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Protein composition of the carboxysomes of *Thiobacillus neapolitanus*

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Abstract. For purifying carboxysomes of *Thiobacillus neapolitanus* an isolation procedure was developed which resulted in carboxysomes free from whole cells, protoplasts and cell fragments. These purified carboxysomes are composed of 8 proteins and at the most of 13 polypeptides. The two most abundant proteins which make up more than 60% of the carboxysomes, are ribulose-1,5-bisphosphate carboxylase and a glycoprotein with a molecular weight of 54,000. The shell of the carboxysomes consists of four glycoproteins, one also with a molecular weight of 54,000. The other proteins are present in minor quantities. Ribulose-1,5-bisphosphate carboxylase is the only enzyme which could be detected in the carboxysomes and 3-phosphoglycerate was the only product formed during incubation with ribulose-1,5-diphosphate and bicarbonate. The supernatant of a broken and centrifuged carboxysome suspension contained the large subunit of ribulose-1,5-bisphosphate carboxylase. The small subunit of ribulose-1,5-bisphosphate carboxylase was found in the pellet together with the shell proteins which indicates that the small subunit of ribulose-1,5-bisphosphate carboxylase is connected to the shell.

Key words: Carboxysomes – Polyhedral bodies – Protein composition – Ribulose-1,5-bisphosphate carboxylase – Shell glycoproteins

Carboxysomes are polyhedral inclusion bodies which are found in many CO_2 fixing microbes. These inclusion bodies contain ribulose-1,5-bisphosphate carboxylase (RuBisCO) (Shively 1974). In *Thiobacillus neapolitanus*, a chemolithoautotrophic organism, carboxysomes appear in large quantities under CO_2 limited growth conditions. These carboxysomes contain more RuBisCO than the cytosol. In CO_2 limited cultures RuBisCO can make up upto 17% of the total cell protein (Beudeker et al. 1981). The native enzyme with a molecular weight of 500,000 (Snead and Shively 1978) consists of eight large and eight small subunits with molecular weights of 56,000 and 13,000. The cytoplasmic and the carboxysomal enzymes are identical. Both enzymes are composed of the large and small subunits, have the same kinetic properties (Cannon and Shively 1983) and appear to be immunologically identical (Lanaras and Codd 1981; Beudeker et al. 1981).

The role of carboxysomes in autotrophic organisms has received considerable attention (for review see Codd and Marsden 1984). Several investigators postulated a role of carboxysomes in CO_2 fixation (Beudeker and Kuenen 1981; Cannon and Shively 1983; Purohit et al. 1976) or suggested a storage accommodation for RuBisCO (Shively 1974; Stewart and Codd 1975; Codd and Stewart 1976). Beudeker and Kuenen (1981) detected in carboxysomal preparations several enzymes of the Calvin cycle and at least 27 polypeptides and suggested that carboxysomes could fix CO_2 via the complete Calvin cycle. However, the 12–15 polypeptides found by Cannon and Shively (1983) in carboxysomes of *T. neapolitanus* and the observation that no activity could be detected of the Calvin cycle enzymes ribose-5-phosphate isomerase, ribulose-5-phosphate kinase and fructose-1,6-bisphosphatase was at variance with this hypothesis.

In this paper we have studied the composition and the nature of the proteins of the carboxysomes of *T. neapolitanus* and estimated the molecular weights of the polypeptides. RuBisCO appears to be the only enzyme present in the purified carboxysome preparations of *T. neapolitanus*. The RuBisCO molecules are surrounded by a shell layer consisting of four glycoproteins.

Materials and methods

Organism and growth conditions

Thiobacillus neapolitanus (strain X) was grown in a 1 l chemostat under CO_2 limitation at 28°C, as described by Kuenen and Veldkamp (1973). The medium contained in demineralized water 2.78 mM K_2HPO_4, 3.67 mM KH_2PO_4, 7.48 mM NH_4Cl, 3.24 mM $MgSO_4 \cdot 7\,H_2O$, 40.29 mM $Na_2S_2O_3 \cdot 5\,H_2O$ and 2 ml per litre of a trace element mixture (Vishniac and Santer 1957). Air was continuously led through the culture. The oxygen concentration was monitored with an oxygen electrode and controlled automatically at 50% air saturation by varying the stirring-rate. CO_2 was removed from the inflowing air by bubbling through soda lime. Limiting CO_2 was supplied via a separate pump. The overflow of the chemostat was stored at 4°C until harvesting of the cells.

Isolation of carboxysomes

Cells were harvested from a 12 l culture by centrifugation (10,000 × *g* for 10 min at 4°C) and resuspended in 5 ml

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Abbreviations. RuBisCO, ribulose-1,5-bisphosphate carboxylase; PMSF, phenylmethylsulfonyl fluoride; PAA gelectrophoresis, polyacrylamide gelelectrophoresis; SDS, sodium dodecyl sulphate; CIE, crossed immunoelectrophoresis; IEF, isoelectric focusing

10 mM Hepes KOH (pH 8.0), 20 mM $MgCl_2$, 1 mM dithiothreitol and 2 mM serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (HMDP buffer). The cell suspension was sonicated (4 times 30 s) in an icebath followed by a high speed centrifugation (27,000 × *g*, 15 min) at 4°C. The centrifugation step is an important step in the isolation procedure of carboxysomes and results in the removal of cells and cell fragments from the sonicated suspension without pelleting carboxysomes. Carboxysomes, obtained by high speed centrifugation (48,200 × *g*) of the sonicated cells, could hardly if ever be freed from contaminating cells, protoplasts and cell fragments. The supernatant (7 ml) containing carboxysomes was carefully separated from the pellet and layered on 2 ml sucrose solution (53% w/w in HMDP buffer, pH 8.0) and centrifuged in a Sorvall SS-34 rotor (48,200 × *g*, 60 min) at 4°C. The pellet was resuspended in about 2 ml of 30% (w/w) sucrose, subsequently layered on 53% sucrose (w/w) both in HMDP buffer pH 8.0 and again centrifuged (48,200 × *g*, 60 min) at 4°C.

Electron microscopy

Carboxysomes were negatively stained with 1% uranylacetate. A pellet of isolated carboxysomes was fixed in 1.5% glutaraldehyde in 0.1 M cacodylate (pH 7.2), followed by postfixation in a solution of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in 0.1 M cacodylate (pH 7.2). Dehydration of the stained carboxysomes was performed in graded series of increasing ethanol concentrations. The water-free carboxysome pellet was embedded in Epon 812 and sectioned with a diamond knife on a LKB III microtome (Bromma, Sweden). Negatively stained and sectioned carboxysomes were examined with a Philips EM 201 or 300.

Gelelectrophoresis

A suspension of carboxysomes (20 mg protein/ml) was diluted once with a buffer containing 0.18 M Tris-HCl (pH 6.8), 12.9% sodium dodecyl sulphate (SDS), 8.6% glycerol and 0.07% bromophenol Blue (Laemmli 1970) and boiled for 2 min. SDS-Polyacrylamide gelelectrophoresis (SDS-PAA gelelectrophoresis) was carried out according to Laemmli and Favre (1973). Silver staining of the gels was performed according to Wray et al. (1981). Glycoproteins in the gels were identified as described by Segrest and Jackson (1972) (PAS-staining).

The following reference proteins were used; cytochrome c (12,500), chymotrypsinogen (25,000), albumine (45,000 and 68,000) and aldolase (158,000).

The relative amounts of the proteins in the gels were scanned with a double beam recording microdensitometer (MK IIIc, Joyce, Loebl and Co., Gateshead-on-Tyne, England).

Isolation of antibodies

Antibodies against the isolated carboxysomes of *T. neapolitanus* and purified RuBisCO were raised and isolated as described previously by Elferink et al. (1979) and Van der Plas et al. (1983).

Crossed immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis of carboxysomes of *T. neapolitanus* was carried out with the method of Smyth et al. (1978). Carboxysomes were solubilized in the presence of 5% Triton X-100. Subsequently the suspension was centrifuged for 10 min in an Eppendorf-centrifuge. The supernatant was used for electrophoresis. The pellet contained unbroken carboxysomes as shown by electron-microscopy.

Electrophoresis of proteins was performed in 1% (w/v) agarose in CIE-buffer (20 mM barbital-HCl, pH 8.6 with 1% Triton X-100) on 5 × 5 cm immunoplates in a water cooled electrophoresis chamber (Van Holm-Nielsen, Copenhagen, Denmark). Gels were run in the first dimension at 2.5 V/cm for 2.75 h and in the second dimension for 16–18 h at 1.5 V/cm. Afterwards the plates were washed three times with 0.1 M NaCl and stained with 0.5% (w/v) Coomassie Brilliant Blue R250 in methanol:acetic acid:water (45:10:45, v/v/v) and after 5 min destained in this mixture without Coomassie Brilliant Blue.

Isoelectric focusing (IEF)

Carboxysomes (15 mg/ml) were mixed with two volumes of a sample preparation buffer, composed of 9,5 M urea (Ultra Pure, Schwarz/Mann Inc., New York, USA), 5% 2-mercaptoethanol, 8% Nonidet P-40, 0,4% Ampholine pH 3.5–10 and 1,6% Ampholine pH 5–7 (Amesz and Nikaido 1976) which results in immediate dissociation of the carboxysomes. The protein solution (40 µg protein per gel) was applied on a 4% polyacrylamide cylindrical gel containing 9 M urea and 4% (v/v) Ampholine pH 5–7, 1% (v/v) Ampholine pH 3.5–10 and 2% Nonidet P-40. The tubes were filled with degassed 0.02 M NaOH and placed in a cylindrical gel apparatus (O'Farrell 1975). The cathode reservoir was filled with degassed 0.02 M NaOH and the anode reservoir with degassed 0.01 M H_3PO_4. Electrophoresis was performed for 10 min at 100 V, for 10 min at 200 V, for 16 h at 300 V and for 4 h at 800 V. The cylindrical gels were removed from the glass tubes with a stream of water and used immediately or frozen at −20°C. For second dimension SDS-PAA gelectrophoresis the cylindrical gels were incubated for 1 h in 50 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 5% 2-mercaptoethanol and 10% (w/v) glycerol. For second dimension CIE the cylindrical gels were incubated in CIE-buffer for 1 h and embedded in 1% agarose on 5 × 15 cm immunoplates.

Protein determination

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumine as a standard.

Isolation of the shell and content of carboxysomes

Carboxysomes are surrounded by a shell, 3–4 nm thick (Shively et al. 1973). This shell has been partly purified by diluting the carboxysomes four times in 10 mM Hepes-KOH (pH 8.0), 20 mM $MgCl_2$, 1 mM dithiothreitol and 2 mM PMSF without sucrose, subsequently followed by sonication (5 × 30 s) and centrifugation (48,200 × *g*, 15 min) at 4°C. The shell (pellet) could now be separated from the content (supernatant).

Isolation of carboxysomal RuBisCO

RuBisCO was isolated with a modified method of Brown et al. (1981). A carboxysome suspension (20 mg/ml) was

centrifuged (48,200 × *g* for 10 min) at 4°C and the pellet was resuspended in 50 mM Hepes, 50 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT (pH 7.0) (HNMED buffer). The suspension was sonicated and disruption of the carboxysomes was followed electronmicroscopically. After at least 10 min sonication the suspension was centrifuged (10,000 × *g* for 5 min) at 4°C and the clear suspension was brought on a high performance liquid chromatography (HPLC) Protein Pack 300-SW column (Waters Associates, 7.5 mm × 300 mm, operating pressure 350 Pa) eluted with HNMED buffer for the first crude separation of proteins. The fractions containing RuBisCO (measured according to Beudeker et al. 1981) were pooled, concentrated in 30% polyethyleneglycol-6000 (PEG-6000) and brought on a DEAE-Si 100 polyol derivative column (Serva, size 3 µm, 7.1 mm × 250 mm, operating pressure 900 Pa). The column was first eluted with 50 mM Tris-HCl and 5 mM 2-mercaptoethanol (pH 7.0) (buffer A) followed by elution with the same buffer containing 45 mM $MgCl_2$. The fractions containing RuBisCO were pooled and concentrated with 30% PEG-6000 in 50 mM Tris-HCl, 5 mM 2-mercaptoethanol and 0.1 M NaCl (pH 7.0) (buffer B). These fractions were again loaded on the DEAE-Si 100 polyol derivative column which was thorougly eluted with buffer B. Subsequently the column was eluted with a linear 0.1 – 0.6 NaCl gradient (in buffer A) and the active fractions were pooled and concentrated with PEG-6000 in buffer A.

Enzyme activities

The purified carboxysomes were immediately used or diluted in 0.1 M Tris-HCl, pH 7.8 and subsequently sonified for 20 × 30 s. The enzyme activities were spectrophotometrically assayed as described by Beudeker et al. (1981). The enzyme activities tested were: phosphoribulokinase (EC 2.7.1.19), D-glyceraldehyde-3-phosphate:NAD oxidoreductase (EC 1.2.1.12), ATP:3-phospho-D-glycerate-1-phosphotransferase (EC 2.7.2.3), fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase (EC 4.1.2.13), D-sedoheptulose-1,7-bisphosphate 1-phosphohydrolase (EC 3.1.3.66), L-malate:NADH oxidoreductase (EC 1.1.1.37), L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1), ATP:AMP phosphotransferase (EC 2.7.4.3). RuBisCO (EC 4.1.1.39) activity was assayed as described by Beudeker et al. (1981).

Product formation

Product formation by carboxysomes incubated in the presence and absence of carbonate and ribulose-1,5-bisphosphate was followed. A 20 µl carboxysome suspension (in HMPD buffer) was added to a reaction mixture (1 ml), containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 6 mM glutathion and 40 mM $NaHCO_3$ and incubated on ice for 30 min. After subsequent incubation for 5 min at 30°C the reaction was started by addition of 0.5 mM ribulose-1,5-diphosphate. The reaction was terminated after 10 min by addition of 0.02 ml 1 M HCl, followed by boiling for 1.5 min. Subsequently, the mixture was centrifuged in an Eppendorf centrifuge for 10 min and the supernatant was used for fluorimetric analysis and thin layer chromatography. With the fluorescence spectrophotometer (Perkin-Elmer 204, Norwalk, USA) D-glycerate-3-phosphate, dihydroxyacetone phosphate and phosphoenolpyruvate were analysed, using a NADH-coupled indicator system described by Thompson and Thomas (1977).

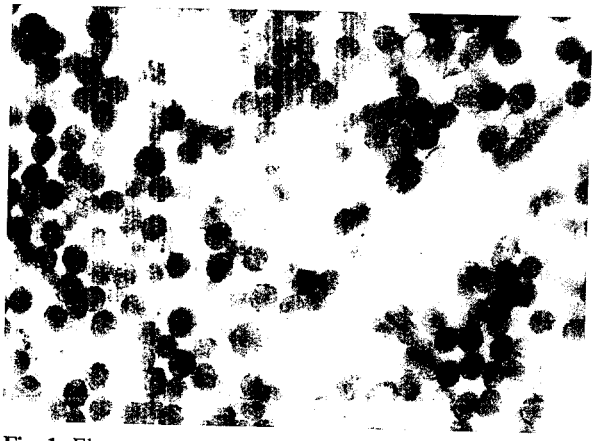

Fig. 1. Electron micrograph of a negatively stained section of carboxysomes of *T. neapolitanus* purified according to the procedure described in Materials and methods. 1 µm = 48.5 mm

Thin layer chromatography was performed on Kieselgel plates (0.25 mm, Merck) using butanol – acetic acid – H_2O (3:3:1, v/v/v) as solvent and ammoniummolybdate-perchloric acid (Wagner et al. 1961) as spray reagent to locate phosphorylated compounds. The supernatants of the carboxysomes, incubated with and without ribulose-1,5-diphosphate and carbonate and the following compounds were subjected to chromatography: D-glycerate-3-phosphate, glyceraldehyde-3-phosphate, glycerate-2-3-diphosphate, glycerate-2-phosphate, glyceric acid, DL-glyceraldehyde, ribulose-1,5-diphosphate, ribulose-5-phosphate, ribose-5-phosphate, ribose, phosphoenolpyruvate, dihydroxyacetone, inorganic phosphate, NAD, NADH, AMP, ADP, ATP, fructose-6-phosphate and fructose-1,6-diphosphate.

Results

Isolation of carboxysomes

For the study of the chemical composition and the physiological properties highly purified carboxysomes are needed. Such a high purification could be achieved by the isolation procedure described in Materials and Methods. With this procedure cells and cell fragments were effectively removed and a white pellet was obtained which contained carboxysomes (about 10 mg protein), free from contaminating membrane fragments. Only carboxysomes and no cells nor membrane fragments were observed in an electronmicrograph of a negatively stained section of a carboxysome pellet (Fig. 1). This carboxysome preparation was analysed for the presence of lipids by Dr. J. A. F. Op den Kamp, Dept. of Biochemistry, University of Utrecht, The Netherlands. The chloroform methanol extract of 1 g of carboxysomes was analysed for lipids. With the conventional phosphate analysis (Rouser et al. 1970) as well as with I_2 staining after thin layer chromatography (Broekhuyse 1969) no (phospho)lipidic material could be detected.

Electron microscopic studies of carboxysomes in whole cells or of isolated carboxysomes revealed that carboxysomes have a polygonal structure. Mostly they were seen as hexagonal bodies (Holthuijzen et al. 1986). The carboxysomes appeared to be quite stable. After storage of the

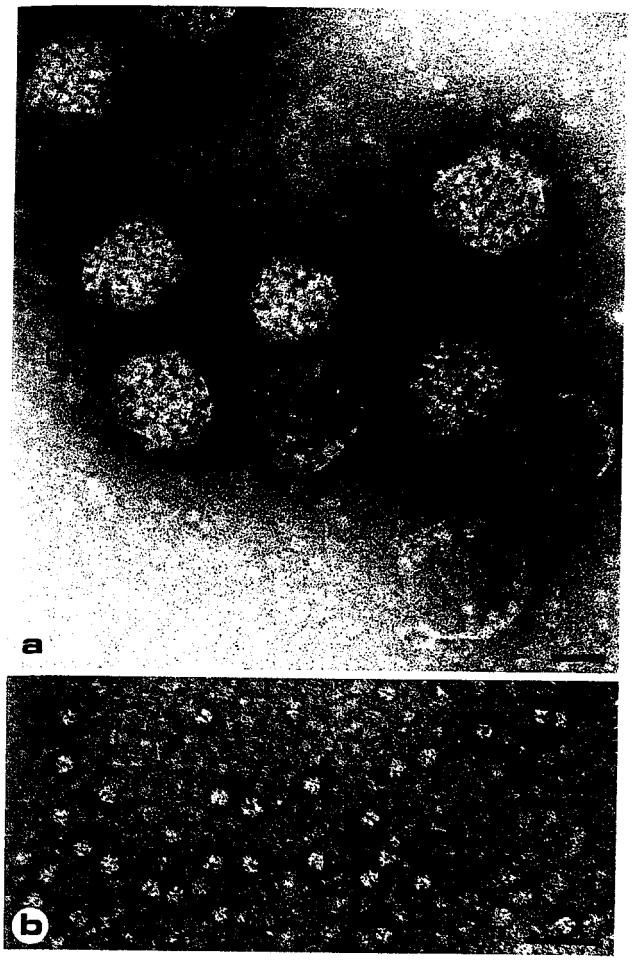

Fig. 2. Electron micrograph of negatively stained (1% uranyl acetate) **a** carboxysomes and **b** RuBisCO molecules from carboxysomes of *T. neapolitanus*. The carboxysomes were stored in 10 mM Hepes KOH (pH 8.0), 20 mM $MgCl_2$ and 1 mM dithiothreitol without protease inhibitor or sucrose for a week at 4°C. The markerbars represent 0.1 μm

carboxysomes in HMDP buffer for 2 months at 4°C the polygonal appearance of the carboxysomes had not changed. Carboxysome preparations stored for a week at 4°C without PMSF or sucrose contained besides intact carboxysomes also broken carboxysomes and free RuBisCO molecules (Fig. 2a). The RuBisCO molecules had a characteristic "doughnut" shape – a slightly depressed ball with a center hole, or a rectangular shape with a densely stained line lengthwise (Fig. 2b). Carboxysomes could be destabilized by long term sonication (10–20 min) in an icebath in the presence of 20 mM EDTA, by dilution of a carboxysome suspension of 60% sucrose 10 times with HMDP buffer without sucrose or by treatment with glycerol. Under these conditions broken carboxysomes were formed in which many RuBisCO molecules were seen leaving the particles. Also empty carboxysomes without RuBisCO molecules were formed and these had sometimes retained the hexagonal appearance (Fig. 2a).

Protein composition of the carboxysomes

1. SDS-PAA gelelectrophoresis. Electrophoresis of purified carboxysomes solubilized with SDS (2%) on polyacrylamide

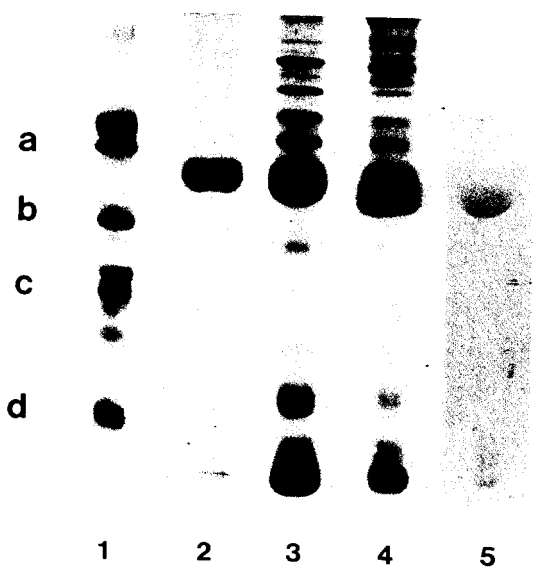

Fig. 3 a–d. SDS-polyacrylamide gelelectrophoresis of solubilized carboxysomes (72 μg) and the supernatant and pellet obtained after centrifugation of partly disrupted carboxysomes (72 μg). *Lane 1:* protein standards are **a** albumine (68,000), **b** albumine (45,000), **c** chymotrypsinogen (25,000) and **d** cytochrome c (12,500). *Lane 2:* the supernatant; *lane 3:* the pellet; *lane 4:* solubilized carboxysomes (72 μg); *lane 5:* solubilized carboxysomes (72 μg); *lane 1–4:* silverstained gels; *lane 5:* PAS-stained gels (see Materials and methods)

gels (10%) revealed 11–13 polypeptide bands (Fig. 3 lane 4). No aggregation was observed after boiling with SDS, indicating that no membrane proteins were present. Four to seven more bands were observed in PAA-gelelectrophoresis patterns of carboxysomes isolated without a protease inhibitor (PMSF) or stored at 4°C without PMSF (data not shown). Three bands of polypeptides with Mr around 56,000, 13,000 and a broad band around 10,000 were found most abundant in the electropherogram. The 56,000 and 13,000 polypeptides corresponded to the large and small subunits of RuBisCO (Cannon and Shively 1983). Some heterogeneity was always found in the area of the large subunit of RuBisCO and molecular weights varying between 54–56,000 were observed (Fig. 3, lane 4). With a specific staining method (PAS-staining) four glycoproteins could be detected (Fig. 3, lane 5). Glycoproteins show an anomalous behaviour on SDS-PAA gels due to a decreased binding of SDS per gram protein (Segrest et al. 1971). To estimate the molecular weights of the glycoproteins a carboxysome suspension was subjected to SDS-PAA gelelectrophoresis on 7.5, 10 and 12.5% polyacrylamide gels. No anomalous mobility of the glycoproteins was found which indicated that carbohydrate content of these proteins was relatively low. The Mr of the glycoproteins could subsequently be estimated to be 120,000, 85,000, 54,000 and around 10,000. One of the glycoproteins had a molecular weight in the same range as that of the large subunit of RuBisCO.

2. Isoelectric focusing (IEF). To discriminate between the large subunits of RuBisCo and the "54,000" glycoprotein isoelectric focusing was applied. With this procedure 8 proteins were found after silver-staining (Fig. 4a). It takes at least 4 h at 800 V to complete electrophoresis and to concentrate the proteins at their isoelectric points. The broad spot

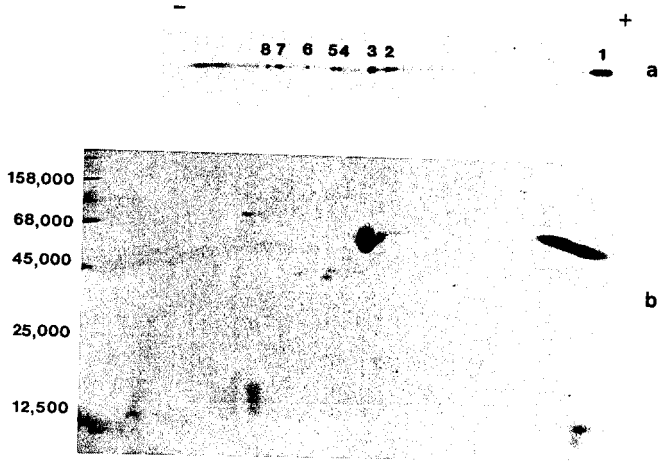

Fig. 4. Isoelectric focusing **a** of purified carboxysomes, followed by SDS-polyacrylamide gelelectrophoresis **b**. The IEF-gel contained 4% and the SDS-PAA gel 10% polyacrylamide. Purified carboxysomes (40 µg protein) were applied in the IEF gel. Protein standards were electrophoresed at the left in the SDS PAA gel. These are from top to bottom aldolase, albumine, albumine, chymotrypsinogen and cytochrome c

of protein 2 shows some heterogeneity. This aspect has not been investigated further. The most abundant proteins in these gels have been numbered 1 and 3. Protein 3 is visible as a white spot in the gel without silver-staining. Sections of the gel containing the proteins were subjected to SDS-PAA gelelectrophoresis (data not shown). Protein 1 appeared to be composed of 2 polypeptides of 56,000 and 13,000. These molecular weights of the polypeptides in protein 1 indicate that this protein is RuBisCO. Some heterogeneity was found in the large subunit of RuBisCO. Protein 3 consisted of one polypeptide of 54,000. PAS-staining indicated that proteins 2 and 3 are glycoproteins.

When the proteins in the IEF gel were electrophoresed in a second dimension in SDS-PAA gels proteins 1 and 3 had the same molarity. This implied that the "54,000" glycoprotein had been hidden by the large subunit of RuBisCO in SDS-PAA gels (Fig. 4).

The ratio between the large subunit of RuBisCO to the "54,000" glycoprotein could roughly be estimated to be 45–55 by scanning of the twodimensional isoelectric focusing gel. Scanning the IEF gel (Fig. 4a) indicated that RuBisCO forms about 30% of the total carboxysomal protein.

3. Crossed immunoelectrophoresis (CIE). A typical crossed immunoelectrophoresis pattern of solubilized carboxysomes is shown in Fig. 5a. Only two major precipitation lines were found. Isolation of carboxysomes in the absence of PMSF resulted in a considerable heterogeneity as indicated by the staining of the whole area under the precipitation lines (A and B).

To identify RuBisCO on the immunoplates antibodies raised against purified RuBisCO were used. Only one symmetrical precipitation line was formed with solubilized carboxysomes (Fig. 5b). This precipitation line corresponds to precipitation line A in Fig. 5a, since precipitation line A increased when purified RuBisCO was added to the solubilized carboxysomes (data not shown).

When the proteins in the IEF gel (Fig. 4a, 6a) were run in the second dimension of the CIE gel three precipitation lines were found (Fig. 6b). These precipitation lines corresponded to band 1 (RuBisCO), band 3 ("54,000" glycoprotein) and one of the minor proteins in the cathode part of the gel. The combined CIE, IEF and SDS-PAA gelelectrophoresis studies indicate that the "54,000" glycoprotein accounts for precipitation line B in the CIE pattern.

Proteins of the shell and the contents of the carboxysomes

Electron microscopic studies revealed that the carboxysomes were only partly disrupted by dilution and sonication. The supernatant obtained after removal of intact carboxysomes and shells contained only RuBisCO, as indicated by electron microscopy and by SDS-PAA gelelectrophoresis (Fig. 3, lane 2). One band with a Mr of 56,000 was present on lane 2, indicating that only the large subunit of RuBisCO was present in the supernatant of the broken carboxysomes. However, no activity could be found in the supernatant containing only the large subunit. PAA gelelectrophoresis of the pellet shows an enrichment of the polypeptides with Mr of 120,000, 85,000, 54,000, 13,000 and 10,000 (Fig. 3, lane 3). Except for the 13,000 protein, the small subunit of RuBisCO, these polypeptides corresponded to the glycoproteins identified above indicating that the shell consists mainly of glycoproteins. It was impossible to purify the shell further due to the rapid desintegration of the shell after sonication of the carboxysomes. CIE of the shell proteins against carboxysomal antibodies yielded only precipitation line B (Fig. 5c). CIE of the supernatant against these antibodies gave rise to precipitation line A.

Enzyme activities and product formation

The only enzyme that could be detected in carboxysomes was RuBisCO. The activity (2.05 µmol/min mg protein) did not change by sonication. None of the many enzymes tested (see Materials and methods) could be found even after prolonged sonication (> 10 min).

The same spots found in thin layer chromatographs of the supernatant and of intact carboxysomes preincubated with ribulose-1,5-diphosphate and carbonate were found in chromatographs of carboxysomes directly mixed with 3-phosphoglycerate. Phosphoenolpyruvate and dihydroxyacetone chromatographed at about the same place as the product(s) of the carboxysomes. Fluorospectrophotometrical analysis of the supernatant of carboxysomes preincubated with ribulose-1,5-diphosphate and bicarbonate revealed only 3-phosphoglycerate and no traces of PEP or dihydroxyacetone phosphate. 3-Phosphoglycerate therefore appears to be the only product formed by the carboxysomes.

Discussion

Knowledge of the protein composition of the carboxysomes is essential for our understanding of the function of these polyhedral inclusion bodies in autotrophic microorganisms. The results of the study described in this paper suggest that two proteins are major components of the carboxysomes of *Thiobacillus neapolitanus*. One protein is the enzyme RuBisCO which appears to be located inside the carboxysomes and is the only enzyme found in the supernatant of disrupted, purified carboxysomes. The other

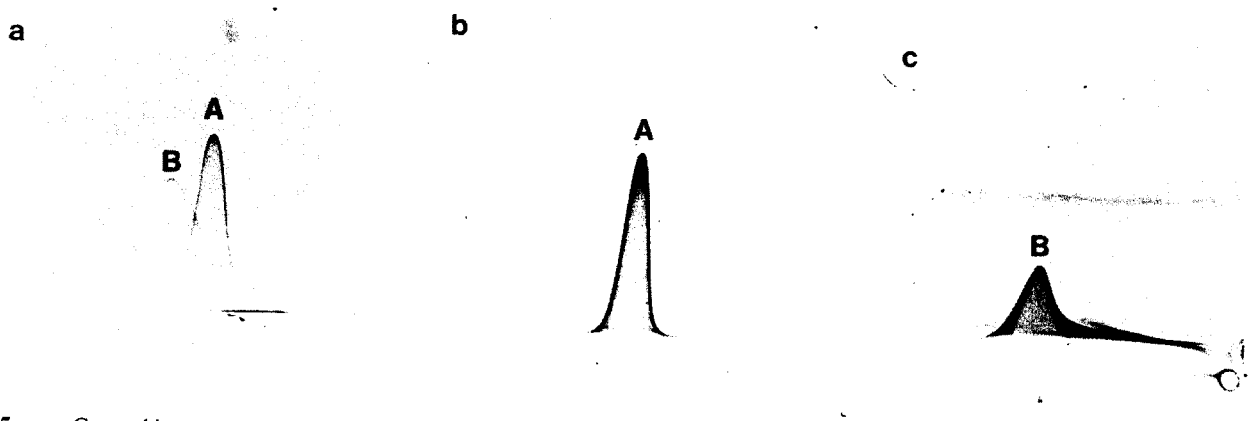

Fig. 5 a–c. Crossed immunoelectrophoresis of solubilized carboxysomes of *T. neapolitanus*. The second dimension contained **a** and **c** 150 μg of rabbit antibody protein raised against purified carboxysomes of *T. neapolitanus*, or **b** 100 μg rabbit antibody protein raised against purified RuBisCO. In **a** and **b** 27 μg of carboxysomal protein and in **c** 12 μg of pellet protein was applied on the gels

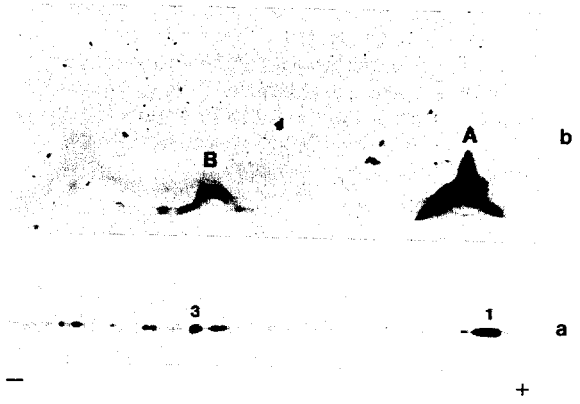

Fig. 6. Isoelectric focusing **a** of purified carboxysomes, followed by crossed immunoelectrophoresis **b**. The IEF gel contained 4% polyacrylamide; the cathode is to the right. On the IEF gels 40 μg carboxysomal protein was applied. The second dimension of the CIE contained 450 μg rabbit raised antibodies

major protein is a glycoprotein which makes up the shell of the carboxysome together with three other glycoproteins.

Our SDS-PAA gelelectrophoresis results resemble those of a homogeneous carboxysome preparation of *T. neapolitanus*, presented by Cannon and Shively (1983). There is also some resemblance between the SDS-PAA gelelectrophoresis patterns of the carboxysomes of *T. neapolitanus* and those of *Nitrobacter agilis* (Biedermann and Westphal 1979) and of *Chlorogloeopsis fritschii* (Lanaras and Codd 1981). The number of polypeptide bands at the most 13, is at variance with the 27 polypeptides found by Beudeker and Kuenen (1981). Most likely the isolation procedure employed by Beudeker and Kuenen (1981) yielded carboxysomes which were contaminated with protoplasts and cell fragments.

In the SDS-PAA gelelectrophoresis patterns of carboxysome preparations of various sources (Cannon and Shively 1983; Lanaras and Codd 1981; Biedermann and Westphal 1979) are the bands of the small and large polypeptides of RuBisCO most obvious. None of these authors noticed the existence of a (glyco)protein with a Mr of 54,000, that runs together with the large subunit of RuBisCO in the SDS-PAA gels. Our results agree with those of Cannon and Shively (1983) and Biedermann and Westphal (1979) who noticed a heterogeneity of the large subunit of RuBisCO. This heterogeneity is to a certain extent explained by the existence of a glycoprotein with a Mr similar to that of the large subunit.

This study supplies strong support for earlier suggestions (Shively et al. 1973; Biedermann and Westphal 1979) that glycoproteins form the outer layer of carboxysomes. Cannon and Shively (1983) presented a SDS-PAA gel of the enriched shell fraction and observed an enrichment of the polypeptides with 10,000 and 15,000. One of these polypeptides is probably the same as the glycoprotein which we found to be a shell protein. The finding that the small subunit of RuBisCO is present in the enriched shell fraction of the carboxysomes is of particular interest. This observation suggests that the small subunit forms the connection between RuBisCO and the shell glycoproteins. The glycoproteins with Mr of 120,000 and 85,000 are found in minor quantities and were probably not seen in the enriched shell fractions of Cannon and Shively (1983). The other proteins present in the carboxysome preparations were found in smaller quantities. At this stage it cannot be excluded that they are part of the carboxysomes.

Our results are consistent with a function of carboxysomes as storage accommodation for RuBisCO (Shively 1974; Stewart and Codd 1975; Codd and Stewart 1976) but at variance with a Calvinosome function as suggested by Beudeker and Kuenen (1981). At present an enzymatic function in vivo of RuBisCO of carboxysomes has not been demonstrated or excluded and an association of carboxysomes with other Calvin cycle enzymes in the cytoplasm is still one of the possibilities. The packaging of molecules inside the glycoprotein shell of the carboxysomes has been the subject of a separate study (Holthuijzen et al. 1986).

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