Immunocytochemical localization and functional determination of phytoene desaturase in photoautotrophic prokaryotes

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The *in situ* location of phytoene desaturase, an enzyme in the carotenoid biosynthetic pathway, has been investigated in the photosynthetic organisms *Rhodobacter capsulatus* SB 1003, *Synechocystis* PCC 6714, and *Anabaena variabilis* ATCC 29413. A post-embedding immunogold electron microscopy procedure using a polyclonal antibody against a phytoene desaturase fusion protein was employed. For all three species most of the gold particles (about 85% in the case of *Anabaena* and *Synechocystis*) were associated with the photosynthetic membranes. Other parts of the cells were only randomly and not significantly labelled. When the anti-phytoene desaturase antibody was omitted, or when pre-immune serum was used, no gold deposition was observed. *In vitro* phytoene desaturase reactions, especially with purified thylakoid membranes from *Synechocystis*, confirmed the nature of phytoene desaturase as an integral protein of the thylakoid membrane.

Introduction

Phytoene desaturase is a key enzyme in the biosynthetic route of carotenoids (Bramley, 1985). It converts phytoene into ζ -carotene and shows feedback inhibition by subsequent carotenes in the pathway (Bramley & Davies, 1976; Sandmann & Kowalczyk, 1989); it is the target for various commercial herbicides (Sandmann & Böger, 1989). Biochemical characterization of this enzyme from photosynthetic organisms has been reported (Clarke *et al.*, 1982; Sandmann & Kowalczyk, 1989), but its purification has not yet been achieved. Nevertheless, the recent progress in the molecular biology of several carotenogenic enzymes, including phytoene desaturase (Armstrong *et al.*, 1989; Bartley & Scolnik, 1989), made it possible to construct a fusion gene with the *lacZ* gene and a 921 bp fragment of the phytoene desaturase gene. Expression in *Escherichia coli* resulted in a fusion protein which was employed to raise polyclonal antibodies. These antibodies inhibit phytoene desaturation and cross-react in Western blots with phytoene desaturase from a photosynthetic bacterium, from a cyanobacterium, and from higher plants (Schmidt *et al.*, 1989).

This antibody preparation and immunocytochemical methods were used in the present investigation to localize phytoene desaturase in the photosynthetic bacterium *Rhodobacter capsulatus* SB 1003, and in the unicellular and the filamentous cyanobacteria *Synechocystis* PCC 6714 and *Anabaena variabilis* ATCC 29413, respectively. Phytoene desaturase activities were also determined in all three organisms and the results obtained by immunogold-labelling studies were confirmed in *Synechocystis* by *in vitro* reactions with purified thylakoids.

Methods

Organisms and growth. Culture conditions for *Synechocystis* PCC 6714 and *Anabaena variabilis* ATCC 29413 were as used previously for other cyanobacteria (Clarke *et al.*, 1982), and those for *Rhodobacter capsulatus* SB 1003 were as described by Marrs (1981). After 2 d of growth, cells were harvested by centrifugation for use in *in vitro* studies and fixation. The *Fusarium* mutant SG4 (from Departamento de Genética, Universidad de Sevilla, Spain), in which phytoene formation is blocked, was grown in 2.4% (w/v) potato-dextrose broth for 5 d.

***In vitro* assays.** Cell-free carotenogenesis by the photosynthetic prokaryotes was carried out in a coupled system using *Fusarium* SG4 extracts as a source of [14 C]geranylgeranyl pyrophosphate (GGPP, synthesized from R-[2- 14 C]mevalonic acid), following the conversion of GGPP into the subsequent carotenes. The details of this assay with membranes from the cyanobacteria are given by Sandmann & Kowalczyk (1989). In the case of *Synechocystis*, thylakoids were

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Abbreviations: GGPP, geranylgeranyl pyrophosphate; PBS, phosphate buffered saline (20 mM-sodium phosphate, 0.9% NaCl, pH 7.4).

purified from a French-press homogenate by centrifugation on a sucrose gradient and the purity of the preparation was determined by its absorption spectrum, as described by Murata & Omata (1988). The membrane preparation and *in vitro* assay for *Rhodobacter* were the same as for cyanobacteria. However, the carotenoid extract was evaporated to dryness, resuspended in toluene and passed through a silica column before application to the HPLC column. The column retains polar compounds which might interfere with the detection of ζ -carotene.

Fixation, embedding and immunocytochemical labelling. The cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, for 2 h at 4 °C, and washed with the buffer. The fixed material was subsequently dehydrated in ethanol (30–100%) and twice in propylene oxide. Samples were embedded in Epon 812 (Polysciences, USA). Ultrathin sections were obtained on a Reichert ultramicrotome using glass knives and placed on gold or nickel grids (200 mesh) covered with carbon-coated Formvar film. The grids were then incubated with the following solutions by floating them (specimen-side down) on the surface of small drops and transferring them from one drop to another with forceps. The sequence of the incubations was: 10 min distilled water; 10 min 10% (v/v) H₂O₂ in distilled water; 10 min NaBH₄ (3 mg ml⁻¹ in distilled water); 10 min 50 mM-glycine in PBS; 5 min 20 mM-NH₄Cl in PBS; 1 h PBS supplemented with 5% (v/v) newborn calf serum, 0.2% (v/v) Tween 20 and 0.5 M-NaCl (PBS/STN); 2 h specific antiserum diluted 1:10 or 1:20 in PBS/STN, or only PBS/STN, in a moist chamber with shaking; 5 min washing in PBS/STN and overnight in the same buffer at 4 °C; 2 h goat anti-rabbit IgG conjugated with 10 nm diameter colloidal gold (Janssen Pharmaceutica, Beerse, Belgium), diluted 1:20 in PBS/STN in a moist chamber with shaking; 3 × 10 min washing in PBS supplemented with Tween 20 and NaCl; 10 min distilled water; 2 h 4% (w/v) uranyl acetate in water; 5 min alkaline lead citrate solution (Reynolds, 1963). The sections were examined with a Zeiss 9S TEM at calibrated magnifications and at an acceleration voltage of 60 kV.

The distance from the antigen to the gold marker – estimated from the size of the antigen–antibody complex (Rohde *et al.*, 1988) – was considered to be 40 nm, and was used as the maximal tolerable distance of a gold particle to be counted as a label for a given cell structure.

Results and Discussion

The antibody raised in rabbits against the β -galactosidase–phytoene desaturase fusion protein has recently been characterized (Schmidt *et al.*, 1989). It recognized a single protein of molecular mass 65 kDa in Western blots of SDS polyacrylamide gels after electrophoresis of detergent-treated cell extracts from *Rhodobacter* and *Synechocystis*. Since β -galactosidase is absent from *Rhodobacter*, cyanobacteria and higher plants, the antibody against the phytoene desaturase fusion protein is suitable for immunodetection of this enzyme on ultrathin sections. We have employed this antibody preparation to investigate the cellular location of phytoene desaturase in photosynthetic prokaryotes using the antibody–gold technique in a post-embedding immunoelectron microscopy procedure. It has recently been demonstrated that the antigenicity of cyanobacterial tissue is retained when using conventional Epon embedding (Bergman *et al.*, 1985), and immunogold labelling has been successfully

used for cyanobacterial cells (Bergman *et al.*, 1985; Hawthornthwaite *et al.*, 1985).

Typical electron micrographs of phytoene desaturase localization using rabbit anti-fusion protein antibodies and colloidal gold-conjugated anti-rabbit IgG are shown in Fig. 1. The ultrathin sections (a) and (b) are from the photosynthetic bacterium *Rhodobacter*, whereas (c) and (d) are from the unicellular cyanobacterium *Synechocystis*. In both organisms, gold labelling of phytoene desaturase (Fig. 1a, c) resulted in the association of gold particles with the photosynthetic membranes, which in the case of *Synechocystis* are intracellular thylakoids. Very few gold particles were located, however, in the cytoplasm and no non-specific background labelling was observed outside the cells.

In the case of *Rhodobacter* it cannot be ruled out that some gold particles located in the cytoplasm could be associated with the intracellular system of membrane-bounded vesicles described for many purple bacteria. This seems, however, rather unlikely in our case because the *Rhodobacter* cultures were grown under such environmental conditions, i.e. aerobic growth under illumination, which not only induce intense carotenoid biosynthesis but also dramatically reduce the abundance of intracellular vesicles. In fact, intracellular membranes are not discernible in our *Rhodobacter* preparations.

In control experiments (Fig. 1b, d), when the anti-phytoene desaturase antibody was omitted, or when pre-immune serum was used, no gold particles were found associated with the cells, thus demonstrating the specificity of the phytoene desaturase antibody. A similar gold-labelling experiment with the phytoene desaturase antibody for the filamentous cyanobacterium *Anabaena variabilis* is shown in Fig. 2. The label was preferentially located over the intracellular thylakoid membranes (Fig.

Table 1. Distribution of colloidal gold particles in the location of phytoene dehydrogenase by immunochemistry

For each species 10 individual cells were examined; 314, 773 and 1133 gold particles were counted in the case of *Rhodobacter*, *Synechocystis* and *Anabaena*, respectively.

	Percentage of gold particles ($\pm$ SD) in:		
	Cytoplasmic membrane region*	Thylakoid membranes	Cytoplasm
<i>Rhodobacter capsulatus</i> SB 1003	78 $\pm$ 5	–	22 $\pm$ 5
<i>Synechocystis</i> PCC 6714	9 $\pm$ 3	85 $\pm$ 6	6 $\pm$ 3
<i>Anabaena variabilis</i> ATCC 29413	9 $\pm$ 4	84 $\pm$ 5	7 $\pm$ 4

* Includes cytoplasmic membrane, periplasmic space and outer membrane.

Fig. 1. Immunocytochemical localization of phytoene desaturase in *Rhodobacter capsulatus* (a, b) and *Synechocystis* PCC 6714 (c, d) cells. Note that in both organisms the majority of the gold particles are located in the photosynthetic membranes (arrows in a and c). No significant labelling is observed in control experiments (b, d). Bars, 0.5 μm (all pictures have the same magnification).

2a) and again non-specific labelling was negligible in the control experiment (Fig. 2b). Statistical data on the distribution of gold labelling particles in immunolabelling experiments are shown in Table 1. Most of the gold particles, about 80%, were observed in the photosynthetic membranes in the species of photosynthetic prokaryotes studied.

The immunochemical results were substantiated by *in vitro* assays (Table 2). For both cyanobacteria, which, in contrast to *Rhodobacter*, form cyclic carotenes, conversion of [^{14}C]GGPP in the carotene pathway to phytoene, ζ -carotene and β -carotene was observed. Other carotenes were not labelled. In the case of *Rhodobacter*, which forms hydroxylated acyclic carotenoids from neurospor-

Fig. 2. Immunocytochemical localization of phytoene desaturase in vegetative cells of *Anabaena variabilis* (a). Note that most of the gold particles appear associated with thylakoids (arrows). No significant labelling is observed in control experiments using preimmune serum (b). Bar, 0.5 μ m.

Table 2. *In vitro* carotene formation from [14 C]GGPP

For details see Methods; 3×10^4 d.p.m. [14 C]GGPP was used as substrate: the results represent typical data taken from a set of experiments; the conversion rate of phytoene varied in the range of $\pm 10\%$.

Organism	Radioactivity (d.p.m.) incorporated into:			Percentage conversion of phytoene
	Phytoene	ζ -Carotene	β -Carotene	
<i>Rhodobacter</i> (membranes)	4890	2401	ND	32.9
<i>Anabaena</i> (membranes)	2238	102	1535	42.2
<i>Synechocystis</i> (membranes)	1355	356	2079	64.2
<i>Synechocystis</i> (purified thylakoids)	1332	1445	384	57.9

ND, Not detectable.

ene, *in vitro* formation up to ζ -carotene was possible. In all cases, the *in vitro* reaction sequence included the phytoene desaturation step. The conversion rates for phytoene desaturase were about 30% for *Rhodobacter*, 40% for *Anabaena*, and about 60% for *Synechocystis*. Thylakoids from *Synechocystis* were purified from the outer membranes and from cytoplasmic contamination by sucrose density-gradient centrifugation. These purified thylakoids showed comparable *in vitro* formation of phytoene and especially ζ -carotene, with less incorpora-

tion of radioactivity into β -carotene. The conversion rate (phytoene to ζ -carotene) for phytoene desaturase was similar to that of the crude membrane preparation. This result clearly demonstrates the association of phytoene desaturase with the thylakoids and supports the immunogold-localization studies. The impaired conversion of ζ -carotene to β -carotene is certainly due to a partial damage of membrane integrity caused by the hypotonic sucrose solutions during density-gradient centrifugation.

The high lipophilicity of the substrates and products of

carotene-converting enzymes imply that phytoene desaturase might be membrane-associated. There has been no previous attempt to determine the subcellular localization of carotenogenic enzymes, due to the lack of suitable antibodies. Indications that phytoene desaturase is a membrane protein were obtained by solubilization studies with crude membrane preparations from *Synechocystis* (Bramley & Sandmann, 1987). Our immunogold-localization studies together with the *in vitro* data clearly indicate that phytoene desaturase might be exclusively localized in photosynthetic membranes of prokaryotic photosynthetic organisms.

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