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Quantification and Intracellular Distribution of Ribulose-1,5-Bisphosphate Carboxylase in *Thiobacillus neapolitanus*, as Related to Possible Functions of Carboxysomes

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Abstract. Ribulose-1,5-bisphosphate carboxylase (RuBPCase) has been quantified by immunological methods in *Thiobacillus neapolitanus* cultivated under various growth conditions in the chemostat at a fixed dilution rate of 0.07 h^{-1}. RuBPCase was a major protein in *T. neapolitanus* accounting for a maximum of 17% of the total protein during CO_2 limitation and for a minimum of 4% during either ammonium- or thiosulfate limitation in the presence of 5% CO_2 (v/v) in the gasphase. The soluble RuBPCase (i. e. in the cytosol) and the particulate RuBPCase (i. e. in the carboxysomes) were shown to be immunologically identical. The intracellular distribution of RuBPCase protein between carboxysomes and cytosol was quantified by rocket immunoelectrophoresis. The particulate RuBPCase content, which correlated with the volume density of carboxysomes, was minimal during ammonium limitation (1.3% of the total protein) and maximal during CO_2 limitation (6.8% of the total protein). A protein storage function of carboxysomes is doubtful since nitrogen starvation did not result in degradation of particulate RuBPCase within 24 h. Proteolysis of RuBPCase was not detected. Carboxysomes, on the other hand, were degraded rapidly (50% within 1 h) after changeover from CO_2 limitation to thiosulfate limitation with excess CO_2. Particulate RuBPCase protein became soluble during this degradation of carboxysomes, but this did not result in an increase in soluble RuBPCase activity. Modification of RuBPCase resulting in a lower true specific activity was suggested to explain this phenomenon. The true specific activity was very similar for soluble and particulate RuBPCase during various steady state growth conditions (about 700 nmol/min · mg RuBPCase protein), with the exception of CO_2-limited growth when the true specific activity of the soluble RuBPCase was extremely low (260 nmol/min · mg protein). When chemostat cultures of *T. neapolitanus* were exposed to different oxygen tensions, neither the intracellular distribution of RuBPCase nor the content of RuBPCase were affected. Short-term labelling experiments showed that during CO_2 limitation, when carboxysomes were most abundant, CO_2 is fixed via the Calvin cycle. The data are assessed in terms of possible functions of carboxysomes.

Key words: Ribulose-1,5-bisphosphate carboxylase – Carboxysomes – *Thiobacillus neapolitanus* – Chemolithotroph – Autotroph

Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; PEP, phosphoenolpyruvate; RIE, rocket immunoelectrophoresis; CIE, crossed immunoelectrophoresis

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The CO_2-fixing enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase (RuBPCase) has been shown to be present in the cytosol and also in the polyhedral bodies of *Thiobacillus neapolitanus* (Shively et al. 1973), *Anabaena cylindrica* (Codd and Stewart 1976), *Chlorogloeopsis fritschii* (Lanaras and Codd 1980) and *Nitrobacter agilis* (Shively et al. 1977). Polyhedral bodies have been observed in all cyanobacteria, all obligately chemolithotrophic thiobacilli, some facultatively chemolithotrophic thiobacilli, in many, but not all of the nitrifying bacteria (Shively 1974) and in the photosynthetic bacterium *Rhodomicrobium vanniellii* (Taylor and Dow 1978). Those polyhedral bodies for which an association with RuBPCase has been demonstrated, have been termed carboxysomes (Shively 1974). It was shown that carboxysomes isolated from *Nitrobacter winogradskyi* and *Nitrobacter agilis* contain double-stranded DNA (Westphal et al. 1979).

The presence of RuBPCase in both the cytosol and in the carboxysomes of various organisms (Shively et al. 1973; Codd and Stewart 1976) has raised the question whether carboxysomes are active in CO_2 fixation as has been suggested for *Thiobacillus intermedius* (Purohit et al. 1976) and/or may have a storage function for RuBPCase (Shively 1974; Stewart and Codd 1975; Codd and Stewart 1976). An important aspect in elucidating the function of carboxysomes is the quantification of carboxysome-bound (particulate) – and soluble RuBPCase – protein during the various growth conditions. A comparison of the quantities of RuBPCase protein in *T. neapolitanus* with the known enzymic activities (Beudeker et al. 1980) might then indicate whether the organelles are active in CO_2 fixation or function as storage bodies. A low specific enzyme activity for the carboxysomes compared with that for the soluble enzyme would indicate that not all of the carboxysomal enzyme was active, or accessible or susceptible to activation (see Lorimer et al. 1977) and would imply a storage function of carboxysomes. The results of these investigations are presented in this paper.

Materials and Methods

Organism and Growth Conditions

Thiobacillus neapolitanus strain X was grown in the chemostat under thiosulfate- and CO_2 limitation as has been described by Kuenen and Veldkamp (1973). CO_2-limited cultures were sparged with air which had been stripped of CO_2 by sodium-asbestos (Merck). CO_2 was supplied to the culture as a 50 mM Na_2CO_3 solution. 1 M NaOH was used for titration during CO_2 limitation whilst 1 M Na_2CO_3 was used during the other growth conditions. Cultivation in the presence of 5% CO_2 (v/v) in the gasmixture was achieved by mixing air with CO_2 from a gas cylinder.

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The basal medium for *T. neapolitanus* cultivation contained (% w/v): NH_4Cl, 0.04; $MgSO_4 \cdot 7H_2O$, 0.08; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $Na_2S_2O_3 \cdot 5H_2O$, 1.0. Two milliliter of a trace element mixture (Vishniac and Santer 1957) were added to a litre of medium. NH_4^+-limited growth was achieved by lowering the NH_4Cl concentration ten-fold. NH_4^+ starvation was carried out by omitting NH_4Cl from the medium. During CO_2 limitation and NH_4^+ limitation the thiosulfate concentration in the medium was chosen such that all of the thiosulfate was consumed by *T. neapolitanus*. This made it possible to change-over from these growth conditions directly to thiosulfate limitation. Complete oxidation of thiothreitol, 20 mM $MgCl_2 \cdot 6H_2O$ and 5 mM $NaHCO_3$ and was possessed a significant overcapacity to oxidize thiosulfate under such growth conditions (Kuenen and Veldkamp 1973).

Enzyme Activity

D-Ribulose-1,5-bisphosphate carboxylase (E.C. 4.1.1.39) activity was determined according to a modification of the method of Lorimer et al. (1977) as detailed by Beudeker et al. (1980). The RuBPCase assay buffer contained 100 mM tris(hydroxymethyl)methylamine (Tris), 5 mM dithiothreitol, 20 mM $MgCl_2 \cdot 6H_2O$ and 5 mM $NaHCO_3$ and was adjusted to pH 8.2 at 20° C.

Preparation of Cell-Free Extracts

Cell harvesting, disruption and the separation of particulate RuBPCase (i.e. in the carboxysomes) from the soluble enzyme was performed according to Beudeker et al. (1980). Cell-free extracts to be used in immunoelectrophoretic studies were solubilized by addition of Triton X100 (final concentration 1% v/v), at 0° C for 60 min.

Enzyme Purification

RuBPCase was purified to electrophoretic homogeneity from CO_2-limited cultures of *T. neapolitanus* as has been described by Snead and Shively (1978) using sucrose density gradient centrifugation combined with DEAE-Sephadex column chromatography.

Antiserum Production

Purified RuBPCase (1 mg protein/ml RuBPCase assay buffer) was emulsified with an equal volume of Freund's adjuvant; 0.3 ml of this mixture was injected subcutaneously into a rabbit at the left and at the right sides in the dorsal area and again intramuscularly into each thigh after 8 days. Blood was taken one week later. The immunoglobulin-containing fraction of the serum was obtained by Ammonium sulfate precipitation (50% w/v; 30 min at 30° C). After centrifugation for 30 min at 48,000 × *g*, 4° C the pellet was resuspended in RuBPCase buffer to half of the serum volume. This preparation was dialyzed overnight at 4° C against RuBPCase assay buffer. The immunoglobulin preparations obtained were stored in small quantities at −20° C and used immediately after thawing.

Crossed Immunoelectrophoresis (CIE)

A modification of the procedure of Axelsen et al. (1973) was used. Tris-glycine buffer (0.09 M Tris; 0.7 M glycine) pH 8.6 containing 1% (v/v) Triton X100 was used throughout and incorporated into all gels. Antiserum preparations were mixed with the agarose solution (1.3%) at 58° C during 2 min before pouring gels for the running in the second dimension. A 7 µl well was punched in the corner of the gel for antigen loading.

Electrophoresis was performed through 3.3 ml gels of 1% (w/v) agarose C (Pharmacia, Denmark) on 5 × 5 cm glass plates in a water-cooled electrophoresis chamber (Van Holm-Nielsen, Copenhagen). Gels were run at 2.5 V/cm for 2.5 h in the first dimension and at 1.5 V/cm for 16–18 h in the second direction. After fixation and washing immunoprecipitates were stained with 0.5% (w/v) Coomassie brilliant blue R 250 (Bio-Rad, Richmond, USA) in a mixture of methanol, water and acetic acid (45/45/10, v/v/v). Gels were destained in a mixture of ethanol, water and acetic acid (45/45/10, v/v/v).

Tandem Crossed Immunoelectrophoresis

Running conditions were as described above for CIE. The intercentre distance between the two 7 µl application wells was 5 mm. Samples were allowed to diffuse for 45 min at room temperature prior to electrophoresis. The wells were filled up with molten agarose after diffusion of the loaded enzyme preparations had occurred (see Axelsen et al. 1973).

Rocket Immunoelectrophoresis (RIE)

Conditions for RIE were as for the second dimension of CIE; 6 wells (7 µl volume) were cut in the gels. The amounts of protein in cell-free extracts added to the wells ranged from 200 to 2,000 ng. Each plate contained 335 µg of the immunoglobulin preparation.

Electron Microscopy

Cells were embedded in Epon after fixation and postfixation as described by Beudeker et al. (1980). Student's t-test was used for statistical analyses at a confidence limit of 0.005%.

Protein Determination

Protein of cell-free extracts in the absence of Triton X100 was determined by the method of Bradford (1976).

Contamination by Other Bacteria

T. neapolitanus cultures were frequently checked for contaminants as described by Kuenen and Veldkamp (1973).

Short-Term Labelling

The kinetics of $H^{14}CO_3$-short-term labelling were determined in a CO_2-limited chemostat ($D = 0.07\ h^{-1}$; $pO_2 = 50\%$ air saturation). Carrier-free $H^{14}CO_3$ (100 µCi/ml) was added to a 300 ml culture of *T. neapolitanus*. Samples containing 60 µg of protein were withdrawn 10, 20, 30, 40 s, 1, 2, 5 and 10 min after addition of the label, into syringes containing ethanol (final concentration 80% v/v). Concentration, separation, identification and counting of the labelling products was carried out by conventional methods as described previously (Schürmann 1969; Döhler and Wegmann 1969).

Results

Qualitative and Quantitative Immunological Studies on Thiobacillus neapolitanus Ribulose-1,5-Bisphophate Carboxylase (RuBPCase)

Crossed immunoelectrophoresis (CIE) of cell-free extracts of *T. neapolitanus* and of purified RuBPCase from this organism yielded similar immunoprecipitation arcs and a typical example is shown in Fig. 1 a. Only one immunoprecipitation line was detected with crude extract indicating that the antibody-preparation raised against *T. neapolitanus* RuBPCase was monospecific. The immunoprecipitation lines formed after tandem crossed immunoelectrophoresis of the particulate and the soluble RuBPCase of *T. neapolitanus* showed complete fusion indicating immunological identity of the carboxysomal and soluble forms of the enzyme (Fig. 1 b). Rocket immunoelectrophoresis (RIE) was used to quantitate the RuBPCase protein in cell-free extracts and a calibration curve was made with increasing amounts of purified *T. neapolitanus* RuBPCase (Fig. 2). Rocket peak heights were directly pro-

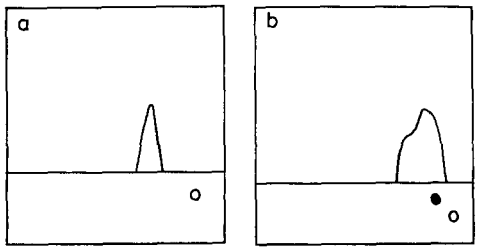

Fig. 1. (a) Typical crossed immunoelectropherogram of a cell-free extract of *Thiobacillus neapolitanus*. Electrophoresis in the first dimension was in the direction of the abscissa. Electrophoresis in the second dimension was in the direction of the ordinate in 1% agarose containing 235 µg of the immunoglobulin preparation (raised against RuBPCase purified from *T. neapolitanus*). For details see Materials and Methods. The anode was at the left and at the top of the figure. **(b)** Tandem crossed immunoelectropherogram of Triton X-100 treated extracts of *T. neapolitanus*. The right hand well contained the particulate enzyme preparation (including solubilized carboxysomes) (○) and the left hand well contained the soluble enzyme preparation (●). The directions of running were as in Fig. 1a. For experimental details see Materials and Methods

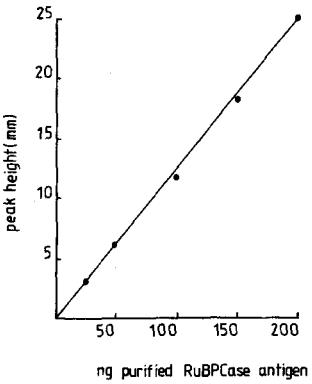

Fig. 2. Peak heights of the immunoprecipitates visualized after rocket immunoelectrophoresis of purified RuBPCase (soluble plus carboxysomal enzyme) through gel containing homologous RuBPCase immunoglobulins. A 5 × 5 cm gel consisting of 3.3 ml of 1% agarose contained 335 µg of the immunoglobulin preparation

portional to the amount of purified RuBPCase applied. Quantitative measurement of the enzyme protein was possible down to 10 ng using this method.

Table 1 shows the total, particulate (carboxysomal) and soluble RuBPCase as percentages of total *T. neapolitanus* protein during various growth conditions. The amount of RuBPCase protein, expressed as a percentage of total protein, differed markedly during different nutrient- and energy limitations. The amount of particulate RuBPCase correlated approximately with the cellular volume density of carboxysomes. During NH_4^+-limited growth it was not possible to quantify the amount of carboxysomes by electron microscopy due to the excessive formation of polyglucose (Beudeker et al. in press). The known activities of both particulate and soluble RuBPCase (also see Beudeker et al. 1980) were used to calculate the *in vitro* true specific activities of RuBPCase (i. e. the catalytic activity per mg RuBPCase protein) in the soluble and particulate fractions (Table 2). True specific activities of the particulate and soluble enzymes were approximately equal and did not vary significantly

Table 1. Quantification and intracellular distribution of D-ribulose-1,5-bisphosphate carboxylase (RuBPCase)-protein between carboxysomes (particulate fraction) and the cytosol (soluble fraction) as determined by rocket immunoelectrophoresis, in relation to the volume density of carboxysomes in *Thiobacillus neapolitanus* grown under different limitations in the chemostat ($D = 0.07\ h^{-1}$; $pO_2 = 50\%$ air saturation)

Growth limiting substrate	RuBPCase content (% of total cell protein)			Volume density of carboxysomes % cellular volume[a]
	Total	Particulate	Soluble	
CO_2	17.1	6.8	10.3	8.7
Thiosulfate	5.2	2.5	2.7	4.4
Thiosulfate (+5% CO_2)	4.1	1.6	2.5	1.5
NH_4^+	4.2	1.3	2.9	n. m.

[a] Data from Beudeker et al. (1980)
n. m. = not measurable due to presence of polyglucose

Table 2. The true in vitro specific activities (activity per mg RuBPCase protein) of soluble and particulate RuBPCase from *T. neapolitanus* grown under different limitations in the chemostat ($D = 0.07\ h^{-1}$; $pO_2 = 50\%$ air saturation)

Growth limiting substrate	True specific activity nmol CO_2 fixed/mg RuBPCase protein	
	Particulate	Soluble
CO_2	1176	262
Thiosulfate	720	778
Thiosulfate (+5% CO_2)	688	720
NH_4^+	846	690

between extracts from thiosulfate-, thiosulfate (+5% CO_2)- and NH_4^+-limited cells. Only in CO_2-limited cell-derived extracts did the activities vary between the particulate and soluble fractions. Variations in true specific activities were not due to low molecular weight inhibitors since dialyzed extracts showed the same activities as nondialyzed extracts. Furthermore assays of mixed extracts showed activities which were fully consistent with the proportional contribution of the components. Thus the specific activity of RuBPCase protein in *T. neapolitanus* carboxysomes can be the same as that of the soluble RuBPCase protein.

Possible Role of Carboxysomes in the Protection of the RuBPCase Reaction from Inhibition by O_2

The RuBPCase enzyme also catalyzes an oxygenase reaction (RuBPOase) yielding phosphoglycollate and phosphoglycerate (Bowes et al. 1971). Phosphoglycollate is metabolized to glycollate and this compound is often found as an excretion product of various autotrophs including *T. neapolitanus* (Cohen et al. 1979). One of our hypotheses concerning the role(s) of carboxysomes was that a protection against this RuBPOase reaction might be provided by compartmentation of the enzyme in the carboxysomes. However, as shown in Table 3 widely different O_2 tensions present during continuous cultivation had no effect either on the quantity or on

Table 3. The effect of different oxygen tensions applied in the chemostat on the quantity and intracellular distribution of RuBPCase in thiosulfate-limited cells of *T. neapolitanus* ($D = 0.07\ h^{-1}$)

pO_2 % air saturation	RuBPCase content (% of total cell protein)		
	Total	Particulate	Soluble
5	5.0	2.5	2.5
50	5.2	2.5	2.7
90	5.2	2.5	2.7

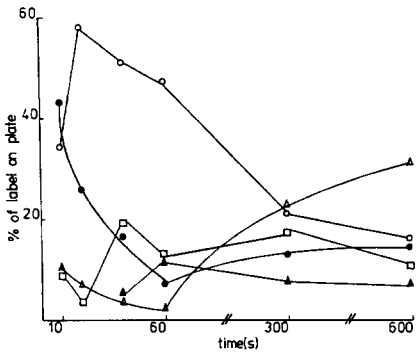

Fig. 3. Short-term kinetics of the incorporation of ^{14}C from $H^{14}CO_3$ into 80% ethanol-soluble cellular compounds by CO_2-limited cells of *T. neapolitanus* ($D = 0.07$; $pO_2 = 50\%$ air saturation). ● Phosphoglycerate; ○ other sugar phosphates; △ glutamate; ▲ sucrose; □ aspartate

the distribution of the RuBPCase protein although the applied different O_2 tensions did lead to varying rates of excretion and presumed production of glycollate (Cohen et al. 1979).

Possible Role of Carboxysomes in a C_4-Plant Type of CO_2 Fixation

Compartmentation of RuBPCase has also been found in C_4-plants (for a recent review see Ray and Black 1979). In such plants CO_2 is primarily fixed into oxaloacetate via phosphoenolpyruvate carboxylase in the mesophyll cells of the leaf. Oxaloacetate (or malate) is transported into the bundle sheath cells which contain RuBPCase. There, C4-acids are decarboxylated and the CO_2 released is fixed by RuBPCase. The C_4 pathway of photosynthesis acts as a mechanism to concentrate CO_2 at the site of RuBPCase and can be viewed as an anatomical and metabolic solution which has evolved to overcome the fundamental sensitivity of the carboxylase reaction to competitive inhibition by O_2 (Hatch 1977). PEP carboxykinase is one of the principle catalysts involved in C_4 acid decarboxylation in C_4 photosynthesis and this enzyme has been found in particulate fractions of *T. neapolitanus* (R. F. Beudeker, unpublished observations). Evidence for the possible role of carboxysomes in a C_4-type of CO_2 fixation mechanism was sought by determining the short-term kinetics of ^{14}C-bicarbonate incorporation into whole cells under conditions of growth when carboxysomes are most abundant (i. e. during CO_2 limitation). As shown in Fig. 3 phosphoglycerate (PGA) accounted for the greatest proportion of the ^{14}C incorporated into soluble products at the earliest sampling time with a pattern typical of primary product formation. After 10–20 s of labelling 80–85% of the ^{14}C incorporated was present in Calvin cycle intermediates (PGA and sugar-phosphates). C_4 dicarboxylic acids (aspartate and malate) did not appear among the major primary labelling products. It is inferred that CO_2 fixation is primarily achieved via the Calvin cycle during CO_2-limited growth of *T. neapolitanus* in the chemostat. These findings thus do not provide evidence for a possible role for carboxysomes in a C_4-plant type of CO_2 fixation.

Possible Role of Carboxysomes in Protein (RuBPCase) Storage

Particulate RuBPCase amounted only to 1.3% of the total protein in NH_4^+-limited cells of *T. neapolitanus* (Table 1). This very low relative amount of the particulate RuBPCase protein during nitrogen-limited growth suggested that the carboxysomes may serve as a nitrogen storage source. To test this possibility cells rich in carboxysomes (CO_2-limited) were transferred to NH_4^+ starvation. However, this did not result in any significant breakdown of the carboxysomes in spite of the fact that excess energy was available to such cells (results not shown). In a set of further experiments NH_4^+-limited cells were changed over either to growth conditions with excess of nitrogen or to nitrogen starvation. Figure 4a shows the effect of addition of NH_4^+ to a NH_4^+-limited *T. neapolitanus* culture. Cells became thiosulfate-limited directly after the addition of NH_4^+. The amount of particulate RuBPCase increased about twofold within 3 h whereas the soluble RuBPCase content remained almost constant. Three hours after the addition of excess NH_4^+ the cells contained a total amount of RuBPCase protein and distribution pattern of the enzyme between cytosol and carboxysomes identical with values found in steady state thiosulfate-limited cells. NH_4^+ starvation of NH_4^+-limited cells, which were still being supplied with thiosulfate for energy generation, did not result in significant changes in either soluble or particulate RuBPCase protein levels (Fig. 4b).

Degradation of Carboxysomes

Carboxysomes, however, are degraded rapidly in intact cells during change-over from CO_2 limitation to thiosulfate limitation in the presence of 5% CO_2 (v/v). The number of carboxysomes per at random diameter as calculated from about 1500 electronmicrographs decreased twofold within 60 min after the change-over (Fig. 5). By wash-out alone only 7% of the carboxysomes would have disappeared during this period ($D = 0.07\ h^{-1}$). Statistical analyses of the data at 0 and at 60 min showed that the differences in number of carboxysomes were significant. Carboxysomes were also degraded (or disintegraded) in the presence of chloramphenicol (25 mg/l) during this change-over indicating that *de novo* enzyme synthesis was not needed for this to occur. The particulate RuBPCase activity also decreased twofold during this period with a time course almost identical with that of the decrease in carboxysome abundance, as apparent from the electron-microscopical observations. Intermediate stages in the loss of carboxysome structure were observed under the electron microscope. The degradation of the carboxysomes always appeared to start in the centre of the bodies. The immunological data reveal that the particulate RuBPCase protein content also decreased during this change-over (Fig. 6), but at a

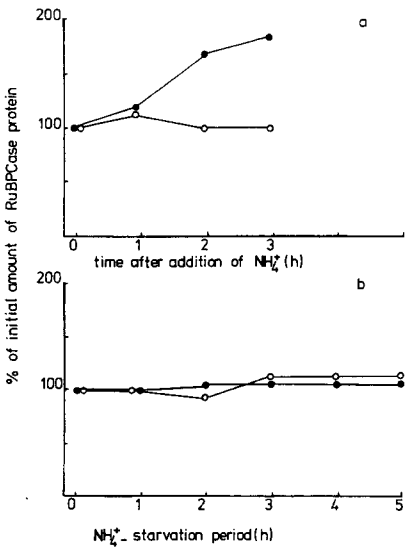

Fig. 4. Intracellular distribution of RuBPCase protein between carboxysomes (●) and cytosol (○) in *T. neapolitanus*, as quantified by rocket immunoelectrophoresis, during change-over from NH_4^+-limitation to thiosulfate-limitation with excess of NH_4^+ (Fig. 4a) and to NH_4^+-starvation (Fig. 4b). $D = 0.07\,h^{-1}$; $pO_2 = 50\%$ air saturation

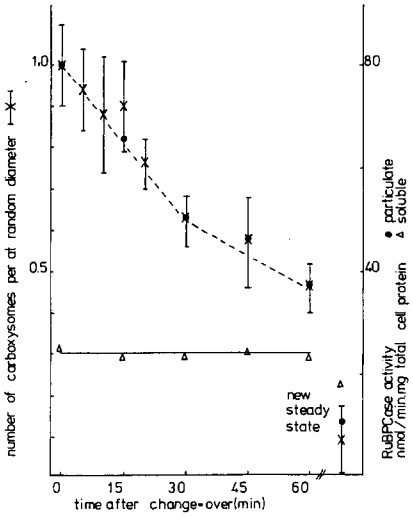

Fig. 5. Activities of particulate (●) and soluble RuBPCase (△) and number of carboxysomes per at random cell diameter (×) in *T. neapolitanus* during change-over from CO_2-limitation to thiosulfate-limitation in the presence of 5% CO_2 ($D = 0.07$; $pO_2 = 50\%$ air saturation)

slower rate than was expected from the rates of decrease of particulate RuBPCase activity and disappearance of carboxysomes (see Fig. 5). This phenomenon might be explained by the continued association of enzymically inactive but immunologically reactive RuBPCase to partially degraded carboxysomes which are no longer recognizable as such, but still may be pelleted during centrifugation. It is possible that the initial decrease in particulate RuBPCase protein content was not due to proteolysis, but a consequence of solubilization of the particulate enzyme, since the soluble RuBPCase content initially increased during this period, whereas the total

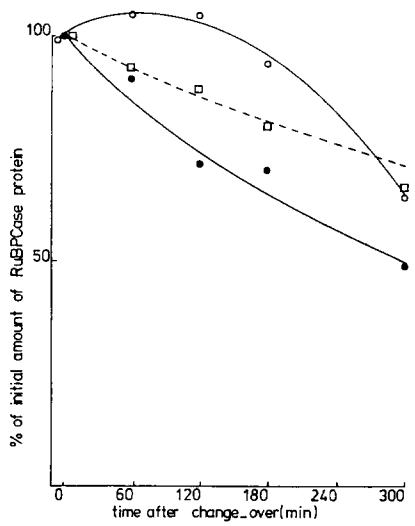

Fig. 6. Quantification and intracellular distribution of RuBPCase protein between carboxysomes (●) and cytosol (○) during change-over from CO_2 limitation to thiosulfate limitation in the presence of 5% CO_2 ($D = 0.07$; $pO_2 = 50\%$ air saturation), expressed as the percentage of the amount present at zero time (100%). The percentage of initial amount of the total RuBPCase protein is represented by □. The dotted line represents the wash-out rate during the change-over experiment

Table 4. The changes in specific activities per mg of RuBPCase protein of soluble and particulate RuBPCase from *T. neapolitanus* during change-over from CO_2 to thiosulfate limitation in the presence of 5% CO_2 ($D = 0.07\,h^{-1}$; $pO_2 = 50\%$ air saturation)

Time after change-over min	True specific activity nmol CO_2 fixed/mg RuBPCase protein	
	Particulate	Soluble
0	1176	262
60	635	220
new steady state	688	720

RuBPCase content remained unchanged (after correction for wash-out). Given the finding that soluble RuBPCase activity remained constant during the first hour after the change-over (even after dialysis of the enzyme preparations) (Fig. 5) it is possible, however, that some kind of modification of the particulate RuBPCase protein occurs during in vivo solubilization resulting in the observed decrease in true specific activity (Table 4). The true specific activity of particulate RuBPCase decreased about twofold within 60 min (Table 4). This decrease in true specificity of particulate RuBPCase is not due to physical breakage of the carboxysomes per se since the in vitro specific activity of the enzyme in intact carboxysomes as measured in Tris-buffer of low ionic strength (0.01 M; see Beudeker et al. 1980) was not changed by disintegration of the organelles in 0.1 M Tris-buffer.

Discussion

The cell disruption and fractionation methods used for purification of the *Thiobacillus neapolitanus* RuBPCase (Snead and Shively 1979) resulted in enzymes being derived

from the soluble and particulate fractions. It is assumed that the antiserum obtained to the resulting electrophoretically homogeneous enzyme contains antibodies against antigenic determinants on the carboxysomal and soluble enzymes. The complete fusion of the immunoprecipitation arcs after tandem-crossed immunoelectrophoresis of particulate and soluble extracts (Fig. 1b) indicates that the solubilized carboxysomal and cytosol RuBPCase pools possess some identical antigenic determinants. The carboxysomal and soluble RuBPCase enzymes have not been purified individually from *T. neapolitanus* and information is not yet available on the comparative molecular characteristics of the two forms of the enzyme. We do not infer that the two forms of the enzyme in freshly-prepared extracts would show complete immunological identity using respective homologeous antisera. It is possible that the carboxysomal enzyme may become modified during extraction and purification and, if so then some carboxysomal RuBPCase antigenic determinants could be lost and/or their accessibility to antibodies reduced.

RuBPCase protein levels in the cytosol and carboxysomal fractions have been quantified by RIE. RuBPCase appears to be a major protein in *T. neapolitanus* with a maximum value of 17% of total protein during CO_2 limitation and a minimum of 4% of total protein during NH_4^+ limitation and thiosulfate limitation in the presence of 5% CO_2 (v/v). RuBPCase constitutes about 10% of total tobacco leaf protein or 50% of chloroplast protein (Wildman and Kwanynen 1978). A value of 3–4% of total protein has been reported in immunological studies on RuBPCase in the cyanobacterium *Aphanothece halophytica* (Cook et al. 1980).

Polyhedral bodies as a whole are among the most readily discernable, but functionally enigmatic, of organelles in autotrophic prokaryotes (see Introduction). So far we have tested 4 hypothetical functions for the bodies in *T. neapolitanus:*

1. Carboxysomes May be Active Sites of CO_2 Fixation. It has already been shown that the volume density of carboxysomes correlates inversely with the maximal CO_2-fixing capacity of whole cells of *T. neapolitanus* during various growth conditions (Beudeker et al. 1980). A low enzyme activity to enzyme protein ratio for the carboxysomal RuBPCase, relative to the cytoplasmic enzyme in extracts, would indicate that not all of the carboxysomal enzyme is capable of being activated and thus maximally catalyzing CO_2 fixation (see Introduction). However, the true specific activity of carboxysomal enzyme in particulate fractions was never found to be significantly less than that of the soluble enzyme, indeed the reverse was found with extracts from CO_2-limited cells (Table 2). The electronmicroscopy data do not support the concept of an active function for the carboxysomes in CO_2 fixation (Beudeker et al. 1980) but equally the present data (Table 2) do not discount the possibility that the carboxysomal enzyme in *T. neapolitanus* is active in CO_2 fixation *in vivo:* carboxysomal RuBPCase is potentially capable of fixing CO_2 at the same rates as the cytosol enzymes.

2. Carboxysomes May Protect RuBPCase from Inhibition by O_2. Although the *T. neapolitanus* enzyme also exhibits RuBP oxygenase activity (Beudeker, unpublished observation) the results shown in Table 3 do not endorse this hypothetical function, since particulate RuBPCase protein remains a constant percentage of total protein during growth under different O_2 tensions. If a protective role of carboxysomes against inhibition by O_2 operated then an increase in the ratio of particulate to soluble RuBPCase with increasing O_2 tensions may have been found. These observations, however, do not rigorously rule out this hypothesis since the cell may simply not be able to control the number of carboxysomes in response to the O_2 tension in its environment. If carboxysomes principally conferred a useful protective role against inhibition by O_2, then it could be argued that they need not have developed in anaerobic autotrophic prokaryotes and indeed we know no reports of polyhedral bodies (carboxysomes) in purple sulphur bacteria. Furthermore, a selective advantage of carboxysomes, if useful for protection against O_2, could have resulted in the development of these organelles in the O_2-requiring hydrogen bacteria, whose RuBPCases typically show oxygenase activity (e. g. Bowien et al. 1976). However, carboxysomes are not known to occur in hydrogen bacteria, and no indication of their functions is readily apparent, at present, from their distribution among different groups of prokaryotic autotrophs.

3. Carboxysomes May be Compartments for CO_2 Fixation Analogous to the Function of Bundle Sheath Cells in C_4 Plants (for review see Ray and Black, 1979). Figure 3 shows, however, that during CO_2-limited growth, when carboxysomes are most abundant in *T. neapolitanus*, CO_2 is fixed via the Calvin cycle. A Calvin cycle for CO_2 fixation was hitherto assumed to be operative in *T. neapolitanus* (Kelly 1971) but had never been tested by means of short-term labelling.

4. Carboxysomes May Serve as Nitrogen Storage Bodies. This possible function is suggested by observations on cyanobacterial polyhedral bodies (Codd and Stewart 1975; Stewart and Codd 1976; van Eykelenburg 1980). Supporting this possibility is the finding that particulate RuBPCase contributed only 1.3% of total protein during NH_4^+-limited growth in *T. neapolitanus*. In addition the particulate RuBPCase as a percentage of total protein increased rapidly after adding of NH_4^+ to NH_4^+-limited cells (Fig. 4a). In contradiction with a function as nitrogen storage bodies are the results of Fig. 4b which show that nitrogen starvation did not result in degradation of either particulate or soluble RuBPCase. In this context it is significant to note that we have failed to demonstrate N_2-fixing capacity in *T. neapolitanus*. The nitrogen reserve material cyanophycin is degraded within 24 h of the onset of nitrogen limitation in the cyanobacterium *Aphanocapsa* 6308 (Allen et al. 1980). However, even in CO_2-limited cells of *T. neapolitanus* which had been without nitrogen for 24 h no degradation of RuBPCase was observed. From these data it is concluded that although *T. neapolitanus* economises on carboxysome protein during nitrogen limitation the organism does not synthesize carboxysomes as a source of storage nitrogen.

The elucidation of carboxysome function will probably depend on the further characterization of these bodies. Tests for the presence of other Calvin cycle enzymes, namely phosphoriboisomerase and phosphoribulokinase in *T. neapolitanus* carboxysomes (Shively and Cannon 1979) and for phosphoribulokinase in *Chlorogloeopsis fritschii* carboxysomes (Lanaras and Codd 1980) have proved negative. Nevertheless it has been estimated that purified carboxysomes of *Nitrobacter agilis* contain at least 7 different polypeptides (Biedermann and Westphal 1979).

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References

Allen MM, Hutchinson F, Weathers PJ (1980) Cyanophycin granule polypeptide formation and degradation in the cyanobacterium *Aphanocapsa* 6308. J Bacteriol 141:687–693

Axelsen NH, Krøll J, Week B (eds) (1973) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand J Immunol 2 Supplement 1. Universites forloget Oslo-Bergen-Tromsø, Norway

Beudeker RF, Cannon GC, Kuenen JG, Shively JM (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

Beudeker RF, Kerver JWM, Kuenen JG (1981) Occurrence, structure and function of intracellular polyglucose in the obligate chemolithotroph *Thiobacillus neapolitanus*. Arch Microbiol 129:221–226

Biedermann M, Westphal K (1979) Chemical composition and stability of NB 1 particles from *Nitrobacter agilis*. Arch Microbiol 121:187–191

Bowes G, Ogren WL, Hageman RH (1971) Phosphoglycollate production catalyzed by ribulosediphosphate carboxylase. Biochem Biophys Res Comm 45:716–722

Bowien B, Mayer F, Codd GA, Schlegel HG (1976) Purification, some properties and quaternary structure of the D-ribulose-1,5-bisphosphate carboxylase of *Alcaligenes eutrophus*. Arch Microbiol 110:157–166

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248–254

Codd GA, Stewart WDP (1976) Polyhedral bodies and ribulose-1,5-diphosphate carboxylase of the blue-green alga *Anabaena cylindrica*. Planta 130:323–326

Cohen Y, de Jonge I, Kuenen JG (1979) Excretion of glycollate by *Thiobacillus neapolitanus* in continuous culture. Arch Microbiol 122:189–194

Cook CM, Codd GA, Stewart WDP (1980) Immunological studies on ribulose bisphosphate carboxylase from cyanobacteria (blue-green algae). Abstracts of the Vth International Congress on Photosynthesis (September 1980) Halkidiki, Greece, p 126

Döhler G, Wegmann K (1969) Untersuchungen zur Photosynthese-Induktion bei *Chlorella* mit Hilfe von radioaktivem CO_2. Planta 89:266–274

Van Eykelenburg C (1980) Ecophysiological studies on *Spirulina platensis*. Effect of temperature, light intensity and nitrate concentration on growth and ultrastructure. Antonie van Leeuwenhoek J Microbiol Serol 46:113–127

Hatch MD (1977) C4-Pathway of photosynthesis: mechanisms and physiological function. Trends in Biochem Sci 2:199–202

Kelly DP (1971) Autotrophy: Concepts of lithotrophic bacteria and their organic metabolism. Ann Rev Microbiol 25:177–210

Kuenen JG, Veldkamp H (1973) Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila*, *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. Arch Microbiol 94:173–190

Lanaras T, Codd GA (1980) D-Ribulose-1,5-bisphosphate carboxylase and carboxysomes of the cyanobacterium *Chlorogloeopsis fritschii*. Society for Gen Microbiol Quarterly 7:102–103

Lorimer GH, Badger MR, Andrews TJ (1977) D-Ribulose-1,5-bisphosphate carboxylase-oxygenase. Analyt Biochem 78:66–75

Purohit K, McFadden BA, Shaykh MM (1976) D-Ribulose-1,5-bisphosphate carboxylase and polyhedral inclusion bodies in *Thiobacillus intermedius*. J Bacteriol 127:516–522

Ray TB, Black CC (1979) The C4 pathway and its regulation. In: Photosynthesis II. Encyclopedia of plant physiology new series Vol 6 (Pirson A, Zimmerman MH, eds). Springer, Berlin Heidelberg New York, pp 77–98

Schürmann P (1969) Separation of phosphate esters and algal extracts by thin-layer electrophoresis and chromatography. J Chromatogr 39:507–509

Shively JM, Ball F, Brown DH, Saunders RE (1973) Functional organelles in prokaryotes: polyhedral inclusions (carboxysomes) of *Thiobacillus neapolitanus*. Science 182:584–586

Shively JM (1974) Inclusion bodies of prokaryotes. Ann Rev Microbiol 28:167–187

Shively JM, Bock E, Westphal K, Cannon GC (1977) Icosohedral inclusions (carboxysomes) of *Nitrobacter agilis*. J Bacteriol 132:673–675

Shively JM, Cannon GC (1979) Carboxysomes, ribulose-1,5-bisphosphate carboxylase and the fixation of carbon dioxide in autotrophic microbes. Abstracts III International Symposium on Photosynthetic Prokaryotes (Oxford) D-20

Snead RM, Shively JM (1978) D-Ribulose-1,5-bisphosphate carboxylase from *Thiobacillus neapolitanus*. Curr Microbiol 1:309–314

Stewart WDP, Codd GA (1975) Polyhedral bodies (carboxysomes) of nitrogen fixing blue-green algae. Br Phycol J 10:273–278

Taylor SC, Dow CS (1978) Ribulose-1,5-bisphosphate carboxylase and carboxysomes in *Rhodomicrobium vannielli*. Proc Soc Gen Microbiol 5:47

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195–213

Westphal K, Bock E, Cannon GC, Shively JM (1979) Deoxyribonucleic acid in nitrobacter carboxysomes. J Bacteriol 140:285–288

Wildman SG, Kwanynen P (1978) Fraction I protein and other products from tobacco for food. In: Photosynthetic carbon assimilation. (Siegelmann HW, Hind G, eds). Plenum Press, New York, pp 1–18

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