Reversible Inactivation and Characterization of Purified Inactivated Form I Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase of *Rhodobacter sphaeroides*

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Form I ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) from *Rhodobacter sphaeroides* is inactivated upon the addition of organic acids to photolithoautotrophically grown cultures. Activity recovers after the dissipation of the organic acid from the culture. The inactivation process depends on both the concentration of the organic compound and the nitrogen status of the cells. The inactivated RubisCO has been purified and was shown to exhibit mobility on both nondenaturing and sodium dodecyl sulfate gels different from that of the active enzyme prepared from cells not treated with organic acids. However, the Michaelis constants for ribulose 1,5-bisphosphate and CO₂ or O₂ were not dramatically altered. Purified inactivated RubisCO could be activated in vitro by increasing the temperature or the levels of Mg(II), and this activation was accompanied by changes in the electrophoretic mobility of the protein. When foreign bacterial RubisCO genes were expressed in an *R. sphaeroides* host strain lacking the ability to synthesize endogenous RubisCO, only slight inactivation of RubisCO activity was attained.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) catalyzes CO₂ fixation in most photosynthetic and chemolithoautotrophic organisms (34). When synthesis is fully derepressed, RubisCO may constitute up to 50% of the soluble protein of the cell (32). However, carbon dioxide fixation is energetically costly, and the amount of material expended by the cell for new protein synthesis is also substantial. Thus, it is essential for CO₂ fixation to be regulated. In addition to CO₂ fixation, purple nonsulfur photosynthetic bacteria catalyze another energy-costly process, nitrogen fixation, which has been intensively investigated genetically and biochemically (9, 23, 24, 30). At this time, a full understanding of the regulation of microbial carbon dioxide fixation is lacking. In higher plants, there are two well-described mechanisms to regulate RubisCO activity in vivo. These involve enzyme-mediated activation of native RubisCO by the enzyme RubisCO activase (31, 40) and, in addition, reversible binding of the intracellular inhibitor 2-carboxy-arabinitol-1-phosphate to carbamylated RubisCO (3, 15). In bacteria, there have been periodic indications that RubisCO might be subject to some form of modification or alteration of its activity in vivo (17, 21, 34), and recent work with the photosynthetic bacterium *Rhodospirillum rubrum* suggests a covalent phosphorylation (26); in this latter study, however, no information relative to how phosphorylation affected RubisCO activity was presented. In other photosynthetic bacteria, particularly *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, two different forms of RubisCO are synthesized (12, 13, 33). The form I enzyme resembles the enzyme that is widely distributed in plants, algae, and most bacteria and contains both large (catalytic) and small subunits (L₈S₈); the form II RubisCO is composed only of large subunits which show little homology to form I large subunits (11, 14, 37). At the molecular level, the form I RubisCO genes of *R. sphaeroides*,

des, *rbcL* and *rbcS*, and the form II RubisCO gene of *R. sphaeroides*, *rbpL*, are found in distinct chromosomal operations (10, 11), and there is evidence to support both independent and interdependent regulation of these genes (6, 11, 16, 19). As for the regulation of RubisCO activity in this organism, it was recently found that the form I enzyme is specifically inactivated when metabolizable organic acids are added to cells growing with CO₂ as the sole source of carbon (20).

The results of the present investigation further characterize the inactivation of the form I RubisCO of *R. sphaeroides* both in vivo and in vitro and give further credence to the possibility that this enzyme is subject to a reversible modification of its activity in the cell.

MATERIALS AND METHODS

Reagents. All biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Agarose and Gel Bond film were from FMC Corp. (Rockland, Me.). Sodium [¹⁴C]bicarbonate (20 mCi/mmol) was from Amersham Corp. (Arlington Heights, Ill.).

Organism and growth. *R. sphaeroides* HR, a strain derived from ATCC 17023 (39), and a form II RubisCO deletion mutant generated from the wild type (7) were grown photolithoautotrophically in Ormerod medium (28) under a gas mixture of 1.5% carbon dioxide in hydrogen as previously described (19). The medium was supplemented with 15 µg of biotin and 1 mg each of nicotinic acid and thiamine hydrochloride per liter. *R. sphaeroides* 16 is a RubisCO-negative strain which lacks the ability to synthesize either form I or form II RubisCO (7). This strain may be complemented to photolithoautotrophic growth by genes encoding foreign RubisCO enzymes expressed from a recently described promoter system (7) or by gene clusters containing foreign RubisCO genes and their original promoters (8). Plasmid pCD102 from *Xanthomonas flavus*, containing the *rbcL* and *rbcS* genes as well as other structural genes important for

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CO₂ fixation and their promoter, was a gift from W. G. Meijer (27). Plasmid pAE312, containing the chromosomally encoded *Alcaligenes eutrophus* *rbcL* and *rbcS* genes and an upstream promoter, was provided by Kjell Andersen (1). Plasmid pRPS-75, containing the *Anacystis nidulans* (*Synechococcus* sp. strain 6301) *rbcL* and *rbcS* genes inserted in a RubisCO expression vector (7), was provided by Deane Falcone of this laboratory. Photosynthetic growth under photolithoautotrophic growth conditions for each plasmid-*R. sphaeroides* 16 construct was performed as previously described (7); pyruvate or water was then added to control cultures. In addition, plasmid pJG336, which contains the form I CO₂ fixation structural gene operon and upstream regulatory sequences of *R. sphaeroides*, (11) was used to complement *Rhodospirillum rubrum* I-19, a RubisCO deletion strain of this organism (8).

Cell permeabilization and crude extract preparation. Photolithoautotrophically grown cells were treated with cetyltrimethyl ammonium bromide to a final concentration of 100 µg/ml. Samples were then incubated in the light for at least 5 min, and the cells were then centrifuged at $3,000 \times g$ at 4°C, resuspended in buffer A (25 mM Tris-HCl [pH 7.2], 1 mM EDTA, 2 mM dithiothreitol, 10 mM MgCl₂). For each assay, 10-µl samples of the cetyltrimethyl ammonium bromide-permeabilized cell suspension were used.

For the preparation of crude extracts, late-exponential-phase cells were harvested by centrifugation at $12,000 \times g$ at 4°C, washed in buffer A, and then resuspended in the same buffer to yield a 20-fold-concentrated cell suspension. The concentrated suspension was then passed twice through a French press at 18,000 lb/in². Cell debris was removed by centrifugation for 15 min at $17,000 \times g$ in a Beckman J2-21 centrifuge at 4°C. The supernatant was then centrifuged at $100,000 \times g$ for 1 h if necessary.

RubisCO inactivation. Freshly prepared stock solutions of filter-sterilized pyruvate or α-ketoglutarate were injected into the culture at the desired time. Equal volumes of water or growth medium were added to control cultures.

Purification of inactivated form I RubisCO. Pyruvate-treated cells were lysed in Mg(II)-free buffer A and loaded onto a green A-agarose column equilibrated with the same buffer as previously described (20); the green A-agarose column (3 by 12 cm) was attached to a DEAE-cellulose column (3 by 15 cm) such that the wash effluent from the green A-agarose column was immediately adsorbed onto the DEAE-cellulose column. After the inactivated form I RubisCO enzyme was loaded onto the DEAE-cellulose column, the green A-agarose column was disconnected and the DEAE column was washed with 200 ml of Mg(II)-free buffer A; elution of the inactivated form I RubisCO with a 0.1 to 0.5 M NaCl gradient in the same buffer followed. Peak enzyme-containing fractions were collected and loaded directly onto a 0.2 to 0.8 M sucrose density gradient as previously reported (20). Form I-containing fractions from the sucrose gradient were then subsequently purified by fast protein liquid anion-exchange chromatography by using a Mono Q HR 10/10 column; the purified inactivated form I RubisCO was eluted with a 0 to 0.4 M NaCl gradient in 50 mM Tris-HCl buffer (pH 8.0) and stored at -70°C. All of the steps were performed at 4°C, except that involving the Mono Q column which was run at room temperature.

RubisCO activity assay. In situ RubisCO activity was measured in permeabilized cells and in crude extracts in vitro at pH 7.2 as previously described (35, 41). One unit of RubisCO activity is defined as the amount of enzyme needed to fix 1 µmol of CO₂ per min at 30°C. All assays represent

duplicate or triplicate determinations. Kinetic constants were obtained by well-described methods at a pH of 8.0 (29).

Quantitation of form I and form II RubisCO in crude extracts. The amount of form I and form II RubisCO enzyme present in crude extracts was determined by rocket electroimmunoassay as previously described (19).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed by published procedures (22) under nondenaturing (6% acrylamide) or denaturing (11% acrylamide) conditions in the presence of sodium dodecyl sulfate (SDS).

Other assays. The concentration of ammonia in the culture was assayed by the method of Chaney and Marbach (4). The concentration of pyruvate was determined by the enzyme-coupled method of Damm et al. (5). Protein concentrations were determined by the method of Lowry et al. (25) with bovine serum albumin as the standard.

RESULTS

Reversible inactivation of RubisCO by pyruvate and α-ketoglutarate in vivo. Previous work had shown that form I RubisCO from *R. sphaeroides* is inactivated after switching cells from photolithoautotrophic to photoheterotrophic growth conditions (20). In this earlier study, inactivation appeared to be irreversible. However, the results presented here indicate that inactivation is reversible but is dependent on both the nitrogen status of the cells and the concentration of the organic carbon added to the culture. In the presence of 5 mM pyruvate, 65 to 70% of the RubisCO activity was lost within the doubling of a culture of strain HR; about 5 h after the initial rapid decrease in activity, there was a marked and steady increase in RubisCO activity which approached the levels of activity found in a culture not treated with pyruvate (Fig. 1). During the time that inactivation and reactivation occurred, the intracellular levels of RubisCO protein paralleled those obtained for a culture not treated with pyruvate, in agreement with previous results (20). In addition, the addition of puromycin did not influence recovery of enzymatic activity (Fig. 2A and D), yet this compound obviously affected gross protein synthesis as manifested by the prevention of increases in cell density (Fig. 2C and F) and marked changes in the level of RubisCO protein (Fig. 2B and E). The inability to demonstrate reversibility in previous experiments by using chloramphenicol appears to be due to the fact that this compound itself leads to a rapid decrease in form I RubisCO activity (data not shown). Reactivation in a form II RubisCO deletion mutant was also independent of the effects of puromycin on de novo protein synthesis, although the inactive enzyme appeared to be unstable between 4 and 8 h after the addition of puromycin and the removal of pyruvate in this strain (Fig. 2D). Considering that there are two RubisCO enzymes in strain HR and the ratio of the form I enzyme to the form II enzyme in this organism is known (19), 70% inactivation of the total RubisCO activity in wild-type strain HR suggests that the form I enzyme has been inhibited to a considerable extent.

These findings led us to carefully examine the culture conditions employed for organic acid-mediated reduction of RubisCO activity. When cells taken from the mid-exponential phase of growth ($A_{660} \leq 0.6$) were used, RubisCO activity in the form II deletion mutant was reduced by more than 90% by either α-ketoglutarate or pyruvate (results not shown). In addition, inactivation was also dependent on the nitrogen status of the cells in that inactivation was irreversible and independent of the presence of pyruvate or α-keto-

FIG. 1. Pyruvate-mediated inactivation of RubisCO in *R. sphaeroides* HR. A culture was grown to mid-exponential phase and split into two separate cultures. At time zero, 5 mM pyruvate was added to one culture (■) and an equal volume of growth medium was added to a second culture (●). At each time point indicated, 10 ml of culture was taken, treated with cetyltrimethyl ammonium bromide, centrifuged, and resuspended in ice-cold buffer A. Samples were then assayed for in situ RubisCO activity as described in Materials and Methods. Activity is expressed in terms of units liter⁻¹ A₆₅₀⁻¹.

glutamate if ammonia was depleted from the culture (initial concentration, 0.5 mM) (Fig. 3A) and/or the organism was grown under ammonia-limiting growth conditions (initial concentration, 5 mM) (Fig. 3B); only in the presence of saturating levels of ammonium (20 mM) was there recovery of form I RubisCO activity to levels that approximated that obtained in a control culture after 24 h (Fig. 3C). These results certainly suggest that the regulation of RubisCO activity depends on the availability of nitrogen. Additional experiments showed that the loss of activity in ammonia-deficient control cultures (Fig. 3A and B) exactly coincided with the depletion of ammonia from the cultures (data not shown).

Time- and temperature-dependent reactivation of inactivated form I RubisCO. Inactivated RubisCO may be separated from vestiges of active form I RubisCO and form II RubisCO after passage of crude extracts through green A-agarose columns (20). It was further found that crude preparations of inactivated form I RubisCO were reactivated in a time-dependent fashion after incubation at 37°C (Fig. 4), and after 90 min the specific activity rose to the value obtained for active crude form I RubisCO obtained from cells not treated with pyruvate. If extra care is taken to rapidly prepare partially purified, inactivated RubisCO from extracts of pyruvate-treated cells, stable specific activities as low as 0.2 U/mg of protein may be obtained, suggesting that the inactivated RubisCO is constantly being reactivated in crude extracts, particularly as the temperature increases, as illustrated for the purified enzyme (see Fig. 7). Inactivated RubisCO was not reactivated by overnight dialysis at 4°C, and the inactivated enzyme was stable indefinitely when crude preparations were frozen at -70°C (20).

Purification of inactivated RubisCO. Because of the pro-

FIG. 2. Reversibility of pyruvate-mediated inactivation of form I RubisCO in the absence of de novo protein synthesis in the wild-type (strain HR) (A, B, and C) and form II RubisCO deletion mutant (D, E, and F) of *R. sphaeroides*. At the cell densities indicated (C and F), 20 mM pyruvate was added. Samples at time zero and after 8 h were taken, treated with cetyltrimethyl ammonium bromide, centrifuged, resuspended in ice-cold buffer A, and assayed for in situ RubisCO activity (A and D) as described in Materials and Methods. The rest of the culture was harvested by centrifugation, resuspended in fresh medium containing 30 mM puromycin, and replaced under growth conditions. Samples were then processed as described above and assayed for in situ RubisCO activity (corresponding to 8.5 and 16.5 h after the addition of pyruvate). All of the procedures for the form II RubisCO deletion strain were the same as those used for the wild-type strain except that two additional samples were taken corresponding to 4 and 12.5 h after the addition of pyruvate. At each time point, samples were also passed twice through a French press and, after centrifugation at 27,000 × g, the supernatant was assayed for RubisCO protein by rocket electroimmunoassay (B and E). Symbols: ●, form I RubisCO protein; ▽, form II RubisCO protein.

pensity of the inactivated enzyme to become activated as crude extracts were processed, we undertook experiments to determine parameters which affected reactivation of inactivated form I RubisCO. Both Mg(II) and temperature were found to influence the reactivation of inactivated form I RubisCO. Thus, Mg(II) was omitted from buffers, and a rapid purification procedure was devised. Because form II RubisCO and active form I RubisCO bind to green A-agarose columns (20), the effluent wash from the green A-agarose column, containing nonadsorbed inactivated form I RubisCO (20), was immediately loaded onto a DEAE-cellulose column by connecting the green A-agarose and DEAE cellulose columns in tandem. This allowed for rapid processing of the inactivated enzyme.

FIG. 3. Loss of RubisCO activity in the presence of 0.5 mM α -ketoglutarate in wild-type *R. sphaeroides*. Levels of ammonium chloride were 0.5 mM (A), 5 mM (B), and 20 mM (C). In all cases, samples were removed from cultures grown in the presence (■) or absence (●) of α -ketoglutarate and subsequently assayed for in situ RubisCO activity. The specific activities of cells at time zero were 12.0, 5.9, and 6.0 U liter⁻¹ A_{650} ⁻¹ in panels A, B, and C, respectively, for cultures not receiving α -ketoglutarate. The specific activities at time zero of cultures supplied with α -ketoglutarate were 11.3, 15.8, and 9.3 U liter⁻¹ A_{650} ⁻¹ in panels A, B, and C, respectively.

After subsequent purification steps involving sucrose density gradient centrifugation and fast protein liquid anion-exchange chromatography, purified inactivated form I RubisCO was obtained. The final specific activity varied, depending on the amount of inactivated enzyme that became reactivated during purification, which, despite the precautions taken, approached from 45 to 55% of the activity of the active form I enzyme.

Characterization of inactivated form I RubisCO. Upon SDS-PAGE, the inactivated form I RubisCO showed considerable aggregation, as significant amounts of the protein barely entered the separating gel. In addition, there was a considerable amount of diffusely stained material between the major RubisCO large-subunit band and the aggregated

FIG. 4. Time-dependent reactivation of inactivated RubisCO in vitro. Extracts ($105,000 \times g$ supernatant fractions) of late-exponential-phase *R. sphaeroides* form II deletion mutant cells that had been treated for 12 h with 10 mM pyruvate (●) or not treated (■) were prepared as described in Materials and Methods. The inactivated RubisCO preparation was further processed through a green A dye affinity column (20). Extracts were incubated at 37°C for the indicated times and then removed for RubisCO determinations.

protein (Fig. 5A). This is in obvious contrast to the active form I RubisCO. In addition, in nondenaturing gels, inactivated form I RubisCO migrated faster than the active form of the enzyme (Fig. 5B). Inactivated form I RubisCO was activated in vitro upon the addition of Mg(II) in a concentration-dependent fashion (Fig. 6), and reactivation was also highly temperature dependent (Fig. 7). Both treatments resulted in conversion of the mobility pattern of the inactivated enzyme to that of the active protein in both SDS (Fig. 5A) and nondenaturing (Fig. 5B) gels. Inactivation of the form I RubisCO did not affect the Michaelis constants for ribulose biphosphate or either of the gaseous substrates, CO₂ or O₂ (Table 1).

Specificity of inactivation system. *R. sphaeroides* 16 is a double-deletion mutant strain incapable of CO₂-dependent growth because of the interruption of the endogenous form I and form II RubisCO genes by antibiotic resistance cartridges. By using a specially constructed promoter-vector, this strain may be complemented to CO₂-dependent growth by expression of foreign RubisCO genes (7). Thus, this host strain and expression vector provided a convenient system to determine the specificity of the *R. sphaeroides* inactivation system for diverse form I RubisCO enzymes. Cyanobacterial RubisCO from *A. nidulans* (*Synechococcus* sp. strain 6301) was found to be expressed at high levels in this system, as previously described (7); however, only 19% inhibition of RubisCO activity was obtained after the addition of 20 mM pyruvate to the culture. *X. flavus* (27) and *A. eutrophus* (1) RubisCO genes were expressed in *R. sphaeroides* 16 by using *Xanthomonas* and *Alcaligenes* promoters, respectively (8). After the addition of pyruvate to the cultures, 27% inhibition was obtained for the *Xanthomonas* enzyme; no inhibition of the *Alcaligenes* enzyme was found. Finally, expression of *R. sphaeroides* form I RubisCO in a

FIG. 5. Electrophoretic analysis of purified active and inactivated form I RubisCO of *R. sphaeroides*. (A) SDS-11% acrylamide gels were electrophoresed at 4°C. Lanes: 1, 6, and 9, active RubisCO; 2, 5, and 8, inactivated RubisCO; 3, inactivated RubisCO treated with 50 mM Mg(II) for 1 h at room temperature; 7, inactivated RubisCO incubated at 37°C for 2 h; 4, commercial protein molecular weight markers. The specific activities of active and inactivated RubisCO were 2.93 and 1.33 U/mg, respectively; 12 µg of active RubisCO and 18 µg of inactivated RubisCO were loaded. (B) Nondenaturing gels (6% acrylamide) were electrophoresed at 4°C. Lanes: 2, 4, 7, and 9, 6 µg of purified active RubisCO (specific activity, 2.93 U/mg); 6 and 8, 9 µg of purified inactivated RubisCO (specific activity, 1.33 U/mg); 1, 3, and 5, 13.4 µg of partially purified inactivated RubisCO (specific activity, 0.67 U/mg). In lane 1, the sample was treated with 50 mM Mg(II) for 1 h at room temperature; in lane 5, the sample was incubated at 37°C for 2 h.

RubisCO deletion strain of *R. rubrum* (8) resulted in no inactivation after the addition of pyruvate to the culture, suggesting that either the inactivation system is not present in *R. rubrum* or the mutation in the host strain somehow disrupts or affects the expression of the inactivation system if present.

DISCUSSION

Previous studies had shown the regulation of form I and form II RubisCO synthesis in *R. sphaeroides* to be complex.

FIG. 6. Mg(II)-dependent reactivation of inactivated RubisCO. Incubation was at room temperature for 1 h prior to assay. The initial specific activities for active (■) and inactivated (●) RubisCO were 2.93 and 1.33 U/mg, respectively.

Under certain conditions, the production of each enzyme is independently controlled (19); in other instances, it may be shown that there are interdependent interactions that modulate control of the expression of the form I and form II RubisCO genes within the two CO₂ fixation structural gene operons of this organism (6, 11, 16). As for the regulation of enzyme activity, it was apparent that only form I RubisCO became inactivated upon the addition of organic compounds to autotrophically grown cultures (20). In this study, we found that the addition of as little as 0.5 mM pyruvate or

FIG. 7. Temperature-dependent reactivation of purified inactivated RubisCO. Incubations were performed at 37°C (▲), 25°C (■), and 4°C (●). The initial specific activity of the inactivated RubisCO was 1.33 U/mg.

TABLE 1. Kinetic constants of purified active and inactivated form I RubisCO of *R. sphaeroides*

RubisCO	K_{RuBP} (μM)	K_{CO_2} (μM)	K_{O_2}	V_{CO_2} (μmol of CO_2 fixed/min/mg)
Active	15.1 ± 2.6	84.0 ± 8.7	640.0 ± 143.0	2.4 ± 0.4
Inactivated	16.0 ± 4.9	60.0 ± 5.0	806.0 ± 101.0	1.3 ± 0.1

α -ketoglutarate had a substantial effect on RubisCO activity and nearly all of the form I RubisCO activity was lost after the addition of 5 mM pyruvate or α -ketoglutarate. This is supported by the 70% inhibition of the total activity of strain HR (Fig. 1), where form II RubisCO is not affected (20). Furthermore, inactivation occurred by some reversible process in vivo since activity recovered soon after the removal of pyruvate in the presence of the protein synthesis inhibitor puromycin. This is also supported by results obtained with the form II deletion strain. It is reasonable to speculate that the inactivation-reativation process somehow depends on the nitrogen status of the cells, reminiscent of the ammonia switch-off, switch-on regulation of nitrogenase activity of purple nonsulfur photosynthetic bacteria (24, 42). Certainly, as demonstrated in Fig. 3, recovery from the pyruvate-mediated inactivation of form I RubisCO is dependent on the level of available ammonia. At low levels of ammonia, activity did not recover; but, at saturating levels, activity rapidly recovered upon depletion of pyruvate from the culture. By using a specific glutamine synthetase inhibitor (2), methionine sulfoximine, we have recently shown that recovery from pyruvate-mediated form I RubisCO inactivation is directly related to the activity of glutamine synthetase (38), the major ammonia assimilatory enzyme of this organism.

Reversibility of RubisCO inactivation was clearly shown in vitro. By monitoring the recovery of RubisCO activity in crude extracts, it was possible to demonstrate that temperature and the levels of Mg(II), a dialyzable factor, and a nondialyzable factor contributed to the reactivation of inactivated form I RubisCO. With the omission of Mg(II) from buffers and rapid purification of crude inactivated form I RubisCO through tandemly connected columns, it was possible to eventually obtain purified inactivated enzyme with a specific activity of about 1, usually 30 to 50% of the activity of the purified active form I enzyme. Specific activities in crude extracts as low as 0.1 to 0.2 U/mg were often obtained for the inactivated enzyme. Inevitably, despite the precautions noted above, the enzyme became partially reactivated during purification. Obviously it would be advantageous to be able to isolate and purify enzyme that was nearly totally inactivated. The purified inactivated RubisCO preparation we have obtained thus far may represent either a mixture of active and inactivated enzyme or a total population of inactivated enzyme molecules that has achieved a partial state of reactivation. The former hypothesis is seemingly supported by the results of SDS-PAGE in which the purified inactive RubisCO was resolved into at least three species: a distinct protein band that comigrated with the large subunit of active form I RubisCO; a second protein species that barely entered the gel, indicative of aggregation; and a third diffuse region that was seen between the two defined protein species. Conceivably, the results of SDS-PAGE may also be explained if variable numbers of large or small subunits of each enzyme molecule were modified. The resolution of the

inactivated and activated RubisCO as distinct bands on nondenaturing gels seemingly favors the second hypothesis, namely, that all of the enzyme molecules are partially inactivated. However, the isolated and purified but only partially inactivated enzyme (specific activity, about 1) may represent enzyme molecules of some intermediate state in the reactivation process that comigrate with totally inactivated enzyme molecules, with the result that a single species was observed on nondenaturing gels. Whatever the nature of the inactivation, beyond an effect on V_{max} , the kinetic properties of the inactivated and active enzymes were indistinguishable.

It is apparent that the purified inactivated RubisCO was not stable at temperatures above the ambient, suggesting that inactivation was not a favorable process in vivo and that the inactivated state of the enzyme must be maintained by some negative effector. Current studies are directed at identifying both positive and negative effectors in crude extracts. It is also not clear what the physiological significance of Mg(II)-induced reactivation might be in vitro, unless this effect might be related to physiological fluxes and compartmentation of this metal during growth. The expression studies using the RubisCO deletion mutant of *R. sphaeroides* indicated that there is some specificity of the inactivation system for the form I enzyme of *R. sphaeroides*, since the *Xanthomonas* and cyanobacterial enzymes were only slightly inhibited and the *Alcaligenes* enzyme was not inhibited at all upon the metabolism of pyruvate in an *R. sphaeroides* background. Complementation of the *R. rubrum* RubisCO deletion mutant with a plasmid containing the form I operon (pJG336), followed by the subsequent addition of pyruvate to photolithoautotrophically grown cells, did not result in inhibition of the form I RubisCO enzyme expressed in the *R. rubrum* background. These results may indicate either that *R. rubrum* does not contain an inactivation system similar to *R. sphaeroides*, that the inactivation system of *R. rubrum* might be activated by the mutation, or that there is some product directed by genetic information found on plasmid pJG336 that prevents pyruvate-mediated inactivation in *R. rubrum*. In addition, the results of this experiment may indicate that the genetic information that specifies inactivation of form I RubisCO is not closely linked to the *rbcL* and *rbcS* genes on plasmid pJG336 or, if there is linkage, expression is somehow masked in the *R. rubrum* background.

It is apparent that either form I or form II RubisCO may be deleted without dramatic effects on photosynthetic cell growth (6). From results obtained in our laboratory (7) and by Hallenbeck et al. (16), it appears that cells that lack both RubisCO enzymes will not grow photosynthetically unless an alternate electron acceptor, such as dimethyl sulfoxide, is supplied. This may be analogous to a situation in which the wild-type organism encounters a highly reduced, carbon-rich environment in nature. Thus, perhaps the relatively stable and less regulated form II RubisCO might have evolved to decrease the reduction potential of the environment by catalyzing the transfer of excess electrons to CO_2 . Under these conditions, carbon dioxide fixation evolved, perhaps, as a secondary function. This might also explain why the *R. sphaeroides* form II enzyme and the *R. rubrum* RubisCO are relatively inefficient catalysts compared to the form I *R. sphaeroides* enzyme and other L_8S_8 RubisCOs (18, 34, 36). This scenario, again, is similar to the situation with nitrogenase in photosynthetic bacteria, which catalyzes the evolution of considerable amounts of hydrogen, by using excess

reductant, when cells are grown with glutamate as the nitrogen source (28, 42).

To further understand in vivo inactivation of form I RubisCO, it is apparent that the nature and mechanism of the putative modification must be characterized. Preliminary results suggest that inactivation might involve the action of some intracellular protein, and experiments concerning this and other aspects of intracellular RubisCO modulation are in progress. The demonstration that inactivation is reversible in vivo and depends on important physiological parameters such as the nitrogen status of the cells lends credence to the assumption that this is an important physiological mechanism to regulate RubisCO activity in this organism.

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