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Interaction of Colloidal Chitin and Chlorophyll a in Ethanol–Water Mixed Solutions

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The interaction of colloidal chitin [a polysaccharide consisting of β-(1-4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues] and chlorophyll a (chl.a) in ethanol–water mixed solutions was investigated by means of vis and UV absorption spectroscopy. The observed facts and discussions are as follows: (i) In ethanol (10%)–water mixed solutions, the reaction rate for complex formation between colloidal chitin and chl.a shows an oscillatory dependence on their concentration ratio. At specific ratios of $7.4\times1, 2, 4, 8\cdots$ of sugar units of chitin to one chl.a molecule, a host–guest complex of colloidal chitin with chl.a is produced. (ii) Another oscillatory phenomenon on the spectral bands of chl.a in the solution is also found as a function of the ethanol concentration. This phenomenon is discussed in terms of cluster formation of ethanol and water molecules in binary mixed solvents. (iii) Upon increasing the ethanol concentration, the host–guest complex of colloidal chitin with chl.a begins to collapse when the ethanol concentration in the solution exceeds a critical value up to which the hydrophilic/hydrophobic hydration equilibrium of the system is attained.

In a previous paper[1)] we reported on the formation of a stable gel of calcium alginate including chlorophyll a (chl.a). The preparation method of the gel[2)] was originally developed in our laboratory while taking into account former investigations on the binding properties of the inclusion compounds, which were formed of polysaccharides, such as alginate or alginic acid and chitin with metal ions, dyes, cholesterol, and radioactive iodine.[3—6)]

In the alginate/chl.a system, an inclusion compound of calcium alginate with chl.a was formed in $MeOH/H_2O$ or $EtOH/H_2O$ solutions. One significant finding regarding this system was that the gel color changed to light green in a solution with an $EtOH/H_2O$ solution having a crucial ratio. This was interpreted as meaning that the water content in the binary mixed solvent had a crucial role in the formation of the gel, since polysaccharide hydrogels are generally composed of a fibrous solid phase, forming a framework with many pores, which are formed by cross-linking of the molecular chain[7)] and act as a container of water molecules.

In the present experiment, we took chitin instead of alginate in order to prepare a simple system for investigating the interaction of saccharide gel with chl.a. Chitin is a polysaccharide consisting of β-(1-4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues, and is a linear-chain compound which maintains a rigid structure with the aid of two kinds of hydrogen bonds. When the strong hydrogen bonds of chitin molecules are weakened, a gel or colloid is produced. Concerning the interaction of chitin with dyes, Mckay et al.[8)] proposed a formation mechanism based on the direct adsorption of dye molecules on chitin chains. However, on the basis of our observation in the alginate/chl.a system, the adsorption mechanism is hardly accepted. This also motivated the present experiment concerning the chitin/chl.a system.

In the first part of this paper, we present the vis and UV absorption spectra of $EtOH/H_2O$ solutions of colloidal chitin and chl.a with various ratios of the sugar unit of chitin vs. chl.a. We found the formation of the precipitate of chitin/chl.a with specific ratios of the sugar unit/chl.a. In the later part, we examined the effect of the water content in an $EtOH/H_2O$ mixed solvent. This enabled us to discuss the host–guest reaction in terms of the equilibrium between hydrophilic and hydrophobic hydrations of the system.

Experimental

An aqueous solution of colloidal chitin, which was prepared according to the following procedure, was mixed with an ethanol solution of chl.a while maintaining a given ratio of the sugar unit/chl.a as well as a given ratio of $EtOH/H_2O$. After precipitation in the solution, the absorption spectrum of the supernatant was taken using a spectrophotometer (Hitachi U-2000).

Preparation of Colloidal Chitin: An aliquot of 5 g of granular chitin (Wako Pure Chemical Co., Ltd.) was dispersed slowly in 200 ml of hydrochloric acid under 5 °C with stirring. The mixture was then gradually heated to 37 °C. After leaving the mixture at 37 °C for 2—3 min, a suspension showing a slight turbidity consisting of chitin was filtered with a G3 glass filter. The filtrate was added into 800 ml of water at 5 °C or lower under stirring. The mixture was stirred for an additional 30 min, and then left in a refrigerator overnight. The generated precipitate was collected by centrifugation and rinsed with water until the suspension became neutral in pH. The whole precipitate was dispersed in 200 ml of water. Its accurate concentration was calculated from the weight of the residue obtained by drying an aliquot of the suspension under reduced pressure.

Materials: Chlorophyll a, isolated from spilurina for biochemical use, was obtained from Wako Pure Chemical Industries, Ltd. Chitin (powder form) isolated from the carapace of the crab was obtained from the same company.

Mixing Ratio of Chitin and Chl.a. To 9 ml of the mixture of colloidal chitin solution and water, 1 ml of 5×10^{-4} mol dm^{-3} chl.a–ethanol solution was added, so that the mixture with the ethanol concentration of 10% with the following ratios of chitin and chl.a (sugar unit number per one chl.a) were prepared: 3.7×(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, and 20). A value of 3.7 was eventually chosen, but 7.4 (3.7×2) was a physically meaningful value. The mixing ratio is discussed in the next section. The absorption spectra of the mixtures were observed 0, 1, 2, and 24 h after mixing. After producing a precipitate, the spectra of the supernatant was measured.

Ethanol Concentration. By the above-mentioned experiment, the optimal mixing ratio of chitin and chl.a was obtained, judging from the color of the precipitate and the sedimentation velocity. Referring to the ratio of 7.4 : 1, the concentration of ethanol in the ethanol–water solution was changed from 30—70% by 1% pitch. The absorption spectra of these mixtures were determined 0, 1, 2, 3, and 24 h after mixing. The concentration was expressed assuming that there was no change in the volume ascribed to mixing.

Results and Discussion

Inclusion Complex of Chitin and Chl.a. When an aqueous solution of colloidal chitin was mixed with a chl.a–ethanol solution, a green precipitate was formed, which was stable only under the dark condition. The precipitate formed from the colloidal chitin + chl.a, maintained its green color in a 10% ethanol–H_2O solution, even under room light, only when the molar ratio of chitin sugar unit to chl.a was adjusted to be 7.4 : 1. This implied that the chitin/chl.a precipitate of the above specific ratio took a stabilized structure comprising chl.a/included H_2O/chitin sugar units. If the molar ratio of chitin sugar units to chl.a was chosen to be 2, 4 or 8 times 7.4, stable precipitate was also produced in a 10% EtOH/H_2O solution, indicating the formation of an inclusion complex of 7.4×n chitin sugar units/chl.a. Hereafter, this ratio is called csu/ch. The specific ratio of the precipitate has also been confirmed by using another chitin (flake form).

Figure 1 shows the observed spectra of ethanol–water solutions of chl.a mixed with colloidal chitin of the csu/ch of 3.7×1, 2, 3, 4, and 5 one hour after mixing. The spectrum of the supernatant containing chl. a with 3.7×1 csu/ch was almost the same as those of the ethanol–water solution of chl.a. This is interpreted as meaning that almost no included complex between chl.a and chitin was formed under this condition. However, in the 3.7×2 csu/ch, the absorption bands of chl.a in the solution became very weak, indicating that most of chl.a in the solution combined with colloidal chitin to form the included complex. If the content of the colloidal chitin was increased in the solution, the spectrum showed the absorption band of chl.a again. However, the peak wavelengths of the bands shifted to the red-side, and, furthermore, their absorbances changed periodically as a function of csu/ch. For example, in a mixture of the 3.7×4 csu/ch, the amount of chl.a remaining in the solution decreased significantly, indicating the formation of another stable included complex. The observed red-shifts of the bands could be attributed to the fact that the chl.a in the solution takes a hydrated or aggregated structure, which will be evidenced in the later part of this paper. In Fig. 1, it can be seen that the spectrum of a mixture of 3.7×5 csu/ch is almost the same as that of the 3.7 csu/ch mixture, except for a small red-shift of the absorption band. Thus, no in-

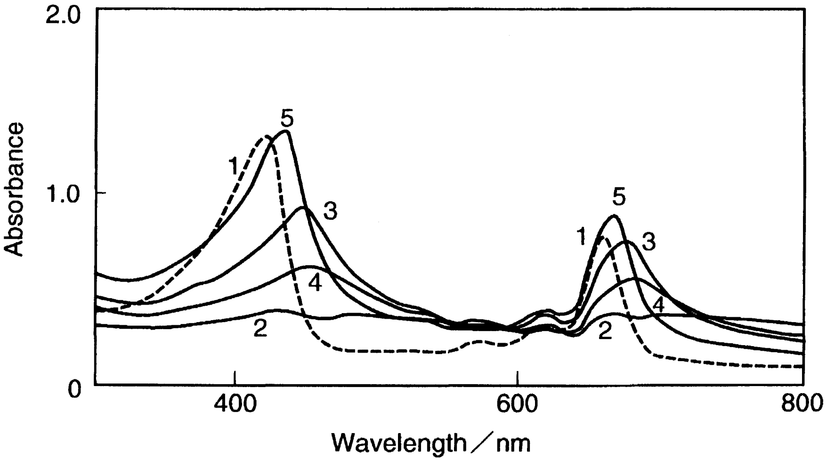

Fig. 1. Absorption spectral changes of 10% ethanol containing colloidal chitin and chl.a in the mixing ratios of 1=3.7×1 : 1, 2=3.7×2 : 1, 3=3.7×3 : 1, 4=3.7×4 : 1, and 5=3.7×5 : 1.

cluded complex was formed in this mixture.

Figure 2 summarizes the absorption spectral shifts and intensity changes of chl.a as a function of the csu/ch ratio. The observed spectral peaks are two typical bands of chl.a, i.e., Soret band and II band (red absorption band) around 420 and 660 nm, respectively. At csu/ch ratios of 7.4, 7.4×2, 7.4×4, and 7.4×8, we note a distinct reduction of the absorption intensities in both bands of the solution, as well as marked shifts of the spectral peaks to the longer wavelength side. The changes in spectra are thus periodic as a function of the csu/ch ratio. In solutions with the above ratios, green precipitates were formed immediately after mixing. The formation of the precipitate occurred most rapidly in a solution with the ratio of 7.4 : 1.

It is said that the central Mg atom of chl.a is electrophilic, i.e., coordinatively unsaturated, and hence has a strong tendency for one or both of the Mg axial positions to be occupied by an electron-pair donor ligand. If the donor is a bifunctional ligand, then cross-linked chl.a species, which may be of colloidal dimensions, can be formed. Water is a particularly important bifunctional ligand, since the oxygen atom can be coordinated to the Mg atom of one chl.a molecule, and the two hydrogen atoms are then available for hydrogen bonding to one (or possibly two) other chl.a molecule. These coordination interactions result in water-linked dimers, $(\text{chl.a H}_2\text{O})_2$. They can also lead to the formation of higher chl.a–water adducts like $(\text{chl.a H}_2\text{O})_n$[9] which show a more red-shifted absorption band at approximately 740 nm, and whose structure is probably similar to the molecular networks of a monolayer characterized in an ethyl chlorophyllide a dihydrate crystal.[10] The aggregation of chl.a with hydration is enhanced by the photoabsorption of chl.a because photoexcited chl.a serves as an electron donor. Moreover, in our system containing colloidal chitin, at the above-mentioned specific ratio the interaction between a colloidal chitin and chl.a would have taken priority in the formation of a green complex. All chl.a–water adducts have been found to be photoactive. Thus, the spectral changes of chl.a remaining in the solution give us significant information about the formation of a chitin–chl.a complex with a ratio of 7.4 : 1.

Another possible explanation for the red-shifts is the formation of self-aggregates of chl.a, i.e., the formation of $(\text{chl.a})_n$, where $n \geqq 2$. In this case, the C-13 keto carbonyl of one chl.a molecule can function as an electron donor to the Mg atom of another chl.a molecule.[10,11]

Addition of Ethanol under Water-Rich Condition. Figures 3 and 4 show the absorption spectra of csu/ch of 7.4 observed in ethanol solutions from 30 to 70% in concentration. In a 44% ethanol solution (the upper frame of Fig. 3) the absorption peak in the ultraviolet band disappears. However, the Soret and II bands shift to much longer wavelength sides and a shoulder appears in the shorter wavelength side of the Soret peak. The precipitate obtained from the 44% solution shows

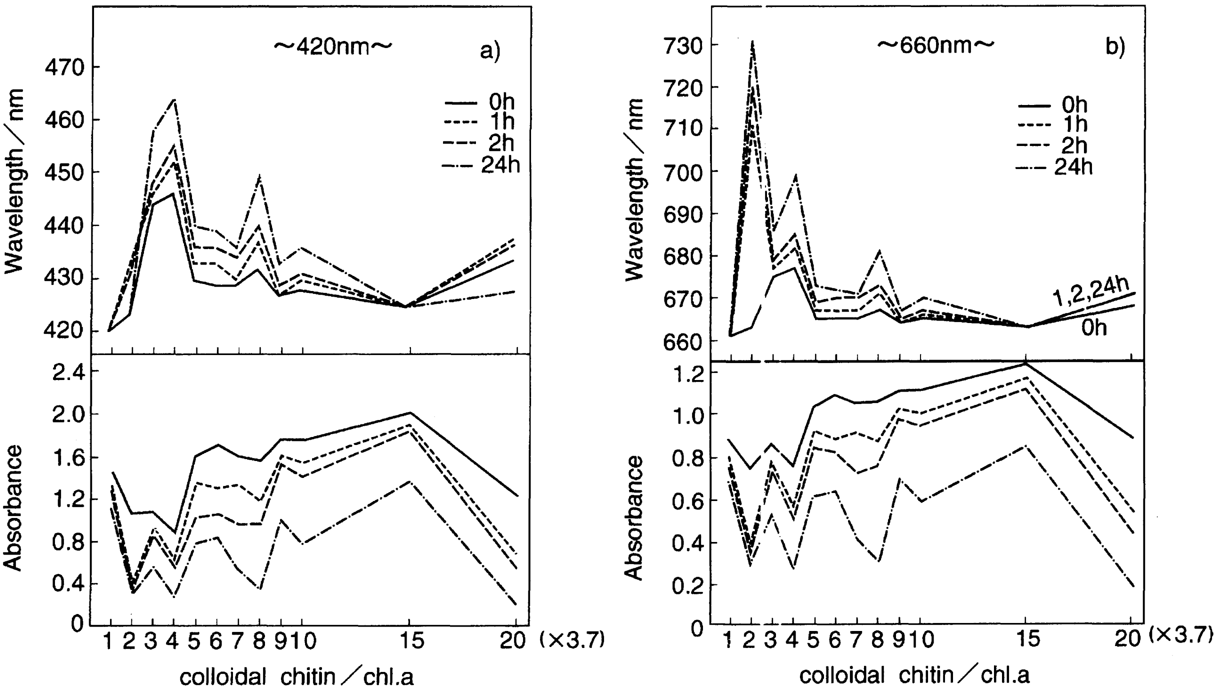

Fig. 2. Absorption spectral changes of 10% ethanol containing colloidal chitin and chl.a in the mixing ratios of 3.7×1,2,3⋯10,15,20 : 1. a) wavelength of the absorption maximum (λ_{max}) and the absorbance of Soret band around at 420 nm. b) λ_{max} and the absorbance II band around at 660 nm. When two peaks appear, the higher one is adopted here.

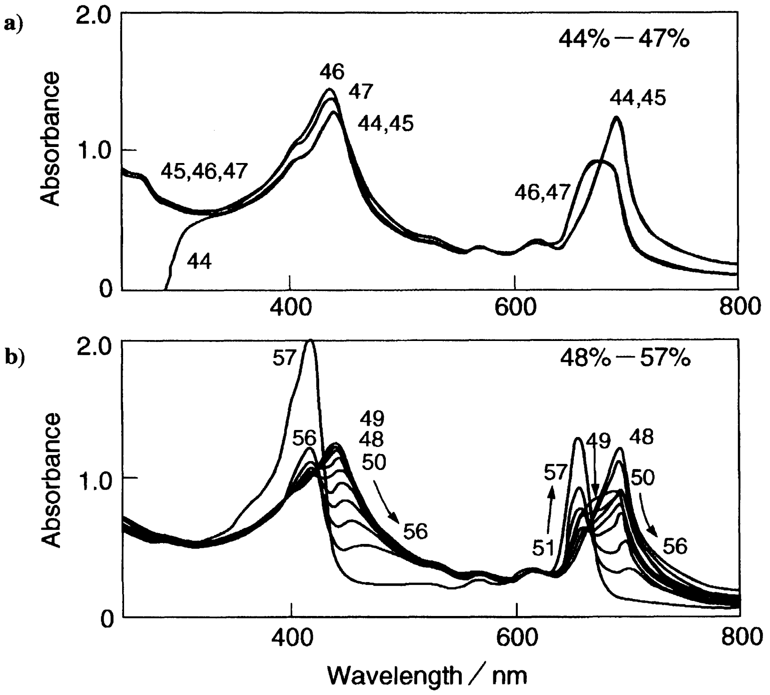

Fig. 3. Absorption spectral changes of 44—57% ethanol containing colloidal chitin and chl.a in the mixing ratios of 7.4 : 1 at 0 h after mixing. a) 44—48% ethanol. b) 49—57% ethanol.

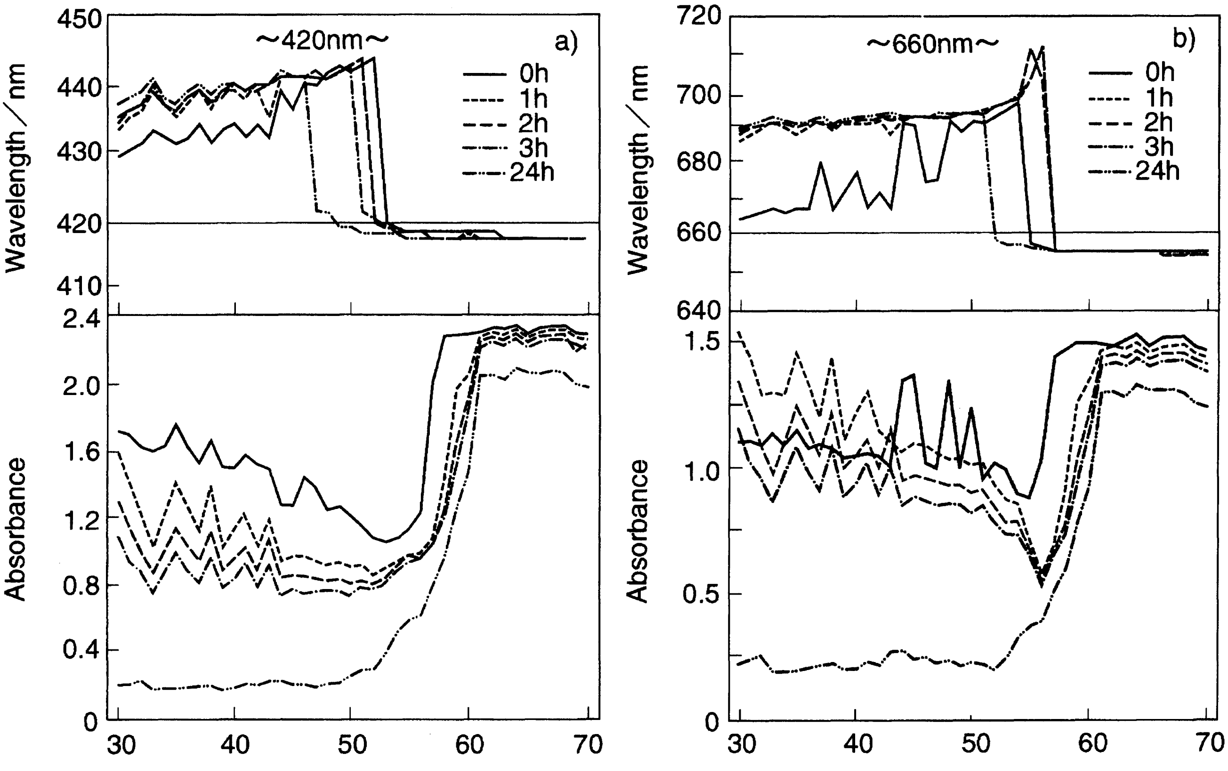

Fig. 4. Absorption spectral changes of 30—70% ethanol containing colloidal chitin and chl.a in the mixing ratios of 7.4 : 1 at 0,1,2,3, and 24 h after mixing. a) wavelength of the absorption maximum ($\lambda_{\max}$) around at 420 nm, b) $\lambda_{\max}$ around at 660 nm. When two peaks appear, the higher one is adopted here.

the brightest green in color among those obtained from solutions of the other ethanol concentrations. This indicates a new equilibrium system of chl.a/EtOH/H_2O formed in the 44% solution.

It can be seen from Figs. 4a and 4b that the spectral shifts and the absorbances of two bands of chl.a change periodically as a function of the ethanol concentration. For example, the red shifts of the Soret band and II band show an oscillatory behavior, and the maximum shift is found at 44—45%. This value corresponds to the cluster formation of [$EtOH\cdot 4H_2O$] or [$EtOH\cdot 8H_2O\cdot EtOH$], since this volume concentration corresponds to the above molecular ratio of EtOH and water. The formation of ethanol hydrate was suggested by Erdey-Gruz et al.,$^{12)}$ who predicted the existence of $EtOH\cdot nH_2O$ (n=4 or 8) in the mixture.

Thus, we speculate that these cluster formations cause a change of the hydrated structure of chl.a in the mixture. In alcohol–water mixed solvents, with decreasing the water content, the ligand-phase structure of water begins to collapse, forming a new type of the water–alcohol structure. In a dehydrated condition, that is, in the ethanol-rich solution, a cross-linked network of chl.a can be formed, as evidenced by the red-shift of II band,$^{11)}$ which is a two-dimensional aggregate of chl.a linked by water molecules. The existence of colloidal chitin in the solution would take some part to realize a dehydrated condition for chl.a molecules near to the chitin molecule, and would accelerate their aggregation on the colloidal chitin to produce a chl.a molecules + colloidal chitin adduct.

Equilibrium State of Hydrophilic/Hydrophobic Hydration. In solutions of more than 50% ethanol, the observed red-shifts of both bands are very large, as shown in Fig. 3. In the 50% ethanol solution, both of the Soret band and the II band split into doublet peaks. As the ethanol fraction increases, the red-side peak loses its intensity, while the blue-side peak grows. These facts indicate that there are two types of hydrated forms of chl.a: one in an ethanol-rich solution and the other in a water-rich solution. According to Agostiano et al.$^{13)}$ the observed II absorption maxima below 660 nm imply the existence of $(\mathrm{chl.a}\cdot 2H_2O)_n$. Therefore, the blue shift from ca. 690 to lower than 660 nm in Fig. 4b can be evidence for formation of dihydrated chl.a aggregates. This hydration of chl.a formed in the ethanol-rich solution can be assigned to be of the hydrophobic type. Another type of hydrated form of chl.a which gives a red-shifted peak is attributed to a hydrophilic interaction.

The transition from the hydrophilic to the hydrophobic hydration is found to start at 52—53% ethanol concentration, judging from the wavelength position of the Soret band. However, from that of the II band, as seen from Fig. 4b, the transition occurs at an ethanol concentration of 56—57%. The critical concentration is lower than that observed from the change in the II absorption band, namely, 56%. At 57% ethanol, just after mixing, the blue-shift of the spectrum could be caused by a hydrophobic hydration. Both of the peaks assigned to be hydrophilic hydration and hydrophobic hydration clearly appear in the 50% ethanol solution, and the transition is reversible in this solution. However, a completely irreversible transition from the hydrophilic to the hydrophobic state is seen in an ethanol solution of 57% or more. A distinct difference in color for the precipitates is clearly observed between the hydrophilic state and the hydrophobic state. In the former state, namely in the ethanol solutions under 56%, the color is green, indicating a strong interaction between chl.a and colloidal chitin. In the other state, the precipitation of non-colored colloidal chitin is found in ethanol solutions of more than 57%.

In a water-rich solution with the colloidal chitin up to the equilibrium state of hydrophilic/hydrophobic hydration, an electron donor–acceptor relation occurs between chl.a and one of the oligomer chains of colloidal chitin, forming 1 : 1 complex between them. Thus, in the ethanol/water system, a subtle change of the water content from the limit where the hydrophilic/hydrophobic equilibrium of the system is attained induces a phase change. The critical concentration, namely 56%, beyond which hydrophilic interaction disappears in the ethanol solution, is considered to be the concentration of the collapse point of the chl.a–chitin complex.

Figure 4 also shows the time-dependence of the spectral changes of chl.a contained in a colloidal chitin solution of 30—70% ethanol concentration. As shown in Fig. 4a, upon increasing the time-lapse after mixing, the critical concentration defined from the Soret band clearly shifts to a lower ethanol concentration side; namely, a slightly blue shift appears in a water-rich solution after a long time lapse, e.g., 24 h. Regarding the shift for the II band, as shown in Fig. 4b, the critical concentration seems to be slightly higher. This cannot be clearly understood at the present stage. However, after 24 h, even in a solution with less than 55% ethanol, hydrophobic hydration occurs for chl.a in the solution. This phenomenon can be interpreted as being a shift of the equilibrium condition between the aggregated or monohydrated(hydrophilic) state and the dihydrated-(hydrophobic) state in a water-rich condition after a long time-lapse in the system.

Formation Mechanism of Chl.a/Chitin Complex. The most significant finding is that only when the csu/ch ratio is $7.4\times n$, is a very clear green precipitate formed in the 10% ethanol–90% water mixture. The value of the ratio 7.4 can be explained by assuming the formation of a cross-linked network comprising saccharide chains of 6—9 chitin oligomers, which are the main components of the colloidal chitin with its diameter of around 30 Å (transpalent colloid). Colloidal chitin consists of oligomers each having an active end group (–H or –OH). This fact has been confirmed by an analysis of chitin oligomers produced by acid hydrolysis with sonication of chitin.$^{14)}$ In water–ethanol solutions, oligomeric chl.a aggregates can be formed on a colloidal chitin comprising oligomer chains face to face through water molecules or active end groups directly. As a result, one chl.a and one of the chitin oligomer, which consists of 6—9 sugar units, may form a complex individually as each having a colloidal dimension. These coordination interactions result in the formation of water-linked dimers, such as [(oligomeric chl.a) (colloidal chitin)]$_2$, and, thus, of a higher complex. At a csu/ch ratio of 7.4×2 or 7.4×4, it is possible to assume the formation of a host–guest like complex, such as $[(\text{chl.a})_n (\text{chitin oligomer})_{2n}]_2$. $[(\text{chl.a})_n (\text{chitin oligomer})_{4n}]_2, \cdots$ in the hydrophilic system. Short-wavelength absorbing complexes of chl.a with colloidal chitin formed in the EtOH/H_2O mixture are most likely small aggregates of chl.a with a changed geometry, as compared to the long-wavelength absorbing (hydrophilic) ones.[15] The formation of the complex is possible up to the condition where the equilibrium state is attained between hydrophilic hydration and hydrophobic hydration.

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