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Reaction mechanism and asymmetric orientation of the reconstituted chloroplast phosphate translocator

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Proteoliposomes loaded with varying levels of internal substrates were used in bisubstrate initial velocity studies to gain insight into the transport mechanism of the reconstituted chloroplast phosphate translocator. The kinetic response to *trans* substrates clearly indicated that the one-to-one exchange mediated by this translocator proceeds via a ping-pong type, and excluded a sequential type of reaction mechanism. It is also shown that reconstitution of the protein leads to an unidirectional orientation of the protein within the liposomes being orientated rightside-out with respect to chloroplasts. Different transport affinities were observed on either side of the membrane and only the outward-facing transport site of the translocator is able to bind inhibitors i.e. pyridoxal 5'-phosphate (PLP) and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS).

Introduction

During photosynthetic CO_2 fixation, the fixed carbon (in form of triose phosphates and 3-phosphoglycerate) is exported from the chloroplasts into the cytosol where it is converted into other substances, e.g., sucrose and amino acids. The inorganic phosphate released during the biosynthetic reactions is shuttled back into the chloroplasts for the formation of ATP. This one-to-one exchange between inorganic phosphate and phosphorylated C3-compounds is mediated by the chloroplast phosphate translocator which represents the most abundant (nuclear-coded) polypeptide of the inner chloroplast envelope membrane (for review, see Ref. 1). In its functional state, the chloroplast phosphate translocator is made up of two identical subunits [2,3]. Recently, the cDNA sequences of both the spinach and the pea phosphate translocator proteins have been determined [4,5]. Each monomer of the phosphate translocator ($M_r \approx 36000$) contain 6–7 membrane-spanning stretches of 20–23 amino acid residues that all can form α-helical structures [5]. Based on the hydropathy plot and on additional information, a three-dimensional structure of the dimeric phosphate translocator protein has been proposed by means of computer-aided-molecular-design [6]. According to this model, the dimeric translocator exhibits an approximately C_2-symmetry and six α-helices of each monomer participate in forming a hydrophilic translocation channel through which the hydrophilic substrates could pass through [6].

In this paper, a reconstituted system [7,8] was employed to decide whether this transport protein uses a simultaneous or a ping-pong reaction, the two basic types of reaction mechanism [9–11]. Bisubstrate initial velocity studies showed that the antiport mediated by the chloroplast phosphate translocator proceeds via a ping-pong mechanism. In addition, it is demonstrated that reconstitution of the chloroplast phosphate translocator results in an unidirectional incorporation of the protein into the liposomal membrane, resulting in a rightside-out orientation of the protein with different substrate affinities on either side of the membrane.

Materials and Methods

Materials. [^{32}P]Phosphate was obtained from Amersham-Buchler (Braunschweig, Germany), 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS), pyridoxal 5'-phosphate (PLP) and soybean phospholipids from Sigma (München, Germany). Amberlite XAD-2 was purchased from Serva (Heidelberg, Germany), Sephadex G-25 from Pharmacia (Freiburg, Germany) and

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Abbreviations: 3-PGA, 3-phosphoglycerate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; PLP, pyridoxal 5'-phosphate; S_{int}, S_{ext}, internal or external substrate, respectively (3-PGA or phosphate).

hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories. All other reagents were of the highest purity available.

Methods. The isolation of the chloroplast phosphate translocator used in the reconstitution experiments was performed by chromatography on hydroxylapatite as described earlier [12]. Liposomes were prepared from acetone-washed soybean phospholipids (100 mg/ml) by sonication for 10 min at 4°C in a medium containing 100 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine (Tricine)-NaOH (pH 7.4), 20 mM NaH_2PO_4 (unless stated otherwise) and 30 mM potassium gluconate. The isolated phosphate translocator protein was added (lipid/detergent ratio ≫ 20) and incorporated into the liposomes by the freeze-thaw technique [13]. After thawing on ice, the proteoliposomes were sonicated (40% line voltage, 20% duty cycle) for 20 s.

The liposomes containing the reconstituted chloroplast phosphate translocator were seperated from the external medium by passing over a Sephadex G-25 column which had been equilibrated with 10 mM Tricine-NaOH (pH 7.4), 100 mM sodium gluconate and 50 mM potassium gluconate. The eluted liposomes were used for transport studies. Transport was initiated by the addition of 10 μl [^{32}P]phosphate or 3-phospho[^{14}C]glycerate [8], respectively, (total volume, 210 μl) and terminated after 20 s by the addition of 40 mM PLP and 1.5 mM DIDS (final concentrations). In control samples, PLP/DIDS 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 [7] preequilibrated with 200 mM sodium acetate. The liposomes were eluted with 1.3 ml of 200 mM

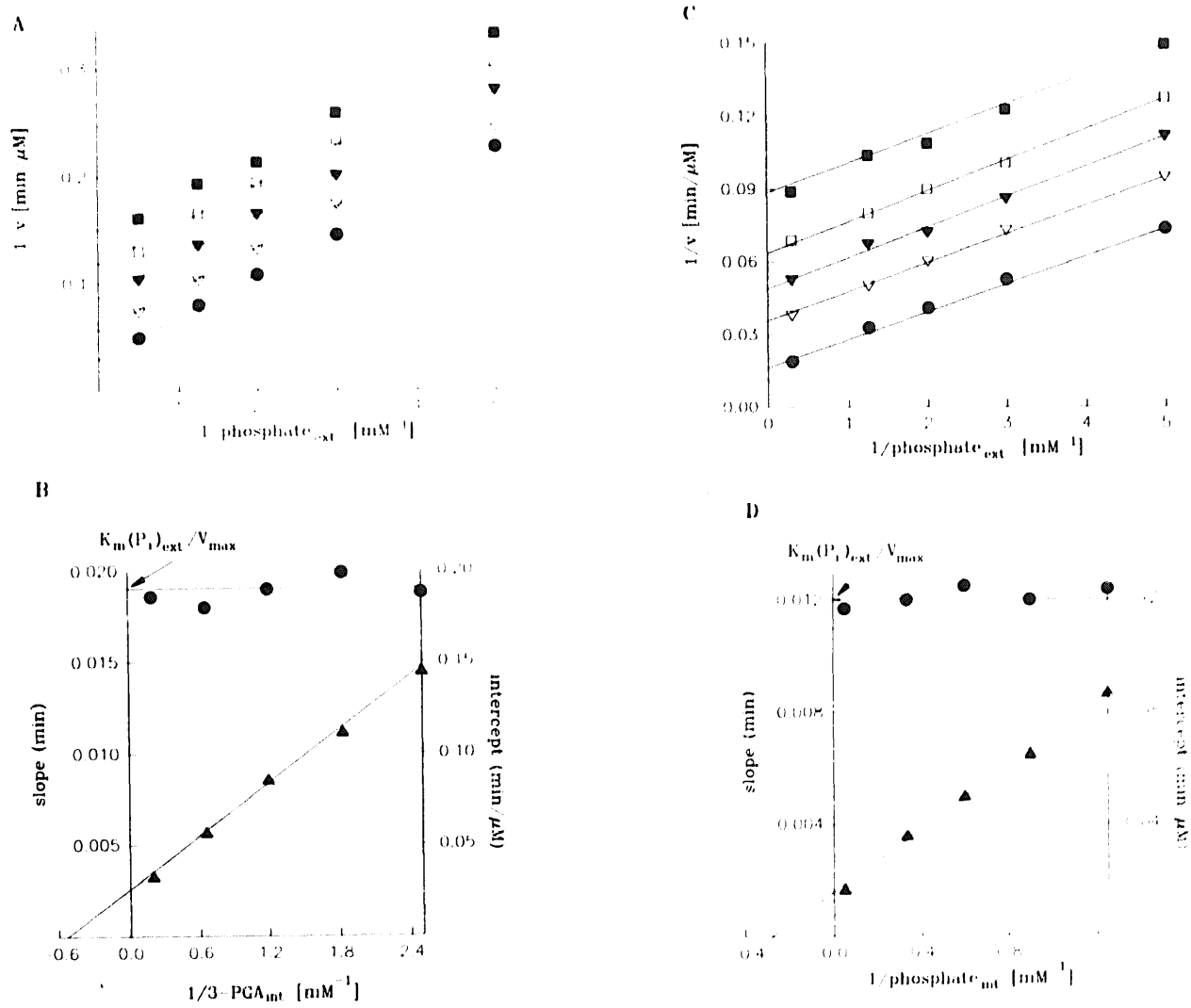

Fig. 1. Bisubstrate analysis of the hetero-exchange (A and B) and the homo-exchange reaction (C and D) mediated by the chloroplast phosphate translocator. (A and C) Lineweaver-Burk plots showing the dependence of external [^{32}P]phosphate uptake on internal 3-PGA (A) or on internal phosphate (C), respectively. The concentrations of internal countersubstrates were: (A) 0.4 (■), 0.55 (□), 0.83 (▼), 1.5 (▽) and 5.0 (●) mM 3-PGA; (C) 0.8 (■), 1.1 (□), 1.67 (▼), 3.0 (▽) and 20.0 (●) mM phosphate. Antiport velocities were calculated from the uptake of [^{32}P]phosphate within 20 s. (B and D) Secondary (slope (●) and intercept (△)) plots of the primary plots A and C, respectively, allowing the calculation of 'concentration-independent' K_m values for phosphate$_{ext}$ (0.78 and 0.73 mM, respectively), 3-PGA$_{int}$ (2.0 mM) and phosphate$_{int}$ (3.44 mM) (see text). V_{max} values were 41 μM/min (hetero-exchange reaction) and 60 μM/min (homo-exchange reaction). P_i, inorganic phosphate. Mean values of four different experiments.

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.

Alternatively, incorporation of the Triton X-100 solubilized translocator protein into the liposomes was achieved by hydrophobic chromatography on amberlite beads according to Ref. 14. Mixed micelles of hydroxylapatite-purified translocator protein, phospholipids and Triton X-100 were passed over columns of amberlite XAD-2. The relevant values are as follows: 16 mg phospholipid/ml, 0.7 mg Triton X-100/mg phospholipid, 0.01 mg hydroxylapatite-purified translocator protein/mg phospholipid, 7 mg Triton X-100/g amberlite XAD-2 'moist beads', 100 mM Tricine (pH 7.4) and 0.5 M sucrose. In addition, the mixture contains varying concentrations of internal substrates (phosphate and 3-PGA) as given in the text. By passing the mixture 15 times over the amberlite column, detergent is removed under the formation of functionally active proteoliposomes. External medium was subsequently removed by chromatography on Sephadex G-25, which had been equilibrated with 0.5 M sucrose, 0.1 M sodium gluconate and 20 mM Tricine-NaOH (pH 7.4). Transport was assayed as described above; external radioactivity was removed by passing the samples over Dowex AG-1X8 preequilibrated with 0.5 M sucrose and 0.11 M sodium acetate. Transport activities are always expressed as volume activities (mol/l vesicles per min), since protein determinations in liposomes are very unreliable.

Other methods. Protein was determined by the Lowry method modified for the presence of Triton X-100 [15].

Results

The question of the transport mechanism of the chloroplast phosphate translocator was addressed by analysing antiport kinetics in a reconstituted system. The kinetic response to *trans* substrates was used to decide whether the reconstituted chloroplast phosphate translocator uses a ping-pong or a sequential reaction, the two basic types of bisubstrate mechanisms: for ping-pong mechanisms the ratio of $K_m(app)/V_{max}(app)$ is constant as internal substrate rises; sequential mechanism show a decrease in the ratio [9–11]. The antiport velocities were determined as [^{32}P]phosphate uptake within 20 s, as a function of substrate concentrations in both the external and the internal compartment. Liposomes were preloaded either with 3-phosphoglycerate (phosphate/3-phosphoglycerate (3-PGA): hetero-exchange reaction) or with phosphate (phosphate/phosphate: homo-exchange reaction). In the hetero-exchange reaction, internal 3-PGA was varied from 0.4 to 5.0 mM, and in the homo-exchange reaction, internal phosphate was varied from from 0.8 to 20 mM. External [^{32}P]phosphate was varied from 0.2 to 2 mM in both experiments. Figs. 1 A and B show the results of a bireactant initial velocity study of the hetero-exchange reaction, and Figs. 1 C and D those of the homoexchange reaction. When the kinetic data were analysed in Lineweaver-Burk plots (Fig. 1, A and C), straight lines with almost identical slopes were obtained for both the hetero- and the homo-exchange reaction. Secondary plots (slopes of the primary curves vs. reciprocal concentrations of the non-varied substrates, Fig. 1, B and D) indicate a

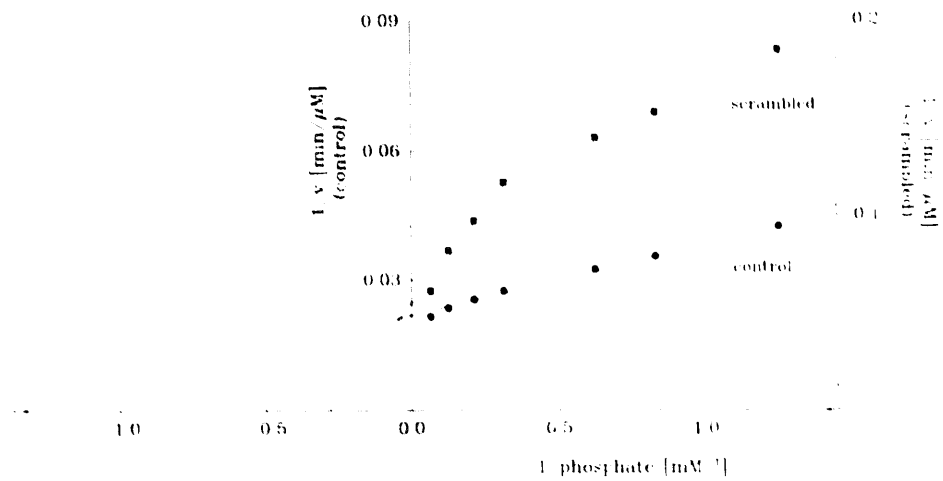

Fig. 2. Effect of scrambling of the proteoliposomes on the concentration dependence of the uptake of [^{32}P]phosphate into reconstituted liposomes (Lineweaver-Burk plot). The liposomes had been preloaded with 100 mM sodium phosphate (pH 7.4) and 20 mM sodium gluconate. External phosphate was varied from 0.8 to 16 mM. Scrambled liposomes (■) were subjected to five freeze-thaw/sonication cycles, whereas control liposomes (●) were freezed/thawed and sonicated only once. Due to the prolonged sonication of the scrambled proteoliposomes, V_{max} decreased by a factor of about 4 from 45 μM/min (control) to 11 μM/min. Mean values of three different experiments.

constant ratio of K_m(app)/V_{max}(app) as internal substrate rises. Thus, changing the substrate concentrations in one compartment has no effect on the transport affinity of the substrate in the opposite compartment. These results clearly exclude a sequential type of reaction mechanism. Instead they are in favor of a ping-pong mechanism which assumes the exposure of the substrate binding site to only one side of the membrane at the same time.

Orientation of the reconstituted phosphate translocator

The 'concentration-independent' K_m value for the external substrate phosphate (i.e. the transport affinity at infinite concentration of the non-varied substrate) can be obtained from secondary plots (Figs. 1 C and D) and was determined to be about 0.75 mM. This value is almost identical with that determined at finite substrate concentrations (not shown) and is obviously lower than that obtained for internal phosphate (K_m (phosphate$_{int}$): 3.44 mM; Fig. 2 D). A similar difference between the substrate affinities at the two different sites of the phosphate translocator was observed for 3-PGA: K_m(app) (3-PGA$_{ext}$): 0.33 mM (not shown), K_m(app) (3-PGA$_{int}$): 2.0 mM (Fig. 1 B). These observations might indicate that the translocator possesses different substrate affinities on either membrane side. This would mean that the functionally active translocator protein is inserted into the proteoliposomes with a definite transmembrane orientation and possesses a lower affinity towards substrates on the internal membrane side compared to the external side. To further assess this issue, K_m(app) (phosphate) values were determined using liposomes which had been subjected to several freeze-thaw/sonication cycles. This procedure should result in scrambling of the translocator orientation within the proteoliposomes and thus should make the internal substrate binding site accessible to external substrate. The results of such experiments are shown in Fig. 2. In control proteoliposomes which had been subjected to only one freeze-thaw/sonication cycle, only one K_m(app) value (0.74 mM) could be detected. In scrambled proteoliposomes, this K_m(app) value was also found (0.73 mM) but, in addition, a second K_m(app) value (3.49 mM) was detected. The first one represents that exposed to the external compartment in non-scrambled liposomes, whereas the second one obviously corresponds to the affinity as determined at the internal membrane surface and which could only be recognized in scrambled proteoliposomes. These results indicate that (i) the translocator is indeed mainly asymmetrically orientated in liposomes and (ii) the substrate affinities of the translocator towards the substrates phosphate and 3-PGA differ on either side of the membrane by a factor of about 5.

To further elucidate the orientation of the translocator in the membrane, the reactivity of both the external and the internal membrane side of the reconstituted phosphate translocator towards inhibitors was tested. By using 4,4'-diisothiocyano(dihydro)stilbene-2,2'-disulfonate (DIDS or H_2DIDS, respectively) and pyridoxal 5'-phosphate (PLP) as inhibitors and labelling reagents, we have shown earlier that the chloroplast phosphate translocator possesses only one accessible substrate-binding site per functional dimer. This substrate binding site can be specifically blocked by both inhibitors competing for the same site [16,17]. For experimental reasons the following experiments were performed using PLP as a membrane-impermeable inhibitor. PLP reacts covalently but, in contrast to DIDS, reversibly [12,16]. Thus, if the reconstituted phosphate translocator has been exposed to PLP on both sides of the membrane, the inhibition of the translocator activity at the external membrane side can specifically be relieved by the addition of amino-group containing substances such as lysine and/or BSA. It may be noted that neither lysine nor BSA have access to the internal liposomal compartment (not shown).

Liposomes were prepared by sonication without (control-liposomes) or in the presence of PLP (PLP-liposomes). The solubilized phosphate translocator was added and inserted into the liposomal membrane by the freeze/thaw-sonication procedure [7,13]. PLP was added externally to the control-liposomes, and both types of proteoliposomes were allowed to react with PLP (i.e., only at the external side, control-proteoliposomes; at both sides of the membrane, PLP-proteoliposomes). Subsequently, both types of proteo-

TABLE I

Inhibition of the reconstituted chloroplast phosphate translocator by pyridoxal 5'-phosphate (PLP) on either side of the membrane

Liposomes were prepared without or in the presence of 0.15 mM PLP (control-liposomes and PLP-liposomes, respectively). The solubilized phosphate translocator protein was added and incorporated into the liposomal membrane by the freeze-thaw/sonication procedure [13]. Control liposomes were then allowed to react with PLP (0.15 mM for 30 min at 4°C) and were subsequently incubated with or without 40 mM lysine plus 2 mg BSA/ml. One half of the PLP-liposomes also received lysine/BSA. After 1 h, all samples were treated with 2 mM KBH_4 for 10 min. After removal of the external medium by chromatography on Sephadex G-25, the amount of [^{32}P]phosphate (0.5 mM) taken up within 20 s by the liposomes was measured. The 100% control value corresponds to 12 μM/min. Mean values of four different experiments.

Additions	Reconstituted transport activity (%)
No addition	= 100
PLP_{ext}	48.1
PLP_{ext}; subsequently removed by lysine/BSA	86.7
PLP_{int} + PLP_{ext}	44.8
PLP_{int} + PLP_{ext} PLP_{ext} subsequently removed by lysine/BSA	80.5

liposomes were treated with lysine to hydrolyze the Schiff base formed between PLP and the phosphate translocator, and to restore (most of) the PLP-inhibited activity at the external membrane side. Transport experiments (Table I) revealed that external PLP led to a substantial inhibition of phosphate uptake into control-proteoliposomes. Treatment of these liposomes with lysine/BSA restored, as expected, most of the PLP-inhibited phosphate transport activity. However, no substantial inhibition of transport activity was observed if PLP was allowed to react only from the internal membrane side, i.e., in PLP-proteoliposomes where the external PLP had subsequently been removed. Likewise, the extent of transport inhibition did not increase under conditions when PLP was present at both sides of the membrane as compared to only the external side. These results clearly demonstrate that the single PLP-binding site contained in the functional dimeric translocator is almost exclusively facing the external side of the membrane and is not accessible from the internal membrane side. Thus, reconstitution of the phosphate translocator in proteoliposomes leads to a virtually completely unidirectional incorporation of the protein into the membranes with the inhibitor binding site orientated to the outside. Most probably, the reconstituted phosphate translocator is preferentially incorporated into the liposomal membrane in the same direction as in chloroplasts (i.e., in a right-side-out orientation) since both the reconstituted phosphate transport activity and the phosphate transport in intact chloroplasts are inhibited by increasing concentrations of externally added PLP and DIDS to about the same extent (Fig. 3). The preferential rightside-out orientation of the reconstituted phosphate translocator is also supported by the findings that the K_m values for both phosphate$_{ext}$ and 3-PGA$_{ext}$ are in the same order of magnitude as the values measured at the cytosolic side of intact chloroplasts (0.2–0.5 mM) [18].

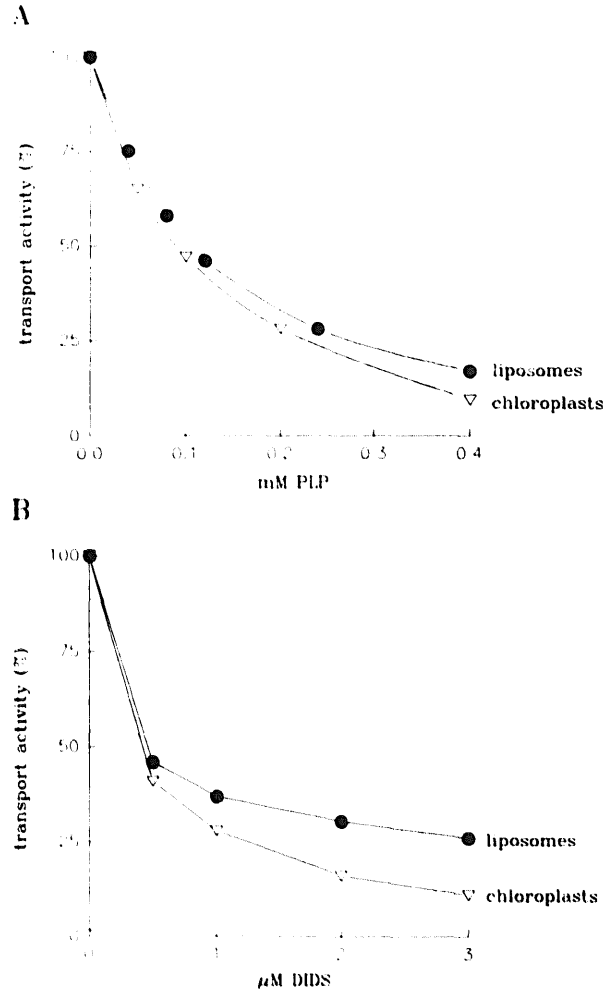

Fig. 3. Influence of PLP and DIDS on the transport of [^{32}P]phosphate into isolated chloroplasts and reconstituted proteoliposomes. Liposomes contained 20 mM internal phosphate. After removal of the external medium by chromatography on Sephadex G-25, the proteoliposomes were incubated for 20 min at 4°C with increasing concentrations of PLP and DIDS as indicated. The reactions were terminated by the addition of 2 mM KBH_4 and 10 mM lysine, respectively. After additional 10 min, uptake of [^{32}P]phosphate (0.5 mM) was measured at 20°C. The control rate was 15 μM/min. Intact spinach chloroplasts were prepared as in Ref. 27. Transport of [^{32}P]phosphate (0.5 mM) using the silicone-oil-layer centrifugation system was measured according to Ref. 28 in a medium containing 0.33 M sorbitol and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-KOH (pH 7.5). The chloroplasts (0.2 mg chlorophyll/ml) were incubated for 20 min at 4°C with the indicated concentrations of inhibitors before the transport was started by the addition of [^{32}P]phosphate (0.5 mM). After 12 s, uptake of [^{32}P]phosphate was terminated by centrifugation of the chloroplasts through the silicone-oil into perchloric acid [28]. The control rate was 32 μmol(mg chlorophyll)$^{-1}$ h^{-1}. Effect of increasing concentrations of PLP (A) and DIDS (B), respectively, on the transport reconstituted in liposomes (●) or in intact spinach chloroplasts (▽), respectively. Mean values of three different experiments.

Discussion

Bisubstrate reactions can be divided into ping-pong and sequential mechanism. The sequential type of transport mechanism involves the binding of two substrate molecules at the same time thus leading to the formation of a ternary complex with the translocator protein, before translocation takes place. In a ping-pong transport cycle, the substrate binding site, being exposed on only one site of the membrane at the same time, is alternating between opposing membrane surfaces, i.e., the first transported substrate has to leave the transport site before the second one is bound.

For antiport translocators, both types of reaction mechanism have been found: a sequential type for several mitochondrial transport systems such as the carriers for aspartate/glutamate [19], oxoglutarate [20] and dicarboxylates (Krämer, R., personal communication) and a ping-pong type for the anion transport system of the human erythrocyte membrane [10,11]. Transport steps involved in both mechanism are expected to be associated with drastic changes of the

translocator conformation. Indeed, by measuring the rotational mobility of the eosin-5-isothiocyanate labelled reconstituted chloroplast phosphate translocator, changes in the velocity of the protein rotation have been measured when the translocator was active in transport [3].

We have used proteoliposomes loaded with varying levels of substrates to gain insight into the kinetic mechanism of the phosphate translocator. The reconstituted system has several advantages as compared to experiments in intact chloroplasts because both the internal and the external compartments are easily accessible to experimental variations. Antiport velocities were determined as a function of substrate concentrations in both the external and internal compartment. For both the hetero-exchange and the homo-exchange reaction, the resulting kinetic pattern show that the ratio $K_m(app)/V_{max}(app)$ (i.e., the slopes of the primary curves in Fig. 1, A and C) is not influenced by the second (non-varied) substrate. These data exclude a sequential type of reaction mechanism and are consistent with a ping-pong mechanism. For an antiport system, this model assumes that the single transport site is alternatively facing either membrane side, thereby translocating substrates in opposite directions. In case of the chloroplast phosphate translocator, the common transport site, however, cannot be interpreted in terms of a certain ensemble of amino acids (or even a single amino acid residue) which is equally well accessible to each side of the membrane. The data presented in this study rather suggest an asymmetry in the structure of the binding site on either membrane site. Only the outward facing transport site appears to be accessible to inhibitors of the phosphate translocator, namely PLP and also DIDS which most probably reacts with the same site of the translocator protein, presumably with Lys-273 (spinach translocator [5]) in the transmembrane helix 6 [6,17]. Thus, PLP and DIDS can be regarded as specific inhibitors for the external (cytosolic) side of the translocator. The structural asymmetry of the binding site obviously results also in a about 5-fold lower affinity towards substrates on the internal membrane side compared to the external side. This can be explained by assuming that substrates bind to the external side of the translocator with a different interphase than when leaving from the opposite side. Both the side-specific inhibition of the transport process by PLP and the different substrate affinities measured on either membrane side are consistent with an asymmetric and unidirectional orientation of the reconstituted and functionally active phosphate translocator in the liposomal membrane. A random orientation of the translocator would have made the translocator equally well accessible towards its inhibitors and, in addition, the side-specific K_m values for both phosphate and 3-PGA should have been detected on both membrane sides. This is obviously not the case: the inhibitor site can only be occupied when facing the external membrane side and the transport affinities turned out to be higher at the external membrane side for both phosphate and 3-PGA (0.74 and 0.33 mM, respectively) as compared to the internal side (3.44 and 2.0 mM, respectively). Remarkably, the lower K_m value at the internal surface side became only visible after randomizaton of the translocator orientation.

Both in the reconstituted system and in chloroplasts, the inhibitor binding sites for the impermeable and covalently reacting inhibitors are about equally well and almost exclusively accessible from the external phase (i.e., the cytosolic side, in case of chloroplasts). In addition, the lower K_m value measured in the external liposomal compartment resembles that of the cytosolic side in intact chloroplasts [18]. In line with this are observations that phosphate transport dependent reactions of CO_2 fixation in chloroplasts can specifically be inhibited by external PLP and DIDS [21,22]. These data strongly support that the reconstituted translocator is orientated rightside-out as compared to its orientation in chloroplasts. A preferential (rightside-out) orientation of reconstituted transport systems is not unusual. It has been reported for several mitochondrial carriers e.g. the mitochondrial aspartate/glutamate carrier [23,24], the oxoglutarate carrier [20] and the uncoupling protein from hamster kidney mitochondria [25] as well as for the anion transport system of the human erythrocyte membrane [26].

It is suggested that, in combination with the 'molecular modelling' of the chloroplast phosphate translocator protein [6], the presented data will provide new insights into structure-function relationships of this translocator protein.

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