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Chlorophyll *a* aggregates stabilized by a synthesized peptide

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Abstract

The affinity of a synthesized amphiphilic peptide to bind Chl has been studied. It was shown that the peptide, which contains histidine, is able to stabilize the dimeric form of Chl in aqueous solution, making the pigment-peptide complex. The interaction between Chl and the amphiphilic peptide, without support of histidine, caused the formation of higher pigment aggregates based on the hydrophobic interaction only. The dimer of Chl ligated to the peptide by the histidine residue showed that the absorption at 685 nm could serve as an in vitro model of the Chl *a* special dimer. The size and molecular weight of the Chl-peptide complex was estimated.

Abbreviations: CD, circular dichroism; Chl, chlorophyll; DLS, dynamic light scattering; FTIR, Fourier transform infrared; GFC, gel filtration chromatography; LALLS, low-angle laser light scattering; THF, tetrahydrofuran

Keywords: Chlorophyll; Histidine; Peptides

1. Introduction

Light harvesting proteins in a photosystem consist of polypeptides and functional molecules, such as chlorophyll (Chl) and carotenoid [1]. Chls and bacteriochlorophylls, that are associated with proteins, are surrounded by hydrophobic amino acids and have at least one ligand from the protein to the central metal atom [2]. Most of them are ligated to the protein by conserved histidine residues through the central Mg atom. The studies of an artificial water-soluble model for the Chl-protein complex contributes to obtaining more precise information and to better understand the nature of the Chl-protein complex in vivo. Early attempts to prepare artificial Chl-protein complexes using synthetic linear polymers or proteins, such as bovine serum albumin and apocatalase, are described in Refs. [3–7]. The two designed, amphiphilic α-helix peptides possess the binding site of Chl and mimic the spectral properties of α-helical photosynthetic proteins. The 30-residue peptides were synthesized to form water-soluble pigment–peptide complexes. In both peptides, the amino acid sequence is almost the same. One of them — peptide L, is amphiphilic and does not contain the histidine residue (NH_2-EEEQQKKLLEELKKLLEELKYLLKEEQKKK-COOH), and in the second one — peptide L/H, the histidine residue was placed in 16 position instead of leucine, in the hydrophobic region of the peptide. It was shown earlier [8], that these amphiphilic peptides form an α-helical structure in aqueous solution and Chl introduced from acetone to peptide L/H solution can form the stable pigment–peptide complex at 0.5 mM peptide concentration. These results also implied that the single molecule of Chl, absorbing at 672 nm, was surrounded or shielded by peptides α-helices [8].

The aim of the present paper is to check the ability of the artificial peptides to bind and stabilize the aggregated form of pigment. It was reported [9] that Chl introduced by tetrahydrofuran (THF) to aqueous solution forms a selective Chl *a* aggregate, absorbing at 685 nm. The 685-nm form was described as a dimer in which the stacking of chlorine rings was supported by a hydrated THF dimer [9]. In order to check whether it is possible to form such a dimer of Chl by coordination of Mg atoms to histidine residues of the peptide, we have used the peptide L/H with, and peptide L without histidine as a control. It would also be interesting to control the Chl forms by changing solvent to introduce the pigment to the peptide solution. In our paper, we show that the Chl *a* forms a stable dimer by interacting with hisidine residue of peptide L/H in buffer solution. The amphiphilic

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interactions are only possible using peptide L and result in the formation of high pigment aggregates at 695 nm. The size and molecular weight of the pigment-peptide complex was also estimated.

2. Materials and methods

Chl *a* isolated from spinach was furnished by Sigma and used without further purification. Peptides were synthesized as described earlier [8,10,11]. Crude peptides were purified by high performance liquid chromatography (Shimadzu) with a reverse-phase column (C18 SynProPep, Shimadzu) [11]. Using Kratos Kompact MALDI 4 V5.1.1 spectrophotometer, the mass spectroscopy spectra were measured to check the molecular weight of synthesized peptides and confirm the purity of used peptides. The theoretically calculated molecular weight was 3786.5 and 3810.5 for peptide L and L/H, respectively. Experimentally obtained data (not shown) were in good agreement with the theoretical results and were equal to 3783.7 and 3806.9 for the peptide L and L/H, respectively.

The peptides (concentration 0.5 mM) were suspended in 30 mM phosphate buffer (pH 7.3), and the pigment dissolved in 100% THF was added to the solutions (the final concentration of THF 6%, v/v) and then stirred for homogeneous dispersion. The samples were kept in the dark at room temperature before the measurements. The samples for FTIR measurements were prepared by evaporating the sample solution under reduced pressure on a gold-evaporated glass slide.

Absorption spectra were measured with a Shimadzu UV-1601 spectrophotometer. Fluorescence spectra were measured using a Hitachi F-4500 spectrofluorometer. The circular dichroism (CD) spectra were measured with a Jasco J-720 spectropolarimeter.

Two methods were also applied to estimate the molecular weight of the peptide and peptide-pigment complex in aqueous solution. The gel filtration chromatography (GFC) of samples was performed on Superdex 75 superfine and the set of proteins from Pharmacia Biotech, were used as a standard. By using the low angle laser light scattering (LALLS) set-up (LS-8000, Tosoh) with refractive index detection, the molecular weight was estimated using ribonuclease as a standard protein. To obtain the size distribution in samples, the dynamic light scattering (DLS) was performed by DLS-6000 AS (Otsuka Electronic).

FTIR spectra were measured at a 4-cm^{-1} resolution by means of Nicolet Magna – IR 760 spectrometer. All measurements were performed at room temperature.

3. Results and discussion

The absorption spectra of Chl introduced from THF to synthesized peptide solutions undergo changes in time. The changes are clearly reflected by the second derivative of absorption spectra (Fig. 1). As follows from these spectra, the time scale and final forms of pigment are different in the case of peptide L (Fig. 1(A)) and L/H (Fig. 1(B)) solutions. In peptide L, the changes of absorption are mainly observed within 3 h of sample preparation, whereas in peptide L/H solution, at least 22 h are necessary to reach the stable absorption of the sample. The final absorption spectra of Chl in solutions with/without peptides are shown in Fig. 2. The

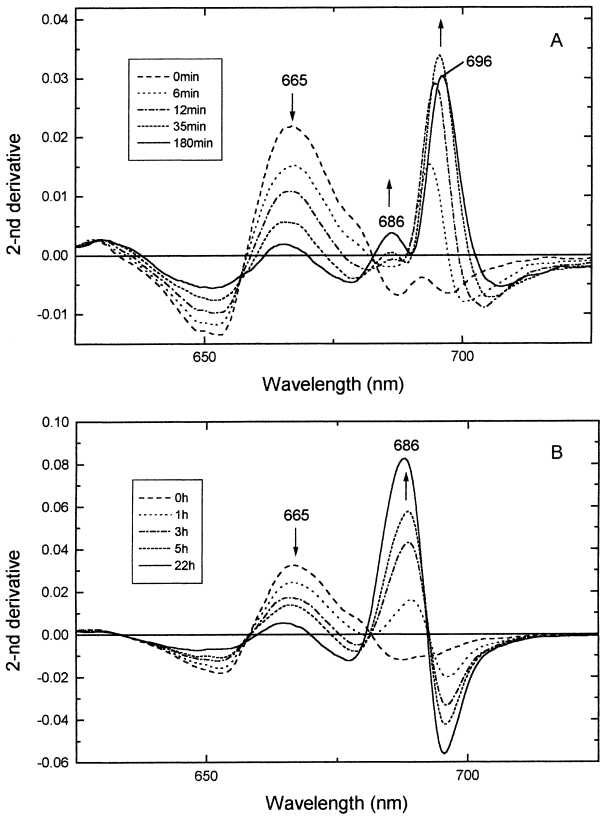

Fig. 1. Changes in second derivative of absorption spectra for Chl *a* in solution of peptide: (A) L and (B) L/H.

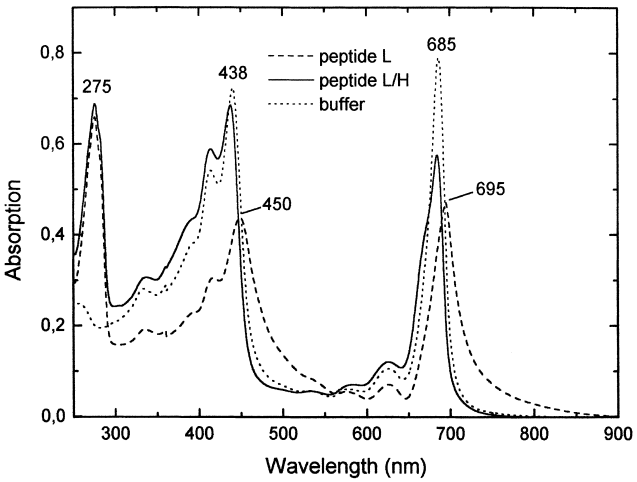

Fig. 2. Stable absorption spectra of Chl *a* in peptides and buffer solution.

spectrum of Chl in 6% THF buffer solution, reported by Uehara et al. [9], was also added as a reference and used to compare their spectral pattern with samples including the synthesized peptides. The Chl in both the buffer and peptide L/H solution produced the same kind of species (Fig. 2). The presence of peptide L in solution caused a shift in the formation of Chl absorption bands to the long wavelength region with maxima at around 450 nm and 695 nm. The maximum in the UV region of absorption at 275 nm belongs to the presence of tyrosine in both peptides.

The second derivative of absorption help us to distinguish which kind of Chl forms, are present in both peptide samples (Fig. 1). These spectra show that the main fraction present in peptide L/H solution absorbs at 686 nm with some admixture of another one at 665 nm. In the case of peptide L solution, Chl is mostly present as a form which absorbs at 696 nm with the small addition of 686-nm and 665-nm forms. Based on the literature, we could ascribe respective maxima to appropriate forms of Chl. The 665 nm peak indicates existence of a Chl monomer, just as in 100% THF [12]; the 696 nm one is characteristic for monohydrated aggregates of Chl $(Chl{\cdot}H_2O)_n$ [13]. The most interesting is the 686-nm component found earlier by Uehara et al. [9] to be a dimer of Chl. The structure of this dimer, involving THF molecules coordinated to a Mg atom, was proposed earlier [7,9]. However, it is rather difficult to make a firm conclusion here about the 686-nm Chl structure in peptide L/H based only upon the results of UV-VIS spectra (see in the following). The samples including peptides were monitored by CD spectra (Fig. 3). The spectral pattern of the CD signal supports the idea that Chl dimer is present in peptide L/H solution (Fig. 3) and the shape of the CD spectrum is similar to that reported by Uehara et al. [9]. This spectrum shows the typical 'S' type CD signal with a positive band at 676 and negative one at 691 nm that resembles the spectrum of a dimer and shows high molar ellipcity. The spectrum of Chl *a* in peptide L solution (Fig. 3) shifted to the red,

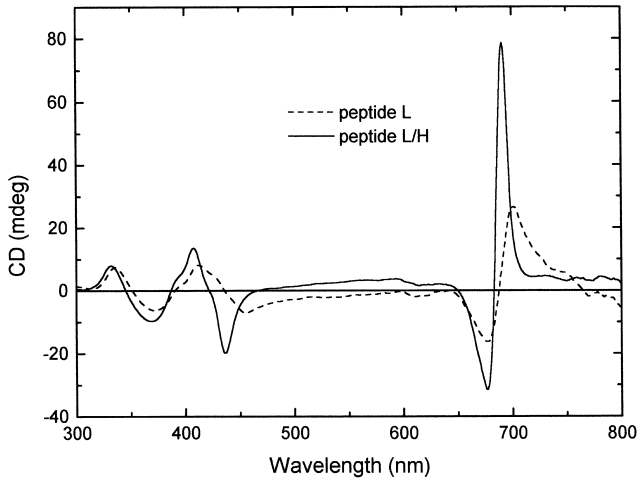

Fig. 3. Circular dichroism spectra of Chl *a* in peptide solution.

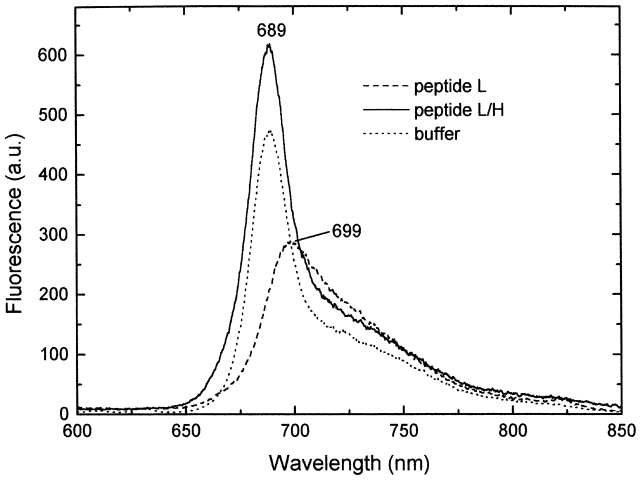

Fig. 4. Fluorescence spectra of Chl *a* in peptides and buffer solution.

decreased in molar ellipticity and displayed CD bands, which may be ascribed to the large Chl *a* aggregate. The CD signal of the samples in the absorption region of the peptide (200–250 nm) was also measured (result not shown). The shape of spectra for peptide L and L/H and the content of α-helical structure (see Ref. [8]) indicated that 6% THF did not disturbed the secondary structure of peptides and their structure still remains unchanged.

The fluorescence spectra of investigated samples are shown in Fig. 4. Similar spectra were observed for Chl in peptide L/H and buffer solution confirmed that the same species of Chl is mostly present in both samples and is responsible for emission. The emission maximum at 689 nm is in good agreement with Stocke's shift between absorption and fluorescence for 685-nm Chl form. Lack of the fluorescence in the long wavelength region reconfirmed (see also Fig. 1(B) and Ref. [9]) that the Chl dimeric form is selectively formed in 6% THF buffer as well as in peptide L/H solution, and the dimer is the last acceptor of energy which is very efficiently emitted as a fluorescence. Chl in peptide L solution emitted relatively weak fluorescence and the maximum is shifted to the red indicating that the first excited state of the 695-nm form resulted in the emission in the 699-nm region.

The GFC measurements (not shown) indicated that the sample of Chl *a* in the peptide L/H solution is not homogenous. Two fractions were detected and Chl was present only in one of them. By the UV detection, it was observed that the first fraction contains the pigment-peptide complex and the second fraction consisted of pure peptide. The estimated molecular weight according to the elution time was ca. 12.4×10^4 for Chl-peptide L/H fraction and $\approx 16.2 \times 10^3$ for peptide fraction. Taking into account the molecular weight of single L/H peptide (3810.5), we could estimate that the pigment-peptide fraction contains around 32.5 peptides (or 26.3 if the weight of Chl is considered). In contrast, the pure peptide fraction consists of some peptide aggregates in which the number of peptides is 4.3.

The average molecular weight of the peptide fraction was also measured using LALLS technique. In this set-up, the detection of the sample was based on the refractive index and direct injection of sample into the light-scattering cell. The sample containing THF solvent could not be measured satisfactory due to the error caused by the solvent refractive index. The molecular weight of pure peptide in buffer solution was estimated to be equal to 14.4×10^3. From this result, the number of peptides creating the aggregate was calculated to be 3.8.

Finally, the DLS measurements for Chl in peptide L/H solution distinguished two pools of molecules differing in size. The measurement of the pure peptide fraction lacked accuracy due to the limitations of the DLS set-up. However, it is clear that the molecular size of the pure peptide fraction, ca. 7.0 nm, is higher than the theoretical length of single peptides (equal to 4.5 nm). It suggests once more that the peptides are probably in some aggregated form, but the number of peptides in aggregation is difficult to estimate. The molecular size of dimer-peptide L/H complex was estimated to be (102.3 ± 26.4) nm. When calculating the size of this fraction, it seems that at least 22.7 monomer of peptides are necessary to form such a complex. The estimation only took the theoretical length of the peptide into account and ignored the size of the Chl porphyrin ring, due to a lack of information about the spatial arrangement. The large pigment–peptide complex was not detectable either for Chl in buffer or for Chl in peptide L solution. This suggests that the complex has to include a peptide moiety and also that it could not be formed without interacting with histidine.

Based on these three types of experiment, i.e. gel filtration, LALLS and DLS, we could speculate that the peptides probably form tightly packed aggregates, because the number of peptides involved in both, pigment–peptide complexes as well as peptide aggregates is lower in the case of DLS than gel filtration and LALLS experiments. Summarizing the results, we can conclude that the pigment-peptide complex is probably based on both, Chl-histidine and/or Chl-hydrophobic interactions involving ca. 23–33 peptides. In contrast, the hydrophobic interaction between the peptide itself is mainly responsible for the aggregate formation, and the number of peptides varied from two (from the DLS experiments) to four (estimated from the molecular weight measurements, GFC and LALLS).

The spectrum of the Chl *a* dimer in peptide L/H solution is highly stable and did not show any changes for at least two days. Uehara et al. [7,9] proposed that the 685-nm form of Chl is a dimer with the structure symmetrical with the oxygen atom of water in the hydrated THF dimer ($THF{\cdot}H_2O{\cdot}THF$). In their model, the oxygen atom is ligated to the central Mg atom of Chl *a*. The dimer in peptide L/H solution shows similar spectral patterns as that found by Uehara et al. [9]. Therefore, the question still remains: which kind of structures has the dimeric form of Chl *a* in 6% THF peptide L/H aqueous solution. It is possible that:

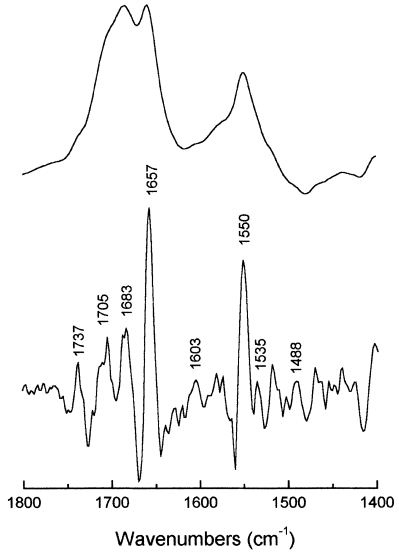

Fig. 5. FTIR spectrum and its second derivative for Chl *a* in peptide L/H solution evaporated on gold plate.

1. L/H peptides surround the dimer which is formed through the interaction of Chl *a* with the hydrated THF dimer; or
2. that the competition between THF molecules and histidine residues occurs and that the structure of the dimer is related to the interactions with both histidine and THF molecules or only histidine residue of peptide L/H.

In order to answer this question the FTIR were performed for the Chl-peptide L/H sample (Fig. 5). The FTIR spectrum was made for the Chl–peptide L/H mixture which was evaporated on a gold plate to dryness under reduced pressure. The maximum intensities were observed at 1657 cm^{-1} for the amide I region, and at 1553 cm^{-1} for the amide II region. According to the spectral analysis of polypeptides of known structure [14], we can conclude that this FTIR spectrum supports the CD results [8], thus indicating that the L/H peptide has the predominant helical structure as was designed and anticipated from the amino acid sequence. Because the peptide signal overlapped the spectral region characteristic for Chl, the second derivative of the FTIR spectrum was made. From this spectrum, we could clearly see the bands related to Chl. The appearance of 1603, 1535 and 1488 cm^{-1} bands assume Chl to be in a five-coordinate form. Also, the ester and keto C=O groups are free, as follows from the 1737, 1705 and 1683 cm^{-1} frequencies. The FTIR spectrum of Chl evaporated from 6% THF buffer was also measured (not shown). The peak at 1662 cm^{-1} observed for this sample was assigned to the keto C=O group coordinated to the Mg atom of another Chl molecule (the 'aggregation peak') discussed in the literature [15]. By evaporating the Chl *a* in 6%THF buffer solution, we disturb the dimer created by ($THF{\cdot}H_2O{\cdot}THF$) and, as a result, the aggregation of Chl is observed. However, in a sample obtained from the 6% THF peptide L/H solution, the five-coordinated Chl

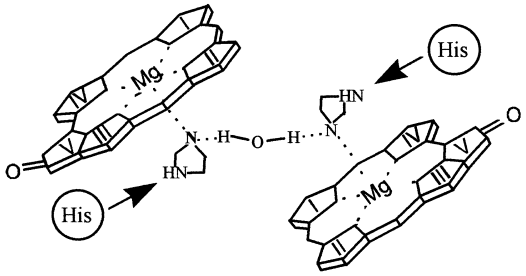

Fig. 6. A tentative molecular model for dimer of Chl *a* ligated by the histidine residue to the peptide L/H.

was observed, suggesting that the histidine residue of peptide L/H may serve as a fifth ligand of the Mg atom. The plausible molecular structure of the Chl *a* dimer ligated to the L/H peptide by the histidine residue is shown in Fig. 6. Based on our results, we modified the model reported by Uehara et al. [9] and we propose that the oxygen of THF molecule is replaced by histidine nitrogen atom of the peptide L/H. In order to obtain the stable Chl *a* dimer, about 22 h is necessary. It suggests that the process is quite complex and mainly related to the competition between the histidine residue and THF molecule, both of which have strong affinity to interact with Chl molecules.

It was also found (result not shown) that pheophytine *a* produced an aggregate, absorbing at ca. 690 nm and emitting fluorescence at ca. 700 nm, in 6%THF peptide solution, independently of the type of peptide used. Such findings suggest once more that the histidine residue is the main force in forming complexes with synthesized peptide L/H. This concept also helps to explain the behavior of the Chl in peptide L solution, where the ligation of the Chl Mg atom does not exist and the environment of Chl is only changed by the presence of amphiphilic peptide L. It seems that the hydrophobic interaction of peptide L is stronger than the ligation of the THF oxygen atom to Chl; therefore, the high Chl aggregates (absorbing at 695 nm) are formed in the presence of competition with the THF hydrated dimer, which stabilizes the Chl in 6% THF buffer solution.

We can also see the advantage of using the synthesized peptide. With the synthesized peptides we can modify their structure and selectively investigate the influence of specific amino acids on the pigment-peptide interaction. Our peptides are stable and also do not undergo denaturation in the presence of organic solvents (in contrast to proteins), which helps us to introduce Chl to aqueous solution by using different solvents and obtain several distinct forms of Chl which are good models for studying the interaction of various pigment forms with synthesized peptides, which mimic the protein properties.

The model of stable Chl *a*-peptide L/H complex is also interesting as an in vitro model for a P-680 special dimer of Chl *a*.

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