FEMS Microbiology Letters 12 (1981) 343–346
Published by Elsevier Biomedical Press

Cytochemical localization of fructose-1,6-bisphosphatase in *Thiobacillus neapolitanus* carboxysomes

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Received 10 August 1981
Accepted 17 August 1981

1. INTRODUCTION

The significance of D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) containing polyhedral bodies, the so-termed carboxysomes, for various autotrophic prokaryotes has been an open question for several years [1–4]. Recently we demonstrated that these organelles, isolated from the obligate chemolithotroph *Thiobacillus neapolitanus*, not only showed activity of RuBPCase but also of all other Calvin cycle enzymes, malate dehydrogenase, adenylate kinase and aspartate aminotransferase [5]. It was suggested that carboxysomes may function as "Calvinosomes" in *T. neapolitanus*. This suggestion postulates that carboxysomes serve as specific organelles for the allocation of reducing power to the integrated Calvin cycle [5]. Since malate stimulated RuBP-dependent $^{14}CO_2$ fixation by intact carboxysomes, whereas addition of NADH had no effect, it was suggested that reducing power travels in the form of malate to the Calvin cycle operative in the carboxysomes. The lack of stimulation of the rate of CO_2 fixation by NADH in carboxysomes was explained by the assumption that the carboxysomal shell constituted a semipermeable barrier. Among the carboxysomal enzymes only activities of RuBPCase, sedoheptulose-1,7-bisphosphate (SBP) aldolase and fructose-1,6-bisphosphate (FBP) aldolase were detectable without the need of sonification of the carboxysomes. Apparently RuBP, SBP and FBP are able to penetrate into intact carboxysomes. This finding enabled us to carry out cytochemical staining experiments on the activity of one of the Calvin cycle enzymes, fructose-1,6-bisphosphatase (FBPase), in carboxysomes by application of a recently developed method which is based on capturing of enzymatically liberated phosphate by cerous ions [6]. FBPase activity was shown in sonified carboxysomes but could not be detected in intact organelles [5]. Since FBP is able to enter the bodies this is most likely due to the complicated assay for the detection of the activity of the enzymes, which includes the presence of auxiliary enzymes converting the reaction product [5].

The assumed semipermeability of the carboxysomal shell prompted us to investigate its substructure with freeze-etching techniques. The results of these investigations are presented in this paper.

2. METHODS

2.1. Organism and growth conditions

T. neapolitanus strain X was cultivated in mineral salts medium in the chemostat under CO_2 limitation as in [3]. The dilution rate was 0.07/h

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and the dissolved oxygen tension was kept at 50% of air saturation.

2.2. *Isolation of carboxysomes*

Carboxysomes were isolated from CO_2-limited *T. neapolitanus* cultures by two successive sucrose gradients as in [5]. Preparations which still contained contaminating membrane fragments were included in the cytochemical staining experiments.

2.3. *Enzyme activity*

D-Fructose-1,6-bisphosphate 1-phosphohydrolase (FBPase EC 3.1.3.11) activity was assayed as in [5].

2.4. *Cytochemical staining*

Unfixed carboxysomes were incubated in 5 ml 0.1 M Tris-maleate buffer pH 9.0 containing 4 mM $MgCl_2$, 1 mM $CeCl_3$ and 2 mM FBP for 60 min at 30°C. After incubation the pellet was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min, subsequently washed for 15 min in 0.1 M cacodylate buffer pH 6.0 and postfixed in 1% OsO_4 + 2% $K_2Cr_2O_7$ for 30 min. After dehydration in a graded alcohol series the organelles were embedded in Epon 812, sectioned with a diamond knife and examined in a Philips EM300.

2.5. *Freeze-etching*

Whole cells were incubated in 10% glycerol for 5 min before freezing in liquid FREON. Isolated carboxysomes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 washed in distilled water and subsequently frozen in FREON. Cells and isolated carboxysomes were also spray-frozen according to the technique of Bachmann [7]. Freeze-fracturing was performed in a Balzer's freeze-etch unit according to the method described by Moor [8].

2.6. *Protein determination*

Protein was determined by the Coomassie blue method using bovine serum albumin as a standard [9].

3. RESULTS AND DISCUSSION

Sonified pure carboxysomes isolated from *T. neapolitanus* showed an FBPase activity of about 45 nmol/min/mg protein. To demonstrate FBPase activity in the carboxysomes cytochemically the effects of aldehyde prefixation and of cerous ions on the enzyme activity were tested in cell-free extracts derived from *T. neapolitanus*. Incubation of such extracts at 0°C with 3% glutaraldehyde inhibited FBPase activity completely within 10 min. Formaldehyde (3%) also strongly inhibited FBPase activity; after 10 min of incubation the activity had been reduced to 12% of the initial activity (Fig. 1). Addition of Ce^{3+} ions to the assay mixture at a final concentration of 1 mM inhibited the FBPase activity only for 30% after 60 min of incubation (Fig. 1). Since both glutaraldehyde and formaldehyde strongly inhibited FBPase activity, prefixation with these compounds had to be omitted. An additional problem for studying the metabolism of organic compounds by intact unfixed cells is that these obligately chemolithotrophic bacteria exhibit a low transport capacity for various organic compounds. It appeared, for example, impossible to introduce the presence of significant amounts of label in *T.*

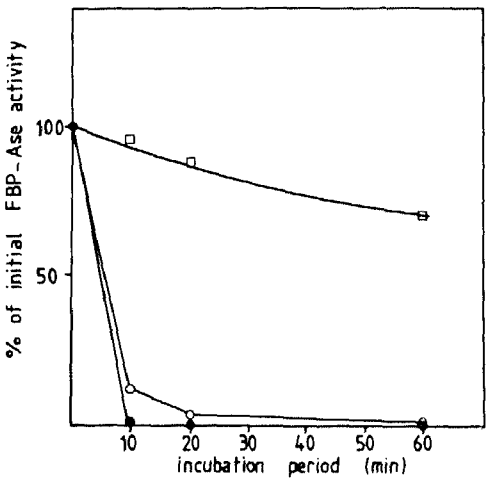
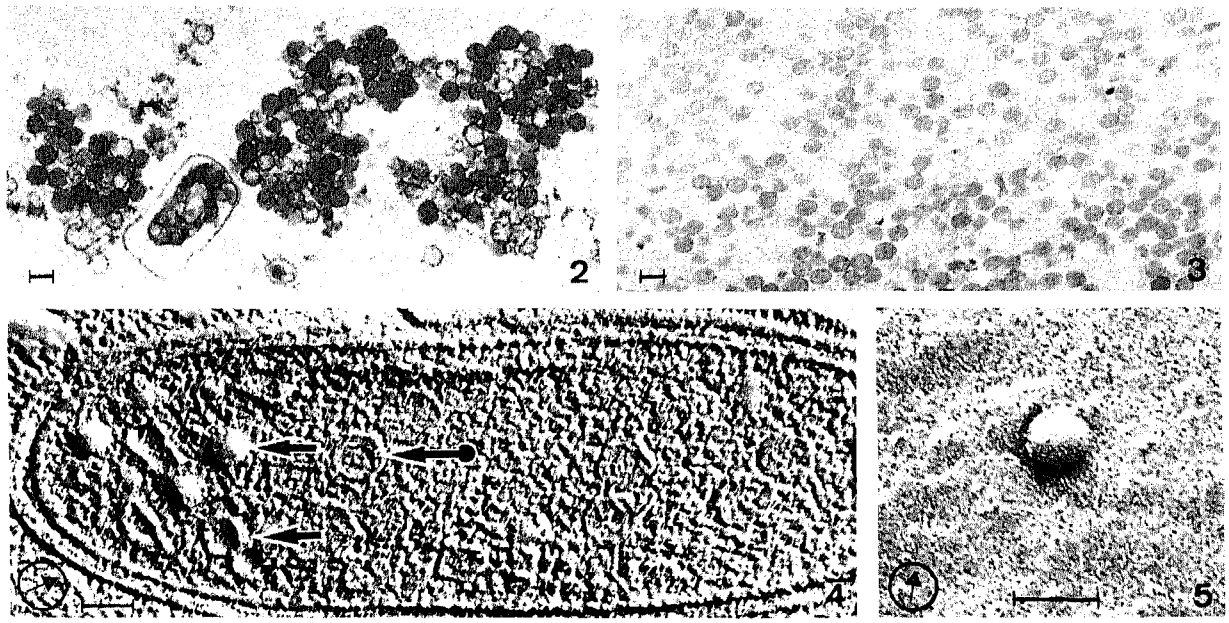


Fig. 1. The effects of cerous ions (□), glutaraldehyde (●) and formaldehyde (○) on FBPase activity in cell-free extracts derived from *Thiobacillus neapolitanus*. For experimental details see text.



Figs. 2 and 3. Cytochemical demonstration of FBPase activity after incubation of isolated carboxysomes with $CeCl_3$ and FBP in the presence of Mg^{2+} ions. The reaction products are localized in the carboxysomal matrix (Fig. 2). These products are absent after similar incubations in the absence of substrate (Fig. 3).

Figs. 4 and 5. Feeze-etch images of intact cells (Fig. 4) and glutaraldehyde-fixed isolated carboxysomes (Fig. 5). In Fig. 4 a number of carboxysomal profiles is observed (dotted arrow) as well as smooth surfaces of the carboxysomal shell site closest to the protoplasm (PS) and of the exoplasmic shell site (ES) (arrows). In Fig. 5 the PS of the shell is observed (nomenclature after [14]). The arrows left under represent the direction of shadowing. The bars represent 100 nm.

neapolitanus cells applying [U-^{14}C]glucose as a tracer molecule [10]. Against this background it is reasonable to assume that FBP would hardly enter intact unfixed cells. However, since FBP can penetrate into isolated carboxysomes [5] the cytochemical experiments were performed on isolated carboxysomes without any pretreatment. Incubation of unfixed carboxysomes with $CeCl_3$ and FBP showed localization of the Ce^{3+} phosphate precipitate in the carboxysomal matrix (Fig. 2). Control experiments performed in the absence of substrate, the absence of Mg^{2+} ions or in the presence of 0.5 mM AMP as a specific inhibitor of *T. neapolitanus* FBPase [11] gave negative results (Fig. 3). The cytochemical demonstration of FBPase activity in isolated carboxysomes from *T. neapolitanus* substantiates the recent enzymic data showing the presence of the integrated Calvin cycle enzymes inside this functional prokaryotic organelle [5].

The functioning of carboxysomes as Calvinosomes requires semipermeability of the carboxysomal shell ([5], see INTRODUCTION). In the electron microscope this shell is visualized as a single dark layer of approx. 350 nm [12]. It was suggested that the carboxysomal shell of *T. neapolitanus* and that of *Nitrobacter agilis* is devoid of lipids and would consist of glycoproteins only [12,13]. To characterize its substructure further freeze-etch experiments were performed. After conventional freeze-etching or after spray-freezing of the cells the carboxysomal shell is rarely observed since the carboxysomes generally are also fractured and only their profile can be examined. When visualized after these techniques the shells showed a smooth appearance (Fig. 4). The resolution of the substructure of the carboxysomal shell was improved after etching of glutaraldehyde-fixed isolated organelles. In these experiments the smooth appearance of the carboxysomal shell was

confirmed (Fig. 5). A smooth surface of membranes in freeze-etch preparations indicates the lack of membrane-bound particles on the fracture face. Such particles which are supposed to be condensed protein complexes are observed for instance in the fracture faces of the cell membranes of *T. neapolitanus* (not shown). Since the carboxysomal shell does not show fracture faces, and the organelles are easily fractured in freeze-etch preparations, it is concluded that the shell does not contain hydrophobic parts like the lipids in cellular membranes. These observations confirm and extend the suggestions made by others that the carboxysomal shell is devoid of lipids [12,13]. Much remains to be learned about the carboxysomal shell glycoproteins affecting semipermeability, however.

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 is gratefully acknowledged for skilful technical assistance. We thank Mr. J. Zagers for performance of the freeze-etching experiments.

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