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Icosahedral Inclusions (Carboxysomes) of *Nitrobacter agilis*

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The icosahedral bodies of *Nitrobacter agilis* are about 120 nm in diameter and, as viewed by electron microscopy, consist of an outer shell enclosing 10-nm particles. The inner 10-nm particle is the enzyme D-ribulose 1,5-bisphosphate carboxylase. The bodies isolated from cells incubated 1 month without nitrite had a specific activity for the enzyme of 0.54 μmol of CO_2 fixed per min per mg of protein.

Autotrophically grown cells of the cyanobacteria, nitrifying bacteria, and thiobacilli contain polyhedral inclusion bodies that vary from 50 to 500 nm in diameter and are situated in the nucleoplasmic region of the cell (8). The bodies of *Thiobacillus neapolitanus* have been isolated and shown to contain the enzyme D-ribulose 1,5-bisphosphate carboxylase (RuBPCase) (EC 4.1.1.39) and have been named carboxysomes (9, 10). Carboxysomes have also been demonstrated in *Anabaena cylindrica* (3).

Hexagonal inclusions of unknown structure and function were described in electron microscopic studies of *Nitrobacter* (5, 11). Bock and his co-workers (1, 2, 6, 12, 13) isolated these bodies and, because of their icosahedral shape, uniform size (120 nm), and preliminary evidence suggesting the presence of nucleic acid, called them phagelike particles (Nb_1).

This report establishes the presence of RuBPCase in the NB_1 particles, which classifies them as carboxysomes.

MATERIALS AND METHODS

Nitrobacter agilis (*nitrobacter* K_1) was grown lithoautotrophically in 30 liters of nitrite medium (pH 7.6 to 7.8) with constant aeration. The nitrite medium contained (in grams per liter of distilled water): $NaNO_2$, 2.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.05; KH_2PO_4, 0.15; $CaCO_3$, 0.07; $FeSO_4$, 0.00015; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.00005. After the nitrite was consumed, an additional 2.0 g of $NaNO_2$ was added per liter. After about 1.5 months, when the nitrite was consumed and the cells had rested for 1 month, the bacteria were harvested by centrifugation, washed twice, and suspended in 10 ml of the following buffer: 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride-0.01 M $MgCl_2 \cdot 2H_2O$, pH 7.5. The polyhedral bodies were isolated as described by Bock (1). The cells were broken by sonic treatment for 6 min at 0°C (20 kHz, Lehfeld sonic oscillator). The nonruptured cells were sedimented by centrifugation for 20 min at 7,000 × *g*, suspended in 10 ml of buffer, and subjected to sonic treatment. This procedure was repeated three times. The polyhedral bodies were sedimented from the combined supernatant fluids by centrifugation for 30 min at 45,000 × *g*, suspended in a small volume of buffer, and layered onto linear gradients of 10 to 40% (wt/wt) sucrose. After centrifugation in a Beckman L5-65 ultracentrifuge (SW40 rotor) for 30 min at 42,000 × *g*, the gradients were scanned using an ISCO gradient fractionator, and the body-containing fractions (at about 20% sucrose) were collected. The volume of the suspension was doubled with buffer, and the bodies were sedimented by centrifugation for 1 h at 120,000 × *g*. The bodies were suspended in buffer and layered onto 10 to 40% (wt/wt) sucrose gradients, centrifuged, and collected as before.

Unfixed samples from the purification were stained with potassium phosphotungstate at pH 7.0 and observed in a Siemens Elmiskop 1.

Assays of RuBPCase were performed as previously described (9). All values were corrected by subtracting background fixation that took place in the absence of ribulose bisphosphate. The radioactivity was measured with Packard Dimilume scintillation fluid and a Berthold BF 5000 liquid scintillation spectrometer equipped with an external standard. Protein was estimated by the method of Lowry et al. (4) with bovine serum albumin as the standard. The specific activity of each sample was calculated as the micromoles of CO_2 fixed per minute per milligram of protein.

RESULTS AND DISCUSSION

The RuBPCase of *N. agilis* copurified with the icosahedral inclusion bodies (Table 1). The first differential centifugation (6,000 × *g*) resulted in a 65% increase in enzyme units. This has been noted in several *N. agilis* preparations, as well as in some *T. neapolitanus* preparations (unpublished data). If the bodies are inactive in CO_2 fixation, i.e., enzyme storage, the increase may be due to the rupturing of

some of the bodies during centrifugation and handling, e.g., suspension of the pellet. However, the increased activity could be due to the elimination of an enzyme inhibitor or body inactivator that sediments at 6,000 × *g*. The second differential centrifugation (40,000 × *g*) consistently resulted in a loss of enzyme activity (46% in this instance). There was a 51% decrease in supernatant activity that was not recovered in the pellet. The bodies sedimented during this procedure, but free enzyme did not. One could hypothesize that there is either a component in the pellet that interferes with the enzyme activity or that there is some factor or environmental state supplied by the supernatant fluid that is required for the inclusion's CO_2-fixing ability. Irreversible enzyme inactivation could account for the loss, but this theory is discounted by the fact that a large increase in activity was noted in the first sucrose gradient (a 23-fold increase in this instance), with the majority of the activity being in the carboxysome fraction (specific activity, 0.18). This supports the earlier contention that an inhibitor or modulator in the pellet interferes with the enzyme activity of the body. However, if one considers that the bodies are inactive in CO_2 fixation, the sucrose may destabilize the bodies, thereby permitting substrate availability to the enzyme. We have noted that sucrose does destabilize the carboxysomes of *T. neapolitanus* (9, 10). The second sucrose gradient further purified the carboxysomes (final specific activity of 0.54). A 15% increase in recoverable enzyme units was noted. The final specific activity is relatively low for pure RuBPCase, but some membrane contamination still was present, and under our current isolation conditions the enzyme is very unstable (90% of the activity is lost in 3 days).

It should be noted that only 53 and 54% of the loaded protein was recovered from the first and second sucrose gradients, respectively. We are reporting only major fractions, i.e., where enzyme activity existed. The rest of the protein was spread throughout the gradient.

The purified bodies (Fig. 1) are similar in structure to those isolated from *T. neapolitanus*. They are about 120 nm in diameter and consist of an outer, monolayer shell surrounding 10-nm particles, i.e., the RuBPCase.

This report demonstrates that the icosahedral bodies of *N. agilis* are carboxysomes (the

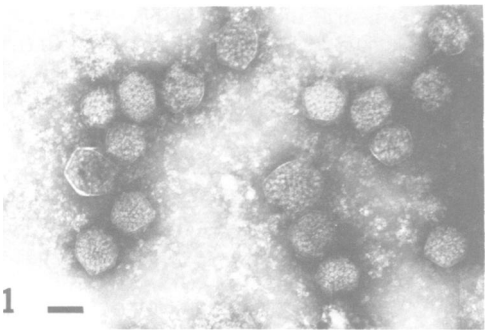

FIG. 1. *Electron micrograph (negative stain) of the purified carboxysomes of N. agilis. The RuBPCase from ruptured bodies can be observed in the background. Bar = 100 nm.*

TABLE 1. *Results of the purification of carboxysomes and RuBPCase from Nitrobacter agilis*

Purification procedure	Fraction	Enzyme units (μmol of CO_2 fixed per min)	Protein (mg)	Sp act (μmol of CO_2 fixed per min per mg of protein)
Sonic treatment	Sonic extract[a]	0.35	93.2	0.004
Differential centrifugation (6,000 × *g*)	Supernatant[a]	0.39	47.4	0.008
	Pellet	0.25	25.5	0.01
Differential centrifugation (40,000 × *g*)	Supernatant	0.19	22.4	0.008
	Pellet[a]	0.02	19.8	0.001
Sucrose gradient 1 (10–40%)	Top	0.01	3.30	0.003
	Carboxysomes[a]	0.41	2.31	0.18
	Pellet	0.04	4.90	0.008
Sucrose gradient 2 (10–40%)	Carboxysomes	0.47	0.87	0.54
	Pellet	0.02	0.37	0.05

[a] Fraction carried forward to next step.

first from a nitrifying bacterium) and, consequently, are like those bodies previously isolated from *T. neapolitanus* (9, 10) and *A. cylindrica* (3). The polyhedral bodies of *Thiobacillus intermedius*, even though they have not been isolated, also appear to be carboxysomes (7). Growth conditions that repress RuBPCase eliminate the polyhedral bodies, and transfer of the culture back to nonrepressive growth conditions permits enzyme and body formation (7). Research is in progress to elucidate the function of the carboxysomes.

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