Biochimica et Biophysica Acta, 1060 (1991) 97–105

ADONIS 000527289100226X

BBABIO 43491

Localization of polypeptides in isolated chlorosomes from green phototrophic bacteria by immuno-gold labeling electron microscopy

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(Received 19 December 1991)
(Revised manuscript received 21 June 1991)

Key words: Chlorosome; Electron microscopy; Immuno-gold labeling; (*Cf. aurantiacus*; *Cb. limicola*; *P. aestuarii*)

Chlorosomes were prepared from *Chloroflexus aurantiacus, Chlorobium limicola* f. *thiosulfatophilum* and *Prosthecochloris aestuarii* by sucrose density gradient centrifugation. Two preparation methods were used. The first was applied to all three organisms and yielded chlorosomes without cytoplasmic membrane (CM) components. The second, which included a cross-linking step, was applied to *Cf. aurantiacus* and *P. aestuarii* only and yielded chlorosomes affixed to small inner CM patches. For the *P. aestuarii* sample this second method also yielded rod-elements, which were released from the inside of the chlorosomes. Antisera, raised against chlorosome polypeptide chains (18, 11 and 5.7 kDa), isolated from CM-free *Cf. aurantiacus* chlorosomes or a mixture of 24 and 24.5 kDa reaction center (RC) polypeptides isolated from *Cf. aurantiacus* inner CM, were incubated with the two different *Cf. aurantiacus* chlorosome samples and analyzed by gold-labeling electron microscopy (EM). Three antisera (anti-18 kDa, anti-11 kDa and anti-5.7 kDa) showed specific interaction with the chlorosomal envelope, whereas the anti-RC serum exhibited specificity for the CM. This result demonstrates for the first time that the 5.7 kDa polypeptide is in the chlorosomal envelope. Previous work had placed the polypeptide as a structural component of the intrachlorosomal rod-elements (Feick, R.G. and Fuller, R.C. (1984) Biochemistry 23, 3693–3700). Affinity-purified antibodies raised against a 7.5 kDa polypeptide isolated from *Cb. limicola* CM-free chlorosomes were incubated with *Cb. limicola* and *P. aestuarii* chlorosome samples. The antibodies showed considerable affinity for the chlorosomal envelopes but far less towards the CM components and hardly any for the rod-elements.

Introduction

The sub-order of the green photosynthetic bacteria can be divided into two main families, the Chloroflexaceae and the Chlorobiaceae. Although these two bacterial families do not seem to be closely related evolutionary [1–3], they both contain a similar light-harvesting apparatus exhibiting a very uncommon structure. The predominant light-harvesting antenna complexes, containing bacteriochlorophyll (BChl) *c*, *d* or *e* (species dependent), are found in specialized organelle-like structures, called chlorosomes, attached to, but not continuous with, the bacterial inner CM. In freeze-fracturing EM studies on chlorosomes from members of both main families of green bacteria (i.e., *Cf. aurantiacus* (Chloroflexaceae) and *Cb. limicola* as well as *P. aestuarii* (Chlorobiaceae)) significant differences were observed, concerning the mode of their connection to the CM and the diameter of rod-elements found inside them (*Cf. aurantiacus* 5.6 nm, *Cb. limicola* and *P. aestuarii* approx. 10 nm) [4–6]. In both the photochemical reaction centers, though structurally different, are placed in the CM neighboring the chlorosome [7,8].

Abbreviations: BChl, bacteriochlorophyll; CCM complex, chlorosome affixed to cytoplasmic membrane patch; CM, cytoplasmic membrane; EM, electron microscopy; GAR10, 10 nm gold conjugated with goat-anti-rabbit antibodies; GPA, grains per area unit; pAg5, 5 nm gold conjugated with protein-A; PBS, potassium phosphate buffer (20 mM, pH 7.4) containing 150 mM NaCl; PMSF, phenylmethanesulfonyl fluoride; RC, reaction center.

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By gel electrophoresis [9,10] and refinement with more sophisticated techniques like amino acid sequencing and plasma desorption mass spectrometry [11–17] (Van Beeumen, J. and Olson, J.M., unpublished data) several chlorosome associated polypeptide chains were identified by their molecular mass. Purified chlorosomes of *Cf. aurantiacus* contained three major polypeptides (relative molecular mass of 18 kDa, 11 kDa and 5.7 kDa), while a fourth (5.8 kDa) was present in minor amounts. An RC polypeptide (an equimolar mixture of two similar polypeptides (24 and 24.5 kDa) [11]) was identified in a purified CM fraction [10]. Samples of CM-free chlorosomes from *Cb. limicola* were shown to contain as their major protein components three different polypeptide chains with relative molecular masses of approx. 6.3, 6.3 and 7.5 kDa, respectively [9,12–14] (Roepstorff, P. and Olson, J.M., unpublished data).

Detailed studies, including the use of cross-linking and proteolytic analysis, resulted in a model of the chlorosome and the associated photosynthetic membrane of *Cf. aurantiacus* [10]. In this model the 5.7 kDa polypeptide is placed inside the chlorosome, associated with rod-like elements, while the 5.8, 11 and 18 kDa proteins are thought to be located in the chlorosomal envelope: the 5.8 kDa polypeptide is specifically localized at the site of attachment to the CM and the two other polypeptides are localized in the remaining area. Further, according to the model the RC protein is embedded in the CM.

In the immuno-EM study, presented here, we used polyclonal antisera [18] for localization of the three major chlorosome polypeptides and the RC polypeptide of *Cf. aurantiacus*. Chlorosomes of *P. aestuarii* and *Cb. limicola* were investigated with purified antibodies against the isolated 7.5 kDa protein of *Cb. limicola* [13,14].

At the moment there are two contradictory hypotheses about the organization of chlorophyll and protein in the intra-chlorosomal rods. One hypothesis proposes that the organization is determined by specific protein molecules on which each BChl*c* molecule is bound to a specific site [16]. The other hypothesis favors the idea that the BChl*c* molecules can form functional aggregates by themselves, after which proteins may play a role in stabilizing the aggregate [19,20]. An answer to the question of where the polypeptides are localized outside and/or inside the chlorosomes would be very helpful for a better understanding of the relation between their structure and function.

Materials and Methods

Culture conditions

This study was carried out with *P. aestuarii* (strain 2K) cells, grown anaerobically in the laboratory of Dr. J. Amesz (Department of Biophysics, Leiden) according to the method reported by Holt et al. [21].

Cf. aurantiacus, strain J-10-fl, was grown phototrophically as described by Pierson and Castenholz [22], in the laboratory of Dr. J. Amesz or according to Cox et al. [23], in the laboratory of Dr. Olson.

Cb. limicola f. *thiosulfatophilum* 6230 (Tassajara) was grown in the laboratory of Dr. Olson using the method described by Olson et al. [24].

Chlorosome preparation

Cells of *P. aestuarii* were harvested by centrifugation (20 min at $8000 \times g$, 4°C). CM-free chlorosomes were prepared by breaking the cells by sonication on melting ice (15 times for 1 min separated by pauses of 1.5 min, using a Branson sonifier B15 at 50 W) in a 10 mM phosphate buffer (pH 7.5), supplemented with sodium azide (0.02%, w/v), PMSF (0.05%, w/v), 10 mM ascorbate and 2 M NaSCN (buffer A). The samples were dialyzed (24 h at 4°C) against a 10 mM Tris-HCl (pH 7.4) buffer containing the same supplementations (buffer B) and purified on a (0–40%) sucrose gradient (4°C) in buffer B (adapted from Ref. 9). Chlorosomes, attached to CM patches, including membrane embedded protein complexes, were isolated from cells of *Cf. aurantiacus* and *P. aestuarii*, that had been cross-linked with dithiobis(succinimidylpropionate) prior to breaking [6]. We will further refer to them as chlorosome-membrane complexes (CCM complexes). After centrifugation of the suspension of fragmented cells to remove large debris the supernatants were dialyzed (24 h at 4°C) against a 0.01 M Tris-HCl (pH 7.4) buffer containing azide and PMSF (buffer C) and centrifuged (4°C) on sucrose (20–60%) gradients in buffer C (adapted from Ref. 9).

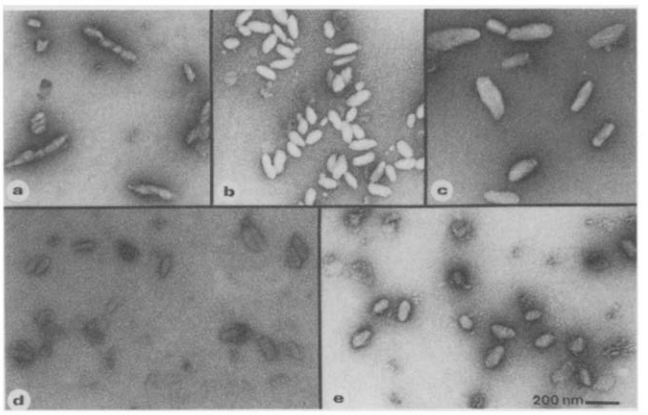

Fig. 1. Purity control of the chlorosome samples used in this study. Chlorosome samples were negatively stained with 1% uranyl acetate in water; (a) CM-free chlorosomes isolated from *Cf. aurantiacus*, (b) CM-free chlorosomes from *P. aestuarii*, (c) CM-free chlorosomes from *Cb. limicola*, (d) CCM complexes from *Cf. aurantiacus*, (e) CCM complexes from *P. aestuarii*.

The pigment bands were removed and their purity assessed by absorption spectroscopy (400–900 nm, on a UVICON 930 UV-VIS spectrometer, KONTRON) and EM (see Fig. 1). When needed selected fractions were subjected to a second gradient centrifugation.

CM-free chlorosomes of *Cb. limicola* and *Cf. aurantiacus* (see Fig. 1), isolated essentially according to Gerola and Olson [9], were a gift from Dr. P.D. Gerola.

Absorption spectra of fractions judged to be pure by EM criteria always showed only the expected peaks. CM-free chlorosome samples gave typical chlorosome BChl*c* spectra free of contaminating membrane pigments (the *Cf. aurantiacus* spectrum also showed a small chlorosome-BChl*a* peak at 797 nm). The spectra of CCM complexes displayed additional absorption peaks at 802 and 865 nm (*Cf. aurantiacus*) or at 810 nm (*P. aestuarii*), typical for pigments associated with the reaction centers and non-chlorosome antenna complexes.

Antisera and purified antibodies

Antisera to Cf. aurantiacus polypeptides. The antisera used in the experiments with *Cf. aurantiacus* were the same as used in earlier studies [18,25].

The antibodies to the 7.5 kDa polypeptide isolated from Cb. limicola. The affinity-purified antibodies to the 7.5 kDa protein from *Cb. limicola* were prepared essentially as described by Mikkelsen and Knudsen [26].

Production and detection

The 7.5 kDa protein was coupled to keyhole-limpet hemocyanin with glutaraldehyde as follows. To 1.5 mg hemocyanin in 100 μl potassium phosphate buffer (0.1 M, pH 8.8) 30 μl glutaraldehyde was added at 25°C. The reaction was allowed to continue overnight at room temperature. Glutaraldehyde that had not reacted was separated from the activated hemocyanin by gel filtration on a short Sephadex G-25 column (3 cm × 0.5 cm) equilibrated with 0.15 M NaCl. To the activated hemocyanin was added 5.2 μl $NaHCO_3$ buffer (1.0 M, pH 9.5) per ml, and 200 μg 7.5 kDa protein. The reaction proceeded overnight at 4°C. Aldehyde groups on the hemocyanin that had not reacted were conjugated to a large excess of glycine. The hemocyanin-protein complex was dialyzed against potassium phosphate buffer (20 mM, pH 7.4) containing 150 mM NaCl (PBS), overnight to remove glycine that had not reacted. The conjugated 7.5 kDa protein-hemocyanin was injected subcutaneously in complete Freund's adjuvant into rabbits. Injections were repeated every 2 weeks with 200 μg of the conjugated 7.5 kDa protein-hemocyanin in complete Freund's adjuvant for 2 months.

Antibody titre against the 7.5 kDa protein was determined by ELISA [27]. Microtitre plates were coated with 7.5 kDa protein (0.4 μg) in 100 μl of $NaHCO_3$ buffer (50 mM, pH 9.5) per well, overnight. After unbound 7.5 kDa protein was washed out with PBS, plates were incubated with a blocking solution (1% bovine serum albumin (BSA) in PBS) for 3 h at 37°C to prevent non-specific binding of the antiserum. Antiserum diluted in 0.1% BSA in PBS was added and incubation carried out overnight. Antibody binding was detected by alkaline phosphatase-conjugated affinity-purified swine-anti-rabbit IgG. Binding was detected by addition of 200 μl substrate solution (1.0 mg of *p*-nitrophenyl phosphate in 0.1 M ethanolamine (pH 9.5) together with 0.5 μM $MgCl_2$). Preimmune serum from the rabbit served as a negative control and gave readings which were less than 1% of those with immune serum at the same dilutions.

Affinity purification of anti-7.5 kDa antibodies

The 7.5 kDa protein (4.0 mg) was coupled to 0.6 g CNBr-activated Sepharose 4B essentially as recommended by the manufacturer (Pharmacia). The 7.5 kDa protein-specific antibody was purified on this column.

Blotting and immunological detection of proteins

Sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) was carried out essentially as described by Schägger and Von Jagow [28]. The slab gels contained a 1.5 cm 4% stacking gel, a 2 cm 10% spacer gel and a 5.5 cm 16.5% separating gel (only this part is shown in Fig. 2A). The molecular mass markers ranged from 6.5 to 43 kDa. The gel was run at approx. 4°C for 4.5 h at 80 W. Casting and running of the gel was done in a MINI-PROTEAN II dual slab cell (Bio-Rad Laboratories, Richmond, CA). Staining was done with Coomassie brilliant blue.

After electrophoresis the proteins were transferred electrophoretically from the gel to nitrocellulose sheets using a Semi Dry Blotter (Kem-En-Tec, Copenhagen) according to the manufacturer's recommendations. After transfer the blots were immersed in 100 ml PBS containing 3% (w/v) BSA and incubated at 37°C for 3 h on a rocking platform. Next the blots were transferred to a solution of 100 ml PBS containing 3% BSA, 0.05% (v/v) Tween-20 and 2.8 μg affinity-purified anti-7.5 kDa IgG per ml, and incubated overnight at room temperature. After washing three times for 5 min with PBS containing 0.05% Tween-20, the blot was transferred to 100 ml PBS containing 3% BSA, 0.05% Tween-20, and alkaline phosphatase-conjugated affinity-purified swine-anti-rabbit IgG (1:1000 dilution) and incubated for 3 h at room temperature. After washing three times for 5 min with 100 ml PBS containing 0.05% Tween-20, and once with 100 ml of ethanolamine (0.1 M, pH 9.0), the alkaline phosphatase activity was detected by the method of Blake et al. [29].

Labeling

The samples (sample concentration: $A_{740/750}$ approx. 2) were applied to specimen grids covered with a carbon-coated formvar film by floating the grids on the sample solution for 3 min. The grids were washed (two times 10 min) on Tris-HCl (20 mM; pH 7.3) buffer containing 150 mM NaCl, 0.1% bovine serum albumin and 20 mM sodium azide (buffer D; i.e., a slight modification (pH 7.3 instead of 8.2) of the buffer in which the gold-label was supplied by the manufacturer; Janssen Pharmaceuticals). The samples were incubated with normal mouse non-specific serum for 1 h followed by washing (three times 10 min) on buffer D. Thereafter, the samples were floated on droplets of specific antisera or on a solution of purified antibodies for 1 h. The samples were washed again three times for 10 min on buffer D and incubated for 1 h with a solution of 10 nm gold particles conjugated with goat-anti-rabbit antibodies (GAR10) (Janssen Pharmaceuticals). After washing (three times 10 min), the samples were negatively stained by floating (twice 3 min) on a solution of uranyl acetate in water (1% w/v). Optimal labeling was found with antibody concentrations of about 0.1 mg/ml and a gold-label concentration corresponding to an A_{520} of approx. 0.25. In the experiments aiming for a more precise antigen localization 5 nm gold particles conjugated with protein-A (pAg5) (Janssen pharmaceuticals, concentration corresponding to $A_{520} = 0.05$) were used instead of GAR10. In those cases the incubation of the samples with normal mouse serum was omitted. All dilutions were in buffer D and the complete procedure was done at room temperature.

Electron microscopy

EM was performed on a JEOL JEM 1200EX operating at 80 kV. The samples were imaged at electron optical magnifications of 24 000 or 55 000 × on sheets of either Kodak Electron Image Film SO-163 or AGFA Scientia 23D56.

Data collection

The gold particles were counted on 3-fold enlarged electron micrographs. The chlorosome area was estimated by counting the number of crossings on chlorosomes using an overlay grid (5 mm × 5 mm, which corresponds to about 70 nm × 70 nm at the sample level). This method leads to a small (15 to 20%) overestimation of the chlorosome area, when compared with exact measurement. The overestimation somewhat compensates for the fact that the chlorosomes are not flat and have a surface area, open for interaction with antibodies, which is larger than their projected area. The labeling was represented as number of gold grains counted per arbitrarily chosen area unit (GPA). When specified parts of chlorosomes were investigated (Table III), the amount of label was related to the area of these particular parts only. In every experiment several hundreds of 'chlorosome-area' units (at least 400 units per sample/antibody combination) were counted. In the experiments with pAg5 only the label distribution and not the labeling efficiency was analyzed.

Image processing

In order to optimize the visibility of the gold grains in Fig. 5, concerning labeling with pAg5, the original

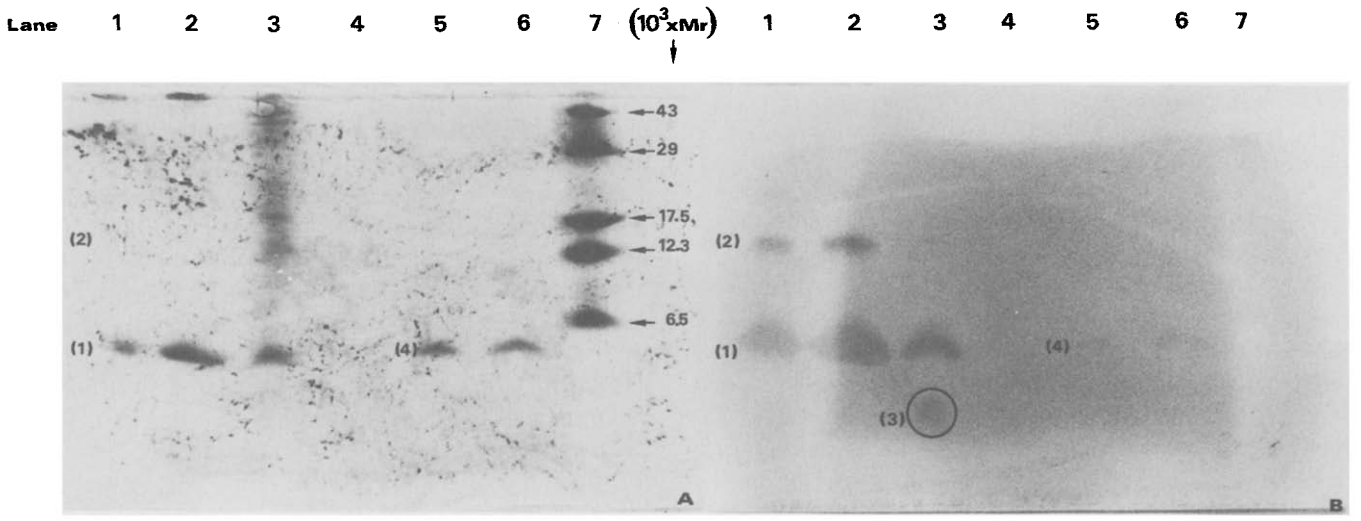

Fig. 2. Specificity check on the antibodies to the 7.5 kDa protein isolated from *Cb. limicola*. (A) polyacrylamide gel lanes 1 and 2 contain a 7.5 kDa protein solution (5 and 10 μl, respectively), lane 3 contains 5 μl desalted *Cb. limicola* chlorosomes (approx. 1 μg BChl*c*), lane 4 is empty, lanes 5 and 6 contain a 6.3 kDa protein solution (5 and 10 μl, respectively), lane 7 contains relative molecular mass markers: Aprotinin (Sigma A-1153; 6.5 kDa); cytochrome *c* (Sigma C-7752; 12.3 kDa); myoglobin (Sigma M-1882; 17.5 kDa); carbonic anhydrase (Sigma C-7500; 29 kDa); ovalbumin (Sigma A-5503; 43 kDa); 4 μg of each standard. (B) The corresponding Western blot. (1) indicates the position of the 7.5 kDa protein monomer, (2) indicates the position of the 7.5 kDa protein dimer, (3) indicates the brown-yellow pigment spot, (4) indicates the position of the 6.3 kDa protein.

negatives have been subjected to image processing. The micrographs were digitized by means of a DATACOPY ccd-camera and image processing software (WIPS), using a step size of approx. 26 μm, corresponding to about 0.5 nm on the specimen level (512 × 512 pixels). The actual treatment enhanced the visibility of features of about 5 nm and densities exceeding a threshold. The treatment was performed on a Convex C1-XP mini-supercomputer using the Imagic software system [30].

Results

Specificity of the affinity-purified anti-7.5 kDa antibodies

Fig. 2 shows the gel and corresponding Western blot demonstrating the specificity of the anti-7.5 kDa antibodies. The Western blot (Fig. 2B, lanes 1 and 2) shows that the 7.5 kDa protein monomer (1) reacts very strongly with the anti-7.5 kDa antibody. It also shows the formation of the 7.5 kDa protein dimer (2), with which the antibody clearly interacts too. The fact that the dimer bands are not visible in the gel (Fig. 2A) demonstrates that they are in extremely low concentration (the band at approx. 15 kDa in lane 3 of the gel must be a polypeptide band comigrating coincidentally with a tiny amount of dimer, which itself is only reflected by a weak band in the blot). It also makes clear how sensitive the antibody is in the corresponding Western blot (Fig. 2B). The brown-yellow spot (3) in lane 3 of the blot results from an almost 100% transfer of pigment from the gel. The very weak bands (4) in lanes 5 and 6 of the blot demonstrate a very low cross reactivity between the antibody to the 7.5 kDa protein and the 6.3 kDa protein. Considering this we believe that the antibody to the 7.5 kDa protein used for gold-labeling in EM can be trusted to label 7.5 kDa protein sites over 90% of the time and 6.3 kDa protein sites less than 10% of the time.

Pre-incubation with mouse normal serum

In the labeling experiments, in which GAR10 was used as a gold-label, the chlorosome samples were first saturated with mouse normal serum to reduce reactions of the rabbit sera with common bacterial antigens. The effect of this precaution was that background labeling was kept extremely low (under 0.15 GPA), while the labeling on the chlorosomes was reduced with about 1.2 GPA. We found that the labeling with non-specific serum was decreased from over 1.9 GPA to consistently less than 0.7 GPA. This made the distinction between positive and negative labeling results rather straightforward, since the counted values did not need significant corrections for non-specific labeling (see Fig. 3).

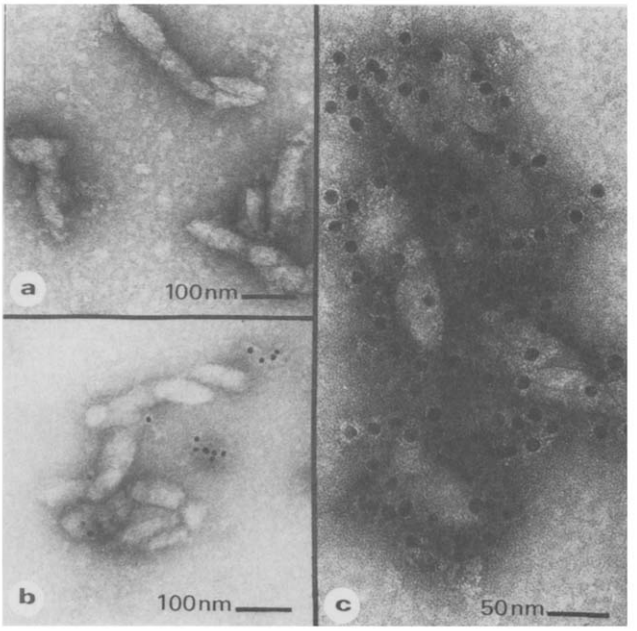

Fig. 3. Controls for the specificity of the GAR10 labeling. All samples (CM-free *Cf. aurantiacus* chlorosomes) were pretreated with normal mouse non-specific serum as described in Materials and Methods. (a) This sample was treated with GAR10 only; (b) this sample was treated with rabbit non-specific serum followed by GAR10 treatment; (c) this sample was treated with one of the specific antisera (anti-18 kDa serum) followed by GAR10 treatment.

Labeling of Cf. aurantiacus chlorosomes

The labeling of CM-free *Cf. aurantiacus* chlorosomes resulted in specific reactions with the antisera against the 5.7, 11 and 18 kDa proteins with high efficiencies. The anti-RC serum showed little labeling, which was only slightly higher than the reaction with the non-specific serum (see Table I and Fig. 4).

In the case of the labeling of *Cf. aurantiacus* CCM complexes we found positive labeling with comparable efficiencies for all of the four specific antisera (see Table I and Fig. 4).

TABLE I

Specific labeling of Cf. aurantiacus chlorosomes with GAR10, using different antisera

Serum used	Specific labeling (GPA) [a]	
	CM-free chlorosomes	CCM complexes
Non-specific	0.6	0.4
Anti-5.7kDa	6.6	5.7
Anti-11kDa	7.9	4.9
Anti-18kDa	8.5	5.2
Anti-RC	1.3	4.5

[a] Background labeling (approx. 0.1) has been subtracted.

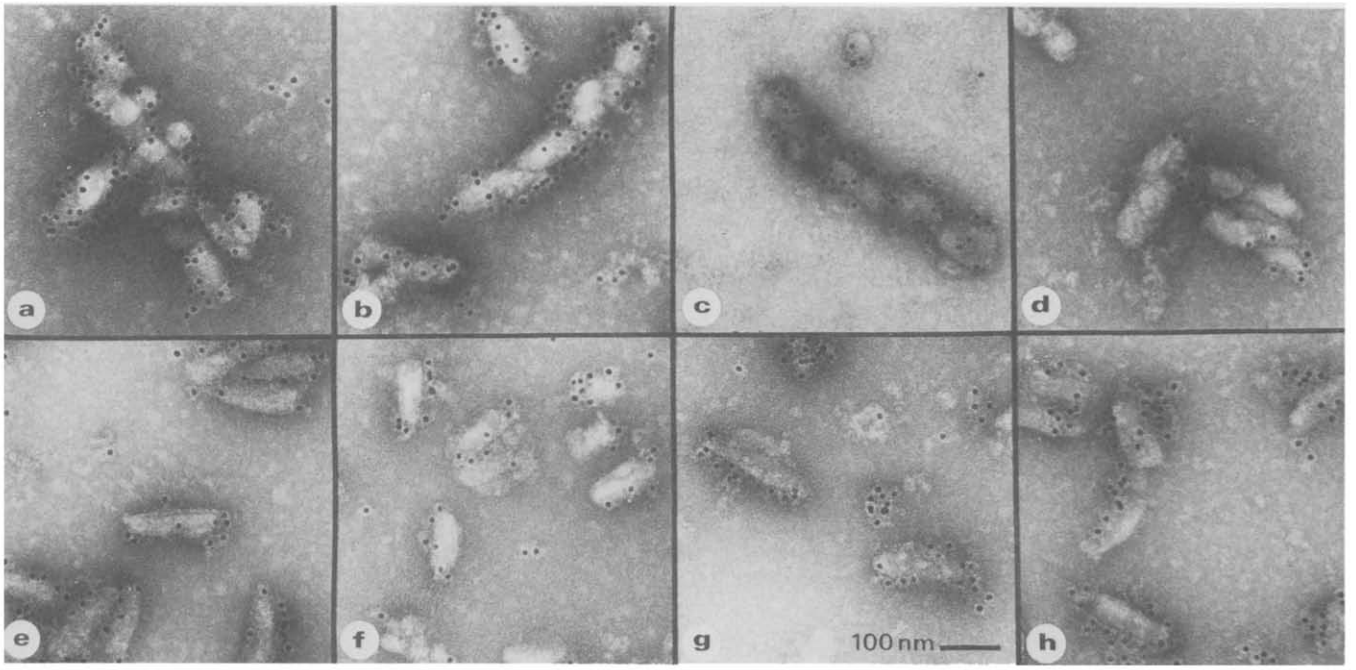

Fig. 4. Illustration of the labeling activity of the specific antisera towards *Cf. aurantiacus* chlorosomes. GAR10 was used as a gold label. (a–d) labeling of CM-free chlorosomes with anti-5.7; anti-11; anti-18 kDa and anti-RC serum respectively; (e–h) labeling of CCM complexes with anti-5.7; anti-11; anti-18 kDa and anti-RC serum.

Refinement of location of the polypeptides in Cf. aurantiacus chlorosomes

Since pAg5 labeling has a resolution superior to GAR10 labeling (resolution better than 20 nm vs. resolution up to 40 nm [31]), we used pAg5 to locate the antigenic sites on CCM complexes more precisely. In this way the location of the polypeptides in the sample could be determined either on the side of the chlorosome facing the cytoplasm or in the CM attached to the chlorosome. For this experiment the conditions were chosen such that the background labeling and labeling with non-specific serum were negligible.

TABLE II

Label distribution over cytoplasmic membrane and the cytoplasmic membrane opposing side of CCM complexes isolated from Cf. aurantiacus, using pAg5 and several antisera

Serum used	Label distribution (%) over	
	cytoplasmic membrane	side opposing cytoplasmic membrane
Non-specific	– [a]	– [a]
Anti-5.7kDa	10	90
Anti-11kDa	1	99
Anti-18kDa	8	92
Anti-RC	94	6

[a] The labeling with non-specific serum on chlorosomes was 0.

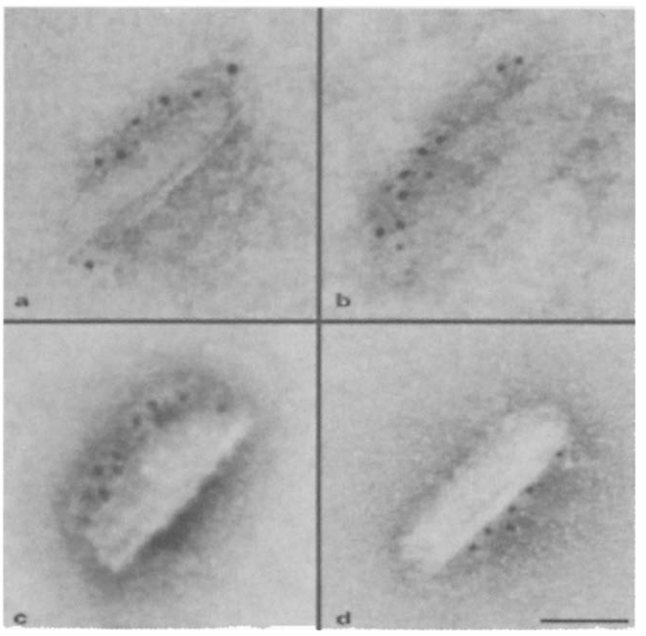

Fig. 5. Examples of side views of labeled *Cf. aurantiacus* chlorosomes showing a cytoplasmic membrane patch clearly on one side of the chlorosome. The chlorosomes were treated with anti-5.7 kDa (a), anti-11 kDa (b), anti-18 kDa (c) or anti-RC serum (d), using pAg5 as a gold label. In the samples treated with anti-5.7 kDa, anti-11 kDa and anti-18 kDa serum a large majority of the label can be found on the side of the chlorosome opposing the CM. In the sample treated with anti-RC serum the label is found associated with the CM exclusively. The bar represents 50 nm.

TABLE III

Labeling of several chlorosome samples isolated from Cb. limicola and P. aestuarii, using purified anti-7.5 kDa antibodies and GAR10

Source	Specific labeling (GPA) [a,b]					
	CM-free chlorosomes	CCM complexes				rods
		total area	CM-free areas	CM-covered areas	non-classifiable	
Cb. limicola	8.4	–	–	–	–	–
P. aestuarii	6.6	4.4	6.0	3.3	4.2	0.3

[a] The labeling with non-specific serum was under 0.3.
[b] Background labeling was approx. 0.2.

The distribution of gold grains over both faces of the chlorosomes could best be observed in views exhibiting the CM patch clearly on only one side of the chlorosome. The experiment with the anti-RC serum showed a convincing majority of the label on the CM side of the chlorosome. In the experiments with the anti-5.7 kDa, anti-11 kDa and anti-18 kDa sera the location of most of the label turned out to be on the other side of the chlorosome, opposite to the CM (see Table II and Fig. 5).

The labeling of P. aestuarii and Cb. limicola chlorosomes

The labeling of CM-free *Cb. limicola* chlorosomes with purified anti-7.5 kDa antibodies resulted in a positive and specific interaction (see Table III and Fig. 6a). With CM-free chlorosomes isolated from *P. aestuarii*, a similar result was found, although the labeling efficiency appeared to be somewhat lower (see Table III and Fig. 6b). For the cross-linked *P. aestuarii* chlorosomes a distinction was made between CM-free chlorosomes, CM-free areas and CM-covered areas of

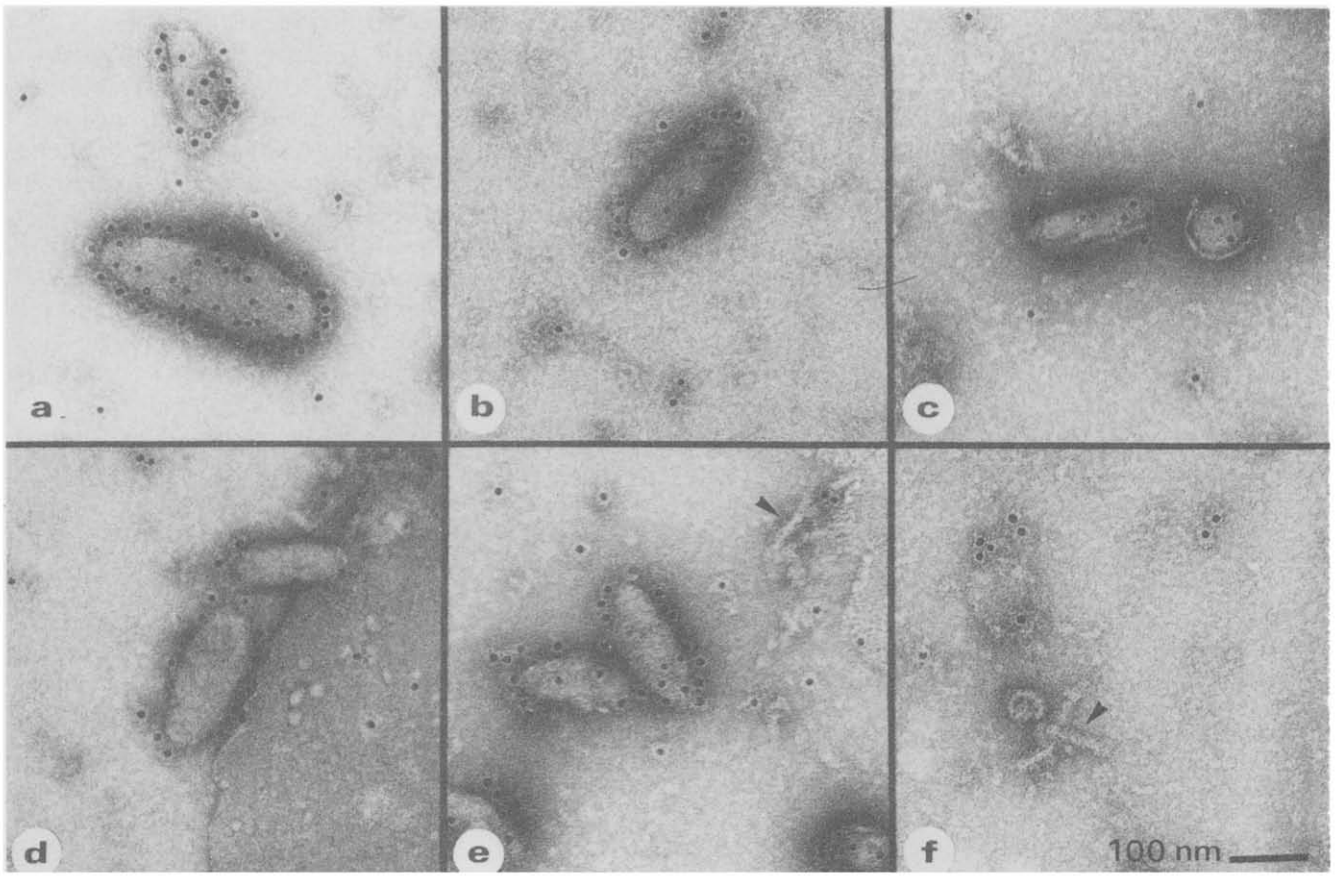

Fig. 6. Several chlorosome samples treated with anti-7.5 kDa antibodies, using GAR10 as a gold label. (a) and (b) Samples of CM-free chlorosomes isolated from *Cb. limicola* and *P. aestuarii*, respectively. Samples of CCM complexes isolated from cross-linked *P. aestuarii* cells, exhibiting side views (c) and top views (d,e) of the chlorosomes as well as rods (e,f, see arrowheads). Note the specific labeling on the chlorosomes, that is absent on the rods.

CCM complexes and a fourth group, which contained those chlorosomes that could not be placed in either of the other groups. The CM-free chlorosomes and the CM-free parts of the CCM complexes had the highest amount of labeling, with a comparable efficiency (see Table III and Fig. 6c–e). The anti-7.5 kDa antibody showed considerably less affinity for the rough areas of chlorosomes covered with CM; the amount of specific label, represented as GPA, was only half as large (see Table III and Fig. 6c–e). Moreover, the way in which the gold particles were arranged not on, but rather around the CM patches suggested that the antigenic sites of the antibody were not in, but only close to the CM. The fourth group had an intermediate labeling efficiency (see Table III and Fig. 6c–e). The sample of cross-linked *P. aestuarii* chlorosomes also contained rod-like elements, released from the inside of the chlorosomes. We found that labeling of these rods with the anti-7.5 kDa antibodies did not exceed the background labeling (see Table III and Fig. 6e–f).

Discussion and Conclusions

Chloroflexus aurantiacus

The 11 and 18 kDa polypeptides. The experiments concerning both the 11 and 18 kDa polypeptide indicate the location of these polypeptides in the chlorosomal envelope. This result was expected since these proteins have been found to be susceptible to proteolytic cleavage and to 3-azido-2,7-naphtalenedisulfonate photo-labeling [10]. In the same study cross-linking experiments made clear that these polypeptides occur in the vicinity of one another. In the model based on those results, the 11 and 18 kDa polypeptides are placed in the chlorosomal envelope and are restricted to the area that is not attached to the CM in vivo. Our results do not indicate such restrictions for the occurrence of these polypeptides. The label distribution observed on CM-free chlorosomes might be explained in terms of a preparation-induced rearrangement of the polypeptides over the chlorosome surface. However, the amount of label found on CM-free chlorosomes is considerably higher than on CCM complexes. This observation indicates that the removal of CM fragments from the attachment sites of the chlorosomes results in the uncovering of a significant fraction of the 11 and 18 kDa polypeptides. It therefore appears that a fraction of these polypeptides is actually located in the area of the chlorosome surface that is covered by the CM in vivo.

The RC polypeptide. Our localization of the RC polypeptide in the CM adjacent to the chlorosome is in good agreement with reports that this polypeptide cannot be detected in CM-free chlorosome samples (isolated with either Miranol or NaSCN) [10,18,32] but is found in a purified CM fraction [10,11,18]. The low, but distinct labeling activity of the anti-RC serum towards the CM-free chlorosomes might be due to minor contamination of the sample with CM components or a cross-reactivity that can not be observed using less sensitive techniques.

The 5.7 kDa polypeptide. Our experimental data indicate that the 5.7 kDa polypeptide occurs in the chlorosomal envelope in amounts almost as high as the 11 and 18 kDa polypeptides. The labeling of the 5.7 kDa polypeptide in the sample of CCM complexes, compared to the CM-free chlorosome sample, is only marginally decreased. This suggests that the CM fragments do not cover antigenic sites that can be recognized by the anti-5.7 kDa serum. So we conclude that the 5.7 kDa polypeptide does not occur on the face of the chlorosome to which the CM is attached.

This location of the 5.7 kDa polypeptide is not in agreement with the findings, summarized in the model presented by Fuller and Feick [10]. In this model, mainly based on cross-linking, photo-labeling and digestion studies, the protein is presented as a component of the rod-like antenna structures inside the chlorosome. According to several authors the protein forms rod-like aggregates onto which the BChl*c* molecules bind at specific binding sites [16,33]. This would mean that the eventual shape of the rods and the orientation of the chromophores is determined by this protein. Proteins isolated from chlorosomes of Chlorobiaceae supposedly can act in a comparable way, even though they do not contain similar chromophore binding sites [15].

According to another view, mainly based on studies of in vitro aggregation of BChl*c* [19,32,34,35] and protein-depleted chlorosomes [32,36,37], BChl*c* molecules can form rod-shaped aggregates by themselves. A role, if any, for a protein would be restricted to stabilization of the rods after their formation.

Since no reactivity of the anti-5.7 kDa serum towards the other chlorosome polypeptides has been found [18,25] we conclude that, unless there are more than one 5.7 kDa polypeptides with different immunogenic characteristics, the 5.7 kDa polypeptide is not restricted to the inside of the chlorosome and at least some of these molecules are embedded in the envelope. The function of these molecules is not known; however, a study of the coding gene predicts that they are synthesized as precursor molecules which have a carboxy-terminal extension [17].

Cb. limicola and P. aestuarii

The antibody labeling experiments with the chlorosomes isolated from *Cb. limicola* and *P. aestuarii* show that the 7.5 kDa polypeptide is located in the chlorosomal envelope of *Cb. limicola* (see Table III). The envelope of *P. aestuarii* chlorosomes probably contains a very similar protein, since it interacts almost equally

(see Table III). The parts of the chlorosomes covered by CM fragments and embedded protein complexes (visible as rosette like structures) show a far lower affinity for the antibodies; therefore, a location of the protein in the CM is improbable.

The specific labeling of the CM-free chlorosomes and the CM-free parts of the CCM complexes is comparable (see Table III). This suggests that sites covered by the CM can interact with the anti-7.5 kDa antibodies after removing the CM. We conclude that the *P. aestuarii* protein, that is similar to the 7.5 kDa protein in *Cb. limicola*, can occur in the chlorosomal envelope without evident constraints concerning the side of the chlorosome.

The rod-like elements, that are normally released from the inside of cross-linked *P. aestuarii* chlorosomes during the cell breaking step in the chlorosome isolation procedure (a higher yield can be obtained by prolonged sonication), do not show a significant affinity for the anti-7.5 kDa antibodies. Thus, no evidence is found for the location of the 7.5 kDa polypeptide in the rods. Whether another polypeptide (the two 6.3 kDa polypeptides [14] are the most likely candidates) can be found in the rods remains a question still to be answered.

Acknowledgements

We wish to thank Prof. P.D. Gerola for his gift of CM-free chlorosomes (*Cf. aurantiacus* and *Cb. limicola*), the group of Prof. J. Amesz for the gift of bacterial cells (*Cf. aurantiacus* and *P. aestuarii*), Drs. T. Wichertjes and M. Miller for valuable discussions and critical reading of the manuscript, W. Keegstra for help with computer facilities and J. Pedersen and K. Gilissen for skillful technical assistance. The work of W.W. was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Science (NWO). J.M.O and J.K. were supported by the Danish Natural Science Research Council.

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