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A Domain Approach to the Adsorption of Complex Proteins: Preliminary Analysis and Application to Albumin*

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Albumin consists of three large domains with differences in electrostatic nature, charge-pH characteristics, and denaturability. The interfacial activity of albumin is due, at least in part, to the interfacial activity of its constituent domains. Consideration of the structure and interfacial activity of the various domains permits new and more precise hypotheses to be developed, with which new and better experiments can be designed. Such hypotheses allow one to evaluate and compare adsorption data, including kinetics and isotherms, adsorbed layer thickness, refractive index, lateral cohesion, multilayer formation, etc.

We feel strongly that each different protein is a unique molecular personality, which must be understood and considered if we are to more fully understand and apply the interfacial behavior of complex proteins.

INTRODUCTION

The adsorption of simple, model proteins at simple, model interfaces is qualitatively understood. We[1-3] and others[4] have hypothesized and shown that adsorption at short contact or residence times can be qualitatively predicted from, and correlated with, the surface chemistry of the protein globule, as deduced from x-ray crystallographic coordinates. Wei and others have demonstrated that adsorption at the air-water interface at long residence times correlates with the conformational stability of the model protein.[1] This correlation has also been suggested by Lyklema and Norde[5] and by others.[2,3,5]

Although much work remains before a reasonable theory is available for the prediction of model protein adsorption at model interfaces, we feel that it is now possible to cautiously and qualitatively approach the problem of the adsorption of complex proteins at model interfaces.

The objective of this paper is to outline an approach to the problem and to consider the adsorption of a »model« complex protein, albumin, at model interfaces. We expect to address other complex proteins in subsequent papers.

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EROM COMPLEXITY TO SIMPLICITY

Although the behavior of 'simple' proteins at 'simple' interfaces is qualitatively understood, the many plasma proteins responsible for blood coagulation and blood »compatibility« are not simple and their 3-D structures are not known. Likewise, many of the tear proteins implicated in contact lens interactions are also complex and structurally undefined.

Complex proteins can be made simpler by treating them as if they are constructed of functional and structural domains[6,7]. Structural domains are generally defined and identified as regions of the protein of relatively high packing density, which can be identified from the x-ray crystallographic coordinates using various algorithms.[8-10]

Rapid progress is being made in the prediction of structural domains from amino acid sequences.[11] Structural and functional domains can also be deduced from enzyme cleavage data[12] and from exon analysis of the DNA sequence.[13] These methods, coupled with careful analysis of the functions of the various domains, have allowed the development of schematics or »cartoons« of the functional structures of complex proteins, including plasma proteins.[13,14]

The identity and thermodynamic autonomy of structural domains can often be deduced from denaturation (unfolding)[15] and calorimetric studies.[16-18] The intrinsic stability of the protein is the free energy of folding and can be obtained from calorimetric or solution (urea or guanidinum chloride) denaturation studies.[1,15,16] Advances in the sensitivity of calorimetry and in the analysis of the scanning curves show, for many complex proteins, that the individual domains are calorimetrically independent and their individual thermal characteristics can be resolved.[17,18]

The »surface chemistry« of proteins can be obtained directly from x-ray crystal structures or from 2D NMR solution structures, when such data are available. By use of molecular computer graphics, one can readily discern the nature of the protein »surface«.[1-4] Such analysis leads to a much better appreciation of the surface chemical virtuosity of a protein. One rapidly begins to appreciate that different »faces« or regions on the protein surface can have very different surface and interface activities.

In the many cases where the three dimensional structure is not known, a simple analysis of the amino acid sequence is often helpful. Today most of the known sequences are organized in protein data banks which can be accessed via computer, using a modem and a telephone line, such as the Protein Identification Resource (PIR) in the USA,[24] and the Institute Pasteur in Europe.[26] Both of these databases contain annotated protein amino acid sequences. To use these data banks effectively, one also needs access to programs which can search the database, extract the raw information and process the data.[26,27] One initial question to ask is how homologous is the sequence in comparison to other known sequences. Homologous proteins may have similarities in main-chain folding and possibly in interfacial behavior. Aligning sequences is particularly useful when one of the homologous proteins has a known tertiary structure.

In addition to homology searches one can predict protein secondary structure from the amino acid sequence.[28] Four common areas of predictions are:

Typical secondary structures (helices, turns, coils);
Trans-membrane helices;
Antigenic sites;
Signal and target sequences.

As the overall accuracy of secondary structure prediction methods is only about 60%, they can only provide a starting point for further, more refined analysis by other methods.

As charged amino acids are generally on the surface of the protein, regions of unusually high electrostatic character, positive or negative, are often clues to particular electrostatic binding characteristics. Such an analysis readily identifies the heparin-binding plasma proteins,[19-21] as they all have domains or amino acid sequences rich in Lys and Arg.

Certain aspects of the surface chemistry can be derived from appropriate ligand-binding studies, especially using fluorescent probes. Probes are available which sense or »report« on many different microenvironments, including charge, potential, and hydrophobicity. Wei[1] recently showed that one such probe can be used to obtain an »apparent surface hydrophobicity« parameter for various model proteins. The surface hydrophobicity of the proteins correlates with their surface activity at the air/water interface.[1]

The interactions of proteins and their protease cleavage fragments with chromatographic surfaces provides clues as to interface characteristics and activities. Affinity chromatography data are especially valuable in identifying »specific« binding properties of proteins and fragments.

The interfacial behavior of a complex protein may be largely dominated by the interfacial activity of only one domain or even a sub-domain. For example, exposure of blood plasma to heparin-Sepharose materials results in the depletion of heparin-binding proteins, such as antithrombin III, with little depletion of the other plasma proteins.[19,20] Although this is an expected result, it suggests that the interfacial activity of the heparin-binding proteins — on a sulfonated surface — is dominated by their richly positive regions or domains.

We propose that the initial adsorption event is a function of the interfacial activity of the various domains or regions of the protein, and primarily on the particular surface chemistry of the domain or region. For many protein — solid surface systems one of the domains can be expected to »dominate« the interfacial activity of the protein, for example:

domains with large hydrophobic patches at hydrophobic surfaces;
anionic protein regions on cationic surfaces; and
cationic protein regions on anionic surfaces.

We further propose that the conformational accommodation (»denaturation«) of the protein at the interface will be a function of the stability of each of the individual protein domains — *i. e.* how »hard« or »soft« are each of the domains.[1,5,29] Domain hardness or softness can be qualitatively assessed by thermal or solution denaturation measurements.[1,15-18]

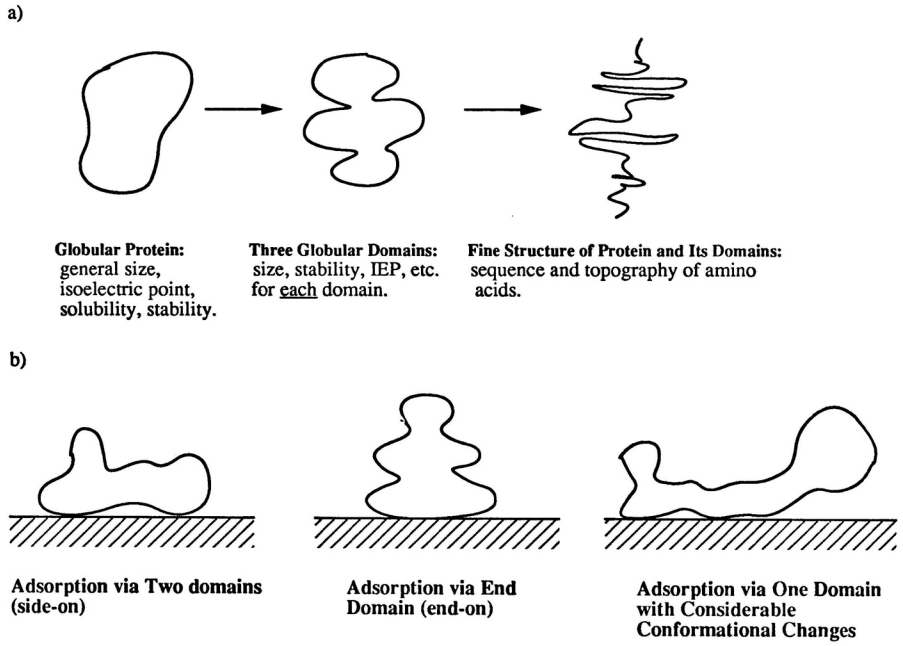

Figure 1. The domain concept for protein interfacial activity.
a) Left: the »classical« approach: proteins are colloidal particles with certain average particle characteristics; center: the domain approach: globular proteins, such as albumin, consist of structural and functional domains interconnected by flexible, hinge-like regions; right: each domain can be treated and modelled to determine its unique properties and characteristics.
b) Multi-domain proteins at a rigid interface; left: adsorption via one of the two domains; center: adsorption via one terminal domain (end on); right: adsorption via one dominant domain which conformationally alters and »spreads« on the surface with time.

Our simplistic model of a complex protein (Figure 1) is that it is a collection of simple domains, tied together via relatively flexible polypeptide segments. Clearly such a model is naive, simplistic, and unrealistic — but it is far better, in our opinion, than treating a protein as an undefined, unknown globule.

APPLICATION TO ALBUMIN

Albumin is a useful protein with which to begin our analysis because it:[34]
contains no carbohydrate;
consists of three fairly distinct domains;
has a high α-helix content;
has many —S—S— bonds;
has important ligand binding properties; and
the 3D x-ray structure is now available for human albumin (35).

Albumin is the major protein component of blood plasma and serum. Its collision rate with surfaces and interfaces is over 7 times greater than that

of any other plasma protein[30]. It is not surprising, therefore, that albumin adsorption dominates the plasma protein adsorption process at short contact times[30]. At longer times adsorbed albumin may be removed from the surface, as other proteins with higher interfacial activity may more strongly interact. For this and other reasons, a complex adsorption hierarchy is observed on most surfaces exposed to blood plasma[30,31]; this phenomenon has been called the »Vroman Effect«[32,33]. What one »sees« on an interface exposed to plasma (or to any other complex protein solution) depends on when one »looks«, that is, the surface composition is — in general — time dependent.

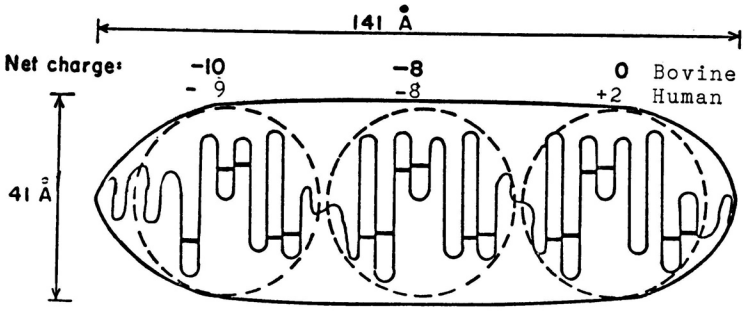

Figure 2. The »tennis ball« model of albumin, showing the three major domains and the disulfide-bonded alpha helical subdomains. Domain I is the N-terminal, domain III the C-terminal. Note the differences in overall charge in the various domains (from Ref 34).

Figure 2 (modified from Ref.[34]) shows the general size, shape, and amino acid sequence folding pattern for albumin. Although bovine and human albumin are similar in general folding properties, their net charge and charge distributions are different. The general shape can be viewed as three tennis balls (the large domains) in a can or cylinder.[34]

The large domains are normally called I, II, and III, going from the N terminal (left in Fig. 2) to the C terminal. The N terminal end binds Cu^{++} and Ni^{++}. The two high affinity fatty acid binding sites are in Domain III and in Domain II near the interface with Domain III (see Fig. 8 in Ref. 34). Other fatty acid sites are located in the center of Domains II and I. There are a variety of binding sites for other ligands.[34] The presence of fatty acid significantly enhances the conformational stability of albumin.

The overall denaturation temperature is increased from about 60° C (defatted) to 80° C (fatted) at pH 7.0[36], which must reflect an increase in stability in Domain III. Domains II and III tend to have higher individual denaturation temperatures than Domain I, probably due largely to their fatty acid binding[36].

Domain I has the highest net charge (—9 for human, while Domain III is +2 in human and zero in bovine albumin, according to Ref. 34. A simulated titration of bovine albumin reveals the estimated net charge on each of the three domains (Figure 3).

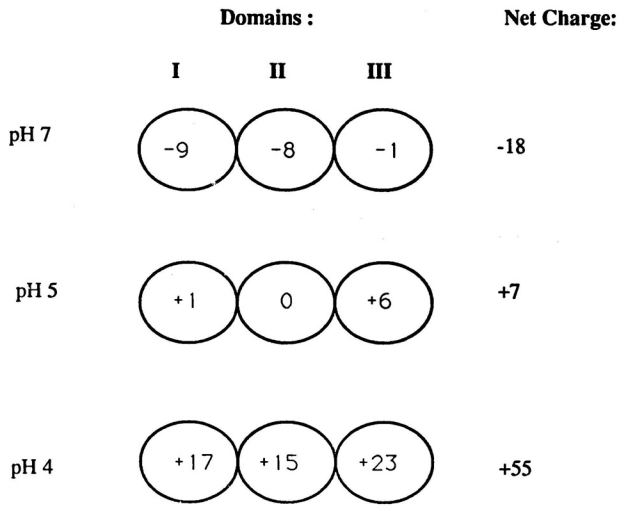

Figure 3. Simulated charge of the three domains in bovine albumin using following pK values*: $pK_{Arg} = 12.48$, $pK_{Lys} = 10.79$, $pK_{His} = 6.00$, $pK_{Asp} = 3.65$, $pK_{Glu} = 4.25$, $pK_{Tyr} = 10.13$, $pK_{Cys} = 8.3$, N-terminal $pK = 9.6$, C-terminal $pK = 2.34$. Newly created N-terminal and C-terminals of each domain were made untitratable by setting the pK equal to 1 or 13, respectively. Note the close agreement with the values reported in Ref. 34 (Figure 2). These results are also in qualitative with Norde's data on the titration of adsorbed and solution human albumin (55).

Albumin undergoes several pH sensitive transitions, especially the N — F transition in going from pH 7 to pH 4 or lower. Most of the expansion of the molecule at lower pH (the F form) is probably the result of expansion or unfolding of Domain III[37].

There is some evidence that the C-terminal (Domain III) may be looser or less compact that the N-terminal (Domain I)[40,41], however the N- terminal region may refold more rapidly than the C-terminal[40]. The evidence suggests that Domains I, II, and III fold and refold somewhat independently[40,41].

Alkyl chain surfactant binding occurs towards the C-terminal, leading to loss in α-helix content; »... the more stable N-terminal unfolds later ...« (Ref. 41, p. 125). Although surfactant treatment leads to a loss in α-helix, it is believed »... that the large loops are the most stable against denaturation by surfactants.« (Ref. 41, p. 125).

Various peptic and tryptic fragments of albumin have been studied.[40,42] The peptic fragments have been exposed to surfactants and to urea and GdnCl denaturants. Urea denaturation is at about 6M, GdnCl denaturation at about 2M[42]. No dramatic differences were observed, although much of Domain III was not represented in these fragments. The thermal denaturation studies of albumin which are available[36] are on different fragments and therefore difficult to compare to the solution denaturation studies.

It is interesting to compare the Brown model of albumin[34,38] with the newly available 3D structure (see Fig. 3 in Ref. 35). Feng, *et al.* recently succeeded in obtaining high resolution scanning tunneling microscopy (STM)

images of individual human albumin molecules on a single crystal graphite surface[39]. Their images exhibit a remarkable similarity to the published 3-D image[35].

TABLE I

Preliminary hypotheses coupling human albumin structural properties to interfacial activity at pH ≈ 7.

Surface	
Positively Charged	Domains I and II are negatively charged and would be preferentially bound. As these domains are less stable than Domain III, one would expect the adsorbed albumin to be more denatured than on other surfaces.
Negatively Charged Surfaces	Domain III is weakly positive and would tend to adsorb; there is little denaturation due to the stability of Domain III when it contains bound fatty acid.
Hydrophobic Surfaces	The first loop in domain I is probably hydrophobic and would tend to bind. As this loop is less stable than other loops, a slow time dependent denaturation can be expected.

Based on an analysis of the available structures and images, Table I presents several very preliminary hypotheses regarding the surface activity of albumin.

ALBUMIN AT MODEL INTERFACES

There is a large literature on the adsorption of human and bovine albumin. Here we briefly review a number of key papers dealing with model surfaces and interfaces.

The Brown model of albumin is based on an association of the hydrophobic faces of the α-helices in the subdomains[34,38]. The amphiphilic nature of these helices suggest that an apolar interface could significanlty disturb the normal hydrophobic association of albumin.

Peptides and proteins often assume amphiphilic secondary structures at oil/water, lipid/water, and air/water interfaces. The apolar nature of the interface may induce an amphiphilic structure or may drive the adsorption and ordering of an existing helical structure[43,44]. The amphiphilicity of known helices can be viewed using the Edmundson »wheel« projection[45] and quantified using Eisenberg's hydrophobic moment analysis[46].

Krebs and Phillips have studied the α-helix contribution to the surface activity of proteins[47] as probed by surface pressure measurements.

There is a strong correlation between helix amphiphilicity and surface activity.

Albumin readily adsorbs at air/water and oil/water interfaces — the resulting decreases in surface and interfacial tensions can be easily monitored.[48-51] Although no work has yet been reported on the surface or interfacial tension behavior of individual albumin fragments, Damodaran and Song[52] have published a most interesting study on the surface activity of bovine serum albumin (BSA) »structural intermediates.« They fully reduced

and denatured BSA and then followed the refolding process by α-helix and dye binding measurements. They could stop the refolding process by blocking the available —SH groups, thus preventing further —S—S— bond formation. In this manner they identified and isolated 7 structural intermediates ranging from 6% (0 bonds) to 30% (~15 —S—S— bonds). »Native« BSA has 17 bonds and 100% »native« structure; percent native structure was based on the extent of dye binding. All of the intermediates are very different from BSA. They could conclude that the rate of adsorption (deduced from the surface tension decrease or spreading pressure increase with time) increases with increasing degree of unfolding, but that the area occupied per molecule at the interface increased with increasing α-helix content.

As albumin's helices exhibit some amphiphilicity, one model is that individual helices or groups of helices can »adsorb«. Even Damodaran and Song's[52] most unfolded intermediate contained some α-helix content. The increase in adsorption rate with degree of unfolding may be a reflection of a greater number of effective collisions. In the more folded intermediates, less collisions would be effective in adsorption, leading to a decreased rate. One can also suggest that if native albumin adsorbs, then the least stable domain can be expected to open up and populate the interface, presumably by adsorption and orientation of its amphiphilic helices.

The earlier air/water interface work by Graham and Phillips[53] using native BSA suggests the adsorption of intact α-helices with loops and turns protruding into the aqueous phase — the adsorbed film thickness being ~50—60 Å (by ellipsometry). Electrostatic interactions appear to play a minimal role at the air/water interface unless one goes to extremes of pH.[53] At air/water, oil/water, and apolar solid/water interfaces the adsorption appears to be dominated by hydrophobic interactions and, probably, by the adsorption of amphiphilic helices.

Watanabe, et al. studied BSA adsorption at cationic monolayers by ellipsometry,[54] concluding that adsorption is maximal at pH ~5, about the isoelectric point (IEP) of BSA. Their study was done using a 0.025M salt concentration, thus the electrical double layer (Debye) length should be ~30 to 40 Å. They showed a strong effect of adsorbed layer thickness with pH, ranging from about 240 Å at pH 4 to roughly 80 Å at pH 6 to 8. We will succumb to the temptation to interpret this result by reference to Fig. 2. Using the »tennis ball« model, BSA at pH 7 and pH 5 would have the electrostatic character given in Figure 3.

At pH 7 at a cationic surface, one could expect domains I and II to bind and domain III to perhaps be oriented into the solution phase (Figure 4). This would result in a monolayer thickness in the range of 50—100 Å, As the pH is decreased, the negative charges on domains I and II decrease, and domain III goes positive, possibly resulting in an end on orientation and a thicker film. Clearly this simple electrostatic argument is not sufficient to explain Watanabe's results.[54]

The behavior at negatively charged surfaces may also be considered via such cartoons. Under these conditions only end on adsorption via domain III may be dominant at pH 7 and 5. At pH 4 each of the three domains is highly positive and side on orientation may be expected (Figure 4).

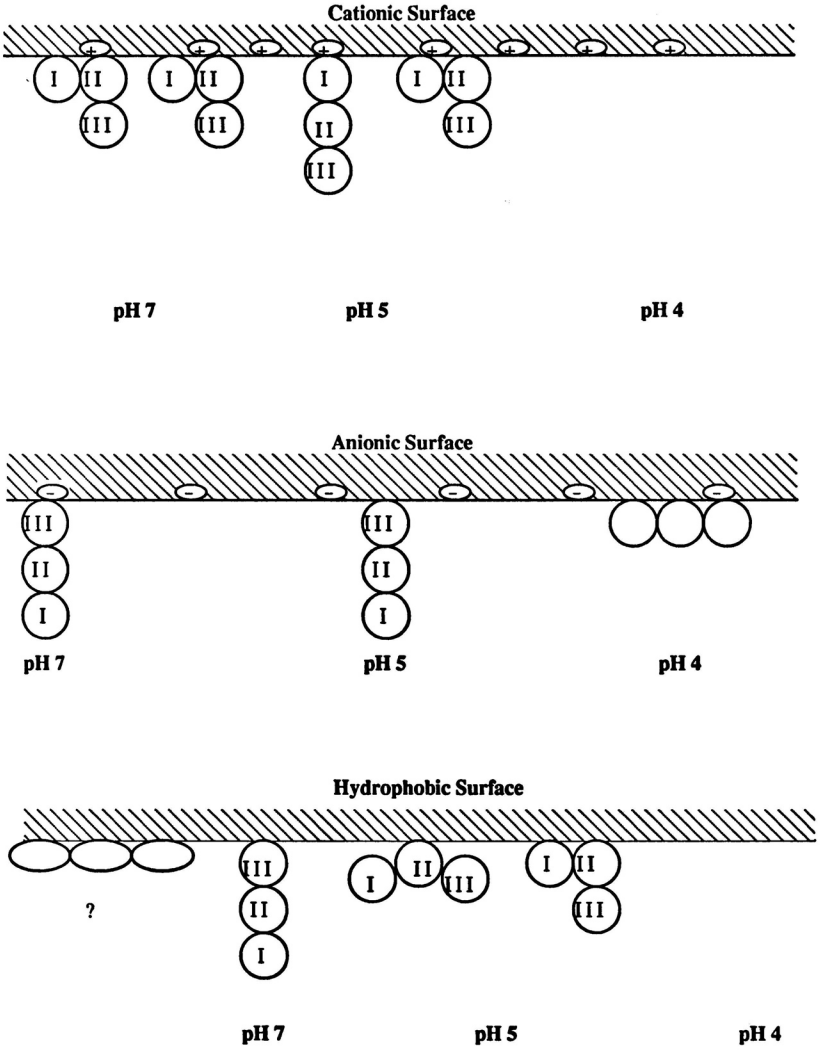

Figure 4. Cartoons and hypotheses related to bovine albumin adsorption on three different surfaces at three different pH values:

Top: Cationic surfaces. At pH 7, domains I and II are highly negatively charged; domain III is approximately neutral. At pH 5, however, domains I and II are nearly neutral, while domain III is positively charged.

Middle: Anionic surfaces. Domain III may facilitate an end on orientation, which would persist to pH 5. At pH 4, each of the domains is positively charged and a side on orientation may be expected.

Bottom: Hydrophobic surfaces. The helix amphilicity may promote a side on adsorption and significant denaturation, sketched on the left. Electrostatically, one would expect domain III nearest the interface because it has the least charge at pH 7. At pH 5, however, Domain II or possibly both Domains I and II might be oriented towards the interface.

As the individual albumin domains are roughly 40 × 40 Å in size, it is clear that only a small part of one of the domains can dominate the electrostatic adsorption process; note that at physiologic ionic strength the Debye length is less than 10 Å.

The presence of various counterions may significantly alter the electrostatic behavior, particularly multivalent anions (PO_4^{-3}, SO_4^{-2}) and cations (Ca^{++}, Mg^{++}, Al^{+++}, etc.). Ions may serve as bridging agents as well as serving to mask surface charged groups on the protein.[55]

It is also clear, however, with reference to Figure 4, that protein-protein lateral interactions can be very important. This is the normal explanation for the maximum in adsorbed amount at the isoelectric point, a behavior, which tends to be more pronounced on hydrophobic surfaces where the adsorptive interaction is predominantly hydrophobic. If we assume that the intrinsic surface hydrophobicity of each of the domains are comparable, then that domain exhibiting the least charge, and thus the least solubility, at a particular pH might tend to be preferentially adsorbed. At pH ~7, the least charge is on domain III, while at pH 5 it is domains I II. Thus as the pH decreases there may be a transition from »end on« to a partial »side on« orientation.

We must examine the actual position and distribution of charges and of hydrophobic residues in each domain and even in each subdomain. Such analyses are in progress.

We will not attempt here to review and analyze the voluminous literature on albumin at solid/liquid interfaces. A more complete analysis of the domain characteristics of albumin and of the albumin adsorption literature will be presented in a later, more complete paper. Our purpose here is merely to set the stage for a domain approach to protein adsorption. With a set of cartoons and hypotheses in mind (Table 1, Figure 4), we are now in a position to wade into the enormous literature on albumin adsorption. Please wish us luck!

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SAŽETAK

Tumačenje adsorpcije kompleksnih proteina teorijom domena: prethodna analiza i primjena na albumin

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Albumin se sastoji od tri različite domene, koje se razlikuju u strukturnom pogledu, karakterističnom odnosu naboja i pH, te prema sklonošću prema denaturaciji. Međupovršinska aktivnost albumina određena je, velikim dijelom, aktivnošću svake od te tri strukturne domene. Uzimajući u obzir međupovršinsku aktivnost svake strukturne jedinice, može se rastumačiti i sveukupna međupovršinska aktivnost albumina. Takav pristup analizi koji se oslanja na strukturne jedinice albumina, omogućuje razvijanje novih i preciznijih pretpostavki, pomoću kojih se mogu dizajnirati novi i bolji eksperimenti. Iste predpostavke dozvoljavaju novu ocjenu i usporedbu dosadašnjih eksperimentalnih podataka za kinetiku adsorpcije i adsorpcijske izoterme, debljinu adsorbiranog sloja i stvaranje višestrukih slojeva, indeks loma, lateralnu koheziju itd. Pristup opisan u radu zasniva se na hipotezi da svaki jednostavni protein ima jedinstvenu molekularnu osobenost, čije je poznavanje preduvjet za tumačenje i korištenje međupovršinske aktivnosti kompleksnih proteina.