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Behavior of Proteins at Interfaces*

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The adsorption and conformational changes of three plasma proteins, (bovine) serum albumin, γ-globulin and fibrinogen, on several (bio)polymer surfaces are reported. A theory is developed which invokes reversible adsorption of the proteins in the initial stages and a time dependent conformational change of adsorbed protein leading to essentially irreversible long-term adsorption. In each case experimental evidence indicates that there is a time--dependent decrease in structural order. It is postulated that the protein unfolds to optimize surface bonding, thus inducing the chemistry of the protein/water interface by bonding at the protein//polymer interface. Cell binding studies support the concept that the plasma proteins unfold to optimize the polymer/protein interaction.

BEHAVIOR OF PROTEINS AT INTERFACES

The behavior of proteins at interfaces is a topic of wide-ranging significance which bears on the properties of substrate bound enzymes and their commercial utilization on one hand, and to biocompatibility of prosthetic implants, artificial kidney machines, etc., on the other. In fact, it is probably fair to say that virtually all protein purification processes involve chromatography and selective adsorption at one stage or another of their preparation and separation. Given this background, it is perhaps surprising that very little is known of the relation between surface binding of proteins and their subsequent structural and functional integrity either in the adsorbed or desorbed state. It is, for example, suspected that structural changes induced in blood proteins as they adsorb at an implant surface dictate virtually all of the subsequent aspects of blood compatibility, yet very little is known of such changes.

Most of the methodology in the surface chemist's arsenal has been directed at understanding the process of protein adsorption; much of the data gathered relate to how much protein adsorbs, adsorption isotherms, competitive adsorption, etc. Thus use of radioactive labelling[1], attenuated total reflectance (A. T. R.) spectroscopy[2], various forms of quantitative analysis[3], fluorescence spectroscopy[4,5], ellipsometry[6], calorimetry[7,8], circular dichroism spectroscopy[9], streaming potential measurements[10], etc., have been applied. Of these techniques,

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only circular dichroism spectroscopy addresses the problem of structural changes of adsorbed proteins more or less directly. Calorimetry could, in principle, also provide information on structural changes in adsorbed proteins if the relation between exotherms and structural denaturation were known. Very indirect information may also be obtained from some of the other techniques, but no consistent theory of protein structural behavior has so far been derived.

Part of the problem arises from the fact that published conclusions vary from monalayer to many multilayer adsorption, irreversible to reversible kinetics, no structural change to complete denaturation. Some of these problems may arise from the choice of substrates which have rarely represented a systematic series of chemically varied materials. Thus adsorption of an enzyme on quartz may have little behavioral relation to adsorption of blood proteins on plastic. In addition some techniques are not easily calibrated.

The work described here is a brief review of a new theory of protein adsorbance and the experimental evidence relating to it. The experimental details will be published elsewhere.[11]

The type of substrate used in this work is a class of biological polymers known as a random copolypeptide. These materials are synthesized by copolymerizing two (or more) amino acids to produce a polymer of more or less random sequence. Since a great many different functional groups may be built into such a polymer, it would seem to have potential as a substrate capable of undergoing specific enzyme binding and commercialization. In fact, most of these materials have been developed in our laboratories as potential biological implant materials or coatings,[12-16] but enzyme localization is an additional important possibility. Since amino acid polymers (proteins) are present as potential substrates in the biological milieu, the use of the random copolypeptides also has biological implications; we have previously mentioned their role as model biological interfaces.[17] Most of the work presented here pertains to $(A_x B_y)_n$ type copolymers, where A is glutamic acid, a glutamate derivative, lysine or a lysine derivative, and B is one of leucine, valine or phenylalanine. In this manner the biopolymers are mainly hydrophobic with a range of acidic, basic or neutral functional groups inserted.

The studies outlined in this paper are concerned with the adsorption and structural changes of three blood proteins, albumin, γ-globulin and fibrinogen. (We have previously reported structural changes in the adsorbed blood proenzyme, Hageman factor.[17])

The detailed structure of these three proteins is unknown; however, certain structural features are well known. All three of the proteins are believed to have highly asymmetric structures. The amino acid sequence of human and bovine serum albumins are known. We have published a proposed structure[5] based on conformational analysis which shows the molecule to be essentially cylindrical with α-helices forming the sides of the cylinder. The molecule is 55—60% α-helical by circular dichroism (CD) measurement and has a molecular weight of 66,248 (bovine) or 66,210 (human).

γ-Globulin is actually a class of antibody immunoglobulins with variable sequence. Some specific γ-globulins have been crystallized and the molecules are mainly irregular in structure with some β-sheet content ($\sim 30\%$). The molecular weight is in the 170,000 range.

Fibrinogen is one of the blood clotting zymogens and consists of three pairs of identical chains (total $M_w \sim 340,000$). The molecule is $\sim 35\%$ α-helical by CD measurement and recent conformational analyses suggest that the α-helical regions consist of a three-strand α-helix coiled coil.

The preceding three proteins represent the majority of protein found in blood serum ($\sim 90\%$) and their surface behavior is particularly relevant to cardiovascular prosthetic development.

EXPERIMENTAL

Glass beads of ca. 0.14 mm diameter were coated with the appropriate polymer by treatment with a solution (usually in chloroform or trifluoroacetic acid) which was allowed to pass through a column containing the beads. Subsequent drying produced a film of ca. 0.5 μ thickness on the beads with a total surface area of ca. 2180 cm².

Aqueous solutions of highest grade protein in various buffer solutions were equilibrated with the substrate for a given period of time at room temperature. The amount adsorbed was calculated from the amount eluted either by ultraviolet absorbance or Lowry determination of protein, or both. Similarly, desorption was achieved by allowing buffer of stated concentration to equilibrate with the surface adsorbed protein for a given period of time and the eluent analyzed as before. Conformation of the protein was assayed before and after adsorption by a JASCO J40A spectropolarimeter.

Two of the proteins (bovine serum albumin, B.S.A., and γ-globulin) were used as received. Bovine fibrinogen (listed as 97% clottable) was further purified by the method of Laki.[18]

RESULTS

The uptake of proteins increases rapidly in the order of a few minutes and generally has reached a »pseudo-equilibrium« after ca. 30 minutes. However, there is a slight but continuous increase in protein uptake after this period. Figure 1 shows the rate of adsorption of B. S. A. in a typical run. The amount of material adsorbed is a function of the amount added in solution, the time of exposure and the method of addition. For example, the amount adsorbed is different if the substrate is exposed to 8 mg/ml of protein or successive additions of 2 mg/ml of protein. This curious behavior, indicative of a complex
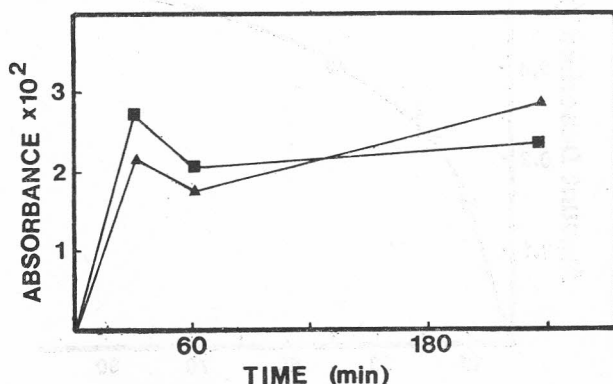

Figure 1. Adsorption of BSA on siliconized glass (■) and [Lys²⁰—Leu⁸⁰] (▲).
The ordinate given in adsorbance units corresponds to 0.01 = 0.35 mg/m² in the present case.
The spike at ~ 10 min is reproducible and perhaps reflects a reorganization and/or a contormational change which modifies the rate of uptake.

adsorption process, is demonstrated in Figure 2. for B. S. A. It can be seen that although the adsorption curves reach a plateau value of ~ 2.5 mg/m², which is close to a side-on monolayer, the actual value depends upon the mode of addition. Furthermore, there is a major »kink« in the adsorption curve at ca. 50% coverage. It has been hypothesized by Fair and Jamieson in our laboratory[19] that this kink corresponds to a reorganization of randomly arranged surface molecules to a more ordered phase, i. e. a surface nucleation process. Fibrinogen also adsorbs in a side-on mode but, surprisingly, γ-globulin adsorbs endon (a phenomenon previously noted on other polymeric surfaces).
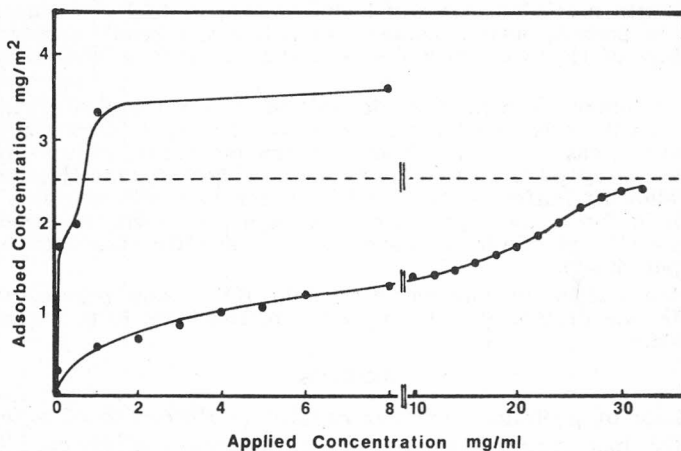

Figure 2. Adsorption isotherms of BSA on [Lys²⁰—Leu⁸⁰].
Upper curve, separate runs equilibrated for 16 h.
Lower curve, successive addition of 2 mg/ml followed by 30 min equilibration.
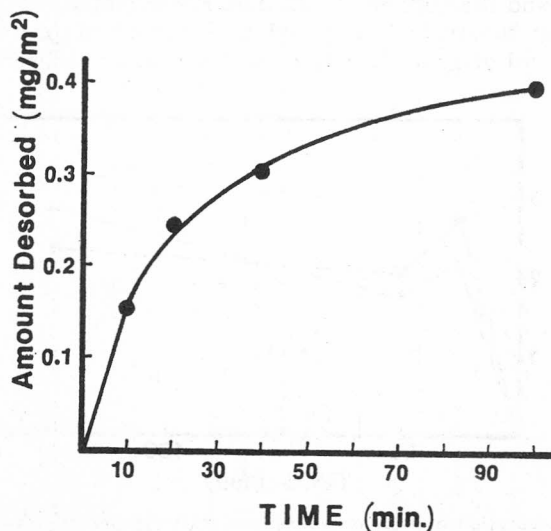

Figure 3. Desorption curve of BSA on siliconized glass beads at pH = 7.4 and ionic strength at 0.13 M. Original amount adsorbed on the surface, after 10 min incubation, was 2.05 mg/m².

Desorption is a very slow process and over a limited time span the process is almost irreversible. However, on standing for several hours it is possible to desorb sufficient quantities of protein to measure. Figure 3. shows a desorption curve for B. S. A. The desorbed material is changed in conformation, the change being in the direction of decreased order, i. e., denaturation of the protein. This change was observed with each of the desorbed proteins: the course of structural change based on decreased circular dichroism ellipticity is shown in Tables I—III.

TABLE I

Change of Adsorbed (Desorbed) γ-Globulin Structure

Time/h	Polymer Substrate % Native Structure* (% Desorbed)			
	Hydrophobic		Basic(+)	Acidic(—)
	Silicone	ZLys^{20} : Leu^{80}	Lys^{20} : Leu^{80}	Glu^{20} : Val^{80}
pH = 7.4/0.26 M				
34	100(37)	100(45)	100(34)	100(37)
106	78(66)	81 —	72(56)	66(59)
274	62(78)	79(67)	79(66)	69(70)
pH = 7.4/0.13 M				
24	76(2)	69(2)	78(2)	85(1)
96	—(5)	56(5)	—(4)	—(4)
168	69(18)	—(12)	63(12)	74(18)
pH = 6.8/0.13 M				
34	59(10)	63(6)	59(7)	59(8)
130	—(38)	—(33)	59(26)	—(31)
178	47(59)	38(39)	38(32)	46(39)

* Based on CD measurements as a function of the $[\Theta]_{217}$ band.

TABLE II

Change of Adsorbed (Desorbed) Fibrinogen Structure

Time/h	Polymer Substrate % Native Structure* (% Desorbed)			
	Hydrophobic		Basic(+)	Acidic(—)
	Silicone	ZLys^{20} : Leu^{80}	Lys^{20} : Leu^{80}	Glu^{20} : Val^{80}
pH = 7.4/0.26 M				
36	42(7)	—(9)	49(2)	52(1)
84	24(86)	41(90)	39(65)	40(64)
128	2(100)	18(100)	13(96)	21(100)
pH = 7.4/0.13 M				
24	51(73)	62(52)	99(66)	87(72)
88	73(89)	43(71)	69(88)	51(95)
100	22(100)	10(89)	12(11)	11(100)

* Based on CD measurements as a function of $[\Theta]_{208}$ band.

TABLE III

Change of Adsorbed (Desorbed) Albumin Structure

Time/h	Polymer Substrate % Native Structure* (% Desorbed)			
	Hydrophobic		Basic(+)	Acidic(—)
	Silicone	ZLys[20] : Leu[80]	Lys[20] : Leu[80]	Glu[20] : Val[80]
pH = 7.4/0.26 M				
15	(18)	(46)	(30)	(35)
22	(32)	(58)	(36)	(55)
24	(44)	(86)	(68)	(68)
pH = 7.4/0.13 M				
40	26(21)	14(35)	16(17)	13(26)
pH = 4.7/0.13 M				
40	86(12)	58(36)	78(19)	48(26)
108	29(21)	38(75)	59(38)	—(51)
204	23(38)	33(—)	29(54)	29(93)

* Based on CD measurements as a function of the $[\Theta]_{208}$ band.

DISCUSSION

The uptake of protein as demonstrated in Figure 1. is much slower than the rate of arrival of protein at the surface by diffusion and thus protein molecules must collide reversibly with the surface. Perhaps the most perplexing aspect of protein adsorption on polymer surfaces is that the protein seems to adhere virtually irreversibly, yet only a limited uptake of protein is achieved from a given concentration of applied solution, which implies reversible adsorption.

We believe that this paradox can be explained by use of the following model.

1) The protein adsorbs reversibly on the surface provided that the residence time on the surface is short.
2) On prolonged contact with the surface, the protein undergoes a conformational change that decreases its rate of desorption.

On the above basis, the rate of adsorption would be Langmuir-like i. e.

$$R_{ads} = k_1 \, c_s A \tag{1}$$

where k_1 is the diffusion controlled rate constant, c_s the solution concentration and A the available surface area. (At high enough c_s, at least one additional layer of protein can adhere and perhaps a methoric layer.)

The rate of desorption is complicated by surface nucleation and rearrangement, but for any given surface state is represented by

$$R_{des} = k_2 \, f \, (\Sigma \, c_i \, t_i) \tag{2}$$

where c_i is the surface concentration adsorbed for a time t_i. Equation (2) is not

readily solved since there is no simple way of determining c_i or t_i. However, if a certain amount of material is adsorbed quickly and any desorbed material is removed as it forms, the rate of desorption may be assessed more readily, i. e.

$$R_{des} = k_2\, c_{ads}\, (1 + k_3\, t^n)^{-1} \qquad (3)$$

In writing this expression it is assumed that there is a time-dependent change of the desorbability of protein, brought about by conformational change, that has the form t^n.

A correlation of equations (1) and (3) indicates then that as $t \to 0$, adsorption is reversible and Langmuirian, whereas with $t \to \infty$ adsorption becomes irreversible.

In order to substantiate this form of process, we have performed desorption experiments of B. S. A. from silicone treated glass spheres and find that the data conform to the relation

$$R_{des} = \frac{7.5 \times 10^{-3}\, c_a}{(1 + 1.22 \times 10^{-3}\, t^{2.2})} \qquad (3b)$$

However, at this time we have insufficient data to assess the general applicability of the equation 3. We hypothesize that adsorbed proteins adjust their conformation to optimize interaction with the substrate: the stronger the interaction the faster the change. Conversely for weakly interacting substrates or more stable proteins, conformation change would be slower or not-existent ($k_3 \to 0$). Thus k_3 will reflect interaction energy between protein and substrate (for a given protein with various substrates) or inherent protein stability (different proteins with the same substrate).

In the preceding approach we have assumed that changes in desorbed protein are an indication of change in adsorbed protein structure. In principle this process should be accessible by surface circular dichroism studies and indeed freshly prepared surfaces (quartz) with adsorbed fibrinogen appear to show relatively intact structure.[9] Unfortunately, longer term studies are complicated by the slow desorption process and difficulties with absolute precision at such low surface concentrations.

We propose then that surface adsorbed proteins undergo structural changes, as shown in Figure 4. Depending on whether the substrate is acidic, basic or hydrophobic, the protein deconvolutes to optimize interactions. This being so, the portion of the protein exposed to solution changes and consequently the binding of ions and other entities should also change. Whereas it should be possible to detect such changes in coated tubes by streaming potential measurements, we have used a somewhat different approach.

If one coats a polymeric surface with protein (preferably a monolayer), equilibrates to achieve optimization of surface interaction and protein conformation and then attempts to adsorb a second entity, the adsorption characteristics of the second entity should reflect the nature of the surface. In this sense the adhesion of negatively charged cells proves to be an interesting probe. On examination of Figure 4., it can be seen that for a negatively charged protein (e. g. B. S. A.), the solution surface of the protein on a negative or neutral surface should be similar, i. e. negatively charged. On a positively charged polymer, however, the protein should turn a positive/hydrophobic surface toward the solution which should be a more »sticky« surface to the

Basic/Hydrophobic

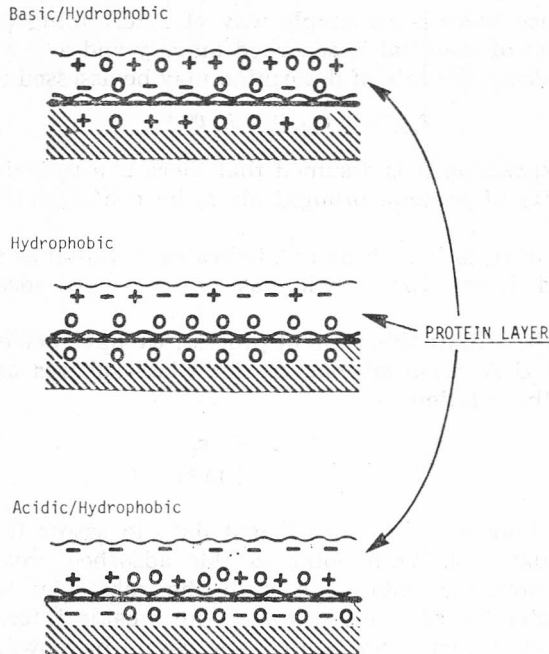Hydrophobic

PROTEIN LAYER

Acidic/Hydrophobic

Figure 4. Schematic diagrams of the proposed process of denaturation at the polymer/water interface.
   If the protein, e. g. albumin, is of low conformational integrit-, a process occurs which optimizes surface interaction.
   The interface between the protein layer and water reflects this process and takes on a characteristic which may be similar (acidic or basic/hydrophobic) or different (hydrophobic) from the underlying substrates.

negatively charged cell than the other two cases. We have previously shown that this is indeed the case[20,21] as is shown in Tables IV and V.

CONCLUSIONS

The conclusions to these studies seem to have considerable implications as follows:

(1) Blood proteins (albumin, γ-globulin, fibrinogen) adsorb on (bio)polymer surfaces and undergo a time-dependent conformational change.
   Since it may be assumed that the body »prefers« surfaces that do not damage or denature proteins, this implies that surfaces which are more hydrophilic or do not bind proteins tightly are more desirable since less damage is caused.

(2) Proteins vary in their stability in the adsorbed state.
   Evidently it is not efficicacious to attempt to immobilize structurally fragile enzymes on highly interacting surfaces by adsorption since the enzyme will rapidly lose activity. Hence sensitive enzymes are most likely to be effective if covalently bound to highly hydrated substrates, whereas highly stable enzymes may be appropriately bound to solid particulate supports by physical adsorption.

TABLE IV

Adhesion of Syrian Hamster Fibroblats (NIL B) To Copolypeptide Films

Substrate	Rate Constants/min^{-1}		% Adhesion	Conditions
	k_1	k_2		
Glass	0.3 ± 0.05	0.006 ± 0.003	100	nc MEM 4X
	0.2 ± 0.14	0.01 ± 0.01	92 ± 5	BSA coated[a,b]
Siliconized Glass	0.33 ± 0.01	0.07 ± 0.004	100	nc MEM 4X
	0.08 ± 0.05	0.09 ± 0.05	43 ± 6	BSA coated
Glu[20]-Val[80]	0.32	0.004	100	nc MEM 4X
	0.1 ± 0.03	0.09 ± 0.05	55 ± 5	BSA coated
Glu(OBzl)[20]-Val[80]	0.38	0.003	100	nc MEM 4X
	0.09 ± 0.07	0.13 ± 0.11	43 ± 4	BSA coated
Lys[20]-Leu[80]	0.23	0.01	96	nc MEM 4X
	0.26	0.01	96	BSA coated
(OBzl)Glu[c]	0.28	.019	96	nc MEM 4X
	0.033	.024	66	BSA coated

[a] Extracted from Soderquist (1977).
[b] BSA = bovine serum albumin.
[c] From present work; for comparison purposes.

TABLE V

Adhesion of Syrian Hamster Fibroblasts (NIL B) to Copolypeptide Films with
γ-Globulins (γG)

Substrate	Rate Constant /min^{-1}		% Adhesion	Conditions
	k_1	k_2		
Glass	0.043	0.042	51	γG coated
Siliconized glass	0.028	0.025	53	γG coated
[Glu[20]-Val[80]]	0.069	0.056	55	γG coated
[(Z)Lys[20]-Leu[80]]	0.05	0.031	60	γG coated
[Glu(OBzl)]	0.055	0.045	50	γG coated
[Lys[20]-Leu[80]]	0.215	0.014	93	γG coated

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SAŽETAK

Ponašanje proteina na granicama faza

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Opisana su opažanja o adsorpciji goveđega serumskog albumina, γ-globulina i fibrinogena na površinama biopolimera, te konformacijske promjene koje prate adsorpciju. Predložena je teorija kojom se tumači brza početna reverzibilna adsorpcija povezana s naknadnim kinetičkim procesima konformacijskih promjena. Konačni rezultat je ireverzibilna adsorpcija. Teorija postulira odmatanje strukture proteina u procesu adsorpcije, kako bi se mogla optimalizirati kemijska veza s površinom. Stanje u graničnom sloju promatra se kao interaktivni odnos stanja na granici faza protein/voda i onoga na granici faza protein/biopolimer. Fenomeni vezivanja proteina na stanične membrane dokazuju osnovnu ispravnost predložene teorije.

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