Pure and Scrambled Self-Aggregates Prepared with Zinc Analogues of Bacteriochlorophylls *c* and *d*

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Zinc analogues of bacteriochlorophylls c *and* d *self-assembled in aqueous media with phospholipids. A methanol solution of zinc chlorin and* α*-lecithin was put in a cellulose tube and the inner methanol solvent was gradually replaced with water by dialysis to form the self-assembled oligomers. Visible absorption spectra of the aqueous solution showed that zinc chlorins formed* J*-aggregates within the hydrophobic core of* α*-lecithin assemblies and that the supramolecular structure of the aggregates depended upon the stereochemistry at the* 3^1*-position and the alkyl substituents at the 8-, 12-, and* 17^4*-positions of the zinc chlorin. When the aqueous aggregates were prepared with a mixture of* 3^1*-epimers and/or 8-, 12-, or* 17^4*-homologues of zinc* 3^1*-hydroxy-*13^1*-oxochlorins, the structurally distinct components coaggregated to make scrambled oligomers. However, during the dialysis, zinc* 3^1*-hydroxy- and* 7^1*-hydroxy-*13^1*-oxochlorins slowly individually aggregated to give two structurally different oligomer units in the cellulose tube. In contrast, if the two zinc chlorin components rapidly self-assembled in an aqueous medium, these components coaggregated to form scrambled oligomers. The present study shows that both the molecular structure of the pigments and the speed of the oligomerization determine the molecular arrangement in chlorosome-type self-assembled oligomers.*

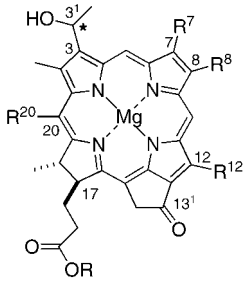
KEYWORDS:

chlorosome · oligomers · photosynthesis · self-assembly · supramolecular chemistry

Introduction

Green photosynthetic bacteria have unique light-harvesting antenna systems called chlorosomes, which are ellipsoid bodies about 100 – 300 nm in length.[1] In a chlorosome, a large number of antenna pigments self-aggregate to form rodlike oligomers surrounded with a monolayer of lipids and some proteins. Absorbed light energy at the antenna pigments is funneled into the pigment – protein complex called the baseplate, which is part of the chlorosomal envelope facing the cytoplasmic membrane.[2, 3] Finally, the excitation energy transfers to a reaction center through intermembraneous pigment – protein complexes.[4] The chlorophyllous pigments contained in chlorosomal oligomers are bacteriochlorophylls (BChls) *c*, *d*, and *e*, which are differentiated by the substituents at the 7- and 20-positions of a chlorin ring (Scheme 1).[5] Most green bacteria have one of the chlorosomal BChl components, and some strains have both BChl *c* and BChl *d* in a cell.[6]

The chlorosomal BChls are easily extracted from cultured cells and the isolated pigments self-aggregate to form chlorosome-like oligomers in nonpolar organic solvents or in aqueous media.[7] The in vitro studies support the theory that no proteins participate in making chlorosomal aggregates, that is, the pigment – pigment interactions alone determine the supramolecular structure of the light-harvesting antenna. The infrared absorption and resonance Raman measurements showed that there is C=O···H–O···Mg bonding between the neighboring BChls in the aggregates.[8, 9] The strong excitonic interaction among BChl molecules results in a significantly red-shifted Qy absorption band and intense circular dichroism (CD) signals. The linear dichroism spectra of isolated chlorosomes showed that the Qy transition moments aligned parallel to the long axis of the chlorosome.[10] These spectroscopic studies support the suggestion that chlorosomal BChls form highly ordered *J*-aggregates.



BChl *c*: R^7 = Me, R^{20} = Me
BChl *d*: R^7 = Me, R^{20} = H
BChl *e*: R^7 = CHO, R^{20} = Me

R^8 = Et, *n*Pr, *i*Bu, *neo*Pentyl
R^{12} = Me, Et
R = stearyl, farnesyl, etc.

Scheme 1. *Structure of bacteriochlorophylls* c, d, *and* e. *The asterisk indicates the* 3^1*-position, which can be either* R *or* S *configuration.*

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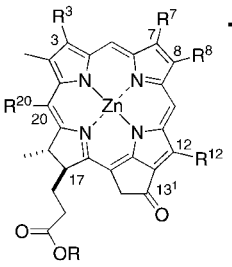
Usually chlorosomal BChls found in green bacteria are a mixture of their homologues (for example, with different substituents at R^8, R^{12}, and R in Scheme 1) and/or 3^1-epimers. Interestingly, the composition of BChl elements is dependent upon the bacterial species and the culturing conditions. For example, chlorosomes of a green filamentous non-sulfur bacterium *Chloroflexus aurantiacus* consist of 3^1-epimeric mixtures of BChl *c* and have several different alkyl chains at the 17-propionate,[11] whereas chlorosomes of green sulfur bacteria consist of mixtures of BChl *c* homologues with different alkyl substituents at the 8- and 12-positions of the chlorin ring.[12, 13] Furthermore, the ratio of the BChl *c* homologues changes when the cell is cultured under different illumination intensity.[14]



	R^3	R^7	R^8	R^{12}	R^{20}	R
1	$CH(CH_3)OH$	Me	Et	Me	H	Me
2a	$CH(CH_3)OH$	Me	Et	Me	Me	Me
2b	$CH(CH_3)OH$	Me	Et	Et	Me	Me
2c	$CH(CH_3)OH$	Me	*n*Pr	Et	Me	Me
2d	$CH(CH_3)OH$	Me	*i*Bu	Et	Me	Me
2e	$CH(CH_3)OH$	Me	Et	Me	Me	stearyl
3	CH_2OH	Me	Et	Me	H	Me
4	$CH{=}CH_2$	CH_2OH	Et	Me	H	phytyl
5	$CH{=}CH_2$	CH_2OH	Et	Me	H	Me

Scheme 2. *Structure of synthetic zinc chlorins* ***1*** *–* ***5****.*

In vitro studies of BChl aggregates have already shown that the absolute configuration at the 3^1-position affected their supramolecular structure. Epimerically pure BChl *c* and zinc analogues were self-assembled in nonpolar organic solvents. The oligomers of the 3^1R and 3^1S epimers gave different absorption spectra.[7b,e, 15, 16a,b] Moreover, the alkyl substituents of chlorosomal BChls also affect the supramolecular structure of the aggregates.[16c,d] The Qy peak of self-aggregates prepared from purified homologues of BChl *c* or *d* in hexane was gradually red-shifted as the 8^2- and/or 12^1-positions were methylated.[7c,d] The aqueous aggregates were formed by the purified homologues of BChl *c* in the presence of galactolipids.[7i] The Qy absorption bands of these aggregates in the aqueous lipid solution were also dependent upon the homologue species. Similar behavior was observed in a natural system; further methylation at the 8^2-position of BChl *d* moved the Qy peak of living cells to a longer wavelength.[6b]

The structural feature of chlorosomal aggregates is roughly understood as a result of numerous studies on in vivo and in vitro aggregates. However, the precise supramolecular structure of the unique antenna system has still not been identified. Recently, much attention has been devoted to the distribution of the epimers and/or homologues of BChls within an in vivo chlorosomal aggregate.[7d] The acid treatment of these aggregates suggested that, in *Chlorobium tepidum*, the BChl *c* homologues possessing different substituents at the 8- and 12-positions are uniformly distributed in a chlorosomal aggregate.[17] In *Chlorobium limicola* grown under a limited-sulfide continuously cultured condition, BChl *c* and *d* molecules are spatially separated and give two different oligomers of each component.[18] However, only a few in vitro models have been prepared with a mixture of structurally different chlorosomal BChls.[7b,d, 16a]

In the present study, self-assembled oligomers were prepared with a mixture of structurally different zinc analogues of BChls *c* and *d*. Mixed or pure synthetic zinc chlorins **1** – **4** (Scheme 2) self-aggregated in an aqueous medium with α-lecithin. The self-aggregates of the zinc analogues are good models for the chlorosomal aggregates, because the artificial aggregates reproduce the supramolecular structure and antenna function of natural chlorosome.[15, 16, 19–21] In addition, the zinc analogues are readily available and relatively stable compared to the natural BChls (magnesium chlorins). Spectroscopic measurements were carried out to examine the structure of the pure and mixed aggregates. The model studies showed that epimers and/or homologues of zinc 3^1-hydroxychlorins spontaneously coaggregate to make scrambled oligomers of structurally different pigments, while, when zinc 3^1- and 7^1-hydroxychlorins were mixed and slowly aggregated, the two pigments individually aggregated to form different oligomeric components. The in vitro model experiments would provide information on aspects of the supramolecular structure and biological formation of chlorosomal oligomers that consist of several BChl components.

Results

Self-aggregation of epimerically pure zinc chlorins in an aqueous lecithin solution

In methanol, synthetic zinc chlorins **1** – **4** showed a Qy absorption band at 652 or 662 nm (Figure 1a and Table 1), which is characteristic for the monomeric form of these compounds. Table 1 shows that neither the stereochemistry at the 3^1-position nor the alkyl substituents at the 8-, 12-, and 17^4-positions affected the optical properties of monomeric zinc chlorins. However, methylation at the 20-position of these chlorins moved the Qy absorption maxima into the red-shifted region, as shown

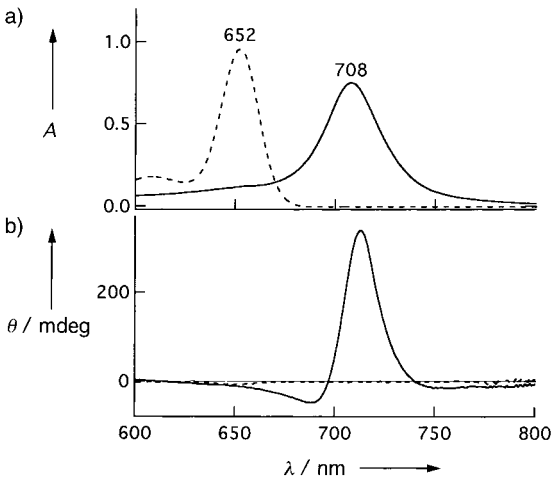


Figure 1. *a) Visible absorption spectra and b) CD spectra of zinc chlorin (3¹R)-**1** in methanol (dashed line) and in an aqueous α-lecithin solution (solid line).*

Table 1. Qy absorption maxima (λ_{max}) and red-shifts caused by oligomerization ($\Delta\tilde{\nu}$) for pure synthetic zinc chlorins and natural BChls c *and* d.

Compound	λ_{max} [nm] monomer[a]	oligomer[b]	$\Delta\tilde{\nu}$ [cm^{-1}]
(3^1R)-**1**	652	708	1210
(3^1S)-**1**	652	720	1450
(3^1R)-**2a**	662	732	1440
(3^1S)-**2a**	662	713	1080
(3^1R)-**2b**	662	724	1290
(3^1R)-**2c**	662	726	1330
(3^1S)-**2d**	662	734	1480
(3^1R)-**2e**	662	720	1220
3	652	746	1930
4	653	714	1310
BChl *c*	669	747[c]	1560
BChl *d*	665	733[c]	1400

[a] In methanol. [b] In an aqueous α-lecithin solution prepared in a cellulose tube, unless otherwise stated. [c] In a natural chlorosome.[2c]

by the different λ_{max} values for the monomers of BChls *c* and *d*. The shift is ascribed to distortion of the chlorin π-plane because of the bulkiness of the 20-methyl group.[16a, 21]

When zinc chlorin (3^1R)-**1** and α-lecithin were mixed in methanol in a cellulose tube ([(3^1R)-**1**] $\approx$ 20 μM and [α-lecithin] $\approx$ 32 μM) and dialyzed in water, the blue methanol solution gradually turned into a green aqueous solution. Although this color change was complete within 30 minutes, the dialysis was continued overnight to minimize the amount of methanol in the tube. During dialysis, the inner volume of the cellulose tube increased, and the zinc chlorin components partly leaked outside the tube or were adsorbed onto the surface of the cellulose. Thus, the final concentration of zinc chlorin within the tube was about 10 μM. The aqueous solution of (3^1R)-**1** showed a Qy absorption band at 708 nm (Figure 1a) and relatively large CD peaks (Figure 1b). These results indicated that zinc chlorin (3^1R)-**1** self-assembled to form *J*-aggregates in the aqueous α-lecithin solution, in the same way as BChls *c* and *d* do in natural chlorosomes.

Although similar observations were found in other synthetic zinc chlorins **1**–**4**, the values $\Delta\tilde{\nu}$ for the shifts caused by oligomerization were dependent on the stereochemistry at the 3^1-position and the alkyl substituents of the zinc chlorins (Table 1). For zinc chlorin **1** (R^{20} = H), the $\Delta\tilde{\nu}$ value of 3^1S epimer (3^1S)-**1** was larger than that of 3^1R epimer (3^1R)-**1**. However, for zinc chlorin **2a** (R^{20} = Me), the 3^1R epimer (3^1R)-**2a** showed a more red-shifted Qy band than the 3^1S epimer (3^1S)-**2a**. Zinc chlorins with the 3^1R configuration which possessed different alkyl substituents at the 8- and 12-positions gave different absorption spectra, with Qy absorption maxima at 732, 724, and 726 nm for (3^1R)-**2a** (8-ethyl-12-methyl), (3^1R)-**2b** (8-ethyl-12-ethyl), and (3^1R)-**2c** (8-propyl-12-ethyl), respectively. The homologues with 3^1S stereochemistry also showed different Qy absorption maxima, with values of 713 and 734 nm for (3^1S)-**2a** (8-ethyl-12-methyl) and (3^1S)-**2d** (8-*iso*-butyl-12-ethyl), respectively.

In addition, the alkyl group on the 17-propionate group also changed the absorption bands of the aggregated zinc chlorins: λ_{max} = 732 nm for (3^1R)-**2a** (R = Me) and 720 nm for (3^1R)-**2e** (R = stearyl). Zinc 3-hydroxymethylchlorin **3** showed a large shift in the Qy absorption band ($\Delta\tilde{\nu}$ = 1930 cm^{-1}) compared to those for the zinc 3-(1-hydroxyethyl)chlorins **1** and **2** ($\Delta\tilde{\nu}$ = 1080–1480 cm^{-1}). Zinc 7-hydroxymethylchlorin **4** gave an absorption maximum at 714 nm and the value of $\Delta\tilde{\nu}$ was smaller than that of the aggregated zinc 3-hydroxymethylchlorin **3**. The absorption spectra of aggregated **3** and **4** in aqueous media were similar to those previously reported for a hexane solution.[9, 20] As compound **5**, the methyl ester of zinc 7-hydroxymethylchlorin, only partly self-aggregated and remained mainly monomeric in an aqueous α-lecithin solution, the equivalent zinc chlorin with a long esterified alkyl group, compound **4** (R = phytyl; $C_{20}H_{39}$) was used in the following experiments.

When the aggregates of zinc chlorins were prepared in the absence of α-lecithin, the precipitates of the chlorins were found within the cellulose tube. Thus, the additive α-lecithin surrounded the zinc chlorin aggregates to stabilize the aqueous oligomers formed.

Self-aggregates prepared with a mixture of epimers and/or homologues of zinc chlorins

Self-assembled oligomers were prepared with a mixture of monomeric zinc chlorin components. Two structurally different zinc chlorins were mixed with α-lecithin in methanol and the mixed solution was dialyzed in water in a cellulose tube. Although the zinc chlorin component was partly lost during dialysis as noted above, HPLC analyses showed the ratio of the two components was unchanged. Self-aggregates of pure (3^1R)-**2a** and (3^1S)-**2a** showed visible absorption maxima at 732 and 713 nm, respectively (Figure 2a, top). The monomers were responsible for the peak which appears at approximately 670 nm in the spectra. The mixed aggregates of (3^1R)-**2a** and (3^1S)-**2a** showed an absorption maximum at 710 nm. The second derivative of the spectrum of the mixed aggregates showed only the broad absorption band around 720 nm (Figure 2a, bottom). Thus, the absorption spectrum of the mixed aggregates (3^1R)-**2a**+(3^1S)-**2a** could not be reproduced by linear combination of the two basic spectra of the pure aggregates, (3^1R)-**2a** and (3^1S)-**2a**. The result indicated that the aqueous aggregates prepared with an equimolar mixture of the 3^1R and 3^1S epimers were not composed of separated pure oligomers but of scrambled oligomers of the two components; that is, the 3^1R and 3^1S epimers coaggregated in an aqueous α-lecithin solution. The absorption spectrum of the mixed aggregates of (3^1R)-**2a** and (3^1S)-**2a** was quite similar to that of the pure (3^1S)-**2a** oligomer, which suggests that the 3^1S epimer might control the arrangement of pigments in the mixed aggregates.

Similarly, self-assembled oligomers were prepared with a mixture of other zinc chlorin homologues. Figure 2b (top) shows the visible absorption spectra of the aggregates of zinc chlorin homologues (3^1R)-**1** and (3^1R)-**2c**, which have different substituents at the 8-, 12-, and 20-positions; triple methylation at the 8^2-, 12^1-, and 20-positions of (3^1R)-**1** gives (3^1R)-**2c**. The individual (3^1R)-**1** and (3^1R)-**2c** aggregates gave absorption maxima at 708 and 726 nm, respectively, while a 1:1 mixture of (3^1R)-**1** and (3^1R)-

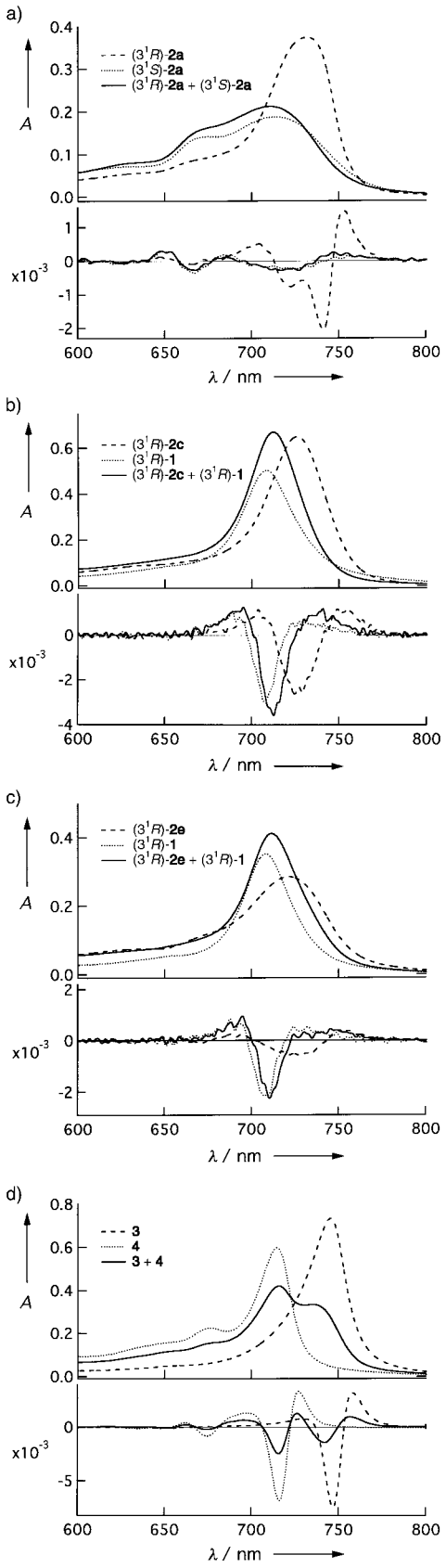


Figure 2. *Visible absorption spectra (top) and the second derivatives of those spectra (bottom) for aqueous aggregates of a pure and equimolar mixture of zinc chlorins prepared by dialysis in a cellulose tube. a) (3¹R)-**2a** and (3¹S)-**2a**; b) (3¹R)-**2c** and (3¹R)-**1**; c) (3¹R)-**2e** and (3¹R)-**1**; d) **3** and **4**.*

2c gave an absorption maximum of 712 nm. The second derivative of absorption spectrum of the mixed aggregates showed the presence of only a single Qy band in the solution, and the spectrum could not be reproduced by linear combination of the two basic spectra. Thus, two homologues of zinc chlorin, (3¹*R*)-**1** and (3¹*R*)-**2c**, coaggregate to produce scrambled oligomers in vitro. A similar result was obtained for mixed zinc chlorin aggregates with different esterified alkyl chains and substituents at the 20-position, formed from (3¹*R*)-**1** and (3¹*R*)-**2e** (Figure 2c).

When zinc 3¹-hydroxychlorin **3** and zinc 7¹-hydroxychlorin **4** were mixed and self-assembled in a cellulose tube, the two distinct components individually aggregated to give two oligomeric components. The pure zinc chlorin aggregates of **3** and **4** showed visible absorption maxima at 745 and 716 nm, respectively (Figure 2d, top). The aggregates prepared with the equimolar mixture of **3** and **4** also gave two spectral components with absorption maxima at 742 and 716 nm. The CD spectral measurements (Figure 3) indicated that the pure **3**

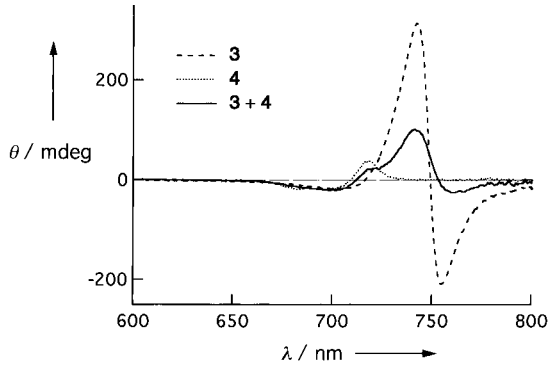


Figure 3. *CD spectra of aqueous aggregates of **3**, **4**, and an equimolar mixture of the two prepared by dialysis in a cellulose tube.*

aggregates gave a large negative band at 754 nm and a large positive band at 742 nm, while pure aggregates of **4** showed a positive band at 718 nm and a broad negative band around 690 nm. The CD spectrum of the mixed aggregates provided two positive bands at 742 and 720 nm and two negative bands at 760 and 690 nm. Thus, the absorption and CD spectra of the mixed aggregates mainly consisted of those of pure **3** and **4** aggregates. The absorption spectra of mixed aggregates prepared with several **3**:**4** ratios were systematically shifted (data not shown). The band at around 740 nm was reduced concomitant with an increase in the shorter wavelength band at around 720 nm as the **3**:**4** ratio was decreased. The mixed aggregates of **3** and **4** were disaggregated when methanol was added to the aqueous solution; Figure 4 shows the absorption spectra measured during this disaggregation. The absorption at 743 nm due to aggregated **3** changed to the absorption for the monomer before the absorption for aggregated **4** disappeared. A small part of the compounds might be coaggregated in the equimolar mixture because the absorption spectrum of the mixture showed a larger amplitude at 714 nm than that at 746 nm, unlike the spectrum expected from the pure aggregates

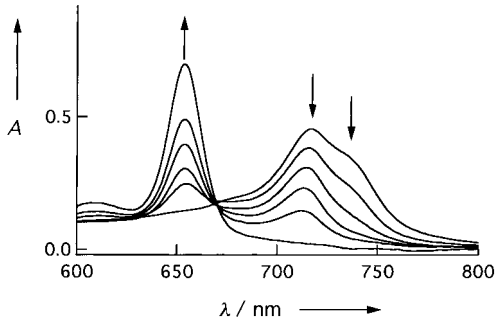


Figure 4. *Visible absorption spectra during the disaggregation of equimolar mixed aggregates of **3** and **4** by addition of methanol. The concentrations of the added methanol were 0, 50, 58, 62, 66, and 72% (v/v).*

(Figure 2d, top). However, the spectral features showed that the zinc chlorins **3** and **4** mostly individually self-assembled to form structurally distinct homooligomers of **3** and **4** in the cellulose tube. The fluorescence emission spectrum of the aggregates from an equimolar mixture of **3** and **4** (Figure 5) could also be represented by the combination of the spectra for pure **3** and **4** aggregates. This indicated little energy exchange between the two oligomers of **3** and **4** and suggested that the aggregated **3** and **4** were spatially separated in the aqueous medium.

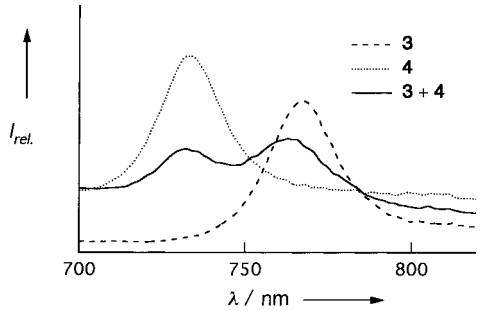


Figure 5. *Corrected fluorescence emission spectra of aggregated **3**, **4**, and their equimolar mixture prepared by dialysis in a cellulose tube. All spectra were measured by excitation with light at 450 nm.*

Zinc chlorins **3** and **4** could give scrambled oligomers by self-organization of the mixture within a short period. When a methanol solution of the mixed zinc chlorins and α-lecithin was dropped into water and shaken vigorously, the blue methanol solution of monomeric zinc chlorins immediately turned into a green aqueous solution.[19c] In this case the self-assembly of zinc chlorins was apparently achieved more rapidly than that by the dialysis procedure described above. The obtained aqueous aggregate was also stable and no precipitates were found in the solution. Figure 6a shows the visible absorption spectra of the pure aggregates of **3** and **4** ($\lambda_{max} = 732$[19c] and 709 nm, respectively) prepared by rapid dilution of the methanol solution with excess water. The aggregates had slightly blue-shifted absorption bands compared to those of the aggregates prepared slowly in a cellulose tube (Figure 2d upper). The mixed aggregates gave a single Qy absorption maximum at 705 nm (Figure 6a and b). The CD spectra of the pure **3** and **4** aggregates

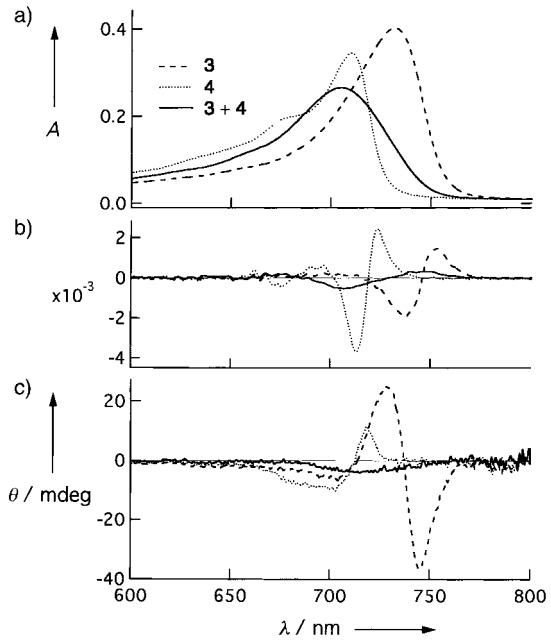


Figure 6. *a) Visible absorption spectra, b) the second derivatives of the visible spectra, and c) CD spectra of aqueous aggregates of **3**, **4**, and an equimolar mixture of **3**+**4** prepared by the rapid dilution method.*

gave relatively large signals around the Qy absorption region (Figure 6c). The mixed aggregates, however, showed a weak and broad negative band at around 715 nm. Thus, the visible absorption and CD spectra of the rapidly prepared mixed aggregates could not be reproduced by combination of the two basic spectra of the pure aggregates. In contrast to the mixed aggregates slowly prepared by dialysis in a cellulose tube, zinc chlorins **3** and **4** coaggregated to form scrambled oligomers when the aggregation was achieved within a short time.

It should be noted that the spectral shapes of the mixed aggregates described above ((3^1*R*)-**2a**+(3^1*S*)-**2a**, (3^1*R*)-**2c**+(3^1*R*)-**1**, (3^1*R*)-**2e**+(3^1*R*)-**1**, and **3**+**4**) were independent of the concentrations used (approximately 5–40 μM). Thus, any significant concentration effect was not observed under the present experimental conditions.

Discussion

Self-assembly of pure zinc chlorins

The present study showed that zinc complexes of 3^1-hydroxy-13^1-oxochlorins **1**–**4** self-organized in an aqueous lipid solution in the same way as BChls *c* and *d* do in chlorosomes. The red-shifted absorption and intense CD bands indicated that zinc chlorin molecules formed *J*-aggregates and were strongly exciton-coupled in an aqueous α-lecithin solution. As can be seen from absorption spectra, zinc chlorin molecules remained in monomeric form in methanol. When the solvating medium was changed from methanol to water, the synthetic pigments self-assembled in the presence of α-lecithin. If the solvating methanol was gradually removed by dialysis, the zinc chlorin oligomers were slowly produced within a cellulose tube. On the

other hand, when a methanol solution of zinc chlorin was rapidly diluted with a large volume of water, the oligomers immediately formed in the aqueous medium. With the dialysis method, the speed of oligomerization can be controlled in vitro.

The supramolecular structure of the chlorosome-like oligomers was dependent upon the speed of oligomerization.[7f] In zinc chlorin **3**, the full width at half maximum (fwhm) of the Qy absorption band for the slowly prepared oligomer (at 520 cm^{-1}; Figure 2d, top) was narrower than that for the rapidly prepared oligomer (at 850 cm^{-1}; Figure 6a). In addition, the CD spectral intensity of the slowly prepared aggregated **3** (Figure 3) was about 10 times larger than that of the rapidly prepared aggregates (Figure 6c). These spectral results demonstrate that the slowly prepared oligomer has a highly ordered arrangement with a long-range chiral feature.[22] In natural chlorosomes, BChl oligomers are gradually produced concomitant with the cell growth.[23] Consequently, the dialysis procedure would be better for making the in vitro model of chlorosomal aggregates.

The spectroscopic measurement of the slowly aggregated zinc chlorins showed that the arrangement of the pigments in the self-assembled oligomers depended on both the stereochemistry at the 3^1-position and the alkyl substituents at the 8-, 12-, 17^4-, and 20-positions. The supramolecular architecture of all the chlorosome-like oligomers would be mainly determined by the same intermolecular linkages, 13-C=O···H–O···Mg or Zn. Spectral diversity of the aggregated zinc chlorin components might be caused by the steric factor of the alkyl groups. In the computer-modeled rodlike structure of the BChl oligomers, the 8- and 12-alkyl substituents are located inside the rod.[24, 25] As the 8- and 12-substituents of each BChl would be close to those of the adjacent BChl, the bulkiness of these substituents would affect the stacking of BChl molecules. Molecular modeling studies showed that the curvature in the semi-rodlike aggregate of dodecameric (3^1R)-**2c** (R^8 = *n*Pr, R^{12} = Et) was smaller than that in the (3^1R)-**1** (R^8 = Et, R^{12} = Me) aggregate: The radii of curvature were 43 Å and 25 Å, respectively. The arrangement of the 3^1-methyl group is also important in stacking the zinc chlorins. The energy-minimized structure of the (3^1R)-**2a** dodecamer gave a well-ordered supramolecular structure compared to that of the (3^1S)-**2a** dodecamer. The estimated diastereomeric difference was consistent with the previous results for (3^1R)-**1** and (3^1S)-**1**.[25] Furthermore, the large esterified alkyl chain also changed the absorption spectra. Molecular modeling studies in aggregated (3^1R)-**2e** (R = strearyl) exhibited that the esterified chains are located outside of the rod. It is suggested that the long alkyl chain at the 17^4-position interacts with hydrophobic region of the surrounding α-lecithin layer, which would change the size and/or the organization of the aqueous oligomers.

Self-assembly of mixed zinc chlorins

Zinc 3^1-hydroxy-13^1-oxochlorins with different alkyl substituents and/or stereochemistry at the 3^1-position preferentially coaggregated to give scrambled oligomers, when the mixture was self-organized in an aqueous solution. The chlorosome-like oligomer is formed through intermolecular hydrogen bonding between the 3^1-hydroxy and 13-keto groups, coordination between the oxygen of the hydroxy group and the central metal atom, $\pi-\pi$ stacking of the chlorin rings, and van der Waals interactions. The model study using the synthetic analogues of BChl *c* showed that the hydrogen bonding and coordination are especially important for formation of the oligomers.[9] As the differences in the absolute configuration at the 3^1-position and the alkyl substituents at the 8- and 12-positions would provide a small contribution to the intermolecular linkage, the epimers and/or homologues of zinc chlorin molecules can coaggregate to give the scrambled oligomers.[16a, 26]

On the other hand, zinc 3^1-hydroxychlorin **3** and zinc 7^1-hydroxychlorin **4** would individually self-organize to give two distinct supramolecular units when the mixed solution was slowly aggregated. The oligomerization of zinc 7^1-hydroxychlorins was partly dependent upon the esterified alkyl chain at the 17^4-position: Zinc chlorin **4** (R = phytyl) afforded larger aggregative and smaller monomeric visible absorption peaks and self-aggregated more than **5** (R = methyl) in an aqueous solution. Both the aggregates of **4** and **5**, however, gave similar absorption peaks around 715 nm. Visible spectra of mixed pigments of **3** and **5** showed their individual self-aggregation and also the presence of other species including the monomer (data not shown); these spectra were more complex than those of **3** and **4**. Figure 2c showed that the zinc chlorins with different esterified alkyl chains at the 17^4-position, (3^1R)-**1** (R = methyl) and (3^1R)-**2e** (R = stearyl), did coaggregate. These phenomena suggested that a large lipophilic side chain at the 17^4-position might improve the stability of aggregates formed, while the difference in the esterified alkyl chain would not be related largely to the individual self-aggregates. The oligomerization of the chlorophyllous pigments would be mainly determined by C=O···H–O···M bonding (where M is the metal). Consequently, the difference in the position of the hydroxy group would be quite important in the organization of the mixed pigments of **3** and **4**. However, zinc chlorins **3** and **4** coaggregated when their mixture was rapidly dispersed into an aqueous medium. These results show that both the structure of pigments and the speed of oligomerization determine whether structurally different chlorophyllous components are scrambled or not. In the build-up of the self-assembled aggregate, a monomer (or an oligomer) would associate with another zinc chlorin component. If one species is attached to the same component, the formed aggregate would be stable. If one component was attached to the structurally different zinc chlorin, however, the formed aggregate would be unstable and the misfitting pigments would be removed. In this way the chlorophyllous pigments correctly organized to make their pure oligomers when zinc 3^1-hydroxychlorin **3** and zinc 7^1-hydroxychlorin **4** were slowly self-assembled. However, if organization of the aggregates is too fast to undo the "misassociation" or the structural difference between the two chlorophyllous components is small, the two distinct zinc chlorins would coaggregate to form a scrambled oligomer.

The Qy absorption bands of the scrambled aggregates prepared with a mixture of zinc chlorin homologues were close

to those of shorter wavelength components of the pure oligomer (Figure 2a – c). This feature suggests that the scrambled oligomers were relatively unstable and could not grow into large oligomers.

BChl aggregates in natural chlorosomes

In a native chlorosome, BChl components self-assemble to make oligomers within a core of the chlorosomal envelope. A question is raised about the supramolecular architecture of chlorosomal oligomers consisting of several epimers and homologues of BChls. The present in vitro model studies gave the following aspects on the self-assembly of metallo-3^1-hydroxy-13^1-oxochlorins connected with C=O···H–O···M bonding: 1) Both the 3^1-stereochemistry and the alkyl substituents affect the supramolecular structure of the oligomer; and 2) zinc 3^1-hydroxy-13^1-oxochlorins with different 3^1-stereochemistry and alkyl substituents easily coaggregate to form the scrambled oligomers. If the chlorosomal aggregates were formed in the same manner as in the present experiments, that is, from a pigment pool consisting of a mixture of several chlorophyllous epimers and homologues, then the native BChl *c* and *d* aggregates were made by scrambling the mixture. Steensgaard et al. reported that the pigment analysis of the acid-treated chlorosomes of *Chlorobium tepidum* suggested that BChl *c* homologues with different substituents at the 8- and 12-positions are uniformly distributed within a chlorosome.[17] This proposal is comparable to the findings in our in vitro experiments. On the other hand, in a chlorosome from a strain of green bacterium, BChl *c* (R^{20} = Me) and BChl *d* (R^{20} = H) molecules were spatially separated within the chlorosomal envelope.[18] The present model study shows, however, that the zinc 20-methylchlorin (3^1*R*)-**2c** (R^{20} = Me) and 20-unsubstituted zinc chlorin (3^1*R*)-**1** (R^{20} = H) coaggregated to form the scrambled aggregates (Figure 2b). The result suggests that the separated aggregates of BChl *c* and *d* found in the natural chlorosome might not be organized from a mixture of BChl *c* and *d* monomers, but are more probably organized stepwise within a chlorosome. First either BChl *c* or *d* self-assembles from the pure pigment pool and then another BChl self-organizes within the chlorosome.

The present study demonstrated that the method of preparation of oligomers determined whether two types of chlorophyllous pigments, zinc 3^1-hydroxychlorins and zinc 7^1-hydroxychlorins, individually aggregated or coaggregated. However, naturally occurring chlorosomal BChls differ only in their alkyl substituents at the 8-, 12-, 17^4-, and 20-positions and in the 3^1-stereochemistry. The main intermolecular linkage, C=O···H–O···Mg, would be a common feature in native chlorosomal aggregates consisting of several BChl homologues and epimers. Thus, the small structural differences found in chlorosomal BChls would provide a small contribution to the oligomerization step.[26] Even if natural chlorosomal aggregates were slowly formed, structurally different BChls would coaggregate to produce the scrambled oligomers composed of a mixture of epimers and homologues of antenna pigments as occurred in the present model experiments.

Experimental Section

Visible absorption, fluorescence emission, and circular dichroism spectra were measured with a Hitachi U-3500 spectrophotometer, a Hitachi F-4500 spectrophotometer, and a Jasco J-720W spectropolarimeter, respectively. ^{1}H NMR spectra were obtained with a Bruker AC-300 spectrometer. Molecular modeling calculations were performed as reported in ref. [25], with PM3 and MM+ in the program package Hyperchem version 5.1 (Hypercube Co.).

Zinc chlorins **1**,[16a] **2a**,[16a] **3**,[9] **4**,[20] and **5**[20] were prepared according to the reported procedures. α-Lecithin (L-α-phosphatidylcholine, from frozen egg yolk, Sigma, USA) was purified by silica gel column chromatography (eluted with methanol/dichloromethane (3:2)) before use.

Zinc methyl 8-ethyl-12-ethyl-, 8-propyl-12-ethyl-, and 8-*iso*-butyl-12-ethylbacteriopheophorbides *c* (2b – d): 8-Ethyl-12-ethyl-, 8-propyl-12-ethyl-, and 8-*iso*-butyl-12-ethyl-BChls *c* were obtained as a mixture from cultured *Chlorobium tepidum* cells.[27] The isolated BChls *c* were transferred to zinc chlorins **2b** – **d** according to the reported procedure.[16a] The epimerically pure homologues, **2b** – **d**, were separated from the mixture by a single reversed-phase HPLC run.[28a] Stereochemistry of the 3^1-position was determined by comparison with the reported ratio for each BChl *c* homologue.[12a, 29]

Compound (3^1*R*)-2b: HPLC:[28a] Retention time = 12 min; ^{1}H NMR (CD_3OD): δ = 9.73, 9.49 (each 1H, s; 5- and 10-H), 6.32 (1H, q, *J* = 7 Hz; 3^1-H), 5.10 (2H, s; 13^2-H_2), 4.55 (1H, q, *J* = 7 Hz; 18-H), 4.03 – 4.09 (1H, m; 17-H), 4.03 (2H, q, *J* = 7 Hz; 12-CH_2), 3.75, 3.46, 3.33, 3.24 (each 3H, s; 2-, 7-, and 20-CH_3, and $COOCH_3$), 3.73 (2H, q, *J* = 7 Hz; 8-CH_2), 2.30 – 2.56, 2.03 – 2.22 (each 2H, m; 17-CH_2CH_2), 2.07 (3H, d, *J* = 7 Hz; 3^1-CH_3), 1.88 (3H, t, *J* = 7 Hz; 12^1-CH_3) 1.69 (3H, t, *J* = 7 Hz; 8^1-CH_3), 1.46 (3H, d, *J* = 7 Hz; 18-CH_3); UV/Vis (CH_3OH): λ_{max} (relative intensity) = 432 (1.00), 626 (0.18), 662 (0.80) nm; MS (FAB): *m/z* found: 656; calcd for $C_{36}H_{40}N_4O_4{}^{64}Zn$: 656 [$M^+$].

Compound (3^1*R*)-2c: HPLC:[28a] Retention time = 14 min; ^{1}H NMR (CD_3OD): δ = 9.75, 9.50 (each 1H, s; 5- and 10-H), 6.33 (1H, q, *J* = 7 Hz; 3^1-H), 5.12 (2H, s; 13^2-H_2), 4.60 (1H, q, *J* = 7 Hz; 18-H), 4.07 – 4.13 (1H, m; 17-H), 4.03 (2H, q, *J* = 7 Hz; 12-CH_2), 3.80, 3.48, 3.36, 3.24 (each 3H, s; 2-, 7-, and 20-CH_3, and $COOCH_3$), 3.71 (2H, t, *J* = 7 Hz; 8-CH_2), 2.35 – 2.59, 2.02 – 2.27 (2H+4H, m; 8^1-CH_2 and 17-CH_2CH_2), 2.08 (3H, d, *J* = 7 Hz; 3^1-CH_3), 1.88 (3H, t, *J* = 7 Hz; 12^1-CH_3), 1.50 (3H, d, *J* = 7 Hz; 18-CH_3), 1.23 (3H, t, *J* = 7 Hz; 8^2-CH_3); UV/Vis (CH_3OH): λ_{max} (relative intensity) = 432 (1.00), 626 (0.18), 662 (0.79) nm; MS (FAB): *m/z* found: 670; calcd for $C_{37}H_{42}N_4O_4{}^{64}Zn$: 670 [$M^+$].

Compound (3^1*S*)-2d: HPLC:[28a] Retention time = 18 min; ^{1}H NMR (CD_3OD): δ = 9.74, 9.49 (each 1H, s; 5- and 10-H), 6.35 (1H, q, *J* = 7 Hz; 3^1-H), 5.14 (2H, s; 13^2-H_2), 4.63 (1H, q, *J* = 7 Hz; 18-H), 4.10 – 4.17 (1H, m; 17-H), 4.04 (2H, q, *J* = 7 Hz; 12-CH_2), 3.82, 3.50, 3.37, 3.25 (each 3H, s; 2-, 7-, and 20-CH_3, and $COOCH_3$), 3.71 (2H, t, *J* = 7 Hz; 8-CH_2), 2.38 – 2.62, 2.14 – 2.34 (3H+2H, m; 8^1-CH and 17-CH_2CH_2), 2.11 (3H, d, *J* = 7 Hz; 3^1-CH_3), 1.89 (3H, t, *J* = 7 Hz; 12^1-CH_3), 1.51 (3H, d, *J* = 7 Hz; 18-CH_3), 1.25 (6H, t, *J* = 7 Hz; 8^2-$(CH_3)_2$); UV/Vis (CH_3OH): λ_{max} (relative intensity) = 432 (1.00), 626 (0.18), 662 (0.79) nm; MS (FAB): *m/z* found: 684; calcd for $C_{38}H_{44}N_4O_4{}^{64}Zn$: 684 [$M^+$].

Zinc stearyl 3^1-*R*-8-ethyl-12-methyl-bacteriopheophorbide *c* ((3^1*R*)-2e): Pheophytization (removal of magnesium) and zinc metallation of BChl *c* extracted from *Chloroflexus aurantiacus*[16a] gave a mixture of zinc bacteriopheophytin *c* with different esterified alkyl chains: stearyl, cetyl, etc.[11] The stearyl ester **2e** was isolated as a major component by reversed-phase HPLC separation[28b] (retention time = 16 min) and the 3^1*R* epimer (3^1*R*)-**2e** was isolated by a successive normal-phase HPLC run[28c] (retention time = 36 min).

^{1}H NMR (C_5D_5N (0.2%) in $CDCl_3$): $\delta = 9.74$, 9.55 (each 1H, s; 5- and 10-H), 6.45 (1H, q, $J = 7$ Hz; 3^1-H), 5.21 (2H, s; 13^2-H_2), 4.53 (1H, q, $J = 7$ Hz; 18-H), 4.03 – 4.10 (1H, m; 17-H), 3.92 (2H, t, $J = 7$ Hz; $COOCH_2$), 3.82, 3.70, 3.42, 3.28 (each 3H, s; 2-, 7-, 12-, and 20-CH_3), 3.77 (2H, t, $J = 7$ Hz; 8-CH_2), 2.26 – 2.44, 1.87 – 2.20 (each 2H, m; 17-CH_2CH_2), 2.13 (3H, d, $J = 7$ Hz; 3^1-CH_3), 1.71 (3H, t, $J = 7$ Hz; 8^1-CH_3), 1.37 (3H, d, $J = 7$ Hz; 18-CH_3), 1.15 – 1.33 (32H, m; $COOCH_2(CH_2)_{16}$), 0.87 (3H, t, $J = 7$ Hz; $COOCH_2(CH_2)_{16}CH_3$); UV/Vis (CH_3OH): λ_{max} (relative intensity) = 433 (1.00), 626 (0.17), 662 (0.75) nm; MS (FAB): *m/z* found: 880; calcd for $C_{52}H_{72}N_4O_4{}^{64}Zn$: 880 [$M^+$].

Dialysis method of aggregate preparation (slow preparation of aggregates): The appropriate zinc chlorin (20 μM) and α-lecithin (32 μM) were mixed in methanol (4 mL). The mixed solution was put into a cellulose tube (Viskase Sales Co.) and dialyzed in water (200 mL) at room temperature. After dialyzing overnight, the aqueous zinc chlorin aggregates were obtained.

Dilution method of aggregate preparation (rapid preparation of aggregates): The appropriate zinc chlorin (10 μM) and α-lecithin (16 μM) were mixed in methanol (40 μL). The mixed solution was dropped into 4 mL of water and shaken vigorously to obtain the aqueous zinc chlorin aggregates.

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