Bilayer Formation of Streptavidin Bridged by Bis(biotinyl) Peptide at the Air/Water Interface

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We report here the formation of a protein double layer at the air/water interface.

An ultrathin protein layer is an attractive system to develop a molecular device based on the function of protein.[1] A convenient method to obtain these protein layers is their preparation at monolayers at the air/liquid interface. For instance, it has been reported that streptavidin (SAv) formed a two-dimensional (2D) crystal underneath a monolayer of biotinyl lipids spread at the air/water interface.[2] In the 2D crystal, two of the four binding sites for biotin in the SAv molecule are occupied by biotin groups of the lipids, and two remaining ones are free for further binding and exposed to the aqueous subphase.[3] Therefore, the SAv layer can be regarded as the template of a highly ordered binding matrix for biotinylated materials. The formation of a monolayer of a biotinylated Fab fragment of a monoclonal antibody underneath a template SAv layer has been reported recently.[4] Concanavalin A, which is a water-soluble protein with specific binding sites for a sugar, was bound to the template with an intervening linker molecule having a biotin group on one end and a sugar moiety on the other, resulting in a protein multilayer.[5] With regard to these results the monolayer technique seems suitable for the assemblage of functional protein multilayers. The ordered structure of the first layer has a template effect on the second protein layer, inducing an ordered structure of the latter. In this regard, a bilayer system of SAv (Figure 1) should be interesting, because the distance between the binding sites of SAv in the second layer is the same as that in the first template layer. Here, it should be noted that a linker molecule to connect the first and second SAv layers should possess a suitable chain length with an appropriate rigidity to avoid coordination of two terminal biotinyl groups with neighboring binding sites of SAv in the first template layer.

Hydrophilic helical peptides take a rigid secondary structure, and the end-to-end distance is variable by changing the number of amino acid residues. Therefore, the helical peptides are suitable for the linker molecule. In the present study, a bis(biotinyl)-helical peptide was used as the linker peptide. It is well-known that the peptide containing 2-aminoisobutyric acid (Aib) residue has a strong tendency to form a helical structure, because the dihedral angles of Aib are constrained in the helical region due to the steric hindrance around the α-carbon atom of Aib.[6]

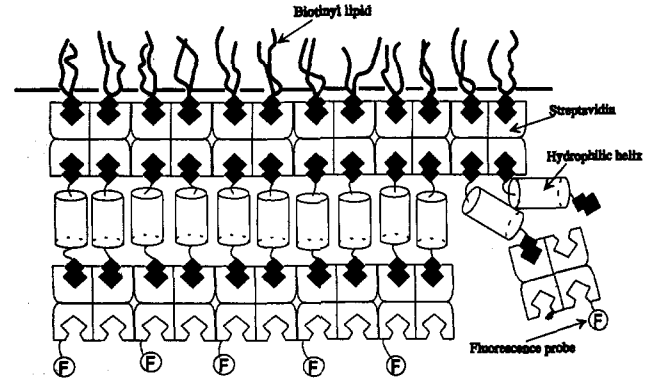

Figure 1. Schematic representation of the SAv bilayer system.

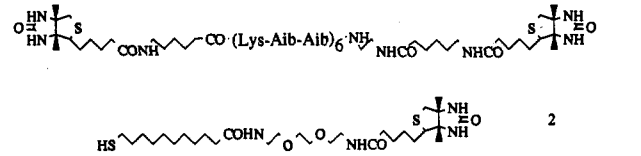

Figure 2. Chemical structure of compounds **1**, bis(biotinyl) helical peptide, and **2**, biotinyl thiol.

Therefore, the linker peptide, **1**, containing Aib residues in the amount of $^2/_3$ of the total residues, was designed and synthesized (Figure 2). The formation of the protein bilayer connected by the linker peptide was investigated by surface plasmon resonance (SPR) and fluorescence microscopy (FM).

The CD spectrum of the peptide linker, **1**, in pH 7.0 buffer solution at a concentration of 3.0×10^{-6} M showed a double-minimum pattern, indicating a helical conformation.[7] Although the pattern was distorted at a higher concentration than 1.5×10^{-4} M in the buffer solution probably because of the aggregation of the peptide, the CD spectra showed a compatible pattern in water, 0.5 M NaCl, pH 11 and pH 4.5 buffer solution at a concentration of 3.0×10^{-6} M.

The protein bilayer was formed at the air/water interface in a small trough (10 cm^2) by the following procedure: (1) The biotinyl lipid was spread on the aqueous subphase (0.5 M NaCl) at a concentration of 500 A^2/molecule. (2) An SAv solution was injected into the subphase, and the temperature was kept at 30 °C for 30 min, followed by cooling to 20 °C. The subphase was replaced with a new aqueous solution. With procedures 1 and 2, the first SAv layer was formed over the whole area of the interface.[4] (3) A 4.2×10^{-8} M aqueous solution of peptide linker **1** was added to the subphase and was incubated at 20 °C for 120 min. The subphase was replaced with brine. (4) The aqueous solution of fluorescein-labeled SAv (FITC–SAv)[8] was injected into the subphase. After incubation at 20 °C for 15 h, bright domains were observed by fluorescence microscopy[9] (Figure 3). The bright domains were not observed in the control experiment which was carried out without operation 3. This result verifies that the first SAv layer was not substituted with FITC–SAv injected in procedure 4, and that the bright domains were formed by the second SAv layer formed underneath the first SAv layer with intervention of the linker peptide **1**. It is notable that the bright domains of the second layer showed fluorescence anisotropy, indicating the formation of a 2D crystal of SAv.[2]

The protein bilayer was examined by SPR.[10] A metal surface with a free electron, e.g., gold, is irradiated through a Kretschmann

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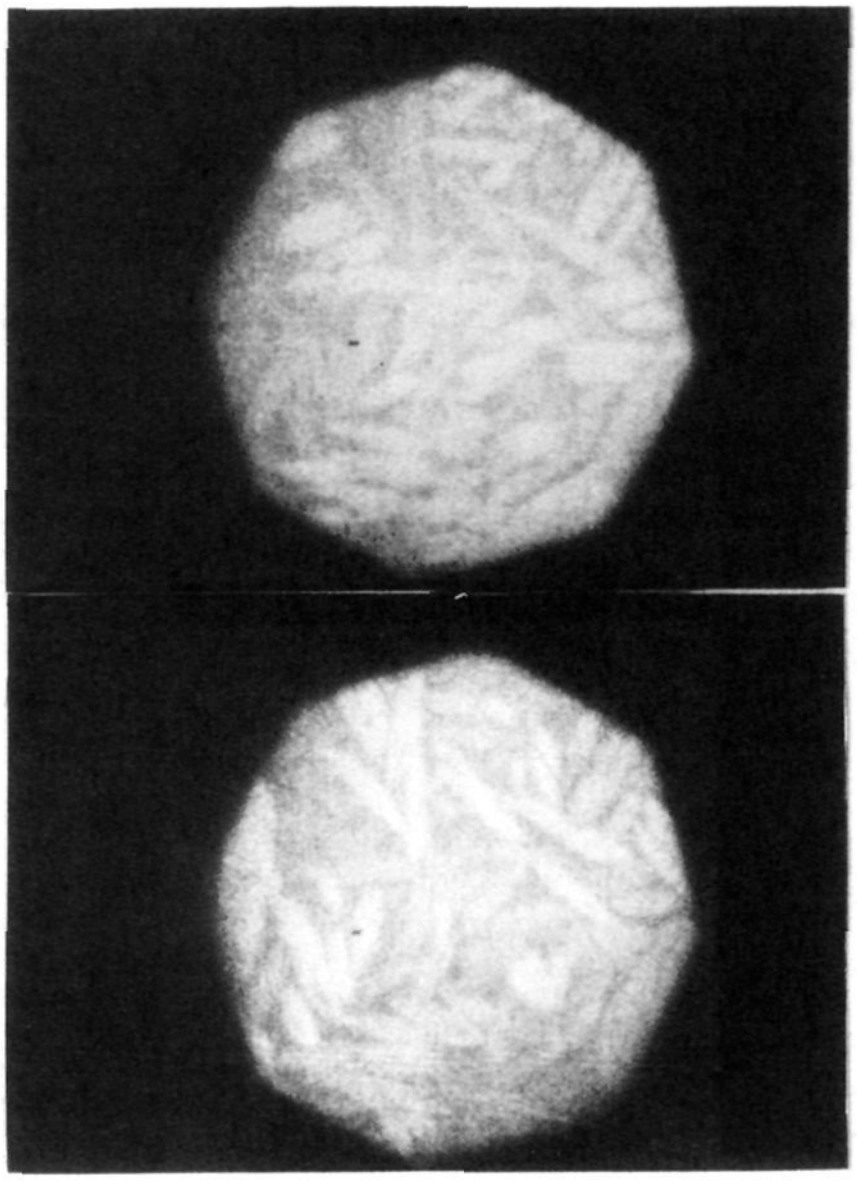

Figure 3. Fluorescence micrograph of SAv bilayer at the air/water interface. The bilayer system: biotinyl lipid, SAv, **1**, and FITC-SAv, illustrated in Figure 1. The polarization of the incident light was shifted by 90° between the two photos.

prism with light. The nonradiative surface is excited electromagnetically (surface plasmon) along the metal/dielectric interface. The mode of excitation depends on the optical properties of the interface. The intensity of the reflected light reached a minimum at the incident angle, at which the energy and momentum of the incident light satisfies conditions for resonantly exciting surface plasmon. When the optical properties of the interface are influenced by adsorption of organic substances etc., the incident angle giving the reflection minimum varies. The thickness of the adsorbed layer can be estimated from the extent of the change of the angle and the refractive index of the layer. The experimental procedure was as follows: (1) adsorption of biotin thiol **2** and 11-mercaptoundecanol on a gold substrate evaporated onto a slide glass (incubation of the glass with a 0.5 mM thiol mixture in a 1:4 molar ratio for 16 h), (2) substitution of the aqueous phase with 1 μM SAv solution and incubation for 30 min, (3) substitution with a 9.1×10^{-5} M aqueous solution of peptide linker **1** and incubation for 15 min, and (4) substitution with 1 μM aqueous SAv solution and incubation for 14 h. Each operation was followed by careful rinsing with water. The thickness of the adsorbed layer after each operation was measured and is summarized in Table 1. It is obvious that the thickness of the organic layer increased with SAv or linker adsorption to the surface. The thickness of the first SAv layer was estimated to be 34 Å, which is comparable to the previous results.[11-13] The increase of the thickness of the second layer was not observed in the control experiment when the inactive SAv, which had been saturated with biotin, was used in place of active SAv in procedure 4. Therefore, SPR observation indicates the formation of a bilayer structure of the protein, in which one protein layer was connected to the other through peptide linker **1**.

Table 1. Thickness Data of the SAv Bilayer Determined with SPR (Å)

+SAv	+**1**	+SAv
34	36	46

The SPR measurement revealed that the thickness of the first SAv layer was 34 Å, which is less than the protein thickness of 45 Å determined by X-ray analysis of SAv. The effective thickness is underestimated because we calculated the thickness by using the effective refraction index of $n = 1.45$, which was experimentally determined for a bulk protein. However, it has been indicated by electron microscopy and image processing that the 2D crystal of SAv formed at the air/water interface contains much water. Therefore, the true refractive index of the SAv layer should be less than 1.45.[11,13] On the other hand, the effective thickness of 10 Å for the second SAv layer indicates that a partial adsorption of SAv occurs on the first SAv layer. The partial adsorption can be explained by coordination of two terminal biotins of a linker peptide with binding sites of SAv. This explanation is supported by the titration experiment of SAv with **1**,[15] showing that SAv bound only 3-fold bis(biotinyl) compound, although SAv bound 4-fold molar biotin.

The length of the helix of **1** is 27 Å, and the radius of the helix section should be larger than 5 Å, assuming a perfect α-helical structure.[16,17] The distance between the biotin-binding sites of the SAv has been reported to be 20 Å,[14] and that of neighboring SAv molecules in the 2D crystal has been reported to be about 45 Å from a 2D projection map of the crystal.[3] Therefore, both biotin moieties of the same peptide linker will not occupy simultaneously the two binding sites of the same SAv molecule nor of the neighboring SAv molecules insofar as the perfect helical conformation of the linker peptide is maintained upon binding to SAv. The backbiting is only possible if the helical structure is disturbed. In order to impose perfectly the second SAv layer underneath the first SAv layer, stabilization of the helical structure of the linker peptide should be the key point for further work.

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(15) The titration was carried out in the following procedure: 30 μL of a 10 mM solution of 2-(*p*-hydroxyphenylazo)benzoic acid in 1 N NaOH was added to an SAv solution (3 mg/10 mL of 0.1 M phosphate buffer). The mixture was diluted by the same volume of 0.1 M phosphate buffer and was placed in a UV–vis cell. The titration curve was obtained from the absorbance at 500 nm of the solution measured as small aliquots of **1** aqueous solution were added.
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