Biochimica et Biophysica Acta, 341 (1974) 187–194

BBA 67172

D-RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE IN *CHLOROBIUM THIOSULFATOPHILUM* TASSAJARA

F. ROBERT TABITA*, BRUCE A. McFADDEN** and NORBERT PFENNIG

Graduate Program in Biochemistry and Department of Chemistry, Washington State University, Pullman, Washington 99163 (U.S.A.) and Institute of Microbiology University of Göttingen, Göttingen (West Germany)

(Received October 22nd, 1973)

SUMMARY

D-Ribulose-1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) was readily detected in extracts of *Chlorobium thiosulfatophilum* Tassajara after transatlantic air delivery. The enzyme, which was moderately unstable, had a specific activity of 7 nmoles CO_2 fixed/min per mg protein when assayed in the presence of 4 mM EDTA. It could be purified 45-fold in one step by centrifugation into a 0.2–0.8 M sucrose density gradient. The enzyme had a molecular weight of $3.61 \cdot 10^5$ when compared with standards after sedimentation into sucrose density gradients. This oligomer of the enzyme was composed of six polypeptide chains (M_r approx. 53 000) revealed by gel electrophoresis in the presence of sodium dodecylsulfate. There was no evidence for a smaller, second type of subunit observed for larger ribulose-1,5-bisphosphate carboxylases.

INTRODUCTION

In 1966 Evans et al. [1] proposed a new ferredoxin-dependent carbon-reduction cycle in *Chlorobium thiosulfatophilum* Tassajara. This cycle involved two new reductive carboxylations catalyzed by pyruvate and α-ketoglutarate synthases and several steps of the tricarboxylic-acid cycle. In addition it required the conversion of pyruvate to phosphoenolpyruvate, carboxylation of the latter, and cleavage of citrate to oxaloacetate plus acetate. All enzymes of the cycle were apparently present in extracts although the presence of citrate lyase was not unequivocally established. Glutamate, presumably derived from the intermediate α-ketoglutarate, contained 75% of the label after 30 s $^{14}CO_2$ fixation in the light by suspensions of *C. thiosulfatophilum*. Unfortunately, the kinetic experiments of Evans et al. did not include fixation times of less than 30 s. Thus, early rates of labeling of glutamate versus 3-phosphoglycerate were not assessed rendering it impossible to evaluate the relative contributions of the reductive carboxylic-acid and Calvin cycles [2]. In earlier experiments on an unspecified

* Present address: Department of Microbiology, University of Texas, Austin, Texas 78712 (U.S.A.)

** The author to whom inquiries should be addressed.

strain of the same species, Smillie et al. [3] reported the presence of all catalysts in the Calvin cycle. It was, therefore, of interest when Buchanan et al. [4] further implicated the reductive carboxylic-acid cycle in CO_2 fixation by *C. thiosulfatophilum* Tassajara, a strain said to be deficient in D-ribulose-1,5-bisphosphate carboxylase.

We now report the occurrence of ribulosebisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) in *C. thiosulfatophilum* Tassajara. Also described are several characteristics of the enzyme including its stability, molecular weight, and quaternary structure.

METHODS

Growth and breakage of the organism

C. thiosulfatophilum Tassajara now called *C. limicola* forma specialis *thiosulfatophilum* [5] strain 6230 (Tassajara) was grown in the absence of organic material with sulfide and thiosulfate as electron donors [6] at 28 °C and a light intensity of 500 ft-candles. Cells were harvested from 22 l of medium in the late exponential phase of growth by centrifugation (approx. 25 g wet wt), resuspended with approx. 50 ml of fresh sulfide medium, and shipped by air freight from Göttingen, West Germany to Pullman, Washington, U.S.A., where all further studies were conducted.

Cells were suspended in an equivalent mass of cold 0.02 M Tris–H_2SO_4 containing 1 mM EDTA, 10 mM $MgCl_2 \cdot 6H_2O$, 5 mM 2-mercaptoethanol, and 50 mM $NaHCO_3$ and which had been adjusted to pH 8.0 at 25 °C. Cells were broken at 2 °C by two passes through a French pressure cell at a pressure of 10 000–15 000 lb/inch^2. The resultant preparation was then centrifuged at 200 000 $\times$ *g* for 60 min at 2 °C and the pellet discarded.

Fractionation and assay for ribulosebisphosphate carboxylase

2 ml of the high-speed supernatant was immediately layered on a discontinuous 0.2–0.8 M sucrose gradient (prepared in the buffer used for cell breakage) consisting of the following steps: 9.0 ml each of 0.2, 0.4 and 0.6 M sucrose with a cushion of 9.5 ml of 0.8 M sucrose. The gradient was centrifuged at 2 °C in a swinging bucket rotor (SW 27) at 27 000 rev./min (131 000 $\times$ *g* at the samples outside edge) for 20 h. Fractions of 1.0 ml were collected and assayed for ribulosebisphosphate carboxylase and absorbance at 280 nm.

Enzyme activity was normally assayed at 30 °C in 0.25-ml reaction mixtures at pH 8.0 containing the following (in μmoles): Tris–HCl, 16; $MgCl_2$, 5; $NaH^{14}CO_3$, 5; and D-ribulose-1,5-bisphosphate, 0.2. The reaction was initiated by the addition of the latter. After 5 min, the reaction was stopped by addition of 0.1 ml of 60% trichloroacetic acid. After several hours to ensure release of unfixed $^{14}CO_2$, samples were counted in a Beckman liquid scintillation counter [7]. One unit of enzyme catalyzed ribulose bisphosphate-dependent CO_2 fixation of 1 μmole CO_2/min. Specific activity is: units enzyme/mg protein [8].

Molecular weight

The molecular weight of ribulosebisphosphate carboxylase from *C. thiosulfatophilum* Tassajara was estimated from sedimentation in sucrose gradient using the relationship: $s_1/s_2 = (M_{r1}/M_{r2})^{\frac{2}{3}}$. This equation derives from the fact that most

enzymes are roughly spherical. The relationship shown between the sedimentation coefficient, s, and molecular weight, M_r, is approximately correct [9].

In all cases s was the known sedimentation coefficient, $s_{20,w}$, of highly purified enzyme standards. The $s_{20,w}$ for the enzyme from *C. thiosulfatophilum* Tassajara was then approximated from its position in the gradient with respect to the standard [10] and became s_2 in the equation shown. The estimate of s_2 in this manner assumes the same partial specific volumes, $\bar{v}$ values, of standards and the unknown. Standards employed were ribulosebisphosphate carboxylase from *Hydrogenomas eutropha* [11], and *Rhodospirillum rubrum* [12] and isocitrate lyase from *Pseudomonas indigofera* [13, 14]. Enzymes from these sources have $\bar{v}$ values of 0.728, 0.727 and 0.730 ml/g, respectively [11, 15, 16]. The $\bar{v}$ values of all ribulosebisphosphate carboxylases examined to date are nearly identical because of the similarities in amino acid composition [17, 18].

Quaternary structure

Gel electrophoresis of native ribulosebisphosphate carboxylases was performed on polyacrylamide gel columns polymerized from 7.5% acrylamide by the general procedure of Davis [19]. Runs were conducted at pH 9.5 (instruction booklet for the Model 6 system of Canalco, Canal Industrial Corp., Rockville, Md.). A current of less than 3 mA/gel was applied at room temperature until a tracking dye (bromphenol blue) was approx. 0.2 cm from the bottom of the gels. Gels were stained with aniline black and destained essentially as described earlier [20].

The subunit structure of the enzyme from *C. thiosulfatophilum* was examined by comparing electrophoretic mobility in sodium dodecylsulfate-containing gels with those for protein standards [21].

RESULTS

Enzyme properties

Ribulosebisphosphate carboxylase was readily detectable in the high-speed supernatant fraction from *C. thiosulfatophilum* Tassajara after transatlantic shipment of cells. The specific activity was in the range of 2.0–4.4 munits/mg. However, no activity could be detected after storage of this fraction at 2 °C for 3 days.

The following specific activities (munits/mg protein) were observed in the freshly prepared high-speed supernatant fraction supplemented as indicated: control (no additions), 4.4; with 4 mM EDTA, 6.8; with 8 mM dithiothreitol, 4.5; with 4 mM EDTA plus 8 mM dithiothreitol, 6.6.

Ribulosebisphosphate carboxylase could be highly purified by simply centrifuging the high-speed supernatant fraction into the 0.2–0.8 M discontinuous sucrose density gradient. The elution profile is shown in Fig. 1. Enzyme in the peak tube had a specific activity of 310 munits/mg when assayed in the presence of 4 mM EDTA.

The enzyme from the gradient showed a broad activity maximum from pH 7.0 through 8.6. The activity was unaffected by 0.1–1.0 mM 6-phospho-D-gluconate in the enzyme-assay mixture supplemented with 4 mM EDTA.

Molecular weight

The profiles of three standard proteins and ribulosebisphosphate carboxylase

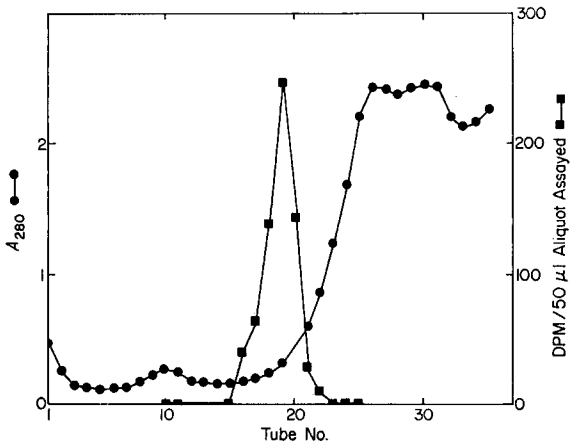

Fig. 1. Fractionation of ribulose bisphosphate carboxylase in the 0.2–0.8 M discontinuous sucrose density gradient. Absolute count rates (dpm) shown on the right ordinate, which are proportional to enzyme activity, have been corrected for background radiation.

from *C. thiosulfatophilum* Tassajara in sucrose density gradients are shown in Fig. 2. From the positions in the gradients, the sedimentation coefficient (in Svedberg units) of the test enzyme was 13.2, 15.8 and 14.2 when compared to the enzyme from *R. rubrum*, *H. eutropha* and isocitrate lyase from P. *indigofera*, respectively. The molecular weights with respect to proteins from each of these three sources were $3.50 \cdot 10^5$, $3.58 \cdot 10^5$ and $3.76 \cdot 10^5$, respectively. The average molecular weight was therefore

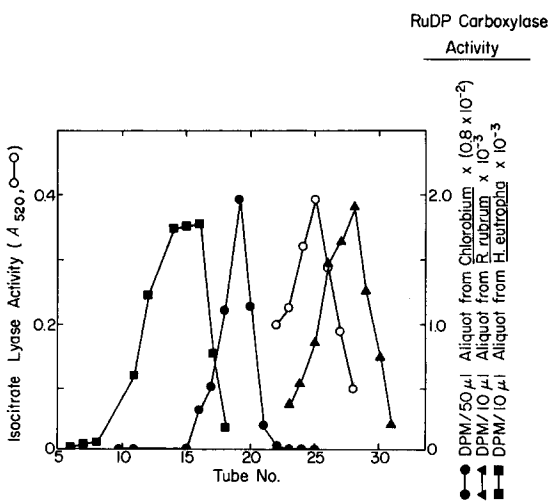

Fig. 2. Distribution of enzymes in the 0.2–0.8 M discontinuous sucrose density gradient. Isocitrate lyase and ribulosebisphosphate (RuDP) carboxylase from *C. thiosulfatophilum* Tassajara were centrifuged into the same gradient. Under identical conditions, ribulosebisphosphate carboxylases from *R. rubrum* and *H. eutropha* were also each centrifuged and collected from gradients. On the left ordinate, $A_{520\ nm}$ is proportional to the activity of isocitrate lyase [14]. On the right ordinate, presentation of data is analogous to that for Fig. 1.

$3.61 \cdot 10^5$ establishing that the enzyme from this green sulfur bacterium was intermediate in size between those from *R. rubrum* (M_r 114 000) [16] and the hydrogen bacteria ($M_r > 500\,000$) [11]. Tending to confirm the intermediate size was the position of the enzyme isolated from the gradient with respect to the other two after electrophoresis in gels polymerized from 7.5% acrylamide (Fig. 3). The enzyme from *C. thiosulfatophilum* Tassajara is nearly pure (Fig. 3) although a light protein load was analyzed.

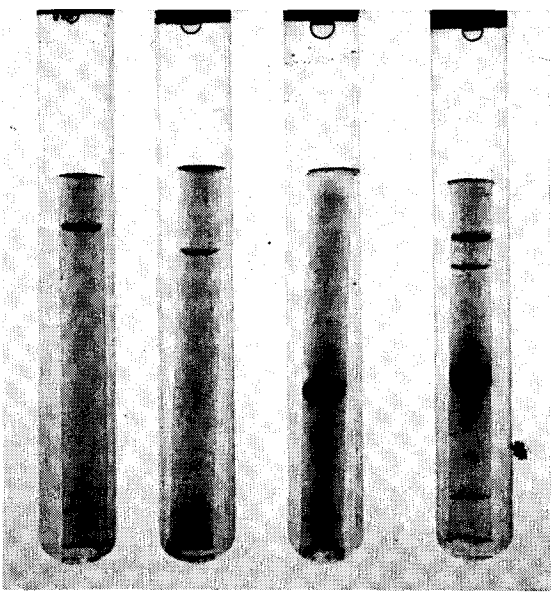

Fig. 3. Gel electrophoretograms of enzymes from peak tubes of gradients depicted in either Fig. 1 or 2. Tracking dye is evident near the bottom of the gels after electrophoresis in gels polymerized from 7.5% acrylamide. Enzymes and amounts added to the gel were from (left to right) *H. eutropha* (10 μg), *C. thiosulfatophilum* Tassajara (10 μg), *R. rubrum* (15 μg), and all three sources (i.e. a mixture).

Quaternary structure

In Fig. 4a the mobilities of several standards and the test enzyme are compared after dissociation and electrophoresis in sodium dodecylsulfate–polyacrylamide gels. The molecular weight of the major protein found after dissociation of ribulosebisphosphate carboxylase from *C. thiosulfatophilum* Tassajara was 53 000. Of particular interest is the fact that this enzyme did not contain a small subunit as evidenced by the lack of a dissociation product in the molecular-weight range of $1 \cdot 10^4$–$2 \cdot 10^4$. Other minor bands presumably reflect impurities.

DISCUSSION

Our finding that *C. thiosulfatophilum* Tassajara contains ribulosebisphosphate carboxylase is entirely consistent with kinetic studies of $^{14}CO_2$ fixation by intact cells [4]. In radioisotopic studies conducted at more physiological concentrations of HCO_3^- and related species (CO_2 and CO_3^{2-}), phosphoglycerate was the principal labeled

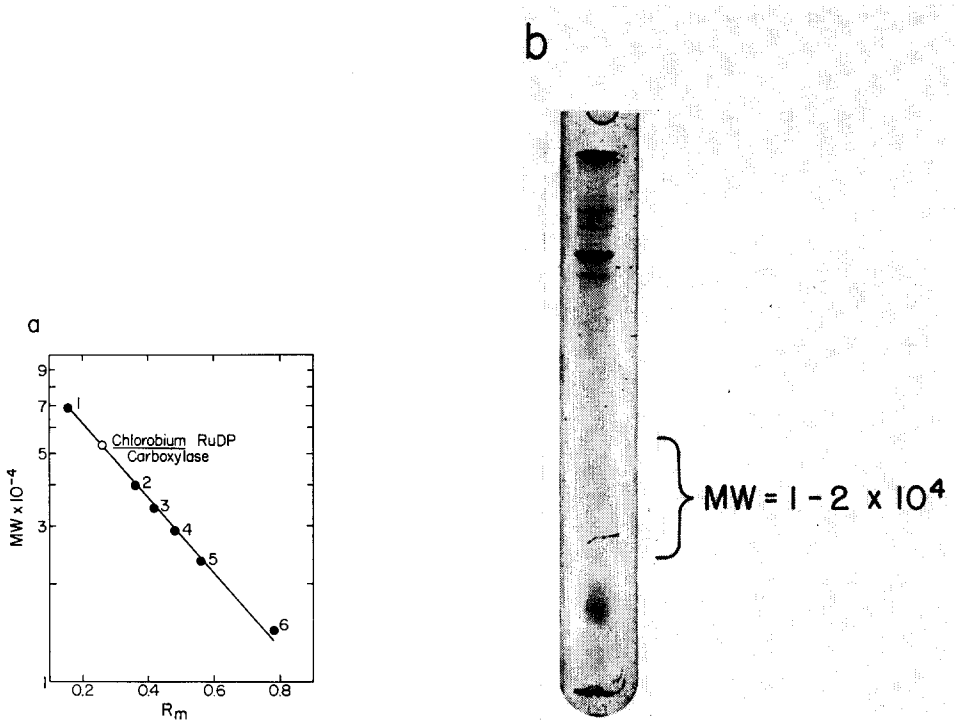

Fig. 4. (a) Log molecular weight versus electrophoretic mobility (R_m) of standards and ribulosebisphosphate (RuDP) carboxylase in polyacrylamide gels containing sodium dodecylsulfate. Standards were: 1, bovine serum albumin; 2, aldolase; 3, pepsin; 4, trypsin; 5, carbonic anhydrase and 6, lysozyme. (b) The photograph to the right shows the gel electrophoretogram after dissociation and electrophoresis of 20 μg of ribulosebisphosphate carboxylase from *C. thiosulfatophilum* Tassajara. Diffuse tracking dye is near the gel bottom. The region corresponding to a M_r of $1 \cdot 10^4$–$2 \cdot 10^4$ is shown next to the gel.

product in the shortest fixation time of 5 s. Moreover, the percentage of radioactivity incorporated into phosphoglycerate plotted against time revealed the steepest negative slope further suggesting that it was the primary product. Buchanan et al. [4] nevertheless suggested that phosphoglycerate was formed via the reductive carboxylic-acid cycle, perhaps mainly because they could not detect ribulosebisphosphate carboxylase in the extracts. Our data establish that the enzyme is present but fairly unstable. Thus the present evidence plus kinetic data strongly suggest that the Calvin cycle and not the reductive carboxylic-acid cycle is the route of CO_2 fixation by one (and perhaps all) species of the green sulfur bacteria. Consistent with this was the recent failure to detect citrate lyase, a key enzyme in the reductive carboxylic-acid cycle, in autotrophically grown *C. thiosulfatophilum* Tassajara [22]. The status of the reductive carboxylic-acid cycle in both heterotrophically and autotrophically grown photosynthetic bacteria has been reviewed quite recently [23].

Our results suggest that ribulosebisphosphate carboxylase comprises about 2% of the soluble protein in *C. thiosulfatophilum* Tassajara. The final specific activity of the enzyme of 0.31 units/mg is lower than that for any other ribulosebisphosphate

carboxylase found to date [23]. In view of the questionable purity and known instability of the present enzyme preparation the value mentioned must be regarded as minimal.

Ribulosebisphosphate carboxylases from a variety of organisms fall into three size categories: (small, M_r approx. 120 000; intermediate, M_r approx. 350 000 and large, M_r approx. 500 000) [18, 23, 24]. Enzymes of small or intermediate size are not inhibited by 6-phospho-D-gluconate [25], a metabolite which probably regulates the activity of ribulosebisphosphate carboxylase in some autotrophs [25, 26]. It is of interest that the enzyme from *C. thiosulfatophilum* Tassajara, which has a molecular weight of about 350 000, is not inhibited by 6-phospho-D-gluconate. Also of interest is the apparent quaternary structure of enzyme from this source. From most sources, ribulosebisphosphate carboxylase consists of two types of subunits ($M_r \cong$ 55 000 and 15 000) [23]. However, it has recently been established that the enzyme from *R. rubrum* (M_r 114 000) is composed of one subunit type (M_r 56 000) [16]. The enzyme described in the present communication also apparently contains one type of subunit. Presumably 6 polypeptides (M_r 53 000) comprise a stable oligomer. Only one other ribulosebisphosphate carboxylase investigated to date may be composed of one type of subunit and this is from the hydrogen bacteria. However, we are reinvestigating the quaternary structure of this enzyme in light of its higher molecular weight, complex dissociation [11], and known inhibition by 6-phospho-D-gluconate [25].

The present rapid and extensive purification of ribulosebisphosphate carboxylase from one of the green sulfur bacteria is successful because the protein has a higher sedimentation coefficient than those of the bulk soluble proteins [27]. This approach coupled with more satisfactory stabilization of the enzyme should open the way to more detailed investigations. Ribulosebisphosphate carboxylase from this source is of deep evolutionary interest because of its intermediate size and unusual quaternary structure.

ACKNOWLEDGEMENTS

We thank NIH for Post-doctoral (1 F02-GM 51026) and Special (1 F03-GM 53698) Fellowships to F.R.T. and B.A.M., respectively. This research was supported by NIH grant GM 19972-04 to B.A.M.

REFERENCES

1 Evans, M. C. W., Buchanan, B. B. and Arnon, D. L. (1966) Proc. Natl. Acad. Sci. U.S. 55, 928–934
2 Calvin, M. (1962) Science 135, 879–889
3 Smillie, R. M., Rigopoulos, N. and Kelly, H. (1962) Biochim. Biophys. Acta 56, 612–614
4 Buchanan, B. B., Schürmann, P. and Shanmugan, K. T. (1972) Biochim. Biophys. Acta 283, 136–145
5 Pfennig, N. and Trüper, H. G. (1971) Int. J. Syst. Bacteriol. 21, 11–14
6 Lippert, K. D. and Pfennig, N. (1969) Arch. Mikrobiol. 65, 29–47
7 Bray, G. A. (1960) Anal. Biochem. 1, 279–285
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
9 Schachman, H. K. (1959) Ultracentrifugation in Biochemistry, Academic Press, Incorporated, New York
10 Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379

11 Kuehn, G. D. and McFadden, B. A. (1969) Biochemistry 8, 2403–2408
12 Tabita, F. R. and McFadden, B. A. J. Biol. Chem., in the press
13 McFadden, B. A. and Howes, W. V. (1963) J. Biol. Chem. 238, 1737–1742
14 McFadden, B. A. (1969) Meth. Enzymol. 13, 163–170
15 Shiio, I., Shiio, T. and McFadden, B. A. (1965) Biochim. Biophys. Acta 96, 114–122
16 Tabita, F. R. and McFadden, B. A. J. Biol. Chem., in the press
17 Cohn, E. J. and Edsall, J. T. (1943) in Proteins, Amino Acids and Peptides (Cohn, E. J. and Edsall, J. T., eds), pp. 370–381, Reinhold, New York
18 Kawashima, N. and Wildman, S. G. (1970) Annu. Rev. Plant Physiol. 21, 325–358
19 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
20 Kuehn, G. D. and McFadden, B. A. (1969) Biochemistry 8, 2394–2402
21 Weber, K., Pringle, J. R. and Osborn, M. (1972) Meth. Enzymol. 26, 3–27
22 Beuscher, N. and Gottschalk, G. (1972) Z. Naturforsch. 27b, 967–973
23 McFadden, B. A. (1973) Bacteriol. Rev., 37, 289–319
24 Anderson, L. E., Price, G. B. and Fuller, R. C. (1968) Science 161, 482–484
25 Tabita, F. R. and McFadden, B. A. (1972) Biochem. Biophys. Res. Commun. 48, 1153–1159
26 Chu, D. K. and Bassham, J. A. (1972) Plant Physiol. 50, 224–227
27 Goldthwaite, J. J. and Bogorad, L. (1971) Anal. Biochem. 41, 57–66