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Induction of changes in the secondary structure of globular proteins by a hydrophobic surface

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Abstract. Circular dichroism, ellipsometry and radiolabeling techniques were employed to study the induction of changes in the secondary structure of BSA, myoglobin and cytochrome *C* by a hydrophobic surface. The results showed that adsorbed protein molecules lose their ordered native structure in the initial stage of adsorption and the structure appears to be a random or disordered conformation. Protein molecules adsorbed in later stages adopt a more ordered secondary structure (α helix and β structure). The changes of secondary structure of globular proteins induced by a hydrophobic surface can be explained by the steric interaction between adsorbed proteins as well as by hydrophobic interactions during the adsorption process. In addition, there is obviously an intermediate stage in which the protein molecules are mainly in the β structure, indicating that for certain proteins, the β structure may be a more stable secondary structure than α helix on the hydrophobic surface.

Key words: Protein adsorption – Globular protein – Hydrophobic surface

Introduction

The development of biocompatible materials and biosensors has prompted studies on the organization of functional biomolecules at interfaces. Protein adsorption, protein-lipid monolayer deposition and covalent binding of protein to a solid surface are conventional methods used to assemble protein onto a solid substrate surface.

Protein adsorption is a rather complicated process. It shows a significant dependence on the solution conditions (temperature, pH and ionic strength), the nature of the solid surface, the individual characteristics of the proteins and other related factors. Protein adsorption has often been thought of as a Langmuir adsorption. The adsorbed amount reaches a plateau value at a critical concentration. Many results have shown that the adsorbed protein molecules form a monolayer or submonolayer on the surface. Wu and Sui (1992) found that the amount of adsorbed BSA on a hydrophobic surface is strongly affected by pH and it approaches a maximum at a pH near the isoelectric point of the protein. This result was explained in terms of the repulsive force between BSA molecules. Lee and Ruckenstein (1988) studied the effect of substrate surface condition on the BSA adsorption in detail. Their results showed that with an increase in surface hydrophobicity the amount of protein adsorbed increased. They also observed that the protein molecules adsorbed on a hydrophilic surface are easily desorbed when the protein solution is removed. The interaction between protein and solid substrate was explained in terms of the surface free energy. In addition, the reversibility of protein adsorption can be used as a measure of the strength of protein interaction with a solid substrate. Schmidt et al. (1990) noted that the protein adsorption is irreversible in the first layer, indicating that these molecules are strongly bound to the surface. In contrast, the molecules are reversibly bound in the second layer, showing that these molecules are loosely adsorbed on the surface. They also observed that lysozyme molecules lost their enzymatic activity in the first layer, but retained enzymatic activity in the second layer.

Since the protein activity depends upon its normal structure characteristics, the denaturation of adsorbed enzyme molecules indicates that the protein has undergone a change in conformation. In other words, an interface-induced conformational change of proteins occurs during the adsorption process. Until now only a few experimental methods have been used to study the conformational change of proteins on adsorption. Soderquist (1980) pioneered the study of protein conformation changes upon adsorption using fluorescence and circular dichroism spectroscopies. Transmission CD spectra of Hageman factor adsorbed on a stack of quartz plates suggested a conformational change. Horsley et al. (1991) studied the effect of adsorption at a silica surface on the conformation of human and hen lysozyme by iodide quenching of protein fluorescence. Their results showed indirectly that both lysozymes were at least partially de-

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natured upon adsorption. Lenk et al. (1991) demonstrated a time-dependent conformational change in adsorbed fibrinogen by ATR-FTIR. An infrared spectral change is indicative of a loss of helical and random structure with time, and an increase in β-structures. Our present work is devoted to the study of the effect of adsorption on protein conformation, especially to understand more about the induction of a secondary structure change in globular proteins by a hydrophobic surface. The secondary structure changes and the determination of surface concentration have been investigated with ellipsometry, radiolabeling techniques and circular dichroism. Qualitative explanations for the secondary structure changes during adsorption are proposed.

Materials

Bovine serum albumin (fatty acid free), myoglobin (type III) and cytochrome C (type III) were purchased from Sigma Chemical Co.. Dichlorodimethylsilane was from Merck Co., FRG. All of the chemicals used in this study were of analytical reagent grade and were used without further purification. $Na^{125}I$ was purchased from the Research Institute of Atomic Energy, Beijing, China. All double-distilled water was from the Microelectronic Research Institute of Tsinghua University, Beijing, China. High quality quartz plates were obtained from the Department of Precise Instrumentation of Tsinghua University with dimensions $20 \times 18 \times 1.2$ mm.

Methods

Hydrophobic treatment of quartz plates

Quartz plates were carefully cleaned by soaking in fresh chromic acid for 24 h and then extensively washing with double-distilled water. The substrates were then dried in air. The cleaned plates were then transferred into 10% dichlorodimethylsilane in trichloroethylene solution for 10 min and subsequently were rinsed with trichloroethylene, acetone and finally ethanol.

Adsorption

The stock solution of proteins (1 mg/ml) was injected into a cuvette containing 100 ml buffer with full agitation using a magnetic stirrer. The treated quartz plates were incubated with proteins in the concentration ranges 1–60 μg/ml (BSA), 50 μg/ml (myoglobin) and 50 μg/ml (cytochrome C) for the desired time. 2 mM PBS (pH 5.0) buffer was used for BSA adsorption, and 0.1 M K_2HPO_4 (pH 7.0) buffer for myoglobin and cytochrome C. All experiments were done at room temperature. In the case of saturated adsorption, 30 min was allowed for all the three proteins.

Determining the secondary structure of a protein adsorbed on a surface

The secondary structures of adsorbed proteins were determined using a Jasco-J500c spectropolarimeter (Jasco Co., Japan). Eight quartz plates were mounted in a home made PTFE sample cell and aligned normal to the incident light beam. Because passage of the light beam through quartz plates may produce birefringence under lateral pressure or reflection which may affect the reliability of CD spectra, control experiments were performed. For each measurement the contributions to the CD spectra from silanized quartz plates were subtracted. The experimental CD was averaged 4 times and then smoothed. In order to assess whether the adsorbed water of the protein layer affects the spectroscopic measurements, the adsorbed plates were kept in air for different periods of time (10, 30 and 120 min) before examination. The CD spectra did not show any change. In our experiments, the CD spectra were recorded within 30 min. The effect of possible variation in adsorbed water was therefore of minor importance.

The directly recorded raw CD spectra can be transformed into mean residue ellipticity $[\theta]$ (deg · cm^2 · dmol^{-1}) using the following formula:

$$[\theta] = M_r \, \alpha / 100 \, c_w \, l$$

where α = observed CD (mdeg.); M_r = mean residue weight (115 for proteins); l = light path (dm); c_w = protein concentration (g/ml). The product $c_w \, l$ is proportional to the surface density in an adsorbed layer, given by $\Gamma \cdot F$, where Γ is the surface concentration of protein on the quartz surface (μg/cm^2) and F is the total number of faces on the quartz plates (there are two faces per plate). Then

$$[\theta] = \frac{1.15 \cdot 10^4 \, \alpha}{\Gamma \cdot F} \tag{1}$$

For the CD spectra of poly-L-lysine films on the surfaces of quartz plates, Stevens et al. (1968) have made a systematic examination. Their results are summarized in Table 1.

The CD spectra of β-structured polypeptide films fall into two categories. In the first, I-β, the spectrum displays a negative band near 219 nm, similar to previous observations on β-structured poly-L-lysine in solution. In the second case, II-β, the CD spectrum is shifted to longer wavelengths with a negative band near 228 nm. The CD spectrum of an α-structured poly-L-lysine film has negative bands at 221 and 209 nm, in excellent agreement with positions established for that conformation in solution. The poly-L-lysine films in the random coil (or disordered) conformation give a negative band at 202 nm. This spectrum is different from that observed in solution which has a negative band at <200 nm. The principles presented in Table 1 were applied to analyze the CD spectra in the next section.

Table 1. Secondary structures of polypeptide films

α-Helix	221(−), 209(−), 201(c-o), 191.5(+)
I-β	219(−), 207(c-o)
II-β	228(−), 217(c-o)
Random coil	202(−)

Wavelength (in nm) of maxima of (+) positive bands; (−) negative bands. (c-o) indicates cross-over position (Stevens et al. 1968)

Determination of surface concentration of adsorbed proteins

The surface concentration of a protein adsorbed on a hydrophobic surface has been determined by two different methods, ellipsometry and ^{125}I radiolabeling technique.

The principle of determining surface concentration by in situ ellipsometry is described in detail elsewhere (Wu and Sui 1992; Kop et al. 1984) and can be summarized as follows. Two polarizer prisms, the polarizer P and the analyzer A, are rotated such that the intensity of monochromatic light ($\lambda = 6328$ Å), reflected on the silicon surface, is kept at a minimum during protein adsorption. The optical constants (n_2, k_2) of the silanized silicon surface can be determined from the initial positions of P and A. Owing to the presence of the silanized layer and initial contamination (e.g., organic or inorganic adsorption from the buffer), the index will be a pseudo-refractive index of the substrate. The ratio of the reflection coefficient for light polarized parallel (R^P) and perpendicular (R^S) to the plane of incidence is given by

$$R^P/R^S = \tan\psi \exp(i\Delta) \tag{2}$$

where Δ and ψ follow directly from the readings of P & A and $i = \sqrt{-1}$. R^P and R^S are dependent on the angle of incidence Φ, the wavelength of light λ, the thickness d and the refractive index n_1 of the adsorbed layer, the refractive index n_0 of the buffer and the optical constants (n_2, k_2) of the silanized silicon surface. Equation (2) was solved by an iterative procedure for d and n_1. The refractive index n_0 of the buffer was measured with an Abbé refractometer.

Because n_1 approaches the refractive index of the pure substance, the surface concentration of adsorbed proteins is estimated from the expression (Kop et al. 1984):

$$\Gamma = d(n_1^2 - l)/[r(n_1^2 + 2) \tag{3}$$

where r is specific refractivity of the protein. The r values for BSA, myoglobin and cytochrome C were estimated from their amino acid composition (Kop et al. 1984).

^{125}I-labeling of proteins was performed using an Iodogen procedure. A small column of Sephadex G-25 was employed to separate the labeled proteins from the unreacted iodide. Only the fraction of labeled proteins free of unreacted iodide was subsequently employed. The amount of adsorbed proteins was determined by gamma radiation counting using a FT613 gamma counter. The surface area of substrate was determined accurately by weighing the plate.

Results

Surface concentration of proteins

As described in the methods section the absolute amount of adsorbed protein can be measured by ellipsometry. Figure 1 shows the surface concentration of adsorbed proteins as a function of time measured by ellipsometry. The surface concentration was estimated according to (3). As shown in Fig. 1, the adsorbed amount is dependent on both protein concentration in bulk solution and the period of adsorption. We can arbitrarily choose a surface concentration by controlling the concentration in bulk solution or the period of adsorption. For BSA with an adsorption condition of 1 μg/ml and 1 min, the surface concentration will be extremely low (0.0213 μg/cm^2). Increasing the concentration in bulk solution to 5 μg/ml, but keeping the period of adsorption unchanged, the surface concentration is 0.076 μg/cm^2. Increasing the concentration in bulk solution further (60 μg/ml), but still keeping the period constant, the surface concentration can reach a value of 0.185 μg/cm^2. Increasing the bulk concentration and the period of adsorption simultaneously (60 μg/ml and 30 min) gives a surface concentration of 0.238 μg/cm^2. For myoglobin adsorption, when the solution concentration is 50 μg/ml and the period of adsorption is 30 s, the surface concentration is 0.0487 μg/cm^2. Increasing the period of adsorption to 1 min and then 30 min gives surface concentrations of 0.101 μg/cm^2 and 0.158 μg/cm^2 respectively. For cytochrome C adsorption, when the concentration in bulk solution is 50 μg/ml and the period of adsorption is 30 s, the surface concentration is 0.0518 μg/cm^2. Increasing the period to 30 min gives a surface concentration of 0.150 μg/cm^2.

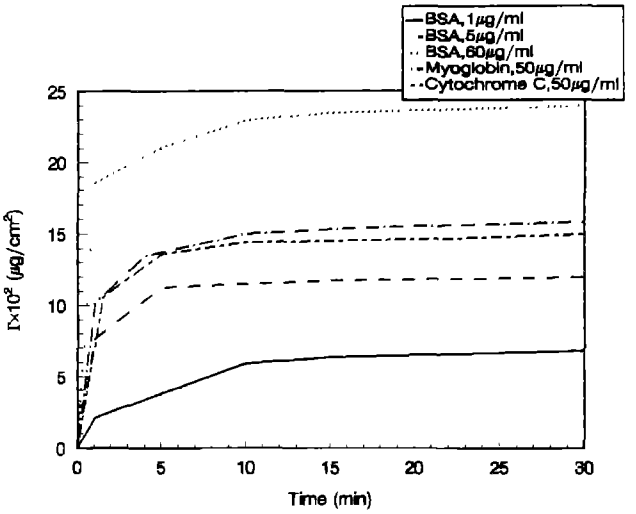

Fig. 1. Surface concentration Γ of adsorbed proteins as a function of time, determined by ellipsometry. Adsorption conditions are indicated in the upper part of the figure

The ellipsometer can only detect the surface concentration on a silicon surface, but we need to determine the surface concentration on the surface of quartz plate. The amounts of adsorption on the surfaces of a silicon wafer and a quartz plate were therefore carefully compared. We measured the surface concentration of BSA adsorbed on the two different surfaces under same adsorption condition (60 μg/ml and 30 min) by the radiolabeling technique. The results of the ^{125}I radiolabeled method show that the surface concentration on the silicon surface is $\Gamma = 1.23 \pm 0.08$ μg/cm^2 and that on the surface of the quartz plate is $\Gamma = 1.30 \pm 0.05$ μg/cm^2. There is no significant difference between them. Thus raw CD spectra were processed by using the surface concentration determined on a silicon surface by ellipsometry, for all experimental conditions.

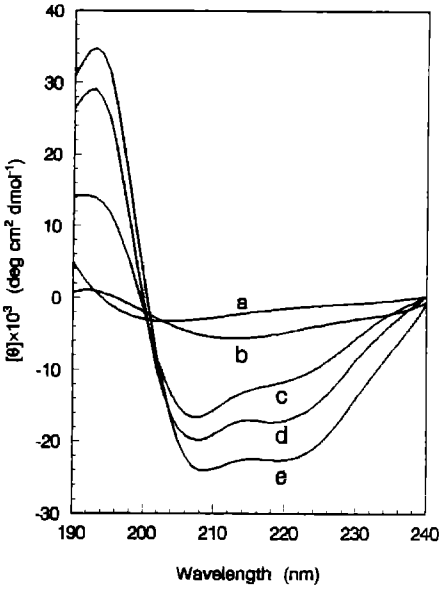

Fig. 2. CD spectra of adsorbed BSA. Buffer: 2 mM PBS pH 5.0. Adsorbed condition: *a* 1 μg/ml, 60 s; *b* 5 μg/ml, 60 s; *c* 60 μg/ml, 60 s; *d* 60 μg/ml, 30 min; *e* 60 μg/ml, BSA in solution

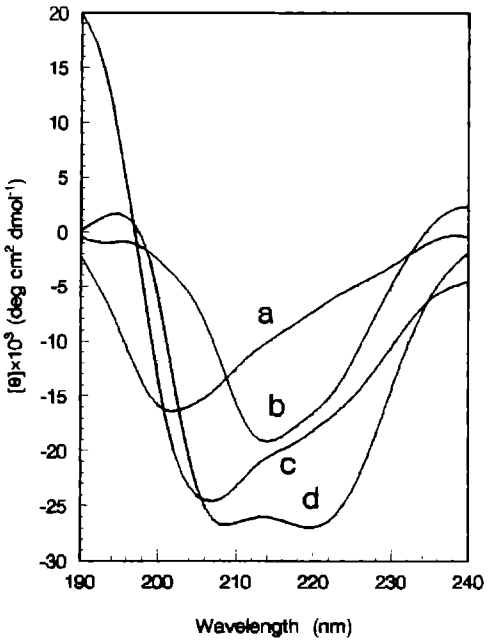

Fig. 3. CD spectra of adsorbed myoglobin. Buffer: 0.1 M K_2HPO_4, pH 7.0. Protein concentration: 50 μg/ml. Time of adsorption process: *a* 30 s; *b* 60 s; *c* 30 min; *d* myoglobin in solution

CD spectra of adsorbed proteins

Figures 2, 3 and 4 show the mean residue ellipticity of BSA, myoglobin and cytochrome C as a function of wavelength in different stages of adsorption. From these figures we can conclude that the hydrophobic surface induces conformational changes in the proteins. Using Table 1, the spectra were analyzed qualitatively as follows. Figure 2a has a negative band near 202 nm. This is characteristic of protein films with random coil conformation. Figure 2b has a minimum at 215 nm and can be assigned to the I-β structure. Because of the presence of some random coil, the 207 nm crossover point of the I-β structure does not appear. Figure 2c displays a double minimum at 209 nm and 221 nm, with partial characteristics of the α helix. In Fig. 2d a stronger negative band at 221 nm implies that the percentage of α helix is greater. In Fig. 2e (BSA in solution), the negative band at 221 nm is almost the same intensity as the band at 209 nm. This means that the percentage of α helix in adsorbed BSA is always less than that of BSA in solution.

Figure 3a shows that myoglobin, at low surface concentration has a negative band at 202 nm, again characteristic of random coil. Figure 3b shows a minimum at 215 nm, attributed to I-β structure. The reason for the absence of a crossover point at 207 nm is probably the presence of some random coil conformation. Figure 3c displays a strong negative band at 209 nm and a weak minimum at 221 nm. This is a mixture of α helix, β structure and random coil. The percentage of α helix increases steadily from Fig. 3b to 3c. Figure 3d is the CD spectrum of myoglobin in solution. This curve has an apparent minimum at 221 nm. Myoglobin has about 80% α helix in solution, this is much more than that of myoglobin adsorbed on the surface, particularly at low surface concentration.

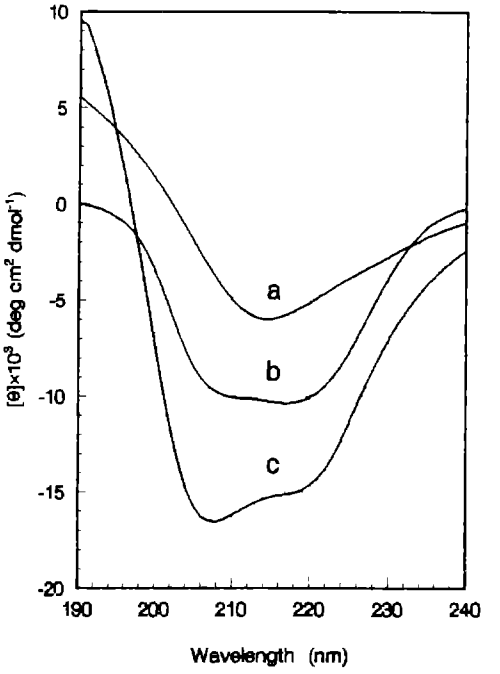

Fig. 4. CD spectra of adsorbed cytochrome C. Buffer: 0.1 M K_2HPO_4, pH 7.0. Protein concentration: 50 μg/ml. Time of adsorption process: *a* 30 s; *c* 30 min; *b* cytochrome C in solution

Figure 4 shows the CD spectra of adsorbed cytochrome C. Figure 4a has a minimum near 215 nm, with a crossover point at 204 nm, close to the crossover point of typical I-β structure. In comparison with BSA and myoglobin, adsorbed cytochrome C has less random coil and more β structure in the initial stage of adsorption. Figure 4c has a double minimum at 208 nm and 221 nm. This means that the secondary structure of cytochrome C is mainly α helix under conditions of saturated adsorp-

tion. Figure 4b is the CD spectrum of cytochrome C in solution. It has a negative band near 217 nm and a shoulder peak at 210 nm. Because the protein has a large β structure content ($\sim$25%), the typical double minimum of the α helix at 208 nm and 221 nm shifts to 210 nm and 217 nm. Cytochrome C contains more β structure in solution than under conditions of saturated adsorption.

Discussion

Mechanism of induction of secondary structure of proteins by a hydrophobic surface

It is clear that the two most important factors determining a protein's conformation are the amino acid sequence and the environmental conditions. The environmental factors include solution polarity (hydrophilic or hydrophobic), temperature, pH, ionic strength and so on. The conformation of native proteins is maintained by three extremely important noncovalent forces: hydrogen bonding, hydrophobic interactions and electrostatic interactions. Changes in environment which affect any one of the three interactions can result in conformational changes in proteins. The protein molecules encounter a completely different environment when they attach to a hydrophobic surface. This special environment may induce changes in secondary structure. At the interface the protein conformation may also be affected by steric interaction between adsorbed proteins. The steric interaction between adsorbed protein molecules derives from the fact that the protein molecules adsorbed in later stages are probably excluded by the protein molecules adsorbed in initial stages.

According to the above analysis, we can summarize the principle dominating the secondary structure changes of adsorbed globular proteins on a hydrophobic surface as follows. As a result of a side chain's preference to interact with a hydrophobic surface, proteins adsorbed in the initial stage lose their ordered structure very quickly and form a secondary structure of mainly random coil. Adsorbed proteins attain a more extended and minimum energy configuration. Proteins adsorbed in this stage have very strong noncovalent binding with the surface. Evidence was presented by Schmidt et al. (1990) that lysozyme is irreversibly adsorbed on the hydrophobic surface in the initial stage. The first adsorbed layer alters the environmental condition of the surface, which may have at least two direct consequences. One is the decrease of the hydrophobicity of the surface. Lee and Ruckenstein (1988) showed that the contact angle $\theta_{0(w)}$ increased after protein adsorption, this means that the hydrophobicity of the surface is reduced. Another is that steric interaction between adsorbed proteins occurs as a result of the presence of the already adsorbed proteins. In view of the above discussion the proteins adsorbed in the later stage therefore have a weaker hydrophobic interaction with the surface than those adsorbed in the early stage, and the steric interaction may encourage the protein molecules to maintain their compact structure. It is thus reasonable that the adsorbed proteins in the outer layer retain more ordered secondary structure (e.g. α helix).

β-structure may be a more stable secondary structure than α helix on the hydrophobic surface

It has been reported that there is an increase in β structure when a protein or polypeptide is adsorbed on a surface. Lu and Park (1991) found that the content of β structure increased upon adsorption of fibrinogen Reedy and Nagaraj (1989) showed that a signal peptide had more β structure on increasing the hydrophobicity of the environment. In the present work, our experiments for all three proteins confirm that there is an intermediate stage in which the protein molecules have mainly β structure during the initial adsorption process (Figs. 2b, 3b and 4a). Because the surface has some degree of hydrophilicity and a weak steric effect after the initial stage, these results suggest that in some stage of protein adsorption a β structure is probably more convenient in that it allows them to lie on the adsorption layer. On increasing the amount of adsorbed protein, the α helix content increases as shown in Fis. 2c–2d, 3c and 4c since the hydrophobicity of the surface becomes weaker and the steric interaction becomes stronger. In the cases of BSA and myoglobin, the component of the secondary structure at saturated adsorption tends toward the native conformation in solution, while cytochrome C has characteristically different properties. The difference may be related to the fact that cytochrome C is a membrane-associated protein.

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