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Solubilization, Partial Purification, and Reconstitution of the Glycolate/Glycerate Transporter from Chloroplast Inner Envelope Membranes[1]

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ABSTRACT

The glycolate/glycerate transporter of spinach (*Spinacia oleracea* L.) chloroplast inner envelope membranes was solubilized by treatment of the membranes with sodium cholate. Mixtures of the cholate extracts and soy asolectin were subjected to gel filtration to remove the detergent. The reconstituted vesicles were frozen, thawed, and sonicated in a buffer that contained 10 millimolar D-glycerate and, usually, [^{3}H]sucrose as an internal space indicator. The dilution of the vesicles into a medium that contained 0.4 millimolar [^{14}C]D-glycerate resulted in a rapid accumulation of labeled glycerate, followed by a much slower loss of [^{14}C]D-glycerate from the vesicles. This behavior is characteristic of counterflow. The accumulation of [^{14}C]D-glycerate was strongly inhibited by $HgCl_2$, which blocks glycolate/glycerate transport in intact chloroplasts. In the absence of proton ionophores, the extent of [^{14}C]glycolate accumulation under similar conditions was much greater than that of [^{14}C]D-glycerate. External glycolate inhibited D-glycerate counterflow and external D-glycerate inhibited glycolate counterflow. The external pH dependence of the efflux of [^{14}C]D-glycerate accumulated in vesicles by counterflow and its inhibition by external L-mandelate are characteristics displayed by glycolate transport in intact chloroplasts. Partial purification of the transporter was achieved by glycerol gradient centrifugation. The solubilized glycolate and glycerate counterflow activities, assayed by reconstitution into vesicles, were found to sediment similarly.

The oxygenation of ribulose-1,5-bisphosphate, catalyzed by Rubisco, produces phosphoglycolate and PGA,[4] a substrate of the photosynthetic carbon reduction cycle. After phosphoglycolate is produced, its two carbons are committed to the "C2" or photorespiratory carbon cycle (see ref. 19 for review). Phosphoglycolate is hydrolyzed to glycolate and Pi in the chloroplast stroma, but glycolate cannot be metabolized in the chloroplast. By way of reactions that occur in the peroxisomes and mitochondria, D-glycerate and CO_2 are produced from two molecules of glycolate. Phosphorylation of D-glycerate in the chloroplast yields PGA, thus reclaiming three of the four carbons lost to the photosynthetic carbon reduction cycle when the two glycolates were formed. Because the reactions of the photorespiratory carbon cycle occur in three different organelles, membrane transport events are essential features of this pathway. In the case of the chloroplast, glycolate must move from the stroma to the cytoplasm across the inner envelope membrane and D-glycerate must go in the opposite direction. Our research has been concerned with these two transport activities. Experiments with intact chloroplasts have shown the transport of glycolate (8, 10) and D-glycerate (12, 22, 23) to be mediated processes. We have found that glycolate and D-glycerate are competitive inhibitors of one another's transport, that each causes counterflow of the other, and that each affords protection to the other's transport against the inhibition caused by an NEM pretreatment of the chloroplasts (11, 12). These results, which were obtained with intact pea chloroplasts, indicated that a single, carrier-type transporter is responsible for the movement of both glycolate and D-glycerate across the chloroplast inner envelope. The present reconstitution study, in which spinach inner envelopes were used as the starting material, provides further confirmation of the existence of a single glycolate/glycerate transporter.

Previous work with intact chloroplasts has defined a number of kinetic and substrate specificity characteristics of the transporter (10–13). Because much of the present work is concerned with confirming these characteristics for the reconstituted system, we will review them briefly here. Unlike, for example, the chloroplast phosphate translocator (2), the glycolate/glycerate transporter does not catalyze a strictly coupled substrate exchange. Unidirectional influx or efflux occurs as a proton symport (proton binds first) or hydroxide antiport (10, 12, 14). Because two glycolates leave the chloroplast for each D-glycerate that enters it during photorespiration, the proton symport activity can be viewed as a means of balancing the charge in the overall transport process. The net efflux of one proton per two glycolates can also be viewed as a means of moving protons produced in the Rubisco oxygenase reaction out of the stroma and into the cytoplasm. It has been suggested that mitochondrial glycine and serine translocators might function as H^+ symporters and thus catalyze the net uptake of one proton per two glycines (derived from two glycolates), (5). There is evidence that a single transporter may catalyze glycine and serine transport in plant mitochon-

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[4] Abbreviations: PGA, D-3-phosphoglycerate; NEM, *N*-ethylmaleimide; CCCP, carbonylcyanide *m*-chlorophenol hydrazone.

dria (27). One H^+ per two glycines is consumed by mitochondrial glycine decarboxylation. The proton symport activity of the glycolate/glycerate transporter could thus be considered part of a system for shuttling protons from the chloroplasts to the mitochondria. A major kinetic consequence of the glycolate/glycerate transporter's mechanism is that the apparent V_{max} is strongly dependent on the pH on the opposite side of the membrane (*trans* pH). Increasing trans pH stimulates the rate of transport (10). Compounds that are good substrates for the carrier (*i.e.* those found to be competitive inhibitors of glycolate transport and found to cause glycolate counterflow) thus far seem to be restricted to two or three carbon 2-hydroxymonocarboxylates (11, 13). These include glycolate, glyoxylate (two hydroxyls on the number 2 carbon when hydrated), glycerate, and lactate. In the case of the chiral, three carbon molecules, the D-isomers are better substrates. Of physiological note in these results is the fact that glycolate and D-glycerate stimulate one another's transport from the opposite side of the membrane.

The transporter can be inhibited by a number of sulfhydryl-modifying reagents including NEM (8, 10), *p*-chloromercuribenzoate (12), and $HgCl_2$ (9, 10). The inhibition by $HgCl_2$ is rapid, and this reagent has been useful as an "inhibitor stop" in transporter assays with intact chloroplasts (10). The covalent modification and inhibition by NEM can be prevented if a transporter substrate is present during the treatment (8, 12, 13). A 35-kD polypeptide of pea chloroplast inner envelopes has been tentatively identified, by a double isotope differential labeling technique ([^{3}H]- or [^{14}C]NEM in the presence or absence of glycolate, D-glycerate, or L-mandelate), as (at least) a component of the transporter (13).

In the present paper we report the conditions for solubilization, reconstitution into liposomes, and assay of the glycolate/glycerate transporter from spinach chloroplast inner envelopes. A partial purification has been achieved by glycerol gradient centrifugation.

MATERIALS AND METHODS

Materials

[^{14}C]D-Glycerate was enzymatically synthesized and purified as described previously (12). [^{14}C]Glycolate was purchased from ICN and [^{3}H]sucrose was from New England Nuclear. Asolectin was from Associated Concentrates (Woodbury, NY) and was purified according to Sone *et al.* (24) before use. Cholic acid (Sigma) was purified according to the method of Kagawa and Racker (15) before conversion to sodium cholate by titration with NaOH. Nitrocellulose filters were from Schleicher and Schuell. All other reagents were the highest grades available commercially.

Inner Envelope Membrane Preparation

Inner envelope membrane vesicles were prepared from intact pea (*Pisum sativum* L.) or spinach (*Spinacia oleracea* L.) chloroplasts essentially as described previously (16), using sucrose step gradients (14, 16). Fractions enriched in inner envelope membranes were removed from the sucrose gradients, pooled, mixed, and stored at either −70°C or under liquid N_2. Preparations could be stored for months without loss of activity.

Detergent Extraction and Reconstitution

Frozen inner envelope membrane fractions were thawed slowly on ice and diluted at least 10-fold with 10 mM Tricine-NaOH (pH 8.0), 2 mM DTT at 4°C. The mixtures were centrifuged 1 h at 50,000 rpm in a Beckman Ti-70 rotor. The pellets were resuspended in 10 mM Tricine-NaOH (pH 8.0), 2 mM DTT to give a protein concentration of 1.25 mg/mL. Sonicated asolectin in the same medium and sodium cholate were added to give final concentrations of 0.2 and 1%, respectively.

The final protein concentration was 1 mg/mL. After 10 min at 4°C, the mixtures were centrifuged in a Beckman Airfuge for 7 min at 90,000*g*. The supernatant fluids were withdrawn.

To prepare reconstituted vesicles, 240 μL of the supernatant were mixed with 108 μL of sonicated asolectin (20 mg/mL), and the mixture was applied to a Sephadex G-50 column (0.8 × 12 cm) at 4°C. The column was equilibrated with 10 mM Hepes-NaOH, pH 8.0. The excluded fraction following the void volume was collected (approximately 0.5 mL) and used for assays.

Transport Assays

The reconstituted vesicles were supplemented with Hepes-NaOH (pH 8.0) to bring the final concentration to 30 mM. D-Glycerate, sodium salt, was added to give a final concentration of 10 mM. [^{3}H]Sucrose (20 μCi, 230 μM) was added to bring the final volume to 550 μL. The [^{3}H]sucrose was omitted for the reconstitutions of the glycerol gradient fractions. The samples in 13-mm Pyrex tubes were frozen in dry ice-ethanol and thawed at room temperature. They were then sonicated for a total of 15 s in three 5-s bursts in a bath-type sonicator (Laboratory Supplies Co., New York). The samples were placed in a −20°C freezer for at least 1 h or as long as overnight. They were thawed at room temperature and sonicated again as described above.

Aliquots (15 μL) of the reconstituted vesicles were diluted to 0.5 mL with a solution that contained 10 mM NaCl, 30 mM buffer (Hepes-NaOH for pH 8.0 or Mops-NaOH for pH 7.0), and either 0.1 mM [^{14}C]D-glycerate (29 μCi/mmol) or 0.1 mM [^{14}C]-glycolate (29 μCi/mmol). The carryover of D-glycerate from the vesicle suspension was 0.3 mM. All buffers were at ice temperature.

At various times, entire samples were applied with a transfer pipet to the center of 25-mm nitrocellulose filters (0.45 or 0.2 μm) and placed in a Millipore filtration manifold under a 25 mm Hg vacuum. The filters were immediately washed with 5 mL of the buffer used during the incubations, except that substrates were not added and 5 mM $HgCl_2$ was present. Filters were placed in scintillation vials and 0.5 mL of 0.1% Triton X-100 added. A xylene/Triton X-100-based scintillation fluid (15 mL) was then added after 30 min and ^{3}H and ^{14}C were determined by a Beckman LS 7500 scintillation counter. The trapping of [^{3}H]sucrose on the filter was a measure of vesicle internal volume. For 15 μL of vesicles the internal space

averaged approximately 0.1 μL. Filter blanks, consisting of filtration of the same incubation media lacking vesicles and washing of the filters, were routinely prepared. Essentially no [^{3}H]sucrose was trapped on the filters. Only 62 ± 24 (n = 10) and 208 ± 103 (n = 3) cpm of [^{14}C]D-glycerate and [^{14}C]glycolate, respectively, were trapped. For a [^{14}C]glycerate or [^{14}C]glycolate internal to external concentration ratio of 2.0, the filters contained approximately 600 cpm above background.

For incubation times of 20 s or less, a modification of the rapid filtration assay developed by Newman *et al.* (18) for assay of counterflow in liposomes reconstituted with the *Escherichia coli* lactose carrier was used. Either 30 or 50 μL of vesicles were applied to the center of a 25-mm nitrocellulose filter, and the vacuum pump was switched on briefly to remove the liquid. Buffer (0.5 mL) of the same composition used for longer incubations (0.1 mM [^{14}C]D-glycerate or glycolate; 0.3 mM unlabeled D-glycerate) was applied dropwise to the center of the filter, at a sufficiently rapid rate to keep the filter wet. At the end of the incubation, the filters were washed, as usual, under full vacuum.

Preliminary experiments were performed to determine the effectiveness of $HgCl_2$ as an inhibitor of efflux during the washing of the filters. Vesicles were loaded with 10 mM D-glycerate and allowed to accumulate [^{14}C]D-glycerate as usual for 30 s at pH 7.0. After application to the filters, the filters were washed with ice-cold medium containing various $HgCl_2$ concentrations. Retention of [^{14}C]D-glycerate by the vesicles when washed with 5 mL of a medium at pH 8.0 for 11 s was maximal at $HgCl_2$ concentrations of 2 mM or greater. When 5 mM $HgCl_2$ was present in the wash medium, the same amount of [^{14}C]D-glycerate was retained when the volume of the wash medium was increased from 5 to 15 mL (11 to 33 s). When the pH of the wash medium was 7.0, the retention of [^{14}C]D-glycerate during a 5-mL wash was the same in the presence or absence of $HgCl_2$ (see Fig. 6A). The retention after washing at pH 7.0 was the same as that of pH 8.0 in the presence of a saturating concentration of $HgCl_2$. *Trans* inhibition of the transporter by protons (10) may account for the slow efflux of D-glycerate at pH 7.0. Unless otherwise noted, filters were washed with 5 mL of a medium that contained 5 mM $HgCl_2$ at the same pH as the medium in which uptake took place.

Glycerol Density Gradient Centrifugation

Continuous 10 to 35% (w/v) glycerol density gradients (10.5 mL) were formed. The gradients also contained 10 mM Tricine-NaOH (pH 8.0), 2 mM DTT, 0.1% asolectin, and 0.4% cholate. Cholate extracts from 2.1 mg of spinach inner envelope vesicle protein, concentrated to 0.5 mL with a 10-kD Centricon filter (Amicon), were applied to the gradients. The tubes were centrifuged at 4°C for 20 h at 192,000g. Fractions of 350 μL were collected from the top. Fraction 32 was prepared by resuspending a small pellet of the bottom of the tube in 350 μL of 35% glycerol in the same buffer as the gradient fractions. Sonicated asolectin (95 μL of 20 mg/mL) was added to each fraction and gel filtration carried out as described above.

Other Procedures

Protein was determined according to the method of Markwell *et al.* (17) with corrections for a contribution of asolectin. Bovine serum albumin was the standard. SDS-PAGE (11% acrylamide) was carried out according to the procedure of Fling and Gregerson (3). Gels were stained with either Coomassie blue or silver (26).

RESULTS

Development of Reconstitution and Transport Assay Procedures

The detergents Triton X-100, octyl glucoside, and sodium cholate were tested for their ability to solubilize proteins from pea inner envelope preparations (Fig. 1). An advantage held by both sodium cholate and octyl glucoside over Triton X-100, due to their high critical micellar concentrations, is the ease with which they can be removed as part of a reconstitution procedure. In preliminary solubilization experiments (K. T. Howitz, unpublished findings), cholate appeared to solubilize efficiently a polypeptide tentatively identified as (at least part of) the glycolate/glycerate carrier of pea inner envelopes (13) and to do so more selectively than octyl glucoside. We therefore concentrated our efforts on cholate in developing reconstitution and transport assay procedures. Proteoliposomes were produced by addition of soy asolectin to cholate solubilized inner envelope proteins, followed by gel filtration to remove cholate. The vesicles were exposed to two cycles of freeze/thaw and sonication. These reconstituted vesicles were found to contain an NEM and L-mandelate-sensitive glycolate efflux activity (13). The assay procedure consisted of preloading the proteoliposomes with [^{14}C]glycolate during the freeze/thaw and sonication steps of the reconstitution procedure and allowing efflux to occur as the vesicles were separated from the external medium on a "centrifuge column" (20) of Seph-

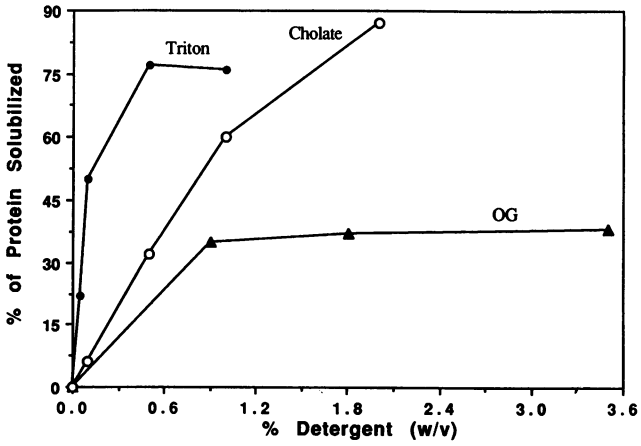

Figure 1. Detergent solubilization of inner envelope membrane proteins. Inner envelope membrane vesicles (1 mg protein/mL in 175 μL) were incubated for 10 min at 4°C in the presence of the detergents shown. The mixtures were centrifuged for 7 min at approximately 90,000g in a Beckman Airfuge and the supernatants assayed for protein. OG, octylglucoside; Triton, Triton X-100.

adex G-50. Unfortunately, approximately 85% of the total glycolate efflux in these assays was insensitive to NEM pretreatment or external L-mandelate (13).

In an effort to obtain a more sensitive assay of reconstituted activity, glycolate/H^+ co-transport activity in reconstituted vesicles preloaded with 8-hydroxypyrene-1,3,6-trisulfonate (Na^+ salt), a membrane-impermeant, fluorescent pH indicator (1), was studied. A similar assay had been used with isolated chloroplast inner envelope vesicles to observe glycolate/H^+ and glycerate/H^+ co-transport and the H^+ transport associated with PGA^{2-}/Pi^{2-} exchange via the phosphate translocator (14). In this system, as with the centrifuge column efflux assays, there was substantial NEM-insensitive transport of glycolate, presumably as glycolic acid diffusion across the lipid bilayer. At pH 7.3, the initial quenching rates due to 8 and 16 mM glycolate additions were, respectively, 42 and 28% sensitive to NEM pretreatment (data not shown). In contrast, the initial quenching rates from 8 mM and 16 mM D-glycerate additions were 100% and 85% inhibited in NEM pretreated vesicles. These results are consistent with the idea that the NEM-insensitive component of the transport is simply nonmediated diffusion of the protonated species across the vesicle membrane. Indeed, we obtained glycolate-induced quenching rates with 8-hydroxypyrene-1,3,6-trisulfonate-loaded vesicles, prepared simply from asolectin, comparable to the NEM-insensitive component obtained with the reconstituted vesicles (K. T. Howitz, unpublished data).

Because the pK_a of glyceric acid is 0.28 unit lower than that of glycolic acid (3.55 *versus* 3.83), the concentration of its protonated form would be 1.9-fold lower at pH 8.0. In addition, because of its larger size and additional hydroxyl, glyceric acid would be expected to have a lower lipid permeability than glycolic acid (25). Although the quenching obtained with D-glycerate in this crude reconstituted system was likely due to transporter activity, the signal was unfortunately too weak to be useful as a routine activity assay in the course of a purification. In preliminary experiments, preparations derived from market spinach were found to be as active as those from pea seedlings. For convenience, market spinach was used as a source for large quantities of intact chloroplasts, and spinach chloroplast inner envelope preparations were used for all of the work reported here.

The transport assay outlined in Figure 2 relies on the phenomenon of counterflow. We have shown previously that the glycolate/glycerate transporter is capable of counterflow and thus can be described as a "carrier" (11). A carrier is defined as a transporter in which the substrate-binding site is accessible alternately at one side of the membrane or the other (see ref. 25 for a review). Counterflow is a kinetic consequence of this type of mechanism, which we have used here to boost the sensitivity of a filtration assay for reconstituted transporter activity. Similar filtration assays of counterflow have been used for assay of the reconstituted *E. coli* lactose carrier (18). Reconstituted vesicles were preloaded with a high concentration of unlabeled transporter substrate (*e.g.* 10 mM sodium D-glycerate). The transport assay was initiated by diluting these vesicles into medium with a low concentration of labeled substrate (*e.g.* 0.4 mM [^{14}C]D-glycerate). If vesicle membranes had incorporated functional transporters, rapid accumulation of internal label to a point above equilibrium with the medium

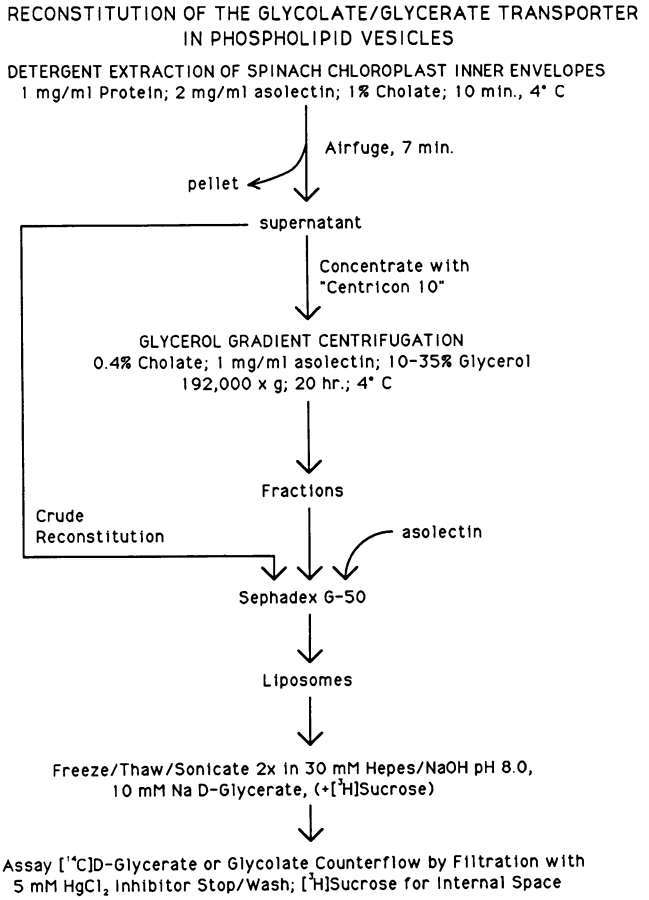

Figure 2. Flow chart for solubilization, partial purification, and reconstitution of the glycolate/glycerate transporter.

(the counterflow maximum), followed by a slower loss of internal label as equilibrium is approached, should be observed. During the initial, "overshoot" phase of such a time course, labeled substrate that has entered the vesicles must compete with a high concentration of internal, unlabeled substrate for binding inwardly oriented transporter sites, whereas the external label faces no competition for outwardly oriented sites. Labeled substrate is driven inward against the overall chemical concentration gradient across the membrane—hence, the term "counterflow." As label accumulates on the inside and unlabeled substrate flows out, the net flow of label is reversed and the transient peak of internal label decays toward equilibrium.

The glycolate/glycerate carrier is a proton symporter (or hydroxide antiporter), and both the transporter kinetics and equilibrium distribution of substrate are dependent on the pH gradient across the membrane (10). A particular concern of ours, with respect to the use of the counterflow assay, was to avoid artifacts that might arise from the nonmediated movement of protonated substrates across the vesicle membrane. For example, if vesicles were preloaded with unlabeled glycolate and then diluted into medium with a low concentration of labeled glycolate, nonmediated diffusion of glycolic acid out of the vesicles could produce a ΔpH across the liposome

membrane. Internal accumulation of the labeled glycolate, in response to the ΔpH, followed by a slow loss of label as leaks broke down the H^+ gradient, could mimic a carrier-mediated counterflow time course. As noted above, at pH 7.3 and 8 mM glycerate, there was no detectable glyceric acid permeation of liposomes. At pH 8 and 10 mM glycerate, the glyceric acid concentration is fivefold lower than at pH 7.3. Counterflow of [^{14}C]D-glycerate into vesicles loaded with 10 mM D-glycerate at pH 8 was therefore used as the primary assay for reconstituted carrier activity. Pains were also taken to confirm the presence of glycolate transport activity, because this is a key feature of the carrier activity in chloroplasts.

Under the conditions used for cholate extraction of chloroplast inner envelopes (see Fig. 2 and "Materials and Methods"), 40 to 50% of the inner envelope protein was solubilized. Approximately 75% of this soluble fraction was incorporated into proteoliposomes in the subsequent reconstitution procedure. The extraction conditions have not been optimized for ionic strength and a low ionic strength buffer (10 mM Hepes/NaOH, pH 8.0, 2 mM DTT) was somewhat arbitrarily chosen. Although this is perhaps unusual for a detergent extraction (for example, see ref. 7), easily measurable transport activity with the combination of extraction, reconstitution, and assay conditions outlined in Figure 2 was obtained. In addition, for reasons that will be discussed below, the low ionic strength may have enhanced the degree of purification achieved by glycerol gradient centrifugation. The reconstitutable transport activity in the crude cholate extract was unchanged after 5 h at 4°C.

The reconstitution procedure consists of a gel filtration step to remove detergent, a freeze/thaw step to increase vesicle size and load the desired internal solutes (21), and a brief sonication to seal the vesicles. A second round of freeze/thaw/sonication was found to increase markedly the measured transport activity (threefold increase in [14C]D-glycerate counterflow, 60 s incubation, pH 8.0). Activity remained stable when subsequent freeze/thaw/sonications were repeated and during storage at −20°C.

Time Courses of Glycerate and Glycolate Counterflow

When reconstituted vesicles preloaded with 10 mM D-glycerate were diluted into a medium containing [^{14}C]D-glycerate, an uptake of [^{14}C]glycerate was observed (Fig. 3A). The [^{14}C]D-glycerate concentration in the interior of the vesicles increased rapidly at first to a value 2.5 times that of the external [^{14}C]D-glycerate concentration. Throughout a much longer time course, the [^{14}C]D-glycerate internal to external concentration ratio decreased. This behavior is characteristic of counterflow. $HgCl_2$ (5 mM) severely inhibited [^{14}C]D-glycerate uptake. If inner envelopes were treated with NEM (10 mM, pH 8.0, 4°C, 1 h) prior to cholate extraction and reconstitution, extents of inhibition similar to the $HgCl_2$ effect were obtained for both D-glycerate and glycolate uptake (data not shown). The sensitivity of transport activity to these two sulfhydryl-modifying reagents is consistent with previous results with intact chloroplasts and inner envelope vesicles (8–10, 12, 14). If the vesicles were loaded at pH 8.0 with unlabeled D-glycerate and diluted into a medium at pH 7.0, the extent of [^{14}C]D-glycerate accumulation was greater than at a medium pH of 8 (Fig. 3B). The increased maximum uptake and slower decline in internal label at lower pH values are characteristic of countertransport experiments with proton symporters (for example, see ref. 6) and are, therefore, consistent with the known kinetic properties of the glycolate/glycerate transporter (10, 11).

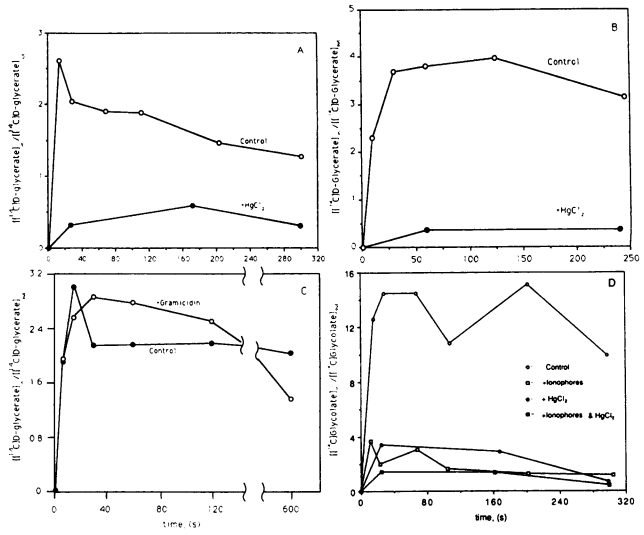

Figure 3. Counterflow of [^{14}C]D-glycerate and [^{14}C]D-glycolate in vesicles reconstituted with unfractionated cholate extracts. All vesicles were loaded with 10 mM D-glycerate and counterflow assayed as described in "Materials and Methods." A, Glycerate counterflow (0.4 mM [^{14}C]D-glycerate) at pH 8.0. $HgCl_2$ (5 mM) was added together with the [^{14}C]glycerate. B, As in A, except that the pH was 7.0. C, As in A, except that 1 μM gramicidin was added where indicated to the vesicles at least 10 min before assay. D, [^{14}C]Glycolate uptake at pH 8.0. The [^{14}C]glycolate concentration was 0.1 mM, and 0.3 mM D-glycerate was carried over as a consequence of the dilution of the D-glycerate-loaded vesicles. +Ionophores, Vesicles were treated for at least 10 min before assay with 1 μM gramicidin, and 4 μM CCCP was present in the assay medium.

The possibility that a ΔpH, produced by net H^+/D-glycerate efflux, contributed to the uptake of labeled D-glycerate was investigated. Time courses of [^{14}C]D-glycerate countertransport, in the presence or absence of a high concentration of the ionophore gramicidin (1 μM), were compared (Fig. 3C). Gramicidin did not markedly affect the level of [^{14}C]D-glycerate accumulation at the counterflow maximum. Control vesicles and those in the presence of gramicidin (1 μM) plus CCCP (4 μM) accumulated identical amounts of [^{14}C]D-glycerate ($[^{14}C]_{in}/[^{14}C]_{out}$ = 2.1) in 30-s incubations (not shown). These results imply that the observed [^{14}C]D-glycerate uptakes are primarily a direct consequence of the glycerate gradient, *i.e.* true counterflow, as opposed to an indirect response to that gradient, mediated by a ΔpH.

Time courses of [^{14}C]glycolate uptake into D-glycerate-loaded vesicles are shown in Figure 3D. Large internal accumulations of glycolate, as great as 15-fold greater than the external concentration, were detected. It should be noted that the external [^{14}C]glycolate concentration was 0.1 mM in these experiments, making the maximum internal glycolate accumulation 1.51 mM ("control," Fig. 3D). Because the external

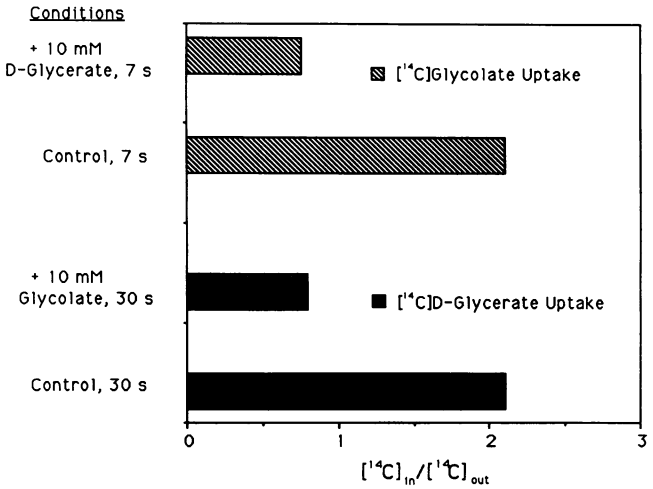

Figure 4. Inhibition of [^{14}C]D-glycerate uptake by glycolate and inhibition of [^{14}C]glycolate uptake by D-glycerate. Vesicles reconstituted with unfractionated cholate extracts were loaded with 10 mM D-glycerate and diluted into a medium (pH 8.0) that contained either 0.4 mM [^{14}C]D-glycerate or 0.1 mM [^{14}C]glycolate and 0.3 mM D-glycerate and the indicated concentrations of D-glycerate or glycolate. All samples were treated with ionophores as described in the legend to Figure 3D.

[^{14}C]D-glycerate concentration was 0.4 mM in the experiment of Figure 3A, the maximum uptake of formerly external D-glycerate was 1.05 mM.

In contrast to the D-glycerate results, glycolate accumulation was dramatically decreased by the presence of ionophores. We noted earlier that nonmediated diffusion of glycolic acid across liposome membranes appears to be much faster than D-glyceric acid diffusion. The differences in glycolate and D-glycerate counterflow, and differences in their ionophore and $HgCl_2$ sensitivities, can be explained by the combined effects of the differences in nonmediated diffusion and differences in the transporter kinetic parameters for the two substrates. It cannot be argued that all, or even most, of the maximum glycolate uptake by the control vesicles (Fig. 3D) is due to a response of nonmediated glycolic acid diffusion to a ΔpH generated by mediated H^+/D-glycerate co-efflux. Even if one assumes that the only internal buffering in the vesicles is the 30 mM Hepes/NaOH (pH 8.0), it can be calculated that the H^+/D-glycerate efflux would come to equilibrium after an 8 mM decline in $[\text{D-glycerate}]_{in}$ and an increase in internal pH to 8.7. A ΔpH of 0.7 could only support a fivefold accumulation of internal glycolate above the external concentration. Furthermore, a transient maximum of nearly fourfold above the external concentration was reached in the presence of ionophores (gramicidin plus CCCP), which prevent formation of a ΔpH. This is not to say that nonmediated glycolic acid diffusion is not occurring, just that D-glycerate-driven glycolate counterflow must be invoked as well to account for the observed glycolate uptakes. The lower internal pH in the ionophore-treated vesicles would, by increasing the proportion of internal glycolate in the acid form, increase an outward nonmediated leak of glycolic acid. This may, at least in part, account for the difference between the control and ionophore-treated samples.

The glycolate and D-glycerate experiments were done by diluting vesicles into media that provided either 0.1 mM [^{14}C] D-glycerate or 0.1 mM [^{14}C]glycolate, with 0.3 mM unlabeled D-glycerate carried over with the preloaded vesicles. Therefore, the much higher internal to external label ratio achieved with [^{14}C]glycolate implies a kinetic discrimination, favoring glycolate uptake over D-glycerate uptake in this system. In our previous work with intact chloroplasts, the kinetic constants for zero *trans* glycolate and D-glycerate uptake at a medium of pH of 7.0 were determined to be similar to one another (11, 12). Constants for D-glycerate efflux, for D-glycerate transport as a function of pH, or for either substrate under equilibrium exchange ("infinite" *trans* or "infinite" *cis* conditions) have yet to be determined. The origin of the discrimination in favor of glycolate will require further investigation. It would be of interest to determine whether there is any asymmetry in the transporter kinetic constants that would favor glycolate efflux and D-glycerate uptake by isolated intact chloroplasts under conditions approximating those that exist during photorespiration.

Response of Reconstituted Transport to Variation of *Cis* and *Trans* Membrane Conditions

Because glycolate and glycerate are both substrates for the transporter, it would be expected that the presence of unlabeled glycolate on the *cis* side of the membrane (the same side as labeled substrate) would inhibit the transport of labeled glycerate and *vice versa*. This has been observed in intact chloroplasts (11, 12). As shown in Figure 4, D-glycerate inhibits its [^{14}C]glycolate uptake and glycolate inhibits [^{14}C]glycerate uptake. These experiments were conducted in the presence of ionophores. Both glycolate and glycerate uptakes were nearly totally dependent on preloading of the vesicles with D-glycerate. Little transport of glycolate or glycerate was observed in vesicles loaded with NaCl instead of D-glycerate (Fig. 5).

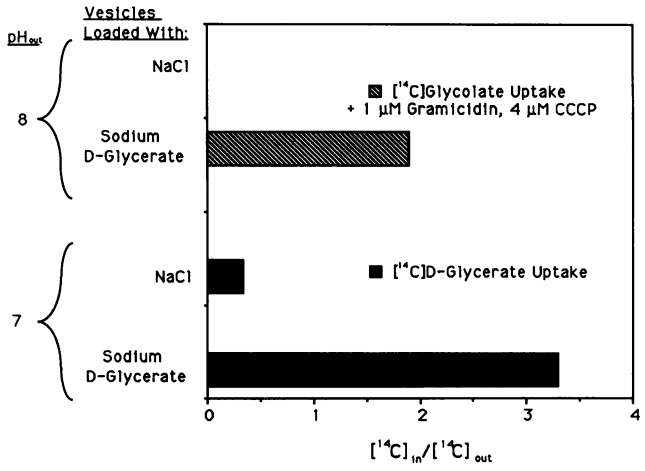

Figure 5. Dependence of [^{14}C]D-glycerate and [^{14}C]glycolate uptakes on loading reconstituted vesicles with D-glycerate. Vesicles were reconstituted with unfractionated cholate extracts and frozen, thawed, and sonicated in the presence of either 10 mM NaCl or 10 mM D-glycerate, Na^+ salt. [^{14}C]Glycolate and [^{14}C]D-glycerate uptakes were assayed under the conditions shown for 30 s.

Efflux of labeled glycerate could be studied by using wash media without $HgCl_2$. Vesicles were allowed to accumulate internal [^{14}C]D-glycerate for 30 s at pH 7.0, before filtration and washing (see legend to Fig. 6); 6 s were required to complete the wash. Because most of the external glycerate is lost early during the wash, efflux under these conditions is essentially under zero *trans* conditions. As expected from the behavior of the transporter in intact chloroplasts, efflux is dependent on the pH of the wash solution and increases with increasing pH of the wash medium (*trans* pH). Mandelate (phenylglycolate) was shown previously to inhibit glycolate transport in intact chloroplasts (13). Although L-mandelate was a weak inhibitor of glycolate uptake when added together with glycolate to the external medium, L-mandelate strongly inhibited glycolate uptake when it was preloaded into intact chloroplasts (*trans* inhibition). Interestingly, D-mandelate was not inhibitory from the *trans* side of the membrane (13). As shown in Figure 6B, L-mandelate inhibits [^{14}C]D-glycerate efflux when it is included in the wash medium. D-Mandelate, however, apparently stimulated efflux to some extent.

Glycerol Density Gradient Centrifugation and SDS-PAGE

Glycerol density gradient centrifugation of cholate extracts proved an effective means for partial purification of the transporter (Fig. 7). The migration of the transport activity to a region of such a high Svedberg value is surprising. As noted in the introduction, differential labeling with NEM indicated that the transporter may include a 35-kD polypeptide (13). This result could suggest that the transporter is a large, oligomeric protein or that it is aggregated or artifactually bound to another protein. The transport activity co-migrates on the gradient with residual Rubisco, which contaminates inner envelope preparations. Under the low ionic strength conditions used, the transporter may form a complex with Rubisco. The fact that glycerate and glycolate transport activity migrates in a similar way on glycerol gradients is further evidence that the two activities are catalyzed by the same transporter.

SDS-PAGE of inner envelope membrane proteins and proteins that reconstitute into liposomes from unfractionated cholate extracts and two fractions from the glycerol gradient are shown in Figure 8. The large subunit of Rubisco (53 kD) is clearly evident in all samples. In this gel system, the small subunit runs at or near the front and is difficult to visualize. From the silver-stained gels, it is apparent that the density gradient centrifugation has led to an enrichment of two species that migrate on the gel as 36- to 38-kD proteins. The identity of the band at 29 kD is unknown, but it could be the phosphate translocator (4). If this is the case, the density gradient procedure enriches glycolate/glycerate transport activity relative to the content of the phosphate translocator.

DISCUSSION

The glycolate/glycerate transporter can be solubilized from inner membrane vesicles, partially purified, and reconstituted into asolectin vesicles. This is only the second transporter of the inner envelope that has been partially purified in active form. The first is the phosphate translocator (4).

The counterflow assay is rapid, convenient, and sensitive.

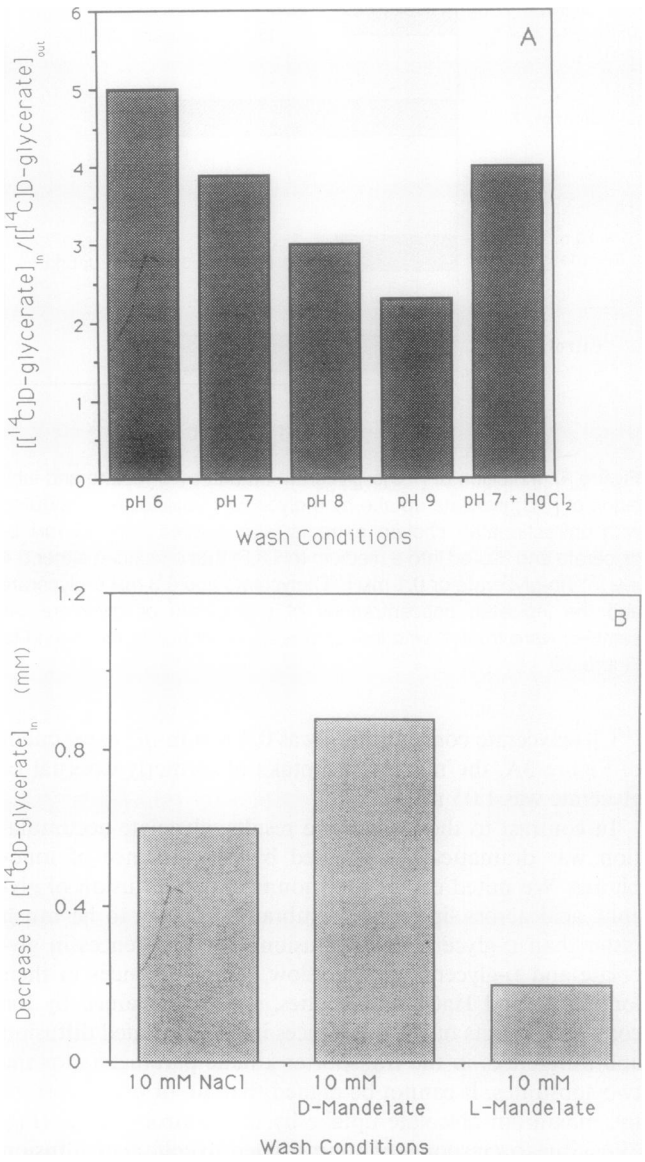

Figure 6. Retention of [^{14}C]D-glycerate in vesicles after counterflow under differing washing conditions. Vesicles were reconstituted with unfractionated cholate extracts and loaded with 10 mM D-glycerate. Counterflow with 0.4 mM [^{14}C]D-glycerate was carried out for 30 s at pH 7.0. This protocol assures that only those vesicles containing active transporters contribute to the efflux of [^{14}C]glycerate. A, After counterflow, the vesicles were washed with 5 mL of media at the pH values shown. The washes were completed in 6 s. The buffers were all present at 30 mM, and 10 mM NaCl was also present. The buffers used were: pH 6.0, Mes-NaOH; pH 7.0, Mops-NaOH; pH 8.0, Hepes-NaOH; pH 9.0, 3-([-2-hydroxy-1,1-bis(hydroxymethyl)ethyl[amino)-1-propanesulfonic acid-NaOH. Where indicated, $HgCl_2$ was present in the wash medium at 5 mM. B, Effects of D- and L-mandelate in the wash medium on the loss of internal [^{14}C]D-glycerate after counterflow. Counterflow was carried out as usual at pH 7.0 for 30 s to give an internal [^{14}C]D-glycerate concentration of 2.0 mM, measured in samples washed with 5 mL of a pH 7.0 medium containing 5 mM $HgCl_2$. Other samples were filtered and washed with media at pH 8.0 with the additions as shown. No $HgCl_2$ was present in these solutions.

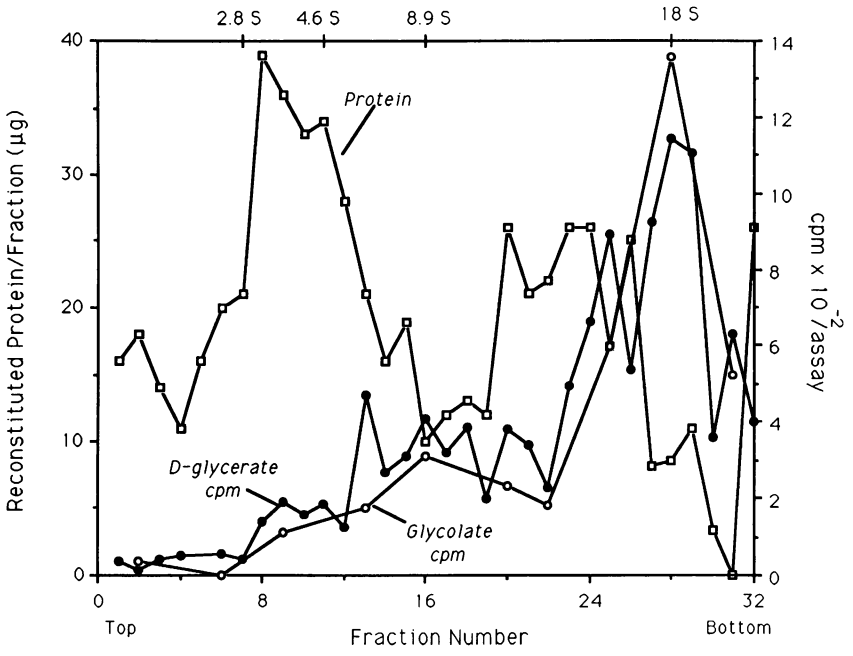

Figure 7. Glycerol density gradient centrifugation of cholate extracts. Continuous (10–35%, w/v) glycerol density gradients were formed, a concentrated cholate extract was applied, and the sample was centrifuged as described in "Materials and Methods." Sedimentation standards were run in a separate gradient in the absence of cholate and asolectin. Standards were: 2.8S bovine carbonic anhydrase; 4.6S bovine serum albumin; 8.9S sweet potato β-amylase; 18S pea Rubisco. Each fraction (0.35 mL) was reconstituted into asolectin vesicles as described in "Materials and Methods." Aliquots were taken for the assay of [^{14}C]D-glycerate and [^{14}C]glycolate counterflow. The protein content of 100-μL aliquots of the reconstituted vesicles was determined after the vesicles were pelleted by centrifugation at 90,000*g* for 20 min in a Beckman Airfuge.

A similar assay was used by Newman *et al.* (18) who solubilized and reconstituted the *lac* carrier of *E. coli*. The filtration assay was not, however, capable of obtaining measurements at the earliest part of the uptake phase of counterflow. The levels of internal label at the first points after time zero in the time courses were always a large percentage of the maximum level measured in that time course and in some cases were the maximum levels. Consequently, it is not possible to estimate initial uptake rates from these experiments. For the same reasons, the values of the counterflow maxima are uncertain and could in some of the experiments be significantly higher than the highest point on a given graph (Fig. 3). Despite these quantitative uncertainties, we are confident, for the following reasons, that the phenomenon that was observed in these experiments was indeed counterflow. Even in the presence of ionophores, with external and internal spaces buffered at the same pH (Fig. 3, B and D) labeled substrate accumulated inside the vesicles to concentrations significantly greater than the medium concentration, and this internal concentration subsequently declined. This accumulation was dependent on internal (*trans*), unlabeled substrate (Fig. 5) and was inhibited by external (*cis*) unlabeled substrate (Fig. 4). The transporter has been shown to catalyze counterflow in intact chloroplasts (11) and to be inhibitable by sulfhydryl-modifying reagents, including $HgCl_2$ (8–10, 12).

There are numerous indications that the transport of [^{14}C] D-glycerate and [^{14}C]glycolate seen in the reconstituted system is a consequence of the glycolate/glycerate transporter characterized in intact chloroplasts. First, counterflow is inhibited by $HgCl_2$. Second, unlabeled glycolate inhibits glycerate counterflow and *vice versa* when present on the *cis* side of the membrane. The results of efflux experiments are also consist-

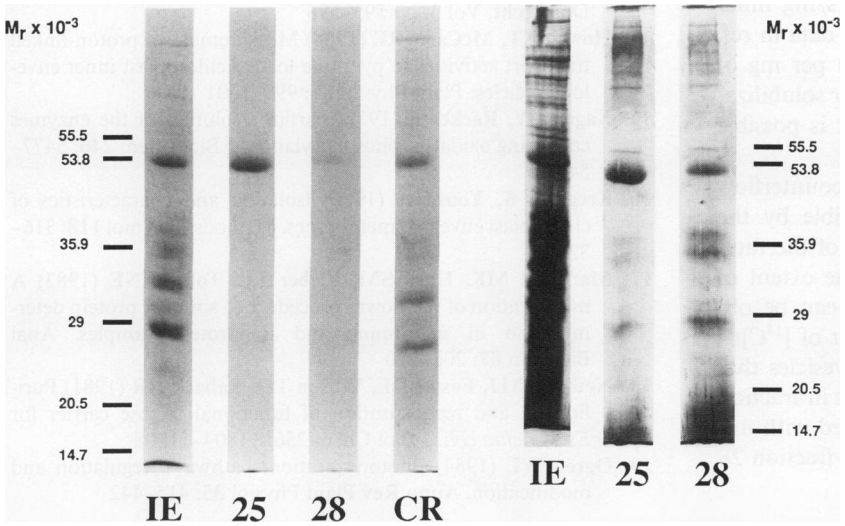

Figure 8. SDS-PAGE of inner envelope membrane proteins and proteins in reconstituted vesicles. SDS-PAGE (11% polyacrylamide) was carried out with 50 μg of inner envelope proteins (IE), 10 μg protein from reconstituted fraction 25 from the glycerol gradient (labeled 25): 5 μg protein from reconstituted fraction 28 of the gradient (labeled 28), and 30 μg of protein from vesicles reconstituted with crude cholate extracts of inner envelopes (CR). The four lanes on the left were stained with Coomassie brilliant blue and the three on the right with silver. The molecular weight standards are the subunits of chloroplast-coupling factor 1 (55.5, 53.8, 35.9, 20.5, and 14.7 kD) and the phosphate translocator of the inner membrane (29 kD).

ent with the operation of the transporter in the vesicles. Efflux is inhibited by $HgCl_2$ and is enhanced by a high *trans* pH. Moreover, L-mandelate, but not D-mandelate, inhibits efflux when present on the *trans* side of the membrane. All of these properties resemble those of the transporter in intact chloroplasts (11–13).

Because of the nature of the assay, it is possible to make only approximate estimates of the rates of transport in the reconstituted vesicles. Such estimates would nevertheless be useful for the sake of comparison to previous measurements of transporter activity in intact chloroplasts. Lower limits for efflux rates can be derived using the data from the experiment described in Figure 6B and the following arguments. The concentration of internal label after the 30 s of preloading at pH 7.0 with [^{14}C]D-glycerate is known from a sample that was filtered and washed with medium containing $HgCl_2$ ([^{14}C] D-glycerate]$_{in}$ = 2.0 mM). The total internal D-glycerate concentration ([^{12}C] + [^{14}C]) at the end of the preloading is not known and depends on what proportion of the original, internal 10 mM [^{12}C]D-glycerate has left the vesicles during the 30 s spent with the external medium containing 0.4 mM [^{14}C]D-glycerate. When the vesicles were subsequently washed for 6 s with medium of pH 8.0 containing no substrate (zero *trans* conditions), 29% of the internal label was lost. What this percentage is equivalent to on a molar basis depends on the total internal D-glycerate concentration, which, in turn, depends on the average [^{12}C]D-glycerate efflux rate during the 30-s preloading period. It can be shown that, for the highest value from the pair of rates to be at a minimum, the two rates should be the same and equal to 8.8 nmol/s/mg of protein. Because there is at least the 2.0 mM [^{14}C]D-glycerate inside the vesicles at the end of the preloading, one can calculate a minimum average efflux rate for the 6 s of zero *trans* efflux of 3.6 nmol/s/mg of protein. This figure would imply a rate of 12.3 nmol/s/mg of protein for the 30-s preloading period. (Calculations were based on the data of Fig. 6B and a value of 27 μg of reconstituted vesicle protein per μL of internal space.) The above are estimates of average efflux rates during the 6- and 30-s periods and as such would be lower than the actual initial rates. In intact chloroplasts, also under zero *trans* conditions, a glycerate uptake rate of 64 nmol/s/mg inner envelope protein was estimated (calculated from data in ref. 12), assuming 100 μg of inner envelope protein per mg of intact chloroplast Chl). Because the conditions for solubilization of transport activity were not optimized, it is possible that the recovery of activity could be improved.

It is difficult to calculate rates of transport from counterflow assays. Even assays at the shortest times possible by the filtration assay are likely to give underestimates of the rates of transport. Nonetheless, some indication of the extent of purification by glycerol gradient centrifugation can be obtained from counterflow assays. The same extent of [^{14}C]D-glycerate accumulation was reached in 30 s in vesicles that were reconstituted in the presence of the proteins in fraction 28 from the glycerol gradient as that reconstituted with unfractionated cholate extracts, despite the fact that fraction 28 had 1/13th the protein content.

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