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Changes in Photorespiratory Enzyme Activity in Response to Limiting CO_2 in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The activity of two photorespiratory enzymes, phosphoglycolate phosphatase (PGPase) and glycolate dehydrogenase (glycolate DH), changes when CO_2-enriched wild-type (WT) *Chlamydomonas reinhardtii* cells are transferred to air levels of CO_2. Adaptation to air levels of CO_2 by *Chlamydomonas* involves induction of a CO_2-concentrating mechanism (CCM) which increases the internal inorganic carbon concentration and suppresses oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. PGPase in cell extracts shows a transient increase in activity that reaches a maximum 3 to 5 hours after transfer and then declines to the original level within 48 hours. The decline in PGPase activity begins at about the time that physiological evidence indicates the CCM is approaching maximal activity. Glycolate DH activity in 24 hour air-adapted WT cells is double that seen in CO_2-enriched cells. Unlike WT, the high-CO_2-requiring mutant, cia-5, does not respond to limiting CO_2 conditions: it does not induce any known aspects of the CCM and it does not show changes in PGPase or glycolate DH activities. Other known mutants of the CCM show patterns of PGPase and glycolate DH activity after transfer to limiting CO_2 which are different from WT and cia-5 but which are consistent with changes in activity being initiated by the same factor that induces the CCM, although secondary regulation must also be involved.

In *Chlamydomonas reinhardtii*, PGPase[2] and glycolate DH are the first two dedicated enzymes in the photorespiratory metabolic pathway (8). PGPase specifically hydrolyzes 2-P-glycolate to glycolate and Pi in the chloroplast (9). It is a critical enzyme because an accumulation of P-glycolate would be expected to inhibit triose phosphate isomerase (2) and carbon recycling in the Calvin cycle. Glycolate DH is a membrane-bound enzyme that oxidizes glycolate to glyoxylate in the mitochondria (5).

Chlamydomonas cells grown under CO_2-enriched conditions (1–5% CO_2) exhibit C_3-like photosynthetic characteristics (6); however, the high CO_2 concentration suppresses oxygenase activity of Rubisco and decreases carbon flow into photorespiratory metabolites. CO_2-enriched cells transferred to air levels of CO_2 excrete glycolate, evidence that they experience significant photorespiratory stress, until they adapt to the lower CO_2 condition by induction of a CCM. The CCM increases the internal C_i concentration (4) which suppresses the oxygenase activity of Rubisco. As a result, fully air-adapted cells do not excrete detectable glycolate except under very low levels of CO_2 (6, 10) or in the presence of inhibitors of glycolate metabolism (13, 14). These fluctuations of carbon flow in the photorespiratory pathway might be expected to affect the activity of photorespiratory enzymes.

Published data have shown that CO_2-enriched and fully air-adapted WT *Chlamydomonas* cells have similar levels of PGPase activity (9, 14), although glycolate DH activity increases significantly after transfer to air levels of CO_2 (14, 22). We have observed that 24-h air-adapted cells consistently have higher PGPase activity than CO_2-enriched cells (24). In this study, we examined enzyme activity at earlier time points to determine whether PGPase shows a transient increase in activity that would correlate with the period of greatest photorespiratory stress and induction of the CCM. We used high-CO_2-requiring mutants deficient in various aspects of the CCM to explore the coordinated regulation of induction of the CCM and changes in photorespiratory enzyme activity.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii, WT strain 2137, the high-CO_2-requiring mutants 18–7F, 12–1C, 16–5K, selected as described previously (20, 21, 23, 24), and the high-CO_2-requiring mutant cia-5 obtained from Dr. J. Moroney (12), were grown photoautotrophically on an orbital shaker under continuous illumination (100 μmol photons m^{-2} s^{-1}). CO_2-enriched cells were cultured in liquid medium (24) with 5% CO_2 in air. Air-adapted cells were cultured in air minimal medium (24). Adapting cells were obtained by transferring CO_2-enriched cells to air minimal medium at time 0.

PGPase Activity

PGPase activity was determined by measuring P-glycolate-dependent Pi release in 20 mM Mes-KOH (pH 6.3) (24). Pi was measured with the Ames reagent (1). NPPase activity at pH 6.3 was determined by the same method using NPP as substrate. Cell enzyme extract was obtained by disrupting

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[2] Abbreviations: PGPase, phosphoglycolate phosphatase; glycolate DH, glycolate dehydrogenase; CCM, CO_2 concentrating mechanism; C_i, dissolved inorganic carbon; WT, wild-type; 18–7F, *pgp*-1–18–7F; 12–1C, *ca*-1–12–1C; 16–5K, *pmp*-1–16–5K; NPP, *p*-nitrophenylphosphate; NPPase, nonspecific phosphatase; DCPIP, 2,6-dichlorophenol indophenol; $K_{0.5}C_i$, concentration of dissolved inorganic carbon required for half-maximal response of photosynthetic O_2 evolution.

cells by sonication. Chl was determined after extraction into 96% (v/v) ethanol (26).

Glycolate DH Activity

Glycolate DH activity was assayed by following the anaerobic reduction of DCPIP at 600 nm (15; K. Suzuki, personal communication). The temperature was maintained at 25°C by water-jacketed cuvette holders in the recording spectrophotometer. Anaerobic conditions were established using the system described by Peterson (16). All reagents and crude enzyme preparations were made anaerobic by repeated vacuum evacuation and argon flushing in stoppered and capped glass serum bottles. Cell enzyme extract was obtained by disrupting cells by sonication in KH_2PO_4 buffer (100 mM, pH 7.0) with 1.0 mM PMSF (final concentration) added just before sonication. Unbroken cells were removed by centrifugation, and the resulting supernatant was centrifuged at 27,000*g* for 30 min. The pellet from the high-speed centrifugation was resuspended in phosphate buffer (100 mM, pH 7.0) with 1.0% Triton X-100 and was sonicated to homogeneity. This sonicated preparation was used for enzyme activity assays. The reaction mixture (final volume of 3 mL) contained 0.08 M Na-PPi buffer (pH 8.6), 0.12 mM DCPIP, and 0.01% Triton X-100. After addition of enzyme to the assay mixture (80–150 μg Chl), an endogenous rate of anaerobic DCPIP reduction was recorded for ≥10 min. The reaction was started by addition of glycolate (5.0 mM final concentration). After 5 or 6 min KCN was added (final concentration of 2.0 mM) to confirm that the glycolate-stimulated rate of DCPIP reduction was due to glycolate DH activity (15). Change in absorbance was converted to DCPIP equivalents using an extinction coefficient of 21.9 at pH 8.6 (3), and a 1:1 relationship between DCPIP reduced and glycolate oxidized was assumed (15). Although it has been standard to include flavin mononucleotide in the reaction mixture (7, 14, 15), we could not detect that it had any effect on anaerobic DCPIP reduction rates in our cells; therefore, we did not use flavin mononucleotide.

^{14}C-Metabolite Labeling

^{14}C-labeling of photosynthetic products was done as described previously (24), except that the cells were labeled for only 3 min. Cells were washed once with CO_2-free Mops-KOH (50 mM, pH 7.0, prepared as described in ref. 24), resuspended in 1 mL of the same buffer, and equilibrated for 1 min in the dark in a Rank O_2 electrode with 200 to 500 μL of additional buffer. $NaH^{14}CO_3$ was added (100 μM or 2.5 mM initial concentration) and the suspension stirred for 1 min before photosynthesis was started by turning on the light (500 μmol photons m^{-2} s^{-1} red light [17]). After 3 min, the cell suspension was removed and immediately injected into an equal volume of 8 M formic acid in ethanol. The suspension was centrifuged and the supernatant fractionated on a cation exchange column (Bio-Rad AG 50W-8X, H^+) into a neutrals plus anions fraction and a basic fraction. After the fractions were dried, the neutrals plus anions fraction was resuspended and characterized by anion exchange HPLC as described in ref. 24 using a KH_2PO_4 gradient on a Spheresorb 5/25 SAX column from Phasesep. Radioactivity was detected with a flow through heterogeneous scintillation counter.

RESULTS

PGPase Activity

As shown in Figure 1A, we observed a transient increase in PGPase activity when CO_2-enriched WT cells were transferred to air levels of CO_2. Maximum activity was seen 3 to 5 h after transfer, and activity declined to the original level within 48 h. When CO_2-enriched cells were transferred to air levels of CO_2 in the dark, there was no increase in PGPase activity (data not shown). PGPase activity in CO_2-enriched cells resuspended in fresh CO_2-minimal medium was unchanged after 5 to 6 h (Fig. 1A), and air-adapted WT cells transferred to fresh air minimal medium showed little change in PGPase activity (Fig. 1A). NPPase activity measured with NPP as substrate was essentially unchanged in both adapting and air-adapted cells during the 48-h time course (Fig. 1A). These data demonstrate that the change in PGPase activity occurred because of a change in CO_2 concentration in the light and not because of transfer or manipulation of the cells.

In 18–7F, a high-CO_2-requiring mutant deficient in PGPase activity (24), there was no change in the residual level of PGPase activity measured when CO_2-enriched cells were transferred to air levels of CO_2 (Fig. 1B), supporting our suggestion (24) that apparent PGPase activity in this mutant is due to nonspecific phosphatase activity. In fact, NPPase activity was consistently greater than PGPase activity in 18–7F (Fig. 1B).

In addition to causing the changes we saw in PGPase activity, transfer of CO_2-enriched *Chlamydomonas* cells to air levels of CO_2 in the light results in induction of a CCM (4). In an effort to explore the relationship between induction of the CCM and changes in PGPase activity, we measured PGPase activity in several high-CO_2-requiring mutants that are deficient in various aspects of the CCM. Two of the mutants, 12–1C and 16–5K, induce some of the characteristics of the CCM (20, 21). The third mutant, cia-5, does not induce any known characteristics of the CCM (12). None of the mutants exhibit the greatly reduced $K_{0.5}C_i$ for photosynthesis that is the end result of induction of the CCM in WT.

In 12–1C, PGPase activity increased after transfer of CO_2-enriched cells to air levels of CO_2 to a maximum approximately 5 h after transfer. Then, activity began to slowly decrease, in a pattern similar to that seen in WT, although activity decreased more slowly in the mutant (Fig. 1C). At all times the level of PGPase activity was significantly higher in 12–1C than in WT, perhaps because of the non-WT phenotype that this mutant exhibits in CO_2-enriched cells (20). NPPase activity was initially about double that seen in WT, but it slowly decreased to WT levels during the 48-h time course (Fig. 1C).

In the mutant 16–5K, PGPase activity also increased after transfer of CO_2-enriched cells to air levels of CO_2 (Fig. 1D). In contrast with 12–1C and WT, PGPase activity did not decline during the 48-h time course in 16–5K. As in WT, NPPase activity levels were essentially unchanged during the 48-h time course (Fig. 1D).

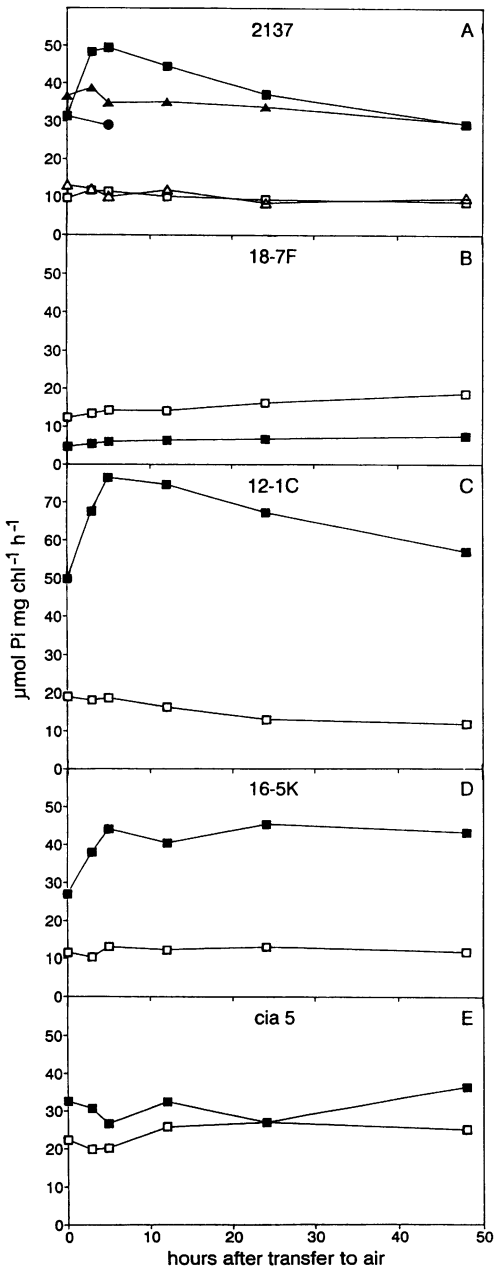

Figure 1. PGPase (■, ▲) and NPPase (□, △) activities in CO_2-enriched (■, □) and air-adapted (▲, △) cells of *C. reinhardtii* WT strain 2137 (A) and high-CO_2-requiring mutants 18–7F (B), 12–1C (C), 16–5K (D), and cia-5 (E) after transfer of cells to air levels of CO_2. A, PGPase activity in CO_2-enriched WT cells (●) after transfer to fresh CO_2-enriched medium. The graphs clearly show a change in the pattern of PGPase activity over time in the different strains when CO_2-enriched cells are transferred to air. The SE of difference between any two strains at a given time (or at different times) is 3.03.

PGPase activity did not respond to air levels of CO_2 in the mutant cia-5 (Fig. 1E). NPPase activity in cia-5 was essentially unchanged during the 48-h time course at levels about double those seen in WT (Fig. 1E). The NPPase activity probably did not account for a significant proportion of the PGPase activity, because if it did, it would indicate a low level of PGPase activity in cia-5. Based on our experience with the 18–7F mutant, we would expect detectable ^{14}C label in P-glycolate in a strain with low PGPase activity (24, Table I), and we did not see this in cia-5 (Table I).

Glycolate DH Activity

In addition to changes in PGPase activity, we observed changes in activity of the second committed enzyme in the photorespiratory pathway, glycolate DH, when CO_2-enriched cells were transferred to air levels of CO_2 (Table II). When we assayed crude enzyme extracts obtained by sonication of whole cells, we observed fairly substantial, nonlinear, endogenous rates of anaerobic DCPIP reduction, especially in CO_2-enriched cells of the mutants (data not shown). The endogenous rates were sometimes greater than the rates seen after addition of glycolate (data not shown). Glycolate DH is a membrane-bound mitochondrial enzyme that is inhibited by KCN (15). When we separated crude cell extracts into soluble and membrane fractions and solubilized the membrane fraction with Triton X-100, endogenous rates in this fraction were much lower than those in unfractionated extracts. In addition, glycolate-stimulated rates observed in the membrane fractions were inhibited by 2 mM KCN to approximately the endogenous rates (data not shown). The rates of anaerobic DCPIP reduction in the soluble fraction were generally unaffected by the addition of glycolate or KCN (data not shown). Therefore, all glycolate DH data reported in this paper were obtained from Triton X-100-solubilized membrane fractions.

In agreement with the data presented by others (14, 22), we consistently observed higher glycolate DH activity in 24-h air-adapted WT cells than in CO_2-enriched cells (Table II). 18–7F was the only mutant of those we examined in which glycolate DH followed the WT pattern and levels of activity (Table II).

Glycolate DH activity in cia-5 was about half that seen in CO_2-enriched WT cells, and the activity did not change in response to limiting CO_2 (Table II). In 12–1C, glycolate DH activity levels were much higher than those in WT, and activity did not increase in 24-h air-adapted cells (Table II). CO_2-enriched cells of 16–5K had glycolate DH activity about equal to WT rates, but activity did not change in response to limiting CO_2 (Table II).

^{14}C-Metabolite Labeling

It has been suggested that a photorespiratory metabolite could be the metabolic signal responsible for induction of the CCM (11, 19), and a reasonable expectation is that enzyme activity might be affected by changes in the concentration of its substrate. In an effort to investigate the possibility that a metabolite signal initiates these responses, we labeled cells with $^{14}CO_2$ and determined the percentage of total acid-stable radioactivity in various photosynthetic products. We did not

detect labeled P-glycolate after 3 min under any conditions except in 18–7F (Table I), which, if it has PGPase activity, has at most 12% of the constitutive PGPase activity found in WT. Therefore, we cannot directly evaluate whether there are changes in P-glycolate that would correlate with induction of the CCM.

We did see differences in the percentage of the total label in glycolate, which indicated that more carbon must be flowing through P-glycolate in WT, 12–1C, 16–5K, and cia-5 under certain conditions (Table I). None of the strains we examined synthesized detectable [^{14}C]glycolate when CO_2-enriched cells were labeled under CO_2-enriched conditions (2.5 mM $NaHCO_3$; Table I). WT, 12–1C, and 16–5K incorporated the greatest percentage of label into glycolate in CO_2-enriched cells labeled for 3 min at approximately air levels of CO_2 (100 μM $NaHCO_3$). Under these conditions, 16–5K and 12–1C incorporated a much greater percentage of label into glycolate than either WT or cia-5 (Table I). Labeled glycolate was undetectable in 24-h air-adapted WT cells after 3 min at approximately air levels of $^{14}CO_2$ (Table I). In 24-h air-adapted cells of 12–1C and 16–5K labeled in 100 μM $NaHCO_3$, the percentage of label in glycolate was much less than in CO_2-enriched cells but was still easily detectable (Table I), and PGPase activity was significantly higher than in WT (Fig. 1, A, C, and D). In the mutant cia-5, 24-h air-adapted cells had a greater percentage of label in glycolate than did CO_2-enriched cells, possibly because of metabolic stress due to lack of induction of the CCM.

Table II. *Glycolate Dehydrogenase Activity in C. reinhardtii WT 2137 and High-CO_2-Requiring Mutants 18-7F, cia-5, 12-1C, and 16-5K*

Cells were grown continuously at 5% CO_2 (CO_2-enriched) or at 5% CO_2 and then for 24 h with air. Number in parentheses is the SE.

Strain	CO_2-Enriched	24-h Air-Adapted
	μmol glycolate mg Chl^{-1} h^{-1}	
WT	0.5 (0.08)	1.1 (0.01)
18-7F	0.6 (0.15)	1.1 (0.17)
cia-5	0.2 (0.17)	0.3 (0.00)
12-1C	1.9 (0.13)	2.0 (0.26)
16-5K	0.7 (0.10)	0.7 (0.15)

DISCUSSION

A significant decrease in the CO_2/O_2 ratio in the light, such as the decrease in the aqueous equilibrium concentration ratio from 6.4 to 0.04 used in this research, will affect the carboxylase/oxygenase ratio of Rubisco and stimulate photorespiration. CO_2-enriched *Chlamydomonas* cells have C_3-like pho-

Table I. *Percentage of Total Acid-Stable ^{14}C Incorporated by C. reinhardtii WT and the High-CO_2-Requiring Mutants 18-7F, cia-5, 12-1C, and 16-5K*

Cells were grown continuously at 5% CO_2 (CO_2-enriched) or at 5% CO_2 and then for 24 h with air before labeling with 100 μM or 2.5 mM $NaH^{14}CO_3$ for 3 min (pH 7.0, 25°C).

	Insoluble	Neutrals	Acids						Basic
			Glycolate	Mono-P[a]	PGA	P-glyc[b]	RuBP[c]	Other	
CO_2-enriched									
100 μM $NaHCO_3$									
WT	50	3	4	12	5	–[d]	7	5	10
18-7F	35	4	–	6	1	35	–	3	11
cia-5	38	4	6	11	9	–	7	3	14
12-1C	18	4	22	14	8	–	14	7	24
16-5K	20	1	24	12	8	–	11	1	19
2.5 mM $NaHCO_3$									
WT	57	5	–	11	7	–	–	4	10
18-7F	52	6	–	7	7	5	–	3	14
cia-5	55	5	–	14	10	–	–	2	12
12-1C	54	4	–	12	10	–	–	4	15
16-5K	54	5	–	16	7	–	1	2	12
24-h air-adapted									
100 μM $NaHCO_3$									
WT	57	7	–	12	8	–	6	2	7
18-7F	31	4	–	14	14	7	10	3	13
cia-5	43	7	12	18	7	–	5	–	7
12-1C	27	2	6	8	3	–	34	2	19
16-5K	43	4	2	10	7	–	26	1	11
2.5 mM $NaHCO_3$									
WT	63	8	–	14	9	–	–	2	7
18-7F	35	5	–	14	15	2	4	4	14
cia-5	45	7	–	22	11	–	1	1	9
12-1C	64	7	–	9	11	–	1	3	8
16-5K	57	7	–	15	11	–	1	3	9

[a] Sugar mono-phosphates. [b] Phosphoglycolate. [c] Ribulose-1,5-bisP. [d] Not detected.

tosynthetic characteristics and thus would experience the greatest photorespiratory stress when first transferred to air levels of CO_2, conditions that might be expected to affect the activity of photorespiratory enzymes. We observed a transient increase in PGPase activity in CO_2-enriched WT cells transferred to air levels of CO_2 that was maximal about 5 h after transfer. We also saw increased glycolate DH activity in 24-h air-adapted WT cells.

The changes in physiological conditions that result in changes in PGPase and glycolate DH activities also induce a CCM in *Chlamydomonas* cells. The CCM results in an internal accumulation of C_i in air-adapted cells (4); consequently, the $K_{0.5}C_i$ for photosynthesis in air-adapted cells is dramatically lower than in CO_2-enriched cells (6, 20). Physiological evidence indicates that the CCM approaches maximal activity within 8 h of transfer (6, 18). After the CCM is operational, carbon flow into the photorespiratory pathway is reduced relative to when the cells are first transferred, and related enzyme activity might be expected to decrease. Indeed, this is the response we see in WT PGPase: following a maximum at 5 h, activity begins to decrease by 12 h after transfer and reaches the initial CO_2-enriched level within 48 h. Previously reported data show little difference in PGPase activity in *Chlamydomonas* cells (9, 14). Because of the logistics involved in running the assays, we do not have any short-term measurements of glycolate DH activity, so we do not know whether this enzyme shows the transient early response that PGPase does.

The CCM does not eliminate carbon flow through the photorespiratory pathway. Although internal CO_2 is increased in the presence of a fully functional CCM to about 20 times that possible from equilibrium with air (4), this represents only 10 to 11% of the CO_2 that would be present in cells in equilibrium with 5% CO_2. We did not detect labeled glycolate in air-adapted WT cells after 3 min, but long-term experiments have shown synthesis of detectable glycolate in air-adapted WT cells (13). Therefore, we might expect photorespiratory enzyme activity to be higher in air-adapted cells than in CO_2-enriched cells. This is the response of PGPase and glycolate DH in 24-h air-adapted WT cells, although within 48 h after transfer, PGPase activity declines to the level seen in CO_2-enriched cells. PGPase activity levels are much higher than those of glycolate DH in both CO_2-enriched and 24-h air-adapted cells. Because P-glycolate is a more toxic product than glycolate, the cells may constitutively maintain high PGPase activity levels. An accumulation of P-glycolate would be expected to inhibit triose phosphate isomerase and thus carbon recycling through the Calvin cycle (2); therefore, its rapid turnover would be important, whereas *Chlamydomonas* cells can excrete excess glycolate.

The relationship among induction of the CCM, changes in PGPase activity, and increased glycolate DH activity in WT *Chlamydomonas* suggests that these responses are initiated by the same signal. In support of the idea of coordinated initiation of these responses, cia-5, which induces no known aspect of the CCM, shows no changes in PGPase or glycolate DH activities. These data suggest that either the signal or its perception must be absent in this mutant. If the signal is a photorespiratory metabolite (11, 19, 24), then the labeling data provide evidence that the signal is present in cia-5.

The two mutants that show partial induction of the CCM, 12–1C and 16–5K, also show a response of PGPase activity to limiting CO_2, supporting the idea of a coordinated regulation. However, glycolate DH does not respond to air levels of CO_2 in these two mutants, suggesting that, if the same signal initiates these responses to limiting CO_2, there must be other factors that affect the regulation of each independently. Because the activity of glycolate DH in CO_2-enriched 12–1C cells is already about double the highest activity we see in air-adapted WT cells, one possibility for the lack of response of glycolate DH in this mutant is that glycolate DH activity is already maximally induced in CO_2-enriched cells. Previous research has provided physiological evidence that both 12–1C and 16–5K have non-WT phenotypes even in CO_2-enriched cells (20, 21, 25). The high activity levels of glycolate DH and PGPase in CO_2-enriched 12–1C cells may be related to this non-WT phenotype.

Because PGPase and glycolate DH in cia-5 do not respond to limiting CO_2, it is unlikely that changes in activities of these enzymes seen in WT and the other mutants are due to nonspecific ionic effects caused by the change in CO_2 concentration. In addition, the labeling data demonstrate that simple substrate-mediated allosteric control cannot be responsible for changes in activity in either enzyme. CO_2-enriched and air-adapted cells of cia-5 synthesize significant amounts of glycolate and, therefore, of P-glycolate; yet neither PGPase nor glycolate DH respond to transfer to air levels of CO_2. 16–5K and 12–1C synthesize a much higher percentage of their fixed carbon as glycolate than does WT in CO_2-enriched cells just transferred to air levels of CO_2 as well as in 24-h air-adapted cells, but the activity of glycolate DH does not respond to limiting CO_2 in these two mutants. In addition, we did not detect labeled glycolate in 18–7F in our short-term labeling studies, yet activity levels of glycolate DH are similar to those in WT, and both strains respond similarly to air levels of CO_2.

If the change in glycolate DH activity is part of the overall adaptive response of *Chlamydomonas* to air levels of CO_2, then the response of 18–7F is consistent with our contention that induction takes place normally in this strain (24). In longer term experiments, K. Suzuki has observed a low level of glycolate excretion by air-adapting cells of 18–7F (personal communication). Dr. Suzuki's data indicate that there must be metabolism of accumulated P-glycolate in 18–7F, presumably by nonspecific phosphatases, although turnover is slow because we did not detect labeled glycolate in 18–7F under our conditions (Table I, 24). We would expect some metabolism over the long term because we have no evidence that the P-glycolate pool continues to increase. Because P-glycolate does turn over in this mutant, if a photorespiratory metabolite provides the metabolic signal for induction of the CCM, the signal would not necessarily have to be P-glycolate as proposed previously (24).

In conclusion, we see changes in PGPase and glycolate DH activities in response to transfer of CO_2-enriched *Chlamydomonas* cells to air levels of CO_2 which suggest that the signal responsible for initiating induction of the CCM is also the signal responsible for initiating changes in PGPase and glycolate DH activities.

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