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Substrate Specificity of the Pea Chloroplast Glycolate Transporter†

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ABSTRACT: The transport of glycolic acid across the chloroplast envelope is a mediated process. Out of a number of metabolites assayed, only two or three carbon 2-hydroxymonocarboxylates significantly inhibited [^{14}C]glycolate uptake by intact pea chloroplasts. These compounds were found to be competitive inhibitors of glycolate uptake, with D-glycerate, D-lactate, and glyoxylate having K_i's approximately equal to the K_m for uninhibited glycolate uptake. L-Glycerate was less inhibitory than D-glycerate, and L-lactate did not appear to inhibit glycolate uptake. D-Glycerate, D-lactate, and glyoxylate caused countertransport of labeled glycolate, indicating that they are transportable substrates of the glycolate carrier. Glycolate and D-glycerate behaved similarly with respect to *N*-ethylmaleimide inhibition of their ability to increase the pH of the medium of a weakly buffered chloroplast suspension. An explanation is provided to resolve the apparent conflict of previous studies [Robinson, S. P. (1982) *Plant Physiol. 70*, 1032], with our conclusion that glycolate and D-glycerate, substrate and product, respectively, of the photorespiratory carbon cycle, are transported by the same carrier.

Glycolate is formed, in illuminated chloroplasts of C_3 plants, by the sequential action of ribulose-1,5-bisphosphate oxygenase and phosphoglycolate phosphatase. For every two glycolate molecules that leave the chloroplast, one glycerate, derived from glycolate by photorespiratory metabolism, enters it. One of the four carbons from the two glycolates is lost as CO_2. Glycerate is converted to 3-phosphoglycerate by the chloroplast glycerate kinase. As 3-phosphoglycerate, the three remaining carbons can reenter the Calvin cycle [see Ogren (1984) for a recent review].

Previous reports from our laboratory have shown the rate of glycolate transport across the envelope of intact pea chloroplasts to saturate with increasing glycolate concentration (Howitz & McCarty, 1983a, 1985). Transport was inhibited by pretreatment of the chloroplasts with *N*-ethylmaleimide (Howitz & McCarty, 1983a, 1985). These results indicated that the transport of glycolate across the chloroplast envelope is carrier mediated. Recently, we have developed multilayered silicon oil centrifugation techniques which have shortened the

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incubation time for glycolate transport measurements to 1 or 2 s (Howitz & McCarty, 1985). Using these methods, we showed that glycolate transport is sufficiently rapid to account for in vivo photorespiratory fluxes. The apparent V_{max} values for glycolate transport were found to be strongly dependent on the trans pH (pH on the opposite side of the membrane from the glycolate) but independent of the cis pH. The apparent K_m depended strongly on the cis pH. These results were most consistent with mobile carrier models for glycolate/hydroxyl antiport or glycolate/proton symport, in which the proton binds the carrier first (Howitz & McCarty, 1985).

Robinson (1982) reported evidence that glycerate uptake by intact spinach chlloroplasts is mediated by a carrier. Subsequently, it was found that illumination of spinach and pea chloroplasts stimulated glycerate uptake in a way that was partially dependent on the increase in the stromal pH (trans pH) (Robinson, 1984).

This report describes investigations of the substrate specificity of the pea chloroplast glycolate transporter. Because of its importance as a photorespiratory product and its structural similarity to glycolate, we have paid particular attention to whether D-glycerate is an alternative substrate of the glycolate carrier. The evidence we will present indicates that the chloroplast glycolate and D-glycerate transport activities are probably due to the same carrier. We will discuss the possible reasons for the apparent conflict between our results and some of Robinson's data on glycerate transport (Robinson, 1982, 1984).

Experimental Procedures

General Procedures. Pea plants (*Pisum sativum* var. progress no. 9) were grown and used to isolate intact chloroplasts as described (Howitz & McCarty, 1985). The solutions used for chloroplast suspensions and Percoll media as well as measurements of [1-^{14}C]glycolate transport and chloroplast internal volume have also been described (Howitz & McCarty, 1985). A brief description of the special silicon oil centrifugation techniques used to obtain rapid glycolate transport measurements is given below. All of the experiments presented here were performed with illuminated chloroplasts and unless otherwise indicated were done at 4 °C. Chlorophyll (Chl)[1] was determined spectrophotometrically (Arnon, 1949).

Rapid Glycolate Transport Measurements. The use of multilayered silicon oil centrifugation techniques to obtain 1- or 2-s incubation times is described in detail elsewhere (Howitz & McCarty, 1985). Briefly, 1-s incubations were obtained by placing a chloroplast suspension into a tube which contained a 7% Percoll layer with [1-^{14}C]glycolate and [^{3}H]sorbitol above a 15% Percoll layer with 4 mM $HgCl_2$ as an inhibitor stop. Two-second incubations were obtained with tubes containing a 10% Percoll layer with the labeled compounds. Thin silicon oil layers separated the Percoll layers from each other and from the chloroplast suspension until the start of centrifugation. Beneath the highest density Percoll layer was the following (top to bottom): silicon oil; 20% (v/v) glycerol and 2% (w/v) trichloroacetic acid; fluorocarbon oil (density = 1.85 g/mL). After centrifugation, the chloroplasts rested on top of the fluorocarbon oil in the glycerol–trichloroacetic acid layer.

Countertransport Experiments. Chloroplast suspensions (100 μL), containing 66 μM [1-^{14}C]glycolate were placed in 400-μL microcentrifuge tubes containing a 75-μL 10% Percoll layer of the same [1-^{14}C]glycolate concentration plus 5.3 μCi/mL [^{3}H]sorbitol. The tubes were illuminated for 5 min, in a water bath at 4 °C before centrifugation in the light at the same temperature. [Both illuminations were >6 × 10^5 erg/(cm^2·s).] Prior to centrifugation, 25 μL of medium containing 66 μM [1-^{14}C]glycolate, either with or without a substance being tested for the induction of countertransport, was stirred into the chloroplast suspension. The glycolate content of chloroplasts at time zero (before the induction of counterflow) was obtained from tubes in which neither the 25-μL addition nor the 10% Percoll layer contained the substance being tested. The 2-s points were obtained from tubes in which the 10% Percoll layer contained the test substance but the 25-μL addition did not. Time points greater than 2 s were obtained with tubes in which both the 10% Percoll layer and the 25-μL addition contained the test substance. The chloroplast suspension medium and Percoll layer pHs were both 7.0.

Medium pH Change Experiments: Inhibitor Pretreatments and Assay Conditions. Isolated pea chloroplasts were suspended (0.4 mg of Chl/mL) in the following medium for inhibitor pretreatments: 330 mM sorbitol; 50 mM Tricine[1]/KOH, pH 7.9; 2 mM EDTA; 1 mM $MgCl_2$; 1 mg/mL bovine serum albumin. All additions were made from stocks containing 330 mM sorbitol. Sodium glycolate or D-glycerate (hemi-calcium salt) was added to aliquots of the chloroplast suspension to a final concentration of 10 mM, prior to the addition of NEM (2 mM). A PCMS concentration of 1 mM was used. Incubation with NEM or PCMS was for 10 min on ice. The NEM incubations were stopped with the addition of dithiothreitol to 2 mM. The PCMS incubation was stopped by dilution and centrifugation of the chloroplasts.

Chloroplast pellets from the inhibitor pretreatments were resuspended in the following medium: 330 mM sorbitol; 0.1 mM HEPES/NaOH, pH 7.0; 25 mM KCl; 1 mM $MgCl_2$. The chloroplasts were pelleted again and resuspended in the same medium. Stirred chloroplast suspension (2.2 mL, 0.17 mg of Chl/mL) were illuminated [1.5 × 10^6 erg/(cm^2·s)] for 5 min in a water-jacketed cell at 20 °C. At this point, 55 μL of 80 mM glycolate or D-glycerate in suspension medium was added to the illuminated chloroplasts (final concentration 2 mM). The medium pH changes due to the glycolate or glycerate additions were measured with a glass combination electrode, a Heath electrometer (EU-200-30) and offset unit (EU-200-02), and a chart recorder. The initial pH of the suspensions was approximately 6.7, and 0.1 pH unit gave full-scale deflection of the recorder pen. The buffering capacity of the suspensions was measured by recording the pH change after the addition of 5 μL of 1 mM HCl.

Results

Metabolite Inhibitors of Glycolate Transport. The effect of various compounds on the chloroplast uptake of [1-^{14}C]-glycolate is presented in Table I. The compounds causing the greatest inhibition of uptake, [^{12}C]glycolate, glycerate, lactate, and glyoxylate, are all two- or three-carbon 2-hydroxymonocarboxylates. (Glyoxylate, when hydrated, has two hydroxyls on C-2.) The 2-hydroxymonocarboxylates of more than three carbons, 2-hydroxybutyrate, 2-hydroxyvalerate, and 2-hydroxycaproate, have relatively little effect. The inhibition by hexanoate, given its five-carbon hydrophobic tail, might be due to uptake of hexanoic acid through the envelope lipid bilayer. Acidification of the stroma lowers the V_{max} for glycolate uptake (Howitz & McCarty, 1985). The slight inhibition by 2-hydroxyvalerate and 2-hydroxycaproate

[1] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; NEM, *N*-ethylmaleimide; PCMS, *p*-(chloromercuri)benzenesulfonic acid; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Chl, chlorophyll; EDTA, ethylenediaminetetraacetic acid.

Table I: Uptake of 0.1 mM [^{14}C]Glycolate after 2 s[a]

addition	[stromal glycolate] (mM)	% of control
none	0.38	100
[^{12}C]glycolate	0.15	38
D-glycerate	0.18	48
DL-glycerate	0.21	54
D-lactate	0.19	51
DL-lactate	0.25	66
glyoxylate	0.12	33
DL-2-hydroxybutyrate	0.36	94
DL-2-hydroxyvalerate	0.33	87
DL-2-hydroxycaproate	0.33	87
hexanoate	0.30	78
pyruvate	0.38	100
2-cyano-4-hydroxycinnamate	0.40	105
2-oxoglutarate	0.36	94
glycine	0.41	108
thioglycolate	0.39	103
acetate	0.35	92
bicarbonate	0.37	97
phosphate	0.45	118
D-3-phosphoglycerate	0.39	103

[a] Measurements were made by centrifuging chloroplasts through a 10% Percoll layer containing [1-^{14}C]glycolate and the indicated additions. The medium pH was 7.0. All additions were made from stocks of the sodium salt, pH 7.0, except for lactates (Li^+ salts) and glycerates (hemi-Ca^{2+} salts). All values are the mean of three determinations except for the control which was the mean of six. All additions, were 0.5 mM, except for acetate, bicarbonate, and phosphate which were added to final concentrations of 1 mM.

could be due to stroma acidification rather than binding to the carrier. Alternatively, these compounds might damage the envelope membrane through their detergent-like properties. Replacement of the 2-hydroxy function by an amine (glycine) a thiol (thioglycolate), a keto group (pyruvate), or a hydrogen (acetate) eliminates the inhibition by two- or three-carbon monocarboxylates. Neither pyruvate nor 2-cyano-4-hydroxycinnamate, an inhibitor of pyruvate transport in mitochondria, erythrocytes (Halestrap & Denton, 1973), and C_4 mesophyll chloroplasts (Huber & Edwards, 1977a), affected glycolate uptake. 2-Oxoglutarate, a substrate of the chloroplast dicarboxylate carrier (Lehner & Heldt, 1978), did not inhibit transport. We have shown previously that malate, another substrate of the dicarboxylate carrier, did not inhibit glycolate uptake (Howitz & McCarty, 1983b). Neither phosphate nor phosphoglycerate, substrates of the chloroplast phosphate translocator (Fliege et al., 1978), was inhibitory. Dihydroxyacetone phosphate, another phosphate translocator substrate, was shown previously not to affect glycolate uptake (Howitz & McCarty, 1983b).

The rate of [1-^{14}C]glycolate uptake, as a function of concentration, was determined in the presence or absence of the various two- or three-carbon 2-hydroxymonocarboxylates (Figure 1). The apparent K_m's and V_{max}'s of transport from these experiments are listed in Table II. Only the apparent K_m's for uptake were significantly affected, indicating that these compounds are competitive inhibitors. Within a given experiment, the K_I's for inhibition by glyoxylate, D-glycerate, and D-lactate (as well as [^{12}C]glycolate) were approximately equal to the K_m for [1-^{14}C]glycolate uptake in the absence of competitors. L-Glycerate has a much higher K_I than D-glycerate, and L-lactate appeared to be noninhibitory.

Countertransport Experiments. The fact that glyoxylate, D-glycerate, and D-lactate are competitive inhibitors suggests that they may be transported by the glycolate carrier. The phenomenon of countertransport [see Kotyk & Janacek (1975) for a review] can be used to determine whether a compound

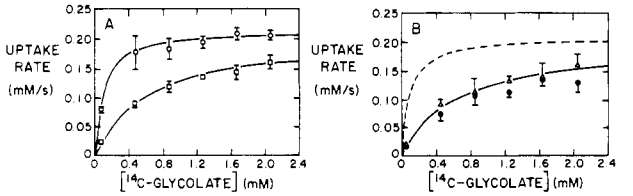

FIGURE 1: Concentration dependence of [1-^{14}C]glycolate uptake rate in the presence of unlabeled 2-hydroxymonocarboxylates. Rate measurements were made by using 1-s incubations as described under Experimental Procedures. The 2-hydroxymonocarboxylates were in the 7% Percoll layers. Results were obtained from experiment 1 (see Table II). The medium pH was 7.0. Curves were obtained from a nonlinear least-squares fit (Marquardt, 1963) to the Michaelis–Menton equation. Uptake rates in mM/s can be converted to μmol/(mg of Chl·h) by multiplying by 90. (A) (○) Control; (□) plus 0.5 mM D-glycerate. (B) (△) Plus 0.5 mM [^{12}C]glycolate; (▲) plus 0.5 mM glyoxylate. The broken line is the best fit to the control data points (see panel A). The solid line is the best fit to the [^{12}C]glycolate points.

Table II: Kinetic Parameters for Glycolate Uptake[a]

expt	additions	apparent K_m[b] (mM)	apparent V_{max} (mM/s)	K_I (mM)
1	none	0.09 ± 0.13	0.21 ± 0.02	
	[^{12}C]glycolate	0.61 ± 0.09	0.20 ± 0.03	0.08
	D-glycerate	0.56 ± 0.04	0.20 ± 0.01	0.09
	glyoxylate	0.54 ± 0.08	0.17 ± 0.03	0.09
2	none	0.20 ± 0.09	0.32 ± 0.02	
	D-glycerate	0.66 ± 0.09	0.32 ± 0.03	0.22
	L-glycerate	0.30 ± 0.08	0.31 ± 0.02	1.0
	D-lactate	0.63 ± 0.08	0.34 ± 0.03	0.23
	L-lactate	0.23 ± 0.26	0.32 ± 0.05	

[a] All inhibitor concentrations were 0.5 mM. [b] Parameters were obtained from the nonlinear least-squares fits to the Michaelis–Menton equation for the data in Figure 1. Errors in K and V are root squared deviations from those curve fits. K_I's were calculated assuming competitive inhibition from the equation $K_m(\text{apparent}) = K_m(1 + [I]/K_i)$, where [I] is the inhibitor concentration.

is a transportable substrate of a mobile carrier. When a labeled compound is in equilibrium across a membrane, the fluxes of the compound in the two directions are equal. When that equilibrium is mediated by a mobile carrier, and when the unlabeled form of the compound or an alternative carrier substrate is added to one side of the membrane, countertransport occurs. Immediately after the addition of the competing substrate to the outside of a membrane-bound compartment, the labeled compound flows out, counter to the previously established equilibrium. This occurs because, just after its addition, the competing substrate is located mostly on the outside, where its competition shuts off the inward, but not the outward, flux of the labeled compound. However, since the competitor is being transported inward, it will increasingly compete against the efflux of the labeled compound. Thus, a second phase is observed in which the labeled compound flows back into the compartment.

As can be seen from Figure 2A, addition of unlabeled glycolate, D-glycerate, D-lactate, or glyoxylate to the outside of chloroplasts equilibrated with [1-^{14}C]glycolate causes counterflow of the labeled glycolate. As a control, D-3-phosphoglycerate, 2-oxoglutarate, and acetate were tested in the same way (Figure 2B). In contrast to the sharp outflow of label, followed by a slow recovery phase observed with the 2-hydroxymonocarboxylates, these compounds caused a much slower and permanent loss of internal [1-^{14}C]glycolate.

Medium pH Change Experiments. The experiments presented above indicate that the transport of glycolate and glycerate through the chloroplast envelope occurs via a common carrier. Despite the fact that radioactively labeled D-glycerate is not commercially available, its uptake by chlo-

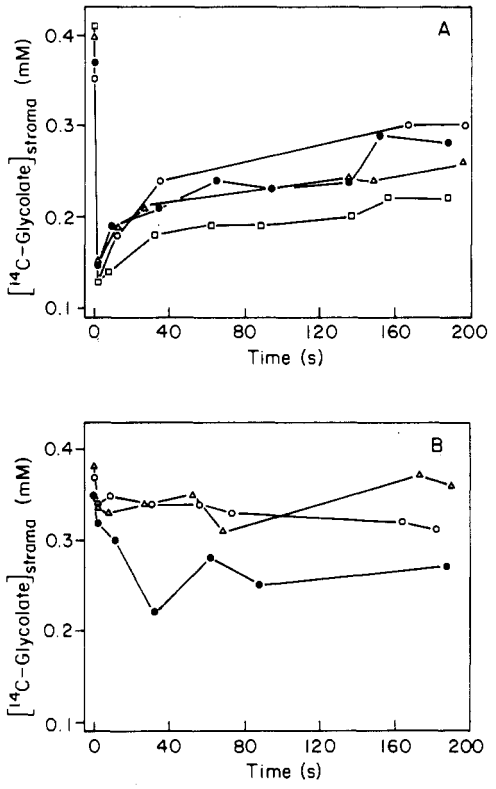

FIGURE 2: Induction of [^{14}C]glycolate countertransport by 2-hydroxymonocarboxylates. Conditions are described under Experimental Procedures. The first time point after zero was 2 s. (A) Additions at time zero: (○) 0.5 mM [^{12}C]glycolate; (△) 0.5 mM D-glycerate; (□) 0.5 mM glyoxylate; (●) 0.5 mM D-lactate. (B) Additions at time zero: (△) 0.5 mM 2-oxoglutarate; (○) 0.5 mM D-3-phosphoglycerate; (●) 1.0 mM acetate.

roplasts may be indirectly monitored. We reported previously (Howitz & McCarty, 1983b) that addition of glycolate to a weakly buffered chloroplast suspension causes an increase in the pH of the suspending medium. Like the uptake of [1-^{14}C]glycolate, this pH change is inhibited by pretreatment of the chloroplasts with NEM, an inhibition prevented by the presence of glycolate during the pretreatment. It has been reported that DL-[^{14}C]glycerate distributes across the chloroplast envelope in a way that is dependent on the ΔpH between the stroma and the medium (Robinson, 1984). This would indicate that, like glycolate, D-glycerate may be transported by proton symport or hydroxyl antiport. Thus, a medium pH increase upon addition of D-glycerate to a chloroplast suspension should be observed. A medium pH increase is observed upon the addition of either glycolate or D-glycerate to a chloroplast suspension (Table III). The pH increase caused by addition of either compound is inhibited to a similar extent by NEM pretreatment of the chloroplasts. Both glycolate and D-glycerate, when present during the NEM pretreatment, partially prevented the inhibition of the pH increase from the addition of either compound during the assay. Pretreatment of the chloroplasts with PCMS, reported to be an inhibitor of glycerate uptake in spinach chloroplasts (Robinson, 1982), yielded a slight inhibition of the pH increase from D-glycerate addition, but not that from glycolate addition.

Table III: Medium Alkalinization from Glycolate or D-Glycerate Uptake[a]

chloroplast pretreatment	addition (2 mM)	pH change	nmol of H^+/mg of Chl	% of control
none	glycolate	0.042	25	100
NEM	glycolate	0.009	5	20
glycolate + NEM	glycolate	0.017	10	40
D-glycerate + NEM	glycolate	0.028	17	68
PCMS	glycolate	0.047	28	112
none	D-glycerate	0.054	32	100
NEM	D-glycerate	0.012	7	22
glycolate + NEM	D-glycerate	0.030	18	56
D-glycerate + NEM	D-glycerate	0.027	16	50
PCMS	D-glycerate	0.042	25	78

[a] pH changes given are the difference between the pH at the time of glycolate or D-glycerate addition and the pH 30 s later. Inhibitor pretreatment and assay conditions are described under Experimental Procedures.

Discussion

The specificity of the metabolite inhibition of glycolate uptake by pea chloroplasts implies strict structural requirements for binding of a compound to the glycolate transporter. Strong inhibition of uptake is obtained with two- or three-carbon 2-hydroxymonocarboxylates. With the chiral three-carbon compounds, lactate and glycerate, markedly greater inhibition is observed with the D isomers. The inhibition by glyoxylate, D-glycerate, and D-lactate is competitive, and the countertransport experiments indicate that these compounds not only bind to the carrier but are also transported by it. The fact that these compounds cause countertransport of glycolate not only shows they are alternative substrates but also confirms our earlier conclusion (Howitz & McCarty, 1985) that glycolate transport can be described by a mobile carrier model. The symmetrical nature of the results with glycolate and D-glycerate in the medium pH change experiments, particularly with regard to NEM inhibition, is further evidence that these two compounds are transported by the same carrier.

The finding that salts of weak acids can inhibit CO_2-dependent O_2 evolution by intact chloroplasts (Enser & Heber, 1980) is frequently cited as evidence of the ability of these compounds to acidify the stroma. It is unlikely, however, that the inhibition of glycolate uptake by 2-hydroxymonocarboxylates is due to this cause. The structural similarity of these compounds to glycolate suggests that the inhibition is specific. Furthermore, we have reported that, lowering the stromal pH (trans pH), decreases the V_{max} and the K_m for uptake of glycolate (Howitz & McCarty, 1985). This contrasts with increased K_m's and unchanged V_{max}'s observed with 2-hydroxymonocarboxylate inhibition. In countertransport experiments, an inhibitor that acted by acidifying the stroma would be expected to cause a gradual and permanent shift in the equilibrium glycolate accumulation as the ΔpH across the envelope was lowered. Such a pattern was observed, especially with acetate, but not with the 2-hydroxymonocarboxylates.

A number of the results reported by Robinson (Robinson, 1982, 1984) on the uptake of DL-[^{14}C]glycerate by spinach and pea chloroplasts are consistent with our results and hence with the idea that glycolate and D-glycerate are transported by the same carrier. Glycolate, glyoxylate, and lactate (isomer not specified) inhibited glycerate uptake (Robinson, 1982). Unlabeled D-glycerate was a much better inhibitor of uptake than was L-glycerate (Robinson, 1982). Glycerate (Robinson, 1984), like glycolate (Howitz & McCarty, 1982, 1985), seemed to distribute across the envelope in a way that equalized the concentration of the undissociated acid on the two sides. Raising the pH of the stroma, by illuminating the chloroplasts, increased the rate of glycerate uptake (Robinson, 1984). This resembles our own finding that increasing the trans pH (stromal pH during uptake) increased the apparent V_{max} of glycolate transport (Howitz & McCarty, 1985). These results with glycerate transport would be consistent with the proton symport or hydroxyl antiport mobile carrier models,

which best describe glycolate transport.

There are, however, a number of apparent conflicts between Robinson's findings with spinach chloroplasts (Robinson, 1982) and the results we have presented here. Dihydroxyacetone phosphate and phosphoglycerate inhibited glycerate uptake; phosphoglycerate appeared to inhibit competitively. Glyoxylate decreased the V_{max} for glycerate uptake and slightly lowered the K_m. Pretreatment of the chloroplasts with 0.1 mM PCMS inhibited glycerate uptake 77%. Some or all of these conflicts may be due to what we consider to be a significant flaw in the methodology used to measure the rate of glycerate uptake. Glycerate uptake rates were measured with 30-s incubations of illuminated chloroplasts at 2 °C (Robinson, 1982). In a published time course of glycerate uptake [Figure 1 (Robinson, 1984)], the amount of ^{14}C as glycerate in the chloroplasts has reached a steady level within 10–15 s. Although the stromal pH was not measured, this level of stromal glycerate was consistent with the expected equilibrium level, dictated by the probable ΔpH across the chloroplast envelope. Accumulation of ^{14}C by the chloroplasts continued, however, because of glycerate metabolism. At 15 s approximately 50% of the ^{14}C in the chloroplasts was as metabolites other than glycerate (Robinson, 1984). If the rate of glycerate metabolism were much faster than glycerate transport, the stromal glycerate level would be well below the predicted equilibrium. The fact that the stromal glycerate concentration is at or near equilibrium after 15 s indicates that the opposite is true; glycerate transport is faster than its metabolism. Therefore, the rate-limiting factor for glycerate uptake in a 30-s incubation is not transport but metabolism. Thus, the inhibition of glycerate uptake by phosphoglycerate and dihydroxyacetone phosphate could have been due to a perturbation of glycerate metabolism rather than an effect on the transporter. Competition for ATP by the phosphoglycerate kinase and phosphoribulokinase reactions and a consequent inhibition of the glycerate kinase reaction are a possible explanation. In this regard, it is perhaps notable that bicarbonate was found to inhibit glycerate uptake (Robinson, 1982). Given the fact that glycerate transport per se was not being measured, the interpretation of the glyoxylate inhibition also becomes obscure. During a 30-s incubation glyoxylate might have time to significantly lower the pH of the stroma (Enser & Heber, 1980; Sicher, 1984). If the glycerate and glycolate transport mechanisms are the same, stromal acidification would act to lower the V_{max} and slightly lower the K_m for glycerate uptake. Acidification of the stroma might also inhibit glycerate metabolism. These effects, plus the possible direct effect of glyoxylate on the transporter, make a straightforward interpretation of Robinson's experiments extremely difficult. PCMS was found to inhibit glycerate uptake in spinach chloroplasts (Robinson, 1982). In our medium pH change experiments, a PCMS pretreatment did not inhibit the pH increase from glycolate addition and only slightly affected that from glycerate addition. The response of the pH electrode is too slow, however, to measure the initial transport rate. We have in fact observed a slight inhibition (27%, 0.95 mM [^{14}C]glycolate) of the [1-^{14}C]glycolate uptake rate after a PCMS pretreatment (0.2 mM, pH 7.0, 4 °C, 15 min). *p*-(Chloromercuri)benzoate, the unsulfonated derivative of PCMS, is a potent inhibitor of glycolate uptake by pea chloroplasts (K. T. Howitz and R. E. McCarty, unpublished observations). Since Robinson's experiment was done with spinach chloroplasts, it is possible that species differences are the cause of the discrepancy. Again, however, the fact that the uptakes of glycerate measured did not necessarily reflect initial transport rates makes interpretation difficult.[2]

The evidence we have presented indicates that the pea chloroplast glycolate transporter is in fact a transporter of several two- and three-carbon 2-hydroxymonocarboxylates. Two of these substrates, glycolate and D-glycerate, flow in opposite directions through the chloroplast envelope in the course of the photorespiratory carbon cycle. These results are consistent with what might be considered a theme in chloroplast transport of photosynthetic and photorespiratory metabolites: transport of substrates related by a common metabolic pathway, in opposite directions, via a common carrier. Triose phosphate, intermediate of the reductive pentose phosphate pathway, can be exchanged for phosphate (which as the γ-P of ATP is incorporated into the pathway) via the phosphate translocator (Fliege et al., 1978). Mesophyll chloroplasts from the C_4 plant *Digitaria sanguinalis* exchange phosphoenolpyruvate (PEP) for phosphate, required for PEP formation, by way of the phosphate translocator (Huber & Edwards, 1977b). Glutamate and 2-oxoglutarate, substrates of the photorespiratory nitrogen cycle, are transported in opposite directions by the same dicarboxylate carrier (Lehner & Heldt, 1978). The glycolate to glycerate stoichiometry in photorespiration is 2:1. However, since the glycolate transporter catalyzes a proton symport or hydroxyl antiport (Howitz & McCarty, 1985), the net fluxes through it would be electroneutral. Even if a transporter mechanism is not a strictly coupled exchange, fluxes of compounds in opposite directions on the same carrier can increase the efficiency of transport by causing a more rapid reorientation of the carrier. For example, we have observed (Howitz & McCarty, 1985) that chloroplasts preloaded with [^{12}C]glycolate took up [1-^{14}C]-glycolate 5 times faster than those which were not preloaded.

Registry No. Glycolic acid, 79-14-1; D-glyceric acid, 6000-40-4; DL-glyceric acid, 600-19-1; D-lactic acid, 10326-41-7; DL-lactic acid, 598-82-3; glyoxylic acid, 298-12-4; DL-2-hydroxybutyric acid, 600-15-7; DL-2-hydroxyvaleric acid, 6450-97-1; DL-2-hydroxycaproic acid, 636-36-2; hexanoic acid, 142-62-1; 2-cyano-4-hydroxycinnamic acid, 96483-15-7; 2-oxoglutaric acid, 328-50-7; glycine, 56-40-6; thioglycolic acid, 68-11-1; acetic acid, 64-19-7; bicarbonate, 71-52-3; phosphate, 14265-44-2; D-3-phosphoglyceric acid, 3443-58-1.

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[2] Recently, we have synthesized D-[1-^{14}C]glycerate. In experiments similar to those reported here for glycolate, glycolate was found to be a competitive inhibitor of glycerate uptake, and only 2-hydroxymonocarboxylates inhibited uptake in 2-s incubations. When 30-s incubations were used, acetate, bicarbonate, phosphate, 3-phosphoglycerate, and triose phosphates also inhibited (K. T. Howitz and R. E. McCarty, unpublished observations).

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Interaction of Fluorescent 3′-[1,5-(Dimethylamino)naphthoyl]adenine Nucleotides with the Solubilized ADP/ATP Carrier†

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ABSTRACT: The binding of the 3′-[1,5-(dimethylamino)naphthoyl] (DAN) derivatives of AMP, ADP, and ATP to the solubilized ADP/ATP carrier is studied, evaluating primarily the fluorescence enhancement and ^{3}H-labeled compound binding. DAN nucleotides also fluoresce when adsorbed to Triton X-100 micelles that are used for solubilization of the carrier. The partition of DAN-AMP between water and Triton X-100 micelles is measured, and it is shown to be shifted toward a higher content in Triton micelles with increasing salt concentration. In order to maintain a low level of fluorescence, the Triton content is decreased. The fraction of DAN nucleotide fluorescence due to carrier binding is determined by the suppression with bongkrekate (BKA). In contrast to the membrane-bound carrier, the solubilized preparation shows an increase of total BKA-sensitive fluorescence by 30–60% upon addition of ATP or ADP. In the solubilized atractylate–protein complex, the ADP-stimulated fluorescence amounts even to 80%. The suppression of fluorescence by BKA is independent of the presence of ADP or ATP, while that by carboxyatractylate (CAT) depends on ADP or ATP. The quantitation with [^{3}H]BKA and [^{3}H]CAT of these ligand interactions with DAN-AMP fluorescence shows that DAN-AMP fluorescence reflects the "m"-state carrier population and its redistribution under the influence of ADP or ATP. Thus, besides the "c"/"m" distribution, the kinetics of the c to m transition in the solubilized carrier also can be determined. The m share is increased to 80% when SO_4, P_i, or pyrophosphate is present during solubilization. The rate of the ADP- or ATP-stimulated transition to the m state is markedly dependent on pH and on the presence of various anions, whereas the extent is little varied. The affinity decreases 4-fold going from DAN-AMP to DAN-ADP and to DAN-ATP (K_D = 0.9, 1.6, and 3.2 μM). Comparison with physical binding of [^{3}H]DAN nucleotides shows that the fluorescence yield of bound DAN-AMP is about 1.4 times higher than that of bound DAN-ATP. DAN substitution causes more than a 100-fold affinity increase for AMP and a 50-fold increase for ADP or ATP, probably because of interaction of the DAN group with a hydrophobic niche. A less specific, low-affinity displacement of DAN nucleotides by GDP, ADP, GTP and ATP (K_i = 1–2 mM) probably reflects primarily the ionic interactions at the binding center.

The 3′-[1,5-(dimethylamino)naphthoyl] derivatives of AMP, ADP, and ATP are valuable fluorescent probes for the ADP/ATP carrier in mitochondrial membranes (Schäfer & Onur, 1980; Schäfer et al., 1980; Klingenberg, 1981a; Mayer et al., 1984). The DAN[1] nucleotides make possible visualization of changes in the binding center of the ADP/ATP carrier associated with the translocation process, which have so far been examined primarily in binding studies of labeled ligands. The DAN nucleotides emit a strong fluorescence on binding to the ADP/ATP carrier only when the binding center is in the "m" state, directed to the m (matrix) side, whereas it does not fluoresce on binding to the center in the "c" state when it is directed to the c (cytosol) side; this was shown both by the sensitivity to the carrier state specific inhibitors CAT and BKA and by studying the access of the DAN nucleotides to the carrier sites in mitochondria vs. submitochondrial particles (Klingenberg et al., 1984).

Whereas the results on the ADP/ATP carrier in the membrane in its original environment are of considerable interest, they are complicated by the presence of other nucleotide binding proteins, by the membrane barrier and uncertain membrane orientation, particularly in submitochondrial particles. Thus, the effects of ADP and ATP on DAN nucleotides binding indicated functional heterogeneity of the binding sites for exchange and for binding. It is likely that this heterogeneity was introduced by the sonication of the membranes.

By using the solubilized and purified ADP/ATP carrier, these complications can be avoided, and less equivocal results on the interaction of the DAN-ATP with the carrier can be

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[1] Abbreviations: ATR, atractylate; BKA, bongkrekate; CAT, carboxyatractylate; DAN-AMP, 3′-[1,5-(dimethylamino)naphthoyl]-adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; LAPAO, lauroylpropylamine oxide; Tris, tris(hydroxymethyl)aminomethane.

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