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Purification and functional reconstitution of the 2-oxoglutarate/malate translocator from spinach chloroplasts

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The chloroplast 2-oxoglutarate/malate translocator was solubilized from envelope membranes by the detergent n-dodecyl β-D-maltoside and purified to apparent homogeneity by anion-exchange chromatography followed by gel permeation chromatography. During the purification procedure, the activity of the translocator was monitored by functional reconstitution into phospholipid vesicles. The purified translocator protein has an apparent molecular mass of about 45 000 as revealed by SDS-PAGE. Based on the specific reconstituted transport activity, the purification was about 31-fold with an overall yield of ≅ 50%. The substrate specificity of the purified translocator closely resembles that described for the native transport system in intact chloroplasts.

Introduction

The inner membrane of the chloroplast envelope is the permeability barrier between the cytosol and the chloroplast stroma and is the site of different translocators coordinating the metabolism in both compartments, e.g., photosynthesis, photorespiration, biosynthesis of sucrose, starch and amino acids (for review see Ref. 1). Several translocators of this membrane have been characterized, but only the triose phosphate/phosphate translocator that mediates the export of the fixed carbon out of the chloroplast has been identified as a membrane component so far [2]. Transport of dicarboxylates across the chloroplast envelope plays an important role during the net synthesis of amino acids and also the photorespiratory NH_3 reassimilation [3,4]. In both pathways, α-keto acids (2-oxoglutarate) are imported into the chloroplasts and glutamate, formed via the glutamine synthetase/glutamate synthase pathway, is released into the cytosol. Recent studies have shown that two different dicarboxylate antiport systems with overlapping specificities are involved in these processes: 2-oxoglutarate is taken up in exchange with stromal malate via the 2-oxoglutarate/malate translocator (which does not transport glutamate) and glutamate is exported from the chloroplasts in exchange with external malate via the glutamate/malate translocator. Thus, malate acts as a counter-ion on both translocators in a cascade-like manner. This results in a 2-oxoglutarate/glutamate exchange with no net malate transport [5–7]. In a mutant from *Arabidopsis thaliana* that is not viable under photorespiratory conditions, a polypeptide has been identified which is possibly associated with the glutamate/malate translocator [8]. Here we describe the purification of the functional 2-oxoglutarate/malate translocator from spinach chloroplasts to apparent homogeneity.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; $C_{12}E_8$, dodecyl octaoxyethylene ether, $C_{12}E_9$, dodecyl nonaoxyethylene ether; $C_{13}E_8$, tridecyl octaoxyethylene ether; $C_{13}E_{10}$, tridecyl decaoxyethylene ether; CMC, critical micellar concentration; LDAO, lauryl(dimethyl)amine oxide; MEGA 8, octanoyl-*N*-methylglucamide; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Materials and Methods

Materials. [^{14}C]Malate was obtained from Amersham-Buchler (Braunschweig, Germany), pyridoxal 5′-phosphate (PLP), mersalyl, soybean phospholipids and n-dodecyl β-D-maltoside from Sigma (München, Germany). TSK DEAE-650 was purchased from Merck (Darmstadt, Germany), the HiLoad Superdex 200 column and Sephadex G-25 from Pharmacia (Freiburg, Germany). All other reagents were the highest grades available commercially.

Isolation of the 2-oxoglutarate / malate translocator. All steps were performed at 4°C. Envelope membranes from spinach chloroplasts (1.5 mg/ml) in 20 mM Tricine buffer (pH 7.5) were solubilized by the detergent n-dodecyl β-D-maltoside (final concentration, 1% v/v unless stated otherwise) and passed over a TSK DEAE-650 column (0.5 bed volumes per ml solubilized protein) which had been equilibrated with 2 bed volumes of 20 mM Tricine buffer (pH 7.5), 1 M sodium acetate and 0.05% n-dodecyl maltoside followed by 4 bed volumes 20 mM Tricine buffer (pH 7.5), 0.05% n-dodecyl maltoside. The unbound fraction containing the enriched translocator protein was adjusted to 0.15 M sodium acetate and further purified by gel chromatography on a HiLoad Superdex 200 column (1.6 × 60 cm) which had been equilibrated with a buffer containing 20 mM Tricine buffer (pH 7.5), 0.15 M sodium acetate and 0.03% n-dodecyl maltoside. The flow rate was 0.5 ml/min and the fraction size was 0.5 ml. Fraction numbers 118–126 contained the purified translocator protein.

Reconstitution of the translocator activity. Liposomes were prepared from acetone-washed soybean phospholipids (100 mg/ml) by sonication for 10 min at 4°C in a medium containing 60 mM Tricine buffer (pH 7.5), 50 mM malate (unless stated otherwise), 75 mM sodium acetate and 30 mM potassium gluconate. Solubilized envelope membranes and fractions from the different chromatography steps were incorporated into the liposomes (lipid/detergent ratio > 20) by the freeze-thaw technique [9]. Incorporation of envelope membranes into liposomes was also achieved without prior solubilization. This procedure, however, resulted in variable reconstituted transport activities. After thawing on ice, the proteoliposomes were sonicated (Branson sonifier with microtip, 40% line voltage, 20% duty cycle) for 20 s. The liposomes containing the reconstituted 2-oxoglutarate/malate translocator were separated from the external medium by passage over a Sephadex G-25 column which had been equilibrated with 10 mM Tricine buffer (pH 7.5), 150 mM sodium gluconate and 90 mM potassium gluconate. The eluted liposomes were used for transport studies. Transport was initiated at 22°C by the addition of 10 μl [^{14}C]malate (final concentration, 0.2 mM unless stated otherwise; specific activity: 3 Ci/mol; total volume, 210 μl) and terminated after 10–20 s by the addition of 45 mM PLP and 8 mM mersalyl (final concentrations). In control samples, PLP/mersalyl was added together with the labelled substrate at time zero. External radioactivity was subsequently removed by passing the suspension over a Dowex AG-1X8 (acetate form, 100–200 mesh) column [10] preequilibrated with 250 mM sodium acetate. The liposomes were eluted with 1.1 ml of 250 mM sodium acetate and the radioactivity was determined by liquid scintillation counting. In control experiments, the linearity with time of the transport was checked in order to confirm that initial rates were measured.

Other methods. SDS-PAGE was performed according to Laemmli [11]. Protein was determined by the Lowry method [12].

Results and Discussion

Effect of different detergents on the reconstituted 2-oxoglutarate / malate transport activity

Transport proteins are supposed to represent integral membrane proteins that are deeply embedded within the membrane and that can only be solubilized with the aid of detergents. Different membranes and transport proteins respond to different detergents differently. Thus, for any particular membrane protein various detergents have to be tested with respect to their solubilization efficiency. Moreover, one has to ensure that the solubilized protein retains (most of) its transport activity, even over longer time periods that might be required for the purification procedure. The chloroplast triose phosphate/phosphate translocator, for example, rapidly loses its transport activity after solubilization with the detergent Triton X-100 [10]. We therefore tested different classes of detergents (e.g., the non-ionic polyoxyethylene octyl phenols, polyoxyethylene alcohols, *N*-methyl glucamides, n-dodecyl maltoside, dimethyl amine oxides and the zwitterionic detergents CHAPS and CHAPSO) with respect to their ability to preserve the reconstituted 2-oxoglutarate/malate transport activity after solubilization of the envelope membrane. In a preceding experiment, the ability of these detergents to solubilize envelope membranes were tested. Envelope membranes (1.5 mg/ml) were solubilized for 10 min with the detergents in concentrations of 0.1–5% and were subsequently centrifuged at 100 000 × *g* for 30 min. Both the sediments and the solubilized fraction were analysed for protein contents. For each detergent, the lowest concentration leading to an almost complete solubilization of the membranes was chosen and the membranes were treated with that particular concentration of the detergent for 1 h. The residual transport activities were then measured by functional reconstitution of the translocator protein. As depicted in Table I, representatives of the polyoxyethylene octyl phenols, polyoxyethylene alcohols and the dimethyl amine oxides led to an almost complete loss of the reconstituted dicarboxylate transport activity. Best preservation of the transport activity was obtained using CHAPS, CHAPSO, MEGA 8 and n-dodecyl maltoside, and this holds true for exposure times even longer than 1 h. These detergents can therefore be used for the solubilization of the translocator protein in a mostly active form. Since these detergents also showed comparable effects on the reconstituted transport activities of the

chloroplast triose phosphate/phosphate translocator and the chloroplast ADP/ATP translocator (not shown) they might be useful for the purification of chloroplast envelope translocator proteins in general. From these detergents, n-dodecyl maltoside has the lowest CMC value (Table I) and was therefore chosen as detergent for solubilization and purification of the 2-oxoglutarate/malate translocator. It may be noted that the presence of substrates or osmolytes (sorbitol, glycerol) during the solubilization procedure was of no marked use to the functional integrity of the translocator protein.

Measurement of the reconstituted 2-oxoglutarate / malate translocator by an inhibitor stop

An essential prerequisite for the measurement of reconstituted transport activities is the availability of an efficient stopping reagent of the transport reaction. This holds especially true for antiporters such as the 2-oxoglutarate/malate transport system which are not as strictly coupled as e.g., the chloroplast triose phosphate/phosphate translocator [13] or the mitochondrial ADP/ATP translocator [14]. Specific inhibitors for the chloroplast dicarboxylate translocators, however, are not known yet. Pyridoxal 5'-phosphate (PLP) which is known to effectively inhibit the chloroplast triose phosphate/phosphate translocator by reacting with an essential lysine residue [15], also inhibits dicarboxylate transport albeit not completely, even at high concentrations. We therefore tested a combination of PLP with several SH-reagents (e.g., mersalyl, 5,5'-dithiobis(2-nitrobenzoic acid), *p*-chloromercuriphenylsulfonic acid) for their ability to instantaneously block the transport reaction in a reconstituted system. A combined addition of 45 mM PLP and 8 mM mersalyl turned out to be most effective even at high external substrate concentrations (up to 2–3 mM). Fig. 1(A) shows the time-course of the uptake of [^{14}C]malate into proteoliposomes. The transport was terminated at various times by adding PLP/mersalyl. The blank value (representing unspecific binding of [^{14}C]malate to the liposomes) was obtained by adding PLP/mersalyl 45 s before zero time. If the inhibitors were added at time zero and [^{14}C]malate 5–60 s afterwards (Fig. 1(B)), none of the measured values significantly differed from the blank value indicating that the transport had been completely stopped in less than 5 s. Thus, the combination of PLP and mersalyl can be used as an efficient stopping reagent for measuring the activity of 2-oxoglutarate/malate translocator in a reconstituted system.

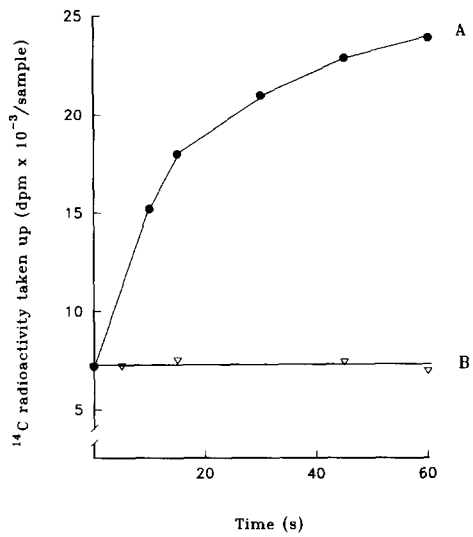

Fig. 1. Inhibition of the reconstituted 2-oxoglutarate/malate translocator by pyridoxal 5'-phosphate/mersalyl. Transport of [^{14}C]malate (1 mM) into the proteoliposomes was terminated at the indicated times by the addition of 45 mM PLP/8 mM mersalyl (A, ●). Alternatively, first PLP/mersalyl was added at time zero, and [^{14}C]malate afterwards at the times indicated (B, ▽). The samples were assayed as described in Materials and Methods.

TABLE I

Effect of different detergents on the reconstituted transport activity of the 2-oxoglutarate / malate translocator

Envelope membranes (1.5 mg/ml) were treated at 4°C with the indicated concentrations of the different detergents. In a preceding experiment, these particular detergent concentrations were shown to result in an almost complete solubilization of the envelope membranes. After 1 h, the solubilized membranes were reconstituted into liposomes and the transport activity of the 2-oxoglutarate/malate translocator was measured as described in Materials and Methods. The 100% exchange activity was 13 nmol/mg protein per min.

Detergent	CMC (% w/v)	Concentration of detergent used (% v/v)	Reconstituted transport activity (% of control)
none	–	–	(100)
Triton X-100	0.015	1	2
$C_{12}E_8$	0.005	1.5	<1
$C_{12}E_9$	0.006	3	<1
$C_{13}E_8$	0.007	3	<1
$C_{13}E_{10}$	0.008	3	<1
CHAPS	0.49	3	31
CHAPSO	0.50	3	19
MEGA 8	1.8	5	18
LDAO	0.035	1	2
n-Dodecyl maltoside	0.01	1	32

Purification of the chloroplast 2-oxoglutarate / malate translocator

Envelope membranes from spinach chloroplast were solubilized by n-dodecyl maltoside and the translocator protein was purified by chromatographic techniques. The different fractions were analysed for malate transport activity by functional reconstitution in liposomes (see above), and the polypeptide pattern by SDS-PAGE. The solubilized membrane proteins (1.5 mg/ml; 1% v/v n-dodecyl maltoside) were first applied to

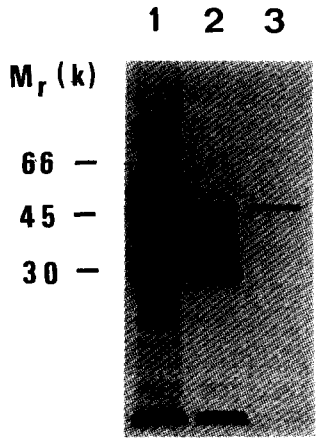

Fig. 2. Purification of the 2-oxoglutarate/malate translocator by chromatography on TSK DEAE-650 followed by chromatography on Superdex 200. Envelope membranes (1.5 mg/ml) were solubilized by the addition of 1% (v/v) n-dodecyl maltoside, passed over an TSK DEAE-650 column and then subjected to chromatography on Superdex 200 as described in Materials and Methods. Aliquots of the different fractions were analysed by SDS-PAGE and silver staining. Lane 1, envelope membrane proteins (5 μg); lane 2, pass-through fraction of the TSK DEAE-650 column (1 μg); lane 3, eluate of the Superdex column (0.08 μg). Relative molecular mass calibration is shown on the left.

chromatography on TSK DEAE-650, a weak anion-exchanger. The 2-oxoglutarate/malate transport activity appeared in the unbound fraction, together with the 29 kDa polypeptide, the triose phosphate/phosphate translocator (Fig. 2). As depicted in Table II, this step resulted in 3.3-fold increase in the reconstituted specific malate transport activity. Further purification of the translocator was accomplished by gel permeation chromatography on a Superdex 200 column which was connected to a FPLC system. The column buffer contained n-dodecyl maltoside just above its critical micellar concentration. This step resulted in an additional 10-fold increase in the reconstituted specific transport activity (Table II) and in a > 98% pure 2-oxoglutarate/malate translocator protein (Fig. 2). The apparent molecular mass of the highly purified translocator is about 45 000–46 000 as revealed by SDS-PAGE. Relative to the initial solubilized envelope membranes (which represent roughly 1% of the total chloroplast protein), the purification was 31-fold with an overall yield of 51% (Table II). The 2-oxoglutarate/malate translocator represents about 3% of the envelope membrane protein and thus about one fourth of the amount of the triose phosphate/phosphate translocator protein [2].

Kinetic constants and substrate specificities of the purified 2-oxoglutarate / malate translocator

The 2-oxoglutarate/malate translocator is specific for the transport of only 2-oxoglutarate, malate and also succinate, fumarate (and glutarate). In contrast, the glutamate/malate translocator transports all these dicarboxylates and, in addition, glutamate (and aspartate) [6,7]. Evidently, the transport of both 2-oxoglutarate and malate could occur on both translocators. In order to obtain basic kinetic data of the purified translocator protein, the dependence of the transport rate on the external [^{14}C]malate concentration was determined, in the absence or presence of unlabelled 2-oxoglutarate or glutamate, respectively. The internal substrate concentration was kept constant at 50 mM malate. The data from typical experiments are shown as double-reciprocal plots in Fig. 3. The apparent K_m and V_{max} values for the transport of malate were 0.21 mM and 0.65 μmol/mg per min, respectively. The K_m(app) value, corresponds to that determined in intact chloroplasts [7] and the V_{max} is of the same order of magnitude as measured for the reconstituted chloroplast triose phosphate/phosphate translocator [2,16]. 2-Oxoglutarate inhibited the transport of malate competitively (K_i(app) value, 0.95 mM). The inhibition by glutamate was rather weak (K_i(app) value, 15 mM) indicating that glutamate is only a very poor substrate for the isolated translocator protein.

To further assess the identity of the purified translocator protein, the dependence of the malate transport activity on internal liposomal counter-anions were investigated in proteoliposomes which had been preloaded with various dicarboxylates. Table III shows data on the substrate specificity of the purified translocator protein. The data indicate that labelled [^{14}C]

TABLE II

Purification of the chloroplast 2-oxoglutarate / malate translocator

The 2-oxoglutarate/malate translocator was purified as described in Materials and Methods. For experimental details see legend to Fig. 2. The data represent means of six separate purifications.

Purification step	Protein (mg)	Transport activity (nmol/min)	Specific transport activity (nmol/mg per min)	Purification factor	Yield (%)
Solubilized envelope membranes	1.54	13.0	8.5	(1)	(100)
TSK DEAE-650 eluate	0.37	10.5	28.1	3.3	80
Chromatography on Superdex 200	0.025	6.7	266	31	51

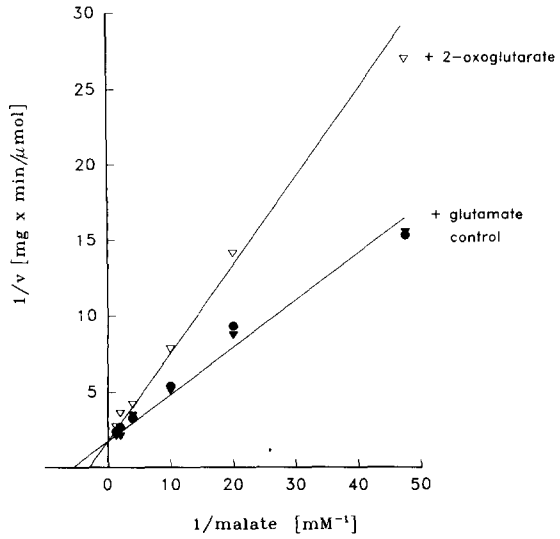

Fig. 3. Dependence of the rate of [^{14}C]malate uptake on the external malate concentration and inhibition by 2-oxoglutarate and glutamate. The purified 2-oxoglutarate/malate translocator (eluate of the Superdex column) was reconstituted into liposomes which had been preloaded with 50 mM malate. The external malate was removed by passage of the proteoliposomes over a Sephadex G-25 column and the transport of increasing concentrations of [^{14}C]malate (0.02–0.75 mM) was assayed without (●) or in the presence of 0.8 mM 2-oxoglutarate (▽) or 1 mM glutamate (▼), respectively. The K_m(app)(malate) value was calculated to be 0.21 mM and the K_i values for glutamate and 2-oxoglutarate as 15 mM and 0.95 mM, respectively (mean values of four experiments). For details see Materials and Methods.

malate can only be exchanged for internal malate, succinate, fumarate, glutarate and 2-oxoglutarate. In contrast, [^{14}C]malate does not significantly exchange against glutamate which observation is in line with the high K_i value for glutamate. Thus, the purified translocator protein discriminates between its physiological substrates and other dicarboxylates and displays the same substrate specificity as has been previously found for the native translocator in intact chloroplasts [5,7]. Taken together, these experiments provide definitive evidence for the identity of the purified protein as the 2-oxoglutarate/malate translocator.

TABLE III

Determination of the substrate specificity of the reconstituted 2-oxoglutarate / malate translocator

Envelope membrane proteins and the purified 2-oxoglutarate/malate translocator, respectively, were reconstituted into liposomes which had been preloaded with various dicarboxylates (50 mM) or potasssium gluconate as indicated. Transport activities were measured as described in Materials and Methods and are given as percentage of the activity measured for proteoliposomes which had been preloaded with malate. The 100% exchange activities (nmol/mg protein per min) were 53 (envelope membranes which had been reconstituted without the addition of detergent) and 263 (purified translocator protein). Mean values of three different experiments.

	Envelope membranes from spinach chloroplasts	Purified translocator protein
Liposomes loaded with:		
Malate	(100)	(100)
Succinate	68	63
Fumarate	49	65
2-Oxoglutarate	46	41
Glutamate	8	10
Aspartate	17	24
Potassium gluconate	< 1	< 1

Conclusion

In this paper we have described a high yield purification of a 45 kDa polypeptide of the chloroplast envelope membrane. Its identity as the 2-oxoglutarate/malate translocator was established by functional reconstitution of the transport activity in liposomes. Like the translocator of intact chloroplasts, the purified translocator protein is specific for 2-oxoglutarate, malate, succinate, fumarate and glutarate and only poorly accepts glutamate as a substrate. Besides the triose phosphate/phosphate translocator [2,17] it represents the second chloroplast transport system which has been identified as a component of the envelope membrane. It functionally resembles the mitochondrial oxoglutarate translocator which catalyzes, as part of the malate/aspartate shuttle, an electroneutral malate^{2-}/2-oxoglutarate^{2-} exchange. The mitochondrial carrier protein has been cloned and sequenced recently [18]. It would be interesting to know whether the chloroplast translocator shares some structural similarities with the mitochondrial carrier protein. Work to obtain sequence information on the chloroplast 2-oxoglutarate/malate translocator is now in progress.

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