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EFFECTS OF PLASMA PROTEIN ADSORPTION ON PROTEIN CONFORMATION AND ACTIVITY

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Abstract - Protein adsorption at solid-liquid interfaces may lead to significant changes in conformation. Such effects can be monitored in situ for native, unlabelled proteins using the total internal reflection spectroscopy method, by monitoring the UV fluorescence of tryptophan side chains at 320-350 nm. Such studies suggest a partial denaturation of human plasma fibronectin on hydrophobic silica and a blue shift for bovine albumin on hydrophilic silica.

INTRODUCTION

The adsorption of proteins at solid(S)-liquid(L) interfaces is important in many fields of scientific and commercial interest. The adsorption process (Figure 1) is dependent on the nature of the protein, the solid-liquid interface, the solution medium, and their interdependencies (1-7). Ideally an adsorption experiment will provide the amount adsorbed as a function of time and concentration, as well as desorption and exchange properties. Note that a protein can adsorb by different mechanisms on different surfaces (Figure 1). In addition to adsorbed amounts and rates, the orientation and conformation of the adsorbed protein are critical (Figure 2). Conformation refers to the secondary (α-helix, β sheet, etc.), tertiary, and quaternary structures.

Certain orientations may make a specific site on the protein inaccessible to ligand, substrate, or antigen. For example, consider the adsorptive immobilization of a specific IgG for a solid-phase immunoassay. The procedure will be optimal if the Fab domains are free to bind antigen.

If the protein's structure is changed due to the adsorption process and/or the new local micro-environment, then it is said to be fully or partially "denatured," meaning that its properties are no longer those of the native protein. Conformational changes can occur due to ligand binding (for example, hemoglobin), substrate binding (many enzymes), heparin binding (antithrombin III), and surface binding (Hageman Factor, fibronectin, albumin, etc.). Although there have been many hypotheses relating to protein adsorption conformational effects, there is little direct data available (next section).

The surface itself may also be conformationally altered or "denatured" due to its interaction with the protein, particularly polymer surfaces which tend to relax or change in response to new environments (8).

CONFORMATIONAL DYNAMICS

Protein folding and denaturation studies show that the normal or native state is only marginally stable. The free energy change involved in the transition to a denatured state is only 5 to 14 kcal/mol (9-11)--an energy corresponding to only a few hydrogen bonds per molecule! Adsorption-free energies of proteins are normally in the range of 5 to 20 kcal/mol (4,12), thus adsorption-induced "denaturation" is highly probable. Proteins in an altered micro-environment, such as adjacent to or on a solid surface, must be expected to be conformationally different from like proteins in the bulk solution.

The fact that a protein molecule and its associated water molecules constitute a very small thermodynamic system means that transient thermal fluctuations result in internal energy fluctuations of the order of 40 kcal/mol (11). This is larger than most ligand-induced conformational changes and can result in significant volume fluctuations. Thus conformational mobility, at least transiently, must be expected.

Proteins are highly dynamic structures which are constantly "sampling" different conformations due to local thermodynamic fluctuations. Some such transient conformations can be stabilized in new micro-environments, such as at a solid-liquid interface.

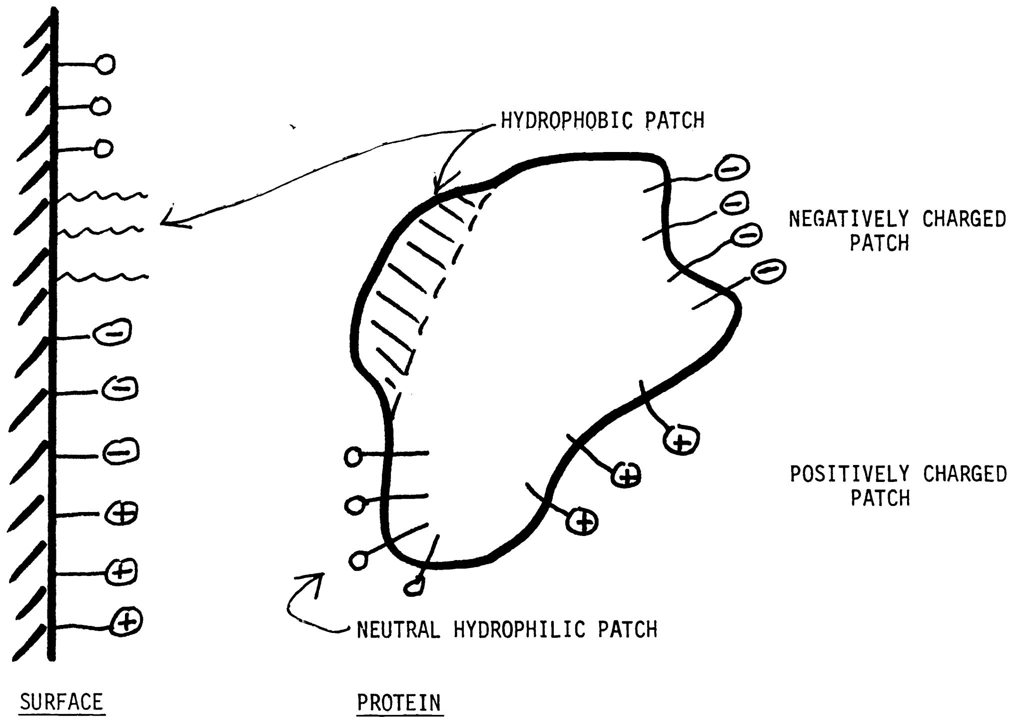

Figure 1. A schematic view of a hypothetical protein of known "surface" structure and properties diffusing towards a solid surface of known structure and properties in a well-defined solution medium.

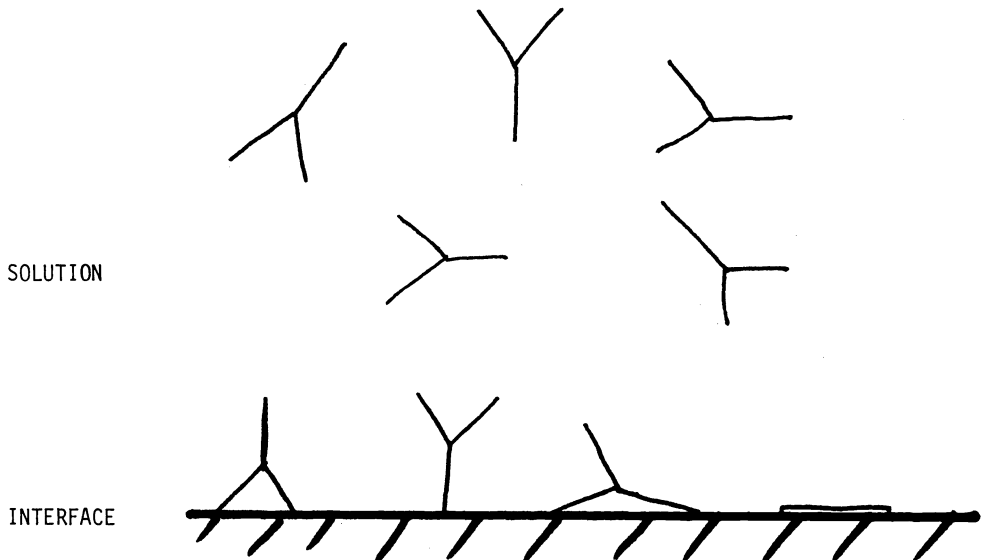

Figure 2. Adsorption of IgG molecules at an interface. The IgG molecule is shown as Y shaped with the arms indicating the Fab fragments and the tail being the Fc portion. Note that many different orientations and conformations are possible.

ADSORPTION & CONFORMATION

Many studies of proteins at air-solution interfaces have indirectly established that the adsorbed proteins undergo detectable conformational changes (7). Similar studies at solid-liquid interfaces are few. We review here only several key studies (see also Ref.6).

Morrissey (6) used transmission infrared spectroscopy to study protein adsorption onto silica particles in a heavy water (D_2O) buffer. By observing the shift in the amide I absorption band, he could deduce the fraction of carbonyl groups involved in the adsorption process, i.e., by bonding to the silica surface. He found that bovine gamma G had a bound fraction of 0.20 at low bulk solution concentrations but only about 0.02 at high solution concentrations. However, neither prothrombin nor bovine serum albumin exhibited a change in bound fraction with concentration.

Parallel experiments with flat silica plates using ellipsometry showed that the gamma G-adsorbed layers had an optical thickness of 140 Å and a surface concentration of 1.7 mg/m^2 at low bulk solution concentration--in concentrated solutions the surface amount was 3.4mg/m^2 with a thickness of 320 Å .

Morrissey suggested that at low solution concentrations the adsorbed protein has sufficient time and "elbow room" to accommodate to its new micro-environment by conformational change resulting in significant hydrogen bonding to the silica surface. At high solution concentrations the collision frequency with the surface is so high that an adsorbed protein has neither the time nor the room to optimize its interaction with the surface. This general interpretation is common in the air/solution field as well (7). Air/water studies show that the protein only needs to get a sufficient "foothold" on the surface to minimize the probability for desorption. Once attached to the surface the rest of the protein is dragged to the interface, thereby optimizing the interaction (7). The foothold need only be roughly 100-200A^2 where the cross sectional area of typical proteins is 1000-10,000A^2. Morrissey's high bulk concentration case may represent a minimum foothold.

Walton and his students have also pioneered the study of protein conformational changes using fluorescence and circular dichroic spectroscopies (13-15). Transmission CD spectra of Hageman Factor adsorbed on a stack of quartz plates suggested conformational changes upon adsorption. CD spectra of proteins eluted after different contact times with the surface show that the degree of conformational change is directly related to the contact time for periods of up to 10 days (15). It is now generally accepted that protein conformational change can be a rather slow event.

Brash has also used CD to study eluted proteins and finds large changes in α-helix content of fibrinogen, perhaps due to enzymatic fragmentation produced by the surface-induced activation of plasminogen to plasmin (16,17).

Transmission fluorescence studies of adsorbed Hageman Factor show fluorescence changes which can be interpreted in terms of conformational and other micro-environmental effects (13,14), although such studies must consider substrate effects on fluorescence which may not directly affect conformational changes (14).

Adsorption may lead to an increase or decrease in titrable groups. Titration data can thus be interpreted in terms of conformational changes (5,6).

Soderquist and Walton (18) showed "an overshoot" in adsorbed amount as a function of time and a kink or inflection in the isotherm at about half saturation. They proposed:

a) Within the first minute of contact adsorption is rapid and reversible--a pseudoequilibrium is present. The protein is adsorbed in a random arrangement at coverages less than 50%.

b) At coverages greater than 50%, surface transitions and ordering may develop which lead to more efficient packing and an increase in adsorbed amount, hence the kink in the isotherm.

c) Given sufficient time adsorbed proteins undergo conformational changes which lead to increased surface interaction. During this process proteins less optimally adsorbed undergo desorption, hence the overshoot in the time curve.

d) The desorption rate decreases with increasing residence time.

e) Desorbed protein may be permanently denatured, suggested by CD results (15,16).

Although this last point may not be general, the others are now accepted as rough "rules-of-thumb" for protein adsorption, though clearly each protein-surface-solution system is unique and may not exhibit all of the features noted.

Soderquist and Walton also showed (18) that as adsorption time goes to zero, the data are compatible with reversible Langmuir adsorption. As residence time goes to infinity, the process becomes more and more irreversible due to the slow conformational changes involved.

The reversibility-irreversibility-hysteresis phenomena have been well modeled and developed by Jennissen (19).

Van Dulm and Norde (20) (in a study of human plasma albumin on negatively charged polystyrene latices) also showed a fast initial adsorption followed by desorption, probably due to conformational change of the adsorbed albumin which induces the release of less well bound protein. This result was observed at pH 4, where the albumin has a net positive charge and was not observed at pH 7.4, where it is highly negative.

The attenuated total reflection (ATR) Fourier transform infrared spectroscopic (FT-IR) studies of protein adsorption by Gendreu, Jakobsen, and others (21) have the potential for direct determination of conformational changes during the adsorption process due to shifts in infrared absorption bands (6).

Total internal reflection fluorescence (TIRF) spectroscopy has recently been applied by several groups (22-27) and complete reviews are now available (22,23). The method can easily follow the kinetics of adsorption using proteins labelled with extrinsic fluors, such as fluorescein or rhodamine. The intrinsic UV fluorescence of tryptophan (Trp) can be used to follow adsorption. The UV approach has the advantage that the tryptophan fluorescence is sensitive to the local micro-environment and no label is required. The major disadvantage of the UV method is the UV photochemical changes which occur, although such changes can be minimized by working at low radiation dose levels (23).

The intrinsic UV fluorescence of proteins is dominated by the tryptophan indole rings. The absorption maximum is 280-290 nm with the fluorescence maximum ranging from 315-355 nm, depending on the local environment of the indole side chains. Quantum yields range from 0.04 to 0.50; 0.10 is a common value. As the local environment polarity or dielectric constant increases, the maximum shifts up to 355 nm, such as for an indole ring in water or buffer. Trp moieties in highly hydrophobic environments fluoresce at 315-320 nm (28). Thus the fluorescence emission maximum (and the quantum yield) provide indirect information as to the local environment of the Trp fluors.

Although a number of proteins of interest (human serum albumin, for example) contain a single Trp, most contain two or more (14,23). Thus the spectrum observed is the sum of all active Trp fluors, making it difficult to deduce the local environment of each fluor. Nevertheless, the UV fluorescence emission spectrum is useful in deducing orientation and/or conformation changes upon adsorption (14,23).

MATERIALS & METHODS

Intrinsic UV TIRF was used; the apparatus has been described (23,27). Care was taken to minimize UV photodegradation by blocking the beam except during the actual measurements and by using continuous rather than pulsed UV sources.

Human plasma fibronectin (Fn) is a large glycoprotein ($M = 460 \times 10^3$) involved in cell adhesion and wound healing, and has heparin and collagen binding properties. It has a unique structure with a high Trp concentration. Most of the Trp are located in disulfide bonded "finger" regions with a unique amino acid homology. The adsorption properties have been studied by a number of groups (reviewed in Ref. 29). It was the first protein for which a UV fluorescence emission shift was observed upon adsorption using the intrinsic TIRF technique (29).

Fn was provided by Mosher (30) and supplied frozen in 1 ml aliquots. It was rapidly thawed by immersion in a 37°C water bath, centrifuged and diluted in PBS or tris buffer, (pH 7.2-7.4). The TIRF flow cell was primed with tris (pH 7.2) buffer. Five ml of the protein solution were injected at 13 ml/min (a shear rate of 210 s^{-1}). Adsorption was monitored for 40 minutes in the static (no flow) mode using an excitation wavelength of 280 nm and an emission of 330 nm. Adsorption was studied for bulk solution concentrations of 0.02, 0.05,and 0.43 mg/ml. Desorption was studied by clearing the bulk protein solution with a rapid buffer flush (60 ml at 60 ml/min; 970 s^{-1}) and then monitoring desorption for 15 minutes in the static mode.

Surface preparation has been described (29). Hydrophobic (n-pentyl triethoxy silane treated) and hydrophilic silica surfaces were used.

Bovine albumin was obtained as crude Fraction V and surely contained higher oligomers as well as some contaminant protein. The material was dissolved in PBS, pH 7.4. All studies were conducted with 1 mg/ml solutions adsorbing onto hydrophilic quartz. Bovine albumin has only 2 Trp, located at positions 134 and 212. On denaturation by heating or in urea solutions the fluorescence emission maximum shifts from 342 to 350 nm and the quantum yield decreases by half (14).

RESULTS & DISCUSSION

Fibronectin adsorption in the static mode from 0.05 mg/ml solution showed very different kinetics for the hydrophobic and hydrophilic surfaces. The data for static and flow adsorption and desorption have been reported (29). The quantity of interest here is the fluorescence maximum, which for Fn on hydrophilic silica is identical to that in bulk solution, suggesting no major conformational change upon adsorption (Table 1).

TABLE 1. Fluorescence maximum of Fn in different environments (from Ref. 29)

Environment	Fluorescence Maximum, nm
Bulk-PBS buffer	321
Bulk-PBS/8M urea	330
Bulk-PBS/3M guanidine Cl	350
Adsorbed on hydrophobic silica	326
Adsorbed on hydrophilic silica	321

The Fn-surface interactions probably involve the charged groups on the surface of the molecule, most likely the highly positively charged heparin binding regions of the molecule (pK=8-9).

Fn adsorbed on hydrophobic silica, however, fluoresces at 326 nm, suggesting a slight denaturation of the molecule. Fn interactions with the hydrophobic surface may involve some of the apolar residues in the protein interior, suggesting a partial denaturation.

Clearly studies on surfaces of a range of charge, polar, and apolar character would be of interest.

Bovine albumin has been studied only on hydrophilic quartz to date. The fluorescence maximum (1 mg/ml BSA in PBS) is 342 nm and shifts to 333 nm upon adsorption. These results suggest that the adsorption of BSA onto silica changes the conformation of the molecule such that the two Trp fluors are in a more hydrophobic environment (23).

Studies using radio-iodinated proteins in the TIRF apparatus permit a direct measurement of the amount adsorbed, thus allowing one to deduce if the quantum yield is also changed on adsorption. Such studies are in progress.

CONCLUSIONS

Intrinsic UV TIRF of the Trp residues in proteins permits one to monitor the adsorption and desorption of single component protein solutions at solid-liquid interfaces. The measurement is made in situ, in a controlled hydrodynamic environment, continuously, using native, unlabelled proteins. More importantly, the fluorescence emission maximum of the adsorbed protein, when compared against fluorescence maxima in different bulk solutions, permits one to deduce micro-environmental changes upon adsorption, which are related (though not straightforwardly) to the orientation and/or conformation of the adsorbed proteins.

We report that human plasma fibronectin adsorbed on hydrophobic and hydrophilic quartz shows maxima of 326 and 321 nm, respectively, suggesting that Fn adsorption on the hydrophobic quartz results in some conformation change of the adsorbed protein. Bovine serum albumin shows a 9 nm blue shift (from 342 nm in solution to 333 nm in the adsorbed state) upon adsorption onto hydrophilic quartz, suggesting a more hydrophobic environment for the Trp as a result of the adsorption process.

These preliminary studies demonstrate that intrinsic TIRF can be used to deduce micro-environmental and possibly conformation changes in adsorbed proteins.

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