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Pigment organization and energy transfer in green bacteria. 2. Circular and linear dichroism spectra of protein-containing and protein-free chlorosomes isolated from *Chloroflexus aurantiacus* strain Ok-70-fl *

Kai Griebenow [1,**], Alfred R. Holzwarth [1], Frank van Mourik [2] and Rienk van Grondelle [2]

[1] *Max-Planck-Institut für Strahlenchemie, Mülheim / Ruhr (F.R.G.)* and [2] *Department of Biophysics, Free University of Amsterdam, Amsterdam (The Netherlands)*

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We have measured the circular dichroism (CD) and linear dichroism (LD) spectra of chlorosomes isolated from *Chloroflexus aurantiacus* strain Ok-70-fl obtained by two different isolation procedures. The gel-electrophoretic filtration procedure yields chlorosomes that are essentially free of BChl a_{790} and proteins, while isolation by sucrose density gradient centrifugation yields the conventional chlorosome preparations. The LD spectra of the two kinds of preparation were very similar. In both cases the Q_y LD signals correspond to an average angle between the BChl-*c*–Q_y transition and the long axis of the chlorosome of approx. $15 \pm 10°$. In contrast to the LD spectra, the CD spectra of different preparations (membranes, BChl-*a*-free chlorosomes, BChl-*a*-containing chlorosomes) show pronounced differences both in the ellipticity as well as in the shape of the spectra and the number of maxima. However, these differences are not caused by the isolation procedure or the detergents used. We show that even freshly prepared membranes (of different, parallel grown batch cultures) give rise to very different CD spectra. The set of different CD spectra we obtained could be simulated well by linear combinations of two basic spectra. This strongly suggests that the variations in the CD spectra are caused by a variation in the relative amounts of two different species, two different types of chlorosome, or possibly by two different types of pigment aggregate within the chlorosomes.

Introduction

The phototrophic bacterium *C. aurantiacus* [1–3] possesses as the main photosynthetic antenna system the so-called chlorosomes [4]. These chlorosomes are flat oblong vesicles with dimensions of approx. $100 \times 30 \times 12$ nm [5], which contain bacteriochlorophyll (BChl) *c* and carotenoids as pigments [2]. The BChl *c* is believed to be organized in rod-shaped elements, which have been observed by electron microscopy [5]. Conventionally, all chlorosomes are isolated by density-gradient centrifugation techniques and contain a small amount of BChl *a* organized in the BChl a_{790} complex [6–10]. A new preparation method called GEF has been reported recently, which results in the isolation of chlorosomes that are free of BChl *a* [11]. These BChl-*a*-free chlorosomes were also essentially free of proteins [10,12]. The isolation of chlorosomes with only very minor or even no protein content supports models for the BChl *c* organization proposed by various authors mainly on the basis of studies from artificial BChl *c* aggregates [13–19]. In addition, a titration study of chlorosomes with 1-hexanol which demonstrates the reversible conversion of aggregated to monomeric BChl *c* supports a model with BChl *c* aggregates in chlorosomes [20].

* Part 1: is Ref. 11: Isolation of native chlorosomes free of bound bacteriochlorophyll *a* from *Chloroflexus aurantiacus* by gel-electrophoretic filtration.

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Abbreviations: BChl, bacteriochlorophyll; CD, circular dichroism; CM, cytoplasmic membrane; DDM, dodecyl β-D-maltoside; GEF, gel-electrophoretic filtration; LD, linear dichroism; LDAO, lauryldimethyl *N*-oxide; SDGC, sucrose density gradient centrifugation; BChl c_s, BChl *c* esterified with stearol.

Correspondence: A.R. Holzwarth, Max-Planck-Institut für Strahlenchemie, Stiftstrasse 34–36, D-4330 Mühlheim/Ruhr, F.R.G.

Present criteria for the native conformation state of the protein-free GEF-chlorosomes are the stationary absorption and fluorescence spectra [11], electron microscopy [11], and time-resolved fluorescence spectra with picosecond time resolution [9,21]. LD spectra of chlorosomes have been reported so far only on conventional BChl-*a*-containing chlorosomes oriented in PVA films [22], polyacrylamide gels [23] or electric fields [24], and have shown that the Q_y transition moments of BChl *c* are preferentially oriented along the long axis of the chlorosome. A detailed analysis in which the degree of orientation of the chlorosome was explicitly calculated yielded an angle of about 20° between the Q_y transition moment and the long axis of the chlorosome [24]. This result was recently confirmed by polarized fluorescence measurements on oriented chlorosomes [25]. Larger angles indicating lower degrees of orientation were found by Van Dorssen et al. [23] (37°) and Betti et al. [22] (40°). Although the presence of two differently oriented transitions in the 740 nm absorption band had been reported by Van Dorssen et al. [23], this result was not confirmed in Ref. 24. The BChl a_{790} protein complex shows a small negative LD signal, indicative of a completely different orientation compared to the BChl *c* 740 nm transition.

The CD spectra of BChl-*a*-containing chlorosomes from *C. aurantiacus* have been reported by several groups, but the published spectra differ widely both in shape and maximal ellipticities. While Betti et al. [22] reported a spectrum with a positive band at 715 nm and a negative band at 750 nm for the BChl-*c*–Q_y transition moment, Van Dorssen et al. [23] found a spectrum with negative bands at 721 nm and 754 nm and a positive band at 736 nm. Olson and co-workers have also studied the CD spectra of chlorosomes [26,27] prepared using various methods. They suggested that the widely varying CD spectra reported for *Chlorobium* chlorosomes in the literature are due to degradation of the chlorosomes and proposed one spectrum as being representive of native chlorosomes [27]. In contrast, we recently found varying CD spectra for chlorosomes of *Chloroflexus* that are still bound to the cytoplasmic membrane [28]. This observation indicates that it is not necessarily the isolation procedure that causes the spectral differences in the CD.

In order to compare the internal structure of protein-containing and protein-free (GEF)-chlorosomes with respect to possible changes of the BChl *c* organization and to shed light on the so far unexplained variations in CD spectra, we applied the sensitive methods of CD and LD spectroscopy.

Materials and Methods

C. aurantiacus strain Ok-70-fl was grown anaerobically in 1-liter bottles at 50°C as batch cultures as described [11]. The light was provided by four Osram L 18W/30 warm white tubes in a distance of 30 cm. Under these conditions cells grow to high density by linear growth [29]. Cells were collected in the linear growth phase after 7 days. Under these conditions cells are adapted to low light because of the high density. About 5 g wet cells were collected from a 1 liter culture. The growth time of 7 days was chosen because under these conditions the BChl *c* content increases linear with the packed cell volume, the BChl *c* to BChl *a* content remains constant, carotenoid content increases linearly, all indicating stable and reproducible conditions [29]. Isolation of the membranes (CM with attached chlorosomes) was carried out as described [11] except that a French press at 1200 lb/in^2 (American Instrument Company) was used for most of the membrane isolations instead of ultrasonication. Conventional chlorosomes containing BChl *a* using the detergents LDAO, DDM, Deriphat-160, or Miranol on the one hand and GEF-chlorosomes free from BChl *a* and proteins on the other hand were isolated as described [9–11]. For GEF-chlorosomes we used LDS concentrations from 0.1% to 5% as already described [11]. For 'normal' chlorosomes we used the optimized detergent concentrations as described in Refs. 9, 10. All preparation steps were carried out at 4°C to reduce or avoid enzymatic processes [30]. Proteinase inhibitors were not added. Stationary absorption and fluorescence spectra were measured as described [11]. CD measurements were performed with the chlorosomes suspended in 20 mM Tris-HCl buffer (pH 8.0). For LD measurements the chlorosomes were oriented by embedding them in a polyacrylamide gel, 15% (w/v), acrylamide : *N*,*N*-methylenebisacrylamide, 29 : 1 in the same Tris-HCl buffer. The gels were compressed by a factor of 1.25, in both the *x* and *y* directions. The LD is measured as the difference in absorption between *z*- and *y*-polarized light (incident along the *x*-axis of the sample).

Both CD and LD measurements were performed on a home-built spectrophotometer that will be described elsewhere (Van Mourik et al., unpublished data). CD spectra were in addition recorded on a Jasco J-20 spectropolarimeter. Both instruments were calibrated using a 0.01% (+)-10-camphorsulfonic acid solution, which has a A_l–A_r of 0.00094 ($\theta = 0.031$ deg cm^{-1}) at 290 nm for a light path of 1 cm (see, for example, the Cary61 manual for Ref. 31). The Jasco J-20 spectropolarimeter was also checked with Chl *a* in ether according to Houssier et al. [31] for the long-wavelength region. We measured a $\Delta\epsilon_{657}$ of -13.7 M^{-1} cm^{-1}, which is nearly identical to the value reported (-13.8 M^{-1} cm^{-1}) [31]. The two CD spectrometers gave similar values $(A_l - A_r)/A_{max}$ for a membrane sample ($+15 \cdot 10^{-4}$ and $+14 \cdot 10^{-4}$, respectively, for the positive band at 730 nm and $-25 \cdot 10^{-4}$ and $-27 \cdot 10^{-4}$, respectively, for the negative band at 750 nm). This spectrum has been

published by us in Ref. 28. The bandwidth used in both the CD and LD measurements was 3 nm. Absorption was measured on a Cary 219 spectrophotometer. All experimental CD spectra were normalized at 740 nm to the corresponding absorbance. Thus the band intensities of all spectra are directly comparable.

Results

LD spectra

The LD and CD spectra have been measured on a large number of independent preparations of both conventional and GEF-chlorosomes (we have checked 10 membrane samples, 27 GEF-samples and 9 conventional chlorosome samples by CD spectroscopy; several of these samples have been measured also by LD spectroscopy). Typical results for the LD spectra of 'normal' chlorosomes, containing proteins and BChl a_{790}, and of GEF-chlorosomes lacking both proteins and BChl *a* are shown in Figs. 1A and B, respectively. The maximum of the LD signal in the NIR region is at 742 nm ± 2 nm for normal BChl-*a*-containing chlorosomes and 739 nm ± 2 nm for the GEF-chlorosomes. The same shift to slightly shorter wavelength of the BChl-*c*–Q_y transition moment is observed also in the absorption spectra [11]. However, the shape and intensity of the LD spectra is essentially identical in the two types of chlorosome. Comparing the absorption and LD spectra it is clear that the BChl-*c*–Q_y transition is highly oriented. Use of the formula derived by Ganago [32] for rod-shaped particles yields an angle of $15 \pm 10°$ between the BChl-*c*–Q_y transition moment and the long axis of the chlorosome in both types of sample. This value indicates the same high degree of organization and orientation of the BChl *c* in both protein-containing and protein-free chlorosomes. The degree of orientation of the 'normal' Miranol preparation is nearly constant over the 740 nm band, strongly suggesting that all Q_y transitions have the same orientation. The polarization degree drops for wavelengths below 680 nm, where some unoriented absorption is apparent. For the GEF-chlorosomes the LD drops more rapidly on the short wavelength side (≤ 720 nm) than does the absorption. Thus, the pigments absorbing at ≤ 720 nm appear to have a somewhat lower polarization. The absorption band at about 670 nm, which is more pronounced in GEF-chlorosomes, does not appear in the LD spectrum. This suggests that it derives from essentially unoriented, i.e., free pigments of BChl *c*. In addition, this drop could be due to a mixing of *x* and *y* polarization in the vibrational shoulder at the short-wavelength side. It was shown by Ebrey et al. [33] that the polarized fluorescence of monomeric BChl *a* decreases in the vibrational shoulder by this reason.

In order to investigate the question of a possible inhomogeneity of the 740 nm absorption band in GEF-chlorosomes more closely, we also measured an LD spectrum at 77 K (Fig. 2), since shoulders in the LD spectrum should be enhanced by lowering the temperature. As can be clearly seen, we did not observe any inhomogeneities of the 740 nm absorption band in this sample. The absorption and LD spectrum are identical in this case.

Comparison of the LD spectra of BChl-*a*-containing and BChl-*a*-free chlorosomes shows that there is essentially no difference in the shapes of the band around 740 nm, with only a minor shift detected. The removal of proteins by the GEF procedure thus does not signifi-

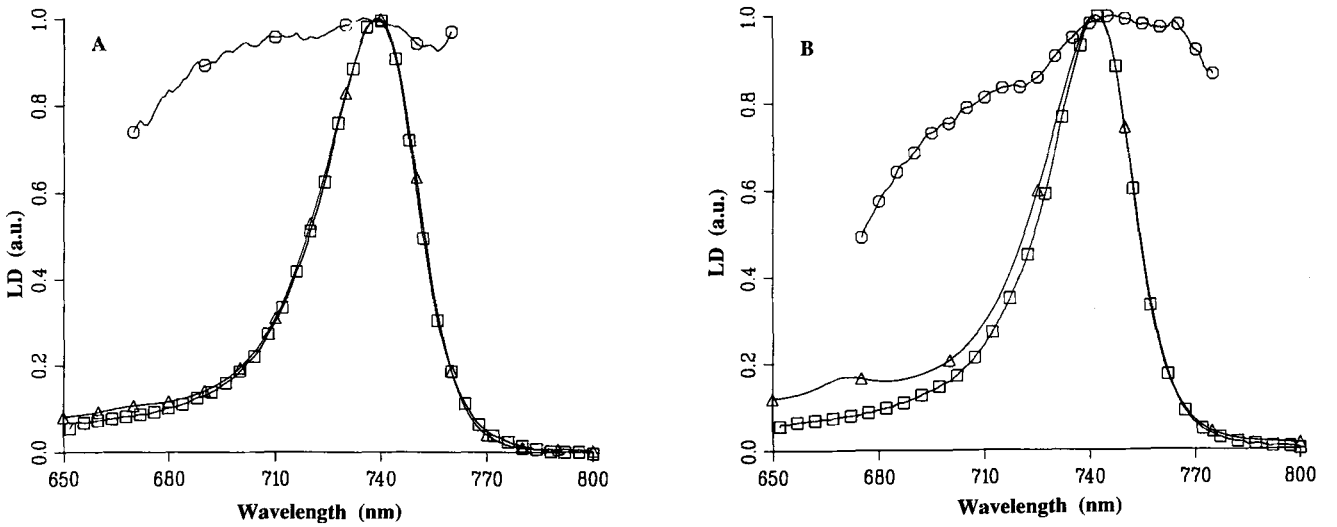

Fig. 1. LD and absorption spectra of protein-containing 'normal' chlorosomes (1A) and of protein-free GEF-chlorosomes (1B) (triangles, absorption spectra, squares, LD spectra). Also included is the ratio between the LD and absorption spectra (circles). The maximum absolute values for the LD/A_{iso} signals in both cases are 0.75. The gels were compressed in the *x* and *y* direction by a factor of 1.25, resulting in a compression factor of $n = 1.56$. The calculation was performed as described in Ref. 24 for compressed gels and rod-shaped particles.

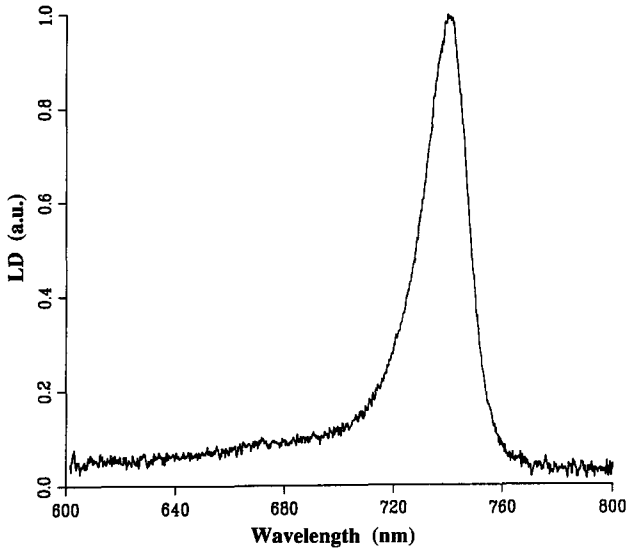

Fig. 2. LD spectrum of protein-free GEF-chlorosomes recorded at 77 K.

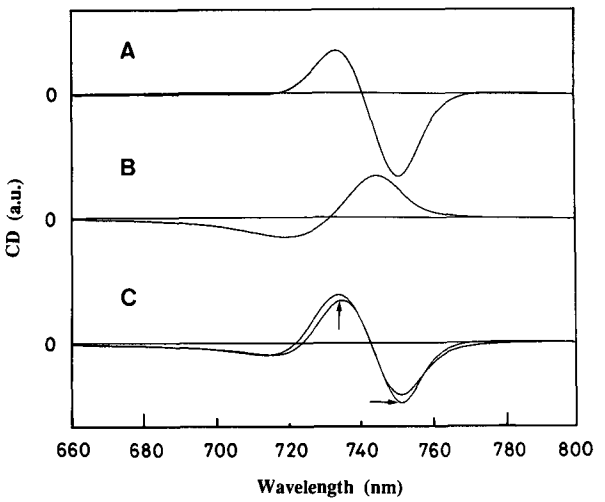

Fig. 3. CD spectra of type I, type II and mixed type (3A–C). Trace A obtained from membranes (type I), trace B from Miranol-prepared (protein-containing) chlorosomes (type II) and trace C of protein-free chlorosomes (mixed type). A linear combination of type I (weighted with a factor of 0.61) and type II (weighted with a factor of 0.39) is shown in 3C (arrows). The resulting spectrum is compared with the experimental measured spectrum 3C.

cantly change the BChl *c* orientation and/or organization.

CD spectra

In contrast to the nearly identical LD absorption spectra for both types of preparation, significant differences in the CD spectra are observed. We have found three different types of CD spectra which we call types I, and II, and mixed type. This nomenclature does not imply, however, that any of the experimental spectra reflect a totally 'pure' form. It simply indicates that the amount of mixing should be greatly different. These three different types of CD spectrum are shown in Fig. 3. Type I is a spectrum with bands at about 730 nm (positive) and 745 nm (negative) (cf. Fig. 3A). Type II has bands at 720–730 nm (negative) and 743–750 nm (positive) (cf. Fig. 3B) and the mixed type bands at 713–716 nm (negative), 730–735 nm (positive) and 749–755 nm (negative) (cf. Fig. 3C). We have repeatedly obtained different types of CD spectrum from membranes isolated under identical conditions. These membranes came from different batches of cells apparently grown under the same conditions and inoculated from the same original culture (Fig. 4). It can be seen from these spectra that the mixed type is not a homogeneous type of CD signal, since the relative band intensities vary. This observation seems to indicate that the isolation procedure is not responsible for variations in the CD signal. Membranes isolated from cells grown in different light conditions as compared to those described here as 'normal' ones, also gave CD signals very similar to those described here in detail. For example membranes isolated from high-light-grown cells (same incident light intensity as described [11] but only very dilute culture and 1 day growth) showed a CD spectrum of the mixed type as did those obtained from low-light cells (very dense culture, 1 week growing in a 1-liter bottle shielded by aluminium foil with an opening spot of 1 cm^2). CD spectra of type I (like that reported by Betti et al. [22]) were observed only once by us for a membrane (see Fig. 3A) and a chlorosome sample. Thus, no clear pattern emerged on what condition may give rise to the various CD spectra found already in the membranes.

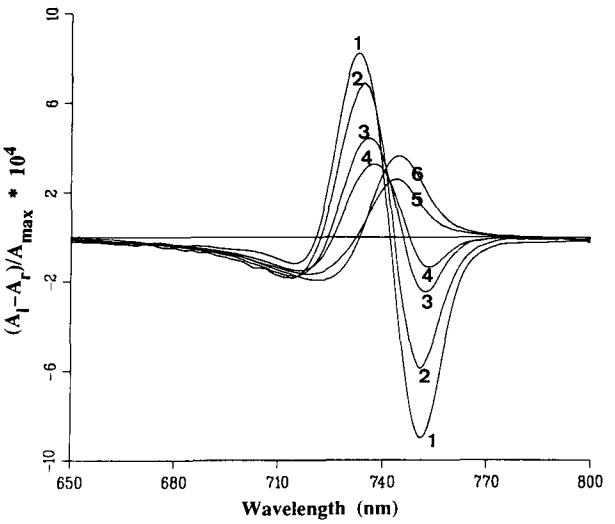

Fig. 4. CD spectra obtained from different membranes as described in detail in the Results section. The membranes were isolated in parallel under the same conditions from cells grown in parallel batches. The spectra shown are of mixed type and type II. The expression $(A_l-A_r)/A_{max}$ means that all CD spectra were normalized to A_{max}, the maximal absorbance, i.e., A_{740}.

It is an important observation that freshly isolated chlorosomes giving rise to one of these three types of CD spectrum always gave the same CD signal as the membranes themselves. In Table I we have listed examples for 'normal' chlorosomes prepared with various detergents, all of which have a type II CD spectrum. For LDS we have presented a detailed analysis in which it is demonstrated that changing the LDS concentration from 0.1% to 5% for isolation has only a minor effect on the CD spectra observed for GEF-chlorosomes prepared from the same membrane batch. This seems to rule out any influence of the isolation procedure and/or of detergent action on the CD signals, which is in contrast to the observations reported for *Chlorobium* chlorosomes [27]. We would like to point out yet again that membranes giving rise to different CD spectra were prepared in parallel using the same isolation conditions. This again rules out the possibility that the isolation procedure is responsible for the observed differences of the CD spectra.

A quantitative analysis of the CD spectra of membranes, normal and GEF-chlorosomes is given in Table I. The $(A_l-A_r)/A_{max}$-values of the observed CD bands of chlorosomes vary by a factor of about 20 in the near-infrared. Also included in Table I are values for a CD spectrum obtained from an artificial aggregate of BChl *c* (the fourth fraction as described by Brune et al. [15] on a reversed phase HPLC-column, identical to BChl c_s but further purified according to Fages et al. [34] to obtain the pure 2a-R epimer), in a solution of *n*-hexane with 2% toluene at a BChl *c* concentration of 40 μM. The aggregate spectra were recorded on the same instrument as the chlorosome spectra.

It is interesting that the CD values obtained by us for the biological samples, although varying over an unusually large range, agree with the similar wide range of

TABLE I

Characterization of the near-infrared CD features of membranes, conventional chlorosomes (SDGC-chlorosomes in various detergents) and GEF-chlorosomes

A_{max} refers to the absorbance at the absorption maximum (740 nm).

Sample	Type of CD	λ_{max}/CD intensity $(A_l-A_r)/A_{max} \times 10000$			
Membranes	I		730/+15.9		743/−25.1
	mixed	715/ −2.6	735/ +6.5		751/ −3.6
	mixed	714/ −2.5	733/+12.0		750/ −9.0
	II	719/ −2.6		745/ +3.9	
	II	722/ −2.9		742/ +5.7	
SDGC-chlorosomes					
Miranol-prep. [a]	II	723/ −1.1		752/ +1.4	
Deriphat-prep. [a]	II	724/ −1.6		750/ +2.6	
DDM-prep. [a]	II	723/ −1.7		750/ +3.0	
LDAO-prep. [a]	II	722/ −1.3		750/ +2.1	
Deriphat-prep.	II	722/ −6.7		749/+13.2	
Miranol-prep.	II	719/ −9		745/+19	
GEF-chlorosomes					
5.0% LDS	mixed	716/ −2.6	732/ +6.0		755/ −3.5
0.1% LDS [c]	mixed	713/ −4.0	732/+14.1		749/−16
1.0% LDS [c]	mixed	713/ −5.5	732/+15		749/−14
5.0% LDS [c]	mixed	713/ −6.0	732/+13		749/−12
5% LDS	II	723/ −5.8		750/+10.9	
0.2% LDS	II	722/ −4.4		750/ +8.9	
0.1% LDS [b]	II	725/−20.8		743/+31.8	
0.2% LDS [b]	II	726/−17.9		744/+24.2	
0.2% LDS [b]	II	725/−24.8		740/+46.9	
0.3% LDS [b]	II	726/−25.5		746/+41.5	
1.0% LDS [b]	II	725/−25.2		750/+43.9	
2.0% LDS [b]	II	725/−24.2		750/+39.2	
5.0% LDS [b]	II	726/−17.9		744/+24.2	
BChl-c_s aggregate [d]	I		730/+60		748/−64.4

[a] All these different SDGC-chlorosomes were prepared from the same membrane batch. It follows that the detergent has no effect on the type of the CD spectrum observed.

[b,c] All these different GEF-chlorosomes were prepared from the same membrane batch, [b] from membranes of type II, [c] from membranes of mixed type.

[d] 40 μM BChl c_s (2a*R*) in *n*-hexane, 2% toluene (v/v).

most of the published ones (see Discussion section). This holds for membranes, protein-containing and protein-free GEF-chlorosomes. The peak intensity of the 740 nm NIR-CD bands of the artificial aggregates is higher than in chlorosomes and these bands are much narrower.

All data shown here are for freshly prepared samples. Changes of the CD spectra were observed over a time-period of several weeks after isolation (sample stored at 4°C) only and in parallel significant changes of the absorption spectrum were observed (the absorption in these cases decreased to less than 10% of the original absorption). During periods typical for preparation and measurement no changes were observed.

In order to determine whether the different types of CD spectrum can be simulated by a set of two basic spectra we took examples of the three different types of spectrum, as shown in Fig. 3A–C (normalized, arbitrary intensities). In Fig. 3C the measured three-banded spectrum is plotted together with a linear combination (arrows) of the other two. The resemblance of the simulated spectrum with the measured spectrum is striking, even though one of the spectra is from membranes while the other two are from isolated chlorosomes. The same linear combination was performed with different membrane spectra we obtained, as shown in Fig. 5A–C. Here the result of the fit is even better. These simulations provide strong indications that the spectra are due to varying mixtures of two different spectral species. The experiments do not indicate whether the species are different types of pigment-aggregate within one chlorosome, or different 'types' of chlorosome. Nevertheless,

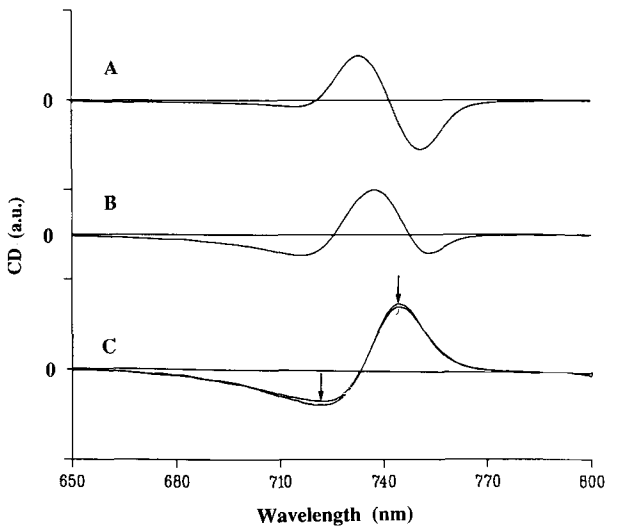

Fig. 5. CD spectra of different membrane samples with mixed type (A and B) and type II (trace C) spectra. Linear combination of both spectra of mixed type is shown in C (arrows). The original spectra were weighted with −0.45 (A) and 0.55 (B) for the linear combination. The result is a typical type II which is compared to the experimental spectrum of a membrane sample.

the type II spectra vary by the relative ratio of the two CD bands and it is therefore clear that they do not represent really pure 'basic' spectra. Our selection into different types can be done only by comparing the shapes and chosing the most extreme cases as 'basic' type I and type II.

Discussion

The LD spectra of BChl *a* and protein-containing chlorosomes and the LD spectra of BChl-*a*-free and protein-free GEF-chlorosomes show the same high orientation in the BChl *c* absorption band, i.e., an angle of $15 \pm 10°$ between the BChl-*c*–Q_y transition moments and the long axis of the chlorosome in both preparations. In our LD measurements we see only minor differences between GEF-chlorosomes and conventional chlorosomes. This result can be explained by assuming that the proteins do not play a significant role in the organization of the BChl *c* pigments. In light of this fact, we might address the question as to whether or not the assumptions made in the analysis of LD results are still valid for such particles. When the structure/shape of chlorosomes is determined by large pigment-aggregates surrounded by a membrane, it could be argued that in the LD experiments, involving gel-deformation, the particle that is being oriented might be the aggregate itself. The fact that Van Amerongen et al. [24] were able to obtain very similar results from LD measurements using both gel and electric field orientation is a very strong indication, however, that the asymmetric shape of the chlorosomes determines the orientational properties in both cases.

The angles calculated from LD measurements are generally dependent both on the model assumptions (i.e., rod vs. disc shape) and to some extent on the size of the particle. Small aggregates with a size comparable to the size of the gel-pores would orient less efficiently than large particles like chlorosomes. This size factor would normally result in an overestimation of the calculated angle (for positive LD values). However, the observed high dichroism values, which are very close to the maximum theoretical value for rod-shaped particles, lead us to the conclusion that the particles that we orient are large and rod shaped.

The average angle between the orientation axis and the BChl *c*-transition dipole has also been determined by polarized fluorescence measurements on oriented chlorosomes [25]. This method gives the same information as LD measurements, but is independent of the degree of orientation and does not depend on the model of Ganago [32]. Notably, in this study, an angle of 17° was determined.

In the past, different LD results have been reported for chlorosomes. When using partially oriented samples, and correcting for this by using the pressing-formulae

derived by Ganago et al. for rod-shaped particles [32], values of 20° have been reported [24] for the angle between the long axis of the particle and the BChl-*c*–Q_y transition dipole. Van Dorssen et al. [23] reported a much larger angle, but in those measurements much larger gel-deformations were used, and in the calculations chlorosomes were assumed to be perfectly oriented. Van Dorssen et al. [23] also reported a shoulder in the LD spectrum at 725 nm that had a higher degree of orientation than the band located at the absorption maximum. In contrast, we occasionally found a lower orientation in that region but no shoulders in the 77 K LD spectrum.

The high degree of BChl *c* orientation as deduced from the LD in the main absorption band points to a conserved organization of BChl *c* in the protein-free GEF-chlorosomes and is in line with the suggestion that the main organizational principle in chlorosomes is the direct chromophore-chromophore interaction and not a chromophore-protein complex. Protein-free chlorosomes show a difference between the LD and the absorption spectra in the region below 690 nm. We assign this absorption to free BChl *c* pigments caused by destruction of some chlorosomes.

In contrast to the LD spectra, the interpretation of the varying CD spectra is very difficult, and we are at present not able to relate these differences to any other external parameters. A wide variety of different chlorosome CD spectra have appeared in the literature so far. The three spectra we found have all been reported before [17,22,23,26,27]. The group of Olson first found the type II spectrum [35], but later reported that the spectrum with three bands should represent the native CD spectrum [26,27]. Blankenship and co-workers reported a type I spectrum [22], but later also published a spectrum with three bands [17]. Finally Van Dorssen et al. published a 77 K CD spectrum of the three-banded type [23].

Apart from the obvious differences in the shape of the CD spectra, the intensities of the spectra published are also very different. The spectrum with three bands of Blankenship et al. [17] is two orders of magnitude smaller than the similar (in shape) spectra in Refs. 26, 27. The intensity of the spectrum measured by Van Dorssen et al. [23] fits well to our data as a medium value. The A_l–A_r values found by Olson and co-workers [26,27] for the three-banded type are at the upper end of values found by us. Surprisingly, Olson and co-workers report that their artificial aggregates have a less intense CD spectrum. In our measurements, and in those of Blankenship et al. [17], the artificial aggregates have a CD signal stronger than that of the chlorosomes. Our aggregates were different, however, since we used the pure BChl c_s (2a*R*) epimer.

We measured the CD spectra of freshly prepared membranes and chlorosomes, isolated from several batches of cells over a time period of more than 1 year. The variations in intensity we found in these independent experiments were large (e.g., $(2\text{–}15)\cdot 10^{-4}$ $(A_l - A_r)/A_{max}$ for the main positive band of the mixed type spectrum) and can be compared to the variations reported in the literature as discussed above. At present we do not have any explanation for the clear differences in intensities found by different research groups and in our own measurements for a large number of independent preparations.

Our linear combination analysis suggests that two 'basic' CD spectra (types I and II, see comment above) are likely to be mixed in different ratios to give the third observed one. Variations or changes of the CD signals due to degradation during the isolation procedure can clearly be ruled out from experiments with intact membranes. At present we are unable to explain the fact that widely different CD spectra can be observed from conventional chlorosomes which do not differ in their fluorescence, absorption and LD spectra. A possible explanation may be that the CD signal comes only from minor parts of the total BChl *c* (otherwise a different organisation should give different LD signals) or from small changes of the angle of the BChl *c* organized in the aggregates. Such small changes were shown by Scherz and Parson [36] to induce different CD signals for a dimer of BPheo *a*. In view of these observations the destructive overlap of different types of CD spectrum in typical native chlorosomes could also explain the smaller CD signal of chlorosomes as compared to that of aggregates. At present and for the time that no good explanations exist for the variations in the CD spectra we suggest that the CD signal should not be used as a parameter for the definition of the quality of a chlorosome preparation. We have found no evidence for a high sensitivity of the CD signal for different detergents (e.g., protein-free chlorosomes prepared from one membrane batch with 0.1% to 5% LDS do show the same type of CD; only minor changes in the CD intensity of the NIR-CD features were observed [28], see also Table I). After the isolation procedure all chlorosomes show the same CD signal as the corresponding membranes. Only degradation over a long period with a drastic decrease of the absorption band at 740 nm introduces some changes.

The observation of variable CD spectra is unusual and contradicts models which assume that the organization of the pigments is determined by a single type of protein complex. If one pigment-protein complex is responsible for binding of BChl *c*, only one CD signal of a constant ellipticity should be observed for the native complex. For example, all pigment-protein complexes isolated from the available wide variety of purple bacteria have a characteristic CD spectrum [37]. It is generally believed that the protein component of these complexes keeps the pigments in the special conforma-

tion which is responsible for the pigment–pigment interactions that cause the CD spectrum. Nevertheless, titrations of BChl-*a*-antenna complexes isolated from purple bacteria with detergents can change the CD spectra by changing the interactions of the protein and the chromophores. In these cases the absorption spectra are also changed [38,39]. This was not observed for different chlorosome samples, since the LD, absorption and fluorescence spectra remain unchanged within the error of the methods.

The fact that the CD spectra of chlorosomes and membranes can be simulated by linear combinations of a set of two 'basic' spectra suggests that the variable CD spectra are due to variable relative amounts of two, perhaps slightly different types of aggregate. These sets could well be different types of aggregate within a single chlorosome. These may be caused by different ratios of the components constituting the interior of the chlorosome – e.g., lipids, carotenoids and of course the BChl *c* pigments [34] themselves. Alternatively, different chlorosomes in a sample could have different spectra, perhaps due to different stages of chlorosome biosynthesis. Chlorosomes presumably are assembled in steps which would mean that different stages of chlorosome synthesis could be present at any given time in cells.

Preliminary results from our studies of different artificial aggregates of BChl c_s show only type I CD spectra for the 740 nm absorbing aggregate. We are thus able to explain type I with artificial aggregates but we are not able to form type II (and thus the mixed type). One interesting question for the future is thus whether type II CD spectra can be obtained also with artificial aggregates of BChl c_s isolated from *C. aurantiacus*. Brune et al. [27] noted that different concentrations of BChl *c* can induce strong differences in the rotational strength of the CD signal and even sig*n*-inversion can be induced by using very low concentrations.

Notwithstanding the problem in the understanding of the CD spectra, the comparison of LD spectra of protein-containing conventional and protein-free GEF-chlorosomes leads us to conclude that the latter are also native chlorosomes with respect to their BChl *c* organization. They both show typical LD absorption and also typical CD spectra. The only major difference (except for the BChl *a* content) between chlorosomes prepared by density-gradient centrifugation and the new GEF-chlorosomes seems to be the lack of proteins in the latter.

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See Hildebrandt et al. [40] for a further criterion by resonance Raman data supporting the BChl *c* organisation in chlorosomes without protein binding.

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