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Properties of the water-soluble chlorophyll-synthetic linear macromolecular complexes

Isamu Inamura [a], Takeo Araki [a], Masaki Shei [a], Tsumoru Nishikawa [a], Hitoshi Shibata [b] and Hideo Ochiai [b]

[a] *Department of Chemistry, Faculty of Science and* [b] *Laboratory of Biochemistry, College of Agriculture, Shimane University, Matsue-shi, Shimane-ken (Japan)*

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The properties of the water-soluble chlorophyll (Chl) *a* or *b*-synthetic linear macromolecular (poly(vinylpyrrolidone)(PVP), poly(ethylene glycol)(PEG) or poly(vinyl alcohol)(PVA)) complexes were investigated. The low-temperature (77 K) fluorescence emission spectra suggested that the Chl *a* or *b*-PVP and Chl *b*-PVA complexes had the similar form of Chl *a* or *b* to that of Chl *a* or *b* in ethyl ether or Triton X-100, probably a monomeric form, while the Chl *a* or *b*-PEG and Chl *a*-PVA complexes contained the several undefined forms of Chl *a* or *b*. The Chl *a*-PVP complex was eluted at the same volume of the corresponding PVP applied independently to a column of Sephadex G-100, but the Chl *a*-PEG and Chl *a*-PVA complexes showed the elution patterns similar to that of Chl *a*-bovine serum albumin (BSA) complex. The Chls in the synthetic linear macromolecular complexes were stable against oxidative stress of photobleaching, but they were easily hydrolyzed by chlorophyllase, as were the Chls in both the BSA complexes and the isolated light-harvesting Chl *a*/*b*-protein complex (LHC). A possible localization of Chls within linear macromolecular complexes was suggested so that the tetrapyrrole ring and phytol group of Chl would be surrounded by a linear macromolecular (PVP) chain or a number of linear macromolecular (PEG or PVA) chains, whereas the hydrophyllic edge of porphyrin ring, adjacent to the phytol group, would be exposed to the environment of a water medium.

Introduction

The chlorophyll-proteins play an important role in the primary process of photosynthesis because all the chlorophyll exists as chlorophyll-proteins in the chloroplast thylakoid membranes [1]. Recently, the molecular organization of the chlorophyll-proteins have been closely investigated, and Murphy [2] presented a schematic model of the possible mode of association of Chl molecules with the membrane-spanning α-helical regions of pigment proteins.

Since we developed the methods to prepare the water-soluble Chl-macromolecular complexes as artificial model compounds of chlorophyll-proteins in vivo, by using synthetic linear macromole-

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; LHC, light-harvesting chlorophyll *a*/*b* protein complex; PVP, poly(vinylpyrrolidone); PEG, poly(ethylene glycol); PVA, poly(vinyl alcohol); BSA, bovine serum albumin.

Correspondence: I. Inamura, Department of Chemistry, Faculty of Science, Shimane University, Matsue-shi, Shimane-ken 690, Japan.

cules (PVP, PEG or PVA) and a naturally-occurring globular macromolecule (BSA) [3–5], the various properties of these complexes have been investigated [6–9]. In the previous study [10], we focused our attention on the Chl-BSA complexes and investigated them by examining the spectral properties, gel chromatography, stability of Chls in the complexes against oxidative stresses such as photobleaching, Fenton reagent and peroxidase-H_2O_2 system, and hydrolysis of Chls in the complexes by chlorophyllase. It was found that BSA molecules in Chl-BSA complexes associated each other by sandwiching phytol moiety of Chl, resulting in a particle of molecular weight higher than $1 \cdot 10^6$. A possible localization of Chls within BSA complex was suggested where the porphyrin moiety of Chl was buried in BSA; however, the hydrophilic edge of porphyrin ring, adjacent to the phytol group, occurred in the hydrophilic region on the surface of a BSA molecule.

In the present paper, the water-soluble Chl-synthetic linear macromolecular complexes were investigated by the examinations similar to those carried out for the Chl-BSA complexes. Because the synthetic linear macromolecular (PVP, PEG or PVA) chains in an aqueous solution are considered to be in random coil state and to have much more flexibility than a globular protein of BSA, it is very interesting to reveal an aggregation of polymer and a localization of Chl within the Chl-synthetic linear macromolecular complexes and to compare them with those within the Chl-BSA complexes. It is also worthwhile to investigate the Chl b-synthetic linear macromolecular complexes, since there is no chlorophyll-protein containing only Chl *b* in vivo. The Chl-BSA complexes were prepared by the new method [5] in the present study, the properties of which were compared with those of the Chl-BSA complexes prepared by the old method [3,4] in the previous investigation [10].

From the results obtained, we could present the possible aggregation of polymer and localization of Chl within the Chl-synthetic linear macromolecular complexes. They likely supported the view originally advanced by Anderson [11,12] and the schematic model recently presented by Murphy [2], for the localization of Chl within intrinsic proteins.

Materials and Methods

Materials. PVP, PEG and PVA were purchased from Tokyo Kasei Kogyo Co., Ltd., Toyo Soda Manufacturing Co., Ltd., and Kuraray Co., Ltd., respectively. PVP was fractionated by the fractional precipitation method using water-acetone. The weight and number average molecular weights (M_{rw} and M_{rn}) of the fractionated PVP sample used in this study were estimated to be 32800 and 15800, respectively, by gel permeation chromatography; $M_{rw}/M_{rn} = 2.08$. PVA was also fractionated by the fractional precipitation method using water-1-propanol. Molecular weights of the fractionated PVA samples were estimated as follows; $M_{rw} = 111400$, $M_{rn} = 78100$, $M_{rw}/M_{rn} = 1.43$ for sample Nos. 4 and 5, and $M_{rw} = 39400$, $M_{rn} = 28700$, $M_{rw}/M_{rn} = 1.37$ for sample No. 6. PEG was used without fractionation; $M_{rw} = 21000$, $M_{rn} = 18800$, $M_{rw}/M_{rn} = 1.12$. BSA was obtained from Armour Pharmaceutical Company (lot No. W81804), and purified by the treatment with charcoal at low pH to remove lipid impurities [13]. Chl *a* and *b* were prepared from spinach leaves by the column chromatographic separations with DEAE-Sepharose CL-6B and Sepharose CL-6B [14]. LHC was isolated from the thylakoids of barley (*Hordeum vulgare*), according to the methods of Burke et al. [15]. The ratio of Chl *a*/Chl *b* in the isolated LHC was determined to be 0.92. Chlorophyllase was extracted with 0.2% Triton X-100 in 50 mM potassium phosphate buffer (pH 7.0) from the acetone-dried thylakoid membranes of *Chrysanthemum cororarium* according to the methods of McFeeter et al. [16].

Preparation of chlorophyll-macromolecular complexes. The Chl-synthetic linear macromolecular complexes and Chl-BSA complexes were prepared by the new method, method A or B, devised by us [5]. The Chl-PVA and Chl-BSA complexes were prepared by the method A as follows. An organic solvent dissolving Chl was added dropwise to an aqueous macromolecular (PVA or BSA) solution with stirring, resulting in the composition of raw material as shown in Table I. The solution was evaporated to a dry green film under a reduced pressure (30 mm Hg) at room-temperature. A small amount of water was added onto the film, and stirred gently at 100 °C (PVA film) or at

TABLE I

COMPOSITION OF RAW MATERIALS, AND ABSORPTION AND FLUORESCENCE PEAKS OF Chl-SYNTHETIC LINEAR MACROMOLECULAR COMPLEXES, Chl-BSA COMPLEXES, Chl IN TRITON X-100 AND Chl IN ETHYL ETHER

The Chl complexes were prepared by the new method, method A or B, reported in our previous paper [5] using organic solvents during preparation process. The solution of which composition is shown in this table was evaporated until a dry film remained. The film was made into a green paste by stirring with a small amount of water, and diluted to a given concentration. Concentrations of Chl are 10 μg/ml for spectroscopic measurements. Molecular weights of polymers are as follows: PVP (Nos. 1–3), $M_{rw} = 32800$, $M_{rw}/M_{rn} = 2.08$; PVA (Nos. 4 and 5), $M_{rw} = 111400$, $M_{rw}/M_{rn} = 1.43$; PVA (No. 6), $M_{rw} = 39400$, $M_{rw}/M_{rn} = 1.37$; PEG (Nos. 7–9), $M_{rw} = 21000$, $M_{rw}/M_{rn} = 1.12$.

Sample number	Method	Composition of raw materials					Absorption at 298 K (λ_{max}, nm)	Fluorescence at 77 K (λ_{max}, nm)
		polymer (mg)	Chl *a* (μg)	Chl *b* (μg)	Organic solvent (ml)	water (ml)		
1	B	PVP 37	1000		EtOH 4.6		670	676
2	B	PVP 36		1000	EtOH 4.5		654	668
3	A	PVP 200	500		acetone 0.5	0.2	674	
4	A	PVA 200	1000		EtOH 0.5	2.0	672	
5	A	PVA 36		1000	EtOH 0.5	4.0	656	666
6	A	PVA 100	250		EtOH 0.25	1.0	673	
7	B	PEG 1120	1000		MeOH 11.0		672	
8	B	PEG 83		100	MeOH 11.0		653	
9	B	PEG 200	500		MeOH 6.0		666	
10	A	BSA 373	1000		EtOH 0.5	3.0	672	
11	A	BSA 184		500	EtOH 0.25	1.5	655	
12		Triton 54	500		Et_2O 2.0	25.0 (buffer)	667	674
13		Triton 54		500	Et_2O 2.0	25.0 (buffer)	649	655
14			100		Et_2O 10.0		660	664
15				100	Et_2O 10.0		642	647

room-temperature (BSA film) until a green paste was formed. The Chl-PVA paste was diluted with water and the Chl-BSA paste diluted with 0.1 M potassium phosphate buffer (pH 6.5) containing 0.2 M NaCl, to a given concentration of the Chl-PVA or Chl-BSA complex solution. They were finally filtered with a glass filter.

The Chl-PVP and Chl-PEG complexes were prepared by the method B as follows. An organic solvent dissolving Chl was added to a polymer/organic solv. solution with stirring, resulting in the composition of raw material as shown in Table I. The solution was evaporated to a dry film under a reduced pressure (30 mm Hg) at room temperature. A small amount of water was added onto the film, and stirred gently at room temperature. The paste was diluted with water and filtered. A sample (No. 3) of Chl-PVP complex, however, was made by method A.

Solubilization of Chl in Triton X-100. Ethyl ether dissolving Chl was added to 50 mM potassium phosphate buffer (pH 7.0) containing Triton X-100. The compositions of the mixture are shown in Table I in the case of sample Nos. 12 and 13. After the mixture was shaken violently, ethyl ether was removed by evaporation under a reduced pressure (30 mm Hg) with continuous shaking until no smelling of ethyl ether. The solution was diluted with the potassium phosphate buffer to a given concentration.

Analytical methods. Absorption spectra at room temperature were measured with a double-beam spectrophotometer, UVIDEC-510 (Japan Spectroscopic Co., Ltd.). Fluorescence emission spectra (uncorrected) at 77 K were measured with a Hitachi fluorescence spectrophotometer 850; excitation wavelengths were 430 nm and 460 nm for Chl *a* and Chl *b* samples, respectively, and a band width of 5 nm.

Gel chromatography. The aqueous solutions (2 ml) of Chl *a*-synthetic linear macromolecular complexes and those (2 ml) of the corresponding polymers were applied to a column (2.6 × 56.5 cm) of Sephadex G-100 equilibrated with distilled water, and eluted with distilled water at a flow rate of 0.2 ml/min. Concentrations of samples applied to the column were 10% wt/vol solution for PVP, PEG and their Chl *a* complexes, and 5% wt/vol solution for PVA and its Chl *a* complex. The content of Chl *a* involved in the eluted fraction was monitored by measuring the absorbance of red peak, and the amount of polymers by weighing the fractions after evaporation which contained sometimes a negligible content of Chl *a* in the case of Chl *a* complexes. The Chl *a* or *b*-BSA complex and BSA solutions (3 ml) were applied to a column (2.6 × 52.5 cm) of Toyopearl HW 60 (Fractogel TSK HW-60) equilibrated with 0.1 M potassium phosphate buffer (pH 6.5) containing 0.2 M NaCl. The procedure was according to the method described in the previous paper [10].

Photobleaching and hydrolysis of chlorophylls. The examination of photobleaching and hydrolysis of Chls in different states were performed according to the method described in the previous paper [10]. Photobleaching experiments were done under white light ($2.7 \cdot 10^2$ J · m^{-2} · s^{-1}) from a projector lamp.

The reaction mixture for determining the rates of hydrolysis of Chls by chlorophyllase, contained 50 mM potassium phosphate buffer (pH 7.5 for an aqueous system or pH 6.5 for 30% acetone system), 0.13 mg of chlorophyllase preparation as indicated above, 26 μM Chl of substrate and 1.4 mM L-ascorbic acid for prevention of possible oxidation of Chlide, in a total volume of 1.4 ml.

After incubation for 5 h at 30 °C in the dark, the amount of Chlide was determined according to the methods described in Ref. 17. The amount of Chl was determined spectroscopically, and the amount of protein according to Bramhall et al. [18].

Results and Discussion

Spectral properties of chlorphyll-synthetic linear macromolecular complexes

Fig. 1 shows the room-temperature (298 K) absorption spectra of Chls in different states, and Table I indicates the wavelengths of absorption peaks in red region. The Chl *a* or *b* in ethyl ether showed an absorption peak in red region at 660 or 642 nm, respectively. The Chl *a* or *b*-synthetic linear macromolecular complexes showed a red peak at longer wavelength by 10–15 nm than the Chl *a* or *b* in ethyl ether. This spectral feature was similar to that of Chl *a* or *b*-BSA complex. Further, the red peaks of the Chl *a* and *b* in the synthetic linear macromolecular complexes were similar to the peak of Chl *a* and to the shoulder of Chl *b* in the isolated LHC, respectively. The Chl *a* or *b* in 0.1% Triton X-100 (sample No. 12 or 13; molar ratio Chl/Triton X-100 = 0.007) showed a red peak at 667 or 649 nm, respectively, which was situated between the wavelength of a red peak of Chl *a* or *b* in ethyl ether and the wavelengths of Chl *a* or *b*-synthetic linear macromolecular complexes. It should be also noted in Table I that the sample (No. 9) of Chl *a*-PEG complex had a red peak at 666 nm, which is a remarkable short wavelength. This fact implies that the wavelength of the red peak of Chl *a*-synthetic linear macromolecular complexes can be shifted to a shorter one by regulating the preparation condition of complexes. In fact, the wavelength of 666 nm is the shortest one that has been observed so far for the red peak of Chl *a*-PEG and Chl *a*-PVP complexes.

Fig. 2 shows the low-temperature (77 K) fluorescence emission spectra of Chls in different states, and Table I indicates the wavelengths of fluorescence peak in red region. The Chl *a* or *b* in ethyl ether showed a fluorescence peak in red region at 664 or 642 nm, respectively. The Chl *a* or *b* in 0.1% Triton X-100 (sample No. 12 or 13) showed a fluorescence peak at 674 or 655 nm, respectively. The Chl *a* or *b*-PVP complex showed a fluorescence peak at 676 or 668 nm, respectively, and the Chl *b*-PVA complex has a peak at 666 nm. As can be seen in Fig. 2, the fluorescence spectra of these Chl *a* or *b*-synthetic linear macromolecular complexes were similar to those of the Chl *a* or *b* in ethyl ether and Triton X-100, though the peaks in red region of the Chl *a* or *b*-synthetic linear macromolecular complexes were of a 12 nm or 24–26 nm longer wavelength than those of the Chl *a* or *b* in ethyl ether, respectively.

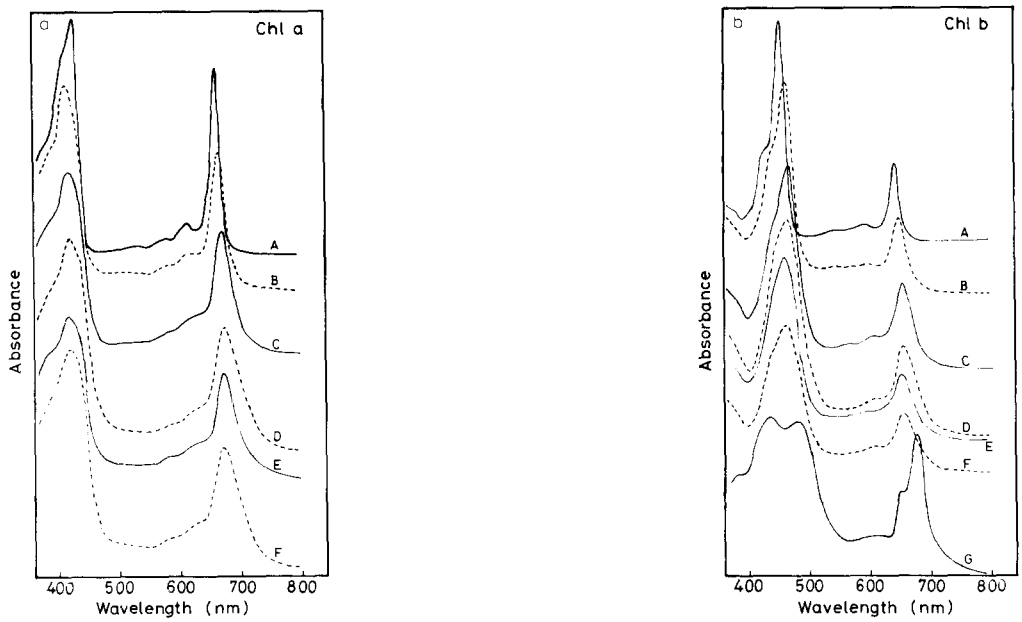

Fig. 1. (a) Room temperature (298 K) absorption spectra of Chl *a*-synthetic linear macromolecular complexes, Chl *a*-BSA complex, Chl *a* in ethyl ether, and Chl *a* in Triton X-100. Concentrations of Chl *a* are 10 μg/ml. The sample numbers indicated in Table I are shown in parentheses. (A) Chl *a* in ethyl ether (No. 14), (B) Chl *a* in 0.1% Trioton X-100 (No. 12), molar ratio of Chl *a*/Triton = 0.007, (C) Chl *a*-PVP complex (No. 1), (D) Chl *a*-PVA complex (No. 4), (E) Chl *a*-PEG complex (No. 7), (F) Chl *a*-BSA complex (No. 10). (b) Room temperature (298 K) absorption spectra of Chl *b*-synthetic linear macromolecular complexes, Chl *b*-BSA complex, Chl *b* in ethyl ether, Chl *b* in Triton X-100, and isolated LHC. Concentration of Chl *b* are 10 μg/ml. (A) Chl *a* in ethyl ether (No. 15), (B) Chl *b* in 0.1% Triton X-100 (No. 13), molar ratio of Chl *b*/Triton = 0.007, (C) Chl *b*-PVP complex (No. 2), (D) Chl *b*-PVA complex (No. 5), (E) Chl *b*-PEG complex (No. 8), (F) Chl *b*-BSA complex (No. 11), (G) isolated LHC.

The fluorescence spectra of the isolated LHC were found to have two prominent peaks; 682 and 697 nm in the case of excitation at 430 nm, 683 and 697 nm in the case of excitation at 460 nm. These peaks are considered to correspond to the absorption peak of Chl *a* and the absorption shoulder of Chl *b* within LHC (Fig. 1). Murata and Satoh [19] reported that LHC II had a fluorescence emission peak at 681 nm at 77 K.

The other samples of Chl *a* or *b*-synthetic linear macromolecular complexes, Chl *a* or *b*-PEG and Chl *a*-PVA complexes, showed the fluorescence emission spectra with a number of very broad peaks having only low fluorescence yields (Fig. 2). The Chl *a* or *b*-BSA complexes also showed the similar fluorescence spectral features. (Fig. 2). The fluorescence spectrum of the Chl *a*-BSA complex almost corresponded to that of the Chl *a*-BSA complex prepared by the old method, which had two prominent peaks, one at 672 and another at 740 nm [10].

From these results of spectral properties, it can be inferred that the Chl *a* or *b*-PVP and Chl *b*-PVA complexes contained the form of Chl *a* or *b* similar to that of Chl *a* or *b* in ethyl ether or in Triton X-100, probably a monomeric form. On the other hand, Chl *a* or *b*-PEG, Chl *a*-PVA and Chl *a* or *b*-BSA complexes had several different and undefined forms of Chl *a* or *b*. It is also emphasized that the Chl *a* or *b*-BSA complexes prepared by the new method were found to show the absorption and fluorescence emission spectra similar to those of the Chl *a* or *b*-BSA complexes obtained by the old method [10].

Gel chromatography

Fig. 3 shows the elution patterns of gel chromatography with Sephadex G-100 of the Chl *a*-

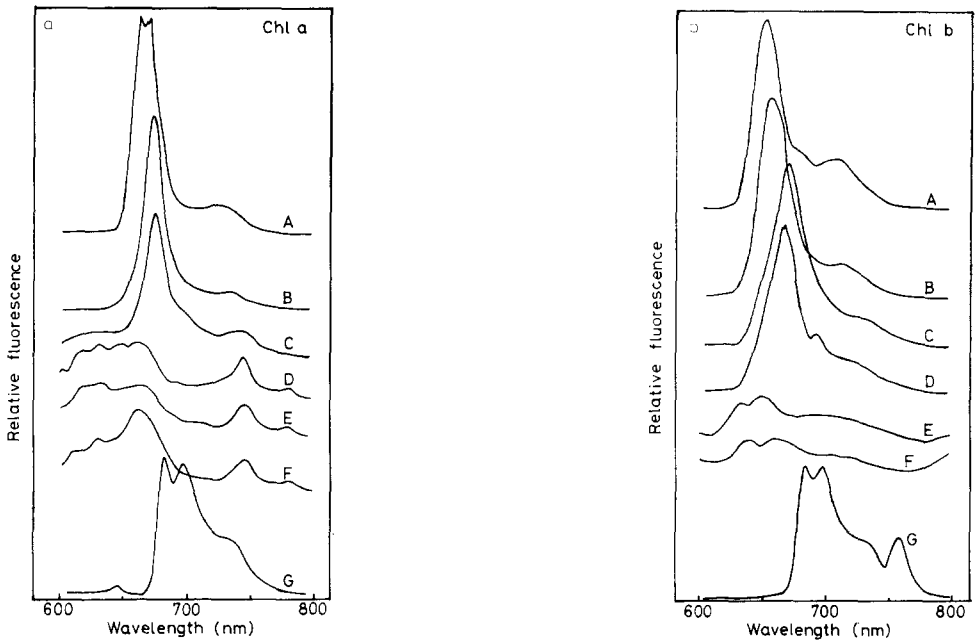

Fig. 2. (a) Low-temperature (77 K) fluorescence emission spectra of Chl *a*-synthetic linear macromolecular complexes, Chl *a*-BSA complex, Chl *a* in ethyl ether, Chl *a* in Triton X-100, and isolated LHC. Concentrations of Chl *a* are 10 μg/ml. The excitation wavelength was 430 nm. (A) Chl *a* in ethyl ether (No. 14), (B) Chl *a* in 0.1% Triton X-100 (No. 12), molar ratio of Chl *a*/Triton = 0.007, (C) Chl *a*-PVP complex (No. 1), (D) Chl *a*-PVA complex (No. 4), (E) Chl *a*-PEG complex (No. 7), (F) Chl *a*-BSA complex (No. 10), (G) isolated LHC. (b) Low-temperature (77 K) fluorescence emission spectra of Chl *b*-synthetic linear macromolecular complexes, Chl *b*-BSA complex, Chl *b* in ethyl ether, Chl *b* in Triton X-100, and isolated LHC. Concentrations of Chl *b* are 10 μg/ml. The excitation wavelength was 460 nm. (A) Chl *b* in ethyl ether (No. 15), (B) Chl *b* in 0.1% Triton X-100 (No. 13), molar ratio of Chl *b*/Triton = 0.007, (C) Chl *b*-PVP complex (No. 2), (D) Chl *b*-PVA complex (No. 5), (E) Chl *b*-PEG complex (No. 8), (F) Chl *b*-BSA complex (No. 11), (G) isolated LHC.

synthetic linear macromolecular complexes and the corresponding macromolecules. The elution pattern of the Chl *a*-PVP complex was quite different from that of Chl *a* or *b*-BSA complexes prepared by the old method [10], which is the same as the elution pattern of the Chl *a* or *b*-BSA complexes (sample No. 10 or 11) obtained by the new method in this study (data not shown). The Chl *a* consisting of Chl *a*-PVP complex was eluted coincidentally at the same volume of the corresponding polymer, PVP ($M_{rw} = 32\,800$) applied independently to the column, and it was eluted in somewhat latter fractions than PVP ($M_{rw} = 93\,000$). This indicates that PVP consisting of the Chl *a*-PVP complex was not an aggregated form but a monomeric form. As can be seen in the figure, the Chl *a*-PVP complex could not be separated from the unreacted PVP, though the PVP component of the complex solution was eluted in somewhat latter fractions than the Chl *a* consisting of Chl *a*-PVP complex.

The Chl *a*-PEG complex showed the elution pattern similar to that of the Chl *a*-BSA complex. The maximum concentration of Chl *a* consisting of the Chl *a*-PEG complex was observed in the former fraction (by nine fractions) than that of the corresponding PEG applied independently to the column. This suggests that PEG consisting of the Chl *a*-PEG complex was an aggregated form. It is also seen in this figure that the Chl *a* consisting of the Chl *a*-PEG complex was eluted in previous fractions than the PEG component of the complex solution which contained both the reacted and unreacted PEG. The Chl *a*-PEG complex was separated from the unreacted PEG in the range of fractions 23–26, because in these fractions the concentration of the PEG component of the complex solution was almost zero and it increased

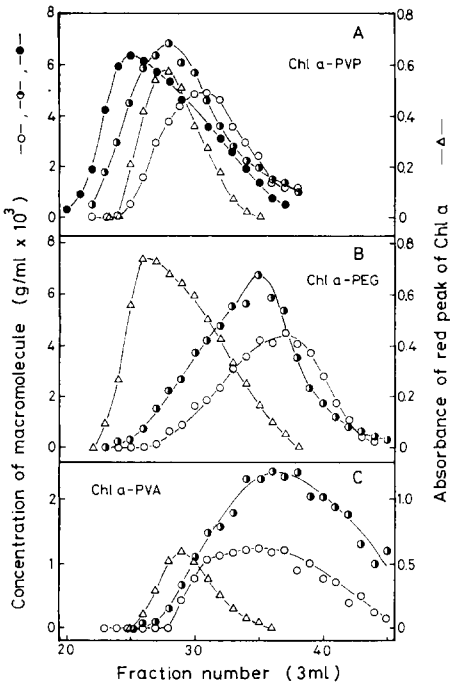

Fig. 3. Elution patterns of column chromatography with Sephadex G-100 of Chl *a*-synthetic linear macromolecular complexes and the corresponding macromolecules. The aqueous solution (2 ml) of sample was applied to a column (2.6×56.5 cm) and eluted with water at a flow rate of 0.2 ml/min. Concentrations of samples applied to the column were 10% wt/vol-solution for PVP, PEG and their Chl *a* complexes, or 5% wt/vol-solution for PVA and its Chl *a* complex. (A) Chl *a*-PVP (M_{rw} = 32800, M_{rw}/M_{rn} = 2.08) complex (No. 3): △, Chl *a* consisting of the complex; ○, PVP component of the complex solution; ◑, PVP (M_{rw} = 32800, M_{rw}/M_{rn} = 2.08); ●, PVP (M_{rw} = 93000, M_{rw}/M_{rn} = 3.44). (B) Chl *a*-PEG (M_{rw} = 21000, M_{rw}/M_{rn} = 1.12) complex (No. 9): △, Chl *a* consisting of the complex: ○, PEG component of the complex solutioni; ◑, PEG (M_{rw} = 21000, M_{rw}/M_{rn} = 1.12). (C) Chl *a*-PVA (M_{rw} = 39400, M_{rw}/M_{rn} = 1.37) complex (No. 6): △, Chl *a* consisting of the complex; ○, PVA component of the complex solution; ◑, PVA (M_{rw} = 39400, M_{rw}/M_{rn} = 1.37).

gradually from fraction 27, indicating the beginning of elution of the unreacted PEG. A narrow distribution of molecular weight of PEG judging from a small value of M_{rw}/M_{rn} seems to favor our interpretation of the elution pattern of Chl *a*-PEG complex. The molecular number of Chl *a* bound to PEG complex expressed on one PEG molecular basis could not yet be determined. It would be determined by measuring more precisely the concentrations of PEG and Chl *a*. Further, the molecular weight of Chl *a*-PEG complex should be measured in future.

The Chl *a*-PVA complex showed the elution pattern similar to that of Chl *a*-PEG complex (Fig. 3c). Therefore, it is inferred that PVA consisting of the Chl-PVA complex was also an aggregated form.

Stability of chlorophylls in the synthetic linear macromolecular complexes against photobleaching

Fig. 4 shows the result of photobleaching of Chl *a* or *b* in different states by irradiation of white light. Illumination caused the marked photobleaching of Chls in acetone, ethanol or Triton X-100, whereas the Chl *a* or *b* in synthetic linear macromolecular complexes were relatively stable, as well as Chls in BSA complexes or LHC.

The photodestruction of Chls under illumination is considered to be caused by singlet oxygen [20,21], so the result described above suggests that the synthetic linear macromolecules may serve as a barrier to protect the tetrapyrrole portion of Chl, the chromophore of Chl, from the singlet oxygen, as well as the globular protein of BSA [10]. The mechanism of protection is considered that the tetrapyrroline ring was surrounded by the synthetic linear macromolecular chains. Further, in view of the results of gel chromatography, it is inferred that the tetrapyrrole ring was surrounded by a linear polymer chain in the case of Chl *a* or *b*-PVP complex, or by a number of linear polymer chains in the case of Chl *a* or *b*-PEG and Chl *a* or *b*-PVA complexes. The relatively high photodegradation of Chl *b* in PVP complex (Fig. 4b) is probably due to a relatively weak protective action of only one polymer chain. It is also interesting to note that an addition of PVP to Chl *a*/EtOH solution had no protective effect on the photodegradation of Chl *a* (Fig. 4a). This implies that the appearance of the protective effects requires a considerably strong interaction of macromolecular chains with tetrapyrrole ring of Chl.

Hydrolysis of chlorophylls in the synthetic linear macromolecular complex by chlorophyllase

Because chlorphyllase hydrolyzes Chls to chlorophyllide (Chlide) and phytol at the carbonyl group of the propionic acid side chain of tetrapyr-

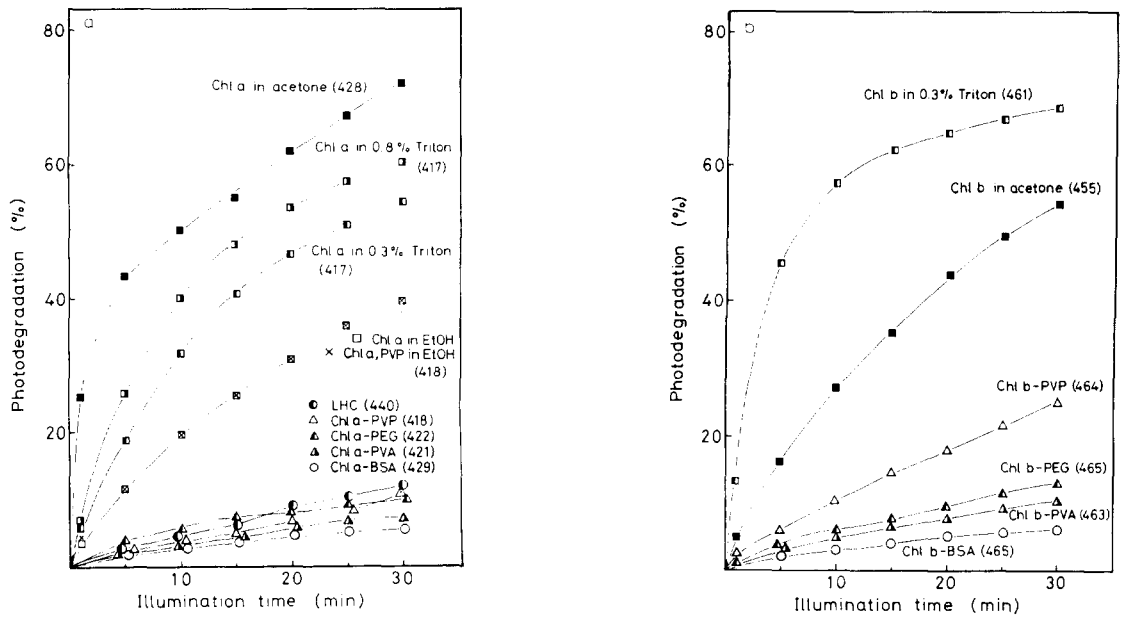

Fig. 4. Photobleaching of Chls in different by irradiation of white light ($2.7 \cdot 10^2$ J·m^{-2}·s^{-1}). The wavelengths monitoring the photobleaching are shown in parentheses. The initial absorbance of blue peak of Chl was adjusted to be 0.5. The Chl-synthetic linear macromolecular complexes were tested in water, while the Chl-BSA complexes and LHC were in 0.1 M potassium phosphate buffer (pH 6.5). The sample numbers of Chl-synthetic linear macromolecular complexes and Chl-BSA complexes are the same as those in Figs. 1 and 2. ×, Chl *a* dissolved in ethanol which dissolved PVP at the concentration equivalent to that of PVP component of Chl *a*-PVP complex (△) solution.

role ring IV, to which phytol is attached, the action of this enzyme would give an information on the localization of this site of Chl in the Chl-synthetic linear macromolecular complexes. If the site was surrounded by synthetic linear macromolecular chains, the rate of hydrolysis would be low or negligible.

Table II shows the results of hydrolysis of Chls in different staes by chlorophyllase. Though the Chl *a* or *b* in 30% acetone was only slowly hydrolyzed by chlorophyllase, the Chl *a* or *b* in synthetic linear macromolecular complexes were rapidly hydrolyzed by chlorophyllase, as well as the Chl *a* or *b* in BSA complexes, LHC and Triton X-100, the results of which were well consistent with those obtained in the previous investigation [10]. Therefore, it could be induced that the hydrophilic edge of porphyrin ring of Chls in the synthetic linear macromolecular complexes was not surrounded by linear polymer chains but exposed to the environment of a water medium.

It is also shown in Table II that the Chl *a*-synthetic linear macromolecular complexes in 30% acetone had the relative activities of chlorophyllase hydrolysis of 2.6–4.5, which were greater than 1.0 for the Chl *a* in 30% acetone and less than 4.0–6.5 for the Chl *a*-synthetic linear macromolecular complexes in a potassium phosphate buffer (pH 7.5). Because the Chl *a*-synthetic linear macromolecular complexes showed little change of absorption spectra by changing their medium from the potassium phosphate buffer to 30% acetone, the binding of Chl *a* with linear polymer chains was not considered to be destroyed even in the medium of 30% acetone. It was revealed therefore that the Chl *a*-synthetic linear macromolecular complexes were more rapidly hydrolyzed by chlorophyllase than the uncomplexed Chl *a* in the common medium of 30% acetone, and the Chl *a*-linear macromolecular complexes in 30% acetone were more slowly hydrolyzed than those in the potassium phosphate buffer. It is also shown in this table that the rate of hydrolysis of Chl *a* or *b* in Triton X-100 became lower as increasing of the

TABLE II

HYDROLYSIS OF CHLOROPHYLLS IN ACETONE, TRITON X-100, SYNTHETIC LINEAR MACROMOLECULAR COMPLEXES, BSA COMPLEX AND LHC BY CHLOROPHYLLASE

The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5 for an aqueous system or pH 6.5 for 30% acetone system), 0.13 mg of chlorophyllase preparation as indicated in the text, 26 μM Chl of substrate and 1.4 mM L-ascorbic acid for prevention of possible oxidation of Chlide, in a total volume of 1.4 ml. In the case of the substrate of Chl in acetone and Triton X-100, the reaction mixture contained also 30% vol-acetone/vol-total mixture and 0.2–0.8% vol-Triton/vol-total mixture, respectively. The individual substrates of Chl were incubated with chlorophyllase in the reaction mixture for 5 h at 30 °C in the dark, then the formed Chlide *a* and/or *b* were determined as described in the text.

Substrate	Activity (pmol Chlide per min per mg protein)		Relative activity
Chl *a* in 30% acetone	Chlide *a*	14.6	1.0
Chl *a* in 0.2% Triton X-100		85.6	5.8
Chl *a* in 0.3% Triton X-100		76.4	5.2
Chl *a* in 0.5% Triton X-100		64.7	4.4
Chl *a* in 0.8% Triton X-100		59.1	4.0
Chl *a*-PVP complex (No. 1)		59.4	4.0
Chl *a*-PVA complex (No. 4)		80.6	5.5
Chl *a*-PEG complex (No. 7)		96.2	6.5
Chl *a*-BSA complex (No. 10)		90.4	6.1
Chl *a*-PVP complex (No. 1) in 30% acetone		48.5	3.3
Chl *a*-PVA complex (No. 4) in 30% acetone		38.9	2.6
Chl *a*-PEG complex (No. 7) in 30% acetone		67.1	4.5
Chl *b* in 30% acetone	Chlide *b*	31.3	1.0
Chl *b* in 0.2% Triton X-100		66.4	2.1
Chl *b* in 0.3% Triton X-100		60.8	1.9
Chl *b* in 0.5% Triton X-100		57.6	1.8
Chl *b* in 0.8% Triton X-100		52.7	1.6
Chl *b*-PVP complex (No. 2)		81.5	2.6
Chl *b*-PVA complex (No. 5)		62.8	2.0
Chl *b*-PEG complex (No. 8)		78.5	2.5
Chl *b*-BSA complex (No. 11)		131.2	4.2
LHC	Chlide *a*	80.0	
	Chlide *b*	57.6	

this table that the rate of hydrolysis of Chl *a* or *b* in Triton X-100 became lower as increasing of the concentration of Triton X-100. This is probably due to a structural change of the micell, since the concentrations of Triton X-100 were greater than the critical micelle concentration, 0.015% (0.24 mM), in all cases.

From the results of the present study, it was concluded that a possible localization of Chls within the synthetic linear macromolecular complexes was as such that the tetrapyrrole ring of Chl was surrounded by a linear macromolecular (PVP) chain or by a number of linear macromolecular (PEG or PVA) chains, whereas the hydrophilic edge of porphyrin ring, adjacent to the phytol group, was exposed to the environment of a water medium. Further, it can be inferred that the phytol group of Chl was also surrounded by the synthetic linear macromolecular chains from the fact that the hydrophobic interaction played an important role in the binding of Chls with the synthetic linear macromolecular chains [3,4].

Our interpretation of the localization of Chls within synthetic linear macromolecular complexes seems to support the model originally advanced by Anderson [11,12] for the localization of Chl within intrinsic proteins. She described that phytol could be associated with the hydrophobic exterior of the intrinsic proteins, one edge of tetrapyrrole macrocycle, adjacent to phytol, would interact at the aqueous membrane surface with the hydrophilic region of the intrinsic protein giving the porphyrin ring to tilt, and the more hydrophobic portion of porphyrin would thus extend further into the hydrophobic region of protein. Recently, Murphy [2] presented the schematic model of the possible mode of association of Chl molecules with the membrane-spanning α-helical region of pigment protein. The outline of this model is as follows: (1) the tetrapyrrole ring system of Chl is oriented at an angle of 30° to the bilayer normal [22,23]; and (2) interacts with the hydrophobic α-helical region of protein having a similar orientation [24] in the thylakoid; (3) Chl molecules span only half of the thylakoid bilayer with the hydrophilic edge of tetrapyrrole ring facing to the aqueous membrane surface; and hence (4) it is possible to fit two Chl molecules into each interhelical domain; (5) the phytol chains are likely oriented to the bilayer normal where they may interact with acyl lipids. Our interpretation of the localization of Chls in the synthetic linear macromolecular complexes seems to be consistent with this model described above. It is also concluded that the property of the Chl-BSA complexes prepared by the new method was almost the same as

that of those obtained by the old method.

It is very interesting to determine the numbers of Chl *a* or *b* bound to the synthetic linear macromolecular complexes expressed on one linear polymer chain basis. Also, the molecular weights of the Chl-synthetic linear macromolecular complexes and Chl-BSA complexes will directly provide valuable information on the size of complex structures. These investigations are now in progress.

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References

1 Markwell, J.P., Thornber, J.P. and Boggs, R.T. (1979) Proc. Natl. Acad. Sci. USA 76, 1233–1235.
2 Murphy, D.J. (1986) Biochim. Biophys. Acta 864, 33–94.
3 Inamura, I., Toki, K. and Araki, T. (1980) Chem. Lett. 1980, 1481–1484.
4 Inamura, I., Ochiai, H., Toki, K., Watanabe, S., Hikino, S. and Araki, T. (1983) Photochem. Photobiol. 38, 37–44.
5 Inamura, I., Ochiai, H., Toki, K., Ishine, Y. and Araki, T. (1985) Chem. Lett. 1985, 381–384.
6 Inamura, I., Ochiai, H., Toki, K. and Araki, T. (1984) Chem. Lett. 1984, 1787–1790.
7 Uehara, K., Shibata, K. and Tanaka, M. (1985) Chem. Lett. 1985, 897–900.
8 Koyama, Y., Umemoto, Y., Akamatsu, A., Uehara, K. and Tanaka, M. (1986) J. Mol. Struct. 146, 273–287.
9 Inamura, I. (1986) Kagaku to Seibutsu (Japan) 24, 702–704.
10 Shibata, H., Ochiai, H., Kawashima, T., Okamoto, T. and Inamura, I. (1986) Biochim. Biophys. Acta 852, 175–182.
11 Anderson, J.M. (1975) Nature 253, 536–537.
12 Anderson, J.M. (1975) Biochim. Biophys. Acta 416, 191–235.
13 Chen, R.F. (1967) J. Biol. Chem. 242, 173–181.
14 Omata, T. and Murata, N. (1983) Plant Cell Physiol. 24, 1093–1100.
15 Burke, J.J., Ditto, C.L. and Arntzen, C.J. (1978) Arch. Biochem. Biophys. 187, 252–263.
16 McFeeter, R.F., Chichester, C.O. and Whiteaker, J.R. (1971) Plant Physiol. 47, 609–618.
17 Tanaka, K., Kakuno, T., Yamashita, J. and Horio, T. (1982) J. Biochem. 92, 1763–1773.
18 Bramhall, S., Noack, N., Wu, M. and Loewenberg, J.R. (1969) Anal. Biochem. 31, 146–148.
19 Murata, N. and Satoh, K. (1986) in Light Emission by Plants and Bacteria (Govindjee, Amesz, J. and Fork, D.C., eds.), pp. 137–159, Academic Press, New York.
20 Koka, P. and Song, P.-S. (1978) Photochem. Photobiol. 28, 509–515.
21 Asada, K., Takahashi, M., Nakano, Y. and Shimada, S. (1982) in Oxygenases and Oxygen Metabolism (Nozaki, M. et al., eds.), pp. 255–266, Academic Press, New York.
22 Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R. (1984) J. Mol. Biol. 179, 125–142.
23 Breton, J. and Nabedryk, E. (1984) FEBS Lett. 176, 355–359.
24 Tapie, P., Choquet, Y., Breton, J., Delepelaire, P. and Wollman, F.-A. (1984) Biochim. Biophys. Acta 767, 57–69.