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ADSORPTION OF PROTEINS AT SOLID-LIQUID INTERFACES

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Abstract

This paper concisely reviews the general principles underlying protein adsorption from aqueous solution onto a solid surface. The discussion includes the various stages of the adsorption process, i.e., transport of the protein molecules towards the surface, the absorbed amount under equilibrium conditions, desorption and re-adsorption. Among the interactions that determine the overall protein adsorption process (1) redistribution of charged groups in the interfacial layer, (2) changes in the hydration of the sorbent and the protein surface, and (3) structural rearrangements in the protein molecule play major roles. Special attention is given to the relation between the structural stability of the protein molecule and its adsorption behaviour.

Key Words: Protein adsorption, solid-liquid interface, adsorption kinetics, thermodynamics of adsorption, electrostatic interaction, hydrophobic interaction, rearrangement in protein structure, protein desorption, α-lactalbumin, lysozyme.

Introduction

Interaction between proteins and solid surfaces is commonly observed, both in natural and synthetic systems. These interactions are of great relevance in, e.g., medical, biotechnological and environmental applications. In many cases, spontaneous adsorption of proteins leads to undesired consequences such as thrombus formation on synthetic cardiovascular implants [76, 77, 81, 82], fouling of hemodialysis membranes, contact lenses and bioprocessing equipment [29], and plaque formation on teeth and dental restoratives [64, 80]. In other cases, protein adsorption is made use of, for instance in drug delivery and controlled drug release systems [4], in diagnostic tests (immunolatices) [39], in biosensors [66] and in protein purification techniques [13].

In all these examples, the influence of the proximity of the sorbent surface on the biological functioning and, because of the structure-function relationship in proteins, on the three-dimensional structure of the protein molecule is of crucial importance. Effective control of the adsorption process requires an understanding of the underlying mechanism, i.e., of the interactions that are involved.

Protein adsorption is an intricate process. Figure 1 shows a schematic outline of the various steps that are involved: transports toward, and binding at the surface, orientation and structure of the adsorbed molecules, reversibility of the sorption process.

In this paper, each of the aspects depicted in Figure 1 will be addressed, emphasizing the relation between protein structure stability and its adsorption behaviour.

Adsorption Kinetics

The rate of transport of a protein molecule from solution towards an interface increases with increasing concentration c_p of the protein in solution. The "reaction" of the protein with the interface, i.e., the actual attachment at the sorbent surface, is independent of c_p. It is, therefore, to be expected that at low c_p and low degree of coverage of the sorbent by the protein, the

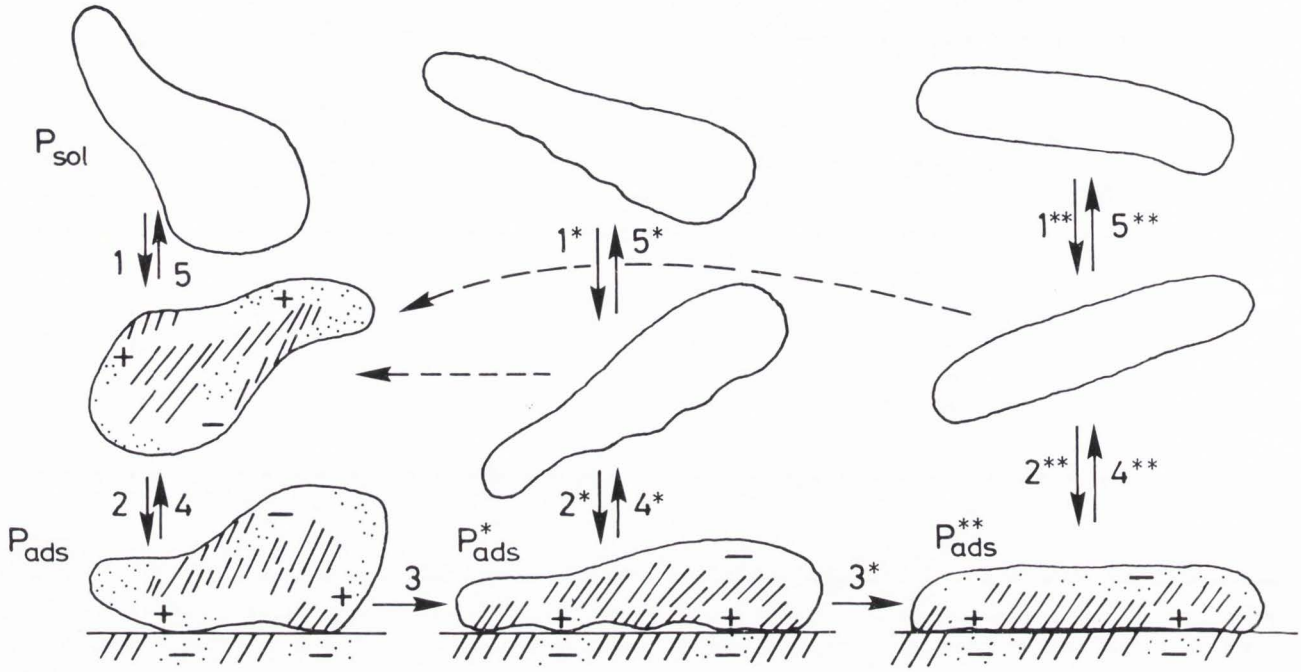

Figure 1. Schematic representation of the protein adsorption process. The native protein is denoted by P (the subscript sol refers to solution and ads to adsorbed state) and the structurally perturbed protein by P* and P**. The following steps are depicted: (**1**). Transport of P from solution towards the surface. (**2**). Attachment at the surface (re-orientation?). (**3**). Structural rearrangement in the adsorbed molecule. (**4**). Detachment from the surface. (**5**). Transport away from the surface.

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transport process controls the rate of adsorption and that at high c_p and high degree of coverage, surface reactions are rate-determining.

The basic transport mechanisms are diffusion and convective transport by laminar or turbulent flow. Under quiescent conditions, protein molecules reach the sorbent surface by stochastic Brownian motion [59]. If they are relatively rapidly attached at the surface, it leads to depletion of protein in the layer adjacent to the surface. The resulting concentration gradient causes protein diffusion from the bulk solution towards the sorbent surface. Under such conditions, the rate of arrival of the protein at the sorbent surface is given by the Ward and Tordai equation [79]

$$J = c_p (D/\pi)^{1/2} t^{-1/2} \qquad (1)$$

where J is the flux of the protein per unit area of sorbent surface; t, the time; D, the diffusion coefficient of the protein in solution; and π is 3.14. Experimental data confirm diffusion-controlled adsorption from non-flowing solutions of low concentration [10, 22, 24, 44].

In practice, however, proteins mostly adsorb from flowing solutions. Here, we will consider laminar flow only and distinguish between (a) a solution tangentially flowing along a surface and (b) an impinging jet flow that hits the surface perpendicularly in a so-called "stagnation point".

For the tangential flow, the protein molecules are transported towards the sorbent surface by simultaneous convection and diffusion and, under conditions of a steady-state concentration boundary layer in the solution adjacent to the sorbent surface, the flux is given by the Lévèque equation [43]

$$J = 0.54 (\dot{\gamma}/yD)^{1/3} D c_p \qquad (2)$$

where $\dot{\gamma}$ is the shear rate at the sorbent surface and y is the distance between the point of observation and the point where the protein solution has reached the surface at t = 0.

For a stagnation point flow, the flux of protein is described by an equation derived by Dabros and Van de Ven [19]

$$J = 0.287 \nu^{-1/3} \phi^{2/3} R^{-5/3} D^{2/3} c_p \qquad (3)$$

where ν is the kinematic viscosity of the solution, ϕ the

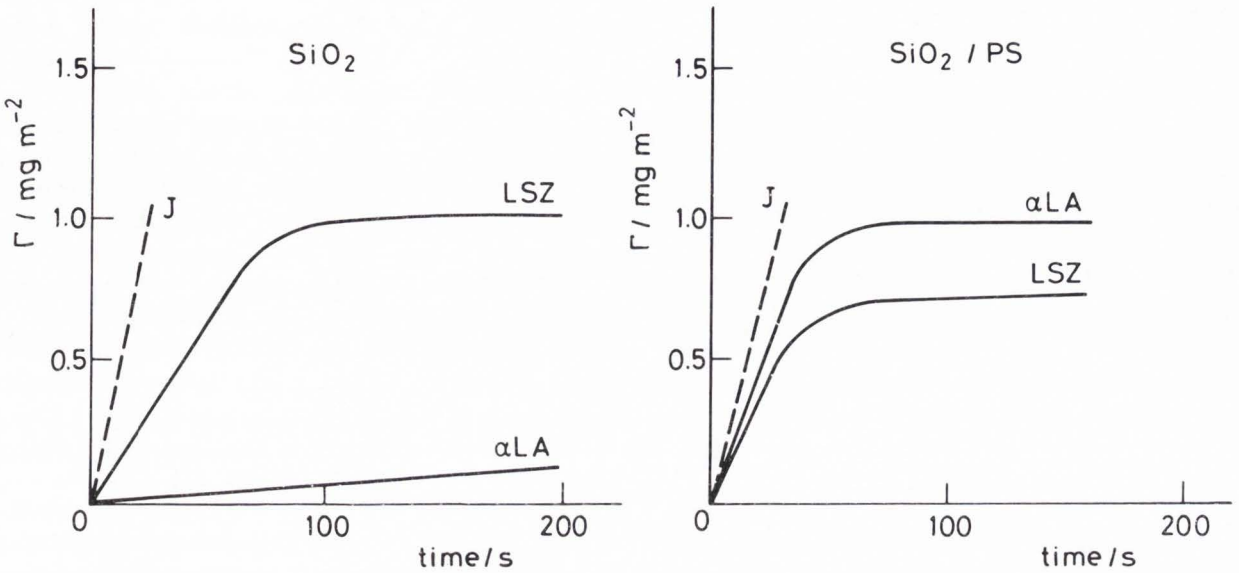

Figure 2. Adsorption of lysozyme and α-lactalbumin from an impinging jet flow (c_p = 1 g dm^{-3}) on hydrophilic silica (left) and on hydrophobic polystyrene-coated silica (right). Adsorbed amount as a function of time, as determined by reflectometry. 0.01 M phosphate buffer pH 7.0; T = 25°C. Dashed line represents the flux of the protein molecules arriving at the surface, according to equation (3).

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volume flux and R the radius of the nozzle of the device that supplies the solution to the sorbent surface. Equation (3) is only valid under the conditions that (a) the nozzle of the supplier is cylindrically shaped, (b) the distance between the nozzle and the surface is much larger than R, (c) the particles in the solution are spherical and (d) interactions between the particles in solution are absent.

Initial adsorption rates (i.e., at conditions of low surface coverage, so that, in principle, each arriving protein molecule can be accommodated at the surface) may be compared with the fluxes calculated using the appropriate equation (1), (2) or (3). Adsorption rates have been measured by various authors [3, 15, 27, 35, 56, 75] applying a wide spectrum of experimental techniques. An extensive review has been given by Ramsden [65].

In general, the initial adsorption rates show the same dependence on the experimental variables as expressed in the corresponding equations for J. However, in many cases the adsorption rate is considerably smaller than the flux towards the surface [11, 22, 31, 58]. It suggests that only a fraction of the molecules that arrive at the surface attach to it. The Gibbs energy, G, associated with that barrier, can be calculated from the retardation factor exp(-G/RT) [22, 58]. The cause for such a barrier could be electrostatic repulsion [22, 58], a hydrodynamic effect [31] or that a fraction of the protein molecules does not collide in the proper orientation that is required for attachment to the surface [49, 58].

By way of example, the adsorption rates for two well-characterized proteins, i.e., hen's egg lysozyme (LSZ) and bovine milk α-lactalbumin (αLA) at both a hydrophilic and a hydrophobic stagnation point, are shown in Figure 2, together with the theoretical fluxes towards the surface. Some relevant characteristics of LSZ and αLA, as well as of the sorbent surfaces, are summarized in Table 1. Note that the proteins are similar as to their molecular dimensions and masses, but differ markedly with respect to their isoelectric points and structural stabilities. Hence, comparative studies with these systems may help to understand the role of hydrophobic dehydration, electrostatic interaction and protein structure stability in the behaviour of proteins at interfaces.

With both LSZ and αLA, the initial adsorption rate is smaller than the flux.

On the hydrophilic silica surface, the adsorbing fraction of αLA is much smaller than that of LSZ. The difference reflects the difference in electrostatic interaction between the proteins and the sorbent, i.e., attraction of LSZ and repulsion of αLA. Even for LSZ, the adsorbing fraction is below unity. It points to the existence of a non-electrostatic "barrier" for adsorption.

On the hydrophobic polystyrene surface, the situation is quite different. First, the adsorbing fraction of LSZ is somewhat larger in spite of less electrostatic attraction. It indicates the "stickiness" of hydrophobic surfaces relative to hydrophilic ones. Though the αLA molecules are electrostatically repelled, the adsorbing

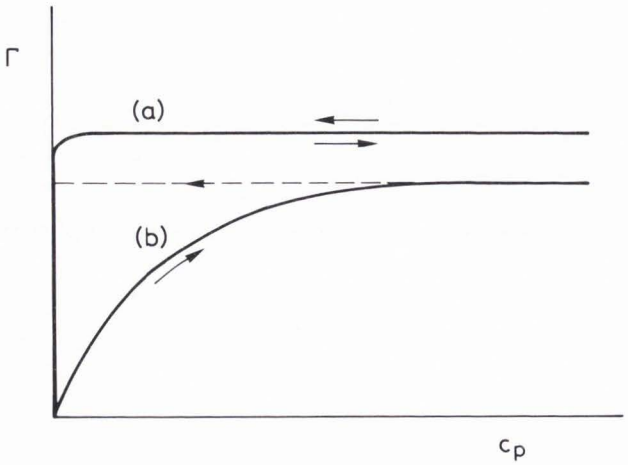

Figure 3. Schematic representation of (a) a high-affinity adsorption isotherm and (b) a non-high-affinity ascending isotherm of which the corresponding descending branch (dashed curve) shows high-affinity. For details, see text.

Table 1. Some physical-chemical properties of the proteins and the sorbent surfaces.

protein	lysozyme	α-lactalbumin
molar mass (D)	14,600	14,200
size (nm^3)	4.5x3.0x3.0	3.7x3.2x2.5
diffusion coefficient ($m^2\ s^{-1}$)	1.04×10^{-10}	1.06×10^{-10}
isoelectric point (pH units)	11.1	4.3
Gibbs energy of denaturation ($J\ g^{-1}$)		
heat	-4.1	-1.5
denaturant	-4.0	-1.9
sorbents	0.01 M phosphate buffer pH 7.0	
	silica	polystyrene-covered silica
thickness of the PS layer (nm)		35
electrokinetic potential (mV)	-48	-21
hydrophobicity (contact angle of a sessile drop of water)	0°	82°

fraction is remarkably high, much higher than that for LSZ. This difference may be related to the relatively low structural stability of αLA molecules, so that structure rearrangements contribute to the adsorption affinity (see below under **Rearrangements in the protein structure**).

After adsorption, the protein molecules may continue to rearrange their structure over a long period of time [5, 38] in order to optimize their interaction with the sorbent surface. It is to be expected that the degree of structural rearrangement and/or the number of molecules that undergo rearrangements depend on the rate of deposition relative to the rate of structural changes. Such long-term structural alterations involves an expanding contact area between the protein molecule and the sorbent surface; it may cause displacement of (later adsorbed) neighbouring molecules. Such a behaviour would show up as a maximum in the adsorbed amount as a function of time [22, 78].

Various authors [7, 10, 45, 78] have attempted to model the dynamics of protein adsorption. Although these models, in one way or another, take into account the various steps depicted in Figure 1, none of them give a satisfactory general description of protein adsorption. It seems that heterogeneity of the sorbent surface and/or the adsorbed protein layer causes the major problem in this respect. For instance, the models should be extended to include several orientations and conformations of the adsorbed protein molecules. Furthermore, because protein adsorption usually proceeds irreversibly (see sections: **Adsorbed Amount** and **Desorption**), the way the protein is supplied to the system may affect the final result.

Adsorbed Amount

The most common way to report adsorbed amounts is in the form of an adsorption isotherm, where the adsorbed amount, Γ, is plotted against c_p. Figure 3 gives schematic representations of the types of isotherms often encountered in protein adsorption. The initial part of the isotherm reflects the affinity between the protein and the sorbent surface. From theory, a high-affinity, type-a, isotherm is to be expected for homodisperse polymers [25, 72]. It is indeed generally found for relatively simple, synthetic polymers. High affinity isotherms are also reported for protein adsorption, but the occurrence of type-b isotherms, reflecting a lower affinity, is not exceptional. Irrespective of the affinity, protein adsorption isotherms develop, as a rule, well-defined plateau-values. These values are usually compatible with, or somewhat lower than, those corresponding to a complete monolayer of native molecules. In some studies [30, 63], it has been observed that at plateau-adsorption a considerable fraction of the sorbent surface is still uncovered. At lower adsorptions, the molecules may be non-uniformly distributed over the surface as well and the distribution may depend on the type of protein and

Table 2. Some physical-chemical properties of the dispersed sorbent particles.

	0.05 M electrolyte				
	Phosphate buffer pH 7.0			Acetate buffer pH 5.5	Borate buffer pH 9.5
	PS-	PS+	Glass	α-$Fe_2O_3^+$	α-$Fe_2O_3^-$
Nature of charged groups	$-OSO_3^-$	$=^+NH-$	$-O^-$	OH_2^+	$-O^-$
Surface charge density (mC m^{-2})	-23	+27	?	?	?
Electrokinetic potential (mV)	-69	+32	-51	+20	-47
Hydrophobicity (contact angle of a sessile drop of water)	82°	82°	0°		hydrophilic
Specific surface area (m^2 g^{-1})	10.0	12.4	0.5	36.0	36.0

type of surface [42, 62]. The application of novel techniques, such as atomic force microscopy, may give more direct information as to the heterogeneity of the adsorbed protein layer.

Another feature, sometimes encountered in protein adsorption, is the occurrence of a step (two plateaus) in the isotherm. Such a step could reflect the formation of a second protein layer, but, as dilution usually does not lead to the level of the first plateau, two-layer adsorption is unlikely. The explanation is then rather a transition in the structure and/or organization of the adsorbed layer [9, 16, 41].

As a rule, dilution does not lead to detectable desorption of the protein (which can be tested only in the case of non high-affinity adsorption). Hence, the ascending and the descending branches of the isotherms do not coincide. The occurrence of such a hysteresis indicates that, at a given c_p, the system has two equilibrium states, one on the ascending branch and the other on the descending branch. These two states are characterized by local minima in the Gibbs energy of the system. It implies that during the adsorption-desorption cycle an irreversible physical change has occurred in the system. In spite of the irreversible nature of protein adsorption, many authors [6, 46, 47, 60] erroneously interpret their experimental data using theories that are based on reversible thermodynamics. The most common example is the determination of the Gibbs energy of adsorption, $\Delta_{ads}g$, by fitting the (ascending) isotherm to the Langmuir or Scatchard equation. For a more detailed treatment of the irreversibility aspects, the reader is referred to reference [51].

Figure 4 shows adsorption isotherms for LSZ and αLA on surfaces of different electrical charge density and hydrophobicity. Relevant characteristics of the sorbent particles are summarized in Table 2. Properties of the proteins are given in Table 1.

At the hydrophobic PS surfaces, both proteins adsorb with high affinity, even under electrostatically unfavorable conditions. It demonstrates that the entropy gain of the water molecules that are released from the hydrophobic hydration layer dominates the adsorption process. The electrostatic interaction between the protein and the sorbent surface is still reflected in the plateau-values of the isotherms.

At the hydrophilic α-Fe_2O_3 surfaces, where dehydration is unfavorable, adsorption of the proteins is expected to be governed by electrostatic interaction. For the positively charged α-Fe_2O_3 surface, this seems to be confirmed. However, at the negatively charged α-Fe_2O_3 surface, αLA does adsorb in spite of overall electrostatic repulsion. Apparently, the adverse effects of hydrophilic dehydration and electrostatic repulsion are outweighed by another contribution that leads to the spontaneous adsorption of αLA. As suggested before in the section **Adsorption Kinetics**, this adsorption promoting contribution is probably associated with structural rearrangements in the protein molecule.

Below, the mechanism of protein-sorbent interaction will be discussed in terms of the contributions of the main forces that drive the adsorption process.

Interactions That Govern Protein Adsorption

Adsorption of proteins from (aqueous) solution on a (solid) surface is the net result of various types of interactions that simultaneously occur between all the components in the system: the protein molecules, the sorbent surface, the solvent (water) and the low-molecular-weight ions. See Figure 5.

For spontaneous adsorption, at constant pressure and temperature, the change in the Gibbs energy must be negative. According to equation (4), this can be achieved by a decrease in the enthalpy and/or an increase in the entropy.

$$\Delta_{ads}g = \Delta_{ads}h - T\Delta_{ads}s \qquad (4)$$

where g, h and s are the Gibbs energy, the enthalpy and

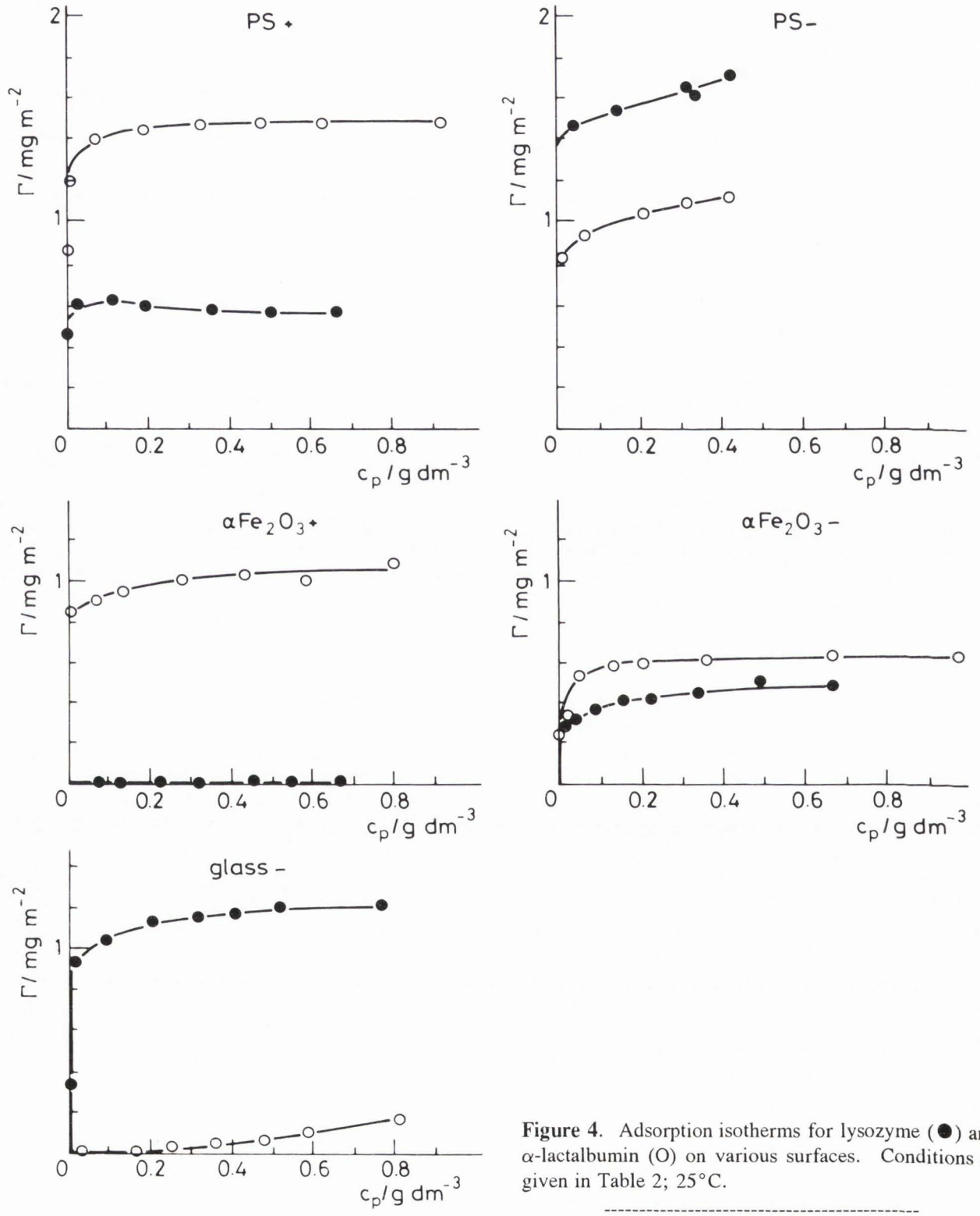

Figure 4. Adsorption isotherms for lysozyme (●) and α-lactalbumin (O) on various surfaces. Conditions as given in Table 2; 25°C.

the entropy per mol of protein and where T is the absolute temperature in K. In the sub-sections below, the contributions from (i) the redistribution of charged groups, (ii) changes in the state of hydration and (iii) structural rearrangements in the protein molecule will be discussed. It may be clear that these contributions are interconnected; for instance, distribution of charge and hydrophobic effects have a strong influence on the protein's structural stability [54].

Redistribution of charged groups

In general, both the protein molecules and the

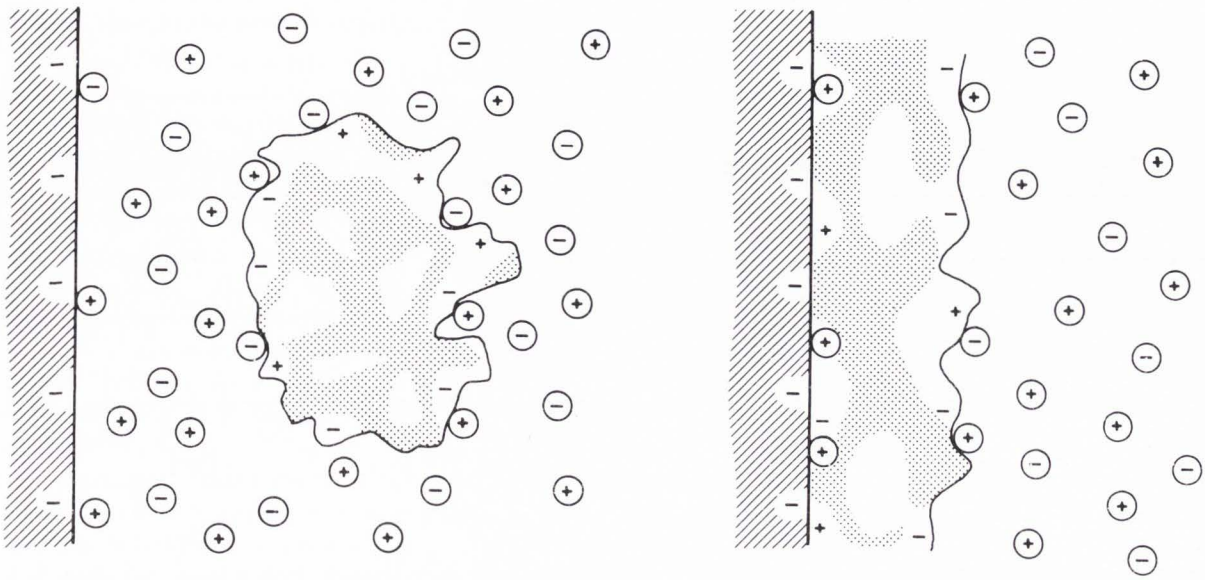

Figure 5. Schematic pictures of a protein molecule in solution and a solid/solution interface (left) and a protein-covered solid surface (right). The charge of the protein originating from (de)protonation of amino acid side groups and the surface charge of the sorbent are indicated by +/-; low-molecular-weight ions are represented by ⊕/⊖. Shaded areas represent hydrophobic regions.

sorbent surface are electrically charged. In an aqueous environment, charged surfaces and protein molecules are surrounded by counterions, which, together with the surface charge, form an electrical double layer. When the protein molecule and the sorbent surface approach each other, their electrical double layers overlap, which gives rise to a redistribution of the counterions.

If the protein and the sorbent surface have opposite charge signs, they attract each other, at least if the charge on the protein and the surface more or less compensate each other. If either one of the two components has a large excess of charge, this would result in a considerable net amount of charge in the contact region between the protein layer and the sorbent surface. This region has a low dielectric permittivity relative to that of water and, therefore, accumulation of charge in such an environment would cause the development of an extremely high electrostatic potential, which is energetically very unfavorable. A similar situation would result upon adsorption of a charged protein on a surface that has the same charge sign. Nevertheless, in many cases it is observed that, in spite of such adverse electrostatic conditions, proteins adsorb spontaneously. Based on a model for the adsorbed protein layer [53], it has been predicted that low-molecular-weight-ions are transferred between the solution and the adsorbed layer to prevent accumulation of net charge in the contact region between the protein and the sorbent surface. The number of ions transferred may be deduced from electrokinetic measurements [32, 52], or, more directly, by tracing labelled ions [21]. By way of example, Figure 6 shows the change in electrokinetic charge, $\Delta_{ads}\sigma_{ek}$, per unit area of sorbent surface, due to ion incorporation in adsorbed layers of LSZ and αLA on a negatively charged PS surface [32]. The data indicate that at pH < 8 the positive charge on LSZ (isoelectric point 11.3) overcompensates the negative charge on the PS surface; therefore, incorporation of negative charge is required to attain an (almost) electrically neutral contact region between the protein and the sorbent surface. At pH 8, positively charged ions are required. Accordingly, co-adsorption of positively charged ions accompanies the adsorption of αLA (isoelectric point 4.3) on the negatively charged PS surface over the entire pH region considered.

As ion incorporation compensates for the charge antagonism between the protein and the sorbent, the resulting contribution from redistribution of charges to $\Delta_{ads}g$ does not exceed values larger than a few tens of RT [55]. Its value and sign depend on the charge distributions and the dielectric constants of the electrical double layers before and after adsorption, respectively [54].

In addition to an electrostatic effect, transferring ions from an aqueous to a non-aqueous protein layer includes a chemical effect as well. This chemical effect is unfavorable and, hence, opposes the overall protein adsorption process [33, 55]. As a consequence, maximum affinity for protein adsorption is observed when the charge on the protein molecule itself just matches the charge on the sorbent surface, so that no additional ions are needed for charge neutralization [26]. The chemical contribution of ion transfer to $\Delta_{ads}g$ can be estimated from model studies on the transfer of ions from aqueous

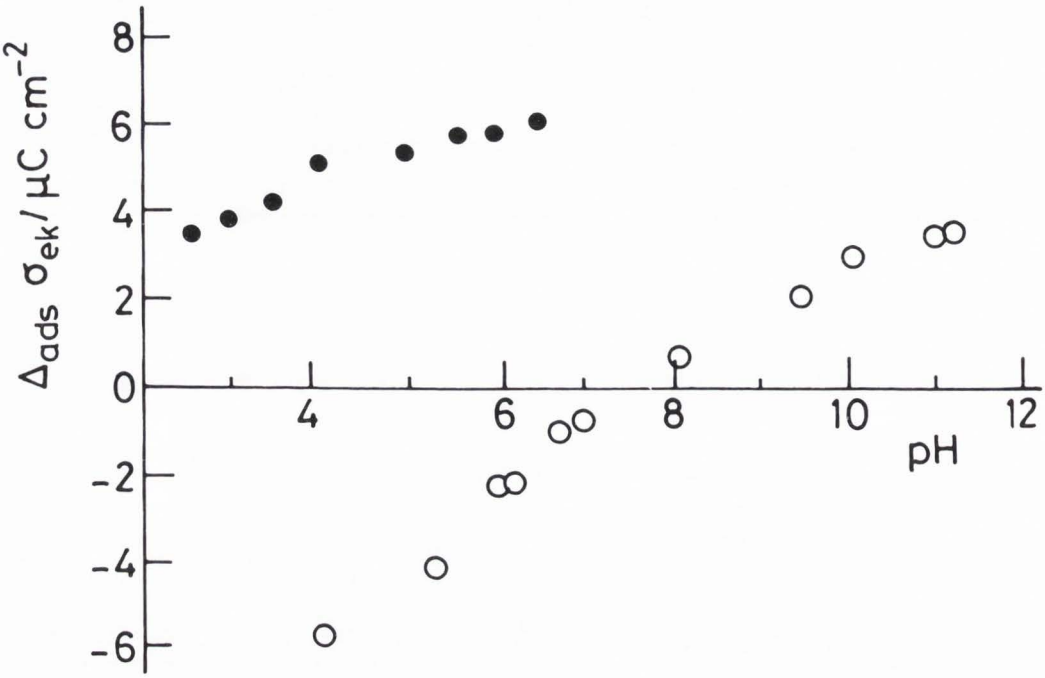

Figure 6. Ion co-adsorption, as reflected by the overall change in the electrokinetic charge density, accompanying the adsorption of lysozyme (O) and α-lactalbumin (●) on negatively charged polystyrene latex. 0.05 M KCl; T = 25°C.

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to non-aqueous media [1, 2, 20]. It usually is in the range of a few to a few tens of RT.

An alternative way to avoid the development of a high electrostatic potential in the adsorbed layer would be the unfolding of the adsorbing protein molecules into a very loose structure that is freely penetrable for water and electrolyte. In such a highly hydrated adsorption layer, the dielectric permittivity would not differ too much from that of the bulk solution. Because of the general observation that globular protein molecules do not form such loose structures, but adsorb in a rather compact form, it is concluded that the chemical effect of ion incorporation is less unfavorable than the exposure of hydrophobic residues of the protein to water, as would occur upon unfolding.

Hydration changes

When the surfaces of the sorbent and the protein are hydrophilic, their hydration is favorable. Then, dehydration would oppose adsorption. If adsorption occurs, some hydration water may be retained between the adsorbed layer and the sorbent surface. However, when (one of) the contacting surface(s) (is) are hydrophobic, dehydration of (that) those surface(s) would stimulate protein adsorption.

Studies related to hydrophobic interaction chromatography have provided substantial evidence that the hydrophobicity of the protein exterior influences protein adsorption at solid water interfaces [28, 67]. Apart from the hydrophobic parts at the aqueous periphery of the protein molecule, its overall hydrophobicity may be relevant for the adsorption behaviour. The overall hydrophobicity influences the protein structure stability, which, in turn, may affect the adsorption (see next subsection). Experimental establishment of the influence of the hydrophobicity, as such, of the sorbent surface is practically impossible because a variation of the hydrophobicity involves a change in the chemical composition and, often, a variation in the surface charge density. Experiments using hydrophobicity gradient surfaces [23] are probably the best to study this matter in more detail.

The contribution of dehydration of a compound to $\Delta_{ads}g$ may be estimated from partition coefficients of (model-)compounds in water/non-aqueous two-phase systems [48]. It has thus been estimated that dehydration of hydrophobic surfaces results in an entropy gain of 20-50 $\mu J\ K^{-1}\ m^{-2}$, which, at 25°C, corresponds to a reduction in the Gibbs energy of 5-15 $mJ\ m^{-2}$. For a protein molecule having a molecular mass of, say 15,000 D (comparable to those of LSZ and αLA) that adsorbs ca. 1 $mg\ m^{-2}$, it corresponds to a contribution to $\Delta_{ads}g$ ranging between -30 RT and -100 RT. It demonstrates that hydrophobic dehydration often overrules electrostatic effects, i.e., the contribution from charge redistribution.

Rearrangements in the protein structure

The densely folded structure of a globular protein molecule in solution is maintained because intramolecular hydrophobic interaction is stronger than intramolecular electrostatic repulsion (at a pH away from the isoelectric point) and reduced conformational entropy of the folded structure [56]. When the protein adsorbs, it changes its environment, which causes a shift in the balance of interactions. This, in turn, may lead to structural rearrangements in the adsorbing protein molecules. For instance, hydrophobic parts of the protein that, in an aqueous environment, are located in the interior of the dissolved molecule, may, after adsorption, be exposed to the sorbent surface where they are still shielded from contact with water. Such a structure rearrangement involves a decrease in *intramolecular* hydrophobic bonding. Because hydrophobic interactions in the protein interior promote the formation of secondary structures as α-helices and β-sheets, a reduction of these interactions may cause a decrease of such secondary structures. This, in turn, leads to an increased conformational entropy.

Various techniques have been used to investigate the structure of adsorbed protein molecules. The most common are spectroscopic methods such as (total internal reflection) fluorescence [12, 17, 36, 74], (Fourier transform) infrared spectroscopy [5, 37, 42], circular dichroism [40, 50], NMR [8] and XPS [61]. It is, however, often a problem to interpret the experimental data quantitatively, in particular to distinguish between orientational and conformational effects. Optical techniques, such as, ellipsometry and reflectometry [3, 18, 69, 70, 71] may also provide information that is conclusive as to structural changes in the adsorbed protein molecules. Recently, differential scanning calorimetry has been used to study denaturation of proteins at surfaces [34, 83].

Based on circular dichroism measurements and infrared spectroscopy considerable losses of ordered secondary structure have been reported [40, 50], the more so the less stable the structure of the native protein molecule is. Furthermore, calorimetry on LSZ and αLA adsorbed on PS and α-Fe_2O_3 surfaces [34] revealed that adsorption induces a loss of enthalpically favorable interactions in the protein molecules. This effect is most pronounced for adsorption at the hydrophobic PS surface and for αLA that has the lowest structural stability. Even though protein molecules may not completely unfold upon adsorption, the break-down of the secondary structure that causes an increased conformational entropy could contribute with several tens of RT to $\Delta_{ads}g$ [33, 54, 55].

The relevance of each of the contributions discussed above, depends on the system. For structurally stable ("hard") proteins, adsorption will be primarily governed by hydrophobic dehydration and electrostatic interaction. For most hydrophobic surfaces, the contribution from dehydration to $\Delta_{ads}g$ exceeds that from charge redistribution (see previous two subsections), so that, as a rule, all proteins adsorb on hydrophobic surfaces even under electrostatically unfavorable conditions. On hydrophilic surfaces, hard proteins adsorb only if they are electrostatically attracted. Proteins that have a low structural stability ("soft" proteins) are more liable to undergo structural changes upon adsorption. The contribution to $\Delta_{ads}g$ from the gain in conformational entropy may outweigh the opposing effects from hydrophilic dehydration and electrostatic repulsion. Under such conditions, the protein adsorbs spontaneously on a hydrophilic, like-charged surface (e.g., negatively charged αLA on negatively charged α-Fe_2O_3, as presented in section **Adsorbed Amount**).

Desorption

Proteins, like other macromolecules, usually adsorb by attaching various segments of each of their molecules to the surface. The fraction of amino acid residues in direct contact with the surface typically is 10%-40%, which, for a protein of 15,000 D molar mass, means attachment of some 15-60 amino acid residues. Even if the contribution to $\Delta_{ads}g$ from each of these contacts is not more than the energy of thermal motion (1 RT), it adds up to several tens of RT per mol of protein. Consequently, diluting the system usually does not lead to desorption of the protein. On the other hand, exchange between adsorbed and dissolved protein molecules, whether or not of the same type, may be possible. In that case, any desorbing segment can be replaced by another adsorbing segment so that the initially adsorbed protein molecule is gradually stripped off from the surface. Similarly, any other surface active substance may displace the protein from the surface [57].

It is very possible that after release from the sorbent surface the structure of the protein molecule differs from the original, native structure as it was before adsorption. Using circular dichroism, various authors have compared the secondary structure in proteins before and after adsorption. It has thus been found that the α-helix content in bovine serum albumin (BSA) desorbed from various surfaces is 15-30% less than in the native state [50, 57, 68], irrespective of the desorption method [57]. For fibrinogen desorbed from glass, an α-helix reduction of 50% has been reported [14] and for albumin, globulin and fibrinogen desorbed from various polypeptides, the α-helix decrease was 80-90%, 20-40% and 0-90%, respectively [73]. On the other hand, LSZ, being a more structurally stable protein, regained its original

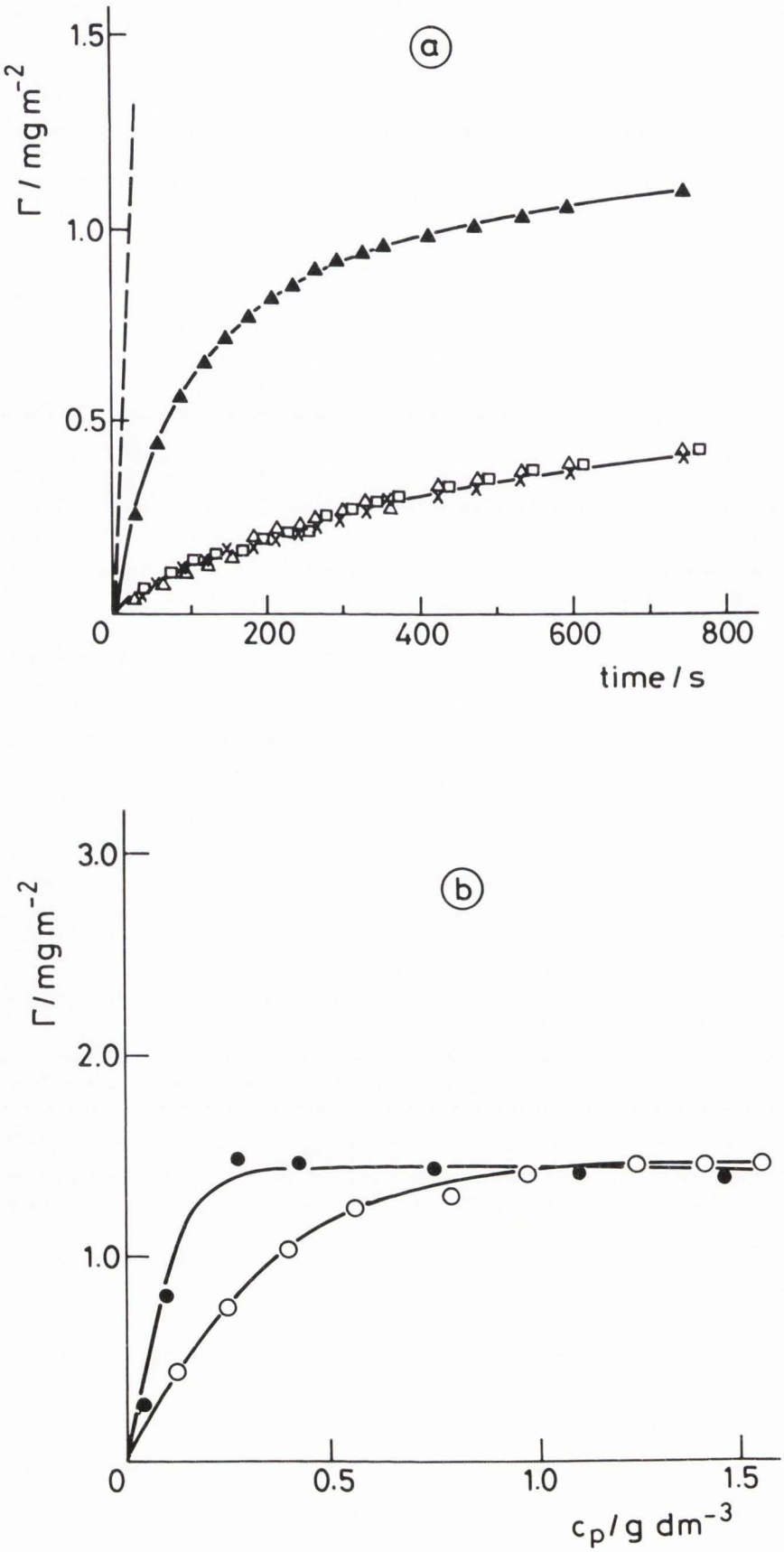

Figure 7. Adsorption of bovine serum albumin (BSA) on silica: (a) adsorbed amounts from an impinging jet flow (c_p = 0.01 g dm^{-3}) where (X) is native BSA, (▲) is BSA previously desorbed from silica by morpholine, and (Δ) and (□) are native BSA pre-exposed (but not adsorbed) to silica and morpholine, respectively; (b) adsorption isotherm for (O) native BSA and for (●) BSA previously desorbed from silica by morpholine. 0.05 phosphate buffer pH 7.0; T = 25°C.

secondary structure after desorption from oxide surfaces [50]. Guoying Yan *et al.* [83] suggest that preceding contact with a polystyrene surface has a destabilizing effect on the structure of dissolved molecules of human serum albumin.

Re-adsorption of Pre-adsorbed Protein

According to the scheme depicted in Figure 1, desorbed molecules may re-adsorb at the same sorbent surface. If the desorbed molecules have not regained their original structure, it is expected that the adsorption characteristics of the pre-adsorbed and subsequently desorbed protein are different from those of the native protein. For BSA and αLA, both "soft" proteins that are likely to undergo structural changes upon adsorption, such an influence has indeed been observed [49]. In the Figures 7a and 7b, this is illustrated for BSA. Both the kinetic data and the adsorption isotherms indicated that the desorbed protein has an increased affinity for adsorption.

Conclusions

The adsorption of proteins from aqueous solution onto a solid surface is the result of an interplay between several subprocesses. Distinction must be made between "hard" proteins, of which the molecules retain most of their conformation upon adsorption, and "soft" proteins that undergo severe structural rearrangements. Adsorption of the hard proteins can be interpreted in terms of electrostatic interactions and (partial) dehydration of the outer surfaces of the sorbent and the protein. The internal structural changes occurring in the adsorbing soft proteins involve an increase in the conformational entropy of the protein molecule and constitute, therefore, an additional driving force for spontaneous adsorption.

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Discussion with Reviewers

R. Hidalgo Alvarez: Protein adsorption is a typical irreversible process. Why is not the thermodynamics of irreversible processes used to describe adsorption processes?

Author: The overall protein adsorption process is a complex process, involving various sub-processes that are linked to each other. The aim of this paper is to estimate the contributions from each of these subprocesses to the Gibbs energy of adsorption, which, at constant temperature as pressure is a measure for the affinity of the protein to adsorb at the surface. The Gibbs energy is a function of state and its change depends on the final and initial states only. Therefore, although the process proceeds irreversibly, the change in the Gibbs energy due to adsorption may as well be calculated using thermodynamics for reversible processes. Irreversible thermodynamics deals with entropy production. Minimum entropy production for the overall process may be estimated from hysteresis of the adsorption isotherms. For this I refer to references [33] and [34].

R. Hidalgo Alvarez: The disagreement between the theoretical and experimental fluxes requires a more extensive discussion. I assume that the theoretical fluxes were calculated using the equation [3].

Author: There is no disagreement between theoretical (calculated, using eq. [3]) and experimental fluxes, simply because we did not determine the flux experimentally. There is a difference between the calculated flux towards the surface and the initial rate of adsorption. However, this difference does not imply disagreement, because it can well be that only a fraction of the molecules that arrive at the surface really attach to it. There may be various reasons for this phenomenon (as mentioned in the section **Adsorption Kinetics** of the paper), such as: electrostatic repulsion between the protein and the sorbent surface, unfavorable dehydration of a hydrophilic surface and unfavorable orientation of the protein molecule when it arrives at the sorbent surface.

R. Hidalgo Alvarez: I would like to know how the author explains the disagreement between the surface charge densities and the electrokinetic potentials of bare PS- and PS+. A greater surface density corresponds with a smaller electrokinetic potential.

Author: The disagreement between the surface charge densities and the electrokinetic potentials of PS- and PS+ (see Table 2) is apparent. The electrokinetic potentials are for PS latexes in 0.05 M phosphate buffer pH 7. Under these conditions, the $H_2PO_4^-$ and $H_2PO_4^{2-}$ have a relatively strong tendency to adsorb to the PS+ surface, thereby reducing the electrokinetic potential. The surface charge densities, given in Table 2, refer to the charge originating from the groups that are covalently linked at the PS surface, i.e., $-OSO_3^-$ and $= +NH$, respectively.

R. Hidalgo Alvarez: The adsorption isotherms of LSZ and αLA on PS- do not develop well-defined plateau values. Please comment.

Author: The reason(s) why the isotherms for LSZ and αLA do not develop a definite plateau value (at $c_p < 0.5$ g dm^{-3}) are not clear. In view of the relatively large amount of LSZ adsorbed, it could be that a second layer of LSZ is built up at the surface. This would be facilitated by the fact that the LSZ-covered PS- particle still has a negative electrokinetic potential, whereas the adsorbing LSZ molecules are positively charged.

R. Hidalgo Alvarez: The change in electrokinetic charge per unit area of sorbent surface, due to ion incorporation in adsorbed layers of LSZ and αLA, has been analyzed only on PS-. What is the change on PS+? How was the electrokinetic potential of the protein-latex calculated? According to Oshima and Kondo {J. Colloid Interface Sci. **130** (1989) 281}, the electrokinetic potential loses its meaning for colloidal particles with a structured surface, since the electrophoretic mobility is insensitive to the precise position of the slipping plane. Does the author trust his results as shown in Figure 6?

Author: We have not made such extensive, pH-dependent protein adsorption studies with PS+. The electrokinetic potentials of the (protein-covered) latex particles were derived from the electrokinetic mobilities, using the theory of O'Brien and White {J. Chem. Soc. Faraday Trans. 2 **74** (1978) 1607}. I agree that if the colloidal particle is covered with an ion-penetrable polymer layer, calculation of the electrokinetic potential becomes more complex. However, based on a variety of literature data, it is generally accepted that after adsorption, the protein molecules remain relatively compact. Ion incorporation is assumed to occur in the contact region between the protein and the sorbent surface. In view of this, I trust our results, shown in Figure 6, in a semi-quantitative way, namely, that they represent the trends by which ion incorporation compensates for the charge antagonism between the protein and the sorbent surface.

R. Hidalgo Alvarez: Hydrophobic dehydration often overrules electrostatic effects. However, it would be very useful to know if only dehydration of hydrophobic surfaces would be sufficient to cause protein adsorption on these surfaces. Otherwise, which is the determining factor in the protein adsorption by hydration changes, the protein or the hydrophobic surface?
Author: Dehydration of a hydrophobic sorbent surface often is the determining factor for protein adsorption. This may be illustrated by the observation that a given protein does adsorb at a hydrophobic surface that has the same charge sign as the protein, whereas adsorption of that protein does not occur at a hydrophilic surface under otherwise similar conditions.

W.G. Pitt: Conformational stability appears to be a significant issue in this paper, regarding which I have some questions.

- How was conformational stability measured?
- Can it be quantified without using adsorption as a measure?
- If conformational stability is measured by DSC, how can you ensure that thermally-induced conformational changes are relevant, similar or analogous to the surface-induced conformational changes?

Author: Adsorption data for LSZ and αLA are compared to illustrate the influence of protein conformational stability on the adsorption behaviour. The conformational stabilities are quantified in terms of the Gibbs energy of unfolding. As shown in Table 1, the conformational stability of LSZ is much greater than that of αLA. This is true for both heat-induced and denaturant (guanidinium chloride)-induced conformational changes. It indicates that the internal coherence in the LSZ molecules is far greater than in the αLA molecules. It is, therefore, likely, although not a priori sure, that αLA is more susceptible to conformational changes upon adsorption. The observation that, in contrast to LSZ, αLA adsorbs on a hydrophilic, like-charged surface (see Figure 4) suggests conformational rearrangements in this protein as a driving force for adsorption. Furthermore, differential scanning calorimetry reveals that the breakdown of ordered structures in adsorbed αLA is much greater than in adsorbed LSZ [34].

K.K. Chittur: At the beginning of **Adsorption Kinetics**, the author states that the intrinsic rate of adsorption of proteins with the surface is independent of bulk concentration. I will not argue with this point, except to say that for some surfaces, even at low c_p, the surface reaction may be rate limiting. It is important to carefully distinguish between the intrinsic rate constant for adsorption which is determined by properties of the protein and the surface and the rate constant that is determined by the analysis of experimental results which almost always include the effect of diffusion, concentration and the surface itself.
Author: I agree that care should be taken when determining the contributions from transport and surface reaction to the adsorption rate. At low c_p and in the initial stage of the adsorption process, i.e., when the degree of coverage of the sorbent surface by the protein is extremely low, the adsorption rate is expected to be controlled by the transport towards the surface. However, whether or not this is really the case can only be experimentally verified under well-defined conditions where the rate of transport is predicted by theory. In the paper, I have discussed a few examples of such conditions.

K.K. Chittur: Under **Adsorption Kinetics**, the author describes events at the liquid solid interface. It is our view that we must, at all times, consider the diffusion from the bulk and surface reaction together, not as separate events. Yes, surface reaction will lead to depletion but only if the reaction is fast and if there is not sufficient protein in the bulk to replace it fast enough. For example, in a system where a protein such as albumin were to adsorb fast, but is also found at a relatively high concentration in the bulk, the depletion at the interface may be very, very short lived, if at all.
Author: Again, I agree with this comment. The discussion in the text is just meant to indicate how transport-limited adsorption rates can be verified.

K.K. Chittur: Towards the end of **Adsorption Kinetics**, the author cites the lack of appropriate models that describe protein adsorption. I agree, however it is important to keep in mind the specific objectives. We would like to distinguish between what we call "macroscopic" models that attempt to describe by reaction diffusion equations the time course of protein adsorption to surfaces. Models that have to include the heterogeneity of surfaces, protein-protein interactions and so on have to rely on microscopic models that need detailed molecular models for both protein and the surface. We must then carefully average molecular interactions to obtain macroscopic estimates, such as rate constants for adsorption and so on.
Author: Yes, and I think that there is a lack of appropriate models of both kinds.

K.K. Chittur: In text, the statement (just before **Adsorbed Amount**): "the way the protein is supplied to the system may affect the final result" needs to be clarified.
Author: The statement "the way the protein is supplied to the system may affect the final result" follows from

the generally observed irreversibility of the adsorption process. In other words, during the time scale of the experiment, the protein molecules do not fully relax to their equilibrium state. This is reflected, for instance, in a hysteresis between the ascending and descending branches of the adsorption isotherm [33]. While the final result does not represent true (thermodynamic) equilibrium it is, in principle, dependent on the history of the system and, therefore, on the way the protein is supplied.

K.K. Chittur: Equation (4) appears to be written for the adsorbed layer. Should not the Δg for the entire system (i.e., adsorbed protein, non-adsorbed protein, water etc.) be considered in the analysis? It is possible that the authors have done that elsewhere, clarification would help.

Author: Equation (4) refers to the overall adsorption process. This process is analyzed in terms of its most relevant contributions, i.e., electrostatic interactions, changes in the state of hydration and structural rearrangements in the protein. By doing so, all the components involved, i.e., the protein, the water, the low molecular weight electrolyte, are taken into account. It is tacitly assumed that the sorbent surface is rigid (does not undergo structural changes) and that the protein solution is ideally diluted, so that further dilution (due to adsorption) does not affect the molar Gibbs energy of the dissolved protein.