CARBOXYSOMES: 'CALVINOSOMES'?

R. F. BEUDEKER and J. G. KUENEN+

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren and *+Laboratory of Microbiology, Delft University of Technology, Julianalaan 67A, 2628 BC Delft, The Netherlands*

Received 19 June 1981

1. Introduction

Examples of compartmentation in prokaryotes are scarce [1]. Those compartments studied in detail, namely heterocysts (review [2]) and gas vacuoles (review [3]) occur only, or mainly, in cyanobacteria. The cyanobacteria also contain carboxysomes (polyhedral bodies) [4,5], organelles of which the function still is unknown. Polyhedral bodies are termed carboxysomes when they have been shown to contain D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) [4]. Carboxysomes occur not only in cyanobacteria but also in many obligate and some facultative chemolithotrophs such as *Thiobacillus* spp. [6,7], *Nitrobacter* spp. [8,9] and *Nitrosomonas* spec. [10].

We have investigated possible functions of carboxysomes in the obligately chemolithotrophic *Thiobacilus neapolitanus* with particular focus on the role of RuBPCase.

The distribution of RuBPCase between cytosol and carboxysomes was studied in detail [11,12]. It was shown that the RuBPCase activity per mg per RuBPCase protein of the carboxysomal enzyme was higher or equal to the enzyme present in the cytosol [12]. This finding as well as the clear demonstration that carboxysomes do not serve as nitrogen storage compounds [12] made it unlikely that carboxysomes would serve as storage bodies. One of the possible functions of carboxysomes which has been considered earlier was compartmentation of the Calvin cycle enzymes but thus far tests for the presence of enzymes such as phosphoribulokinase and ribose-5-phosphate isomerase had proved negative [13,14]. The clear presence of highly active RuBPCase in the carboxysomes prompted us to reinvestigate this possibility using purified carboxysomes. This paper demonstrates the presence of all relevant Calvin cycle enzymes as well as malate dehydrogenase, aspartate aminotransferase and adenylate kinase in the carboxysomes of *T. neapolitanus*. On the basis of these findings we present a model of carboxysome functioning, showing the carboxysome as one of the few functional organelles in the prokaryotic cell.

Address correspondence to J. G. K.

2. Materials and methods

2.1. *Growth and harvesting of cells*

Thiobacilus neapolitanus strain X was grown under CO_2-limiting conditions as in a chemostat as in [11]. Cells were collected by overflow from the chemostat into a receiving bottle at 4°C. Cells were harvested by centrifugation for 40 min at 20 000 × *g* (4°C) and washed in TMD buffer (10 mM Tris (hydroxymethyl)methylamine (Tris) containing 5 mM dithiotreitol (DTT) and 20 mM $MgCl_2 \cdot 6\ H_2O$) that had been adjusted to pH 8.2 at 20°C with HCl.

2.2. *Isolation of carboxysomes*

The method used is a modification of isolation procedures in [4,15]. To obtain 4.5 mg of pure carboxysome protein it was necessary to start with 150 mg cell protein from CO_2-limited cells which contain high numbers of carboxysomes [11]. Washed concentrated (40 mg protein/ml TMD buffer) cell suspensions were sonified in the absence of ballotini beads, a procedure which did not disrupture carboxysomes [11]. Non-ruptured cells, membrane fragments and carboxysomes were pelleted by centrifugation for 60 min at 48 000 × *g* (4°C). The pellet was resus-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

pended in 1 ml of 40% (w/w) sucrose in TMD buffer and layered subsequently on a 10 ml linear gradient of 40–66% (w/w) sucrose in TMD buffer. Ultracentrifugation was carried out at 25 000 rev./min (4°C) in a Beckman SW 41 rotor over 18 h. Fractions containing 0.5 ml sucrose were collected from the top using a fraction collector. The fractions containing highest RuBPCase activity were layered once again on a 40–66% (w/w) sucrose gradient in TMD buffer. This second gradient was subjected to ultracentrifugation at 35 000 rev./min (4°C) in a Beckman SW 41 rotor over 30 h. Fractions were collected as above. The fraction containing highest RuBPCase activity was tested on purity by electron microscopy, using uranylacetate for direct negative staining. Electron micrographs were taken with a Philip EM 300.

2.3. *Enzyme activities*

The purified carboxysomes were resuspended (final conc. 1%, v/v) in 0.1 M Tris buffer adjusted to the appropriate pH and subsequently sonified for 30 s. D-Ribulose-1,5-bisphosphate carboxylase (RuBPCase, EC 4.1.1.39) activity was assayed as in [11]. ATP: D-ribulose-5-phosphate-1-phosphotransferase (phosphoribulokinase, EC 2.7.1.19) activity was determined as for RuBPCase using ribulose-5-phosphate (final conc. 0.67 mM) in the presence of 4.95 mM ATP as a substrate instead of RuBP. D-Ribose-5-phosphate ketol-isomerase (ribose-5-phosphate isomerase, EC 5.3.1.6) and D-ribulose-5-phosphate 3-epimerase (xylulose-5-phosphate epimerase, EC 5.1.3.1) were determined as described for phosphoribulokinase using ribose-5-phosphate or xylulose-5-phosphate (final conc. 0.67 mM) as a substrate instead of ribulose-5-phosphate. Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase (SBP aldolase, EC 4.1.2.13), D-sedoheptulose-1,7-bisphosphate 1-phosphohydrolase (EC 3.1.3.66), L-malate:NAD oxidoreductase (malate dehydrogenase EC 1.1.1.37), L-asparate:2-oxoglutarate aminotransferase (aspartate aminotransferase, EC 2.6.1.1), ATP:AMP phosphotransferase (adenylate kinase, EC 2.7.4.3), D-glyceraldehyde-3-phosphate:NAD oxidoreductase (glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12), ATP:3-phospho-D-glycerate-1-phosphotransferase (3-phosphoglycerate kinase, EC 2.7.2.3), D-glyceraldehyde 3-phosphate ketol isomerase (triosephosphate isomerase, EC 5.3.1.1), sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glyceraldehyde transferase (transketolase, EC 2.2.1.1) were assayed using standard methods. Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase (FBP aldolase, EC 4.1.2.13) activity was measured after preincubation at 37°C during 10 min in the presence of Fe^{2+} and cysteine [16]. The cleavage of FBP was followed at 30°C in 1 ml containing 50 μmol Tris–maleate buffer (pH 7.5), 0.12 μmol NADH, 4.5 U triosephosphate isomerase, 0.36 U α-glycerolphosphate dehydrogenase. The reaction was started by addition of 2 μmol FBP. D-fructose-1,6-bisphosphate 1-phosphohydrolase (FBPase, EC 3.1.3.11) activity was followed by a coupled assay in a 1 ml mixture containing 50 μmol Tris–maleate buffer (pH 9.0), 5 μmol $MgCl_2$, 1 U glucose-6-phosphate isomerase, 1 U glucose-6-phosphate dehydrogenase, 0.4 μmol NADP. The reaction was started by addition of 2 μmol FBP.

2.4. *Sodium dodecylsulfate (SDS)–polyacrylamide slab gel electrophoresis*

Slab gel electrophoresis was performed with 60 μg purified carboxysome protein which was denatured according to standard procedures [17]. A 3% (w/v) stacking gel and a 12.5% (w/v) running gel was employed in the procedure. The method used has been described [17].

2.5. *Determination of the ratio of particulate and soluble enzyme activities*

The distribution of enzyme activities between the particulate (i.e., in the carboxysomes or associated with membranes) and soluble (i.e., in the cytosol) fractions was determined as in [11].

2.6. *Protein determination*

Protein in cell free extracts was determined by the Coomassie blue method [18] using bovine serum albumin as a standard.

3. Results and discussion

Carboxysomes were purified by two successive sucrose gradient centrifugations from CO_2-limited chemostat grown *T. neapolitanus* cells which contain high numbers of carboxysomes (fig.1a,b). Highest specific RuBPCase activity was found in fraction 14 (fig.1b) containing 57% (w/w) sucrose. This fraction was not visibly contaminated with other cellular material as judged by electron microscopy. Without further treatment of the carboxysomes only RuBPCase,

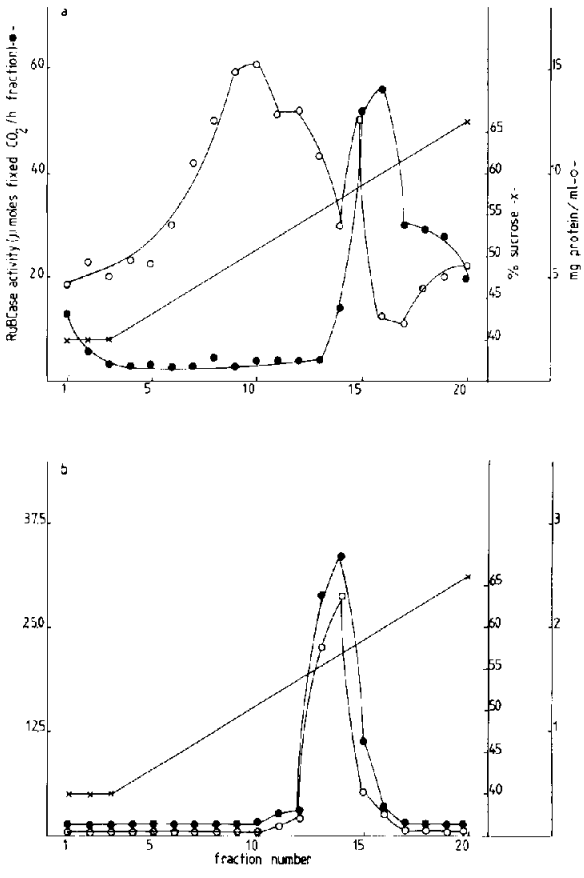

Fig.1. Purification of carboxysomes from CO_2-limited *T. neapolitanus* cells by two successive sucrose gradients. After the first sucrose gradient highest specific RuBPCase activity was observed in fractions 16 and 17 (fig.1A) which, however, still contained membrane fragments as judged by electron microscopy. These two fractions were subsequently layered on a second sucrose gradient which was run for a longer period and at a higher speed (see section 2). Electron microscopical investigations of fraction 14 (fig.1B) which contained highest specific RuBPCase activity showed that this fraction contained carboxysomes only.

fructose-1,6-bisphosphate (FBP) aldolase and sedoheptulose-1,7-bisphosphate (SBP) aldolase activity could be detected (table 1). However, after ultrasonic treatment of the carboxysomes all enzymes required for the operation of the Calvin cycle could be detected in this fraction. The absence of activity of most enzymes before sonification clearly indicated that these activities were not due to contamination of the carboxysome preparation with cytoplasmatic enzymes. It is very likely that previous unsuccessful attempts to detect other Calvin cycle enzymes in purified carboxysomes is due to their latency, which makes the unmasking of their activity for example by sonical treatment a prerequisite for their detection.

Interestingly, carboxysomes contained not only the Calvin cycle enzymes but also activities of malate dehydrogenase, aspartate aminotransferase and adenylase kinase (table 1). This suggests the possible operation of a shuttle mechanism for NADH transport analogous to that found in chloroplasts and mammalian mitochondria. In agreement with such a shuttle mechanism was the finding that addition of malate to intact carboxysomes increased the rate of RuBP dependent $^{14}CO_2$ fixation by 30% whilst addition of NADH did not result in higher activity. These findings suggest that the carboxysomal shell which has been shown to contain glycoproteins without any lipids [15] might possess selective permeability.

On the basis of these findings a model of carboxysome functioning has been constructed. Fig.2 shows that carboxysomes may be a unique prokaryotic organel for CO_2 fixation and for the allocation of reducing power to the integrated enzymes of the Calvin cycle. Thus, one might say that these carboxysomes in fact are 'Calvinosomes'. In fig.2 the product of the Calvin cycle leaving the carboxysome is assumed to be a 3-carbon compound such as glyceraldehyde phosphate (GAP), but as yet we cannot exclude other possibilities. In our scheme reducing power produced in the cytoplasmic membrane by reversed electron transport would be used to reduce oxaloacetate (OAA) to malate which then would travel to the carboxysomes. Subsequent dehydrogenation of malate inside the carboxysomes would yield NADH which is necessary for the reduction of diphosphoglycerate to glyceraldehyde phosphate (GAP) by GAP dehydrogenase. The presence of aspartate aminotransferase inside the carboxysomes would prevent accumulation of OAA which otherwise would inhibit malate dehydrogenase activity. Addition of OAA indeed prevented stimulation of RuBP-dependent $^{14}CO_2$ fixation by intact carboxysomes (table 2). The functioning of the shuttle mechanism might in this way ensure the presence of sufficiently high concentrations of NADH required for CO_2 fixation inside these organelles. The ATP required for the phosphorylation of phosphoglycerate and ribulose-5-phosphate in the carboxysome would originate from the cytosol. The presence of adenylate kinase in the organel would allow to regain 1 mole-

Table 1
Demonstration of the presence of all Calvin cycle enzymes in the carboxysomes of *Thiobacillus neapolitanus* as well as the distribution of the enzyme activities between the particulate (i.e., carboxysomes and membrane fragments) and the soluble (i.e., cytosol) fraction of a cell-free extract derived from CO_2-limited *T. neapolitanus* cells grown in the chemostat ($D = 0.07\ h^{-1}$)

	Soluble (nmol . min^{-1}. mg total cell protein^{-1})	Particulate (nmol . min^{-1}. mg total cell protein^{-1})	Pure carboxysomes	
			Sonified (nmol . min^{-1}. mg protein^{-1})	Non-sonified (nmol . min^{-1}. mg protein^{-1})
Ribulose-1,5-bisphosphate carboxylase	27	80	540	+
3-Phosphoglycerate kinase	327	105	398	–
Glyceraldehyde-3-phosphate dehydrogenase	1841	50	110	–
Triosephosphate isomerase	271	36	67	–
Fructose-1,6-bisphosphate aldolase	50	351	115	+
Fructose-1,6-bisphosphatase	171	57	42	–
Transketolase	22	5	25	–
Sedoheptulose-1,7-bisphosphate aldolase	318	20	76	+
Sedoheptulose-1,7-bisphosphatase	28	6	41	
Xylulose-5-phosphate epimerase	51	28	125	–
Ribose-5-phosphate isomerase	15	6	120	–
Phosphoribulokinase	14	17	570	–
Malate dehydrogenase	153	34	220	–
Aspartate aminotransferase	39	36	80	
Adenylate kinase	66	144	160	–

Particulate and soluble fractions were obtained as in [11]. Detectable enzyme activity in non-sonified pure carboxysomes is indicated with +, whereas the absence of measurable activity in the same preparation is indicated by –. The activities of malate dehydrogenase, aspartate aminotransferase and adenylate kinase which are also present in the carboxysomes have been included in the table

cule of ATP from 2 molecules of ADP which are produced in the Calvin cycle. The stimulatory effects of ADP and ATP on RuBP-dependent $^{14}CO_2$ fixation by intact carboxysomes are consistent with the operation of carboxysomes as 'Calvinosomes' (table 2).

One might speculate that the distribution of carboxysomes among prokaryotes can be explained according to fig.2. Most chemo(litho)autotrophic bacteria contain carboxysomes, but these bodies are absent in autotrophic hydrogen bacteria. The chemolithotrophs other than hydrogen bacteria must expend much energy for the generation of NADH through the reversed electron transport, since in most cases the electron donor is coupled to the electron-transport chain at the cytochrome-*c* level. In contrast, some hydrogen bacteria can directly reduce NAD^+ whereas others may produce reducing power at the flavine level, which still would be energetically more favourable than the situation in other chemolithotrophs. These considerations would imply that the chemolithotrophs might be unable to produce sufficiently high concentrations of NADH to run the Calvin cycle at the required rate unless they use the carboxysomes. Furthermore the presence of carboxysomes in cyanobacteria might be explained in an analogous way considering their ecological niche. They predominate in environments with a low light intensity which indicates that these organisms migth also be limited in their growth by the availability of reducing power, which, in an analogous way, would necessitate the presence of carboxysomes.

All enzymes present in carboxysomes also occur in the cytosol (table 1) though in different relative activities. Therefore CO_2 fixation may also occur in the cytosol and complicated regulation mechanisms control their relative contribution to total CO_2 fixation. It should be realized, however, that most of the Calvin cycle enzymes in the cytosol are required for the oxidative pentose phosphate cycle which has been shown to be involved in the metabolism of intracellular polyglucose [19]. The key enzymes of the oxidative pentose phosphate cycle (i.e., glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were not detectable in the sonified

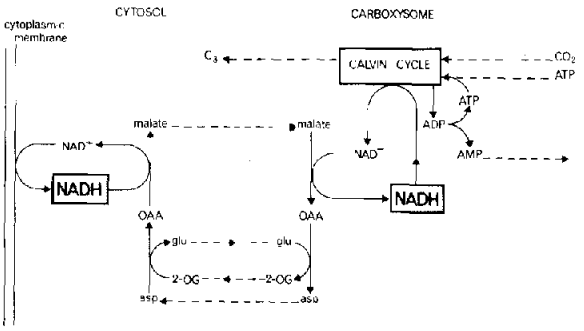

Fig.2. Hypothetical functioning of carboxysomes as 'Calvinosomes' in autotrophic prokaryotes based on the presence of enzyme activities in purified carboxysomes. For details see text. *Abbreviations:* OAA, oxaloacetate, glu, glutamate; asp, aspartate; 2-OG, 2-oxoglutarate.

carboxysomes of *T. neapolitanus* whereas their activities (52 and 77 nmol . min^{-1} . mg $protein^{-1}$, respectively) were detected easily in cell-free extracts. Naturally, the enzymes needed for the shuttle mechanism should be present both in the cytosol and in the carboxysomes. The presence of FBPase both in the carboxysomes and in the cytosol of *T. neapolitanus* could also be demonstrated by cytochemical staining: details will be presented elsewhere.

SDS–Polyacrylamide slab gel electrophoresis revealed the presence of at least 27 polypeptides in *T. neapolitanus* carboxysomes. This number would be high enough to account for the different enzyme activities detected, but obviously this is a minimum and further work will be needed to relate these polypeptides to the activities found.

As pointed out above we consider it highly likely that carboxysomes of other prokaryotes have the same or at least similar properties. While this remains to be established a whole new field of research seems to open up with many important questions to be answered, for example concerning the role of the shell as a semipermeable barrier.

Acknowledgements

The investigations were supported by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). Mr A. de Kievit and Mr M. Veenhuis are gratefully acknowledged for their electron microscopic research. We thank Dr J. P. van Dijken for stimulating discussions.

Table 2

The effects of metabolites on the rate of RuBP-dependent $^{14}CO_2$ fixation by intact carboxysomes present in the particulate fraction of a cell free extract derived from CO_2-limited *Thiobacillus neapolitanus* cells, grown in the chemostat (D = 0.07 h^{-1}; dissolved oxygen tension = 50% of air saturation)

Compound added	Stimulation (%)
None	0
Malate	30
NADH	0
Malate + NADH	30
Malate + ADP	36
Malate + ATP	42
Malate + ADP + ATP	42
Malate + ADP + ATP + OAA	0
OAA	0

The final concentrations of malate, ADP, ATP, NADH and oxaloacetate (OAA) were 0.67 mM. The control (0% stimulation) incorporated 180 nmol CO_2 . min^{-1} . mg $protein^{-1}$

References

[1] Shively, J. M. (1974) Annu. Rev. Microbiol. 28, 167–187.
[2] Haselkorn, R. (1978) Annu. Rev. Plant Physiol. 29, 319–344.
[3] Walsby, A. E. (1978) in: Relations between structure and function in the prokaryotic cell (SGM 28) (Stanier, R. Y. et al. eds) pp. 327–357, Cambridge University Press, London.
[4] Codd, G. A. and Stewart, W. D. P. (1976) Planta 130, 323–326.
[5] Stewart, W. D. P. and Codd, G. A. (1975) Brit. Phycol. J. 10, 273–278.
[6] Shively, J. M., Ball, F., Brown, D. H. and Saunders, R. E. (1973) Science 182, 584–586.
[7] Shively, J. M., Decker, G. L. and Greenawalt, J. W. (1970) J. Bacteriol. 101, 618–627.
[8] Shively, J. M., Bock, E., Westphal, K. and Cannon, G. C. (1977) J. Bacteriol. 132, 673–675.
[9] Westphal, K., Bock, E., Cannon, G. C. and Shively, J. M. (1979) J. Bacteriol. 140, 285–288.
[10] Harms, H., Koops, H. P., Martiny, H. and Wullenweber, M. (1981) Arch. Microbiol. 128, 280–281.

[11] Beudeker, R. F., Cannon, G. C., Kuenen, J. G. and Shively, J. M. (1980) Arch. Microbiol. 124, 185–189.
[12] Beudeker, R. F., Codd, G. A. and Kuenen, J. G. (1981) Arch. Microbiol. in press.
[13] Shively, J. M. and Cannon, G. C. (1979) in: abstracts IIIrd Symp. Photosynthetic Prokaryotes (Oxford) D20.
[14] Lanaras, T. and Codd, G. A. (1980) Soc. Gen. Microbiol. Quart. 7, 102–103.
[15] Biedermann, M. and Westphal, K. (1979) Arch. Microbiol. 121, 187–191.
[16] Willard, J. M. and Gibbs, M. (1968) Plant Physiol. 43, 793–798.
[17] Laemli, U. K. (1970) Nature 227, 680–685.
[18] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[19] Beudeker, R. F., Kerver, J. W. M. and Kuenen, J. G. (1981) Arch. Microbiol. 129, 221–226.