. The resulting gradient fractions were then assayed for the distribution of the subcellular compartments (marked by specific marker enzymes) and the localization of the expressed cTPT (determined by functional reconstitution into artificial membranes). The peaks of activity for the plasma membrane marker, the mitochondrial marker, and the vacuole marker were clearly distinct (Fig. 2). The ER marker was present as two peaks: the one at higher density cofractionated with the activity of the mitochondrial marker and probably represents the rough ER. The peak at the lower density presumably represents smooth ER but could not be detected in all experiments. The ER activity at the higher density could not be separated from the mitochondrial marker, even by a differential centrifugation step at 8000-12,000 × g. Actually, it appears to be difficult to separate mitochondria and rough ER on the basis of their bouyant densities (17). The activity of the cTPT was clearly absent from plasma membranes, the smooth ER, and vacuolar membranes but was localized to the mitochondrial membrane and the rough ER. Thus, it appears that the cTPT is expressed in yeast internal membranes but it cannot be determined whether it is localized to mitochondrial membranes, to those of the rough ER, or to both.

**Functional Reconstitution of the Expressed cTPT.** We have earlier presented evidence that the cDNA clone for E29 encodes the cTPT (16). The question therefore arises whether the expression of mature E29 in transformed yeast cells is indeed linked to the appearance of the same phosphate transport characteristics as observed for the authentic translocator protein. Intact cells from control and transformed yeast, however, did not show any difference in phosphate transport activity, further indicating the absence of the expressed cTPT from the plasma membrane (data not shown). The 100,000 × g membrane fraction of the yeast transformant NSP-PT7 was therefore solubilized by the addition of detergent and directly reconstituted into liposomes containing inorganic phosphate as the exchangeable anion. This proteoliposomal system was then used for measuring phosphate transport activity (13). [32P]Phosphate transport into the reconstituted liposomes was linear with time for about 60 sec at a rate of 15 nmol/mg of protein per minute (Fig. 3), which rate exceeds the endogenous phosphate transport activity of control transformants by a factor of 60–100. These results demonstrate the functional expression of the cTPT protein in transformed cells harboring the pEVPl1-mPtra plasmid. We further addressed the question whether the substrate specificity of the cTPT is preserved in the transformed yeast cells. Therefore liposomes were loaded with triose phosphate, phosphoglycerate, or phosphoenolpyruvate. The authentic cTPT catalyzes a strict counterexchange of inorganic phosphate, 3-phosphoglycerate, and triose phosphate and accepts at its binding site only inorganic phosphate and C3 compounds with the phosphate bound to C-3 (triol phosphate, 3-phosphoglycerate), whereas C3 compounds in which the phosphate is attached to C-2 (phosphoenolpyruvate and 2-phosphoglycerate) show only poor interaction with the translocator (1). Expressed phosphate transport activity which was reconstituted from membranes of transformed yeast cells displayed transport characteristics identical to those of the authentic protein (Table 1): (i) virtually no phosphate uptake occurred in the absence of a suitable countersubstrate within the liposomes, demonstrating the antiport function of the reconstituted translocator (1), and (ii) the substrate specificities of expressed E29 from both *Sch. pombe* and *S. cerevisiae* cells were indistinguishable from that of the cTPT. Note that, based on the specific reconstituted transport activity, the expression level in *S. cerevisiae* was lower by a factor of 3–5 than that obtained in *Sch. pombe*.

**Construction, Expression, and Purification of cTPT-His.** To facilitate efficient purification of the expressed cTPT, a recombinant mature translocator protein with a C-terminal affinity tag of six histidine residues was engineered by
overlap extension using the PCR (4). The resulting fusion product was then cloned into the yeast expression vector pEVP11 and used to transform *Sch. pombe* cells. The histidine-tagged translocator (TPT-His$_8$), like the authentic translocator, could be labeled with $[^3$H]DIDS. As expected, it had a slightly higher apparent $M_r$, 30,000, than the authentic protein ($M_r$, 29,000; Fig. 4, lanes 6 and 7). The recombinant cTPT-His$_8$ was extracted from yeast membranes (representing about 60% of the total protein) by the addition of the nonionic detergent n-dodecyl maltoside. The solubilized proteins were then incubated for 1 hr with Ni$^{2+}$-NTA-agarose, which is highly selective for proteins containing a stretch of histidine residues (5). To prevent detergent-dependent denaturation of transport activity, high concentrations of inorganic phosphate were included in all buffers. This led to a substantial stabilization of the functional transport activity during the purification procedure (data not shown). The binding buffer also contained low concentrations of imidazole to reduce nonspecific binding. Imidazole structurally mimics the histidine side chain and competes with proteins for binding to the Ni$^{2+}$-NTA resin (18). The resin was then packed into a column which was extensively washed with imidazole-containing buffer. Different concentrations of imidazole were tested for their ability to remove contaminating proteins without eluting the cTPT-His$_8$ protein. Best results were obtained when both the binding buffer and the washing buffer contained 10 mM imidazole (Fig. 4, lanes 3 and 8). The TPT-His$_8$ protein and the $[^3$H]DIDS-labeled TPT-His$_8$ were finally eluted from the column with 100 mM imidazole. The eluted protein was $>95\%$ pure as revealed by SDS/PAGE (Fig. 4, lanes 4 and 9), with an overall yield of about 25%. An ~60-fold purification was achieved for both the functional translocator and the $[^3$H]DIDS-labeled TPT-His$_8$ as determined from the reconstituted specific transport activity and the $[^3$H]DIDS/protein ratio, respectively (Table 2). Based on the purification factor, the expressed cTPT protein represents about 1.8% of the membrane fraction, corresponding to about 1% of total yeast protein. Remarkably, the transport characteristics of the purified histidine-tagged translocator were identical to that of the authentic protein, with markedly lower transport affinities for 2-phosphoglycerate and phosphoenolpyruvate as compared to triose phosphate and 3-phosphoglycerate (Table 1).

**DISCUSSION**

Several prokaryotic and eukaryotic organisms can be used for heterologous gene expression. *Escherichia coli*, which is commonly used as a host for expression of foreign proteins, failed to express either the precursor or the mature cTPT (or even part of it), most probably because of the toxicity of the cTPT gene product. Expression in *E. coli* was achieved only if the cTPT cDNA was fused to the β-galactosidase gene, resulting in an overexpressed β-galactosidase fusion product. This protein could be isolated from inclusion bodies, however, in a nonfunctional state (unpublished results). Work from several laboratories led to the notion that it is difficult

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**Table 1. Determination of substrate specificities of the cTPT expressed in yeast cells**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Spinach chloroplast membranes</th>
<th>Membranes from transformants</th>
<th>Purified cTPT-His$_8$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Sch. pombe</em></td>
<td><em>S. cerevisiae</em></td>
<td><em>Sch. pombe</em></td>
</tr>
<tr>
<td>Triose phosphate</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>86.2</td>
<td>76.3</td>
<td>73.5</td>
</tr>
<tr>
<td>2-Phosphoglycerate</td>
<td>14.7</td>
<td>18.3</td>
<td>31.5</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>18.5</td>
<td>22.9</td>
<td>38.5</td>
</tr>
<tr>
<td>Potassium glutonate</td>
<td>1.8</td>
<td>2.2</td>
<td>16.7</td>
</tr>
</tbody>
</table>

The cTPT from transformed *Sch. pombe* or *S. cerevisiae* cells, or the purified cTPT-His$_8$ protein, was reconstituted into liposomes which had been preloaded with 20 mM substrates as indicated. Transport activities were measured as described in Materials and Methods and are given as a percentage of the activity measured for proteoliposomes preloaded with triose phosphate. For comparison, the substrate specificity of the spinach cTPT is given in column 2. The 100% exchange activities (nmol per mg of protein per min) were 220 (envelope membranes from spinach chloroplasts), 13.1 and 3.2 (membranes from NSP-PT7 (*Sch. pombe*) and NSC-PT4 (*S. cerevisiae*), respectively), and 970 (purified cTPT-His$_8$).
to (over)express eukaryotic authentic membrane proteins in prokaryotic organism (19). In contrast, both Sch. pombe and S. cerevisiae have been used for the expression of functional plasma membrane proteins—e.g., the hexose transporter (20) and the ATPase (21) of plant plasma membranes. Except for the hexose transporter and the mammalian Na⁺,K⁺-ATPase (22), it has not been established whether these proteins are indeed correctly targeted to the plasma membrane. At least, the plant plasma membrane ATPase appears to be trapped in the yeast ER (21). Our results demonstrate that the yeast system can also be successfully used for the expression of functional membrane proteins having no routing or targeting signals, such as the mature form of the cTPT. The functionality of the expressed translocator activity was tested by reconstitution of total yeast cell membrane proteins into liposomes. The expressed translocator possessed transport characteristics identical to those of the authentic protein. The system thus opens the way to study, by site-directed mutagenesis, the role of individual amino acids that constitute the translocation pore.

Fractionation studies of the transformed Sch. pombe cells have shown that this translocator is associated with yeast mitochondrial membranes and/or membranes of the rough ER. Its presence in mitochondrial membranes, however, appears more likely since mitochondria from yeast and also Neurospora crassa showed in vitro an energy- and receptor-independent association of the mature cTPT which had been synthesized from its cDNA (unpublished results).

The cTPT represents about 1% of the total protein from the transformed Sch. pombe cells. This level is comparable to that of soluble mammalian proteins expressed in yeast. To facilitate production of a purified cTPT, a recombinant translocator protein with a C-terminal His₆ tag was engineered, and despite the low level of expression, the cTPT-His₆ protein can be purified to apparent homogeneity by a single chromatography step on Ni²⁺-NTA-agarose. Furthermore, purification can easily be scaled up to generate the quantities of protein necessary for structure determination, for example. This approach might also be useful for translocators from other plant organelles or transport proteins from bacterial and animal systems. It may be noted that E29 has been suggested not to represent the cTPT but the receptor for nuclearencoded precursor proteins to be imported into chloroplasts. This assertion by Schnell et al. (23) has been disproved by us recently (16). The expression of a cTPT gene associated with that of the activity of the cTPT as shown in this paper provides unambiguous and definitive evidence for the identity of E29 and the cTPT and argues against its assignment as the chloroplast import receptor. In addition, data from transgenic potato plants with a reduced TPT activity due to antisense repression are in full agreement with the role of E29 as the cTPT (unpublished data). Thus, it remains only a speculation that the cTPT might have an additional role in the overall process of protein import.

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Table 2. Purification of the cTPT-His₆ from transformed Sch. pombe on Ni²⁺-NTA-agarose

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein, mg</th>
<th>Specific transport activity, nmol/(mg-min)</th>
<th>[³H]DIDS-labeled translocator, dpm × 10⁻³/mg</th>
<th>Purification factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized membranes</td>
<td>2.7</td>
<td>27</td>
<td>7.1</td>
<td>1</td>
</tr>
<tr>
<td>Eluted translocator protein</td>
<td>0.011</td>
<td>1800</td>
<td>407</td>
<td>67 (57)</td>
</tr>
<tr>
<td>(100 mM imidazole)</td>
<td></td>
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*Value in parentheses refers to [³H]DIDS-labeled TPT-His₆.