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In Situ Assay of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in *Thiobacillus neapolitanus*

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Cells permeabilized with chloroform yielded ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activities nearly equal to those of cell extracts, thus indicating that both cytoplasmic and carboxysomal RuBisCO are functional in situ. The carboxysomal and cytoplasmic RuBisCO both form the CO_2-Mg^{2+}-enzyme ternary complex, as evidenced by stabilization with 2-C-carboxy-D-arabinitol-1,5-bisphosphate (CABP), a potent competitive inhibitor of RuBisCO. The data are consistent with the hypothesis that the carboxysome is functional in carbon dioxide fixation.

All cyanobacteria thus far examined and many but not all chemoautotrophic bacteria contain polyhedral inclusion bodies (6, 19, 23). The inclusions have been isolated from *Thiobacillus neapolitanus*, a number of *Nitrobacter* spp., *Anabaena cylindrica*, *Chlorogloeopsis fritschii*, and *Nitrosomonas* sp., shown to contain ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and thus labeled carboxysomes (6, 7, 9, 13, 19, 20, 22, 23). The organisms also contain cytoplasmic (noncarboxysomal) RuBisCO, but the enzymes appear to be identical (5, 13, 24). RuBisCO is the only enzymatic activity thus far identified in the carboxysome (5, 6, 12, 23). Although considerable information is available regarding the structure and composition of the carboxysome, the function has not been resolved (3, 5, 6, 12, 13, 23). A number of researchers contend that carboxysomal RuBisCO represents an inactive storage form and the activity of the isolated carboxysome is created as an artifact of purification (5, 6, 23). However, evidence is accumulating which supports the theory that the carboxysome is not only active in carbon dioxide fixation but essential for the survival of the organism. Cells of *T. neapolitanus* grown under carbon dioxide limitation produce elevated quantities of RuBisCO, much of which is sequestered into the carboxysomes (1, 2, 10). This is suggestive that the carboxysome is functional in carbon dioxide fixation; i.e., it seems unlikely that cells under a stress condition would spend extensive quantities of nutrients and energy to synthesize the enzyme and then store it in an inactive state. More recently, Holthuijzen (10) demonstrated that these carbon dioxide-starved cells have a carbon dioxide-fixing capacity beyond that expected of the cytoplasmic enzyme. Recently, Friedberg et al. (8) created mutants in a gene located in the 5′-flanking region of the large subunit gene of RuBisCO in *Synechococcus* sp. The cells possess aberrant carboxysomes, and these cultures require elevated levels of carbon dioxide for growth.

T. neapolitanus was grown in a chemostat at a dilution rate of 0.07 to 0.1 h^{-1} in the medium of Vishniac and Santer (26) as described earlier (5). Protein was determined by the method of Lowry et al. (16), using grade I bovine serum albumin as the standard.

2-C-carboxy-D-arabinitol-1,5-bisphosphate (CABP) was synthesized as described by Pierce (18). Ribulose-1,5-bisphosphate (tetrasodium salt), grade I bovine serum albumin, and Sephadex G-75-120 were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium [^{14}C]bicarbonate was purchased from New England Nuclear, Boston, Mass. All other chemicals were from Fisher Scientific, Waycross, Ga.

Cells were made permeable to exogenously supplied substrates by using a modification of a method described by Tabita et al. (25). Cells from 100 ml of culture were collected by centrifugation at 12,000 × *g* for 20 min. The pellet was resuspended in 2.5 ml of cold 50 mM Tris-HCl–1 mM dithioerythritol (TD) buffer at pH 8.0 and placed in a chilled 15-ml conical glass tube. One volume of cold chloroform was added, and the tube was vortexed (Genie vortexer; Fisher Scientific) at full speed for 2.5 min and allowed to stand on ice for 15 min. The upper aqueous phase was removed and used for experimentation.

Cell extracts of both cells and permeabilized cells were prepared by sonication (three 30-s bursts with 60-s cooling periods), using a cell sonic oscillator equipped with a microtip (Heat Systems, Inc.).

RuBisCO activity was determined as described by Snead and Shively (24). A unit of activity equals 1 μmol of CO_2 fixed per min at room temperature.

Carboxysomes were purified by differential and sucrose density gradient centrifugation and preparative agarose gel electrophoresis as described by Cannon and Shively (5).

Purified carboxysomes were dialyzed against large volumes of CO_2-free TD buffer to remove CO_2 and Mg^{2+}. The TD buffer was made CO_2 free by sparging with nitrogen for 1 h at pH 4.0 and then adjusting the pH to 8.0 by the addition of freshly boiled 2 M NaOH. The RuBisCO of the carboxysomes (0.1 to 0.5 mg of protein) was then activated in 20 mM $NaH^{14}CO_3$ (2.25 Ci/mol) and 10 mM $MgCl_2$ in TD buffer for 30 min. A three- to fivefold excess of CABP was added; the mixture was incubated for 1 h and then passed over a Sephadex G-75-120 column (0.7 by 30 cm) equilibrated with TD buffer. Fractions (0.5 ml) were collected, and the radioactivity was determined by scintillation counting (Beckman LS-3133P). Permeabilized cells (3.5 mg of protein) were washed twice in CO_2-free TD buffer and subjected to the same activation and trapping procedure described for carboxysomes except that the Sephadex column was 1.2 by 50 cm.

The permeabilized CABP-treated cells were centrifuged,

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TABLE 1. RuBisCO activity in cells and purified carboxysomes of *T. neapolitanus*

Sample	U of activity/ mg of protein
Whole cells	0
Sonicated cells	0.17
$CHCl_3$-treated (permeabilized) cells	0.16
Permeabilized and sonicated cells	0.15
Permeabilized and CABP-treated (25 mM) cells	
CABP not removed	0
Unbound CABP removed	0
Carboxysomes	1.6
CABP-treated carboxysomes	
CABP not removed	0
Unbound CABP removed	0

resuspended in 1 ml of TD buffer, and sonicated (see above). The cell extract was layered on the surface of a 10 to 65% linear sucrose gradient (12 ml) and centrifuged in a Beckman SW41 rotor at 30,000 rpm for 18 h (20). A control sample of cells was treated identically, excluding CABP treatment. After centrifugation, the gradients were fractionated by means of an ISCO model 6 density gradient fractionator. The radioactivity of each fraction for the CABP-treated cells and RuBisCO activity for the control cells were determined.

Essentially all of the cytoplasmic and carboxysomal RuBisCO of *T. neapolitanus* becomes available to exogenously supplied substrates when the cells are made permeable by chloroform treatment (Table 1). CABP could also freely permeate these cells; i.e., complete inhibition of RuBisCO was observed when assays were performed in the presence of CABP. RuBisCO activity of purified carboxysomes was also found to be totally inhibited when assayed in the presence of CABP. In both permeabilized cells and purified carboxysomes, the inhibition could not be reversed by the removal of unbound CABP, which implies the formation of a stable ternary complex (17, 18).

Carboxysomes activated with $^{14}CO_2$ and Mg^{2+}, treated with CABP, and subsequently passed over a gel filtration column retained bound $^{14}CO_2$, confirming the formation of a stable ternary complex (Fig. 1).

Sucrose density gradient centrifugation of cell extracts created from permeabilized $^{14}CO_2$-Mg^{2+}-RuBisCO-activated cells treated with CABP revealed two peaks of bound $^{14}CO_2$ (Fig. 2). These peaks corresponded to cytoplasmic and carboxysomal RuBisCO as determined from control gradients.

The investigation of carboxysomal RuBisCO activity has been limited to isolated particles (4, 5, 7, 9, 13, 20, 22). It is possible that the isolation procedures alter the carboxysomes such that they become active in carbon dioxide fixation. However, this seems unlikely, since carboxysomes isolated from freshly grown cells of *T. neapolitanus* harvested from the late logarithmic growth phase are very stable (4, 5, 12). Strong detergents or denaturants are required to destroy their structure.

The permeabilized cells described by Tabita et al. (25) for cyanobacteria facilitate the assay of RuBisCO in minimally perturbed cells by allowing the passage of substrates and effectors through the cell wall and membrane. Our results demonstrate that all of the RuBisCO found to be active in sonicated cell extracts is also active in permeabilized cells. This finding supports the contention that sonication does not create carboxysomes active in carbon dioxide fixation. Biedermann and Westphal (3) reported the absence of lipids in the carboxysome monolayer shell. Furthermore, they found no discernible alterations in the structure of chloroform-treated carboxysomes. It is therefore unlikely that the chloroform treatment significantly affects the inclusions. This view is supported by the fact that nearly all of the RuBisCO, both cytoplasmic and carboxysomal, remains active after the chloroform treatment. We were also able to demonstrate that CABP, a potent, essentially irreversible inhibitor of RuBisCO, rendered all of the enzyme inactive.

To be functional, RuBisCO must first be activated by carbon dioxide and a divalent metal cation, e.g., Mg^{2+} (15). This activation results in the formation of a CO_2-Mg^{2+}-enzyme ternary complex. This complex is stabilized by CABP. By using $^{14}CO_2$, one can use this complex to exper-

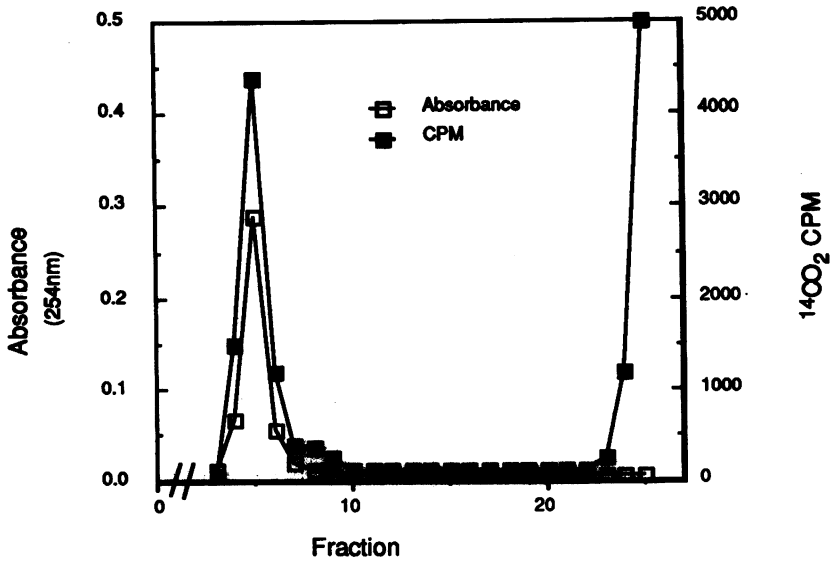

FIG. 1. Formation of the CO_2-Mg^{2+}-CABP complex by purified carboxysomes.

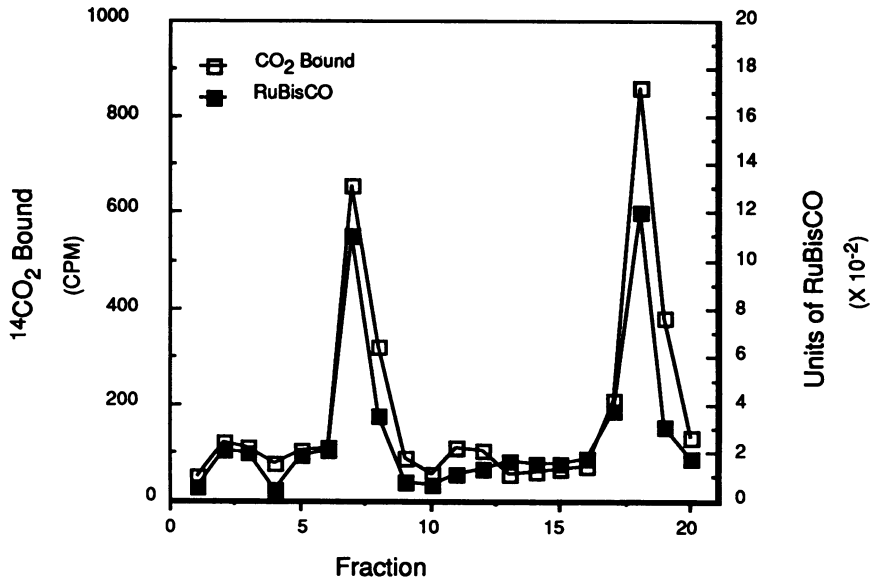

FIG. 2. Separation of $^{14}CO_2$-CABP-labeled cytoplasmic and carboxysomal RuBisCO on a 10 to 65% linear sucrose gradient. The control gradient (fractions assayed for RuBisCO activity) was identical except for the $^{14}CO_2$-CABP treatment. Top of gradients is on the left.

imentally determine the amount of activated enzyme in any given quantity of RuBisCO.

After first showing that the RuBisCO of isolated carboxysomes, as well as purified cytoplasmic RuBisCO (data not presented), formed the ternary complex, we used the permeabilized cells to demonstrate that both RuBisCO forms are components of the complex in situ. Because of other cellular components in the sucrose gradients, it was impossible to determine the $^{14}CO_2$-to-RuBisCO protein ratio; however, the amount of $^{14}CO_2$ bound per unit of RuBisCO activity determined in control gradients (CABP untreated but otherwise identical cells) was very similar for the carboxysomal and cytoplasmic enzymes. It is also worth noting that if one assumes that 62% of the carboxysomal protein is RuBisCO (5), the amount of bound $^{14}CO_2$ to RuBisCO promoter was calculated to be approximately 1.

This study demonstrates that the RuBisCO of carboxysomes in situ is similar to cytoplasmic RuBisCO with regard to both carbon dioxide-fixing potential and the activation process. Our data are consistent with other information which supports the hypothesis that the carboxysome is indeed capable of carbon dioxide fixation, i.e., is a functional prokaryotic organelle (1, 2, 8, 10).

REFERENCES

1. **Beudeker, R. F., G. C. Cannon, J. G. Kuenen, and J. M. Shively.** 1980. Relations between D-ribulose-1,5-bisphosphate carboxylase, carboxysomes and CO_2 fixing capacity in the obligate chemolithotroph *Thiobacillus neapolitanus* grown under different limitations in the chemostat. Arch. Microbiol. **124**:185–189.
2. **Beudeker, R. F., G. A. Codd, and J. G. Kuenen.** 1981. Quantification and intracellular distribution of ribulose-1,5-bisphosphate carboxylase in *Thiobacillus neapolitanus*, as related to possible functions of carboxysomes. Arch. Microbiol. **129**:361–367.
3. **Biedermann, M., and K. Westphal.** 1979. Chemical composition and stability of NB_1 particles from *Nitrobacter agilis*. Arch. Microbiol. **121**:187–191.
4. **Cannon, G. C.** 1982. Carboxysomes and CO_2 fixation in *Thiobacillus neapolitanus*. Ph.D. dissertation. Clemson University, Clemson, S.C.
5. **Cannon, G. C., and J. M. Shively.** 1983. Characterization of a homogeneous preparation of carboxysomes from *Thiobacillus neapolitanus*. Arch. Microbiol. **134**:52–59.
6. **Codd, G. A.** 1988. Carboxysomes and ribulose bisphosphate carboxylase/oxygenase. Adv. Microb. Physiol. **29**:115–164.
7. **Codd, G. A., and W. D. P. Stewart.** 1976. Polyhedral bodies and ribulose 1-5-diphosphate carboxylase of the blue-green alga *Anabaena cylindrica*. Planta **130**:323–326.
8. **Friedberg, D., A. Kaplan, R. Ariel, M. Kessel, and J. Seijffers.** 1989. The 5'-flanking region of the gene encoding the large subunit of ribulose-1-5-bisphosphate carboxylase/oxygenase is crucial for growth of the cyanobacteria *Synechococcus* sp. strain PCC 7942 at the level of CO_2 in the air. J. Bacteriol. **171**:6069–6076.
9. **Harms, H., H. P. Koops, H. Martiny, and M. Wullenweber.** 1981. D-ribulose-1,5-bisphosphate carboxylase and polyhedral inclusion bodies in *Nitrosomonas* spec. Arch. Microbiol. **128**: 280–281.
10. **Holthuijzen, Y. A.** 1986. Carboxysomes in *Thiobacillus neapolitanus*. Ph.D. dissertation. University of Groningen, Groningen, The Netherlands.
11. **Holthuijzen, Y. A., J. F. L. van Breemen, W. N. Konings, and E. F. J. van Bruggen.** 1986. Electron microscopic studies of carboxysomes of *Thiobacillus neapolitanus*. Arch. Microbiol. **144**:258–262.
12. **Holthuijzen, Y. A., J. F. L. van Breemen, J. G. Kuenen, and W. N. Konings.** 1986. Protein composition of the carboxysomes of *Thiobacillus neapolitanus*. Arch. Microbiol. **144**:398–404.
13. **Lanaras, T., and G. Codd.** 1981. Structural and immunoelectrophoretic comparison of soluble and particulate ribulose bisphosphate carboxylases from the cyanobacterium *Chlorogloeopsis fritschii*. Arch. Microbiol. **130**:213–217.
14. **Lanaras, T., A. M. Hawthorn, and G. A. Codd.** 1985. Localization of carbonic anhydrase in the cyanobacterium *Chlorogloeopsis fritschii*. FEMS Microbiol. Lett. **26**:285–288.
15. **Lorimer, G. H.** 1981. The carboxylation and oxygenation of ribulose-1,5-bisphosphate: the primary events in photosynthesis and photorespiration. Annu. Rev. Plant Physiol. **32**:349–385.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Miziorko, H. M., and R. C. Sealy.** 1980. Characterization of ribulose bisphosphate carboxylase-carbon dioxide-divalent cation-carboxypentitol bisphosphate complex. Biochemistry **19:**

1167–1172.
18. **Pierce, J., N. E. Tolbert, and R. Barker.** 1980. Interaction of ribulosebisphosphate carboxylase/oxygenase with transition-state analogs. Biochemistry **14**:934–942.
19. **Shively, J. M.** 1974. Inclusion bodies of prokaryotes. Annu. Rev. Microbiol. **28**:167–187.
20. **Shively, J. M., F. Ball, D. H. Brown, and R. E. Saunders.** 1973. Functional organelles in prokaryotes: polyhedral inclusions (carboxysomes) of *Thiobacillus neapolitanus*. Science **182**:584–586.
21. **Shively, J. M., F. L. Ball, and B. W. Kline.** 1973. Electron microscopy of the carboxysomes (polyhedral bodies) of *Thiobacillus neapolitanus*. J. Bacteriol. **116**:1405–1411.
22. **Shively, J. M., E. Bock, K. Westphal, and G. C. Cannon.** 1977. Icosahedral inclusions (carboxysomes) of *Nitrobacter agilis*. J. Bacteriol. **132**:673–675.
23. **Shively, J. M., D. A. Bryant, F. C. Fuller, A. E. Konopka, S. E. Stevens, Jr., and W. R. Strohl.** 1988. Functional inclusions in prokaryotic cells. Int. Rev. Cytol. **113**:35–100.
24. **Snead, R. M., and J. M. Shively.** 1978. D-Ribulose-1,5-bisphosphate carboxylase from *Thiobacillus neapolitanus*. Curr. Microbiol. **1**:309–314.
25. **Tabita, F. R., P. Caruso, and W. Whitman.** 1978. Facile assay of enzymes unique to the Calvin cycle in intact cells, with special reference to ribulose-1,5-bisphosphate carboxylase. Anal. Biochem. **84**:462–472.
26. **Vishniac, W., and M. Santer.** 1957. The thiobacilli. Bacteriol. Rev. **21**:195–213.