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Genetic and physiological analysis of the CO_2-concentrating system of *Chlamydomonas reinhardii*

Martin H. Spalding*, Robert J. Spreitzer** and William L. Ogren
Department of Agronomy, University of Illinois, and U.S. Department of Agriculture, Agricultural Research Service, Urbana, IL 61801, USA

Abstract. When grown photoautotrophically at air levels of CO_2, *Chlamydomonas reinhardii* possesses a system involving active transport of inorganic carbon which increases the intracellular CO_2 concentration considerably above ambient, thereby stimulating photosynthetic CO_2 assimilation. In previous investigations, two mutant strains of this unicellular green alga deficient in some component of this CO_2-concentrating system were recovered as strains requiring high levels of CO_2 to support photoautotrophic growth. One of the mutants, *ca*-1-12-1C, is a leaky (nonstringent) CO_2-requiring strain deficient in carbonic anhydrase (EC 4.2.1.1) activity, while the other, *pmp*-1-16-5K, is a stringent CO_2-requiring strain deficient in inorganic carbon transport. In the present study a double mutant (*ca pmp*) was constructed to investigate the physiological and biochemical interaction of the two mutations. The two mutations are unlinked and inherited in a Mendelian fashion. The double mutant was found to have a leaky CO_2-requiring phenotype, indicating that the mutation *ca*-1 overcomes the stringent CO_2-requirement conferred by the mutation *pmp*-1. Several physiological characteristics of the double mutant were very similar to the carbonic-anhydrase-deficient mutant, including high CO_2 compensation concentration, photosynthetic CO_2 response curve, and deficiency of carbonic-anhydrase activity. However, the labeling pattern of metabolites during photosynthesis in $^{14}CO_2$ was more like that of the bicarbonate-transport-deficient mutant, and accumulation of internal inorganic carbon was intermediate between that of the two original mutants. These data indicate a previously unsuspected complexity in the *Chlamydomonas* CO_2-concentrating system.

Present address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA
***Present address:* Départment de Biologie Moléculaire, Université de Genève, CH-1211 Genève 4, Switzerland

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Introduction

Chlamydomonas reinhardii Dangeard and other unicellular green algae posses a CO_2-concentrating system which increases their photosynthetic efficiency at CO_2 concentrations which otherwise would be limiting (Findenegg 1976; Badger et al. 1980; Beardall and Raven 1981; Zenvirth and Kaplan 1981). The mechanism responsible for this effect requires energy (Badger et al. 1980), is light dependent (Spalding and Ogren 1982; Spalding et al. 1983a), results in an accumulation of intracellular inorganic carbon to a concentration several-fold higher than that in the external medium (Badger et al. 1980; Beardall and Raven 1981; Zenvirth and Kaplan 1981), involves a saturable transport system for inorganic carbon (Spalding and Ogren 1983), probably involves the electrogenic transport of bicarbonate (Kaplan et al. 1982), and includes carbonic anhydrase as an essential component of the system (Spalding et al. 1983a).

Two *C. reinhardii* mutants deficient in some portion of the CO_2-concentrating system have been described (Spalding et al. 1983a, b). Based largely on the physiological characteristics of one of these, *ca-1*, a mutant deficient in carbonic-anhydrase activity, the major role of carbonic anhydrase in the system was concluded to be the dehydration of transported bicarbonate to supply CO_2 for photosynthesis (Spalding et al. 1983a). A second mutation affecting this system, *pmp*-1, has

been characterized as resulting in a deficiency in bicarbonate transport (Spalding et al. 1983b). With the work reported in this paper we have extended previous investigations of these two mutations by constructing the double mutant, *ca pmp*, in order to characterize in more detail the genetics of the mutations and to investigate their physiological and biochemical interactions.

Materials and methods

Algal strains and culture conditions. Chlamydomonas reinhardii strain CC 221 *mt-*, supplied by the *Chlamydomonas* stock center at Duke University (Durham, N.C., USA), was used as wild-type *mt-* in crosses. Strains CC 221, wild-type 2137 *mt+* (Spreitzer and Mets 1981), and *pf*-2 *mt-* (paralyzed flagella, centromere marker) were maintained on acetate medium (Spreitzer and Mets 1981) at 45 µmol (photons) m^{-2} s^{-1} (General Electric, Cleveland, O., USA, cool-white fluorescent lamps) and 25° C. Mutant strains *ca*-1-12-1C (Spalding et al. 1983a; Spreitzer and Mets 1981) and *pmp*-1-16-5K (Spalding et al. 1983b) and all progeny from crosses involving these mutant strains were maintained on acetate medium in the dark. For biochemical and physiological analyses all strains were grown photoautotrophically at 150 µmol (photons) m^{-2} s^{-1} (General Electric Power-Groove Fluorescent lamps) with CO_2 enrichment (5% CO_2 in air) to logarithmic phase, then were transferred to standard atmospheric conditions 2 d prior to analysis. Removal of CO_2 enrichment induces the CO_2-concentrating system of *C. reinhardii* (Spalding and Ogren 1982).

Genetic analyses. Gamete induction, zygote maturation and germination, and tetrad analysis were performed as described in Spreitzer and Mets (1981). Tetrads were scored as parental ditype (PD), nonparental ditype (NPD), or tetratype (T) following replica-plating at 80 µmol (photons) m^{-1} s^{-1} on minimal medium, minimal medium and 5% CO_2 and acetate medium, and on acetate medium in the dark.

Physiological and biochemical analyses. Photosynthetic O_2 exchange, $^{14}CO_2$-product labeling and analysis, and infrared CO_2 gas-exchange analysis were performed as described in Spalding et al. (1983a). Inorganic carbon uptake and accumulation and simultaneous carbon fixation were determined using silicone-oil filtering centrifugation (Badger et al. 1980). Extract preparation, assay of carbonic-anhydrase (EC 4.2.1.1) activity, and calculation of carbonic-anhydrase enzyme units were performed as described previously (Spalding and Ogren 1982). Chlorophyll was determined after extraction into 96% ethanol (Wintermans and De Mots 1965).

Results and discussion

Genetic analysis. From the data for the first two crosses in Table 1, gene-centromere distances were calculated to be 28 map units for *ca*-1 (cross No. 1) and 6 map units for *pmp*-1 (cross No. 2). In order to determine whether these two mutations are linked, a cross between *ca*-1 and *pmp*-1 was performed (cross No. 3). Out of 37 tetrads scored, 20 tetratype tetrads were identified by their 3:1 pattern of segregation for CO_2-requiring: wild-type phenotypes. The expected frequency of tetratype tetrads for unlinked genes was calculated as:

Table 1. Tetrad analysis of *Chlamydomonas reinhardii ca*-1 and *pmp*-1 mutants. PD, Parental ditype; NPD, nonparental ditype; T, tetratype; CD, centromere distance

Cross	PD	NPD	T	CD
1. $ca\ pf\text{-}2^+\ mt+ \times ca^+\ pf\text{-}2\ mt-$	8	4	15	28
2. $pmp\ pf\text{-}2\ mt+ \times pmp^+\ pf\text{-}2^+\ mt-$	22	17	5	6
3. $ca^+ pmp\ pf\text{-}2\ mt+ \times ca\ pmp^+\ pf\text{-}2^+\ mt-$				
Score *pmp-ca*	7	10	20	
Score *ca-pf*	8	10	19	26

$$(1\text{-}t_1)t_2+(1\text{-}t_2)t_1+0.5(t_1)(t_2)=0.58$$

where t_1 and t_2 are the frequencies of second-division segregation (proportion of tetratype tetrads) of *ca* and *pmp* respectively (from crosses No. 1 and 2, Table 1). The solution of this equation, 0.58, indicated that 21.5 tetratype tetrads would be expected for unlinked genes. χ^2 analysis ($P>0.70$) of the observed and expected values of (PD+NPD) and T indicated that the number of tetratype tetrads arising from the cross was consistent with the frequencies of second-division segregation for *ca* and *pmp* alone. This demonstrates that *ca* and *pmp* segregate in a normal Mendelian pattern and are not linked, a conclusion further supported by the ratio PD:NPD being close to unity. These results also confirm the gene-centromere distances calculated from crosses No. 1 and 2.

Phenotype of the double mutant. Two different, readily discernible phenotypes could be recognized among the CO_2-requiring progeny from crosses between the *ca*-1 and *pmp*-1 mutants. In each tetrad two progeny were of a leaky (non-stringent) CO_2-requiring phenotype (CO_2R-leaky), similar to that of the *ca*-1 mutant (Spalding et al. 1983a). In all parental ditype and tetratype tetrads the additional CO_2-requiring progeny were of a stringent phenotype (CO_2R-stringent) with regard to their CO_2 requirement, similar to that of the *pmp*-1 mutant (Spalding et al. 1983b). In order to assess whether the CO_2R-leaky phenotype corresponded to *ca*, the tetrads from the double-mutant cross were scored for this phenotype and for the *pf*-2 centromere marker (cross No. 3, Table 1). Contingency χ^2 analysis ($P>0.90$) of the values (PD+NPD) and T in the double-mutant cross and cross No. 1 indicated that the CO_2R-leaky phenotype accurately reproduced the second-division segregation frequency of *ca*. Thus, progeny that carry both mutations (*ca pmp*) have a leaky CO_2-requiring pheno-

Table 2. Physiological analysis of tetrads from a cross between *C. reinhardii pmp*-1 and *ca*-1 mutants

Tetrad progeny	Phenotype[a]	Total internal inorganic carbon[b] (mM)	Photosynthetics rate[c] (μmol ^{14}C mg^{-1} Chl h^{-1})	Carbonic anhydrase (units mg^{-1} Chl)	CO_2 compensation concentration (50% O_2) (μl l^{-1})	genotype
2-1	CO_2R-stringent	0.50	35	493	<10	*ca*⁺ *pmp*
2-2	wt	2.65	142	679	<10	*ca*⁺ *pmp*⁺
2-3	CO_2R-leaky	4.20	11	133	325	*ca pmp*
2-4	CO_2R-leaky	10.75	14	97	309	*ca pmp*⁺
10-1	wt	2.90	147	842	<10	*ca*⁺ *pmp*⁺
10-2	CO_2R-leaky	3.20	14	192	339	*ca pmp*
10-3	CO_2R-leaky	4.05	9	157	298	*ca pmp*
10-4	wt	2.25	129	1009	<10	*ca*⁺ *pmp*⁺
12-1	wt	3.15	131	703	<10	*ca*⁺ *pmp*⁺
12-2	CO_2R-stringent	0.60	29	572	<10	*ca*⁺ *pmp*
12-3	CO_2R-leaky	9.25	12	106	244	*ca pmp*⁺
12-4	CO_2R-leaky	3.05	13	124	225	*ca pmp*

[a] Abbreviations: wt, wild type; CO_2R, required elevated (5%) CO_2 for photoautotrophic growth
[b] Determined after a 30-s incubation at pH 7.0, 25 C, 700 μmol (photons) m^{-2} s^{-1}, and 80 μM $NaH^{14}CO_3$ (initial concentration)
[c] Measured as acid-stable ^{14}C incorporation during 30 s incubation at pH 7.0, 25° C, 700 μmol (photons) m^{-2} s^{-1}, and 80 μM $NaH^{14}CO_3$ (initial concentration)

type similar to that of *ca*-1 alone. This is true even though *pmp*-1, when alone, results in a stringent CO_2-requiring phenotype.

Physiological characterization of the tetrads. Three tetrads were chosen for analysis of the physiological characteristics of the progeny from the cross between the *ca*-1 and *pmp*-1 mutants. From these analyses (Table 2) it was possible to determine the genotype[1] of each of the progeny using several physiological characteristics. Progeny of the genotype ca^+ *pmp* have characteristics of the parent *pmp*-1 mutant: stringent requirement for CO_2 enrichment, no appreciable inorganic-carbon accumulation, low photosynthetic rate, normal (wild-type) carbonic-anhydrase activity, and normal (wild-type) CO_2 compensation concentration (Spalding et al. 1983b). Progeny with the genotype *ca* pmp^+ have characteristics of the parent *ca*-1 mutant: nonstringent requirement for CO_2 enrichment, abnormally high inorganic-carbon accumulation, low photosynthetic rate, diminished carbonic-anhydrase activity, and very high CO_2 compensation concentration (Spalding et al. 1983a). Characteristics of progeny with the double-mutant genotype, *ca pmp,* were similar to those fo *ca* pmp^+ (carbonic-anhydrase deficient) except that the accumulation of inorganic carbon was only slightly higher than that of wild type. These characteristics of the double mutant were confirmed by the analysis of a nonparental ditype tetrad (tetrad No. 10, Table 2) in which both CO_2-requiring progeny must be double mutants.

More detailed physiological analyses were carried out with one of the tetratype tetrads (tetrad 12) consisting of progeny representing all four possible genotypes: ca^+ pmp^+ (wild type), ca^+ *pmp* (transport deficient), *ca* pmp^+ (carbonic-anhydrase deficient), and *ca pmp* (double mutant) (Table 2). The CO_2 response curve of photosynthesis for cells from each of the four progeny (Fig. 1) was consistent with the assigned genotype. The responses of *ca* pmp^+, ca^+ *pmp,* and ca^+ pmp^+ were nearly identical to those previously reported for mutant and wild-type strains of the same genotypes (Spalding et al. 1983a, b). The CO_2 response of photosynthesis in the double mutant *ca pmp* was virtually indistinguishable from that of *ca* pmp^+ (carbonic-anhydrase deficient). The maximum photosynthetic rates for cells from all four progeny at saturating CO_2 concentrations were very similar (data not shown). Similar correlations were observed in the O_2 response of photosynthesis and the CO_2 compensation concentration (Table 3). Progeny 12-1 (ca^+ pmp^+; wild type), 12-2 (ca^+ *pmp;* transport deficient) and 12-3 (*ca* pmp^+; carbonic-anhydrase deficient) responded in a manner similar to that of wild type and the parent mutant strains of the same genotypes (Spalding et al. 1983a, b), and the double mutant (*ca pmp;* 12-4) responded very much like *ca* pmp^+ (12-3).

[1] Genotype notation: ca^+ pmp^+ = wild type at both loci; *ca* pmp^+ = mutant at *ca* locus; ca^+ *pmp* = mutant at *pmp* locus; *ca pmp* = mutant at both loci

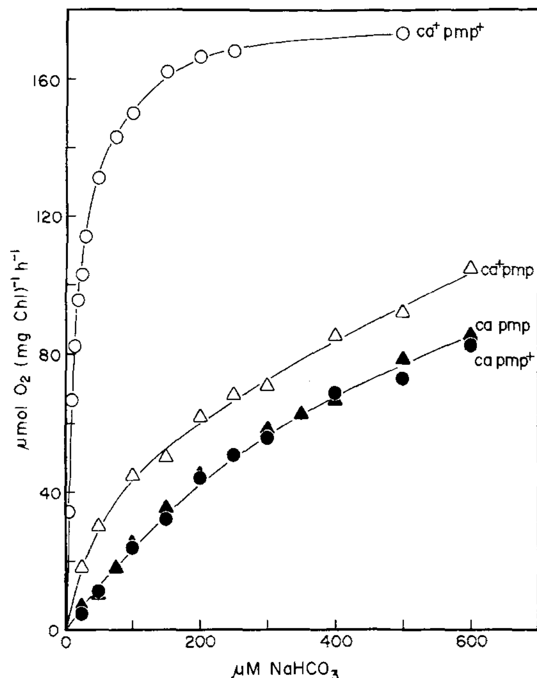

Fig. 1. Response of CO_2-dependent photosynthetic O_2 evolution to $NaHCO_3$ concentration (pH 7.0) in progeny of a tetrad from a cross between the *ca*-1 and *pmp*-1 mutants of *C. reinhardii*

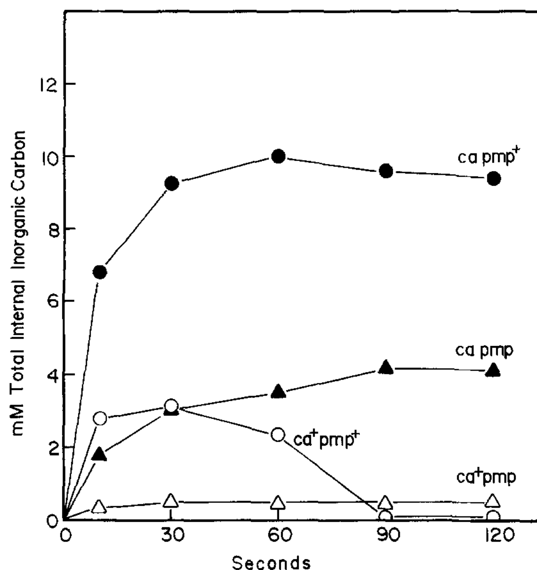

Fig. 2. Time course of internal accumulation of inorganic carbon in progeny of a tetrad from a cross between the *ca*-1 and *pmp*-1 mutants of *C. reinhardii*. The initial external inorganic carbon concentration was 80 µM at pH 7.0

Based on our current understanding of the CO_2-concentrating system of *C. reinhardii*, the photosynthetic rate of the double mutant would be expected to be no higher than that of the carbonic-anhydrase-deficient mutant (*ca pmp*$^+$) at any given external CO_2 concentration. The photosynthetic rate is dependent on the internal CO_2 concentration, which is limited in both *ca pmp*$^+$ and the double mutant (*ca pmp*) by the rate of dehydration of internal bicarbonate to CO_2 as the consequence of a deficiency in carbonic-anhydrase activity (Spalding et al. 1983a). This CO_2 limitation also explains the similarity in O_2 inhibition of photosynthesis between *ca pmp*$^+$ and *ca pmp*, since O_2 inhibition is determined by the internal CO_2 and O_2 concentrations (Laing et al. 1974). It is not evident, however, why the CO_2 compensation concentration for the double mutant was similar to that of the carbonic-anhydrase-deficient mutant, since it is not even clear why the CO_2 compensation concentrations of the carbonic-anhydrase-deficient and transport-deficient mutants are so different (see Spalding et al. 1983a, b). One can speculate, however, that either the abnormal bicarbonate accumulation or the functional disconnection between the bicarbonate and dissolved CO_2 pools inside the cells is somehow responsible for the elevated compensation concentrations in both *ca pmp*$^+$ and *ca pmp*.

The time courses of inorganic-carbon uptake for the progeny of tetrad 12 demonstrate the functional operation of the CO_2-concentrating system in each (Fig. 2). Accumulation of inorganic carbon in *ca*$^+$ *pmp*$^+$ (wild type) was similar to that pre-

Table 3. Effect of O_2 on photosynthetic rate and CO_2 compensation concentration in cells of progeny from a cross between *C. reinhardii pmp*-1 and *ca*-1 mutants

Tetrad progeny	Genotype	CO_2 compensation concentration (µl l^{-1})			Photosynthetic rate[a] (µmol CO_2 mg^{-1} Chl h^{-1})		
		2% O_2	21% O_2	50% O_2	2% O_2	21% O_2	50% O_2
12–1	*ca*$^+$ *pmp*$^+$	<10	<10	<10	142	145	141
12–2	*ca*$^+$ *pmp*	<10	<10	<10	66	37	21
12–3	*ca pmp*$^+$	19	31	244	28	14	4
12–4	*ca pmp*	23	38	225	28	15	9

[a] Measured as CO_2 uptake at 350 µl l^{-1} CO_2 with infra-red gas analysis

Table 4. Products of photosynthetic $^{14}CO_2$ fixation by *C. reinhardii* wild type and a *ca pmp* double mutant. Determinations after 5 min in $NaH^{14}CO_3$ at pH 7.0, 25° C, 700 μmol (photons) m^{-2} s^{-1}, and at an O_2 concentration of 227–315 μM. Cell density was adjusted so that approx. 50% of the CO_2 was fixed in 5 min when the initial $NaHCO_3$ concentration was 100 μM. SMP, Sugar monophosphates; PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate

Fraction	100 μM $NaHCO_3$ (% incorporated ^{14}C)		2.5 mM $NaHCO_3$ (% incorporated ^{14}C)	
	Wild type[a]	*ca pmp*	Wild type[a]	*ca pmp*
Insoluble	49.5	25.3	37.6	46.6
Neutral	2.4	1.2	4.7	2.9
Acids:				
Glycolate	1.0	10.4	0.3	0.3
SMP	10.7	8.7	16.9	11.2
Malate	3.7	10.7	5.4	8.3
PGA	3.1	6.5	5.8	5.4
RuBP	6.0	5.6	1.6	1.9
Amino acids:				
Aspartate	3.0	2.8	3.5	1.6
Glutamate	5.5	3.9	6.1	5.6
Glycine	1.8	7.9	1.5	0.9
Alanine	2.5	5.0	1.1	1.3
Serine	2.4	3.5	3.2	1.9

[a] Strain 2137 *mt*+

viously observed in wild type, in which a maximum level of 2–3 mM internal inorganic carbon was associated with a rapid rate of photosynthesis which quickly utilized all the inorganic carbon in the medium (Spalding et al. 1983a). No substantial inorganic-carbon accumulation occurred in ca^+ *pmp* (transport deficient) and the rate of photosynthesis (Tables 2, 3) was correspondingly low. Reduced photosynthesis and a lack of inorganic carbon accumulation were previously observed in the *pmp*-1 mutant, and arose from a deficiency in the transport of inorganic carbon into the cell (Spalding et al. 1983b). In *ca* pmp^+ (carbonic-anhydrase deficient) the level of internal inorganic carbon was found to be very high, but the photosynthetic rate was low, nevertheless (Tables 2, 3). A similar accumulation of inorganic carbon in the *ca*-1 mutant was concluded to result from accumulation of transported bicarbonate which underwent dehydration to CO_2 very slowly because of a deficiency in carbonic-anhydrase activity (Spalding et al. 1983a).

The maximum inorganic-carbon accumulation in the double mutant *ca pmp* (Fig. 2; Table 2) was slightly higher than that observed in ca^+ pmp^+ (wild type) and previously in wild type (Spalding et al. 1983a, b), but was associated with a much reduced rate of photosynthesis (Tables 2, 3). In this double-mutant strain, the mutation *ca*-1 resulted in a deficiency in carbonic-anhydrase activity (Spalding et al. 1983a) which greatly reduced the rate of dehydration of bicarbonate to CO_2 and led to the accumulation of transported bicarbonate. Since transport of bicarbonate is reduced in the double mutant because of the mutation *pmp*-1 (Spalding et al. 1983b), the accumulation of inorganic carbon is much slower and not as extensive as that seen with the *ca*-1 mutation alone. This observation has been mimicked by chemical inhibition of carbonic anhydrase in the transport-deficient mutant, and the time course of inorganic-carbon accumulation was very similar to that observed in the present study with the double mutant (Spalding et al. 1983b).

The labeling pattern of photosynthetic products for the double mutant compared with the wild type showed increased labeling of the photorespiratory metabolites glycolate, glycine, and serine (Table 4). This labeling pattern is very similar to that of the *pmp*-1 mutant under the same conditions (Spalding et al. 1983b). The pattern of the *ca*-1 mutant, however, was somewhat different, with a much larger accumulation (35% of total) of label found in glycolate (Spalding et al. 1983a) compared with only 10% for the double mutant and 6% for the *pmp*-1 mutant. As was observed previously with each of the individual mutations, the labeling pattern of the double mutant at 2.5 mM $NaHCO_3$ was not substantially different from that of wild type, indicating that the differences observed at 100 μM $NaHCO_3$ were the result of CO_2 limitation in the double mutant (see Spalding et al. 1983a, b). It is not clear why the labeling patterns of the *ca*-1 and *pmp*-1 mutants are different since both appear to be similarly CO_2-limited. It is therefore difficult to assess the significance of the similarity between the labeling patterns of the double mutant and the *pmp*-1 mutant. The most obvious difference between these two and the *ca*-1 mutant is the very high internal inorganic-carbon (presumably bicarbonate) concentration in the *ca*-1 mutant. Since nearly all of the labeled glycolate of the *ca*-1 mutant is found outside the cell, it is possible that the glycolate anion is excreted to offset the large accumulation of anions (bicarbonate) inside the cell. In any event, the labeling data do represent one characteristic in which the double mutant is more similar to the *pmp*-1 than to the *ca*-1 mutant.

Conclusions. In most respects the characteristics of the double mutant (*ca pmp*) of *C. reinhardii* are

consistent with our current understanding of the CO_2-concentrating system of this alga, namely active transport of bicarbonate across the plasma membrane, followed by dehydration of the bicarbonate via carbonic anhydrase to provide a near-saturating internal CO_2 concentration for ribulose 1,5-bisphosphate carboxylase/oxygenase. There are, however, a few characteristics which we do not currently understand. Although the photosynthetic rate at air levels of CO_2 and O_2 of *ca*$^+$ *pmp* (transport deficient) is higher than either *ca pmp*$^+$ (carbonic-anhydrase deficient) or *ca pmp* (double mutant) (Fig. 1), both *ca pmp*$^+$ and *ca pmp* are capable of slow growth without CO_2 enrichment, but *ca*$^+$*pmp* is stringently dependent on CO_2 enrichment for photoautotrophic growth (Table 2). Thus the mutation *ca*-1 overrides in some way the stringent CO_2 requirement conferred by the mutation *pmp*-1. This apparent anomaly may be related to the photosensitivity commonly associated with photosynthesis-deficient mutants (Spreitzer and Mets 1981), since the *pmp*-1 mutant is much more photosensitive when grown on acetate in the light than are either the *ca*-1 mutant or the double mutant (Spalding et al. 1983a, b). This indicates that the *ca*-1 mutation might confer some protection against photosensitivity. Although an undetected suppressor of photosensitivity (Spreitzer and Ogren 1983) might also explain the relatively low photosensitivity of *ca pmp*$^+$ and *ca pmp*, this seems unlikely since no segregation of light-sensitivity and non-light-sensitivity was ever observed in any cross involving the *ca*-1 mutant.

The unexpected masking of the *pmp*-1 stringent CO_2-requiring phenotype by *ca*-1 in the double mutant and the differences between the *ca*-1 and *pmp*-1 mutants with regard to CO_2 compensation concentrations and labeling patterns indicate that we do not fully understand the algal CO_2-concentrating mechanism. A better understanding of how this CO_2-concentrating system operates and how it is regulated may be gained by analyzing additional mutants deficient in components of the system and by evaluating the interactions of these mutations.

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