Kinetic properties of ribulose biphosphate carboxylase/oxygenase from *Thiobacillus thyasiris*, the putative symbiont of *Thyasira flexuosa* (Montagu), a bivalve mussel

C. M. COOK,¹* T. LANARAS,¹ A. P. WOOD,² G. A. CODD³ and D. P. KELLY²

¹Department of Botany, University of Thessaloniki, P.O. Box 109, GR-540 06 Thessaloniki, Greece

²Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK

³Department of Biological Sciences, University of Dundee, Dundee, DD1 4HN, UK

(Received 2 November 1990; revised 5 March 1991; accepted 26 March 1991)

Some kinetic properties of ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase from *Thiobacillus thyasiris*, a marine, facultatively heterotrophic, sulphur-oxidizing bacterium and putative symbiont of *Thyasira flexuosa* (Montagu), a bivalve mussel, have been determined. The kinetic parameters for the CO₂/Mg²⁺-activated enzyme were: $K_m(\text{RuBP})$ 24.3 µM, $K_m(\text{CO}_2)$ 125.5 µM, $K_m(\text{O}_2)$ 900 µM and $K_m(\text{Mg}^{2+})$ 1.53 mM. The low CO₂ affinity suggests that *T. thyasiris* may possess a CO₂-concentrating mechanism. RuBP oxygenase activity was inhibited by increasing CO₂ concentration. Divalent metal ions were essential for RuBP carboxylase activity; activity of the Mg²⁺-free enzyme could be restored by the addition of Mg²⁺, Mn²⁺ or Ca²⁺. The pH optimum was 7.8. The temperature optimum for RuBP carboxylase activity was 55 °C, although the enzyme rapidly lost activity at this temperature. An Arrhenius plot was biphasic, with a break at 40 °C. The activation energies were $55.5 \times 10^3 \text{ J mol}^{-1}$ and $32.9 \times 10^3 \text{ J mol}^{-1}$ over the temperature ranges 10–40 °C and 40–55 °C, respectively. Q_{10} was 2.12 for any 10 °C increment between 10–40 °C, and 1.47 between 40–55 °C. RuBP carboxylase activity was stable at 35 °C, the optimum growth temperature of *T. thyasiris* and at 7.5 °C, the temperature of the habitat of *Thyasira flexuosa*, but the activity was 40% and 3.5%, respectively, of the potential activity at 55 °C. RuBP carboxylase activity was stimulated by NaCl concentrations of up to 0.3 M, with a maximum (33%), occurring between 0.1 and 0.2 M-NaCl. At higher concentrations of NaCl (>0.3 M) RuBP carboxylase activity was inhibited.

Introduction

Symbioses between marine invertebrates and endosymbiotic chemoautotrophic sulphur bacteria are now well documented in several phyla of worms and in five families of bivalve molluscs, found in a variety of habitats where reduced chemical species are present (Cavanaugh, 1985; Southward, 1987). In members of the Pogonophora, gutless marine worms, the symbionts are internally localized in the trophosome, a highly vascularised organ, where they are supplied with oxygen and other metabolites (Southward & Southward, 1988). In most molluscs, with the exception of the thyasirids, the bacteria are located in the epidermal cells of the gills (Southward, 1987). In thyasirids and some oligochaetous annelids, the bacteria are located under the cuticle of the epidermal cells (Southward, 1986). Epidermally located

bacteria are in close proximity to the external environment and the necessary metabolites CO₂, O₂ and sulphide.

It has been estimated that chemoautotrophic symbionts may contribute from 50–100% of the host carbon, either through the provision of extracellular products or by the digestion of the symbionts themselves (Southward, 1986, 1987). However, further investigation of the nutritional basis of the symbioses requires the identification and characterization of the symbionts. Their isolation in pure culture has proved difficult; however, the putative symbiont from *Thyasira flexuosa* (Montagu), a thyasirid bivalve mussel from a low-sulphide environment, has recently been isolated and characterized (Wood & Kelly, 1989). The symbiont, *Thiobacillus thyasiris*, is a novel, marine, facultatively heterotrophic *Thiobacillus* unlike any previously described. Autotrophically grown cultures contained ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) and excreted up to 20% of their fixed carbon into the

Abbreviations: RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate.

medium. These extracellular products would presumably be available for use by the host.

In view of the important role played by autotrophy in the nutrition of the animal host (Southward, 1987), it was of interest to examine the kinetic properties of the primary CO₂-fixing enzyme, RuBisCO. A wide species variation has been found in the kinetic properties of RuBisCO, which appears to be determined partly by past evolutionary pressures and partly by the present physico-chemical environment in which the enzyme functions (Yeoh *et al.*, 1981; Jordan & Ogren, 1981, 1983). Apart from several reports of RuBP carboxylase activity in symbiont-containing host tissue (see Southward, 1987) data on RuBisCO kinetics are limited to temperature optimum and K_m (RuBP), determined in extracts of symbiont-containing tissues (Fisher & Hand, 1984; Williams *et al.*, 1988). In this study, some kinetic properties of RuBisCO from *T. thyasiris* have been determined. To our knowledge, this is the first report on purified RuBisCO from an organism isolated from a marine invertebrate-chemoautotrophic bacterial symbiosis.

Methods

Growth of *Thiobacillus thyasiris*. *T. thyasiris*, deposited with the Deutsche Sammlung von Microorganismen, Braunschweig, FRG, (DSM 5322), was grown mixotrophically in chemostat culture at dilution rates of 0.012–0.038 h⁻¹ on a medium described previously (Wood & Kelly, 1989), containing thiosulphate (20 mM) and acetate (2 or 10 mM) at pH 7.5 and 30 °C, and gassed with CO₂ in air (2.5% v/v). Cells were harvested by centrifugation at 23000 g for 10 min at 5 °C, washed in 0.055 M-sodium phosphate buffer (pH 8.0) containing 0.43 M-NaCl, recentrifuged, and stored at -20 °C.

Preparation of cell-free extracts and purification of ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39). The procedure, carried out at 4 °C, was essentially that of Bowien (1977) with the following modifications. Deep-frozen *T. thyasiris* cells were resuspended in 3 vols isolation buffer (10 mM-Tris, 0.5 mM-EDTA-Na₂, 50 mM-NaHCO₃, 10 mM-MgCl₂ and 14.2 mM-2-mercaptoethanol; pH 8.2). The cells were broken by sonication (6 × 30 s) with an MSE Soniprep 150 at 26 amplitude microns and the resulting preparation was centrifuged at 40000 g for 1 h. The supernatant was fractionated by additions of a saturated solution of (NH₄)₂SO₄ (pH 7.6) and the bulk of the RuBP carboxylase activity was precipitated in the 30–70% saturated (NH₄)₂SO₄ fraction. This was resuspended in, and dialysed against, isolation buffer, and sedimented into linear 0.2 M–0.8 M-sucrose gradients (32 ml) by centrifugation in a swing-out rotor at 23000 g for 15 h, in a minor modification of the method of Goldthwaite & Bogorad (1971). Gradients were fractionated and the fractions (1 ml) monitored for protein and RuBP carboxylase activity. Those fractions containing peak activity were pooled, and dialysed against isolation buffer containing 10 mM-Tris, 0.5 mM-EDTA-Na₂, 10 mM-NaHCO₃, 5 mM-MgCl₂ and 14.2 mM-2-mercaptoethanol, pH 8.2. This preparation was used for the kinetic studies and showed a single protein band when analysed by native gel electrophoresis. The specific activity of the purified enzyme was 0.85 μmol min⁻¹ (mg protein)⁻¹. Protein was determined according to the method of Bradford (1976). A slow loss of activity was observed on storage at 4 °C.

Assay of ribulose 1,5-bisphosphate carboxylase/oxygenase. RuBP carboxylase and RuBP oxygenase activities were measured essentially as described by Pierce *et al.* (1982). Enzyme (0.38 mg ml⁻¹) was activated in the presence of 10 or 100 mM-Bicine, 0.5 mM-EDTA-Na₂, 10 mM-NaHCO₃, 5 mM-MgCl₂ and 1 mM-DTT (pH 8.2) for 30 min at 30 °C, prior to assay in either carboxylase or oxygenase assays.

RuBP carboxylase activity was determined as the incorporation of [¹⁴C]bicarbonate into acid-stable products. The assay (0.25 ml) contained 100 mM-Bicine (pH 8.2), 0.5 mM-EDTA-Na₂, 30 mM-NaH¹⁴CO₃ (specific radioactivity 3.7 × 10³ Bq μmol⁻¹), 10 mM-MgCl₂, 1.0 mM-DTT and 0.5 mM-RuBP. Assays (at 30 °C) were initiated either by activated enzyme or RuBP and terminated by the addition of 2 M-HCl (final concentration 0.57 M). Assay time was dependent on the amount of protein or the activity of the enzyme added. RuBP carboxylase activity was proportional to protein concentration (up to 0.16 mg ml⁻¹) when assayed for 2 min, indicating that protein concentration was the limiting factor in the assay. The gradient, corresponding to specific activity, was 0.7 μmol min⁻¹ (mg protein)⁻¹.

RuBP oxygenase activity was measured at 30 °C using a Rank Pt-Ag oxygen electrode. The assay (1 ml) contained 100 mM-Bicine (pH 8.2), 0.5 mM-EDTA-Na₂, 10 mM-MgCl₂, 1.0 mM-DTT and 0.5 mM-RuBP. Assays were initiated by the addition of activated enzyme. The final concentration in the assay of NaHCO₃ carried over with the activated enzyme was 0.5 mM. Oxygen concentration in the assay was adjusted by gassing with either 100% N₂ or O₂.

Kinetic parameters. RuBP carboxylase or oxygenase activity was measured over a range of substrate and cofactor concentrations; RuBP (0.01–0.4 mM), HCO₃⁻ (0.4–12.4 mM), O₂ (0.1–0.9 mM) and Mg²⁺ (0.2–2.0 mM). Assays (in triplicate) were initiated with activated enzyme to give final protein concentrations of 0.075, 0.015, 0.019 and 0.015 mg ml⁻¹, respectively, in the above experiments. Apparent K_m values were determined from double reciprocal plots (Lineweaver–Burk) or Eadie–Hofstee plots.

To determine the apparent K_m (CO₂), all solutions were freshly prepared in MilliQ-beaked water, and buffer was adjusted with 50% (w/v) NaOH. Enzyme was activated with H¹⁴CO₃⁻ and the carry over of H¹⁴CO₃⁻ in the assay gave a final concentration of 0.4 mM. The concentration of CO₂ at pH 8.2 and 30 °C was calculated using equations and constants (Buch, 1960) for zero ionic strength. The RuBP carboxylase activity of the enzyme used in the determination of apparent K_m (O₂) was 57 nmol CO₂ min⁻¹ (mg protein)⁻¹. The equilibrium concentration of oxygen in air-saturated water at a given temperature was calculated from the data of Truesdale *et al.* (1955).

Cofactor specificity. Chloride salts of the divalent metal ions (Ca²⁺, Co²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Zn²⁺, Fe²⁺) were prepared in 100 mM-Bicine buffer, pH 8.2. RuBisCO free of metal ions was prepared by extensive dialysis against 10 mM-Tris (pH 8.2) containing 0.5 mM-EDTA-Na₂ and 14.2 mM-2-mercaptoethanol. The protein concentration was determined after dialysis. RuBP carboxylase activity was 139.6 nmol min⁻¹ (mg protein)⁻¹ before dialysis, and was not detectable after dialysis.

Enzyme free of metal ions was incubated in assay mixture (not containing MgCl₂) containing a final concentration of 20 mM of one of each of the metal ions listed, for 30 min at 30 °C. Assays were initiated with RuBP, terminated after 5 min, and the protein concentration in the assay was 0.051 mg ml⁻¹. The RuBP carboxylase activity recovered in the presence of MgCl₂ was 57.2 nmol min⁻¹ (mg protein)⁻¹; 41% of the activity prior to dialysis.

pH optimum. RuBP carboxylase activity was assayed (in triplicate) in Bicine (100 mM) over the pH range 7.4–9.2 and in HEPES (100 mM) over the pH range 7.0–8.2, in increments of 0.2 pH unit. Assays were initiated with enzyme activated at pH 8.2 and the concentration of

buffer carried over into the assay was 1 mM. Substrates were saturating at each pH, and the protein concentration in the assay was 0.038 mg ml⁻¹.

Temperature optimum. RuBP carboxylase activity was assayed (in duplicate) over the temperature range 5–75 °C, in increments of 5 °C. Assays were initiated with enzyme activated at 30 °C and terminated after 2 min, thus minimizing the effects of thermal denaturation during the assay. The protein concentration in the assay was 0.075 mg ml⁻¹.

Thermostability. RuBisCO was incubated at 55, 35 and 7.5 °C under activating conditions. Samples were removed at intervals and incubated for 5 min at 30 °C prior to assay. Enzyme incubated at 55 °C was cooled immediately on ice at the given time, before incubation at 30 °C and assay. Assays were initiated with RuBP and terminated after 5 min. The protein concentration in the assay was 0.075 mg ml⁻¹.

Effect of NaCl. RuBP carboxylase activity was measured in the presence of NaCl over a range of concentrations (0–0.3 M and 0–1.0 M). Assays were initiated by the addition of activated enzyme (final concentration in assay 0.038 mg ml⁻¹) and were terminated after 10 min.

Results

The apparent K_m values for the substrates RuBP, CO₂ and O₂, and for the cofactor Mg²⁺ were determined for CO₂/Mg²⁺-activated RuBisCO from *T. thyasiris* (Table 1). In accordance with the competitive nature of the substrates CO₂ and O₂ in other RuBisCOs, where $K_m(\text{CO}_2)$ carboxylase = $K_i(\text{CO}_2)$ oxygenase (Laing *et al.* 1974), increasing concentrations of added HCO₃⁻ inhibited *T. thyasiris* RuBP oxygenase activity. Fifty percent inhibition was observed at 3.2 mM-HCO₃⁻ (41.3 µM-CO₂) (pH 8.2, 30 °C and atmospheric oxygen concentration).

Divalent metal ions were essential for RuBP carboxylase activity, since enzyme activity was not detectable when enzyme was dialysed free of Mg²⁺. Activity (41% of the initial activity) was recovered after the readdition of 20 mM-MgCl₂. The addition of the chloride salts of Mn²⁺ and Ca²⁺ (20 mM) to the Mg²⁺-free enzyme supported 12.8 and 5.4% respectively, of the RuBP carboxylase activity recovered in the presence of Mg²⁺. The other divalent metal ions tested (Co²⁺, Cu²⁺, Ni²⁺,

Fig. 1. Arrhenius plot for *Thiobacillus thyasiris* RuBP carboxylase activity. A break occurs at 40 °C. Enzyme activity was expressed as nmol min⁻¹ (mg⁻¹ protein)⁻¹.

Zn²⁺, Fe²⁺) were ineffective in restoring the activity of the Mg²⁺-free enzyme.

The relationship between RuBP carboxylase activity and pH was broad over the pH range 7.0–9.0, with a 20% loss of activity having occurred at pH 7.0 and pH 8.8. The optimum pH for activity was 7.8. The relationship between RuBP carboxylase activity and temperature was fairly sharp, with an optimum occurring at 55 °C. Above 55 °C, the decline in activity was quite rapid, reflecting the increasing influence of temperature on enzyme denaturation relative to the increase in reaction rate.

The activation energy (E_a) for the RuBP carboxylase reaction was determined from an Arrhenius plot and a break occurred at 40 °C (Fig. 1). Over the temperature range 10–40 °C, E_a was 55.5×10^3 J mol⁻¹ and Q_{10} 2.12 (for any 10 °C increment) and over the temperature range 40–55 °C, E_a was 32.9×10^3 J mol⁻¹ and Q_{10} 1.47 (for any 10 °C increment).

The thermostability of *T. thyasiris* RuBisCO was investigated (Fig. 2). Despite the relatively high temperature optimum, RuBP carboxylase activity was unstable when incubated for prolonged periods at 55 °C. Incubation at 55 °C for 10 min reduced RuBP carboxylase activity by 50%, and after 30 min no activity was detected. Enzyme activity was fairly stable to incubation at 35 °C, the temperature optimum for the growth of the symbiont *T. thyasiris* as a free-living organism, at which about 10% of the RuBP carboxylase activity was lost over a 2.5 h period. At 7.5 °C, the temperature of the habitat in which the bivalve *Thyasira flexuosa* is found, the RuBP carboxylase activity remained unchanged over the same period. However, at 35 °C and 7.5 °C, RuBP carboxylase activity represented only 40% and 3.5%, respectively, of the potential activity at 55 °C.

Table 1. Kinetic parameters determined for CO₂/Mg²⁺-activated *Thiobacillus thyasiris* RuBP carboxylase and oxygenase activities

Parameter	RuBP carboxylase activity			RuBP oxygenase activity
	RuBP	Mg ²⁺	CO ₂	O ₂
K_m (µM)	24.3	1530.0	125.5	900.0
V_{max} [nmol min ⁻¹ (mg protein) ⁻¹]	331.7	262.6	164.8	336.0

Fig. 2. Thermostability of *Thiobacillus thyasiris* RuBP carboxylase activity at 55 °C (●), 35 °C (○) and 7.5 °C (△). See Methods for experimental details. The initial RuBP carboxylase activity was $0.112 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Fig. 3. The effect of NaCl on *Thiobacillus thyasiris* RuBP carboxylase activity over the concentration ranges 0 to 0.3 M (○) and 0 to 1.0 M (●). The initial RuBP carboxylase activities, in the absence of added NaCl, were 0.070 and $0.061 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively.

T. thyasiris was found to be slightly halophilic when grown autotrophically on thiosulphate, requiring about 0.5 M-NaCl , and extremely halotolerant when grown on acetate, tolerating up to 3.0 M-NaCl (Wood & Kelly, 1989). The effect of NaCl concentration on RuBP carboxylase activity was examined. NaCl concentrations of up to 0.3 M in the assay stimulated activity, with a maximum stimulation of 33% occurring between 0.1 – 0.2 M-NaCl (Fig. 3). At NaCl concentrations greater than 0.3 M , RuBP carboxylase activity was inhibited (Fig. 3). At 0.58 M-NaCl carboxylase activity was 50%; however, even at 1.0 M-NaCl , 13% of the original activity was still detectable.

Discussion

A wide variation has been observed in the kinetic properties, primarily of $K_m(\text{CO}_2)$ and $K_m(\text{O}_2)$, of RuBisCO isolated from phylogenetically diverse sources (Yeoh *et al.*, 1981; Jordan & Ogren, 1981, 1983). An increased affinity of the enzyme for CO_2 in more advanced photosynthetic organisms has been seen as an adaptation to a decrease in atmospheric CO_2 and increase in O_2 during the evolution of photosynthesis (Yeoh *et al.*, 1981; Jordan & Ogren, 1981, 1983). A correlation has also been observed between the possession of a CO_2 -concentrating mechanism, as found in many cyanobacteria and algae, and C_4 plants, and a RuBisCO with low CO_2 affinity (Yeoh *et al.*, 1981). The value of $K_m(\text{CO}_2)$ determined for *T. thyasiris* RuBisCO ($125.5 \mu\text{M}$) demonstrated that the enzyme had a low affinity for CO_2 similar (80 – $290 \mu\text{M}$) to values determined for cyanobacterial RuBisCOs (Badger, 1980; Yeoh *et al.*, 1981; Jordan & Ogren, 1981), and suggests that *T. thyasiris* may possess a CO_2 -concentrating mechanism which maintains high internal CO_2 levels at the site of CO_2 fixation. Values of $K_m(\text{CO}_2)$ for other *Thiobacillus* RuBisCOs are few and varied (values converted to CO_2 from HCO_3^- concentration according to Buch, 1960): *T. novellus*, $147 \mu\text{M}$ (McCarthy & Charles, 1975); *T. versutus* (formerly *Thiobacillus* A2), $52.5 \mu\text{M}$ (Charles & White, 1976a); and *T. neapolitanus*, $18.7 \mu\text{M}$ (Snead & Shively, 1978). These values may reflect the diverse nutritional capacities, methods of acquisition of inorganic carbon, or the presence or absence of carboxysomes (inclusion bodies containing RuBisCO).

Species-dependent variation in the affinity of RuBisCO for O_2 was observed by Jordan & Ogren (1981). The *T. thyasiris* RuBisCO demonstrated a low affinity for O_2 [$K_m(\text{O}_2)$ $900 \mu\text{M}$], similar (990 – $1220 \mu\text{M}$) to values determined for cyanobacterial RuBisCOs (Badger, 1980; Jordan & Ogren, 1981), and represents the first report of $K_m(\text{O}_2)$ for a *Thiobacillus* enzyme. The low affinities for both CO_2 and O_2 of *T. thyasiris* and cyanobacterial RuBisCOs may reflect the common aerobic, prokaryotic nature of these organisms. *T. thyasiris* probably experiences conditions almost identical to the external environment (low CO_2 and high O_2) in the symbiosis, being located under the cuticle of the epidermal gill cells. The low O_2 affinity would be advantageous under aerobic conditions. Symbiont RuBisCOs may exhibit different $K_m(\text{CO}_2)$ and $K_m(\text{O}_2)$ values, depending on their intracellular location and the ambient CO_2 and O_2 concentration in the surrounding host tissue. There is evidence that low levels of free O_2 are in fact maintained in the vicinity of some symbionts. It has been proposed that pigment granules and an epithelium rich in

Table 2. Parameters of temperature, and activation energies of ribulose 1,5-bisphosphate carboxylase/oxygenase from various microorganisms

Organism	RuBisCO temperature optimum (°C)	Break temperature (°C)	Activation energy (kJ mol ⁻¹)	Temperature range (°C)	Reference
<i>Thiobacillus thyasiris</i>	55	40	55.5 32.9	10–40 40–55	This study
<i>Thiobacillus novellus</i>	50	40–50	78.9 37.1	20–40 40–50	McCarthy & Charles (1975)
<i>Thiobacillus versutus</i> (formally A2)	50	29	51.6 4.4	10–29 29–50	Charles & White (1976b)
<i>Anabaena variabilis</i>	–	–	65.3	15–40	Badger (1980)
<i>Cyanidium caldarium</i>	42.5	36	83.6 15.2	20–36 36–45	Ford (1979)
<i>Chlorella</i> spp.	35	20	65.9 5.8	2.5–20 20–30	Ford (1979)

mitochondria play an O₂-scavenging role in the protection of O₂-sensitive enzymes in the bacteriocytes of *Lucina floridana*, a bivalve mollusc (Fisher & Hand, 1984). In the vestimentiferan pogonophore, *Riftia pachyptila*, O₂ is bound to haemoglobin thereby protecting the symbionts from O₂ inhibition of CO₂-fixation (Fisher *et al.*, 1989).

Although taxonomic patterns have been observed in K_m (RuBP) values, there was no correlation with K_m (CO₂), photosynthetic pathway or natural habitat, with a wide variation in values occurring within any one phylum (Yeoh *et al.*, 1981). The K_m (RuBP) value determined for *T. thyasiris* RuBisCO (24.3 µM) is comparable to values from *Anabaena variabilis* (Badger, 1980) and spinach (Yeoh *et al.*, 1981). K_m (RuBP) values for RuBisCO from sulphur-oxidizing bacteria show some variation: *T. novellus*, 14.8 µM (McCarthy & Charles, 1975); *T. neapolitanus*, 92 µM (Snead & Shively, 1978); *T. versutus*, 122 µM (Charles & White, 1976a); and 65 µM for the symbiont of *R. pachyptila* (Williams *et al.*, 1988), but all fall within the range (10–136 µM) exhibited by terrestrial versions of the enzyme (Yeoh *et al.*, 1981). In contrast, the K_m (RuBP) for RuBisCO of the symbiont of *L. floridana* was reported to be 380 µM (Fisher & Hand, 1984).

In nature, Mg²⁺ plays a dual role in the activation and catalysis of RuBisCO (Miziorko & Lorimer, 1983). The K_m (Mg²⁺) for catalysis was 1.53 mM for *T. thyasiris* RuBisCO. For other *Thiobacillus* RuBisCOs, the following values have been reported: *T. novellus*, 0.61 mM (McCarthy & Charles, 1975); *T. versutus*, 0.02 mM (Charles & White, 1976a); and *T. neapolitanus*, 0.59 mM (Snead & Shively, 1978). Divalent metal ions were essential for *T. thyasiris* RuBP carboxylase activity and Mg²⁺ was partially replaceable by Mn²⁺ and Ca²⁺. RuBisCOs appear to vary in their ability to substitute Mg²⁺ with other divalent metal ions such as Mn²⁺, Co²⁺

and Ni²⁺ (Miziorko & Lorimer, 1983). Among the other *Thiobacillus* enzymes examined, *T. versutus* RuBP carboxylase activity was supported by Mg²⁺ and Mn²⁺ (Charles & White, 1976a), while the enzymes from *T. novellus* (McCarthy & Charles, 1975) and *T. neapolitanus* (Snead & Shively, 1978) were specific for Mg²⁺.

The pH optimum of 7.8 for *T. thyasiris* RuBP carboxylase activity is consistent with the range 7.8–8.2 determined for other RuBisCOs (Charles & White, 1976a; Badger, 1980). The temperature optimum of *T. thyasiris* RuBisCO (55 °C) is consistent with the temperature optima reported for other *Thiobacillus* RuBisCOs (Table 2), but higher than those from the thermophiles *Cyanidium caldarium* (42 °C) (Ford, 1979) and *Chromatium tepidum* (50 °C) (Heda & Madigan, 1988). This is not indicative of a thermophilic characteristic since whereas the *Cyanidium* and *Chromatium* enzymes were stable at their temperature optima, *T. thyasiris* RuBisCO was not. Many RuBisCOs examined have demonstrated a biphasic Arrhenius plot (Table 2), which has been associated with a conformational change in the enzyme molecule. The activation energies determined for *T. thyasiris* RuBisCO are similar to those reported for other *Thiobacillus* RuBisCOs (Table 2).

T. thyasiris is slightly halophilic and extremely halotolerant, depending on growth conditions (Wood & Kelly, 1989). The stimulation of *T. thyasiris* RuBP carboxylase activity *in vitro* by up to 0.3 M-NaCl is unlike the response to NaCl reported for other RuBisCOs examined to date, where NaCl has proved inhibitory even to the enzyme from halophiles (Charles & White, 1976a; Tabita & McFadden, 1976; Weber *et al.*, 1977; Takabe *et al.*, 1984). Although inhibited by 0.3 M-NaCl and above, *T. thyasiris* RuBisCO demonstrated a greater tolerance to NaCl than other RuBisCOs. It is not evident whether low NaCl concentrations are required for *T. thyasiris* RuBisCO stability or activity, or both. NaCl

(0.3 M) has been shown to stabilize the quaternary structure of *Aphanothece halophytica* RuBisCO *in vitro*; however, enzyme activity was drastically inhibited (Takabe *et al.*, 1984). The stimulation of *T. thyasiris* RuBP carboxylase activity *in vitro* by NaCl may reflect the *in vivo* intracellular level of Na⁺ or the cytoplasmic ionic strength, or may mimic the effect of other intracellular compounds. A similar pattern of NaCl stimulation and inhibition has been reported for glutamine synthetase from the halotolerant cyanobacterium *Synechocystis* sp. DUN52, where maximum stimulation occurred at 0.3 M-NaCl *in vitro* and the intracellular Na⁺ concentration was 0.145 M (Warr *et al.*, 1984).

This determination of some kinetic properties of RuBisCO from *T. thyasiris* represents the first report on the purified enzyme from a symbiotic *Thiobacillus*. The data presented will prove valuable in comparative studies of the kinetic properties of RuBisCO from other symbionts of marine invertebrate-chemoautotrophic bacterial symbioses and free-living chemoautotrophic bacteria. Variations arising from the ambient CO₂ and O₂ concentrations experienced by the bacterial symbiont or free-living bacteria, will be of particular interest with respect to the nutritional bases of the symbioses.

T. Lanaras wishes to thank the British Council for the award of a long-term Research Fellowship (SAL/2510). A. P. Wood acknowledges support from the Leverhulme Trust.

References

- BADGER, M. R. (1980). Kinetic properties of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Anabaena variabilis*. *Archives of Biochemistry and Biophysics* **201**, 247–254.
- BOWEN, B. (1977). D-Ribulose 1,5-bisphosphate carboxylase from *Paracoccus denitrificans*. *FEMS Microbiology Letters* **2**, 263–266.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BUCH, K. (1960). Dissoziation der kohlen-saure, gleichgewichte und puffersysteme. In *Handbuch der Pflanzenphysiologie*, vol. 1, pp. 1–11. Edited by W. Ruhland. Berlin: Springer-Verlag.
- CAVANAUGH, C. M. (1985). Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments. *Bulletin of the Biological Society of Washington* **6**, 373–388.
- CHARLES, A. M. & WHITE, B. (1976a). Ribulose biphosphate carboxylase from *Thiobacillus* A2. Its purification and properties. *Archives of Microbiology* **108**, 195–202.
- CHARLES, A. M. & WHITE, B. (1976b). Physical properties and metabolite regulation of ribulose biphosphate carboxylase from *Thiobacillus* A2. *Archives of Microbiology* **108**, 203–209.
- FISHER, M. R. & HAND, S. C. (1984). Chemoautotrophic symbionts in the bivalve *Lucina floridana* from seagrass beds. *Biological Bulletin* **167**, 445–459.
- FISHER, C. R., CHILDRESS, J. J. & MINNICH, E. (1989). Autotrophic carbon fixation by the chemoautotrophic symbionts of *Riftia pachyptila*. *Biological Bulletin* **177**, 372–385.
- FORD, T. W. (1979). Ribulose 1,5-bisphosphate carboxylase from the thermophilic, acidophilic alga, *Cyanidium caldarium* (Geitler). Purification, characterisation and thermostability of the enzyme. *Biochimica et Biophysica Acta* **569**, 239–248.
- GOLDTHWAITE, J. J. & BOGORAD, L. (1971). A one-step method for the isolation and determination of leaf ribulose-1,5-diphosphate carboxylase. *Analytical Biochemistry* **41**, 57–66.
- HEDA, G. D. & MADIGAN, M. T. (1988). Thermal properties and oxygenase activity of ribulose-1,5-bisphosphate carboxylase from the thermophilic purple bacterium, *Chromatium tepidum*. *FEMS Microbiology Letters* **51**, 45–50.
- JORDAN, D. B. & OGREN, W. L. (1981). Species variation in the specificity of ribulose biphosphate carboxylase/oxygenase. *Nature, London* **291**, 513–515.
- JORDAN, D. B. & OGREN, W. L. (1983). Species variation in kinetic properties of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Archives of Biochemistry and Biophysics* **227**, 425–433.
- LAING, W. A., OGREN, W. L. & HAGEMAN, R. H. (1974). Regulation of soybean net photosynthetic CO₂ fixation by the interaction of CO₂, O₂ and ribulose 1,5-bisphosphate carboxylase. *Plant Physiology* **54**, 678–685.
- MCCARTHY, J. T. & CHARLES, A. M. (1975). Properties and regulation of ribulose diphosphate carboxylase from *Thiobacillus novellus*. *Archives of Microbiology* **105**, 51–59.
- MIZIORKO, H. M. & LORIMER, G. H. (1983). Ribulose-1,5-bisphosphate carboxylase-oxygenase. *Annual Review of Biochemistry* **52**, 507–535.
- PIERCE, J., MCCURRY, S. D., MULLIGAN, R. M. & TOLBERT, N. E. (1982). Activation and assay of ribulose-1,5-bisphosphate carboxylase/oxygenase. In *Methods in Enzymology*, vol. 89, pp. 47–55. Edited by W. A. Wood. New York: Academic Press.
- SNEAD, R. M. & SHIVELY, J. M. (1978). D-Ribulose-1,5-bisphosphate carboxylase from *Thiobacillus neapolitanus*. *Current Microbiology* **1**, 309–314.
- SOUTHWARD, E. C. (1986). Gill symbionts in thyasirids and other bivalve molluscs. *Journal of the Marine Biological Association of the United Kingdom* **66**, 889–914.
- SOUTHWARD, E. C. (1987). Contribution of symbiotic chemoautotrophs to the nutrition of benthic invertebrates. In *Microbes in the Sea*, pp. 83–118. Edited by M. A. Sleight. Chichester: Ellis Horwood.
- SOUTHWARD, A. J. & SOUTHWARD, E. C. (1988). Pogonophora: Tube-worms dependent on endosymbiotic bacteria. *Animal and Plant Sciences* **1**, 203–207.
- TABITA, F. R. & MCFADDEN, B. A. (1976). Molecular and catalytic properties of ribulose 1,5-bisphosphate carboxylase from the photosynthetic extreme halophile *Ectothiorhodospira halophila*. *Journal of Bacteriology* **126**, 1271–1277.
- TAKABE, T., RAI, A. N. & AKAZAWA, T. (1984). Interaction of constituent subunits in ribulose 1,5-bisphosphate carboxylase from *Aphanothece halophytica*. *Archives of Biochemistry and Biophysics* **229**, 202–211.
- TRUESDALE, G. A., DOWNING, A. L. & LOWDEN, G. F. (1955). The solubility of oxygen in pure water and seawater. *Journal of Applied Chemistry* **5**, 53–62.
- WARR, S. R. C., REED, R. H. & STEWART, W. D. P. (1984). Osmotic adjustment of cyanobacteria: the effects of NaCl, KCl, sucrose and glycine betaine on glutamine synthetase activity in a marine and halotolerant strain. *Journal of General Microbiology* **130**, 2169–2175.
- WEBER, D. J., ANDERSEN, W. R., HESS, S., HANSEN, D. J. & GUNASEKARAN, M. (1977). Ribulose-1,5-bisphosphate carboxylase from plants adapted to extreme environments. *Plant and Cell Physiology* **18**, 693–699.
- WILLIAMS, C. A., NELSON, D. C., FARAH, B. A., JANNASCH, H. W. & SHIVELY, J. M. (1988). Ribulose biphosphate carboxylase of the procaryotic symbiont of a hydrothermal vent tube-worm: kinetics, activity and gene hybridization. *FEMS Microbiology Letters* **50**, 107–112.
- WOOD, A. P. & KELLY, D. P. (1989). Isolation and physiological characterisation of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa*. *Archives of Microbiology* **152**, 160–166.
- YEOH, H.-H., BADGER, M. R. & WATSON, L. (1981). Variations in kinetic properties of ribulose-1,5-bisphosphate carboxylases among plants. *Plant Physiology* **67**, 1151–1155.