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Ribulose-1,5-bisphosphate carboxylase of thermophilic hydrogen-oxidizing microorganism *Bacillus schlegelii*

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Summary: Ribulose—1,5-bisphosphate carboxylase was isolated from thermophilic hydrogen-oxidizing *Bacillus schlegelii*. Molecular mass of the native enzyme is 560 000 and optimal reaction temperature is 70°C. K_m value for ribulose 1,5-bisphosphate is 0.27 mM. The carboxylase activity of the enzyme is dependent on Mg^{2+} with the optimum at 10 mM. The enzyme is an oligomer of L_8S_8 type with M_r of large subunits and small subunits of 56 000 and 14 000, respectively. Negatively stained enzyme has regular polygonal shape in top view, 12 nm in diameter, with central electron dense patch.

Ribulose-1,5-bisphosphate carboxylases (RuBPCase) have been isolated and characterized to varying extents from more than 50 different prokaryotes. All preparations of RuBPCase are bifunctional enzymes, catalyzing the carboxylation and oxygenation of ribulose-1,5-bisphosphate (1). Most bacterial RuBPCase are high M_r proteins composed of large (55 000-64 000) and small (10 000-15 000) subunits with a total M_r of (500 000-640 000). The large subunits contain the active site for carboxylation and oxygenation (2). The enzyme must be activated at a specific site, distinct from the catalytic site. This activation requires the carbamylation of the ε-amino group of lysine 201 of the large subunits and stabilization with a divalent cation before the catalytic reaction (3).

In autotrophic bacteria a number of reports have led to the general impression that RuBPCase is composed of L_8S_8 structure (4-8). Relatively little information, however, is available on the enzyme from thermophilic bacteria. Thermophilic purple sulfur bacterium *Chromatium tepidum* was shown to produce L_8S_8 RuBPCase stable at the temperature 60°C (9). Crude extracts of RuBPCase of *Thermothrix thiopara* stimulated the ribulose 1,5-bisphosphate dependent fixation of CO_2 at 83°C (10). No information concerning the structure of the enzyme or component involved in thermal stability of the enzyme is available. In the present paper we

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examined molecular and functional properties of RuBPCase from the thermophilic hydrogen oxidizing microorganism *Bacillus schlegelii*. The purified enzyme is an oligomer with the optimum temperature for carboxylation reaction at 70°C.

Materials and Methods

Bacillus schlegelii is an endospore forming hydrogen oxidizing microorganisms, growing in a mineral medium under the atmosphere $H_2/O_2/CO_2$ (7:2:1) at temperature 70°C (11).

Preparation of Ribulose-1,5-bisphosphate carboxylase: Frozen cells were disintegrated by grinding with glass beads and the homogenate was extracted with standard buffer containing 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 20 mM $NaHCO_3$, 20 mM NH_4Cl and 6 mM 2-mercaptoethanol. The mixture was centrifuged at 8 000 xg for 10 min to remove glass and unbroken cells. The supernatant was centrifuged for 30 min at 30 000 xg. Membrane fraction was discarded and supernatant was precipitated with protamine sulphate (0.05 mg/1 mg proteins) and the precipitate was removed by centrifugation at 20 000 xg for 20 min. The supernatant was precipitated by ammonium sulphate to 25% saturation. The mixture was centrifuged at 20 000 xg for 20 min and the sediment was discarded. To supernatant solution ammonium sulphate was added to 40% saturation. The precipitate was collected by centrifugation, solubilized and dialyzed in the standard buffer. After low speed centrifugation to remove precipitated material the solution was introduced to a 5-30% linear sucrose gradient and centrifuged at 22 000 rpm for 24 h in an SW 27 rotor. The fractions sedimenting between 14S and 18S were saved, concentrated and dialyzed in standard buffer without NH_4Cl. The precipitated material was removed and the solution was layered onto 10-65% linear sucrose gradient and centrifuged in an SW50.1 rotor at 30 000 rpm for 18 h and 4°C. Fractions were collected and those containing RuBPCase activity were pooled dialyzed and concentrated by ultrafiltration.

Asay of RuBPCase activity: RuBPCase activity was measured in a 100 μl reaction mixture containing 100 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (0.1 mCi/mmol),0.8 mM ribulose 1,5-bisphosphate by incorporation of $^{14}CO_2$ into acid-stable product (12). The assay mixtures and controls without ribulose 1,5-bisphosphate were incubated at 70°C for 10 min and the reaction was terminated with 50 μl of concentrated HCl.

Electron microscopy: Five μl of suçrose gradient fraction containing enzyme particles were pipetted onto EM grids coated with glow-discharge activated carbon support film (13). After 30 s of adsorption, the excess fluid was removed with filter paper and simultaneously a negative stain (2% uranyl acetate in H_2O) was added. After additional 30 s, the most of the stain was drained off with filter paper and the grids were air dried. The specimens were examined in Philips CM12/STEM electron microscope at a magnification of 45 000 and 80 kV. The magnification was calibrated using ferritin (Koch-Light, horse spleen ferritin) and replica greating (Balzers).

Other methods: For the molecular mass determination gel filtration (14) was used. Antibodies against the RuBPCase from *Alcaligenes eutrophus* was obtained from prof.Bowien (University Gottingen, FRG).

Results

In order to gain a more detailed information on molecular and functional properties of RuBPCace from *Bacillus schlegelii* the enzyme

Table I. Purification of RuBPCase from *Bacillus schlegelii*

Fraction	Proteins	Total activity	Specific activity	Recovery
	mg	U	U/mg	%
Membrane free supernatant	1052	94,7	0.090	100.0
Protamine sulphate supernatant	782	89.1	0.114	94.2
Ammonium sulphate precipitate	205	75.1	0.366	79.2
First sucrose gradient	42	34.4	0.82	36.4
Second sucrose gradient	14	18.5	1.32	19.5

Units of the enzyme activity are expressed as $\mu mol^{14}CO_2$ incorporated min^{-1}.

was isolated and purified to near homogeneity by the modified method of Bowien at al. (15). Individual purification steps are shown in Table I. Precipitation of proteins with protamine sulphate and ammonium sulphate greatly reduced total proteins in the active fraction. After two sucrose density gradients, more than 95% pure enzyme was obtained (Fig.1A slot 3).

Molecular mass and subunits composition. The native enzyme migrate in 4% polyacrylamide gel (in absence of SDS) as a single band (Fig.1B). Molecular mass of native enzyme estimated by gel filtration on Sephacryl S-300 HR was calculated to be M_r = 560 000. After denaturation of the

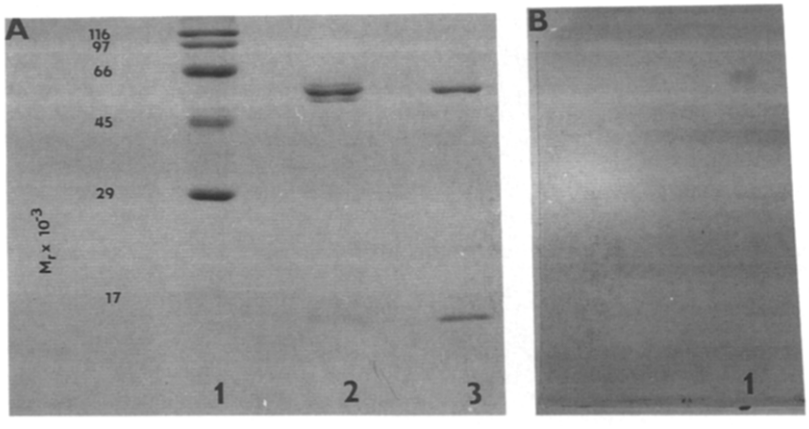

Fig.1A. SDS-PAGE electrophoresis of pooled fractions of RuBPCase from sucrose gradients. (1)-Standards of M_r, RuBPCase from the first (2), and second (3) sucrose gradients.

Fig.1B. Electrophoretic analysis of native RuBPCase under non-denaturing conditions in 4% polyacrylamide gel. (1)-RuBPCase of *B. schlegelii*.

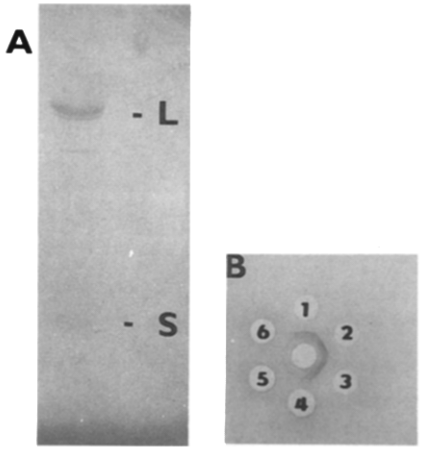

Fig.2A. Immunoblotting of the large and small subunits of RuBPCase of B. schlegelii. Large (L) and small (S) subunits were separated on the SDS-PAGE and transfered to nitrocellulose membrane. The binding of antiRuBPCase antibody of *A. eutrophus* to RuBPCase of *B. schlegelii* was identified using peroxidase-labelled second antibody.

Fig.2B. Double immunodiffusion of RuBPCase preparations of A. eutrophus and B. schlegelii. The central well contained antiRuBPCase antibody of *A. eutrophus* and peripheral wells (1-3) 1,2 and 5 μg of RuBPCase of *A. eutrophus*. Wells (4-6) 1,2 and 6 μg RuBPCase of *B. schlegelii*.

enzyme for 5 min at 100°C in 10% SDS-1% 2-mercaptoethanol two polypeptides of M_r = 56 000 and 14 000 were identified on SDS-PAGE (Fig.1 slot 3). These results suggest that the enzyme from *B.schlegelii* is an oligomer of eight large and eight small subunits (L_8S_8). The molar ratio of S and L subunits is about 1:1. The polypeptides were transferred to nitrocellulose membrane and tested for immunological reactivity with antibody against RuBPCase of the mesophilic bacterium *Alcaligenes eutrophus*. Result of immunoblots (Fig.2A) and double diffusion assay (Fig.2B) indicated structural similarities in both large and small subunits of RuBPCase from mesophilic and extreme thermophilic cells examined. Carboxylase activity of RuBPCase is dependent on the presence of Mg^{2+} with the optimum at 10 mM. Other divalent cations (Mn^{2+},Co^{2+} or Ca^{2+}) at 5 mM have no effect on the activity and at 10 mM Mn^{2+} supported only 25% of the Mg^{2+} dependent carboxylase activity. K_m for carboxylase activity is 0.27 mM. This value is lower than K_m values obtained for the enzymes from mesophilic sources (8,9).

Molecular properties and thermal stability of the enzyme. On electron micrographs of the negatively stained enzyme (Fig.3), two distinct subpopulations of molecular images can be recognized: top views (ring shaped, end-on views) and side views (rectangular structures, side-on views). The top views are characterized by regular polygonal

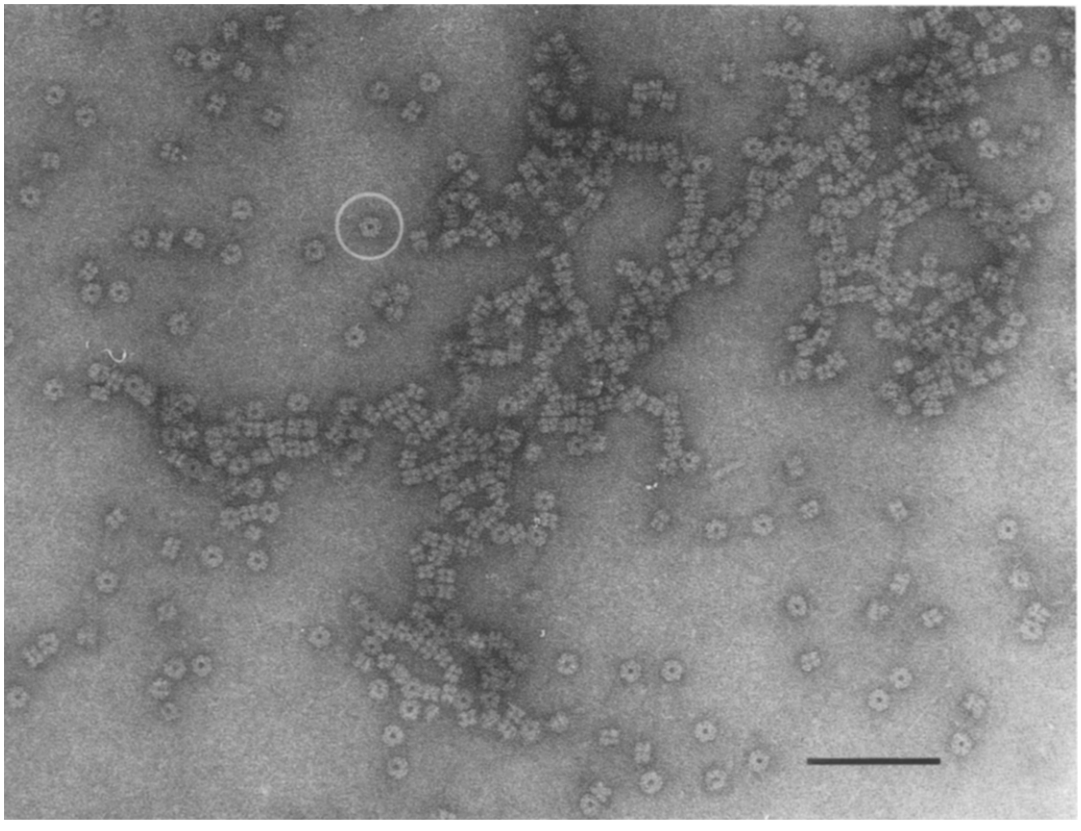

Fig.3. **Negatively stained RuBPCase of *B. schlegelii*.** The typical top view of RuBPCase is circled. The scale bar, 100 nm.

shape, 12 nm in diameter, with central electron dense patch. The side views are approximatelly rectangular, 10 nm in length and 12 nm in width, with a characteristic four-part projection. The particles tend to form long chains and/or aggregates. No changes in these two subpopulations of molecular images were found in samples prepared after incubation of the enzyme at 70°C.

As shown in Fig.4 the minimum temperature for enzyme activity is 40°C. Under in vitro conditions the optimum temperature for the carboxylase activity of the enzyme is 70°C. This temperature correspond to optimum temperature for growth (70°C) of *B. schlegelii*. The enzyme is rapidly inactivated at temperature higher than 75°C.

Discussion

The data presented in this paper show that RuBPCase of *B. schlegelii* is a thermally stable protein with the optimum activity at 70°C. The purification procedure employed involve two sucrose gradient

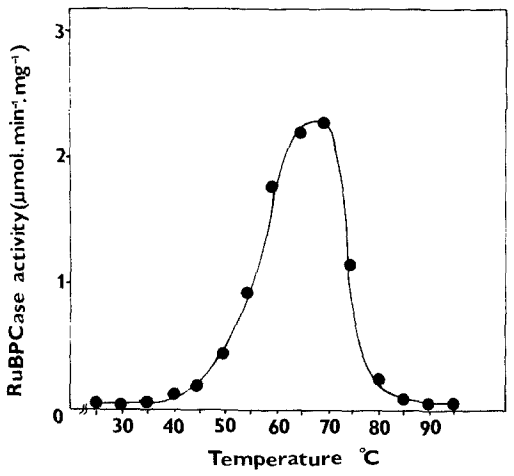

Fig.4. Thermal stability of RuBPCase of B. schlegelii. The enzyme was preincubated for 10 min at 25°C to 95°C. The reaction was initated by ribulose-1,5-bisphosphate and stopped by the addition of HCl. The composition of reaction mixtures is described in Materials and Methods.

centrifugations. As shown in Fig.1A, the second sucrose gradient (slot 3) removed several lighty contaminants from the first gradient (slot 2). When purified enzyme was analyzed under non-denaturing conditions, only one protein band was identified in polyacrylamide gel (Fig.1B) and one peak of activity was obtained upon gel filtration on Sephacryl S-300 HR. After thermal denaturation of the enzyme in the sample buffer, besides two main polypeptide M_r 56 000 (L subunit) and 14 000 (S subunit) a polypeptide M_r 49 000, reactive with the anti-RuBPCase antibody appers (Fig.2A). This minor polypeptide originating in the presence or absence of phenylmethyl sulfonyl fluoride is rather a degradation product of L subunit during thermal inactivation of the enzyme before SDS electrophoresis than a heterogenous L subunit as supposed for RuBPCase from several sources (9,16-18). The experiments reported here also demonstrate, that thermal stability of RuBPCase of *B.schlegelii* is not accompanied by an extensive structural changes of the enzyme. This suggestion is supported by the results of immunological experiments indicating structural relationship between the enzyme of mesophilic and thermophilic species.

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