Monomolecular Layer of the Alkylated Cytochrome b_{562} Formed at the Air/Water Interface

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A method is provided for forming a monomolecular layer of cytochrome b_{562} at the air/water interface by introducing dialkyl chains onto a water-soluble cytochrome b_{562}. The purpose of the present study was to form densely and/or regularly packed two-dimensional arrays of protein molecules. Protoheme, which is a prosthetic group of cytochrome b_{562}, was extracted from cytochrome b_{562}, and then dialkylated(dioctadecylated) with the synthetic-chemical procedure. This dioctadecylated heme was reconstituted with apo-cytochrome b_{562} in dimethyl sulfoxide–water mixtures in vitro. The reconstitution reaction was successful when the reaction time was greater than 50 min, the dimethyl sulfoxide content was 63% and the temperature was maintained at 10 °C. Surface pressure–area per molecule isotherm of the reconstituted dioctadecylated holo-cytochrome b_{562} was measured and this clearly shows the monolayer formation of the water-soluble protein on the water subphase. The coefficient of compressibility of the protein film was estimated and is discussed. It is found that reconstituted dioctadecylated holo-cytochrome b_{562} forms a stable and closely packed monomolecular layer on the water surface.

The two-dimensional crystallization of proteins has become a basic and important technique for applications in structural biology and material science. Some proteins have been crystallized in two-dimensions by utilizing interfaces.[1—13] Fromherz reported the adsorption of proteins from a solution onto a lipid monolayer at the air/water interface to obtain a crystalline film of proteins.[3] A two-dimensional crystallization technique of proteins on a monolayer was developed by Uzgiris and Kornberg.[4] Yoshimura et al. prepared two-dimensional crystals of ferritin and F_1-ATPase on a clean mercury surface in an oxygen atmosphere and investigated its crystallinity using electron crystallography.[5] Furuno et al. studied the binding of ferritin molecules to a charged polypeptide layer of poly-1-benzyl-L-histidine spread at the air/water interface due to an electrostatic interaction and observed its protein film with a scanning electron microscope.[7] Aoyama et al. crystallized ferritin, catalase, chaperonin, and 50S ribosome at liquid/liquid interface.[11] Although lots of papers on the fabrication of two-dimensional arrays of proteins have been published, a general method of constructing two-dimensional protein crystals has not been determined.

Heme proteins are a particularly interesting class due to the unique electronic and optical properties associated with the porphyrin-based prosthetic group. Cytochrome b_{562}, which is one of the heme proteins, has been chosen as a model protein for the fabrication of two-dimensional crystals and its application to bioelectronic devices. Cytochrome b_{562} is a small (M_r 12000) water-soluble protein found in *Escherichia coli*, consisting of 106 amino acid residues.[14] The structure of the *Escherichia coli* cytochrome b_{562} has been revealed by X-ray crystallography to a resolution of 0.25 nm[15,16] and it is found that cytochrome b_{562} consists of four α-helices. The prosthetic group of cytochrome b_{562}, protoheme, is noncovalently bound and is ligated to the polypeptide chain through methionine 7 on the N-terminal helix and histidine 102 on the C-terminal helix. Therefore, protoheme can be easily extracted from the holo-protein, modified with a synthetic-chemical procedure, and then reconstituted with an original apo-protein.

When crystallizing molecules in two-dimensions, the Langmuir–Blodgett method is widely utilized.[17] If the protein molecules have an amphiphilic property, one can spread the protein molecules directly onto the water surface, and then protein molecules can be crystallized in two dimensions, on the analogy of lipids on the water surface. As mentioned above, the protoheme, prosthetic group of cytochrome b_{562} is not covalently bound to the protein, and can therefore be removed and replaced in vitro by other prosthetic groups, such as modified heme. Therefore, we synthesized protoheme with long alkyl chains (octadecyl chains in this study), and reconstituted holo-cytochrome b_{562} from apo-cytochrome b_{562} and the modified heme. This gives the water-soluble cytochrome b_{562} an amphiphilic property. Compared

to the adsorption method by which the protein orientation is difficult to predict, this alkylated protein can be regularly oriented in two dimensions due to the fixed configuration of alkyl chains and peptide chain. Therefore, we are able to report the surface property of the dioctadecylated cytochrome b_{562} directly spread on the water surface.

Experimental

Dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), and chloroform ($CHCl_3$) used in this study were spectrometric grade and were used without further purification. Sodium dithionite was purchased from Merck and was used as a reducing reagent of the oxidized heme. Inorganic chemicals were purchased from Kanto.

Cytochrome b_{562} was obtained from *Escherichia coli* strain TB-1 harboring pNS 207 grown in LB medium.[18] The harvested cells were resuspended in 10 mM Tris/1 mM EDTA (M = mol dm^{-3}), pH 8.0, buffer and were subjected to a $CHCl_3$ extraction.[19] The supernatant solution containing cytochrome b_{562} was titrated to pH 4.55 and was stirred for a minimum of 30 min at 4 °C. The precipitate was removed by centrifugation, and the supernatant solution was loaded onto a carboxymethyl cellulose ion-exchange column chromatography (Whatman) equilibrated with the 50 mM KH_2PO_4/0.1 mM EDTA, pH 4.55, buffer and eluted with a salt gradient of 0—200 mM KCl. The eluted fractions were concentrated, and then applied to a Sephadex G-50 gel filtration column chromatograph (Pharmacia) equilibrated with buffer containing 50 mM Tris/0.1 mM EDTA, pH 8.0. The protein, cytochrome b_{562}, used in this study had an absorption ratio of 6.1 for A_{418}/A_{280} in the oxidized state, which is an index of the purification.

The apo-cytochrome b_{562} (peptide chain) was prepared at 4 °C using the butanone extraction method.[20] Typically, the cytochrome b_{562} solution was titrated to pH 2.0 by adding 1.0 M of precooled HCl. An equal volume of butanone (Kanto), precooled to 4 °C, was used repeatedly to extract the red protoheme until the butanone layer became colorless. The apo-protein was dialyzed against pure water (Milli Q, Millipore Milli Q system) for 3 d with several pure water changes. The obtained apo-cytochrome b_{562} contained no holo-protein, as indicated from the Soret band peak.

The dioctadecylated heme shown in Fig. 1 was synthesized from the reaction of hemin chloride and octadecylamine. The hemin chloride (Funakoshi) was dissolved into pyridine at 0 °C, and then dicyclohexylcarbodiimide (Kanto) was added. The solution was kept at 0 °C for 2 h with stirring. The octadecylamine (Kanto), dissolved in pyridine at 0 °C, was gently added to hemin chloride solution. The reaction mixture was kept at 0 °C for 24 h under stirring to complete the reaction. The mixture was filtrated to remove any insoluble material, *N,N'*-dicyclohexylurea, and then the filtrate was completely evaporated. The residue was then purified with hexane, $CHCl_3$, ethanol, DMF, and pyridine to remove any unmodified hemin, *N,N'*-dicyclohexylurea, and octadecylamine. The dioctadecylated heme was purified on a silica-gel chromatography (Merck), using 9 : 1 of $CHCl_3$ and methanol mixture as solvent. After the purification, IR and fast atom bombardment (FAB) mass spectrum were measured to confirm the chemical structure of the modified heme. ν_{max} (KBr)/cm^{-1} 3326, 2927, 2852, 1627, 1575, 1534, 1463, 1448, 1378-720. *m/z* (FAB, positive mode, matrix: 3-nitrobenzyl alcohol) Found: *m/z* 1118.8. Calcd: M, 1119.5.

UV-vis spectra were measured by Hitachi U-3300 spectrophotometer equipped with temperature controller (Taitec). Reduced state of native and reconstituted cytochrome b_{562} was given by adding an aliquot of sodium dithionite to the oxidized solution.

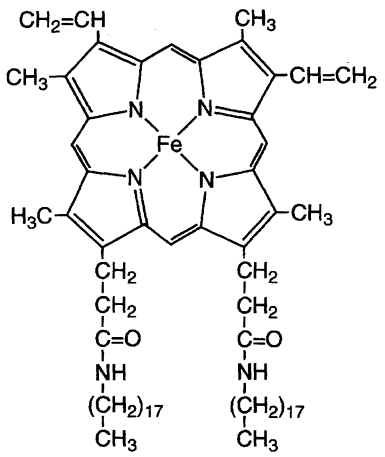

Fig. 1. Chemical structure of the dioctadecylated heme used in this study.

Surface pressure–area per molecule (π–A) isotherms were recorded using a home-made trough (area 8.8×10^{-2} m^2) with a Wilhelmy-type microbalance using filter paper. Milli Q water was used for the subphase buffer. Subphase buffer was filtered to remove any organic impurity from inorganic chemicals by using the octadecyl cartridge filter (Tosoh). In our experiments, the protein solution was spread onto a water surface and then allowed 10 min left before being compressed. The compression rate was maintained at 5.1×10^{-3} nm^2 $molecule^{-1}$ s^{-1} using a microcomputer. Although it is difficult to spread protein solution onto a water surface, loss of the compound (protein molecules) on water surface was negligible due to the reproducibility of the data.

Results and Discussion

Native cytochrome b_{562} shows absorption bands at 562 (α-band), 532 (β-band), and 427 nm (Soret band), in the reduced state and at 564 (α-band), 531 (β-band), and 418 nm (Soret band) in the oxidized state, as shown in Fig. 2.

The reconstitution of a holo-protein from an apo-cytochrome b_{562} and a native heme can be achieved by a reaction in an alkaline pH buffer.[21] Since a dioctadecylated heme is insoluble in water, a mixture of DMSO and Tris-buffer was used as the solvent for the reconstitution reaction. Figure 3 shows the effect of different solvents containing different proportions of DMSO and Tris-buffers on the reconstitution reaction. The reconstitution reaction mixture was composed of equimolar amounts of apo-cytochrome b_{562} and dioctadecylated heme, and UV-vis spectra were recorded at 17 °C after 30 min from the start of the reconstitution reaction. The absorption spectrum of the dioctadecylated heme in an organic solvent, such as DMSO, was almost the same as that of native heme, which was very similar to spectrum (A) in Fig. 3, and the absorption maximum was from around 390 to 400 nm. On the other hand, the absorption band at 427 nm is characteristic to the holo-protein after the reconstitution reaction. That is, the higher is the intensity of 427 nm, the higher is the fraction of reconstitution. As can be seen from Fig. 3, the reconstitution reaction was successful when the DMSO component of the solvent ranging between 55 and 75% and was optimum at a DMSO concentration of 63%.

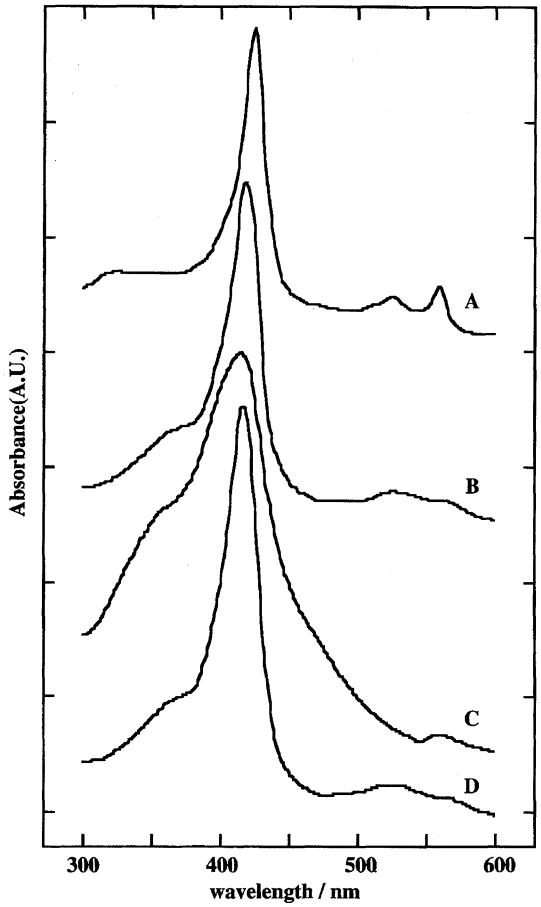

Fig. 2. UV-vis spectra of native cytochrome b_{562} and dioctadecylated holo-cytochrome b_{562}. A: native cytochrome b_{562} in the reduced form in 50 mM Tris/0.1 mM EDTA, pH 8.0 at R.T. The reduced form was obtained by the addition of a grain of sodium dithionite into the protein solution. B: native cytochrome b_{562} in the oxidized form in same buffer as line A at R.T. C: dioctadecylated holo-cytochrome b_{562} just after the reconstitution reaction in 63% DMSO at 10 °C for 24 h, D: dioctadecylated holo-cytochrome b_{562} in pure water after the removal of the unreacted dioctadecylated heme and salts by the dialysis.

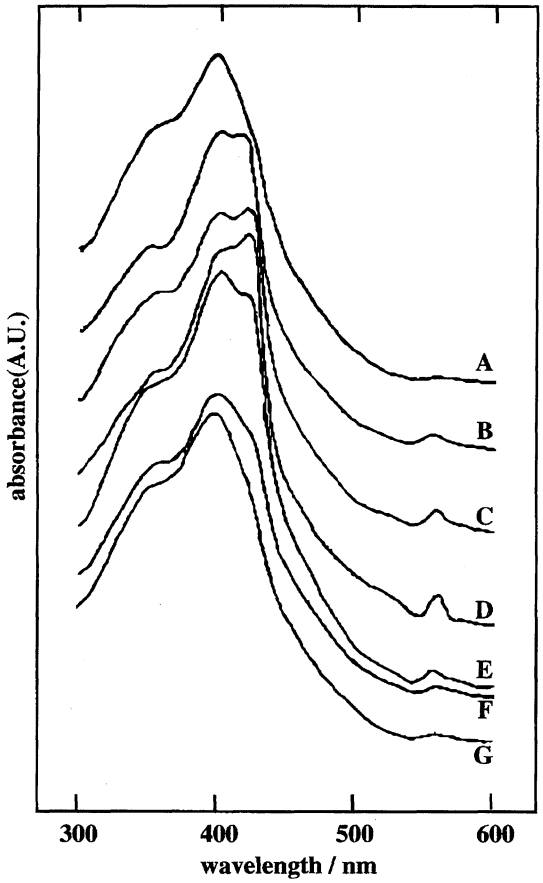

Fig. 3. UV-vis spectra of the mixtures of apo-cytochrome b_{562} and dioctadecylated heme in solutions containing various volumes of DMSO. Equimolar of apo-cytochrome b_{562} in 50 mM Tris/0.1 mM EDTA, pH 8.0 and the dioctadecylated heme in DMSO were mixed at 17 °C. A: 90%, B: 75%, C: 65%, D: 63%, E: 55%, F: 45%, G: 30%. UV-vis spectra were recorded at 17 °C after 30 min from the start of reconstitution reaction.

Figure 4 shows the effect of the temperature on the reconstitution reaction. The reconstitution reaction mixture was composed of equimolar amounts of apo-cytochrome b_{562} and the dioctadecylated heme dissolved in 63% DMSO, and UV-vis spectra were recorded after 30 min from the start of the reconstitution reaction. The shoulder at around 427 nm appears below 35 °C, which shows evidence of reconstitution. It is clear that the lower is the temperature of the reconstitution reaction, the higher does the fraction of the reconstitution reaction become. Since the melting point of DMSO is 18.4 °C, we could not examine the temperature effect below 10 °C.

Figure 5 shows the reaction-time dependence on the reconstitution, monitored by the characteristic Soret band at 427 nm. A reconstitution reaction was initiated by the addition of dioctadecylated heme in DMSO to apo-cytochrome b_{562} in Tris-buffer at 10 °C. As a result, it was found that the reconstitution reaction takes at least 50 min to complete. Itagaki et al. studied the reconstitution reaction of holo-cytochrome b_{562} from an apo-cytochrome b_{562} and the native protoheme and reported the kinetics of the formation of holo-cytochrome b_{562}.[21] According to their results, the reconstitution reaction takes only 1 min to complete. Therefore, the reconstitution of holo-cytochrome b_{562} from apo-cytochrome b_{562} and dioctadecylated heme is a rather slow reaction compared to that of native protoheme. This is probably caused by a decrease in the mobility of the heme moiety due to (1) longer alkyl chains, (2) higher viscosity of the solvent (DMSO), and (3) lower temperature, such as 10 °C.

Figures 2 (C) and (D) show the UV-vis spectra of the dioctadecylated holo-cytochrome b_{562} prepared by the reconstitution reaction under the optimum conditions obtained above. The reconstitution reaction mixture was composed of apo-cytochrome b_{562} in Tris-buffer and 10-fold concentration of dioctadecylated heme in DMSO and maintained for 24 h to complete the reaction. The mixture solution after the reconstitution reaction was dialyzed against pure water

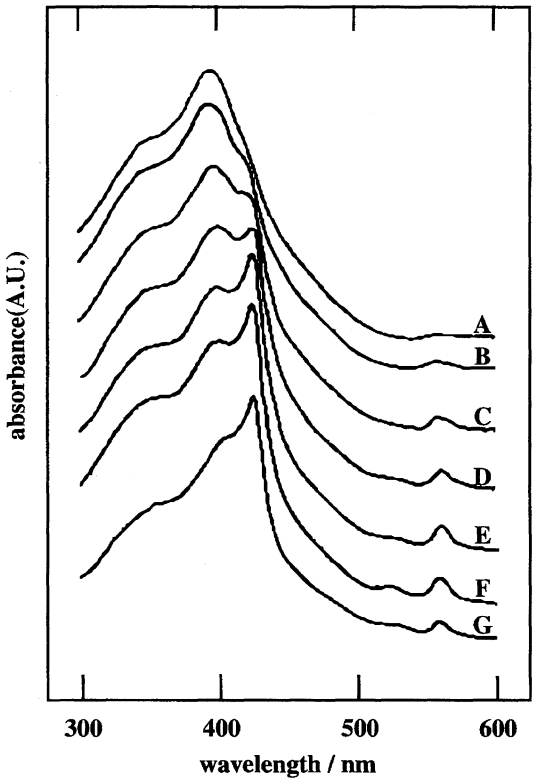

Fig. 4. UV-vis spectra of the mixtures of apo-cytochrome b_{562} and dioctadecylated heme in 63% DMSO solution at various temperatures. Equimolar of apo-cytochrome b_{562} in 50 mM Tris/0.1 mM EDTA, pH 8.0 and the dioctadecylated heme in DMSO were mixed. A: 40 °C, B: 35 °C, C: 30 °C, D: 25 °C, E: 20 °C, F: 15 °C, G: 10 °C. UV-vis spectra were recorded after 30 min from the start of reconstitution reaction.

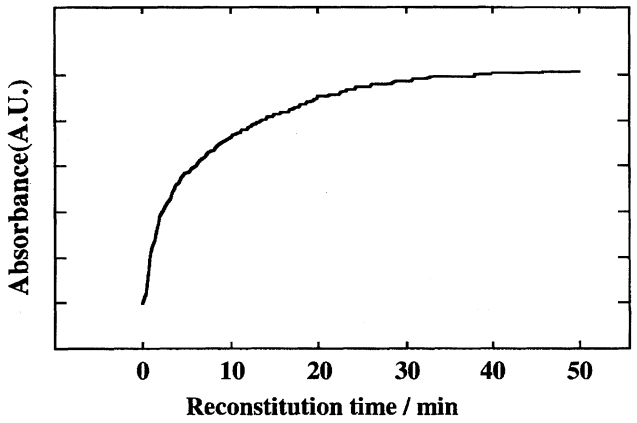

Fig. 5. The absorbance change of Soret band at 427 nm with the reconstitution reaction time. Reconstitution reaction was initiated by the addition of dioctadecylated heme in DMSO to apo-cytochrome b_{562} in 50 mM Tris/0.1 mM EDTA, pH 8.0, buffer. Equimolar of apo-cytochrome b_{562} in 50 mM Tris/0.1 mM EDTA, pH 8.0 and the dioctadecylated heme in DMSO were mixed at 10 °C.

with several changes for 3 d, and then centrifuged to remove any insoluble material in water (unreacted dioctadecylated heme). Spectrum (C) was measured just after the reconstitution reaction. This spectrum shows the overlapping of reconstituted dioctadecylated holo-cytochrome b_{562} and unreacted dioctadecylated heme. The UV-vis spectrum of the dioctadecylated holo-cytochrome b_{562} after the dialysis is shown in Fig. 2(D). The obtained spectrum is identical to native cytochrome b_{562} in the oxidized state shown in Fig. 2(B), showing that the reconstitution reaction was successful.

Figure 6 shows the π–A isotherms of native cytochrome b_{562}, apo-cytochrome b_{562}, and reconstituted dioctadecylated holo-cytochrome b_{562} spread on 0.25 mM $CdCl_2$, pH 7.7, at 21 °C. Lines (A) and (B) are the isotherms of native cytochrome b_{562} and apo-cytochrome b_{562}, respectively. Both isotherms show almost no surface-pressure, i.e., no surface-active property of the samples. This means that both samples were dissolved in the water subphase. On the other hand, it is clear that line (C), which is a result of dioctadecylated holo-cytochrome b_{562}, shows the surface-active property. The surface pressure reached above 25 mN m^{-1}. The surface-pressure of ca. 25 mN m^{-1} represented in Fig. 6(C) is thought to be an inherent character of the dioctadecylated holo-cytochrome b_{562}. We also checked the stability of the monomolecular layer of the dioctadecylated holo-cytochrome b_{562}. Once the surface pressure reached 25 mN m^{-1} the barrier was fixed and the surface pressure was

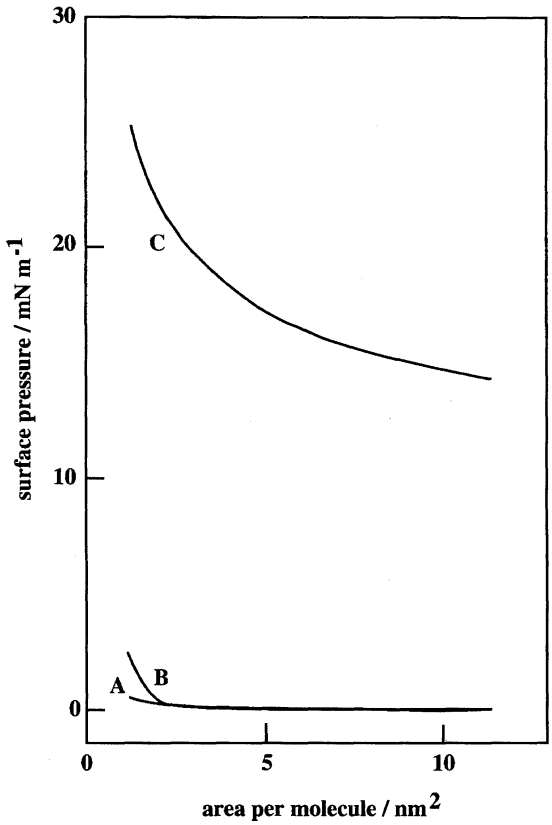

Fig. 6. π–A isotherms of native cytochrome b_{562}, apo-cytochrome b_{562}, and dioctadecylated holo-cytochrome b_{562} spread on a subphase containing 0.25 mM $CdCl_2$, pH 7.7, at 21 °C. Spreading solutions were A: native cytochrome b_{562} in pure water, B: apo-cytochrome b_{562} in pure water, C: dioctadecylated holo-cytochrome b_{562} in pure water.

then monitored continuously as a function of time to see any change. The surface-pressure gradually decreased during 20 min and showed a stable value of ca. 20 mN m^{-1} (data not shown), which indicates that the dioctadecylated holo-cytochrome b_{562} forms a stable monomolecular layer on the water subphase. This decrease of the surface pressure (relaxation process in the monolayer) is commonly observed in Langmuir–Blodgett studies. The limiting area which is the most compressed area of the protein film, is one of clues that allows us to understand the molecular structure on the water surface. This area is normally obtained by extrapolating the approximately linear portion of the isotherm to the abscissa. However, the π–A isotherm is bent in many cases, the linear part of which is ambiguous. Therefore, Bull et al. suggested that the coefficient of compressibility be used to characterize protein films in the high-pressure region on the isotherm.[22–24] The coefficient of compressibility (δ), of a spread protein film is given by

$$\delta = -\frac{1}{A}\frac{dA}{d\pi}, \quad (1)$$

where A is the area per protein molecule in nm^2 and π is the surface pressure in mN m^{-1}. When the coefficient of compressibility is plotted against the area per protein molecule, a well-defined minimum is usually observed in this plot, which allows the area and pressure at minimum compressibility to be assigned. Therefore, the minimum compressibility of a protein film will correspond to a state where protein molecules are densely packed at the air/water interface without a partial collapse of the film occurring. The coefficient of compressibility of the dioctadecylated holo-cytochrome b_{562} against the area per protein molecule is therefore derived from Fig. 6(C) and Eq. 1, and it is shown in Fig. 7. This compressibility curve has an inflection point at 4.3 nm^2 of area per protein molecule and a minimum at 2.1 nm^2. The surface pressure at 2.1 nm^2 in Fig. 6(C) is 21.4 mN m^{-1}. Figure 8 shows a schematic representation for the arrangement of the dioctadecylated cytochrome b_{562} on the water surface, although we do not have any evidence for the orientation of the dialkyl chains normal to the water surface. Here, we calculated the molecular area of the dioctadecylated holo-cytochrome b_{562} from the data of three-dimensional structure of native cytochrome b_{562}[16] by a computer simulation. For the calculation, the protein shape was assumed to be an ellipsoid of revolution with a major axis of 5 nm and a minor

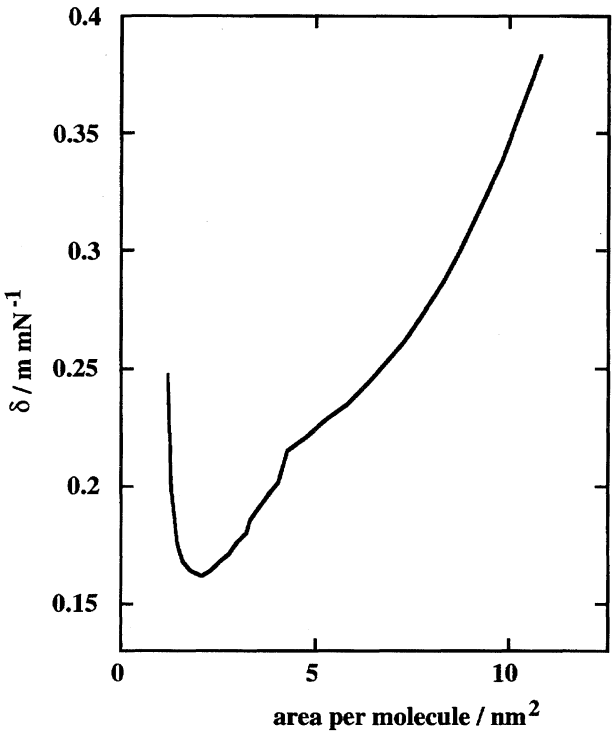

Fig. 7. Compressibility curve for dioctadecylated holo-cytochrome b_{562} spread on a subphase containing 0.25 mM $CdCl_2$, pH 7.7, at 21 °C. This curve was derived from the π–A isotherm shown in Fig. 6(C) and Eq. 1 in the text.

axis of 2.5 nm. The angle between the major axis of ellipsoid and the direction of propionates in heme molecule was 55 degrees. Although the heme molecule is noncovalently bound to the peptide, it is ligated to the peptide chain through methionine 7 and histidine 102. Therefore, the angle of the heme face is fixed against the major axis of the ellipsoid (protein molecule). If we assume that the dialkyl chains orient normal to the water surface, the dioctadecylated holo- cytochrome b_{562} has to tilt with a certain angle to it. Thus, the projected area would be the molecular area of the isolated protein molecule on the water surface, which is 9.5 nm^2 (Fig. 8(a)). At the stage of Fig. 8(b), the projected area per protein molecule is 7.2 nm^2, because molecules overlap with each other. At higher surface pressure region in the process of compression of the monolayer (Fig. 8(b) → (c)), the tilt angle between the alkyl chains and peptide may

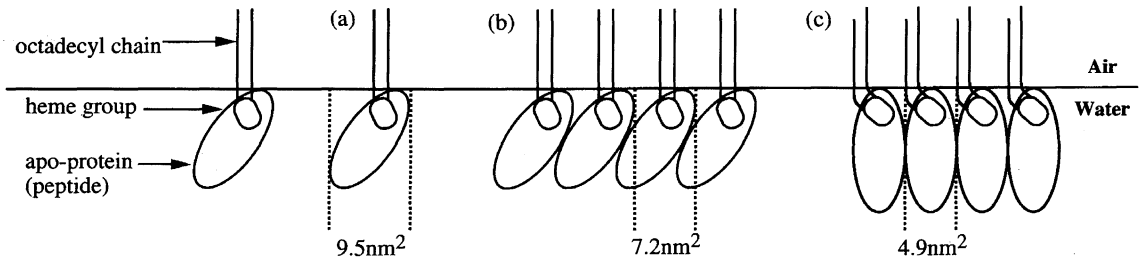

Fig. 8. Schematic representation for the arrangement of the dioctadecylated cytochrome b_{562} at the air/water interface. (a) at the lower surface pressure (before compression and the beginning of the compression), (b) at the middle stage of the compression, (c) at the higher surface pressure.

be altered due to the flexibility of the alkyl chains, consequently the protein molecules would stand up-right. If we assume hexagonal packing on the water surface, the area per protein molecule is 4.9 nm^2, which may be associated with the inflection point at 4.3 nm^2 in Fig. 7. Judging from the minimum compressibility value in Fig. 7, the protein films can be packed further to an area of 2.1 nm^2 per protein molecule, then partial collapse of the protein monolayer may occur. It has been reported that lipid molecules in monolayer on water have cross section of 0.19 nm^2.[17] Therefore, the cross-sectional area of two alkyl chains, 0.38 nm^2, is too small compared to that of the protein molecule. Thus, the hydrophobic interaction between two alkyl chains does not contribute for the packing of the protein. They just help water-soluble protein molecules float on the water surface.

Direct spreading of the water soluble proteins on water surfaces to form protein films has been studied for decades.[9,22—27] In most of cases protein molecules are expanded with the unfolding (denaturation) of the native structure. Then, the limiting area per protein molecule and the thickness of the molecule in monolayer tend to be much larger and thinner, respectively. Gallant et al. investigated the state of the photosystem II core complex in a monolayer at the gas-water interface.[27] They studied the structure of the protein molecules on the water surface as functions of the initial surface-pressure and the incubation time before compression. They concluded that the native structure of the protein can be maintained when the protein film is compressed from a relatively high initial surface-pressure and after a shorter incubation time. This is the reason why we measured the π–A isotherm of the reconstituted dioctadecylated holo-cytochrome b_{562} after incubation time of 10 min and from the relatively high initial surface pressure, to avoid the denaturation of the protein as possible. Although we do not have any direct information on the tertiary structure, the data of the π–A isotherms indicate that the protein molecules in the monolayer examined in this study appear to maintain their native structure. The important finding in this study is that we could form a protein monolayer by direct-alkylation of the water-soluble protein.

We have chosen cytochrome b_{562}, an electron-transfer protein, as a target protein for two-dimensional crystallization for the development of bioelectronic materials. A site-specific modification of the protein, direct-alkylation, has been achieved, which gave the water-soluble protein an amphiphilic property. This is the first study in which protein films have been formed by direct-alkylation of the protein molecules.

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