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Molecular and Catalytic Properties of Ribulose 1,5-Bisphosphate Carboxylase from the Photosynthetic Extreme Halophile *Ectothiorhodospira halophila*

F. ROBERT TABITA AND BRUCE A. McFADDEN*

*Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712, and Program in Biochemistry and Biophysics in the Department of Chemistry, Washington State University, Pullman, Washington 99163**

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D-Ribulose 1,5-bisphosphate (RuBP) carboxylase has been purified from the photosynthetic extreme halophile *Ectothiorhodospira halophila*. Despite a growth requirement for almost saturating sodium chloride in the medium, both crude and homogeneous preparations of RuBP carboxylase obtained from this organism were inhibited by salts. Sedimentation equilibrium analyses showed the enzyme to be large (molecular weight: 601,000). The protein was composed of two types of polypeptide chains of 56,000 and of 18,000 daltons. The small subunit appeared to be considerably larger than the small subunit obtained from the RuBP carboxylase isolated from *Chromatium*, an organism related to *E. halophila*. Amino acid analyses of hydrolysates of both *E. halophilia* and *Chromatium* RuBP carboxylases were very similar. Initial velocity experiments showed that the *E. halophila* RuBP carboxylase had a K_m for ribulose diphosphate of 0.07 mM and a K_m for HCO_3^- of 10 mM. Moreover, 6-phospho-D-gluconate was found to markedly inhibit the *E. halophila* carboxylase; a K_i for phosphogluconate of 0.14 mM was determined.

In recent years, considerable interest has focused on the key enzyme of the reductive pentose phosphate pathway (Calvin cycle)-D-ribulose 1,5-bisphosphate (RuBP) carboxylase (3-phospho-D-glycerate carboxylase [dimerizing], EC 4.1.1.39). This enzyme, which catalyzes primary carbon dioxide fixation in all autotrophic species (8), is composed of large subunits, each of about 55,000 daltons, and, in addition, in eukaryota also contains smaller subunits of approximately 15,000 daltons (for a recent review see reference 9). The presence of the large subunit is invariant among all organisms that assimilate carbon dioxide as their sole or major source of carbon. Recent investigations, however, have shown the small subunit to be absent from the protein isolated from the prokaryota *Rhodospirillum rubrum* (19) *Chlorobium limicola f. thiosulfatophilum* (20), and blue-green algae (21, 22). In contrast, recent work by Akazawa et al. has shown that *Chromatium vinosum*, a photosynthetic purple sulfur bacterium, contains a two-subunit type RuBP carboxylase with the larger subunit having a molecular weight of 56,000 (1). The function of the small subunit, at this time, is unknown, although this group has proposed that the small subunit has a regulatory function (12, 13, 23).

In the present communication we describe the RuBP carboxylase from *Ectothiorhodospira halophila*, an organism that is in the same family, the *Chromatiaceae*, as is *Chromatium*. Physiologically, *E. halophila* is of special interest in that it is the only extremely halophilic photosynthetic bacterium thus far described.

MATERIALS AND METHODS

Reagents. All biochemicals and enzymes were purchased from Sigma Chemical Co. $Na^{14}CO_3$ (20 mCi/mmol) was purchased from Nuclear-Chicago Corp. All more common compounds were reagent grade quality. Purity of chemicals as indicated by the vendors was taken into account in calculating concentrations.

Growth of *E. halophila*. A culture of *E. halophila* was provided by J. Raymond and maintained in the thiosulfate-succinate media of Raymond and Sistrom (14) to which was added 0.5% $NaHCO_3$. Inocula for 1-liter bottles were cultured in completely filled 75-ml screw-cap tubes; one 75-ml preculture served to inoculate a 1-liter prescription bottle of the above medium. Culture bottles were placed in a transparent water bath maintained at 45 C and incubated at this temperature before banks of 100-Watt incandescent bulbs at a light intensity of 200-foot candles. Cultures were harvested between the mid- to late-exponential phase of growth. The resultant cell paste was then washed in TEMMB buffer (pH 8.0,

25 C) containing 20 mM tris(hydroxymethyl)aminomethane-SO_4, 1 mM disodium ethylenediaminetetraacetic acid, 10 mM $MgCl_2 \cdot 6H_2O$, 5 mM 2-mercaptoethanol, and 50 mM $NaHCO_3$ supplemented with 4.0 M NaCl and stored at −20 C until needed.

Purification of ribulose 1,5-bisphosphate carboxylase. Upon thawing, the cell paste was suspended in an equal volume of TEMMB and passed two times through a chilled French pressure cell at 2 C at a pressure of 10,000 to 15,000 lb/in^2. Unbroken cells and debris were removed by centrifugation at 30,000 × *g* for 30 min at 2 C, and the supernatant fluid was decanted. The supernatant fluid was then subjected to ultracentrifugation at 105,000 × *g* for 60 min. The resulting deep red supernatant fraction was then carefully removed with a Pasteur pipette and dialyzed against TEMMB. NaCl was not required to maintain enzyme stability as substantiated in Results. Two milliliters of the high-speed supernatant fraction were immediately layered onto a discontinuous 0.2 to 0.8 M sucrose gradient (prepared in TEMMB) as described previously (16). After centrifugation at 2 C for 20 to 24 h in the SW27 rotor, fractions of 1.0 ml were collected and assayed for RuBP carboxylase activity and absorbance at 280 nm.

Assay. The assay for RuBP carboxylase was as previously described (10). One enzyme unit is the amount of enzyme catalyzing the RuBP-dependent carboxylation of 1 μmol of RuBP in 1 min at 30 C and pH 8.0. Specific activity is expressed as units per milligram of protein. The method of Lowry et al. (7) was used to estimate protein.

Electrophoresis. The procedures for polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate(SDS)-disc gel electrophoresis were as described in a previous report (18).

Molecular weight determination. Sedimentation to equilibrium using the high-speed meniscus-depletion method of Yphantis (26) was used to determine the molecular weight of the *E. halophila* RuBP carboxylase. Experiments were conducted as previously reported for the molecular weight determination of *Hydrogenomonas* RuBP carboxylase (6). The value for the partial specific volume of *E. halophila* RuBP carboxylase was calculated from the amino acid composition (15) and was determined to be 0.729.

Amino acid analyses. Prior to amino acid analysis, RuBP carboxylase from *E. halophila* was exhaustively dialyzed against four changes of 1,000 volumes of Pyrex glass-distilled water. Aliquots of the dialyzed enzyme solution were then transferred to hydrolysis vials. Hydrolysis was performed in 6 N HCl in vacuo under N_2 at 100 to 113 C for 12, 24, and 48 h. All hydrolysates were analyzed with a Beckman 121 automatic amino acid analyzer. Half-cystine and methionine were determined after performic acid oxidation of the protein for 3 h at 0 C followed by acid hydrolysis (4).

RESULTS

Purification of RuBP carboxylase. Using the procedure of sucrose density gradient centrifugation, RuBP carboxylase was easily purified to a state of apparent homogeneity (Fig. 1). It was found that omission of NaCl from the TEMMB buffer had no effect upon the enzyme in the crude cell-free preparation or in the course of purification. Peak fractions recovered in a yield of 43% had a specific activity of 1.34, representing about a 90-fold purification over that of the 105,000-g supernatant fraction (usually 0.012 to 0.016). RuBP carboxylase therefore comprises about 1% of the total "soluble" protein in *E. halophila* when grown as described if it is assumed that there are no activators or inhibitors in the soluble fraction which are removed during purification. The product obtained was homogeneous by the criterion of polyacrylamide gel electrophoresis (Fig. 1). One stained band was obtained in gels polymerized from several concentrations of acrylamide and upon electrophoresis at pH 7.0 (not shown).

Molecular weight. Results obtained from sedimentation equilibrium studies (Fig. 2) provided additional evidence for the homogeneity

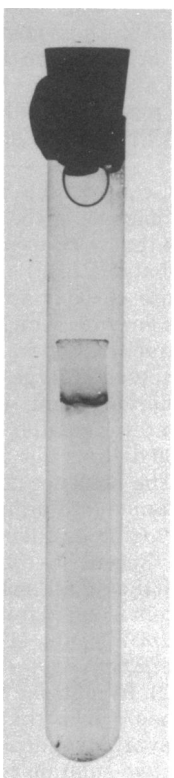

FIG. 1. *Ribulose bisphosphate carboxylase stained with aniline blue-black after electrophoresis in gel polymerized from 7.5% acrylamide. Migration was toward the anode disposed at the bottom of the gel column.*

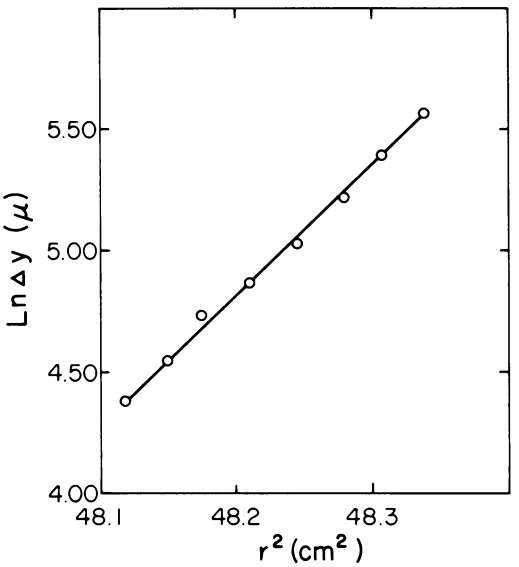

FIG. 2. *Distribution of ribulose bisphosphate carboxylase in the bottom half of a solution column after sedimentation at 12,000 rpm at 20 C to equilibrium. Fringes in the top half of the solution column were flat.*

of the *E. halophila* RuBP carboxylase. Using these data, the molecular weight for this protein was calculated to be 601,000 ± 1.7% using a $\bar{v}$ of 0.729 calculated from the amino acid analysis. These results establish that this enzyme has the highest molecular weight of any RuBP carboxylase thus far described (9, 10).

Quaternary structure. The *E. halophila* RuBP carboxylase was denatured in the presence of SDS and 2-mercaptoethanol and then subjected to SDS-electrophoresis according to the procedure of Weber et al. (24). The electrophoretogram indicated the presence of two kinds of polypeptide chains in the native protein (Fig. 3). The band for the smaller polypeptide was broader because of its greater mobility; moreover, although it was definitely Coomassie-blue positive, the stained peptide lost color during gel storage. The mobilities of the two polypeptides were compared to several standards (Fig. 3) to estimate the molecular weights of the two dissimilar subunits. In addition, homogeneous RuBP carboxylase from *Chromatium vinosum*, also prepared by sucrose density gradient centrifugation (16), was dissociated along with the standards and the *E. halophila* enzyme. The results (Fig. 3) show the large subunit of the enzyme from *E. halophila* to exhibit a molecular weight of 56,000, whereas the small subunit has a molecular weight of 18,000. These data are in contrast to the results obtained for the dissociated *Chromatium* enzyme, which has a 56,000-dalton large subunit but a small subunit of only 15,000 daltons.

Amino acid analysis. Amino acid analyses are depicted in Table 1. It is evident that the *E. halophila* carboxylase closely resembles the *C. vinosum* enzyme in composition, with the possible exception of glutamate, half-cystine, and methionine.

Catalytic properties. Initial velocity studies and subsequent double reciprocal plots show the *E. halophila* RuBP carboxylase to exhibit the following kinetic constants: K_m for RuBP = 0.07 mM and K_m for HCO_3^- = 10 mM. Of interest was an examination of the mechanism of inhibition by 6-phosphogluconate, a ligand known to inhibit the *E. halophila* enzyme (17). The results of kinetic experiments are shown in Fig. 4. The inhibition with respect to RuBP appears to be of the linear noncompetitive type. Slope and intercept replots were linear. The K_i for phosphogluconate was found to be 0.14 mM.

Effect of salt. Because the *E. halophila* enzyme had been isolated from an extreme halophile, it was of interest to examine the effects of NaCl and KCl on enzyme activity, since, presumably, this organism maintains a high intracellular salt content. Surprisingly, both NaCl and KCl were inhibitory (Fig. 5); 1 M salt caused 90% inhibition of RuBP carboxylase activity. Moreover, the inhibition by NaCl was readily reversible as evidenced by the recovery of normal enzymatic activity when the stock enzyme solution containing 4 M NaCl had been extensively diluted before assay. These results were obtained with the high-speed supernatant (105,000 × *g*) fraction derived from crude extracts as well.

DISCUSSION

E. halophila is the only photosynthetic extreme halophile thus far described. As a photosynthetic organism *E. halophila* must reduce carbon dioxide into utilizable organic matter in order to grow. As part of a study concerned with the mechanism of carbon dioxide assimilation and the evolution of autotrophy, the elucidation of structural and catalytic properties of RuBP carboxylase from *E. halophila* is of interest. The sedimentation equilibrium studies described in the present communication establish that the *E. halophila* carboxylase has a molecular weight of 601,000. The enzyme from *E. halophila* contains two dissimilar polypeptides—a large subunit of 56,000 daltons and a small subunit of 18,000 daltons. Parallel examination of the size of the small subunit from *C. vinosum*, a related member of the *Chromati-*

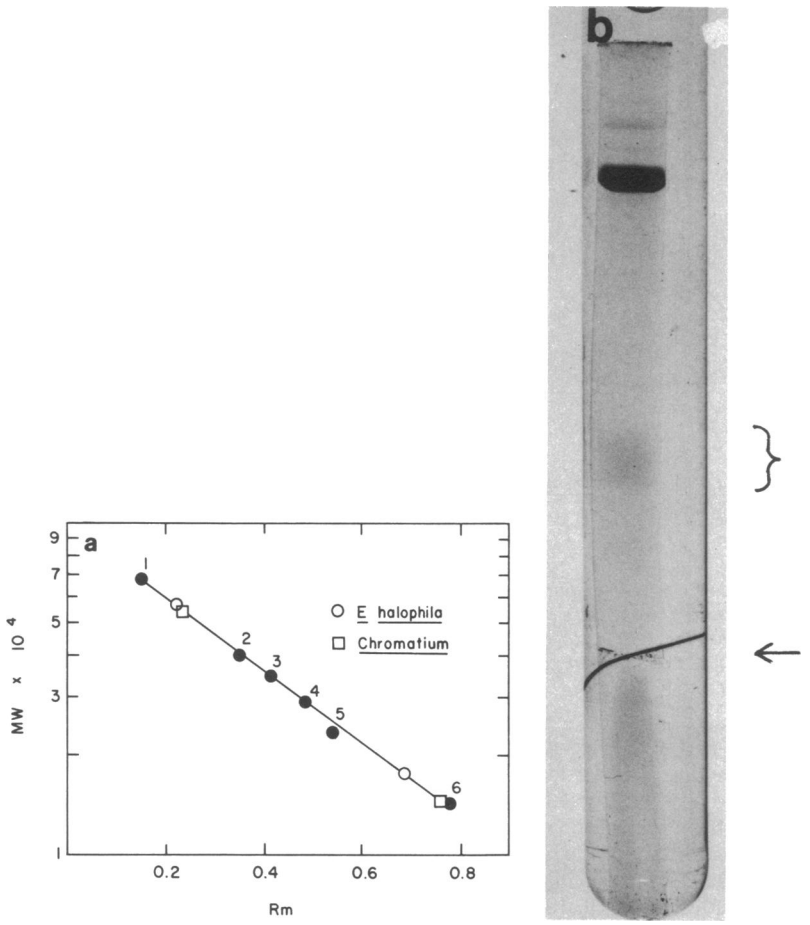

FIG. 3. *(A) Mobilities of standards (closed symbols) and ribulose bisphosphate carboxylase (open symbols) after dissociation with SDS. Standards were: 1, bovine serum albumin; 2, aldolase; 3, pepsin; 4, carbonic anhydrase; 5, trypsin; and 6, lysozyme. (B) Photograph of gel electrophoretogram of SDS-dissociated, Coomassie blue-stained ribulose bisophosphate carboxylase form E. halophila. The smaller polypeptide is bracketed and an arrow marks the ion front.*

aceae, reveals a significantly lower molecular weight of 15,000. If one assumes that there are eight large and eight small subunits, as there are in *Chromatium vinosum* (23) and spinach RuBP carboxylase (25), a calculated molecular weight of 592,000 is obtained for the protein from *E. halophila*, a value quite close to the molecular weight determined by sedimentation equilibrium. A parallel calculation based upon the presently determined subunit molecular weights for *C. vinosum* yields a value of 568,000—in excellent agreement with the directly determined value of 550,000 (1). Thus, it appears that the *E. halophila* carboxylase is larger than the *C. vinosum* enzyme and the enzyme from other sources by virtue of having larger small subunits.

Amino acid analyses show that the *E. halophila* carboxylase closely resembles the *C. vinosum* carboxylase. Moreover, using the statistical technique of Marchalonis and Weltman to compare amino acid compositions (11) an SΔQ value of 14 is found when the *Chromatium* and *E. halophila* proteins are compared. These authors introduced the parameter SΔQ, which is the sum of the squares of individual differences in mole percent content of each amino acid in two proteins. Where comparisons were made for 16 amino acids, the analysis of over 5,000 pairs of proteins revealed that for 98% of unrelated proteins SΔQ was >100. In no case was SΔQ < 50 found for unrelated proteins. The median SΔQ values for various vertebrate hemoglobins, immunoglobins, and cytochrome *c*'s

TABLE 1. *Amino acid composition of RuBP carboxylases from E. halophila and Chromatium D*

Amino acid	*E. halophila*			Residues/ 600,000 daltons[a]	*C. vinosum*			Residues/ 550,000 daltons[a]
	Nanomoles after hydrolysys of				Nanomoles after hydrolysis of			
	12 h	24 h	48 h		12 h	24 h	48 h	
Asp	17.2	15.6	16.7	518	51.3	53.5	55.0	509
Thr	7.5	6.5	7.7	215	23.9	25.3	27.3	244
Ser		8.3	7.3	283[b]	24.2	24.4	25.5	236[b]
Glu	20.8	19.9	21.7	655		56.0	60.4	534
Pro	8.5			267	24.2	24.6	25.6	240
Gly	19.6	17.6	18.6	585	47.8	50.4		499
Ala	17.9	16.2	17.5	541	53.7	54.8		537
Val	7.6	9.2	13.4	421[c]	27.5	31.0	37.6	359[c]
Ile	6.0	6.6	8.2	257[c]	16.9	21.0	27.6	264[c]
Leu	13.8	12.7	13.3	419	34.6	38.3	44.5	427[c]
Tyr	5.3	5.3	5.6	170	14.0	15.0	16.1	144
Phe	5.8	5.9	5.8	183	15.5	16.8	18.3	162
Lys	10.7		8.8	305	25.4	25.0	27.4	248
His		3.8	4.4	129		12.3	13.4	116
Arg	8.6	8.5	9.2	276		31.6	33.9	284
Cys[d]				35				97
Met[d]				239				101

[a] Assumes a 95% recovery of amino acids which is the average recovery obtained for many hydrolysates examined by the analyst. The calculation was made by: multiplying each corrected recovery by the residue molecular weight to yield nanograms of a given residue; summation of the latter for all amino acids to yield mass of protein; division of the latter by molecular weight to yield nanomoles of protein analyzed; estimation of residues per molecule (right-hand column). In this analysis the yield of ammonia in the hydrolysate was ignored.

[b] Residues estimated from extrapolation to zero time.

[c] Residues estimated from the 48-h value.

[d] Residues estimated after performic acid oxidation as described.

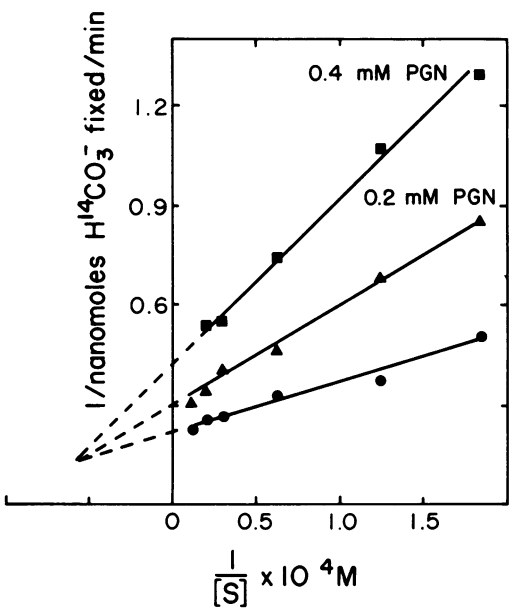

FIG. 4. *Double reciprocal plots of velocity versus substrate concentration in the absence and presence of 6-phospho-D-gluconate (added 5 min prior to initiation of the reaction by addition of RuBP).*

were 80, 30, and 20, respectively. A value of SΔQ < 50 between two proteins is taken to indicate relatedness or homology of primary structure. The lower the SΔQ the greater is the degree of relatedness. The maximum SΔQ obtained for hydrolysates of the same protein analyzed in different laboratories was 4, suggesting that this value is a lower meaningful limit. The value of 14 mentioned with respect to the data in Table 1 implies striking homology between RuBP carboxylase from halophilic and nonhalophilic members of *Chromatiaceae*.

Interestingly, the enzyme from *E. halophila* was strongly inhibited by low concentrations of 6-phospho-D-gluconate. However, the pattern of inhibition obtained with respect to ribulose 1,5-bisphosphate was noncompetitive with respect to RuBP in contrast with the competitive inhibition obtained using spinach and *H. eutropha* RuBP carboxylase (16). Perhaps the relatively large size of the small subunit influences the pattern of inhibition obtained with phosphogluconate. It should be noted that these assays were performed by first preincubating enzyme with saturating levels of magnesium and bicarbonate and fixed variable concentrations of phosphogluconate followed by initiation of ca-

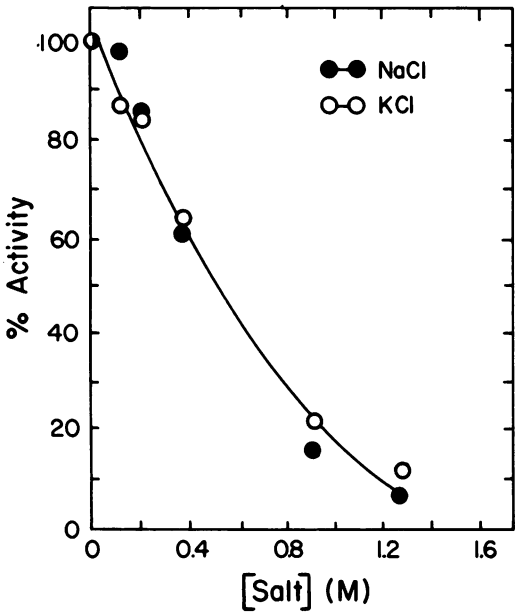

FIG. 5. *Effects of salt added 5 min prior to initiation of the reaction with RuBP.*

talysis with ribulose diphosphate; the order of addition of cofactors, substrates, and inhibitors markedly affects the rate of the reaction (3, 5, 18).

Lastly, the unexpected inhibition by salt of the *E. halophila* RuBP carboxylase, a major enzyme in this organism, is noteworthy. As an extreme halophile, *E. halophila* requires a medium nearly saturated with NaCl in order to grow (14). Moreover, previous workers have shown that species of *Halobacterium* actively concentrate potassium chloride intracellularly and maintain a fairly high intracellular concentration of sodium chloride (2). The inhibition by NaCl and KCl of a soluble enzyme such as RuBP carboxylase may reflect a low intracellular level of these salts within *E. halophila*. In this connection it is of interest that two other soluble enzymes derived from *E. halophila*, nicotinamide adenine dinucleotide phosphate-linked isocitrate and malate dehydrogenases, are similarly inhibited by NaCl. Perhaps, this organism merely requires high concentrations of salt to maintain the structural integrity of the cell envelope. Certainly, more detailed investigations of several other enzymes and a study of the cell envelope of this organism will be of interest.

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