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Morphology and spectroscopy of chlorosomes from *Chlorobium tepidum* by alcohol treatments

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Abstract

Chlorosomes from *Chlorobium tepidum* have been treated with alcohol-saturated buffers, followed by dilution to the buffers with half the saturated concentrations. Morphologic changes during this process have been statistically investigated by dynamic light scattering technique combined with electron microscopy to obtain the complete information on shape, size and distribution, while spectral properties have been studied by absorption, CD and magnetic circular dichroism. Three alcohols (1-hexanol, 1-butanol and phenol) have been found to produce nearly reversible conversion of absorption spectra despite the more than 15-times difference in the alcohol concentration. It is shown that the degree of saturation, not the alcohol concentration, is the key factor for the complete conversion of bacteriochlorophyll *c* in chlorosomes from the aggregated state to the monomeric form. We have observed substantial changes in the shape, size and distribution at each step of the treatment with 1-hexanol, indicating that the whole process is morphologically irreversible. Comparison of the morphologic changes with the corresponding spectroscopic behaviour suggests that the relative overall size rather than the shape and distribution may be a more important factor affecting the spectral properties.

Keywords: Antenna; Chlorosome; Morphology; Hydrodynamic diameter; Alcohol treatment; (Green bacterium)

1. Introduction

Chlorosomes are known as major light-harvesting apparatus in green photosynthetic bacteria [1–3] and are attached to the cytoplasmic side of the inner cell membrane [4,5]. The light energy absorbed by chlorosomes is transferred to membrane antenna pigments, via the so-called baseplate, and finally to the reaction centre located in cytoplasmic membrane [6]. The main light-absorbing pigment in chlorosomes has been determined to be bacteriochlorophyll *c* (BChl *c*, or BChl *d*, *e* in some species) [7] which has an in vivo absorption maximum at about 740 nm in the near-infrared region, corresponding to a red-shift of $\approx$ 70–80 nm from that of its monomeric form as observed in polar organic solvents. Some small amounts of other pigments, such as carotenoid and BChl *a*, have also been found in the chlorosomes as minor components.

The origin of the red-shift of in vivo BChl *c* has attracted a great deal of interest from researchers and has led to a debate on the interaction mode and the role of proteins in the organisation of BChl *c* molecules in chlorosome. This has subsequently formed one of the central topics in the current study of this area. Feick and Fuller [8] identified at least three proteins from *Chloroflexus aurantiacus* chlorosomes with molecular weight ranging from 3700 to 18000. Among these proteins, the two larger ones are located in the chlorosome envelope and are accessible to proteinase K proteolyses, while a small polypeptide is located in the chlorosome interior and associated with the antenna BChl *c*. Based on the information of amino acid sequence, Wechsler et al. [9] proposed a pigment-protein model with 7 BChl *c* molecules coordinated to glutamine and asparagine residues of the 3.7 kDa polypeptide in an α-helical conformation. They suggested that the rod elements observed by electron microscope were formed by the aggregates of these small structural units. Further evidence has been reported supporting the concept that protein(s) does play an essential role in the functional arrangement of BChl *c* in chlorosomes [10–13]. However,

Abbreviations: BChl, bacteriochlorophyll; DLS, dynamic light scattering; CD, circular dichroism; MCD, magnetic circular dichroism.

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the pigment-protein model has been challenged, since its appearance by other experimental results. It is shown that isolated BChl *c* molecules form aggregates in non-polar solvents with spectral properties closely resembling those of native chlorosomes [14–18]. This suggests that the BChls *c* in vivo are present in an aggregate form without direct involvement of protein [6,19,20]. This idea has also been supported by the result of a protein free preparation [21–23] in which the protein removed chlorosomes showed an essentially similar spectroscopic behaviour to that of conventional chlorosomes. Wullink et al. [24] has provided further evidence showing that the small polypeptide previously determined as BChl *c*-bound protein is in the chlorosome envelope.

On the other hand, the debate has brought about several new findings. One of them is the conversion, found by Brune et al. [16], of the 740 nm absorption maximum of BChl *c* in aqueous chlorosomes to a monomeric form at 670 nm by addition of small amounts of hexanol or octanol, and the restoration of the original peak after removing the alcohols by dilution with fresh buffer. This process was confirmed later to be spectroscopically reversible [25] and used as an alternative method for investigating the functional role of proteins [13]. Despite the spectral similarity, however, morphologic change of the chlorosomes and its relationship with spectroscopic behaviour during the process are not well known, although early morphologic study by electron microscopy has been proven to be indispensable to understanding of the ultrastructure of chlorosomes. In 1964, Cohen-Bazire et al. [26] reported that several strains of *Chlorobium* cells contain flat, elongated vesicles, each surrounded by a single-layered envelope of about 3 nm thickness. More detailed suprastructures for both *Chloroflexus* and *Chlorobium* chlorosomes were studied by Staehelin et al. [4,5], revealing that both systems possess the same basic structural elements and organisation, but all of the components have smaller dimensions in *Chloroflexus* compared to those in *Chlorobium*. Both types of chlorosome consist of a core filled with ≈ 10–30 rod-shaped elements which are further made up of globular subunits with a periodicity of 6 nm. The overall size of chlorosomes was found to vary in a considerably wide range with average dimensions of $100 \times 32 \times 12$ nm for *Chloroflexus* and $140 \times 45 \times 18$ nm for *Chlorobium*, respectively [27]. There are also some differences between the two bacteria in pigmentation and chemical composition of chlorosomes [28], as well as the morphology of rod element, envelope and baseplate.

In the present study, we report results on the morphologic changes of chlorosomes isolated from *Chlorobium tepidum* by alcohol treatments along with the corresponding spectral properties. *Chlorobium tepidum* is a thermophilic green sulfur bacterium and is known to have an additional trimeric BChl *a*-containing protein (named as FMO protein after Fenna, Mathews and Olson) between the baseplate of chlorosome and cytoplasmic membrane [29]. While there seem to be a relatively large number of studies on the chlorosomes from *Chloroflexus aurantiacus* and in most cases *Chlorobium limicola* has been used as a counterpart in the comparative study, only little information about the morphology and spectral characteristic has been reported for *Chlorobium tepidum*. In this work, we used negative staining electron microscopy to obtain the local information on the shape and size of chlorosomes at different stages of alcohol treatment. At the same time, dynamic light scattering (DLS) was applied to gain statistical information on the distribution of hydrodynamic diameter of particles, a measure of the size for chlorosomes. For spectroscopic measurements, magnetic circular dichroism (MCD) was used in addition to absorption and circular dichroism (CD). The intensities and signs of MCD are known to be very sensitive to changes in electronic state of chromophores [30] and these are practically useful for detecting very weak electron transitions (the chlorin Q_x band) as the transitions will be significantly enchanced by mixing with closely related states of *x*- or *y*-polarization in the case of BChl *c*.

2. Materials and methods

2.1. Chlorosome isolation

Cells of *Chlorobium tepidum* were grown as described by Wahlund et al. [31]. Chlorosomes were isolated by the method of Gerola and Olson [32] using 2 M NaSCN, 10 mM potassium phosphate (pH 7.0), and purified by ultracentrifugation on a continuous sucrose density gradient. The upper layer fraction was collected and dialysed with 20 mM potassium phosphate buffer (pH 7.2). Fresh chlorosome samples were used throughout the experiments.

2.2. Alcohol treatments

Three alcohols (1-hexanol, 1-butanol and phenol) were used in the treatment of chlorosomes. Buffer saturated with each alcohol was prepared at room temperature by adding certain amount of alcohol to 20 mM potassium phosphate solutions in a separating funnel. After shaking, the solutions were allowed to stand for at least 30 min before separation of the alcohol saturated buffers. Alcohol concentrations in the saturated solutions were estimated to be 59 mM for 1-hexanol [25], 910 mM for 1-butanol [33] and 900 mM for phenol [34], respectively. Chlorosomes were treated with the alcohol-saturated buffer by adding the buffer to a small volume of a concentrated chlorosome suspension. The alcohol-treated chlorosomes were diluted in fresh buffer to a final alcohol concentration equal to the half saturated concentration. The diluting procedure was done gradually, and the diluted samples were left in dark for at least 30 min before various measurements.

2.3. Spectroscopy

Absorption spectra from 250 nm to 900 nm were measured using a Beckman DU-640 spectrophotometer with a 1 cm quartz cuvette. Both CD and MCD spectra were recorded on a Jasco J-720w spectropolarimeter with a cuvette of 1 cm path length. The wavelength range was set from 400 nm to 800 nm, unless otherwise stated. The scan speed was 20 nm/min, the band width 1.0 nm and the response 1 sec. Both instruments were calibrated using a standard Nd glass that has an absorption peak at 585.5 nm. For MCD measurement, an electromagnet was used to produce an external magnetic field of 1.5 T. Since the direction of the magnetic field can be reversed by reversing the direction of current, we define here the positive signal when the magnetic field vector is parallel to the direction of propagation of the light, and the negative signal when the vector is anti-parallel. Final MCD spectra were obtained by subtracting the negative signals from the positive ones and correspond to those obtained under a magnetic field of 3.0 T. All spectroscopic measurements were conducted at room temperature.

2.4. Electron microscopy

Transmission electron microscopy was performed on a Hitachi H-800 operating at 150 kV. The samples were negatively stained with 4% sodium phosphowolframate (pH 7.2) on collodion-coated 400 mesh copper grids with carbon reinforcement.

2.5. Dynamic light scattering (DLS)

Principle of light scattering spectroscopy is based on the fact that there exist microscopic fluctuations in the local density of particles in solution due to Brownian motion. In the scattering experiments an incident laser beam of photons of wave vector $\mathbf{k}_0$ is scattered by these local density fluctuations. By choosing a particular scattering angle θ, one is able to select a particular spatial Fourier component. In general, the translational diffusion coefficient D is related to the friction factor f by

$$D = kT/f \quad (1)$$

where k is the Boltzmann constant and T is absolute temperature. For a spherical particle of hydrodynamic diameter d_0 and solution viscosity η, the simple Stokes formula yields

$$f = 3\pi\eta d_0 \quad (2)$$

For a general prolate ellipsoid of axial ratio $z = a/b\ (> 1)$ and effective spherical diameter $d = (ab^2)^{1/3}$, the following formula of Perrin [35] should be used:

$$f = 3\pi\eta d \left\{ \frac{(z^2-1)^{1/2}}{z^{1/3} \ln\left[z + (z^2-1)^{1/2}\right]} \right\} \quad (3)$$

Distribution of hydrodynamic diameters of chlorosomes was measured on an Otsuka Electronics DLS-700 dynamic light scattering spectrophotometer equipped with a 5 mW He–Ne laser (632.8 nm wavelength). A photomultiplier was set at scattering angle of 90° and the scattered light was analysed by an autocorrelator with a sampling time of 4 μs and correlation channel of 1024. Since the results of DLS are very sensitive to the temperature, all measurements were carried out at 25°C and controlled to within 0.1°C by means of an electric heating and cooling circulator. An equilibration time was about 15 min. The instrument was examined by using two kinds of standard monodispersed microparticles with controlled size and shape (Polybead, 100 nm and 50 nm). Scattering cell was cleaned by flushing several times with distilled water filtered by a high-flow 0.2 μm membrane filter. After cleaning, approximately 4 ml of chlorosome solution was passed through the cell via a 0.45 μm membrane filter. Computer analysis of the time decay of the autocorrelation function using the methods of histogram and cumulant [36] provides a characterisation of the distribution of correlation times associated with the polydispersity of the chlorosome particles. Further details of the theory and methods of DLS can be found in Refs. [37] and [38].

3. Results

3.1. Absorption spectra

We started with absorption measurement to investigate the changes of chlorosomes in the process of alcohol treatment. Fig. 1 shows the absorption spectra of chlorosomes at different stages of 1-hexanol treatment. Chlorosomes in phosphate buffer shows an absorption peak at 743 nm in the near infrared region, a characteristic of

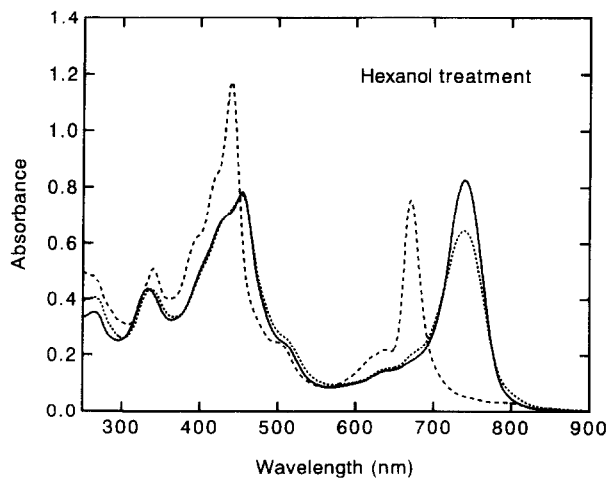

Fig. 1. Absorption spectra of chlorosomes in phosphate buffer of pH 7.2 (solid line), in 1-hexanol saturated (59 mM) buffer (dashed line), and in 29.5 mM 1-hexanol buffer after the hexanol treatment (dotted line). All spectra have been normalised to give zero absorbance at 900 nm.

aggregated form of BChl *c* molecules. After treatment with 1-hexanol saturated buffer (59 mM), the 743 nm peak disappeared, instead a 671 nm peak with a similar height but only half the bandwidth has been observed, corresponding to the monomeric form of BChl *c* in polar organic solvents. A very weak shoulder that can not be distinguished from the spectrum of untreated chlorosomes can be seen around 800 nm in the spectrum of the hexanol-treated sample. This may represent a small amount of BChl *a* [16,32] existing in the chlorosomes which were unaffected by the hexanol treatment [25]. Time variation for this process is shown in Fig. 2. It can be seen that the reaction was almost completed within 15 min and the most drastic changes occurred over the first 5 min. A small peak at 702 nm was observed at the very early stage (within 3 min) of the hexanol treatment, which might correspond to an intermediate form of BChl *c* between the aggregate and the monomer. When the hexanol-treated chlorosomes were diluted into a solution with 29.5 mM (half the saturated concentration) of 1-hexanol, a peak closely resembling that in the original spectrum of chlorosomes was observed at 741 nm, as shown in Fig. 1, only slightly blue-shifted and broadened compared with the original spectrum. These results are in good agreement with those previously reported by Brune et al. [16] and Matsuura et al. [25].

Since it has become clear that the addition and removal of hexanol can cause an apparently reversible conversion of the spectrum of BChl *c* molecules in chlorosome, it is of interest to see how the alcohols with different molecular structures and properties could affect the process. Fig. 3 shows the absorption spectra of chlorosomes by the treatment of 1-butanol which has similar structure but shorter chain length and is more than 15 times higher in solubility compared to 1-hexanol. The 743 nm peak was converted to

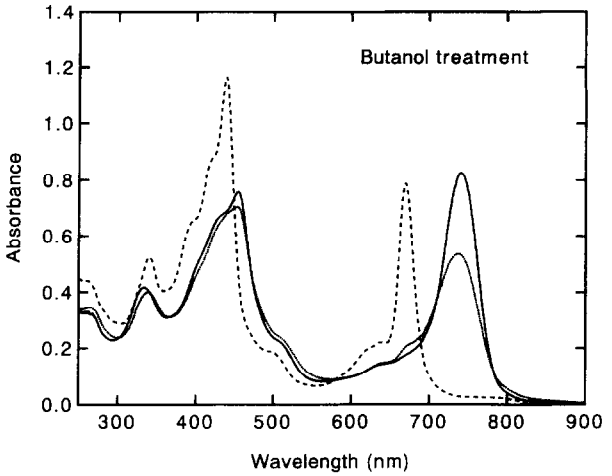

Fig. 3. Absorption spectra of chlorosomes in phosphate buffer of pH 7.2 (solid line), in 1-butanol saturated (910 mM) buffer (dashed line), and in 455 mM 1-butanol buffer after the butanol treatment (dotted line). All spectra have been normalised to give zero absorbance at 900 nm.

670 nm by the treatment of 1-butanol saturated buffer and then was restored to 738 nm by dilution to a solution with 450 mM 1-butanol concentration. Qualitatively, the spectroscopic behaviour is very similar to that of the treatment with 1-hexanol, except for a shoulder at 670 nm and a slight blue-shift observed in the restored spectrum in respect to the original peak. The minor differences indicate an incomplete recovery and might be accounted for by changes in position or increased hydration of BChl *c* molecules in the chlorosomes during addition and removal of alcohol [16]. It is noteworthy that even nearly saturated 1-butanol buffer failed to convert the 743 nm peak to 670 nm completely. This suggests that the degree of saturation, not the concentration of alcohol, is the key factor for the

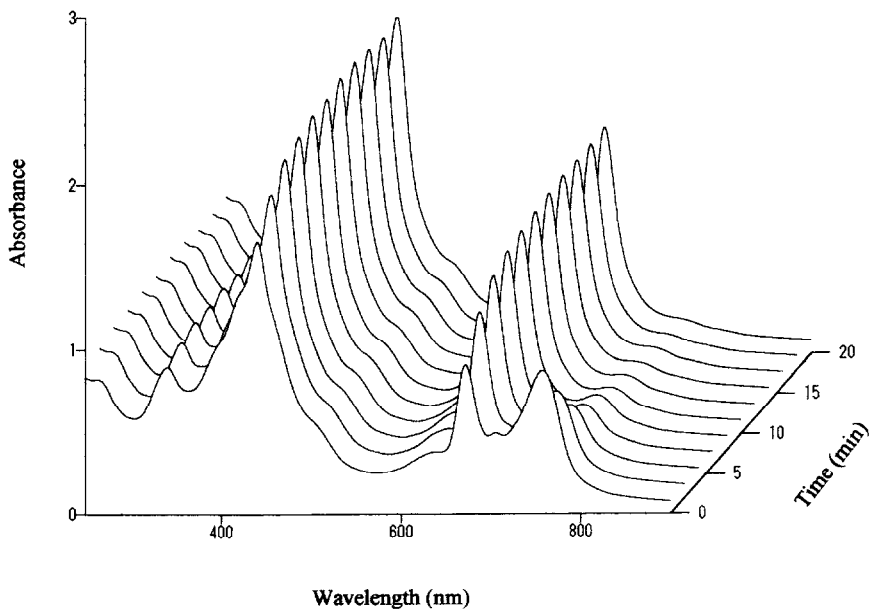

Fig. 2. Time variation of the absorption spectrum of chlorosomes when treated with saturated (59 mM) 1-hexanol buffer.

complete conversion. The result can be understood by assuming that only the unhydrated alcohol molecules are effective in altering the aggregated structure of bacteriochlorophyll *c* in chlorosome, while below the saturated concentration all alcohols are considered to be hydrated by water molecules and be unable to coordinate to the Mg atom of bacteriochlorophyll *c*. A similar result has been obtained for the experiment with phenol treatment, as shown in Fig. 4. The solubility of the aromatic alcohol is as high as that of 1-butanol, and the phenol-saturated buffer was found to be necessary for the complete conversion. For the converted spectrum, the absorbance around 280 nm was much higher and the peak at 673 nm was about ≈ 2–3 nm red-shifted compared to those of 1-hexanol- and 1-butanol-treated samples. These might correspond to some small differences in the structure between these alcohol-treated chlorosomes.

3.2. Electron microscopy

Observation by electron microscope provides the most direct way for visualising morphology and its changes of chlorosomes, which consequently could be closely related to the structural organisation of pigments inside the chlorosome. Fig. 5 shows electron micrographs of native chlorosomes along with those treated with 1-hexanol saturated buffer. Untreated chlorosomes have elongated bodies of uniform shape and size with rounded ends and typical dimension of 150 nm × 60 nm (Fig. 5a), similar to those observed by Staehelin et al. [5]. After treatment with 1-hexanol saturated buffer, remarkable changes have been found in size, shape and distribution of the chlorosomes. The originally rice-grain-shaped chlorosome particles became stick-like form with sharp ends and increased more than two times in length (Fig. 5b). A considerable variation was observed in the size and shape of the hexanol-

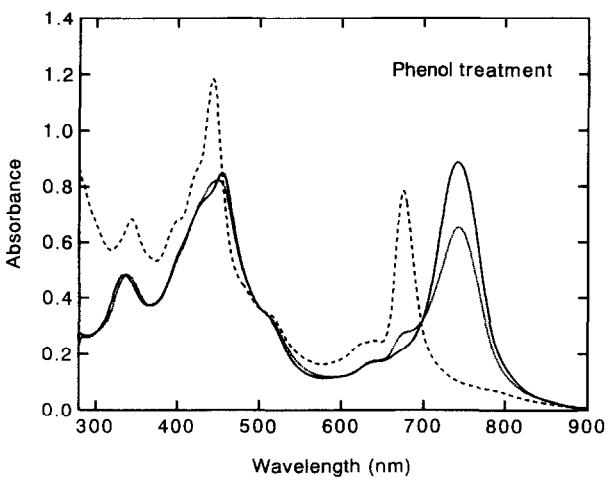

Fig. 4. Absorption spectra of chlorosomes in phosphate buffer of pH 7.2 (solid line), in phenol-saturated (900 mM) buffer (dashed line), and in 450 mM phenol buffer after the phenol treatment (dotted line). All spectra have been normalised to give zero absorbance at 900 nm.

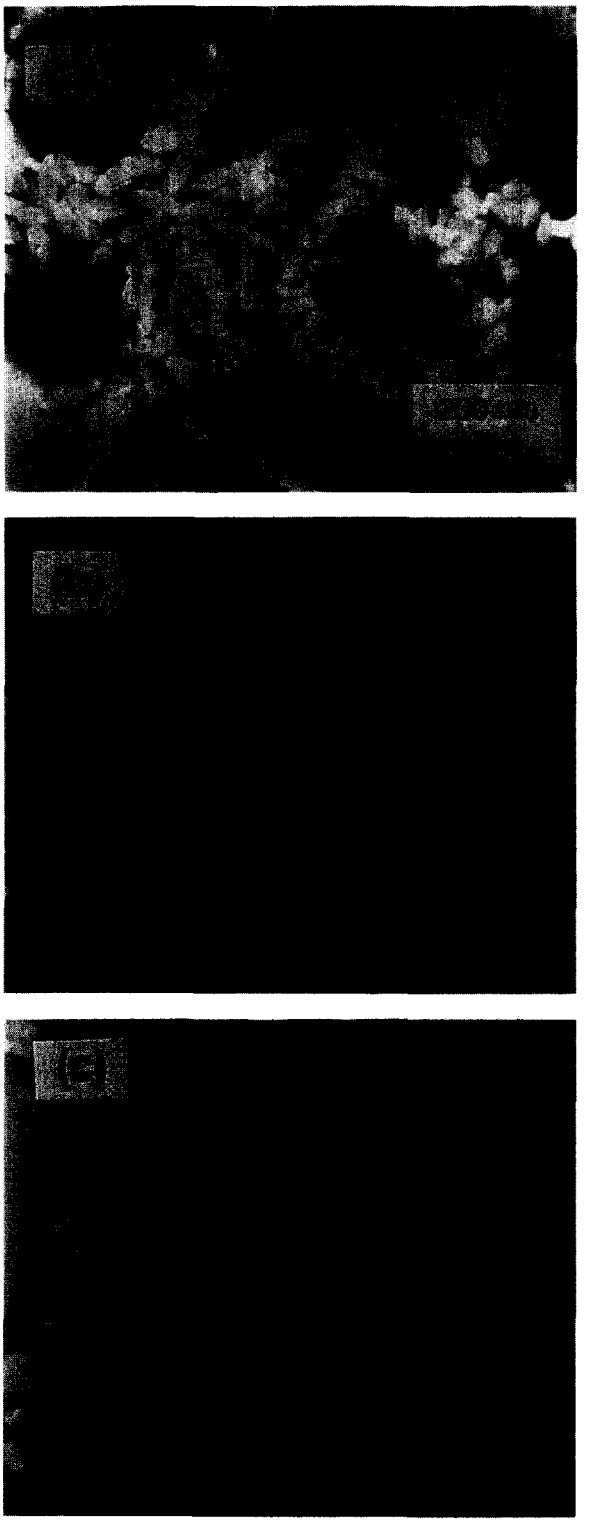

Fig. 5. Negatively stained electron micrographs of various chlorosome samples. (a) Untreated chlorosomes in phosphate buffer (pH 7.2). (b) Chlorosomes in 1-hexanol saturated (59 mM) buffer. (c) Chlorosomes in 29.5 mM 1-hexanol buffer after the hexanol treatment. Note that the scale bar in (a) also applies to (b) and (c).

treated chlorosomes. Similar behaviour has been reported by Lehmann et al. [13] for the chlorosomes isolated from *Chloroflexus aurantiacus*, which have somewhat smaller

size than those of *Chlorobium tepidum*. But in their case, only about 50% increase in the length was observed for the hexanol-treated chlorosomes, while treatment with proteinase K in the saturated 1-hexanol buffer resulted in an increase by a factor of 2 in size (both length and width) and this is comparable with the result of this study. Dilution of the hexanol-treated chlorosomes to a solution of 29.5 mM 1-hexanol brought about further changes in the size and shape (Fig. 5c) which were also different from those of untreated chlorosomes. The chlorosomes were more rounded than the original with a mix of larger and smaller particles varying in shape. The overall size was of about the same order of that for native chlorosomes. Apparently, significant changes in the morphology of chlorosomes have been caused by the hexanol treatment at each step and these changes seem to be irreversible.

3.3. Dynamic light scattering

In order to describe more quantitatively the morphology of chlorosomes, we employed dynamic light scattering (DLS) spectroscopy to determine the hydrodynamic diameter, diffusion coefficient and size distribution. Fig. 6 shows the size distribution histograms of the chlorosomes at different stages of 1-hexanol treatment. The fresh chlorosomes have a narrow distribution centred at 105 nm in the hydrodynamic diameter (Fig. 6a). This result is in good agreement with the observed uniformity for the same sample by electron microscopy (see Fig. 5a). By treatment with the 1-hexanol saturated buffer, the distribution shifted toward greater hydrodynamic diameters, peaked at about 240 nm, and became much broad (Fig. 6b). After the following dilution to 29.5 mM 1-hexanol buffer, the distribution was further broadened with the average value back to about 180 nm. The behaviour observed from the changes in hydrodynamic diameter during this process is well consistent with the trend revealed by electron microscopy that fresh chlorosome particles with uniform shape increased more than two times in length by treatment with 59 mM 1-hexanol and then changed back to an intermediate average size in the following treatment with 29.5 mM 1-hexanol.

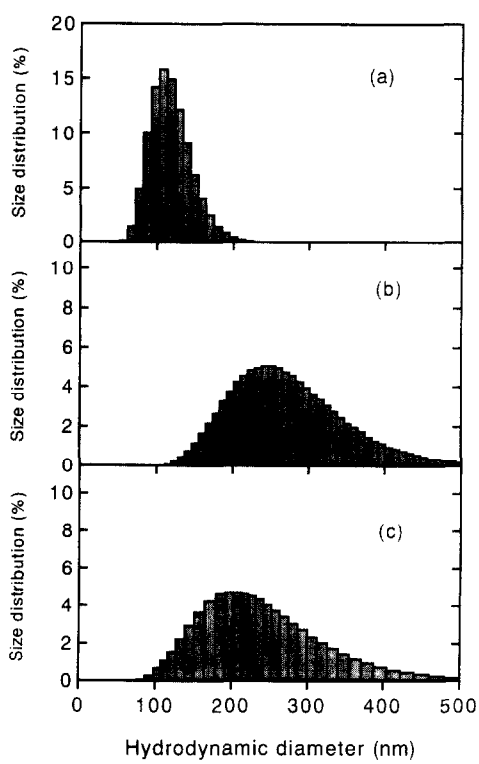

Fig. 6. Distributions of hydrodynamic diameter determined by histogram method of dynamic light scattering for the chlorosomes treated with hexanol. (a) Untreated chlorosomes in phosphate buffer (pH 7.2). (b) Chlorosomes in 1-hexanol saturated (59 mM) buffer. (c) Chlorosomes in 29.5 mM 1-hexanol buffer after the hexanol treatment.

Table 1
Statistical parameters determined by the cumulant method of dynamic light scattering for the chlorosome samples at different stages of 1-hexanol treatment

Sample	d (nm)	$D_{av} \times 10^8$ (cm^2/s)	$[K_2/(K_1)^2] \times 10^2$
Untreated	105	4.64	2.82
59 mM 1-hexanol	248	1.97	4.24
29.5 mM 1-hexanol	183	2.68	6.78

The observed autocorrelation functions were fit by two cumulants K_1 and K_2, where K_1 is proportional to the mean diffusion coefficient D_{av} and inversely proportional to the average hydrodynamic diameter d; and K_2, normalised by $(K_1)^2$, is a quantitative measure of chlorosome particle polydispersity. The chlorosomes were first treated with 59 mM 1-hexanol buffer (saturated), then followed by dilution to 29.5 mM 1-hexanol buffer.

Table 1 summarises some statistical parameters determined by cumulant method for each sample in the process. In the present experiments, the observed autocorrelation functions can be fit by two cumulants with a precision of better than 98% (typically 99%). This precision could not be improved upon with the addition of further cumulants. The first cumulant K_1 is directly proportional to the mean diffusion coefficient D_{av}, and the second cumulant K_2, usually normalised by $(K_1)^2$, is a good measure of the relative width of the distribution. Corresponding to the changes in distribution of Fig. 6 determined by histogram method, Table 1 shows that the average hydrodynamic diameter first increased from 105 nm to 248 nm and then decreased to 183 nm, with the similar trend for the mean diffusion coefficient, and the second cumulant increased constantly during the hexanol treatments corresponding to a continuous increase in the width of the distribution.

3.4. CD and MCD

Fig. 7 shows CD spectra of control chlorosomes and the chlorosomes treated with 1-hexanol. For the untreated chlorosomes, there seem to exist two CD components with

relatively close proportion but reserved signs around 740 nm (Fig. 7a). The negative CD band is located at the longer wavelength region. Similar shape has been found for a different sample from *Chlorobium tepidum* and *Chloroflexus aurantiacus* [3,15,23,39]. With treatment by 59 mM 1-hexanol, the spectrum changed completely, only a very weak signal of negative sign appeared around 670 nm, which was assigned as Q_y band of monomeric BChl *c* (Fig. 7b). In the following dilution to 29.5 mM 1-hexanol buffer, the CD spectrum shows a roughly similar shape to that of the original. Some differences can also be observed: there was only one dominant component around 740 nm corresponding to the positive CD band as was found for the original chlorosomes, and the rotational strength recovered to 60%. These may be due to an incomplete recovery at the final step of the hexanol treatment.

Fig. 8 shows the corresponding MCD spectra of chlorosomes under the same conditions as described in Fig. 7. For the native chlorosomes (Fig. 8a), there is broad positive peak around 740 nm assigned as the Q_y band of aggregated BChl *c*, and another broad maximum of opposite sign around 622 nm assigned as Q_x or a mix of Q_x and Q_{y1-0} of BChl *c*. It is noted that the Q_y band appears to be composed of several components. When treated with 59 mM 1-hexanol, all MCD bands became much narrower, having the same shape as monomeric BChl *c* (Fig. 8b). The 740 band shifted to 672 nm and the 622 nm band split

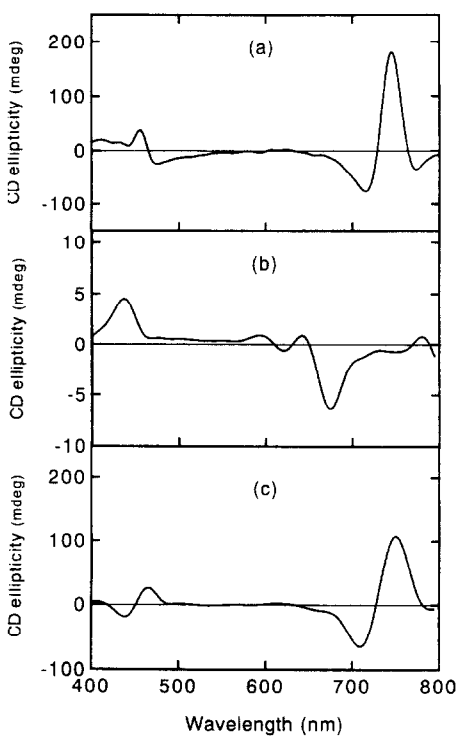

Fig. 7. CD spectra of chlorosomes treated with hexanol. (a) Untreated chlorosomes in phosphate buffer pH 7.2. (b) Chlorosomes in 1-hexanol saturated (59 mM) buffer. (c) Chlorosomes in 29.5 mM 1-hexanol buffer after the hexanol treatment. The concentration of chlorosomes corresponds to an absorbance of 1.0 for the Q_y band in near-infrared region.

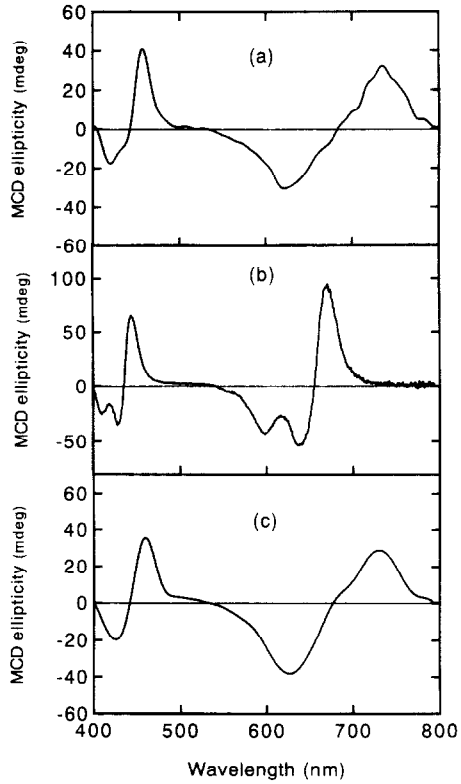

Fig. 8. MCD spectra of chlorosomes treated with hexanol. (a) Untreated chlorosomes in phosphate buffer (pH 7.2). (b) Chlorosomes in 1-hexanol saturated (59 mM) buffer. (c) Chlorosomes in 29.5 mM 1-hexanol buffer after the hexanol treatment. The concentration of chlorosomes corresponds to an absorbance of 1.0 for the Q_y band in near infrared region. All spectra were measured under an equivalent magnetic field of 3.0 T.

into two bands: Q_x at 599 nm and Q_{y1-0} at 638 nm. The Q_{y1-0} band was stronger than that of Q_x. By the following dilution up to 29.5 mM 1-hexanol, the MCD spectrum showed a similar but more uniform shape with almost the same intensity compared with that of the native chlorosomes. A broader band shape in 29.5 mM hexanol than that in 59 mM indicates that plural components in the native chlorosomes were also recovered by dilution of hexanol concentration. The MCD results obtained are consistent with those from CD, but show more detailed features in the spectrum, especially for the Q_x and Q_{y1-0} bands that are usually very weak in the absorption and CD spectra.

4. Discussion

The result of electron microscopy shows significant changes in the size, shape and distribution of chlorosomes during the process of 1-hexanol treatment. In order to investigate if the monomeric BChl *c* molecules were liberated from chlorosomes by the hexanol treatment, we centrifuged the sample at $150\,000 \times g$ for 1 h and found only a small amount of upper layer fraction. This fraction was also examined by electron microscope and showed

similar shape to that of the bottom fraction (Fig. 5b), but somewhat smaller sizes. This suggests that most BChl *c* molecules were still confined in the chlorosome particles and penetration of 1-hexanol molecules through the chlorosome envelope might occur and result in a large elongation in the particle bodies and probably a further coordination of hydroxyl group of hexanol to the magnesium atom of BChl *c* as pointed out by Brune et al. [16] and Matsuura et al. [25]. The expansion of the size could cause a substantial separation between the pigments that originally exist in an aggregated form, and this may explain the spectroscopic features of a monomer form BChl *c* observed for the hexanol-treated sample.

After dilution, the size of chlorosomes was almost changed back to the original, but with different shape and distribution. In the meantime, all spectra were nearly restored. This implies that the relative overall size, rather than the shape or distribution, of chlorosomes may be a more important factor affecting the spectroscopic behaviour. It is also obvious that the whole process of hexanol treatment is morphologically irreversible. Compared with the restored optical properties, one can speculate that a particular optical property may correspond to more than one molecular organisation.

Miller et al. [19] reported that both protein-containing and protein-free chlorosomes of *Chloroflexus aurantiacus* obtained by treatment with lithium dodecyl sulfate of $\approx 0.5–1.0\%$ revealed rounded shape with almost unchanged size. In this case, all the treated chlorosomes had a similar absorption spectrum to that of the control chlorosomes, and the observed spheres of $\approx 50–70$ nm in diameter were assumed to be micelles of BChl *c* released from the chlorosomes during the detergent treatment. Lehmann et al. [13] found that there was no significant difference in both size and shape between the native chlorosomes and the proteolytically treated chlorosomes with trypsin. The absorption and CD spectra were also not altered by the treatment. When chlorosomes were treated with 59 mM 1-hexanol buffer and proteinase K in the same buffer, both length and width were increased up to 2 times. Meanwhile, the 740 nm band shifted to 670 nm and an irreversible giant CD signal formed by using the combination of 1-hexanol and proteinase K. These results are in accord with those of this study and support that the change in size could be reflected by the changes in spectrum.

Electron microscopy is useful for visualising the size and shape of a limited number of chlorosome particles but lacks statistical accuracy. So far, estimation of the size has relied on the measurement from micrographs for a very small number of objects [13] and therefore contains relatively large uncertainty. DLS has been proven in this study to be a complementary tool to the electron microscopy and gives much more detailed, quantitative information on the morphology. In the case of chlorosomes, Eq. (3) was used to evaluate the dimension. It is important to note that the dependence of friction factor f on axial ratio $z = a/b$ is very weak. For axial ratios between 1 and 2 the term in brackets of Eq. (3) ranges from 1.00 to 1.04. For the native chlorosomes, if we use the average hydrodynamic diameter $d = 105$ nm determined by DLS (Table 1) and assume that the width (short axis) is 80 nm from the micrograph, then the length (long axis) is estimated to be 160 nm. This is in good agreement with the value that we can measure from the micrograph. Similarly, an average length of about 700 nm was estimated for the extremely prolate chlorosomes treated with 59 mM 1-hexanol. Since the chlorosome particles obtained by dilution to 29.5 mM 1-hexanol can be approximated to have a nearly rounded shape, the average hydrodynamic diameter of 183 nm represents the actual dimension.

It is interesting to find that different alcohols in molecular structure and property can completely change the BChl *c* in chlorosomes into monomeric form as long as the saturated buffers are used. The difference in alcohol concentration at saturation was about 15-fold in this study, and all alcohols produced nearly reversible absorption spectra. This result may be explained by the hydration of alcohols in aqueous solution, since most alcohol molecules in unsaturated solution are thought to be consumed for the hydration and have no ability to interact with chlorosomes. It has been shown recently that aggregates of BChl *c* containing small amounts of BChl *a* and lipids have an absorption spectrum remarkably similar to that of intact chlorosomes [40] and the 740 nm band is reversibly converted to 670 nm when treated with saturated 1-hexanol buffer without disruption of the aggregated state.

The CD spectrum of untreated chlorosomes shows a two-component characteristic in the near infrared region (Fig. 7a). Similar CD shape has been reported for the chlorosomes in normal buffer and in 0.45% 1-hexanol buffer [41], the observed spectra can be simulated by a sum of two exciton-type components. Brune et al. [39] argued that the instability could caused a hypsochromic shift during the preparation process. On the other hand, Griebenow et al. [23] suggested that two basic CD spectra were mixed in different ratios to form any intermediate spectrum and the variations of CD signal is intrinsic rather than due to degradation in the isolation procedure. For the recovered CD spectrum (Fig. 7c), the rotational strength was about 60% that of untreated chlorosomes. At present, there is no definitive information on the relationship between BChl *c* arrangement and the CD strength, although theoretically several factors can be considered. Considering that the average dimension of the recovered chlorosomes was larger than that of untreated ones, difference in the dimension might be responsible for the decrease in rotational strength since the dimension is thought to be proportional to compactness of BChl *c* molecules within the chlorosome, and the compactness might be one of the factors influencing the CD intensity. The compactness can also be considered to reflect the size of BChl *c* oligomers (clusters). Lehmann et al. [13] attributed the giant CD

signals obtained by treatment with 1-hexanol and proteinase K to an increase in array of interacting BChl *c* molecules and suggested the involvement of 5.7 kDa protein in BChl *c* organisation as the protein should be digested by the treatment.

In the MCD spectrum of untreated chlorosomes (Fig. 8a), one can find some shoulders in the positive peak of near infrared region, but these cannot be clearly seen in the individual scans under positive and negative magnetic fields. This indicates that the MCD is very sensitive to the signals with slight differences in spectral properties. Since BChl *c* is basically a chlorin, the monomer form MCD shows a spectrum closely resembling that of Chl *a* in ether [42] and MCD rotational strength was increased three times compared with the native form of BChl *c* in chlorosomes. In a separate experiment using extracted BChl *c*, we have found that the MCD intensity decreases and peak position moves to a longer wavelength as BChl *c* monomers form dimer, tetramer, oligomer and aggregate in organic solvents. Further applications of MCD to study the spectral properties of various BChl *c* aggregates will be presented in a forthcoming paper.

5. Conclusions

Chlorosomes from *Chlorobium tepidum* have been treated with three alcohol aqueous solutions with saturated concentrations, followed by dilution to the buffers with half the saturated concentrations. Both morphologic and spectroscopic measurements have been carried out at each step of this process. Several conclusions reached in this study are summarised as follows. (a) The degree of saturation, not the alcohol concentration, is the key factor for the complete conversion of bacteriochlorophyll *c* in chlorosomes from aggregated state to monomeric form. This means that only the unhydrated alcohol molecules are effective in altering the aggregated structure of bacteriochlorophyll *c* in chlorosome. (b) Substantial changes in the shape, size and distribution have been observed at each step of the treatment with 1-hexanol, indicating that the whole process is morphologically irreversible, while spectroscopically this process appears to be reversible. (c) Comparison of the morphologic changes with the corresponding spectroscopic behaviour suggests that the relative overall size rather than the shape and distribution may be a more important factor affecting the spectral properties. (d) It is first shown by MCD spectra that there are more than four components existing in the Q region of untreated chlorosomes, in accord with previous observation.

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