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Immunocytochemical Localization of Phosphoribulose Kinase in the Cyanelles of *Cyanophora paradoxa* and *Glaucocystis nostochinearum*[1]

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ABSTRACT

The distribution of phosphoribulose kinase (PRK) in the cyanelles of *Cyanophora paradoxa* Korschikoff and *Glaucocystis nostochinearum* Itzigsohn was studied by protein A-gold immunoelectron microscopy. In both endocyanomes, antiserum against PRK heavily labeled the thylakoid region of the cyanelles, whereas little or no label was present over the carboxysomes. Antiserum against ribulose 1,5-bisphosphate carboxylase/oxygenase by contrast heavily labeled the carboxysomes of each endocyanome. *In vitro* studies of PRK distribution in cell-free extracts of *C. paradoxa* showed that 93% of the enzyme was in the soluble fraction. Quantitative immunoelectron microscopy showed that more than 99% of the PRK in the cyanelle of *C. paradoxa* was localized in the thylakoid region. We conclude that the carboxysomes of cyanelles like the carboxysomes of autotrophic prokaryotes and the pyrenoids of green algal chloroplasts do not contain phosphoribulose kinase.

RuBisCo[3] and PRK are unique and essential enzymes of the reductive pentose phosphate cycle (Calvin cycle) of CO_2 assimilation. RuBisCO is responsible for the initial steps of CO_2 fixation and photorespiration since this bifunctional enzyme catalyzes the carboxylation and oxygenation of RuBP. PRK precedes RuBisCO in the Calvin cycle and catalyzes the regeneration of RuBP from ribulose 5-P (5).

In apparently all cyanobacteria and some colorless sulfur-oxidizing bacteria and nitrifying bacteria, a variable proportion of the cellular complement of RuBisCO is located in electron-dense polyhedral bodies called carboxysomes (6, 20). Under some physiological conditions, much of the RuBisCO in these organisms is soluble, but between 10 and almost 100% of the enzyme may be carboxysomal (1, 14). Besides RuBisCO, carboxysomes contain several cryptic polypeptides. The possibility that carboxysomes also contain PRK has been considered, although this has been discounted in the colorless sulfur-oxidizer *Thiobacillus neapolitanus* and the cyanobacterium *Chlorogloeopsis fritschii* on the basis of *in vitro* fractionation studies with both organisms (4, 10, 18) and immunoelectron microscopy of *C. fritschii* cell sections (7).

Electron-dense inclusion bodies are a feature of several cyanelles (6, 11). The likely origins of cyanelles from endosymbiotic ancestral photosynthetic prokaryotes contributes to the interest in these structures, which may represent an intermediate stage in the origins of chloroplasts (8). We have recently shown by immunoelectron microscopy that the central dense body in the cyanelles of *Cyanophora paradoxa* and the terminal electron-dense lamella-free region in the cyanelles of *Glaucocystis nostochinearum* also contain RuBisCO (16) and thus are probably functionally equivalent to the carboxysomes of autotrophic prokaryotes. In the present study we wished to determine whether or not PRK is present in the carboxysomes of cyanelles. We show by *in vitro* fractionation of whole cell extracts and by immunoelectron microscopy that PRK is localized throughout the thylakoid region of the cyanelles of *C. paradoxa* and *G. nostochinearum* and that no enzyme is localized in the carboxysomes.

MATERIALS AND METHODS

Cell Culture. *Cyanophora paradoxa* Korschikoff used for the immunocytochemical studies was obtained from the University of Texas Culture Collection of Algae (UTEX LB555). *Glaucocystis nostochinearum* Itzigsohn was obtained from the Carolina Biological Supply Co., Burlington, NC (No. 15-1790). The cultures were maintained as described previously (16).

The stock culture of *C. paradoxa* used for the enzyme assays was obtained from Dr. L. Provasoli. Cells were grown photoautotrophically in the nitrate medium of Bothe and Floener (2) as described previously (14) and harvested in the late logarithmic phase of growth.

Antisera. Antiserum against PRK purified from *Chlorogloeopsis fritschii* (17) was raised in rabbits as described previously (18). Null serum was collected by bleeding the rabbit prior to the injection of PRK. Antiserum prepared against the large subunit of RuBisCO from tobacco (15) was kindly given us by Jacqueline Fleck (IBMC-CNRS, Strasbourg).

Immunoelectron Microscopy. In order to localize PRK, stationary phase cells of *C. paradoxa* and 16 day old cells of *G. nostochinearum* were fixed in 1.5 to 3.0% (v/v) glutaraldehyde in culture medium for 1.5 to 2 h at 4°C and embedded in Lowicryl K4M as detailed earlier (12). Pale gold sections were cut and mounted on Formvar-coated nickel grids. Immunostaining was performed by placing the grids section-side down on drops of the following solutions: PBS, 15 min; 1% (w/v) oval-

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[3] Abbreviations: RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); PRK, phosphoribulose kinase (EC 2.7.1.19); RuBP, ribulose 1,5-bisphosphate; LSU, large subunit of RuBisCO.

bumin, 15 min; PRK antiserum, diluted 1:100 in PBS, 30 min to 2 h; rinse in jets of PBS; protein A-gold, prepared as described previously (16), 30 min. Sections were post-stained with aqueous uranyl acetate and lead citrate and viewed in a Philips EM410 electron microscope. As a control, the antibody solution was replaced by null serum, diluted 1:100 in PBS.

In order to localize RuBisCO, cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Spurr's epoxy resin. Sections were treated with saturated sodium metaperiodate and then labeled with anti-LSU as detailed previously (16).

Quantitative Evaluation of Immunoelectron Micrographs. The amount of labeling over various cell compartments was compared by determining the number of gold particles per μm^2. Area determinations were made on a Zeiss MOP-3. To determine volume, the length and width of the cyanelles and carboxysomes of *C. paradoxa* were measured on 48 micrographs in which the organelles were judged to be cut in medial or near medial section, and volumes were calculated using the formula for an oblate spheroid.

PRK Assays. Cell-free extracts of *C. paradoxa* containing soluble and particulate (inclusion body) proteins were obtained as 40,000*g* for 1 h supernatant and pellet fractions as detailed earlier (13, 18). PRK activity was measured by coupling the formation of RuBP to the subsequent incorporation of $NaH^{14}CO_3$ into acid-stable material in the presence of excess exogenous RuBisCO as described previously (17). In tests to determine the effects of *C. fritschii* PRK antiserum on the activity of the *C. paradoxa* enzyme, 200 μl of antiserum or null serum were incubated with the soluble and particulate cell-free extracts for 15 min at 30°C before assaying. Controls were run minus ribulose 5-P. Protein was determined by the method of Bradford (3) using bovine serum albumin as a standard.

RESULTS

Figure 1 shows a cyanelle of *C. paradoxa* labeled with anti-PRK. Numerous gold particles are localized throughout the thylakoid region, whereas only a few scattered particles are present over the dense central body. The cytoplasm of the cell appears unlabeled. Figure 2 shows the contrasting pattern of labeling obtained with antiserum to the large subunit of RuBisCO. The dense body in the center of the cyanelle is heavily labeled, thus identifying it as a carboxysome. Only scattered gold particles are present over the thylakoid region of the cyanelle or

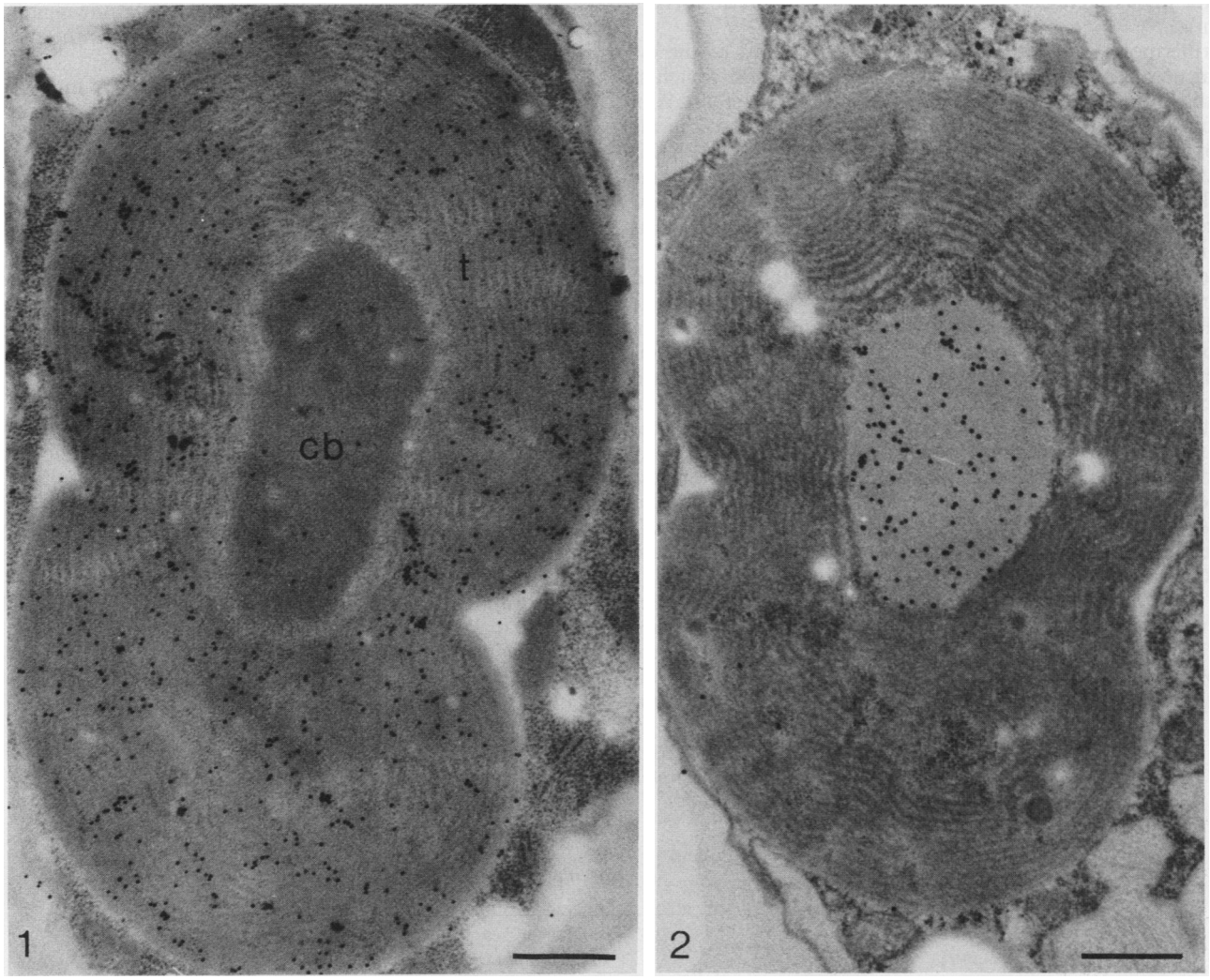

FIG. 1. Labeling of *C. paradoxa* with anti-PRK. The gold particles are concentrated over the thylakoid region (t) of the cyanelle. Little label is seen over the carboxysome (cb) (×28,600; bar = 0.5 μm).

FIG. 2. Labeling of *C. paradoxa* with anti-LSU. The carboxysome of the cyanelle is heavily labeled. Only a few gold particles are present over the thylakoid region (×28,000; bar = 0.5 μm).

Table I. *Density of Labeling over Various Cell Compartments in C. paradoxa*

	Cyanelle		Host Cell	n^a
	Carboxysome	Thylakoid region		
	gold particles per μm^2 ± SEM			
Anti-PRK	8.1 ± 0.6	45.9 ± 2.1	3.7 ± 0.4	23
Null serum	0.6 ± 0.4	0.7 ± 0.1	0.6 ± 0.2	13

[a] n = number of micrographs evaluated.

the cytoplasm of the host cell.

Table I compares the density of labeling of the thylakoid region, carboxysome, and host cell cytoplasm by antiserum against PRK. The enzyme, being exclusive to the Calvin cycle, is not found outside the photosynthetic cyanelle, and therefore any gold particles counted over the host cell are considered as background. The labeling over the carboxysome is significantly higher than that over the host cell ($P < 0.01$) which suggests that carboxysomes may also contain PRK. However, we believe that the labeling of the carboxysome by anti-PRK is nonspecific, since the labeling intensity obtained with increasing antibody dilutions decreases more markedly over the carboxysome than over the thylakoid region and the host cell cytoplasm (results not shown).

Furthermore, calculations based on the volumes of the cyanellar compartments indicate that a labeling intensity such as that shown in Table I corresponds to a distribution of 99.5% of the total PRK in the thylakoid region and of only 0.5% in the carboxysome. We therefore assume that all the cyanellar PRK is located in the thylakoid region. In Table I and in the volume calculations, it was not possible to assess separately the narrow nucleoplasmic region surrounding the carboxysome and it has been included in the thylakoid region.

The distribution of PRK activity between the soluble and particulate fractions of *C. paradoxa* cyanelles is shown in Table II. Ninety-three per cent of the activity detected was present in the soluble fraction. The specific activity of the soluble PRK was 15 times higher than that of the small amount of enzyme detected in the particulate fraction. The location of the remaining 7% of detectable activity in the particulate fraction is unclear, but the immunoelectron microscopy studies indicate that this activity would be associated with the thylakoids, rather than the carboxysomes. The inhibitory effect of the *C. fritschii* PRK antiserum against the *C. paradoxa* cyanelle PRK is also shown in Table II, confirming the cross-reactivity of the cyanobacterial antiserum with the cyanellar protein.

In *G. nostochinearum* the cyanelles are elongate structures which have a dense lamella-free region at one extremity. Figures 3 and 4 show the labeling pattern observed with anti-PRK. Figure 5 shows the pattern of labeling observed with anti-LSU. Anti-PRK heavily labels the thylakoid region of the cyanelles, whereas the lamella-free region of each cyanelle and the background cytoplasm are unlabeled (Figs. 3 and 4). A few scattered gold particles are present over the nucleoplasmic region of the cyanelle (Fig. 4). Anti-LSU by contrast densely labels the lamella-free regions of the cyanelles (Fig. 5), which suggests that they are equivalent to carboxysomes. Only a few scattered gold particles are observed over the rest of the cyanelle, and the cytoplasm of the host cell appears unlabeled.

Table III compares the labeling of the different cyanellar compartments and the host cell cytoplasm of *G. nostochinearum* by anti-PRK. At the shorter labeling time, the labeling of the lamella-free region was not significantly higher than the background labeling of the host cell cytoplasm ($P > 0.05$). After a longer incubation period with anti-PRK, the labeling was higher over the lamella-free region than over the background. We believe that this labeling is nonspecific since prolonged incubation with antibodies increases nonspecific binding. We conclude that PRK is not present in the carboxysomes of *G. nostochinearum*. In this species, it was possible to measure the labeling of the nucleoplasmic region separately from that of the thylakoid region, although it was often difficult to determine the exact boundaries of the nucleoplasmic region due to the poor contrast of these Lowicryl-embedded cells. Nonetheless, low levels of labeling were observed over the nucleoplasmic region. One would expect some PRK to be present in the nucleoplasmic region, for PRK is likely to be coded by cyanelle DNA and synthesized on the abundant ribosomes found in this region.

As a control, antiserum to PRK was replaced by null serum. No specific labeling was observed in either *C. paradoxa* or *G. nostochinearum* (Tables I and III). The controls for the experiments with anti-LSU and the quantification of these results are given in a previous publication (16).

DISCUSSION

In this study we demonstrate that the Calvin-cycle enzyme PRK is localized throughout the thylakoid region of the cyanelles of two endocyanomes and is absent from the carboxysomes. Quantitative immunoelectron microscopy showed that virtually all the PRK in the cyanelle of *C. paradoxa* is localized in the thylakoid region (99.5%) whereas only 0.5% is found in the carboxysome. We conclude, therefore, that no PRK is present in the carboxysome of *C. paradoxa*. These findings were supported by the results of *in vitro* PRK distribution assays. Similarly, in *G. nostochinearum* after 1 h labeling time, the lamella-free region was unlabeled, with most of the PRK being found in the thylakoid region. Only a small amount is localized in the nucleoplasm.

Our results agree with those obtained by Marsden *et al.* (18) on the cyanobacterium *C. fritschii*. Upon cell fractionation, they found that 95% of the PRK activity was soluble, and that the remaining activity, although pelletable, was not associated with the carboxysome fraction. An electron microscopic study confirmed that PRK was present throughout the cytoplasm of *C.*

Table II. *Distribution of PRK Activity in Extracts of the Cyanelles of C. paradoxa and Inhibition of Cyanelle PRK by Antiserum to Enzyme from Cyanobacterium Chlorogloeopsis fritschii*

Cyanelle Fraction	Volume	Protein	Phosphoribulose Kinase		
			Specific activity	Distribution of activity	Inhibition by 200 μl PRK antiserum
	ml	*mg·ml^{-1}*	*μmol·min^{-1}·mg^{-1} protein*	%	%
40,000*g* × 1 h supernatant	10.0	7.38	0.570	93.15	91.6
40,000*g* × 1 h pellet	5.0	8.77	0.039	6.85	92.3

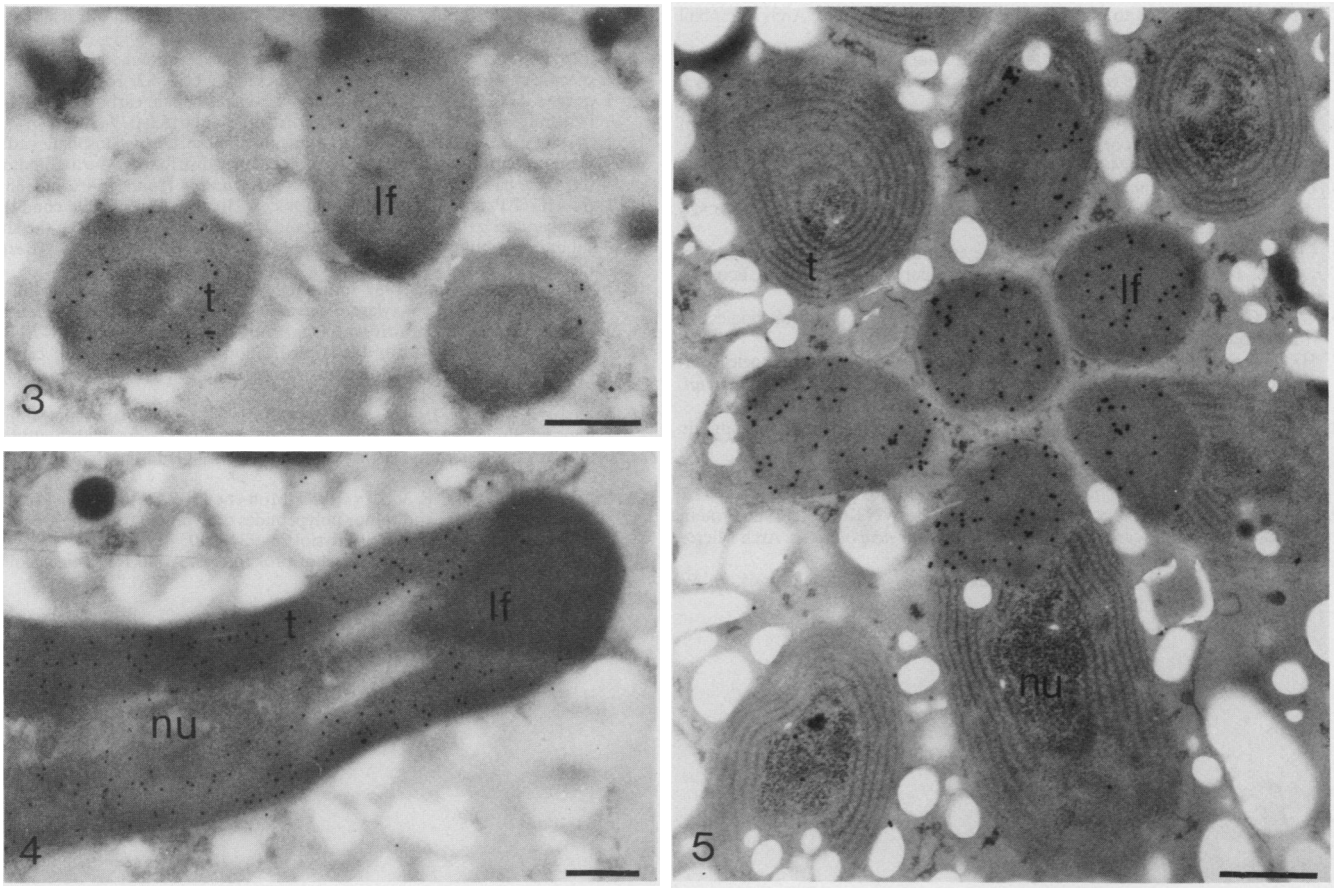

FIG. 3. Labeling of *G. nostochinearum* with anti-PRK (1 h). Labeling is restricted to the thylakoid region (t) of each cyanelle. The lamella-free regions (lf) are unlabeled (×22,750; bar = 0.5 μm).

FIG. 4. Labeling of *G. nostochinearum* with anti-PRK (2 h). The thylakoid region (t) of the cyanelle is heavily labeled whereas the lamella-free region (lf) is unlabeled. Scattered gold particles are present over the nucleoplasm (nu) (×19,250; bar = 0.5 μm).

FIG. 5. Labeling of *G. nostochinearum* with anti-LSU. The lamella-free regions (lf) of the cyanelles are intensely labeled. The typical arrangement of cyanelles in clusters, with the lamella-free regions facing each other, can be seen in this micrograph. nu, nucleoplasm; t, thylakloid region (×22,750; bar = 0.5 μm).

Table III. *Density of Labeling over Various Cell Compartments in G. nostochinearum*

	Cyanelle			Host Cell	n[a]
	Lamella-free region	Thylakoid region	Nucleoplasm		
	gold particles per μm² ± SEM				
Anti-PRK					
1 h	3.0 ± 0.4	23.3 ± 1.3	8.1 ± 1.8	2.3 ± 0.4	20
2 h	7.3 ± 1.4	36.4 ± 1.8	8.1 ± 1.0	1.4 ± 0.3	14
Null serum (2 h)	2.2 ± 1.0	1.5 ± 0.3	1.6 ± 0.8	0.9 ± 0.2	14

[a] n = number of micrographs evaluated.

fritschii, but absent from the carboxysomes (7). Our data demonstrate that PRK, an essential enzyme of the Calvin cycle, is not present in cyanelle carboxysomes. In this respect, these inclusions resemble the carboxysomes of autotrophic prokaryotes. An early study (9) on the composition of pyrenoids, the electron-dense inclusions found in the chloroplasts of many algal species, suggested that they contained PRK in addition to RuBisCO. However, recently Satoh *et al.* (19) have shown conclusively that the pyrenoid of the green alga *Bryopsis maxima* does not contain PRK and thus is similar in composition to the carboxysomes of cyanelles and prokaryotes.

Several possible functions for carboxysomes exist, including an active role in CO_2 fixation, a storage function and a role to protect RuBisCO from inhibition by O_2 (1, 6, 14). The labeling of RuBisCO in the *C. paradoxa* and *G. nostochinearum* cyanelles has indicated that all, or almost all, of this enzyme is located in the carboxysomes (16) (Figs. 2 and 5). If this is so, then an active role for the cyanelle carboxysomes in CO_2 fixation may exist. If cyanelle carboxysomes are a site of CO_2 fixation *in vivo*, then the present data indicate that RuBP would have to enter the carboxysomes, since these structures lack the RuBP-regenerating enzyme, PRK.

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