The 2-Oxoglutarate/Malate Translocator of Chloroplast Envelope Membranes: Molecular Cloning of a Transporter Containing a 12-Helix Motif and Expression of the Functional Protein in Yeast Cells[†,‡]

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ABSTRACT: The 2-oxoglutarate/malate translocator of spinach chloroplasts transports carbon skeletons into chloroplasts for net glutamate synthesis. A sequence of a endoprotease Lys-C peptide derived from the purified protein allowed the design of an oligonucleotide which was then used for a hybridization screening of a cDNA library. A 1945 bp insert of 1 of the isolated clones codes for the entire 569 amino acid residues of the precursor protein corresponding to a molecular mass of 60 288 Da. There was no significant homology to the mitochondrial 2-oxoglutarate/malate carrier from bovine heart or to any other known protein. The translocator protein is composed of a hydrophilic N-terminal region (the transit peptide) with a length of about 90−100 amino acid residues which shows, in contrast to presequences of other known envelope membrane proteins, typical features of higher plant chloroplast transit sequences. The mature protein contains 12 putative transmembrane segments in α-helical conformation. It is suggested that this translocator, in contrast to other known transporters of organellar origin which are all homodimers with a 6+6 helix folding pattern, may function as a monomer. The *in vitro* synthesized precursor protein is directed to chloroplasts where it is inserted into the chloroplast envelope membrane in a protease-resistant manner. The cDNA coding for the precursor protein was cloned into the yeast expression vector pEVP11, and this construct was used to transform cells from the fission yeast *Schizosaccharomyces pombe*. The 2-oxoglutarate/malate translocator could be functionally expressed in the transformed yeast cells, and the recombinant protein showed substrate specificities identical to those of the authentic chloroplast protein.

Chloroplasts are enclosed by two membranes, the outer and the inner envelope membrane. The inner membrane is the actual permeability barrier between the cytosol and the chloroplast stroma and the site of specific transport systems connecting both compartments [for reviews, see Flügge and Heldt (1991) and Joyard et al. (1991)]. So far, very few genes encoding envelope proteins of either membrane have been isolated. A specific function could be attributed to only three of the cloned genes: (i) the triosephosphate/phosphate translocator, a protein of the inner membrane which mediates the export of fixed carbon from the chloroplast in exchange for inorganic phosphate (Flügge et al., 1989, 1991; Loddenkötter et al., 1993); (ii) a Ca^{2+}-ATPase of the inner envelope membrane (Huang et al., 1993); and (iii) a voltage-dependent anion channel (porin) of the outer envelope membrane (Fischer et al., 1994a).

We recently described the purification of the functional 2-oxoglutarate/malate translocator from spinach chloroplasts (Menzlaff & Flügge, 1993), which is involved in the transport of carbon skeletons into the chloroplast for the synthesis of glutamate and thus plays an important role in the biosynthesis of amino acids (Woo & Osmond, 1982). In this pathway, α-keto acids are imported into the chloroplast, and glutamate, deriving from the glutamine synthetase/glutamate synthase cycle, is released into the cytosol. Two different dicarboxylate antiport systems with overlapping substrate specificities are involved in this process: the 2-oxoglutarate/malate translocator imports 2-oxoglutarate in exchange for stromal malate, but transports glutamate only to a low extent. Export of glutamate from the chloroplast in exchange for malate is catalyzed by the glutamate/malate translocator. Malate is the counterion for both translocators, resulting in 2-oxoglutarate/glutamate exchange without net malate transport (Woo et al., 1987; Flügge et al., 1988).

Here we present the nucleotide sequence of a cDNA clone encoding the spinach chloroplast 2-oxoglutarate/malate translocator and its deduced amino acid sequence. The protein contains a 12-helix motif and probably functions as a monomer. The entire precursor protein was functionally expressed in yeast cells and showed substrate specificities identical to those of the authentic protein, providing definitive evidence for the identity of the cDNA clone with the assigned function. Furthermore, the energy-dependent insertion of the *in vitro* synthesized protein into the chloroplast envelope membrane is demonstrated.

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[‡] The nucleotide sequence of clone 211 has been submitted to the EMBL Data Bank under Accession No. U13238.
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MATERIALS AND METHODS

Materials. Radiochemicals were obtained from Amersham-Buchler (Braunschweig, FRG). Reagents and enzymes for recombinant DNA techniques were obtained from Pharmacia LKB (Freiburg, Germany), Boehringer (Mannheim, Germany), or New England Biolabs, if not stated otherwise.

Cloning and Sequencing Procedures. For the preparation of the 2-oxoglutarate/malate translocator, envelope membranes of spinach chloroplasts (12 mg of protein) were extracted with 40 mL of ice-cold chloroform/methanol (2:1, v/v) for 15 min on ice. The mixture was centrifuged (12000*g* for 15 min), and the supernatant containing hydrophobic membrane proteins was evaporated to dryness, delipidated with ethanol/diethyl ether (1:1, v/v), washed once with diethyl ether, and then subjected to preparative SDS–PAGE (Laemmli, 1970). The protein band migrating at 45 kDa (containing the translocator protein; Menzlaff & Flügge, 1993) was excised from the copper-stained gel (Lee et al., 1987). After destaining and desalting of the immobilized protein, the 45 kDa protein was digested with endoproteinase Lys-C while still in the polyacrylamide matrix. The resulting peptides were eluted and then purified by reverse-phase HPLC (Eckerskorn & Lottspeich, 1989) and sequenced in a gas-phase sequencer (Eckerskorn et al., 1988). One mixed oligonucleotide probe was modeled on the basis of the sequence of one peptide obtained [ITXF(S?)ENVVK]: 5′-ATIACITGITTY(T/A)(C/G)IGARAAYGTIGTIAA-3′, where R is A or G and where Y is C or T. The γ-^{32}P-labeled oligonucleotide was then used for plaque hybridization screening (Benton & Davies, 1977) of a spinach leaf cDNA library in λgt10 (Flügge et al., 1989). Positive plaques were purified, and the inserts were subcloned into the Bluescript vector for sequencing at both 5′ and 3′ ends (Sanger et al., 1977). In addition, full-length cDNAs were digested with the restriction enzymes *Ava*I, *Dde*I, *Hae*II, *Hin*dII, *Nco*I, and *Rsa*I, and the fragments obtained were also sequenced from both ends.

Heterologous Expression of the 2-Oxoglutarate/Malate Translocator Gene and Reconstitution of Transport Activity. The Bluescript vector containing the DNA coding for the precursor of the 2-oxoglutarate/malate translocator (pBSC-211) was digested with *Sal*I and treated with T4-DNA polymerase. A *Sal*I-filled/*Bam*HI-cut fragment was then cloned downstream of the alcohol dehydrogenase promoter of *Schizosaccharomyces pombe* into the *Sac*I-filled/*Bam*HI-cut yeast expression vector pEVP11 (Russel & Nurse, 1986). The resulting plasmid, pEVP-211, was used to transform cells from *S. pombe*. Leu+ transformants were selected on agar minimal selection plates [2% (w/v) glucose, 0.35% (w/v) yeast nitrogen base without amino acids] and subsequently grown in the same medium to an OD_{600} of 1.0 [for details, see Loddenkötter et al. (1993)].

Cells from a 50 mL culture were collected by centrifugation (3000*g* for 5 min), resuspended in 0.2 mL of ice-cold buffer A [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1 mM PMSF], and disrupted by vigorous agitation for 2 min with glass beads in a tissue homogenizer (Braun, Melsungen, Germany). Glass beads and cell debris were removed by centrifugation (1 min at 600*g*), and the supernatant was diluted 4-fold (buffer A) and then adjusted to 0.5% (v/v) Triton X-100. Subsequent incorporation of the translocator protein into liposomes was achieved by the freeze–thaw procedure (Kasahara & Hinkle, 1977). The liposomes had been prepared from acetone-washed soybean phospholipids (200 mg/mL) by sonication at 4 °C for 10 min in the presence of 200 mM Tricine–NaOH (pH 7.6), 40 mM malate (if not stated otherwise), and 60 mM potassium gluconate. The external substrate was removed by chromatography on Sephadex G-25 which had been equilibrated with 10 mM Tricine–NaOH (pH 7.6), 100 mM sodium gluconate, and 50 mM potassium gluconate. The eluted liposomes were used for transport studies. Reconstituted malate transport activity was measured by the inhibitor stop method as described previously (Menzlaff & Flügge, 1993).

Protein Import Assay. The cloned DNA for the 2-oxoglutarate/malate translocator was linearized with *Sma*I and then transcribed using T3 RNA polymerase according to the instructions given by the manufacturer (Boehringer, Mannheim). Translation was performed in a reticulocyte lysate (Promega, Heidelberg) using [^{35}S]methionine for labeling. The *in vitro* synthesized protein was used for import studies into isolated spinach chloroplasts. The import assay contained 250 mM sorbitol, 10 mM methionine, 25 mM potassium gluconate, 0.2% (w/v) bovine serum albumin, 2 mM $MgSO_4$, 50 mM HEPES–KOH (pH 8.0), and purified spinach chloroplasts equivalent to 0.2 mg of chlorophyll (final volume, 0.3 mL). Other additions are indicated in the legend to Figure 4. The import reaction was allowed to proceed for 20 min at 20 °C. The chloroplasts were then washed twice in 0.33 M sorbitol, 50 mM HEPES–KOH (pH 8.0), 5 mM EGTA, and 2 mM EDTA and subfractionated into the different compartments (Flügge et al., 1989), and the envelope fraction was analyzed by SDS–PAGE (Laemmli, 1970) and fluorography. To assess the association of the 2-oxoglutarate/malate translocator with the membrane, envelope membranes containing the inserted processed protein were resuspended either in 0.2 mL of 100 mM Na_2CO_3 (pH 11.5) or in 0.1 mL of 100 mM NaOH and incubated for 30 min at 0 °C (Fujiki et al., 1982). The samples were then centrifuged at 160000*g* for 60 min. The supernatant of the NaOH sample was subsequently neutralized by the addition of 0.1 M HCl. The proteins were precipitated with trichloroacetic acid (final concentration, 10%, v/v) and were analyzed by SDS–PAGE and fluorography.

RESULTS AND DISCUSSION

Molecular Cloning of the 2-Oxoglutarate/Malate Translocator. We have recently shown that the 2-oxoglutarate/malate translocator of spinach chloroplasts is represented by an inner envelope membrane protein with an apparent molecular mass of 45 kDa (Menzlaff & Flügge, 1993). To obtain the protein in an amount sufficient for sequence analysis, total envelope membranes were subjected to extraction by chloroform/methanol (C/M). The delipidated soluble C/M fraction which contained the 45 kDa protein and, in addition, proteins with molecular masses of 43, 34, and the 29 kDa triosephosphate/phosphate translocator (Figure 1, see Materials and Methods) was separated on preparative SDS–polyacrylamide gels and stained with copper chloride, and the 45 kDa band was excised from the gel. The gel slices were subsequently digested with endoproteinase Lys-C since the N-terminus of the protein appeared to be blocked (unpublished observations). On the basis of the sequence of one peptide obtained [LITXF(S?)ENVVK], a mixed oligonucleotide was synthesized which was then used to

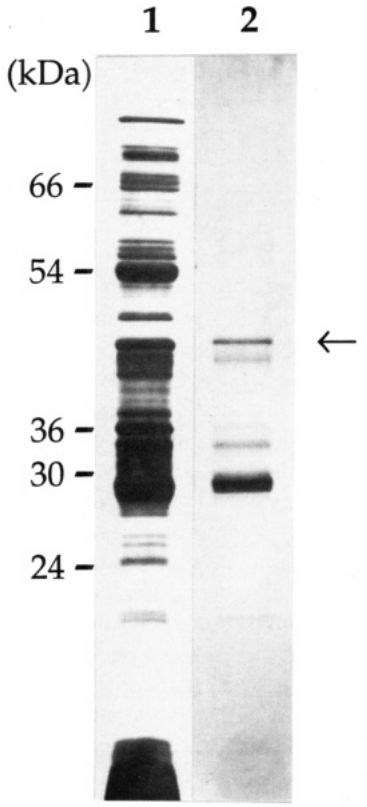

FIGURE 1: Chloroform/methanol extraction of chloroplast envelope membranes for preparation of the 2-oxoglutarate/malate translocator. Spinach envelope membranes were extracted with a mixture of ice-cold chloroform and methanol as described under Materials and Methods, and an aliquot of the chloroform/methanol soluble fraction (3 μg) was analyzed by SDS−PAGE (lane 2). Lane 1, total envelope membranes. Molecular masses are indicated at the left. The position of the 45 kDa protein representing the 2-oxoglutarate/malate translocator is marked by an arrow.

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MASMALSLTS SPTYSLSFRS LPSLKPLSKS QPSISLPSLR SNASKSPSLS HKHFLSPPSL
LLPHKLKPIS ASSPTNPPPP PAPVPSPAPV SAPAQVQPWQ GASIKPLLAS ILTGVIIWFI
PTPEGVSRNA WQLLAIFLST IVGIITQPLP LGAVALMGLG ASVLTKTLTF SAAPSAPGDP
IPWLIALAFF FARGFIKTGL GNRIAYQFVK LPGSSSLGLG YSLVFSEALL APAIPSVSAR
AGGIFLPLVK SLCIACGSNV GDGTERKLGA WLMLTCFQTS VISSSMFLTA MAANPLSATL
TFNTIGKAIG WMDWAKAAFV PGLVSLIVVP LLLYVVYPPE IKSSPDAPRL AKEKLDKMGP
MTKNESIMAV TLLLTVGLWV FGGKLGVDAV TAAILGLSVL LITGVVTWKE CLAESVAWDT
LTWFAALIAM AGYLNKYGLI TWFSENVVKV VGGLGLSWQM SFGVLVLLYF YSHYFFASGA
AHIGAMFTAF LSVASALGTP PFLAAIVLSF LSNLMGGLTH YGIGSAPVFY GANYVPLPQW
WGYGFLISIV NLIIWLGVGG LWWKAIGLW
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FIGURE 2: Deduced amino acid sequence of the 2-oxoglutarate/malate translocator from spinach chloroplasts. The position of the putative processing site is marked by an arrowhead, and that of the endoproteinase Lys-C peptide is underlined.

screen a spinach leaf cDNA library. Several different clones were obtained, two of which (clones 211 and 81) contained inserts close in length to that of the mRNA as estimated by Northern hybridization (not shown). Sequencing of both clones revealed that they indeed contained identical and full-length cDNAs, each of them 1945 bp in length. The endoproteinase Lys-C peptide could be found at amino acid positions 439−449 (LITWFSENVVK). The nucleotide sequence of clone 211 has been submitted to the EMBL Data Bank and is available under Accession No. U13238. The deduced amino acid sequence is shown in Figure 2. Clone 211 comprises a 14 bp 5′-untranslated sequence, a 1707 bp coding region, and 245 bp at the noncoding 3′-region, not including the poly(A) tail. A heptanucleotide, ACAATGG, representing the consensus eukaryotic ribosome initiation

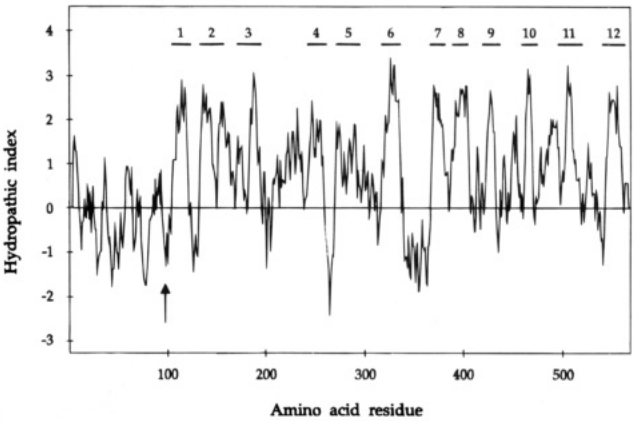

FIGURE 3: Hydrophobicity distribution analysis of the deduced amino acid sequence of the 2-oxoglutarate/malate translocator from spinach chloroplasts. Each hydropathic value is an average of 11 successive residues (hydrophilic, negative values; hydrophobic, positive values). The predicted 12 membrane-spanning α-helices are indicated by bars (1−12) and the putative processing site by an arrow.

sequence (Kozak, 1984) was present at nucleotide positions 11−17.

Analysis of the Amino Acid Sequence. The open reading frame of clone 211 codes for a protein of 569 amino acid residues with a predicted relative molecular mass of 60 288 daltons (Figure 2). A comparison of the translocator sequence with those in the EMBL Nucleotide Sequence Database and the SWISS-PROT Sequence Database revealed no significant homologies to any known protein. Remarkably, there was even no detectable homology to the mitochondrial 2-oxoglutarate/malate carrier from bovine heart (Runswick et al., 1990), although both proteins resemble each other in having similar transport characteristics and substrate specificities.

Analysis of the smoothed hydropathy profile (Kyte & Doolittle, 1982; Bangham, 1988) of the amino acid sequence of the 2-oxoglutarate/malate translocator using the program pSAAM (Crofts, 1992) with a 7-residue span (smoothed 7 × 2) demonstrates its highly hydrophobic nature, which continues through almost the whole sequence except the first amino-terminal 100 amino acid residues (Figure 3). This region of the protein comprises the transit peptide containing targeting information for directing the protein to chloroplasts. It possesses an uncharged N-terminus rich in hydroxylated amino acid residues, a central positively charged domain lacking acidic amino acid residues, and a C-terminal domain with a high potential to form an amphiphilic β-strand. Thus, it resembles typical transit sequences of nuclear-encoded proteins destined for the chloroplast stroma (von Heijne et al., 1989). Interestingly, the transit sequence of the 2-oxoglutarate/malate translocator differs from the presequences of other chloroplast inner envelope membrane proteins sequenced so far (triosephosphate/phosphate translocator: Flügge et al., 1989; Willey et al., 1991; Fischer et al., 1994b; 37 kDa protein: Dreses-Werringloer et al., 1991; Ca^{2+}-ATPase: Huang et al., 1993) which do not possess the typical features of higher plant chloroplast transit sequences, but show the potential to form a positively charged amphiphilic α-helix like mitochondrial presequences.

We could not yet determine the exact start of the mature protein, but the sharp transition between the hydrophilic N-terminal region and the hydrophobic part of the protein

Table 1: Amino Acid Composition of the Putative Mature 2-Oxoglutarate/Malate Translocator from Spinach Chloroplasts

polar amino acids	no. (%)	nonpolar amino acids	no. (%)
Arg	6 (1.3)	Phe	30 (6.3)
Lys	18 (3.8)	Leu	69 (14.5)
Asp	7 (1.5)	Ile	34 (7.1)
Asn	11 (2.3)	Val	39 (8.2)
Glu	9 (1.9)	Trp	19 (4.0)
Gln	9 (1.9)	Ala	54 (11.3)
His	3 (0.6)	Tyr	13 (2.7)
Thr	27 (5.7)	Gly	50 (10.5)
Ser	38 (8.0)	Met	12 (2.5)
		Cys	4 (0.8)
		Pro	24 (5.1)
subtotal	128 (27.0)	subtotal	348 (73.0)

at amino acid positions ~90−95 (see Figure 3) presumably marks the processing site between the amino-terminal extra peptide and the mature protein. In addition, the sequence -Val-Ser-Ala-Pro-Ala- (amino acid residues 90−94) almost fits the consensus cleavage site motif (-Val/Ile)-X-(Ala/Cys)↓Ala- that is contained in a number of chloroplast transit peptides (Gavel & von Heijne, 1990). We therefore assume that processing occurs between amino acids 93 and 94 (Pro-93, Ala-94; -Val-Ser-Ala-Pro↓Ala-). The chloroplast 2-oxoglutarate/malate translocator thus possess an extremely long transit sequence containing features for directing the adjacent mature protein to chloroplasts.

Analysis of the amino acid composition of the putative mature translocator protein revealed an overall polarity index (Capaldi & Vanderkooi, 1973) of only 27% (Table 1). This value is even lower than that for the chloroplast triosephosphate/phosphate translocators (Fischer et al., 1994a), thus emphasizing the highly hydrophobic nature of this translocator protein. It has an about 2-fold excess of basic amino acids (5.7%) as compared to acidic residues (3.4%), resulting in an isoelectric point of 9.6. The putative mature part of the translocator contains 13−14 regions of strong hydrophobicity which might be able to traverse the envelope membrane. Analysis of the sequence with the program MEMSAT (Jones et al., 1994) revealed, however, the presence of only 12 transmembrane helices (1: 101−120, score 2.13; 2: 133−157, score 2.82; 3: 168−192, score 2.77; 4: 241−261, score 1.86; 5: 268−292, score 1.80; 6: 317−337, score 3.89; 7: 367−383, score 2.86; 8: 390−406, score 3.13; 9: 416−434, score 2.01; 10: 461−477, score 1.03; 11: 487−511, score 3.29; 12: 540−563, score 3.74; see Figure 3). We therefore assume that the chloroplast 2-oxoglutarate/malate translocator is composed of 12 hydrophobic segments in α-helical conformation that traverse the membrane in a zig-zag fashion connected by hydrophilic domains.

Hydropathy analysis made it possible to clearly identify two main groups of membrane transporters (Maloney, 1990; Nikaido & Saier, 1992; Higgins, 1992): The larger group includes examples from both prokaryotes and eukaryotes and has 12 transmembrane segments which can span the membrane in an α-helical conformation. The 12 α-helices are often found as paired bundles of 6 helices each, separated by a hydrophilic loop. Members of this group may function as monomers (Costello et al., 1987; Lindenthal & Schubert, 1991). The other group comprises, without any known exception, exchange transporters from mitochondria and chloroplasts. These transporters have 5−7 transmembrane segments and probably function as homodimers with a 6+6 helix folding pattern (Hackenberg & Klingenberg, 1980; Lin et al., 1980; Wagner et al., 1989; Capobianco et al., 1991; Wallmeier et al., 1992; Palmieri et al., 1993; Kaplan & Mayor, 1993). As a result, both groups of transporters have a comparable number of membrane-spanning segments in the functional state. According to the predicted folding pattern of the chloroplast 2-oxoglutarate/malate translocator, this protein obviously belongs to the first group of transporters having 12 hydrophobic transmembrane α-helices. As evident from Figure 3 and usually found for transporters containing 12 membrane-spanning segments (Maloney, 1990), the helices are arranged in such a way that a cluster of 6 helices each is separated by an intervening loop which represents the only longer sequence of hydrophilic amino acid residues present within the mature protein (amino acids 340−366). This translocator thus represents the first example of a translocator of organellar origin with such a membrane topology. It can thus be emphasized that transporters from chloropasts and mitochondria differ in various aspects: (i) there is a close relation of mitochondrial transporters to each other on a structural basis, suggesting that these transporters are derived from a common anchestor (Aquila et al., 1985; Walker & Runswick, 1993). On the other hand, transporters from chloroplasts greatly diverge from each other. These organelles obviously possess the 2 different types of translocator proteins: homodimers having a 6+6 transmembrane helix pattern (e.g., the triosephosphate/phosphate translocator; Wagner et al., 1989; Wallmeier et al., 1992) and transporters having 12 transmembrane segments as the 2-oxoglutarate/malate translocator. In analogy to other members belonging to this group, it is suggested that the chloroplast 2-oxoglutarate/malate translocator operates as a monomer. There is, however, no definitive experimental proof yet for this conclusion. However, during gel permeation chromatography, the functional translocator (molecular mass of the mature protein, ~50 kDa) almost copurifies with the dimeric triosephosphate/phosphate translocator (Flügge & Menzlaff, 1993), an observation that leads one to assume that the latter (dimeric) translocator is close in size to the (monomeric) 2-oxoglutarate/malate translocator. (ii) Taking the 2-oxoglutarate/malate translocator as an example, chloroplasts and mitochondria evidently possess completely different translocators for identical physiologial functions, e.g., the exchange of 2-oxoglutarate for malate.

Import of the 2-Oxoglutarate/Malate Translocator into Chloroplasts. As outlined above, the transit peptide of the 2-oxoglutarate/malate translocator is extremely long and differs from those of other inner envelope membrane proteins in having typical characteristics of soluble chloroplast proteins. We therefore studied the uptake of the precursor protein into spinach chloroplasts (Figure 4). The protein was synthesized by *in vitro* transcripton/translation in the presence of [^{35}S]methionine and added to intact chloroplasts. The chloroplasts were subsequently fractionated into a soluble fraction and fractions containing thylakoids and envelope membranes, respectively (Flügge et al., 1989). The imported protein was completely absent from the stromal fraction, and the processed mature protein (apparent molecular mass, 45 kDa) could only be found in the envelope compartment. Insertion into the envelope membrane under both dark and light conditions was strictly dependent on ATP (lane 3 and lanes 5 and 6); no import was observed if either ATP was

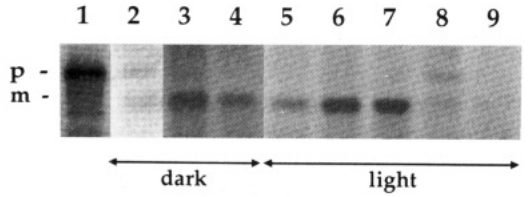

FIGURE 4: Import of the ^{35}S-labeled 2-oxoglutarate/malate translocator into chloroplasts. Intact spinach chloroplasts (0.6 mg of chlorophyll/mL) were preincubated for 15 min with import buffer (Materials and Methods) either in the dark (lanes 2−4) or in the light (lanes 5−9) and in the presence of 10 units of apyrase (lanes 2 and 5), 2 mM ATP (lanes 3, 4 and 6, 7), and 5 μM valinomycin and 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (lane 8). Sample 9 (lane 9) contained chloroplasts which had been pretreated with thermolysin (30 μg/mL). After import, samples 4 and 7 (lanes 4, 7) were further treated with thermolysin (100 μg/mL) in the presence of 1 mM $CaCl_2$ for 30 min. Envelope membranes were separated from the stroma and the thylakoids as described (Flügge et al., 1989) and analyzed by SDS−PAGE and fluorography. Lane 1, *in vitro* synthesized precursor protein. p and m represent the precursor and the mature form, respectively.

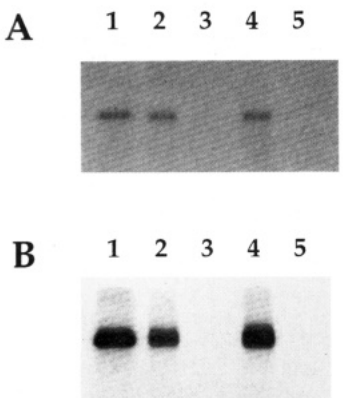

FIGURE 5: 2-Oxoglutarate/malate translocator is membrane-associated. Envelope membranes containing the inserted and processed 2-oxoglutarate/malate translocator (A) or the triosephosphate/phosphate translocator (B) were treated with either 0.1 M NaOH (lanes 2, 3) or 0.1 M Na_2CO_3, pH 11.5 (lanes 4, 5), as described under Materials and Methods. The membrane pellets (lanes 2, 4) and the supernatants (lanes 3, 5) were subsequently analyzed by SDS−PAGE and fluorography. Lane 1, untreated membranes.

omitted (dark conditions, lane 2) or the production of photosynthetically generated ATP was prevented by uncouplers (lane 8). Subsequent treatment of the chloroplasts with the protease thermolysin revealed that the processed mature form was protease-resistantly inserted into the envelope membrane (lanes 4 and 7). Pretreatment of the chloroplasts with thermolysin led to a complete loss of binding and import, indicating the requirement for proteinaceous surface receptors (lane 9). To assess the membrane association of the imported protein, envelope membranes containing the processed mature translocator were treated with carbonate or 0.1 M NaOH. Such treatments are known to disrupt protein−protein interactions and to extract soluble and peripheral membrane proteins only, while integral membrane proteins remain in the membrane sheets (Fujiki et al., 1982). As is the case for the chloroplast triosephosphate/phosphate translocator (another inner envelope membrane protein, used as control), the imported 2-oxoglutarate/malate translocator was completely found in the membrane pellet after incubation of the envelopes at pH 11.5 (sodium carbonate) and even after treatment with 0.1 M NaOH (Figure 5). These findings indicate that the inserted and processed protein was deeply embedded into the envelope membrane.

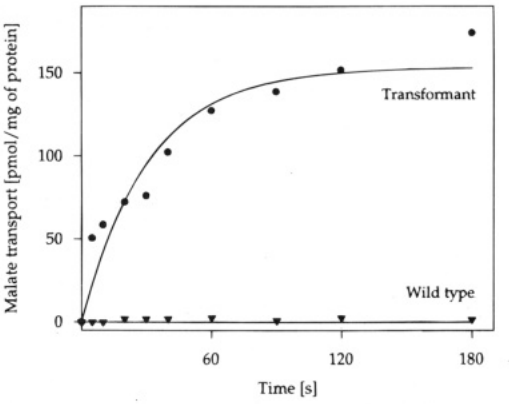

FIGURE 6: Time course of the uptake of [^{14}C]malate into reconstituted liposomes containing *S. pombe* cell membranes. Both control cells and transformed *S. pombe* cells harboring the plasmid pEVP-211 (SP-DC3 cells) were solubilized by the addition of Triton X-100 (final concentration, 0.5% w/v) and reconstituted in liposomes containing 20 mM malate (final concentration; for details, see Materials and Methods). (▼) Control cells; (●) transformed *S. pombe* cells (SP-DC3 cells).

It can be concluded that, although the 2-oxoglutarate/malate translocator possesses a typical chloroplast transit sequence, its import characteristics closely resemble those described for other inner envelope membrane proteins (Flügge et al., 1989, Dreses-Werringloer et al., 1991).

Functional Expression of the 2-Oxoglutarate/Malate Translocator in S. pombe. In order to confirm the identity of the cloned 2-oxoglutarate/malate translocator cDNA and to further characterize the function of the translocator protein, the DNA coding for the translocator protein precursor was expressed in a heterologous system. We have recently shown that the mature chloroplast triosephosphate/phosphate translocators could be functionally produced in the fission yeast *Schizosaccharomyces pombe* and in baker's yeast *Saccharomyces cerevisiae*, although these chloroplast translocators did not contain any routing or targeting signals (Loddenkötter et al., 1993; Fischer et al., 1994a). It had turned out that the expression of the phosphate translocator activity was 3−5 times higher in *S. pombe* cells than in *S. cerevisiae* cells. Therefore, *S. pombe* was chosen as the host for the 2-oxoglutarate/malate translocator. Since we were not absolutely certain about the exact start of the mature 2-oxoglutarate/malate translocator protein, cDNA-211 coding for the entire precursor protein was cloned into the yeast expression vector pEVP11 under the control of the *S. pombe* alcohol dehydrogenase promoter (Russel & Nurse, 1986). The resulting plasmid, pEVP-211, was then used to transform cells from *S. pombe*.

Cells from both Leu+ transformants (SP-DC3 cells containing plasmid pEVP-211) and control cells which had been transformed with pEVP11 only were disrupted by agitation with glass beads. Total protein was solubilized by the addition of detergent and directly reconstituted into phospholipid vesicles which had been preloaded with malate as a counterion for transport. These proteoliposomes were used for measuring malate transport activity. As shown in Figure 6, [^{14}C]malate transport into the reconstituted liposomes was linear for ≈40 s at a rate of ≈0.24 nmol (mg of protein)$^{-1}$ min^{-1}, a rate that exceeds the endogenous malate transport activity of the control transformants by at least 2 orders of magnitude.

Table 2: Substrate Specificities of the Reconstituted 2-Oxoglutarate/Malate Translocator[a]

liposomes loaded with	recombinant protein (transformed SP-DC3 cells)	envelope membranes from spinach chloroplasts	purified translocator protein[b]
malate	(100)	(100)	(100)
fumarate	119 (±24)	72 (±2)	65
succinate	83 (±12)	74 (±2)	63
2-oxoglutarate	44 (±14)	46 (±2)	41
aspartate	13 (±4)	12 (±1)	24
glutamate	3 (±1)	6 (±1)	10
no substrate	1 (±1)	1 (±1)	1

[a] Total yeast protein, envelope membrane proteins, and the purified 2-oxoglutarate/malate translocator, respectively, were reconstituted into liposomes which had been preloaded with the indicated dicarboxylates or potassium gluconate (no substrate) as described under Material and Methods. Transport activities were measured (Materials and Methods) and are given as the percentage of the activity measured for proteoliposomes which had been preloaded with malate; standard deviations are given in parentheses. The 100% exchange activities (nanomoles per milligram of protein per minute) were 0.8 (recombinant protein), 21.4 (envelope membranes), and 263 (purified translocator protein). Means of 2−4 different experiments. [b] Data from Menzlaff and Flügge (1993).

These results demonstrate the expression of the functional 2-oxoglutarate/malate translocator protein in *S. pombe* cells harboring the pEVP11-211 plasmid. The activity of the recombinant translocator could reliably be assessed even by reconstituting total yeast cell protein since transport measurements were almost not affected by the activities of endogenous yeast (mitochondrial) dicarboxylate carriers. It can further be concluded that expression in the heterologous yeast system can be achieved with an entire chloroplast precursor protein containing a typical chloroplast transit sequence.

To further assess the identity of the expressed translocator, we studied the dependence of the malate transport activity on internal liposomal counterions. Since chloroplast transport systems mediate an almost obligatory exchange of metabolites (Flügge & Heldt, 1991), transport in phospholipid vesicles is expected to be strictly dependent on the presence of an exchangeable substrate within the liposomes. Table 2 shows the transport characteristics of the expressed and reconstituted 2-oxoglutarate/malate translocator. For comparison, Table 2 also contains data on the substrate specificities of the purified authentic translocator protein (Menzlaff & Flügge, 1993) and of a total envelope membrane protein fraction. It is obvious that the dicarboxylate transport activities expressed in transformed yeast cells displayed transport characteristics almost identical to those of the authentic protein (Woo et al., 1987; Flügge et al., 1988; Flügge & Menzlaff, 1993): Malate could only be exchanged for internal succinate, fumarate, and 2-oxoglutarate (and malate) but not for aspartate and glutamate, respectively. These results provide definitive evidence for the identity of cDNA clone 211 as the 2-oxoglutarate/malate translocator. Virtually no malate uptake occurred in the absence of a suitable counter-substrate within the liposomes which demonstrates the strict counter-exchange function of the translocator. The activity of the expressed translocator protein is thus in accordance with its proposed function as a supplier of the chloroplasts with carbon skeletons for assimilation of nitrogen.

Perspectives. In a mutant from *Arabidopsis thaliana*, which is not viable under photorespiratory conditions, a polypeptide has been identified that is possibly associated with the chloroplast glutamate/aspartate translocator (Somerville & Somerville, 1985). Mutants lacking this transport component of the envelope membrane only survived when grown under high CO_2. The availability of the cDNA clone encoding the chloroplast 2-oxoglutarate/malate translocator now enables us to study the underlying mechanisms in more detail by means of antisense repression or overexpression of the translocator protein in transgenic plants. We have recently described a method which allows rapid screening of metabolite transport activities in crude plant tissue homogenates, thus bypassing the necessity to isolate intact organelles (Flügge & Weber, 1994). Work is now in progress to produce transgenic plants with altered activities of the 2-oxoglutarate/malate translocator.

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