The 5.6-Kilodalton Protein in Isolated Chlorosomes of *Chloroflexus aurantiacus* Strain Ok-70-fl is a Degradation Product

Kai Griebenow, Alfred R. Holzwarth, and Kurt Schaffner

Max-Planck-Institut für Strahlenchemie,
D-4330 Mülheim a. d. Ruhr, Bundesrepublik Deutschland

Chlorosomes containing BChl a_{790} have been isolated from *Chloroflexus aurantiacus* on sucrose density gradients using the detergents Miranol, Deriphat, N,N-dimethyldodecyl-amine-N-oxide, and dodecyl-β-D-maltoside. All freshly prepared samples either lack the poly-peptide of approximately 5 kDa, which appears identical with the 5.6-kDa protein previously assigned the role of BChl *c*-binding [R. G. Feick and R. C. Fuller, Biochemistry **23**, 3693−3700 (1984)], or they contain only a minor amount thereof. This polypeptide accumulates in the chlorosomes *in vitro* at room temperature within 24 h after isolation. The reaction cannot be prevented simply by addition of the protease inhibitors benzamidine, ε-caproic acid, and phenylmethylsulfonyl fluoride. However, upon denaturation, as required for gel electrophore-sis, of the freshly isolated chlorosome sample the formation of the 5-kDa polypeptide is inhibit-ed. We conclude that this species, *viz.* 5.6-kDa protein, is a degradation product of another − as yet unidentified − protein present in the chlorosome preparations. Despite the pronounced proteolytic activity which affords the 5-kDa fragment, the native absorption and fluorescence properties of BChl *c* and BChl *a* are essentially not changed in these chlorosome preparations.

Introduction

The Chlorobiaceae and Chloroflexaceae both contain so-called chlorosomes as the main light-harvesting antenna system [1]. Chlorosomes are vesicles with the dimensions of approx. $12 \times 30 \times 100$ nm in *Chloroflexus aurantiacus* [2]. They con-tain bacteriochlorophyll (BChl) *c* as the main light-harvesting chromophore. BChl *a* is present in the chlorosomes in a small amount (about 4% of the total BChl [3]) when isolated by SDGC, but it is removed by GEF [4], and by treatment of the isolated chlorosomes with SDS [5, 6].

The organization of BChl *c* in chlorosomes is still a matter of debate. One of the models for the chlorosomes of *C. aurantiacus* [7] assumes binding of BChl *c* to a 5.6-kDa protein [3, 7, 8]. Six dimers of this protein, each with 10−16 BChl *c* molecules

attached, were proposed to constitute one unit of the rod elements which had been detected by elec-tron microscopy [2]. However, Schmidt *et al.* [9] did not report the 5.6-kDa polypeptide in the chlo-rosomes of *C. aurantiacus* strain Ok-70-fl. They rather found proteins with M_r values of 10, 15, 40, and 57 kDa only.

An alternative organizational model is based on the finding that isolated BChl *c* molecules *in vitro* form aggregates whose spectroscopic properties in appropriate solvents match those of BChl *c in vivo* [10−14]. Interestingly, Olson and coworkers [6] note that 3 proteins in the 10−20-kDa region are missing in SDS-treated BChl *a*-free chlorosomes of *C. aurantiacus*. We have recently found that the GEF procedure [4] either eliminates all proteins in the chlorosomes, or it reduces them to concentra-tions which exclude their role as BChl *c*-binding proteins [15, 16] as proposed by Wechsler *et al.* [7]. Estimates of the 5.6-kDa protein-to-BChl *c* ratio in our BChl *a*-free GEF chlorosomes indicate at least a 25−40-fold excess of BChl *c* [15, 16]. This constitutes the first biochemical evidence against relevant antenna functions by this protein and − together with other data [4, 17, 18] − provided evi-dence in favour of chromophore-chromophore interactions as the main organizational principle in chlorosomes.

This paper demonstrates that also in conventional chlorosome preparations – isolated by SDGC – which are generally accepted as "normal" intact chlorosomes [3, 7, 8], the 5.6-kDa polypeptide initially is not present in any appreciable amount. Rather, this polypeptide is shown to be a post-isolation degradation product and thus cannot function as a BChl *c*-binding protein *in vivo*.

Materials and Methods

The growing of the cells (*C. aurantiacus* strain Ok-70-fl, Deutsche Sammlung von Mikroorganismen No. 636) and isolation of membranes and chlorosomes have been described [4, 15]. Often two sucrose density gradient steps ($T = 4$ °C) were performed using the conditions reported in [15]. The pigment content and the stationary absorption and fluorescence spectra were measured according to [4]. The Pharmacia PhastSystem (8−25% SDS gradient gels) was used for PAGE, and silver staining was performed according to Heukeshoven *et al.* [19]. It was verified to have a minimal sensitivity of 1 ng/band (often below 0.5 ng/band) [15, 16].

Protein analyses of the chlorosomes as a function of time after isolation were carried out from stock solutions prepared by diluting samples of different Miranol-isolated chlorosomes with 20 mM Tris-HCl buffer (pH 8.0) to an absorbance of 2.3−2.4 at 740 nm (which corresponds to about 23−24 nmol BChl *c*/ml). The whole procedure was carried out at room temperature. Degradation was stopped by 1:1 dilution with incubation buffer (containing 5% SDS, 1% mercaptoethanol, and a trace of bromophenolic blue [15]) and heating for 10 min to 100 °C. The samples were then stored at room temperature until PAGE was performed.

For the absorbance and fluorescence measurements the isolated chlorosomes were diluted with Tris-HCl buffer (pH 8.0) to a concentration of approximately 2.3 nmol BChl *c*/ml, and the first measurement was started within 15 min after isolation.

In the inhibition experiments, the protease inhibitors were applied at concentrations of 1 mg/ml for PMSF and benzamidine, and 0.33 mg/ml for ε-caproic acid. PMSF was added in powder form (alcohols such as, *e.g.*, ethanol used as a solvent in

[8], may reduce the absorbance A_{740} by destroying the aggregates in the chlorosomes [13]).

In order to estimate the relative concentrations of the proteins on silver-stained gels [19] and also of those of different gels, the gels were photographed under identical conditions, and the resulting negatives were scanned with a Sigma FTR 20 scanner. The absolute protein concentration is difficult to determine from silver staining [15]. Calibration with the 5.6-kDa polypeptide reference sample established a minimal sensitivity of 1 ng per band.

Gel scans as in Fig. 2 are from the same gel and can be compared directly.

The following molecular weight markers were used: Phosphorylase (92.5 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor from soy bean (21 kDa), cytochrome *c* (12.5 kDa), and trypsin inhibitor from lung (6.5 kDa). All marker proteins were obtained from Serva. The M_r values were calculated according to Weber *et al.* [20]. In most analyses an additional set of marker proteins was used as well as described previously [15, 21].

Results

The protein patterns of different chlorosome preparations, isolated by SDGC using the detergents Miranol [3, 22], Deriphat [22], LDAO [21], and DDM [21], have been analyzed. In addition to PAGE analysis all samples were further characterized by absorption spectra, by fluorescence spectra and relative fluorescence intensities, and by the pigment content.

Densitometric scans for several PAGE samples are presented in Fig. 1 for the critical region up to 20 kDa. The ratios of six clearly detectable polypeptides depend strongly on both the detergent used and on the details of the preparation. The molecular weights of the polypeptides corresponding to peaks 2−5, which were determined with low-molecular weight markers (Serva markers; see Materials and Methods), ranged approximately from 5 to 14 kDa. The small polypeptide of *ca.* 5 kDa was identified by PAGE with the 5.6-kDa reference protein [7]. Peak 1 in Fig. 1 appeared only occasionally in minor amounts and may represent small protein fragments in the isolated chlorosomes. In addition to the proteins of peaks 2−5
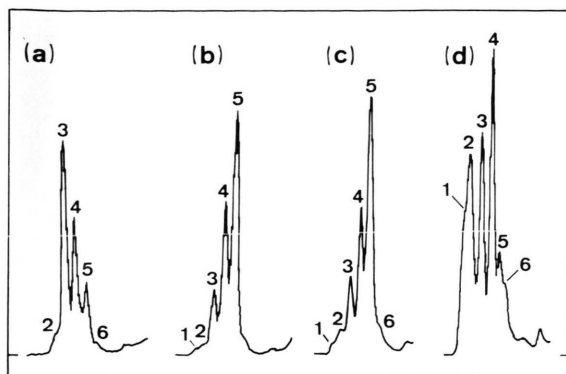
Fig. 1. PAGE densitograms (8–25% acrylamide, silver staining [15]) of the M_r range up to 22 kDa, showing protein peaks 1–6 of different isolated chlorosome preparations. (a) Preparation with LDAO, (b) with Miranol, (c) with Deriphat (preparations a–c represent pure chlorosomes of SDG band 2); (d) Miranol preparation of SDG band-3 chlorosomes which are contaminated with residual membrane proteins [15]. All gels were adjusted to equal BChl *c* concentrations. Molecular weights of *ca.* 5 kDa were determined for peak 2 (corresponding to the 5.6-kDa protein of Wechsler *et al.* [7]; see text), of 7 kDa for peak 3, 10 kDa for 4, and 14 kDa for 5.

the smallest polypeptide (number 2 as in Figs. 1 and 3) accumulates strongly during the first 24 h at room temperature (*cf.* Figs. 2a and 2b). There appears no significant further accumulation during the subsequent 24-h period (Fig. 2c). Concomitant with the increase of peak 2, peaks 3 (7 kDa) and 5 (14 kDa) decrease, while often 4 (10 kDa) remains practically unchanged. Additionally, the proteins with M_r values higher than approx. 20 kDa also show strong reductions. After 48 h their concentration has become quite small. It is important to note in this regard that the staining sensitivity for these proteins is much higher than for the smaller fragments; this is evident from a comparison with

contaminations by other proteins with higher molecular weights could regularly be detected by the very sensitive silver staining. It should be noted here that silver staining − when performed as described in Materials and Methods − may be more sensitive for proteins of higher molecular weights than for low-molecular weight proteins by a factor of up to 10.

The present M_r values for the main proteins differ slightly from the M_r values which we have reported previously [15, 16]. This difference is due to an M_r calculation based on different marker proteins, particularly in the low-molecular weight region where the determination of absolute M_r values is more difficult.

A striking and very important observation in our PAGE analysis of pure chlorosomes is that in most preparations the *ca.* 5-kDa polypeptide (*i.e.,* peak 2 in Fig. 1a–c) is missing. In contrast, chlorosome fractions contaminated with cytoplasmic membrane (as detected by absorption and fluorescence) often contain small polypeptides (including, *e.g.,* peak 2 in Fig. 1d) in relatively high concentrations (see also [15]).

The gel scans of Fig. 2, run at time intervals after the chlorosome isolation show that the peak of
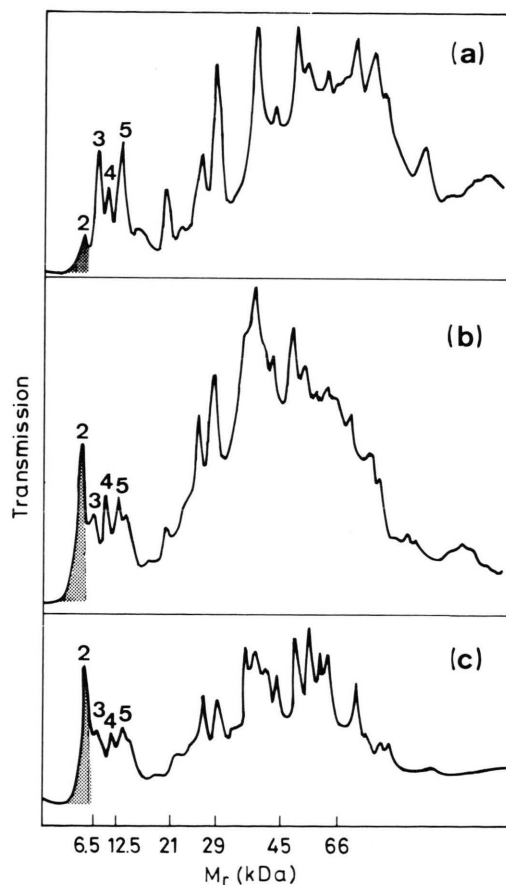

Fig. 2. PAGE densitograms (8–25% acrylamide, silver staining) of chlorosomes 0 h (a), 24 h (b) and 48 h (c) after isolation with Miranol, in the absence of any added proteolytic inhibitor. The shaded peak 2 corresponds to the 5.6-kDa protein. The transmission amplitudes of all three densitograms (a–c) can be directly compared.

the results obtained with 1-mm gels in [15]. The only protein with a higher M_r value which seems to be stable is one of about 40 kDa. It is possibly identical with the 40-kDa protein reported by Schmidt *et al.* [9].

Important observations were also made in a time-dependent PAGE study in the presence of proteolytic inhibitors. For example, Fig. 3 shows that, in the presence of benzamidine, the two major proteins of 7 and 14 kDa (bands 3 and 5) still decrease in concentration, and that the *ca.* 5-kDa polypeptide still increases throughout the 48-h period after isolation. These changes with time, evidently a protein degradation process despite the presence of benzamidine, could be stopped by incubation at 100 °C in buffer containing SDS and mercaptoethanol. PAGE comparison of such samples showed the same pattern 0 h and 48 h after incubation. Surprisingly, however, common protease inhibitors such as, *e.g.*, PMSF, benzamidine, and ε-caproic acid are not capable of suppressing the reaction.

Absorption and fluorescence properties such as those compiled in Table I are sensitive to the integrity of the BChl *c*-organization and have been used in the past to define the quality of chlorosomes. We now find that neither the absorption nor the fluorescence spectra and the relative fluorescence intensities ($\lambda_{exc} = 460$ nm, $\lambda_{em} = 750$ nm) reveal any significant difference between fresh and 24-h old samples of chlorosomes, irrespective of the absence or presence of proteolytic inhibitors.
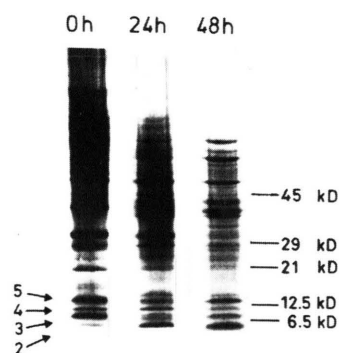

Fig. 3. PAGE (8–25% acrylamide, silver staining) of chlorosomes, 0, 24 and 48 h after isolation with Miranol. The samples were kept in the dark at room temperature in the presence of benzamidine. Numbers 2–5 of the lowest molecular bands correspond to those of the peaks in the densitograms of Fig. 1.

Table I. Absorption of chlorosomes isolated with Miranol in the absence and presence of benzamidine.

Time after isolation	Absorbance*	
	at 740 nm	at 790 nm
0 h	0.255	0.015
24 h	0.250	0.015
48 h	0.245	0.015

* The error in the absorbance is 0.005 units. Within this error, the values were the same for runs with and without added benzamidine. Analogous results were obtained also with PMSF and ε-caproic acid.

Discussion

The time course of the appearance of band 2 and its absence in freshly isolated chlorosomes illustrates unequivocally that the corresponding polypeptide, which in PAGE proved indistinguishable from the 5.6-kDa protein [7], cannot function as a BChl *c*-binding protein. Rather, the protein appears to be formed in a proteolytic cleavage reaction occurring after isolation. Consequently, the proposal [7, 8] that the BChl *c* molecules are complexed to the 5.6-kDa protein does not apply to the chlorosomes of the *C. aurantiacus* strain Ok-70-fl. Of course, the 5-kDa fragment of band 2 could fortuitously coincide only in M_r with the known 5.6-kDa entity. Although a coincidence of this sort is unlikely, it would not invalidate our conclusions in any way. Such a coincidence would simply mean that the 5.6-kDa protein is neither contained nor formed in these chlorosomes. We should like to note here that our identification of the 5.6-kDa protein on the gels is straightforward and does not simply rely on the comparison with the pure 5.6-kDa reference sample. In fact two further criteria can be used for identification: i) the 5.6-kDa protein is the lowest molecular weight protein on the gels of Feick *et al.* [8] and also on our gels, and ii) this polypeptide is the only dominant one in that M_r region which at longer times (48 h) proves stable against proteolytic cleavage.

One pitfall which might have led to erroneous conclusions in previous studies is that Feick *et al.* [8] had not been aware of the fact that PMSF could not have acted as an inhibitor in their proteolytic digestion experiments.

Since the protein degradation rapidly progresses already within the first 5-h period (results not

shown), it is in place here to stress the necessity that in digestion experiments the degradation is suppressed before exogenic proteolytic enzymes are added. Otherwise, the *ca.* 5-kDa fragment accumulates noticeably during the experiment independent of exogenic proteases.

Another observation deserves mention here. Mechanical stress, *e.g.* slight stirring, seems to damage the chlorosome samples as indicated by a decrease in absorption at 740 nm. We therefore avoided stirring for any prolonged periods. Furthermore, pelleting (60 min at 45,000 rpm; Ti 70 rotor) did not remove the *ca.* 5-kDa polypeptide from the chlorosomes, which suggests that this fragment is located either in the chlorosome interior or in the chlorosome envelope.

The high proteolytic activity leading to the post-isolation formation of the 5.6-kDa protein does not change the optical properties of these chlorosomes. In their proteolytic digestion experiments Feick *et al.* [3, 8] excluded as potential BChl *c*-binding proteins all other proteins except for the 5.6-kDa protein. Owing to the much higher sensitivity of our PAGE system, we are observing an appreciably larger number of proteins being associated with BChl *a*-containing chlorosomes than other authors. Since only a few of these proteins are among those checked by Feick *et al.* [3, 8], the remaining might *a priori* still function as BChl *c*-binding proteins. However, this is excluded from our previous report [16] which demonstrated that further purification of these samples by GEF ultimately affords essentially *protein*-(and BChl *a*-) *free* species with intact chlorosome absorption and fluorescence properties. This conforms, of course, with other evidence, including the similarity of the spectroscopic properties of artificial BChl *c* aggregates in organic solvents and of BChl *c in vivo* [4, 10–15], and it strengthens the concept of direct chromophore-chromophore binding [16] as opposed to the proposed involvement of structural protein-BChl *c* complexes. It is appropriate to recall here that attempts of isolating such complexes from chlorosomes of any origin have failed so far.

In view of the strong evidence in favour of the direct BChl *c* chromophore-chromophore model the question which proteins do occur in native chlorosomes, and which function such proteins perform (*e.g.*, binding of BChl *a* in the presumed BChl a_{790} complex), remains unanswered.

Finally, one should not ignore the (perhaps far-fetched) possibility that the 5.6-kDa protein, characterized by Wechsler *et al.* [7] as a component of strain J-10-fl, neither occurs nor is formed at all in the chlorosomes of strain Ok-70-fl. However, in view of the far-reaching analogies in the properties of the chlorosomes from both strains (absorption and fluorescence spectra [18, 23], BChl *c* distribution pattern in reverse-phase high-performance liquid chromatography [13, 24]), an essentially identical organization of the BChl-*c* antennae in both strains is more likely.

Comments on the problem of M_r determinations

A reliable M_r determination of small proteins is notoriously difficult. For example, we have obtained values ranging from about 4 to 11 kDa for the 5.6-kDa fragment depending on the marker proteins employed. It is therefore quite possible that the small proteins (peaks 1 and 2) found in highly contaminated fractions arise from the $B_{806-866}$ antenna complex. The two proteins attributed to this complex have M_r values of 4.9 and 6.3 kDa [25, 26], which could have rendered their distinction from a 5.6-kDa protein difficult.

Our M_r determinations for the low-molecular weight region now gave values of 7, 10 and 14 kDa (bands 3, 4, and 5, Fig. 1) for the dominant components in the Miranol preparations. These values differ somewhat from those reported previously [15, 16] for technical reasons. If we allow for the possibility that the 7- and 10-kDa proteins were not resolved in the past, our results are in agreement with the values of 10 and 15 kDa of Schmidt *et al.* [9] but differ from those reported by the group of Feick [3, 8]. One may consequently argue now that the *ca.* 5-kDa polypeptide must have also been missing on the gels of Schmidt *et al.* [9].

[1] J. M. Olson, Biochim. Biophys. Acta **594**, 33–51 (1980).

[2] L. A. Staehelin, J. R. Golecki, R. C. Fuller, and G. Drews, Arch. Mikrobiol. **119**, 269–277 (1978).

[3] R. G. Feick, M. Fitzpatrick, and R. C. Fuller, J. Bacteriol. **150**, 905–915 (1982).

[4] K. Griebenow and A. R. Holzwarth, Biochim. Biophys. Acta **973**, 235–240 (1989).

[5] J. M. Olson, D. C. Brune, and P. D. Gerola, Biology of Membrane-Bound Complexes in Phototropic Bacteria (G. Drews, ed.), in press, Plenum Press, New York 1990.

[6] D. C. Brune, P. D. Gerola, and J. M. Olson, Photosynth. Res., in press (1990).

[7] T. Wechsler, F. Suter, R. C. Fuller, and H. Zuber, FEBS Lett. **181**, 173–178 (1985).

[8] R. G. Feick and R. C. Fuller, Biochemistry **23**, 3693–3700 (1984).

[9] K. Schmidt, M. Maarzahl, and F. Mayer, Arch. Microbiol. **127**, 87–97 (1980).

[10] M. I. Bystrova, I. N. Mal'gosheva, and A. A. Krasnovskii, Mol. Biol. **13**, 440–451 (1979).

[11] K. M. Smith and K. A. Kehrs, J. Am. Chem. Soc. **105**, 1387–1389 (1983).

[12] M. Lutz and G. van Brakel, in: Green Photosynthetic Bacteria (J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper, eds.), pp. 23–34, Plenum Press, New York 1988.

[13] D. C. Brune, T. Nazawa, and R. E. Blankenship, Biochemistry **26**, 8644–8652 (1987).

[14] D. C. Brune, G. H. King, and R. E. Blankenship, in: Photosynthetic Light-Harvesting Systems (H. Scheer, ed.), pp. 141–151, de Gruyter, Berlin 1988.

[15] K. Griebenow and A. R. Holzwarth, in: Molecular Biology of Membrane-Bound Complexes in Phototropic Bacteria (G. Drews, ed.), in press, Plenum Press, New York 1990.

[16] A. R. Holzwarth, K. Griebenow, and K. Schaffner, Z. Naturforsch. **45c**, 35–38 (1990).

[17] V. Van Mourik, K. Griebenow, B. van Haeringen, A. R. Holzwarth, and R. van Grondelle, Proc. VIIIth Intern. Congr. Photosynthesis, Stockholm, in press (1990).

[18] A. R. Holzwarth, M. G. Müller, and K. Griebenow, J. Photochem. Photobiol. B **5**, 457–465 (1990).

[19] J. Heukeshoven and R. Dernick, Electrophoresis **9**, 28–32 (1988).

[20] K. Weber and M. Osborn, J. Biol. Chem. **244**, 4406–4412 (1969).

[21] K. Griebenow, Diploma Thesis, Philipps-Universität Marburg/MPI Strahlenchemie, Mülheim a.d. Ruhr 1988.

[22] J. P. Markwell, C. D. Miles, R. T. Boggs, and J. P. Thornber, FEBS Lett. **99**, 11–14 (1979).

[23] R. J. Van Dorssen, H. Vasmel, and J. Amesz, Photosynth. Res. **9**, 33–45 (1986).

[24] F. Fages, N. Griebenow, K. Griebenow, A. R. Holzwarth, and K. Schaffner, J. Chem. Soc., Perkin Trans. 1, in press (1990).

[25] T. Wechsler, R. Brunisholz, F. Suter, R. C. Fuller, and H. Zuber, FEBS Lett. **191**, 34–38 (1985).

[26] T. D. Wechsler, R. A. Brunisholz, F. Suter, G. Frank, and H. Zuber, FEBS Lett. **210**, 189–194 (1987).