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Joseph McGuire

Adsorption kinetic data recorded for α-lactalbumin, β-casein, β-lactoglobulin, bovine serum albumin and lysozyme at silianized silica surfaces of low and high hydrophobicity, along with a simple model for adsorption and surfactant-mediated elution of protein, were used to analyze the removal of each protein by sodium dodecylsulfate (SDS) and dodecyltrimethylammonium bromide (DTAB) at each surface. The model relates resistance to surfactant elution to two rate constants: one governing conversion of removable protein to a nonremovable form (s_1), and one governing removal of protein by the surfactant (k_s). Elution of each protein from hydrophobic silica with SDS was interpreted as providing information relevant to protein-surface binding strength, or s_1; i.e., protein-specific differences in removal were a result of SDS adsorption to the surface and displacement of surface-bound protein, as opposed to solubilization driven by SDS binding to the protein. SDS-mediated removal of protein from surfaces of lower

hydrophobicity were interpreted as generally proceeding according to a similar, displacement mechanism. The model indicated that data recorded for DTAB-mediated elution at each surface were generally less representative of protein-surface behavior, and more a function of k_s, where differences in surfactant attachment to protein and solubilization appeared to play an important role in protein removal. Under controlled conditions use of the model would allow identification of cases where k_s in particularly protein specific, and illustrates the point that in such cases surfactant-mediated elution of a protein may reveal little about its surface behavior.

Elution of Adsorbed Proteins at Hydrophobic and Hydrophilic Surfaces by Dodecyltrimethylammonium Bromide and Sodium Dodecyl Sulfate

By

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Pravina Vinaraphong, Author

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1. INTRODUCTION

Understanding the behavior of proteins at solid surfaces is important in several areas of industry, medicine, and biology. Proteins play a major role in the fouling of both membrane and heat-exchange surfaces in the food industry, especially in dairy processing, and in general much study has been done on the surface activity of food proteins (Arnebrant et al., 1985, 1987; Arnebrant and Nylander, 1986; Krisdhasima and McGuire, 1991). The attachment of microorganisms to hard surfaces is often mediated by protein interactions to form a biofilm. Biofilms in steam condensers, heat exchangers, or on industrial fermenter heating/cooling coils, can significantly reduce heat transfer efficiencies. Moreover, these biofilms can increase fluid frictional resistance causing both increased power consumption and maintenance costs due to periodic system shut-downs (Bryers, 1987). Recently, preadsorption of batericidal proteins as a barrier to bacterial adhesion to the surface was recently reported (Daeschel et al., 1992).

Interaction of the surfaces of medical devices with surrounding fluids leads to protein adsorption and other interfacial processes that can be desirable or detrimental. Protein adsorption is also important in blood coagulation (Horbett, 1982; Solderquist and Walton, 1980). The separation and purification of proteins is usually accomplished by

solid-liquid chromatography. Protein chromatography depends on interaction of protein with the chromatographic support, generally by hydrophobic, ion exchange, or charge-transfer mechanisms.

In the instances and applications of protein adsorption stated above, the relevant fluids including blood plasma, milk and other fluid foods usually contain a number of different proteins. Often, the course of events occuring when a surface is contacted by a particular fluid differ from what experiments with single-component protein solutions would predict. However, it is difficult to understand the behavior of multi-component protein solutions at contact surfaces because, among other reasons, proteins can exist in multiple states on a surface (Horbett and Brash, 1987). Some strong evidence for this includes numerous observations indicating the presence of weakly and tightly bound proteins; for example, rinsing a surface after protein contact does not remove all the protein from the surface. In addition, there are plausible mechanisms to explain how and why proteins could reside at interfaces in more than one way (Andrade et al., 1992; Krisdhasima et al., 1992). The origin of these thoughts is from early attempts to understand changes in the detergent elutability of adsorbed proteins. Desorption induced by surfactant solutions can yield substantial information on protein-surface interactions and how their binding strength is affected by protein and surface properties; for example, decreases in surfactant elutability of protein from an adsorbed layer are observed as protein-surface contact time increases (Bohnert and Horbett, 1986). Thus, surfactant elution of adsorbed protein from a surface can be used as a tool to probe the "state" of adsorbed protein molecules (Rapoza and Horbett, 1990). However, using different

surfactant-protein-surface combinations may also involve different protein removal mechanisms (Wahlgren and Arnebrant, 1991).

Since 1950, the term surfactant has become universally accepted to describe organic substances with certain characteristic features related to amphipathicity, solubility, their adsorptive behavior and orientation at interfaces, and micelle formation. The term detergent is often used interchangeably with surfactant. More often, detergent refers to a combination of surfactants with other substances, organic, or inorganic, formulated to enhance functional performance, specifically cleaning, over that of the surfactant alone.

Surfactants have been widely used in the purification and characterizaton of proteins, as in SDS PAGE electrophoresis. Renaturation of proteins from SDS solutions is also an area of interest and importance in protein-surfactant interactions.

The objective of this research was to gain a better understanding of molecular properties affecting protein adsorption by studying removal of a set of well-characterized proteins from hydrophobic and hydrophilic surfaces as mediated by a cationic and an anionic surfactant of similar hydrocarbon chain length. Results were interpreted with reference to a model developed from a simple mechanism for protein adsorption and surfactant-mediated elution. In this way, differences in kinetic rate constants governing a protein's ability to attain a more tightly bound state, and a surfactant's ability to remove it, could be discussed in terms of protein, surfactant and surface properties. In this work, two surfactants, sodium dodecylsulfate and dodecyltrimethylammonium bromide, were used to remove α-lactalbumin, β-lactoglobulin, β-casein, bovine serum albumin (BSA) and lysozyme that had been adsorbed to hydrophobic and hydrophilic silica surfaces from

single-component solutions. Following will be a brief literature review on the physical properties of these proteins, adsorption from protein-surfactant mixtures, and surfactant interactions with adsorbed protein, to help understand the remainder of the thesis.

2. REVIEW OF LITERATURE

2.1 Physical and Chemical Properties of Bovine Serum Albumin, β-Lactoglobulin, α Lactalbumin, β-Casein and Lysozyme

Bovine serum albumin (BSA) is one of the more abundant blood proteins with molecular weight 66,300 Da. It is a transport protein with the ability to bind a wide variety of biological material, for example, fatty acids which bind primarily through hydrophobic interaction with the protein. Interaction with molecules such as fatty acids can lead to changes in the conformation of BSA indicating that the protein possesses considerable flexibility (Peter, 1985). The conformation of BSA isolated from milk has not been investigated, but extensive investigations of this protein isolated from bovine blood serum have been made (Whitney, 1988). There is some variability in the conclusions reached by various workers concerning the structure of BSA. Brown (1977) proposed two possible models based on the primary sequence of the protein. He demonstrated that the molecule could possess a triple domain structure with three very similar domains: residues 1-190, 191-382, and 383-582. Each domain could then consist of five helical rods of about equal length arranged either in a parallel or antiparallel manner. His second model consisted of the following: (1) a lone subdomain (1-101); (2) a pair of antiparallel subdomains, with their hydrophobic faces toward each other (113-287); (3) another pair of subdomains (314-484); and (4) a lone subdomain (512-582). These structures of both model are supported by the observed helical content of BSA (54-68%) (Reed et al., 1975) and by the location of the proline residues and reactive binding sites (Anderson *et al.*, 1971; Tayler and Vatz, 1973; Taylor *et al.*, 1975). Other investigators suggest two to nine domains (Foster, 1977). As the pH of BSA is lowered below its isoelectric point (pH

5.1), numerous changes in its physical and chemical properties occur. At pH 7, BSA has a negative charge of -18 units.

β-Lactoglobulin has a molecular weight of 18400 Da and is the major whey protein, with a concentration in milk of around 3.2 g/liter (Walstra *et al.*, 1984). It binds vitamin A and one of its functions could be as a transport protein for this substance (Papiz *et al.*, 1986). It has been reported that several other substances interact with β-lactoglobulin, e.g. fatty acids and small surfactants. These interactions are both hydrophobic and electrostatic in character. In the pH range from 5.2 to 7.5, all genetic variants of β-lactoglobulin investigated have been observed to exist primarily as dimers (Whitney, 1988). β-Lactoglobulin can associate to form dimers and higher oligomers, with dimers being the dominant form at pH 7.0 (Swaisgood, 1982). At pH 7.0, it has a negative net charge of -5 units. Mckenzie (1967) postulated approximately 33% α-helix, 33% β-configuration, and 33% disordered chain, while Townend *et al.* (1967) proposed 10% α-helix, 47% β-conformation and 43% disordered chain for the β-lactoglobulin molecule. While the numbers are somewhat variable, the interpretation proposed by Townend and co-workers are in general agreement with those predicted from the primary sequence (Deckmyn *et al.*, 1978).

α-Lactalbumin is a whey protein with molecular weight 14,200 Da. It is found in milk at a concentration of around 1.2 g/liter (Walstra and *et al.*, 1984). α-Lactalbumin was shown to exist primarily as a nearly spherical, compact globular monomer in neutral and alkaline media. It participates in the synthesis of lactose by modifying the enzyme galactosyl transferase (Swaisgood, 1982). Brown *et al.* (1969) noted the similarity of the primary sequence of α-lactalbumin and hen's egg-white lysozyme as well as the similarity in their functional properties and proposed a structure for α-lactalbumin based on the mainchain conformation of lysozyme; 47 out of 123 amino acids are identical and a number of others are conservatively replaced. However, the proteins differ greatly with respect to isoelectric point (α-lactalbumin pI = 4.8, lysozyme pI = 11) and the structure of α-

lactalbumin is less stable and more expanded than that of lysozyme. The charge at pH 7.0 of α-lactalbumin is -3 units compared to +8 for lysozyme (Imoto *et al.*, 1970).

Optical rotary dispersion studies of Herscovits and Mescanti (1965) indicated a tightly folded molecule with about 40% α-helix (Whitney, 1988). Circular dichroism spectra suggest 26% α-helix, 14% β-configuration, and 60% random coil (Whitney, 1988). The latter configuration is similar to the secondary structure of lysozyme (Robbins and Holmes 1970; Barel *et al.* 1972). The disulfide bonds in α-lactalbumin, as predicted from the expanded model, are more rapidly reduced, and therefore more accessible than those in lysozyme (Iyer and Klee, 1973). At alkaline pH, although no observable association or aggregation occurs some changes in conformation are observed.

β-casein is a single chained, fibrous protein of molecular weight 24,000 Da that has no disulfide bonds (Swaisgood, 1982). The major portion of β-casein is in an unordered structure, with regions of stable structure and large regions of marginal stability which have a high degree of segmental motion (Swaisgood, 1982). This structure makes unequivocable determination of the shape and dimension of the β-casein molecule difficult. The N-terminal 21-amino acid sequence of β-casein contains one-third of the charged residues at pH 7, and this portion of the protein is highly solvated and flexible. The remainder of the molecule is non polar and very hydrophobic, making β-casein distinctly amphiphilic (Swaisgood, 1982). At pH 7, β-casein has a negative net charge of -12 units. β-Casein also aggregates at temperature above 4°C, with the degree of aggregation increasing as the temperature is raised.

Lysozyme is an enzyme with bacteriocidal and lytic action (Reiter,1985). The type used in the present work is from hen egg white with molecular weight 14100 Da (Gekko and Hasegawa, 1986) but different types of lysozyme exist, for example, in milk and in tears (Walstra, 1984). It is involved in the hydrolysis of β(1-4) glycosidic linkages of carbohydrates in the cell walls (Imoto, 1972). Lysozyme can form dimers and higher oligomers above pH 4.5 and at high protein concentrations. At pH 7.0 it has a positive net

charge of +8 units. The isoelectric point of lysozyme is 11 (Gekko *et al.*, 1986). Imoto *et al.* (1972) postulated that lysozyme has approximately 29% α-helix and 16% β-configuration.

2.2 Surfactant Interactions with Adsorbed Protein

Bohnert and Horbett (1986) studied the SDS-mediated elution of adsorbed baboon albumin and fibrinogen at polymer surfaces. They found that the strength of protein-surface binding increased slowly over a period of days at room temperature. Thus, the elutability of proteins by surfactant is influenced by the residence time of the adsorbed proteins which can be correlated to time dependent conformational changes. Proteins were found to be much less elutable as both time and temperature of storage increased. The loss of elutability indicated strengthened binding, probably due to protein denaturation. In any case, the results suggested that changes in the protein-surface interaction continue to occur long after the adsorption process is over. The heterogeneity in protein binding to surfaces indicated by partial buffer desorption or partial detergent elution can be attributed to the fact that proteins continue to increase their degree of interaction once on the surface. The effect of surfactants on adsorbed proteins is also dependent on surface properties as shown by Elwing *et al.* (1987). Rapoza and Horbett (1990) continued to examine the effect of adsorption and elution conditions on the elutability of baboon fibrinogen and albumin from polyethylene and polystyrene. They found that at below a certain threshold surfactant concentration, very little elution occurs, and elution after a 2 h adsorption is incomplete, even when the surfactant concentration is far greater than the threshold value. They also observed that decreasing the size of the surfactant's hydrophobic group by two carbons will need an increase in an order of magnitude of the surfactant concentration to obtain the equivalent elution ability. Wahlgren *et al.* (1993b) investigated the elutability of adsorbed fibrinogen by anionic

(SDS) and cationic (dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB), and cethyltrimethylammonium bromide (CTAB)) surfactants at wettability gradient silica surfaces. They observed that at the hydrophobic end of the gradient surfaces, fraction of adsorbed protein was removed by all the surfactants while only SDS removed the protein at the hydrophilic end. The lack of the removal of protein by all cationic surfactants at the hydrophilic end may be due to fibrinogen increasing its contact area with the surface. The influence of cationic surfactant can be observed at the hydrophobic end where the elution ability increases slightly in the order DTAB, TTAB, CTAB after a long exposure time.

In other work, Wahlgren *et al.* (1993a) attempted to relate elutability, as measured by in situ ellipsometry, with the molecular properties of six model proteins: BSA, cytochrome c, β-lactoglobulin, α-lactalbumin, lysozyme and ovalbumin. The influence of protein properties on the extent of desorption upon rinsing with buffer and the extent of elution was found to differ, which indicated that the mechanisms behind protein removal are not the same. Further, they indicated that size, charge, temperature of denaturation and adiabatic compressibility influenced elutability at hydrophilic, negatively-charged silica surfaces, while size and shell hydrophobicity influence elutability at hydrophobic silica surfaces.

Wahlgren *et al.* (1991) studied the interaction between SDS and CTAB with β-lactoglobulin and lysozyme at silicon oxide, chromium oxide, nickel oxide, and silanized silica surfaces. They observed that elution was most complete at the silanized, hydrophobic silica surface; for the oxide surfaces the extent of elution decreased in the order silica, chromium oxide, then nickel oxide. The effect of surfactants on adsorbed protein films (with no protein initially present in solution) were summarized as proceeding according to one of four adsorption/displacement models: (1) removal of the protein upon addition of surfactants (2) replacement of protein by the surfactants (3) reversible

adsorption of the surfactant to the surface with adsorbed protein (4) partial removal of protein according to models 1 and 2.

Krisdhasima et al. (1993) interpret SDS-mediated elutability of milk proteins: α-lactalbumin, β-casein, β-lactoglobulin, and BSA with reference to a simple kinetic model for protein adsorption. The kinetic rate constants defining initial arrival and surface-induced unfolding for these proteins were ranked in order of magnitude. BSA exhibit the highest unfolding rate constant followed by those of β-casein, α-lactalbumin, and β-lactoglobulin. Ranking the intial arrival rate constants, α-lactalbumin exhibit the highest magnitude followed by β-lactoglobulin, β-casein, and BSA. The relative magnitudes of these rate constants were found to be consistent with molecular properties shown to affect the surface activity of each protein.

Since Horbett and coworkers (Horbett, 1981; Horbett and Weathersby, 1981) initiated the use of surfactant as a tool to study protein adsorption, many investigators (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990; Wahlgren *et al.* ,1991, 1993a, 1993b, Krisdhasima *et al.*, 1993) have applied this method to investigation of the binding strength of adsorbed protein. Although the elutability of the protein from the surface in surfactant solution reveals a range of binding strengths that is quite sensitive to protein and surface types, due to the complexity of the elution process, differences in the type of proteins and surfaces could be attributed to different removal mechanisms. (Adsorbed protein can be removed by either displacing of protein by surfactant at the adsorption site or formation of a protein-surfactant complex with less affinity for the surface (Wahlgren *et al.*, 1991).) Several qualitative interpretations for elution of adsorbed protein have been reported (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990; Wahlgren *et al.* ,1991, 1993a, 1993b). More work in this area with a focus on quantitative interpretation of elutability in terms of kinetic rate constants associated with protein adsorption and removal mechanisms is needed to more fully understand the removal of protein by surfactants.

2.3 Adsorption from Protein-Surfactant Mixtures

The effect of surfactants on proteins adsorbed at a surface as well as the adsorption from protein-surfactant mixtures are of interest in study of the binding strength of adsorbed proteins. The interaction of surfactants and proteins at the air-water interface have been reviewed by Dickinson *et al.* (1989), who point out that there are several parallels between these interactions and the behavior at a hydrophobic solid surface. At concentrations on the order of the critical micelle concentration (CMC) and higher, surfactant molecules are most likely to dominate the interface due to their greater ability to reduce interfacial tension. The formation of protein-surfactant complexes which may differ in properties from those of the protein and surfactant alone, could, however influence the adsorption behavior. Such formation of the complex also depends on surfactant concentration.

The character of SDS binding to β-lactoglobulin is strongly dependent on the concentration of surfactant (Wahlgren *et al.*, 1992). At low SDS concentration, SDS is bound to β-lactoglobulin by electrostatic interaction at two sites per dimer; at higher surfactant concentrations the amount may increase to 22 molecules per dimer (Jones *et al.*, 1976). These bind to the protein in a highly cooperative adsorption, which is characteristic for hydrophobic interaction (Jones *et al.*, 1976).

There is also evident that at high SDS concentration there is further binding of surfactant molecules to protein in a micelle-like state (Jones *et al.*, 1976). The binding of SDS molecules might change the conformation of protein molecules. At high SDS concentrations, it is suggested that the SDS molecules form a shell around β-lactoglobulin molecule and alter its conformation (Reynolds *et al.*, 1970).

The adsorption from mixtures of surfactant and fibrinogen was studied by Wahlgren *et al.* (1993b) and they reported that the effects of cationic and anionic surfactants were quite different. The adsorption of fibrinogen was increased in the presence of

alkyltrimethylammonium bromide (cationic surfactants), especially on the hydrophilic silica surface, but decreased in the presence of SDS anionic surfactant. In the case of alkyltrimethylammonium bromides, they observed that the mass of fibrinogen remaining after rinsing with buffer increased with decreasing length of surfactant hydrocarbon chain. Wahlgren *et al.* (1991) studied the adsorption of mixtures of β-lactoglobulin and lysozyme with each of two ionic surfactants: cetyltrimethylammonium bromide (CTAB) and SDS at solid surfaces. Mixtures of SDS and protein lead to either no adsorption at all or the preferential adsorption of SDS on all surfaces studied. From the mixtures of CTAB and each protein, the complexes adsorbed to hydrophilic metal oxide surfaces while at a hydrophobic, silanized silica surface, CTAB adsorbed preferentially. Differences observed in adsorption behavior among the protein-surfactant mixtures could be explained by dissimilarity in surfactant binding to protein in solution and to the protein adsorbed onto the surface (Wahlgren *et al.*, 1991).

3. METHODS

3.1 Protein Solution Preparation

All proteins were purchased from Sigma Chemical Co. (St. Louis, MO). α-Lactalbumin (type III, L-6010, Lot 128F8140), β-lactoglobulin (L-0130, Lot 51H7210) , bovine serum albumin (A-7906, Lot 15F0112), and β-casein (C-6905, Lot 40H9510) were of the highest native pure grade prepared from bovine milk. Lysozyme was from chicken egg white (L-6876, Lot 111H7010). Proteins were independently weighed and dissolved in a sodium phosphate buffer solution. Buffer solutions (pH 7.0) were prepared by mixing a solution of 0.01 M sodium phosphate monobasic monohydrate and 0.01 M sodium phosphate dibasic (EM Science, Cherry Hill, N.J.). Sodium azide (EM Scinece, Cherry Hill, N.J.), used as an antimicrobial agent, was added to the solutions at a concentration of 0.02% (mass per volume) prior to mixing. Both buffer and protein solutions were filtered (0.45 μm type GV, Millipore Corp., Bedford, MA) prior to use to removed undissolved material and other impurities such as bacterial cells. Protein solution was prepared with the concentration of the molar equivalent of 1.000 mg/ml β-lactoglobulin (27.22 μM).

3.2 Surface Preparation

All surfaces were prepared from a single type of silicon (Si) wafer (hyperpure, type N, resistivity .05-.5 Ohm/cm) purchased from Wafernet (San Jose, CA). First, the Si wafers were cut into small plates of approximately 1 x 2 cm using a tungsten pen. They were subsequently treated to exhibit hydrophilic or hydrophobic surfaces.

The following treatment was slightly modified from the method as described by Jönsson *et al.* (1982). Each small Si plate was placed into a test tube into which 5 ml of the mixture NH_4OH:H_2O_2:H_2O (1:1:5) was added followed by heating to 80°C in a water bath for 15 min. The Si plates were then rinsed with 20 ml of distilled, deionized water (Corning Mega Pure System ™, Corning, NY) followed by immersion in 5 ml HCl:H_2O_2:H_2O (1:1:5) for 15 min at 80°C. Each plate was then rinsed with 30 ml distilled-deionized water. In order to maintain some stability in the hydrophilicity of the surface, each Si plate was stored in 20 ml of a 50 % ethanol/water solution. Ammonium hydroxide, hydrochoric acid, hydrogen peroxide, and 100% ethanol were purchased from Mallinckrodt Inc. (Paris, KY).

The washed Si plates were rinsed with 40 ml distilled deionized water, then dried with N_2. The surfaces were then stored in a desiccator for approximately 24 hours. Dried plates were then immersed in a stirred solution of dichlorodimethylsilane (DDS) in xylene for 1 hour. The degree of silanization was controlled by varying the concentration of DDS. The concentrations used in this work were 0.010% DDS (for "hydrophilic" surfaces) and 0.100 % DDS (for "hydrophobic" surfaces) in xylene. Finally, the silanized silica surfaces were sequentially rinsed in 100 ml xylene, acetone, and ethanol. The plates were then dried with nitrogen and kept in a desiccator. Dichlorodimethylsilane (DDS) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Xylene and acetone were purchased from Mallinckrodt Inc. (Paris, KY).

3.3 Adsorption Kinetics

Protein adsorption was monitored, *in situ*, with ellipsometry (Model L104 SA, Gaertner Scientific Corp., Chicago, IL) (Krisdhasima *et al.*, 1992). Silanized, bare surfaces were glued (Cyanoacrylate Adhesive, Columbus, OH) onto a stainless steel plate with a rubber mat fixed underneath, and placed into a fused quartz trapezoid cuvette (Hellma Cells,

Jamaica, NY). The cuvette has a volume of about 35 ml; its fused quartz windows were placed perpendicular to the incident and reflected beams (angle of incidence = 70°). The ellipsometer sample stage was then adjusted to obtain a maximum in reflected light intensity. Filtered buffer solution (30 ml) was then injected into the cuvette. The surface was left to equilibrate with the buffer for 30 min. Fine adjustments of the stage were conducted in parallel with ellipsometric measurements of bare surface optical constant until steady values were obtained. Final measurements of bare surface properties were then recorded. The buffer solution was carefully removed from the cuvette and replaced with 30 ml filtered protein solution. The ellipsometric measurements were recorded every 30 s for 8 h under static conditions, i.e., no stirring and no flow. A computer program based on McCrackin's calculation procedure (Krisdhasima *et al.*, 1992a) was used to import data that has been stored on a disk to determine protein film refractive index and thickness, which were then used to calculate the adsorbed mass of protein according to the Lorentz-Lorenz relationship (Cuypers *et al.*, 1983). The required molecular weight: molar refractivity ratios (M/A) were calculated to be 3.816 g/ml for α-lactalbumin, 3.8140 g/ml for β-casein, 3.796 g/ml for β-lactoglobulin, 3.837 g/ml for bovine serum albumin (BSA), and 3.841 g/ml for lysozyme (Pethig, 1979; Suttiprasit and McGuire, 1992). The partial specific volumes (ν) for each protein are 0.733 ml/g for α-lactalbumin, 0.748 ml/g for β-casein, 0.751 ml/g for β-lactoglobulin, 0.729 ml/g for BSA and 0.761 ml/g for lysozyme (Pethig, 1979). The protein adsorption kinetic parameters were consequently calculated using a non-linear regression routine within the computer program. At least three replicate kinetic tests were performed with each protein at each type of surface.

3.4 Sodium Dodecyl Sulfate (SDS) and Dodecyltrimethyl Ammonium Bromide (DTAB) - Mediated Elutability

The experiments were performed according to Krisdhasima *et al.*(1993). In each test, the silica surface was placed in the trapezoid cuvette with liquid transfer ports on the top of the cuvette. Optical properties of the clean surface were recorded as constant after a 30 min equilibration in 30 ml buffer. Twenty-five ml of the original buffer was then pumped (mini-tubing pump, VWR Scientific, 60 rpm) out of the cuvette, at which time protein solution was passed through the cuvette at the rate of 10 ml/min for 6 min. The volume of protein solution was allowed to reach 30 ml, and contact was maintained for 90 min. Rinsing proceeded by first removing 25 ml of the protein solution at the rate of 10 ml/min using peristaltic pump, then flowing buffer through the cuvette for 15 min. The volume of buffer was allowed to reach 30 ml and adsorbed protein contact with buffer was maintained for an additional 30 min. Twenty-five ml of this incubation buffer was then pumped out of the cuvette, after which time surfactant was introduced at the rate of 10 ml/min for 6 min. DTAB and SDS were each used at a concentration of 0.1040 M, which is equivalent to 3% wt/volume of SDS in buffer solutionand well above the CMC of each surfactant. The volume of surfactant solution was allowed to reach 30 ml and contact was maintained for an additional 30 min. Twenty-five ml of the surfactant solution was then pumped out of the cuvette, after which time buffer was introduced at the rate of 10 ml/min for 15 min. The volume of buffer was allowed to reach 30 ml, and contact was maintained for an additional 30 min. Throughout each test, ellipsometric measurements were recorded every 15 s.

4. RESULTS AND DISCUSSION

4.1 Adsorption Kinetics

The adsorption kinetics of BSA, β-lactoglobulin, α-lactalbumin, and β-casein were performed earlier and are discussed in Appendix A, which is the published version of that work. Lysozyme kinetics were performed here in order to compare its behavior at hydrophobic and hydrophilic surfaces with that of the milk proteins. Thus, it will be instructive to briefly discuss the behavior of the milk proteins along with that of lysozyme. All kinetic experiments were performed over a period of 8 h. Kinetic data were then fit to the following equation:

$$\Gamma = a_3 + a_1\exp(-r_1 t) + a_2\exp(-r_2 t) \qquad (4.1)$$

where Γ is the adsorbed mass of protein ($\mu g/cm^2$) at time t (min), and a_1, a_2, and a_3 are coefficients related to the final value of adsorbed mass. The parameters r_1 and r_2 are functions of the rate constants k_1, k_{-1}, and s_1 defined in the mechanism for protein adsorption shown in Fig. 4.1 (Krisdhasima *et al.*, 1992; 1993). The mechanism consists of two steps that account for generally observed protein adsorption behavior. In step 1, corresponding to short contact time, the protein molecule reversibly adsorbs to the surface; *i.e.*, it can be readily removed. In step 2, a surface-induced conformational change takes place in which the reversibly adsorbed molecule is changed to an

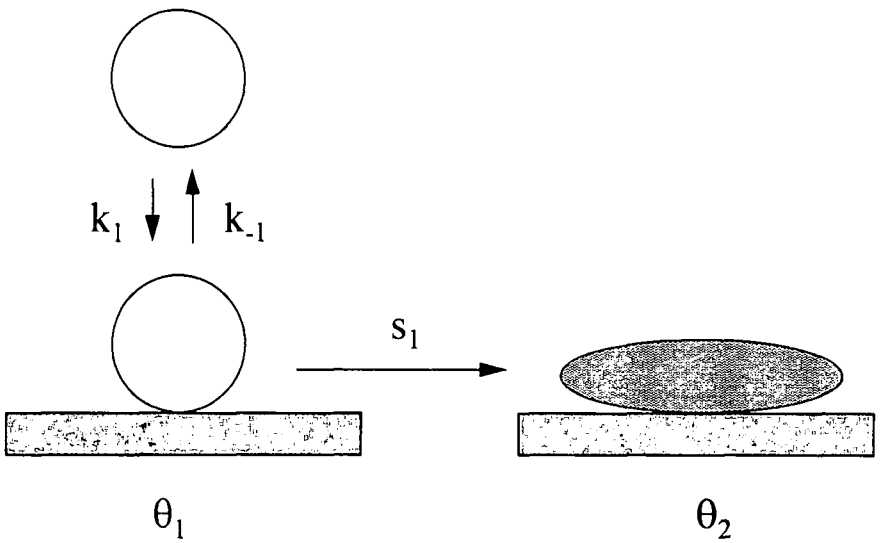

Figure 4.1 A simple two-step mechanism for protein adsorption

irreversibly adsorbed form. According to the mechanism, r_1 and r_2 can be presented as (Krisdhasima *et al.*, 1992):

$$r_1 + r_2 = -(k_1C + k_{-1} + s_1) \quad (4.2)$$

and

$$r_1 r_2 = s_1 k_1 C \quad (4.3)$$

where C is protein concentration. Unfortunately, the rate constants k_1, k_{-1}, and s_1 can not be obtained explicitly. However, the term s_1k_1C could yield information about the relative affinity of a protein molecule for a contact surface: a large value of s_1k_1C indicating a high affinity. Values of s_1k_1C calculated from the averaged value of r_1 and r_2 for each protein-surface contact are listed in Table 4.1.

Table 4.1 Averaged values of r_1 and r_2 determined for each protein at hydrophobic and hydrophilic surfaces, along with the value of s_1k_1C calculated according to Eq. (4.3).

Protein	Surface	$r_1 \times 10^3$	r_2	$s_1k_1C \times 10^2$
BSA	hydrophobic	5.18	2.36	1.22
BSA	hydrophilic	7.70	1.19	0.92
β-lactoglobulin	hydrophobic	9.20	1.98	1.82
β-lactoglobulin	hydrophilic	6.74	2.18	1.47
α-lactalbumin	hydrophobic	23.92	3.03	7.25
α-lactalbumin	hydrophilic	2.34	0.43	0.10
β-casein	hydrophobic	5.10	3.27	1.67
β-casein	hydrophilic	9.94	2.04	2.03
lysozyme	hydrophobic	6.60	1.68	1.11
lysozyme	hydrophilic	5.23	2.84	1.49

Representative plots of the adsorption kinetics exhibited by protein at each surface are presented in Figs. 4.2-4.6.

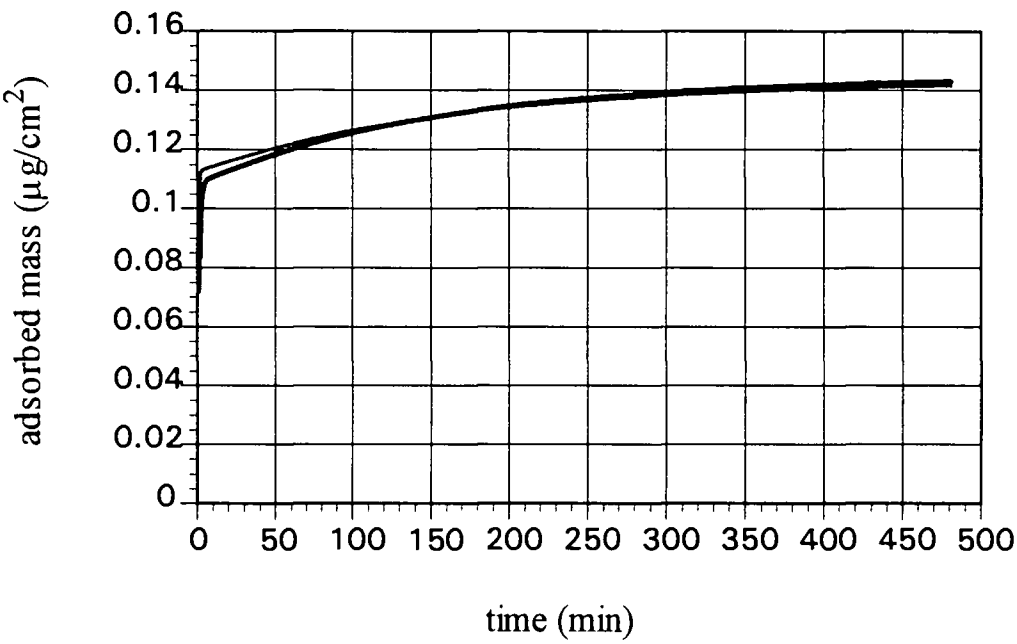

Figure 4.2 Comparison of BSA adsorption kinetics at hydrophobic (light line) and hydrophilic (heavy line) surfaces.

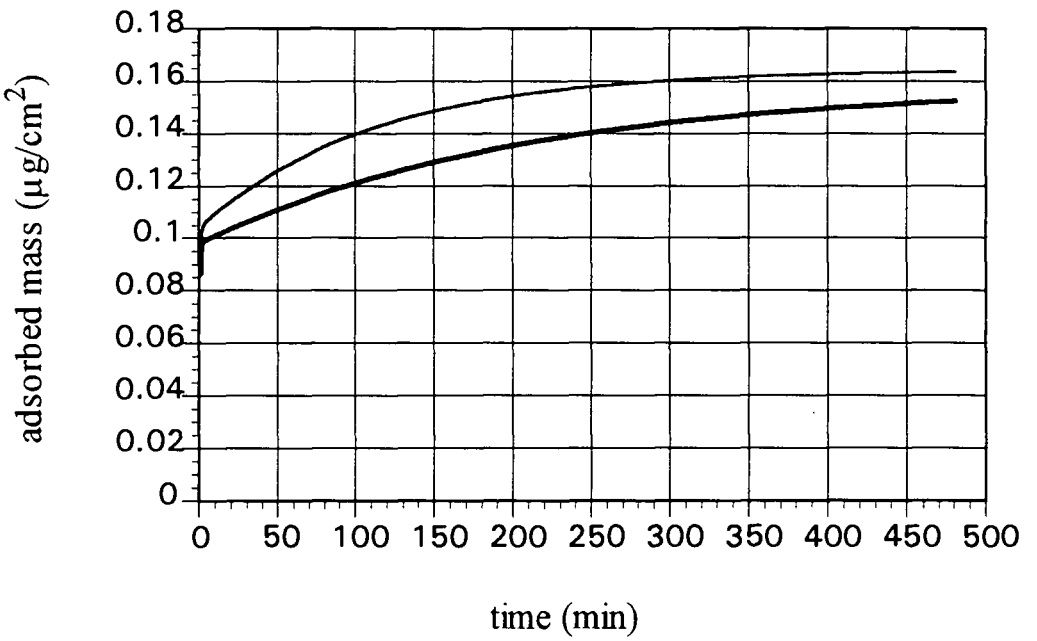

Figure 4.3 Comparison of β-lactoglobulin adsorption kinetics at hydrophobic (light line) and hydrophilic (heavy line) surfaces.

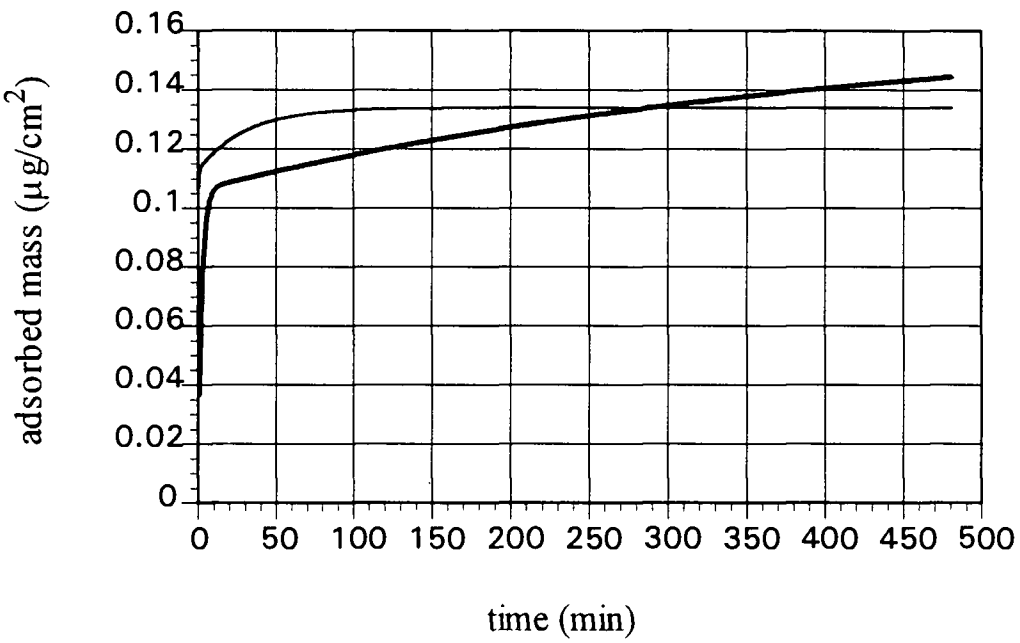

Figure 4.4 Comparison of α-lactalbumin adsorption kinetics at hydrophobic (light line) and hydrophilic (heavy line) surfaces.

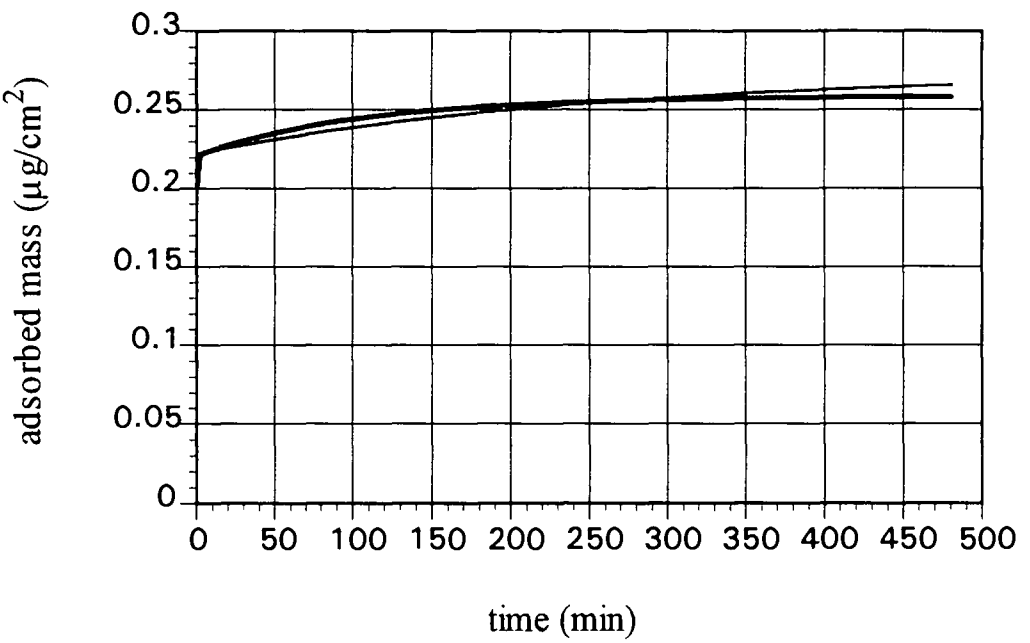

Figure 4.5 Comparison of β-casein adsorption kinetics at hydrophobic (light line) and hydrophilic (heavy line) surfaces.

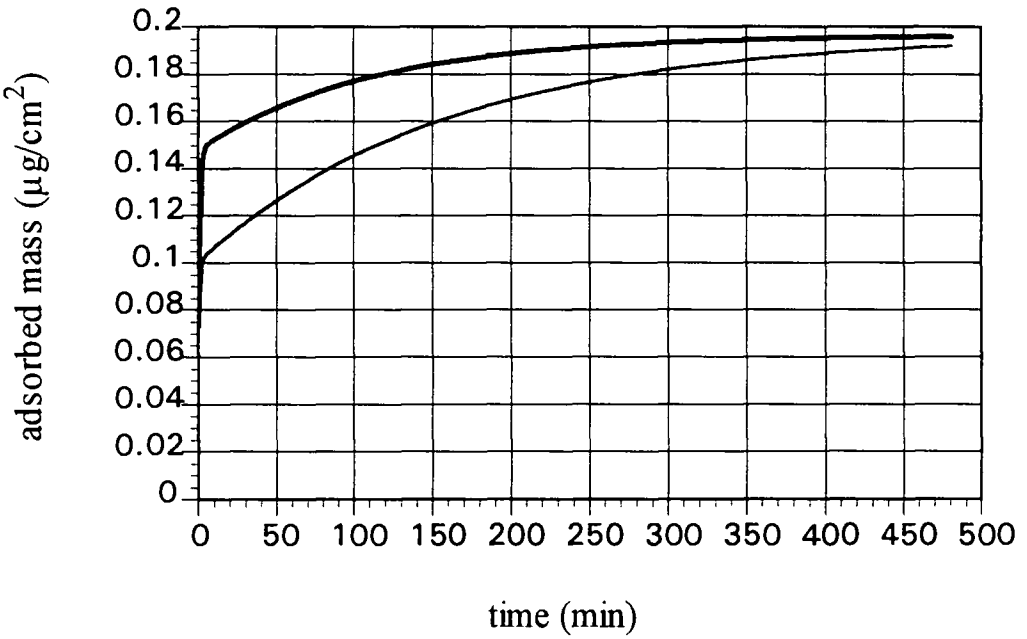

Figure 4.6 Comparison of lysozyme adsorption kinetics at hydrophobic (light line) and hydrophilic (heavy line) surfaces.

BSA adsorption kinetics were observed to be quite similar on hydrophobic and hydrophilic surfaces, although the initial slope was greater at the hydrophobic surface. This is consistent with the larger value of s_1k_1C estimated for the hydrophobic surface. A difference in behavior is more pronounced concerning β-lactoglobulin adsorption to each type of surface. β-Lactoglobulin adsorbed much faster, with a larger value of s_1k_1C, at the hydrophobic surface. The value of s_1k_1C for α-lactalbumin measured at the hydrophobic surface would indicate much higher affinity for that surface than that exibited by other proteins. At the hydrophilic surface, on the other hand, s_1k_1C of α-lactalbumin is an order of magnitude lower than that of the other proteins. This is taken to indicate that hydrophobic interaction played a major role in α-lactalbumin adsorption. β-Casein apparently shows a higher affinity for the hydrophilic surface, consistent with the fact that s_1k_1C for β-casein was greater at the hydrophilic surface than that estimated for the hydrophobic surface. In addition, the adsorbed mass of β-casein on each type of surface was substantially greater than corresponding values for the other proteins. Lysozyme adsorption exihibited a substantially different behavior from the other globular proteins. In particular, the adsorption rate at the hydrophilic surface was faster than that on the hydrophobic surface. This could be partly due to the electrostatic attraction between the lysozyme molecule (positive charge) and the hydrophilic surface (negative charge). Wahlgren *et al.* (1993) measured a faster adsorption rate on hydrophobic as opposed to hydrophilic (unsilanized) silica. However, the hydrophilic surface used in this study has much more hydrophobic character than the hydrophilic silica surface used by Wahlgren *et al.*, while it still possesses greater negative charge than the hydrophobic surface. Both

hydrophobic association and electrostatic attraction might serve to increase lysozyme affinity to the hydrophilic surface in this case. The magnitude of s_1k_1C of lysozyme is relatively low compared to the other proteins. This may be due to the relatively high stability of the lysozyme molecule, which is higher than other globular proteins used in this study (Appendix B).

4.2 Elutability

As described in Appendix A, removal of adsorbed protein by a surfactant, under controlled conditions of duration of adsorption, rinsing, and incubation with buffer and surfactant, can be generally described by a model originally developed for bulk-surface exchange reactions at an interface involving two different types of protein proposed by Lundström and Elwing (1990). In this study, protein was contacted with each surface for 90 min, rinsed with buffer for 15 min, incubated with buffer for an additional 30 min, incubated with surfactant for 30 min, then followed by another 15 min rinsing with buffer and incubation in buffer for an additional 30 min. Representative plots of each of these experiments are shown in Figs. 4.7 - 4.16. With reference to Fig. 4.1, θ_1 can be taken to denote the fractional surface coverage of removable protein, and θ_2 the fractional surface coverage that is not removable. During incubation in buffer both types of fractional surface coverage can be expressed as (Krisdhasima *et al.* 1993):

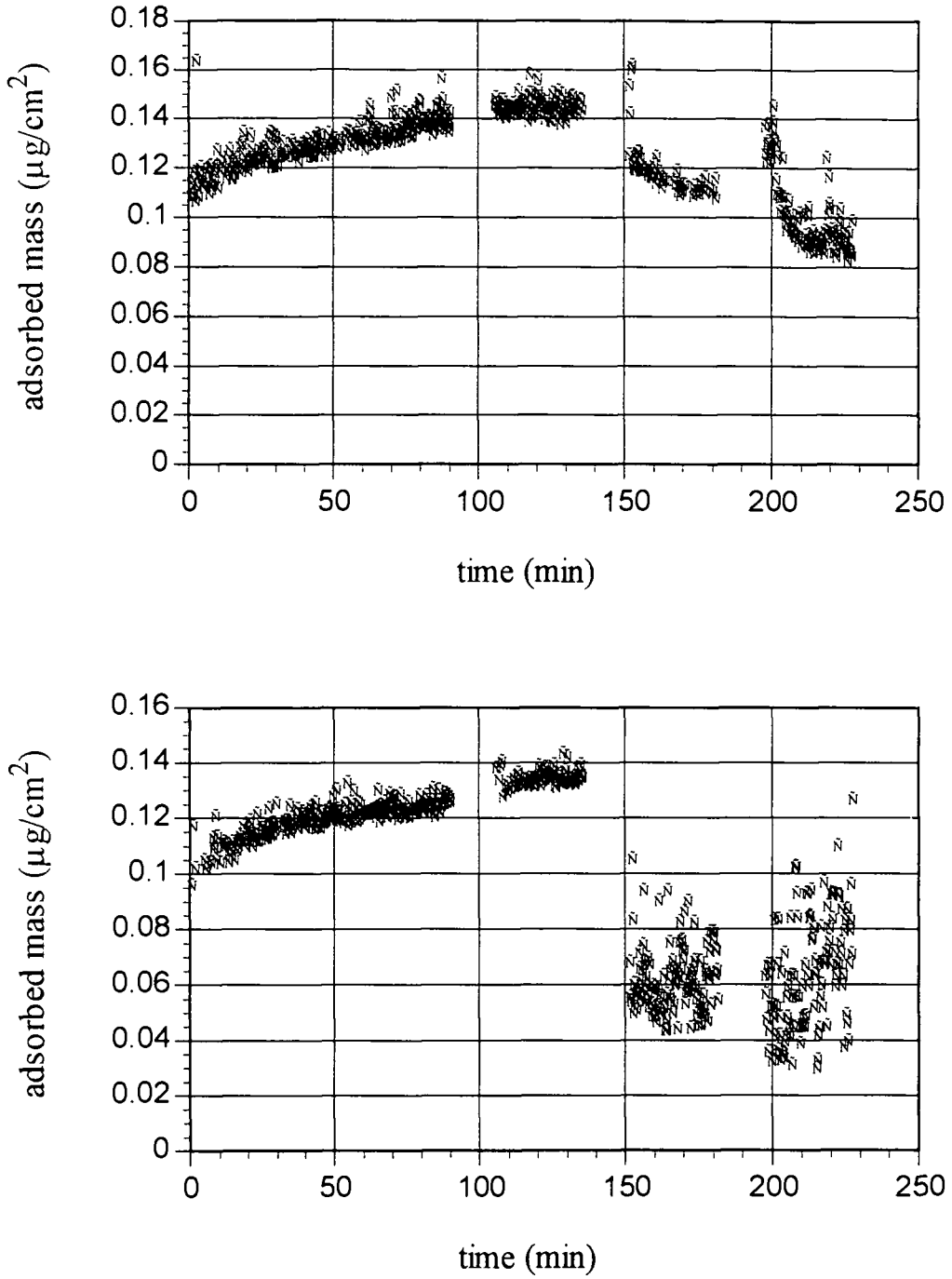

Figure 4.7 Representative plots of the change in adsorbed mass of BSA on hydrophobic (top) and hydrophilic (bottom) surfaces using SDS as surfactant.

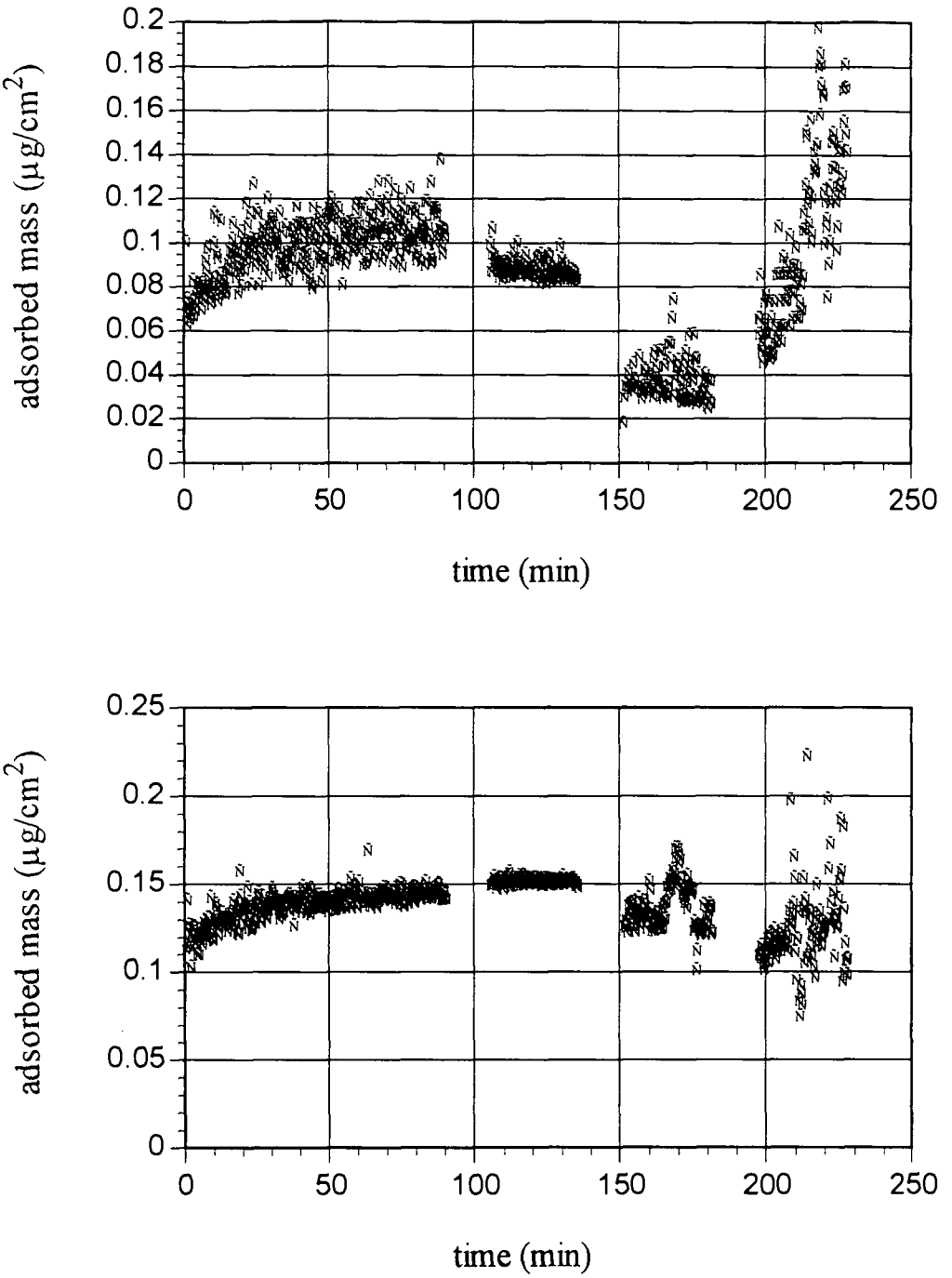

Figure 4.8 Representative plots of the change in adsorbed mass of BSA on hydrophobic (top) and hydrophilic (bottom) surfaces using DTAB as surfactant.

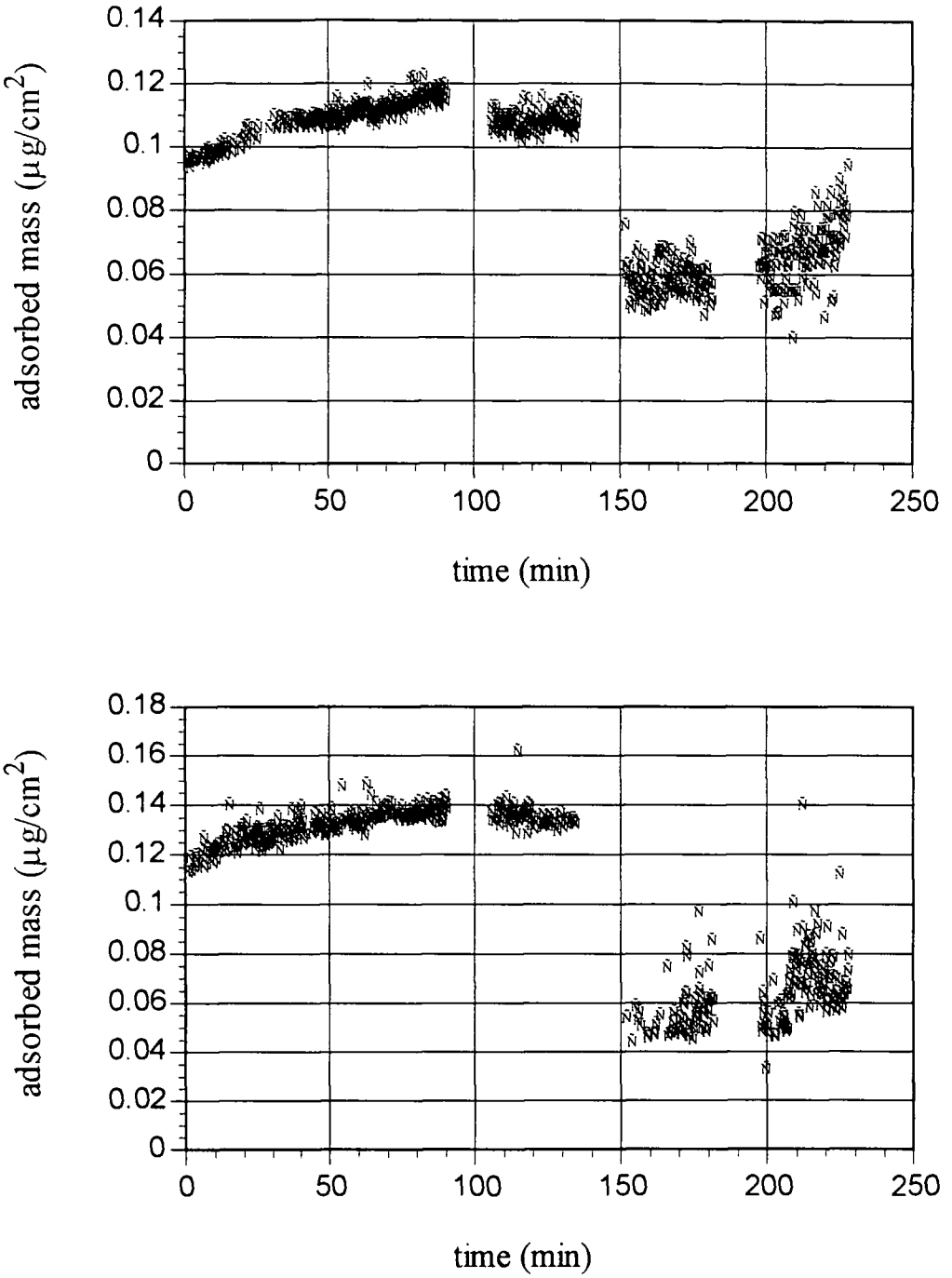

Figure 4.9 Representative plots of the change in adsorbed mass of β-lactoglobulin on hydrophobic (top) and hydrophilic (bottom) surfaces using SDS as surfactant.

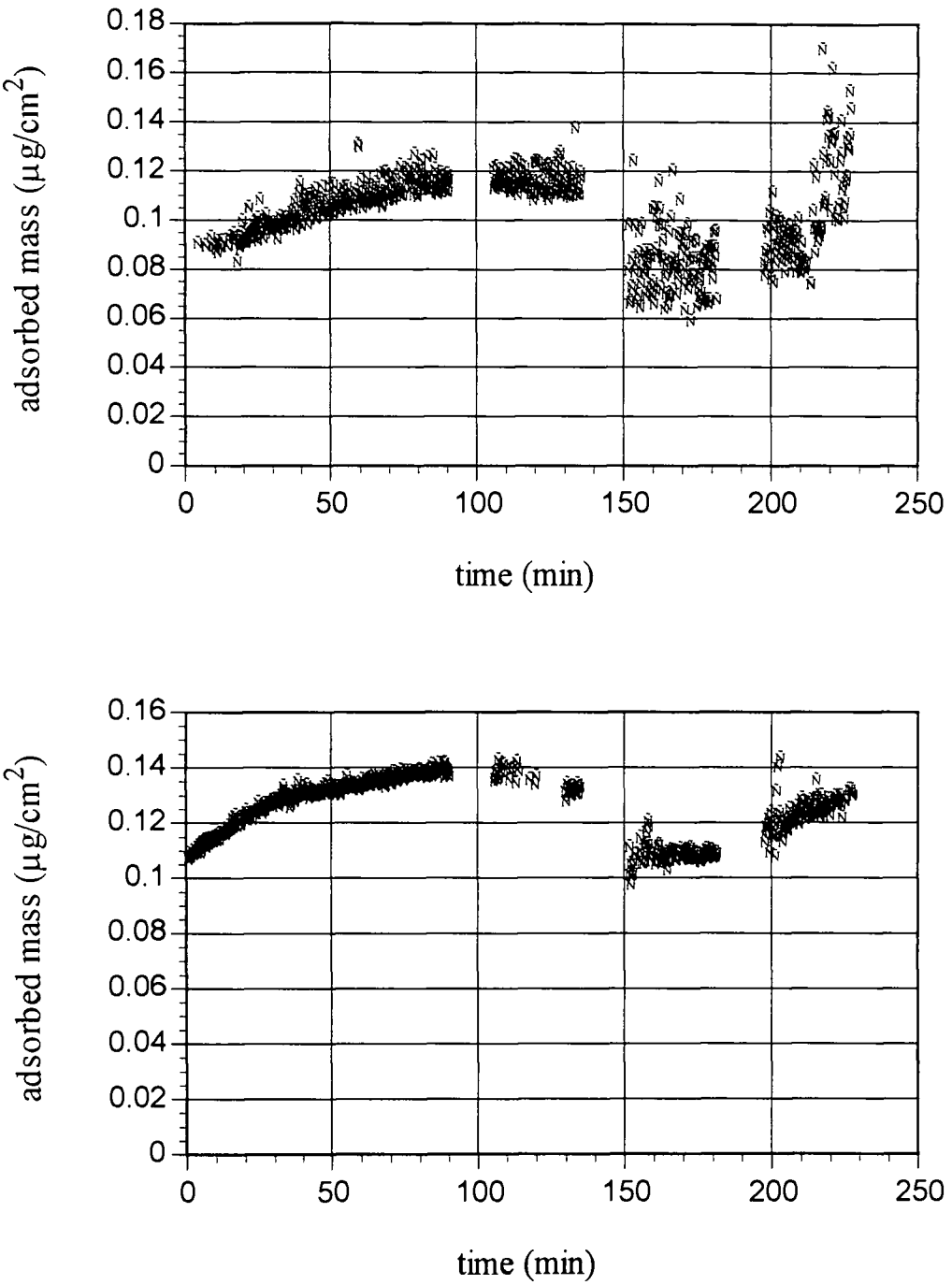

Figure 4.10 Representative plots of the change in adsorbed mass of β-lactoglobulin on hydrophobic (top) and hydrophilic (bottom) surfaces using DTAB as surfactant.

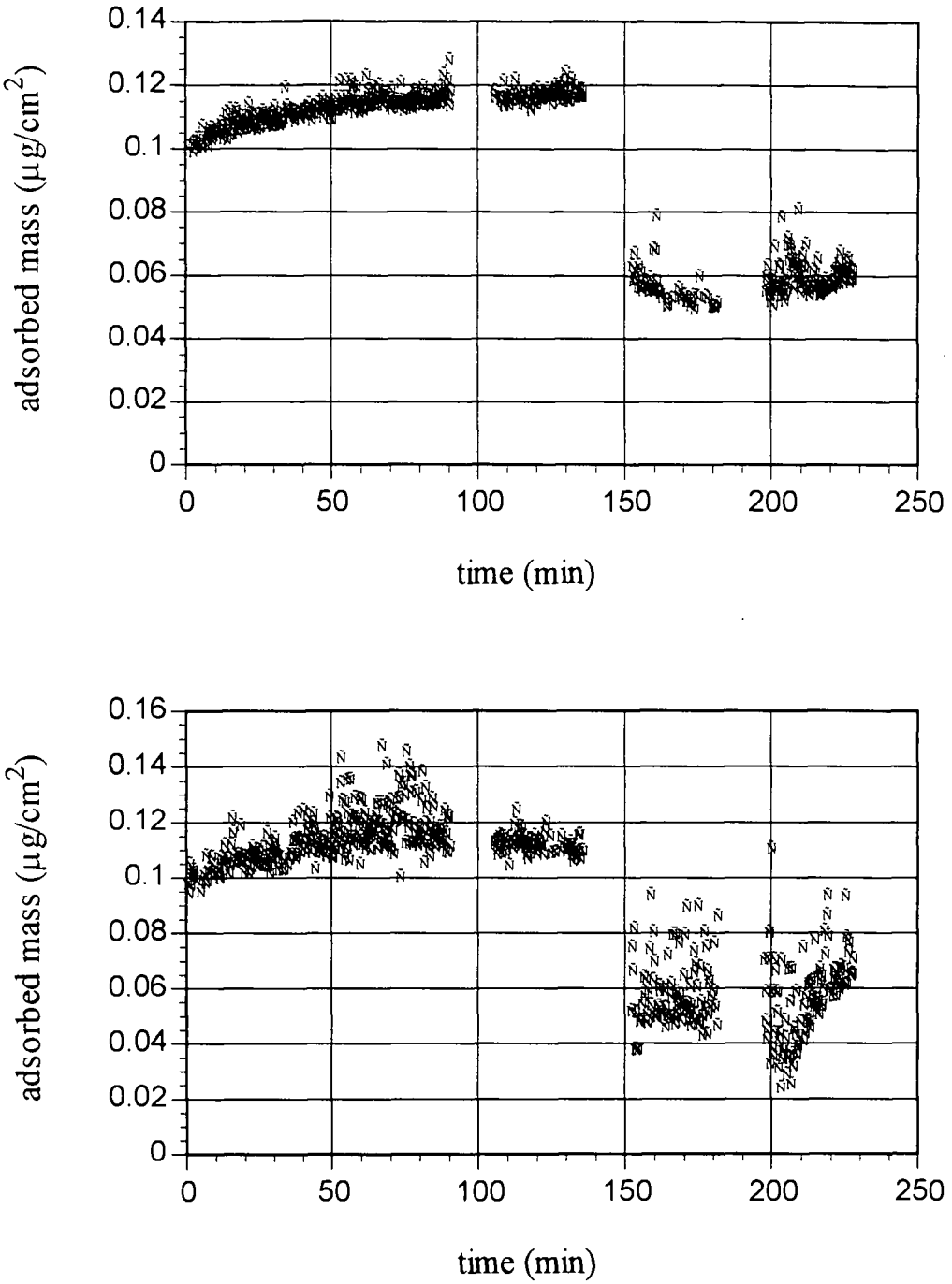

Figure 4.11 Representative plots of the change in adsorbed mass of α-lactalbumin on hydrophobic (top) and hydrophilic (bottom) surfaces using SDS as surfactant.

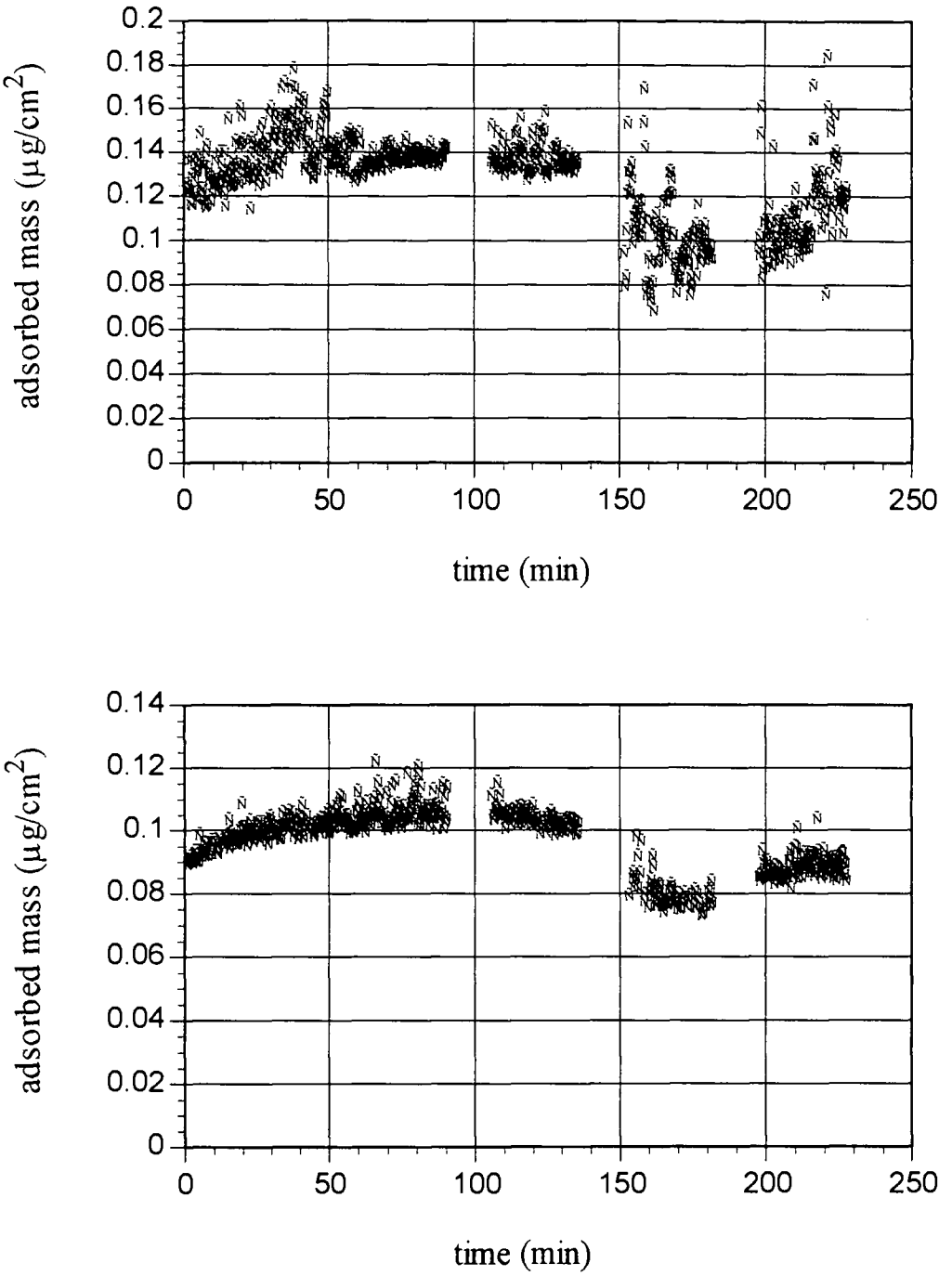

Figure 4.12 Representative plots of the change in adsorbed mass of α-lactalbumin on hydrophobic (top) and hydrophilic (bottom) surfaces using DTAB as surfactant.

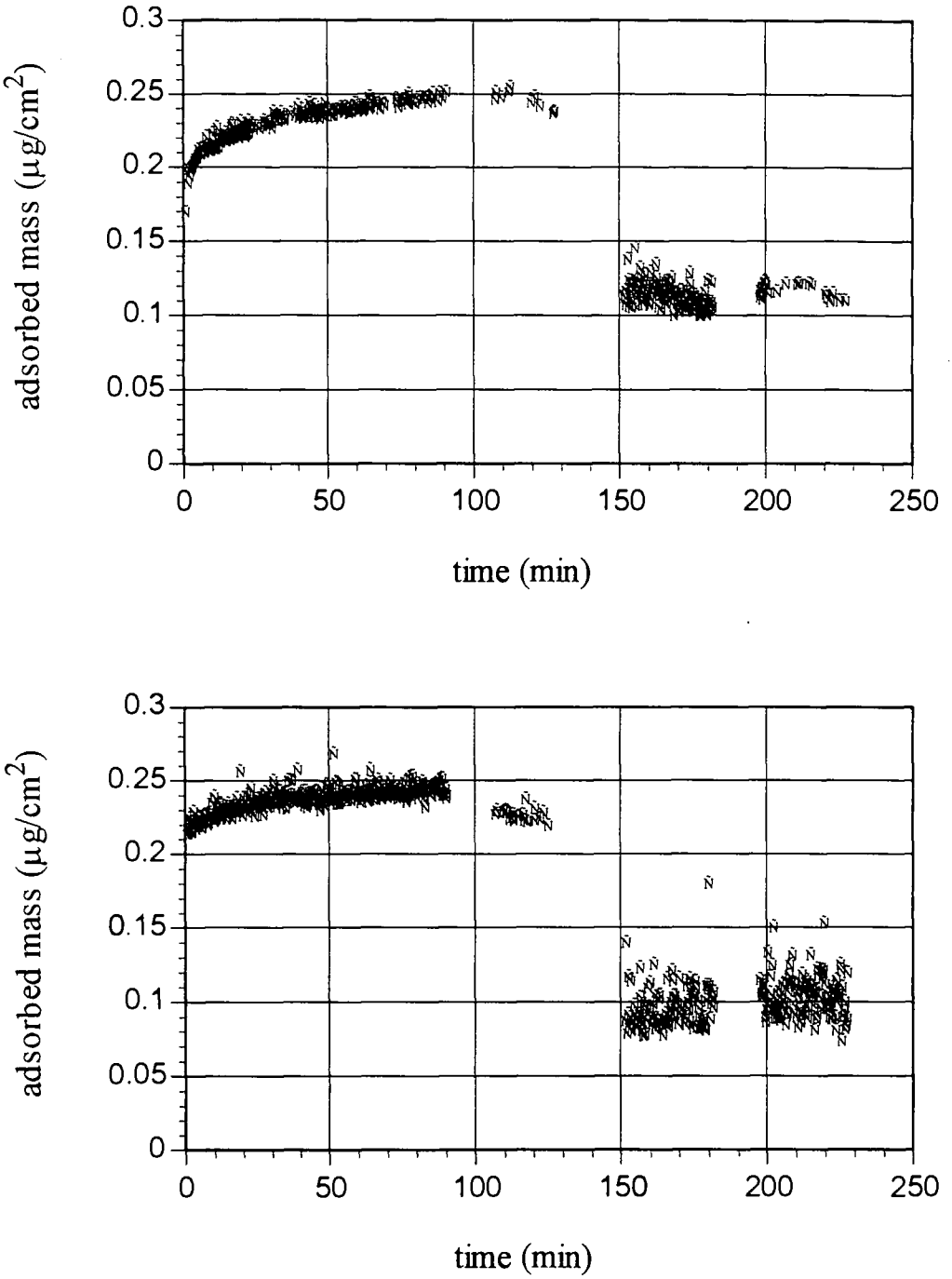

Figure 4.13 Representative plots of the change in adsorbed mass of β-casein on hydrophobic (top) and hydrophilic (bottom) using SDS as surfactant.

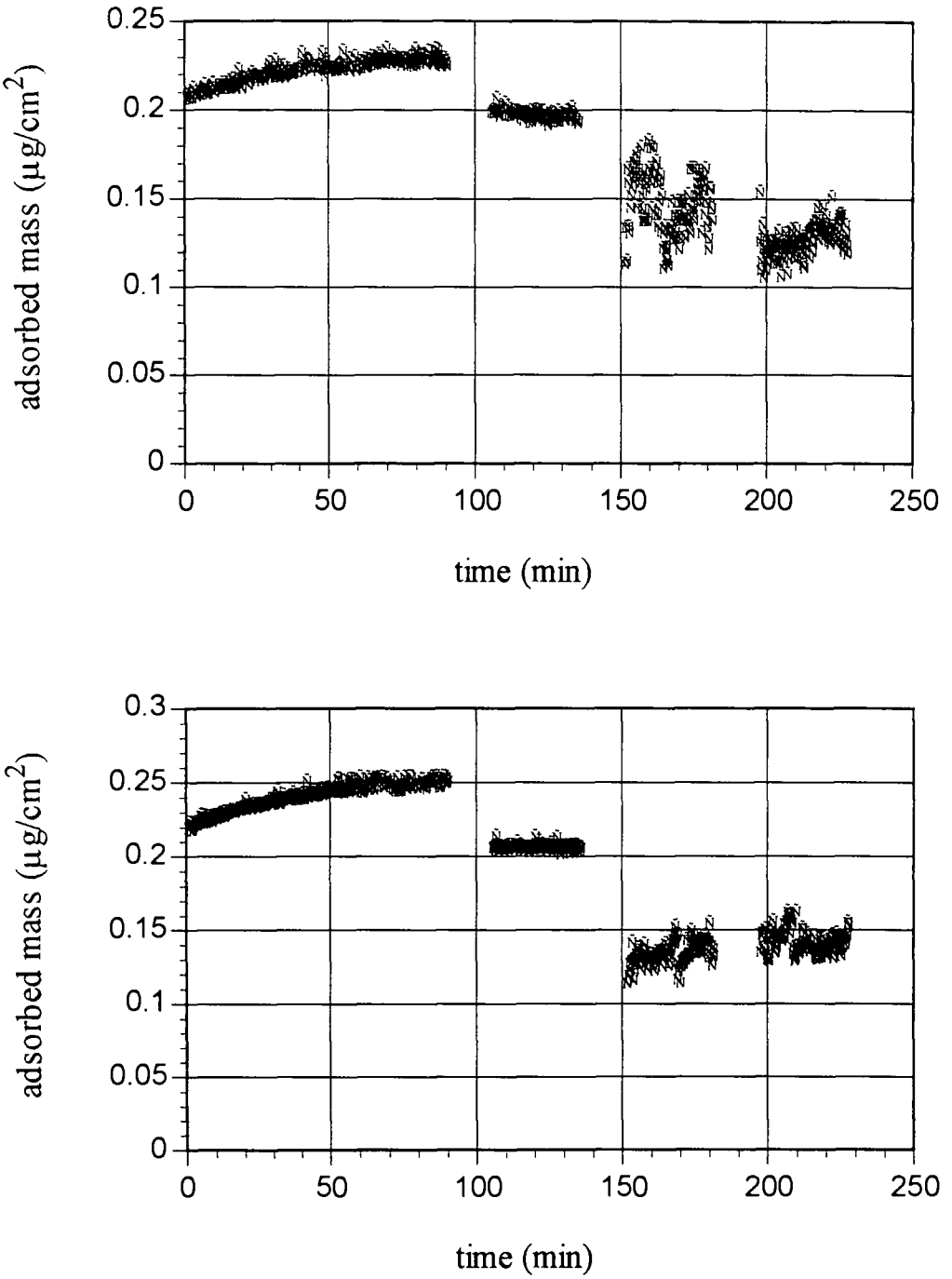

Figure 4.14 Representative plots of the change in adsorbed mass of β-casein on hydrophobic (top) and hydrophilic (bottom) using DTAB as surfactant.

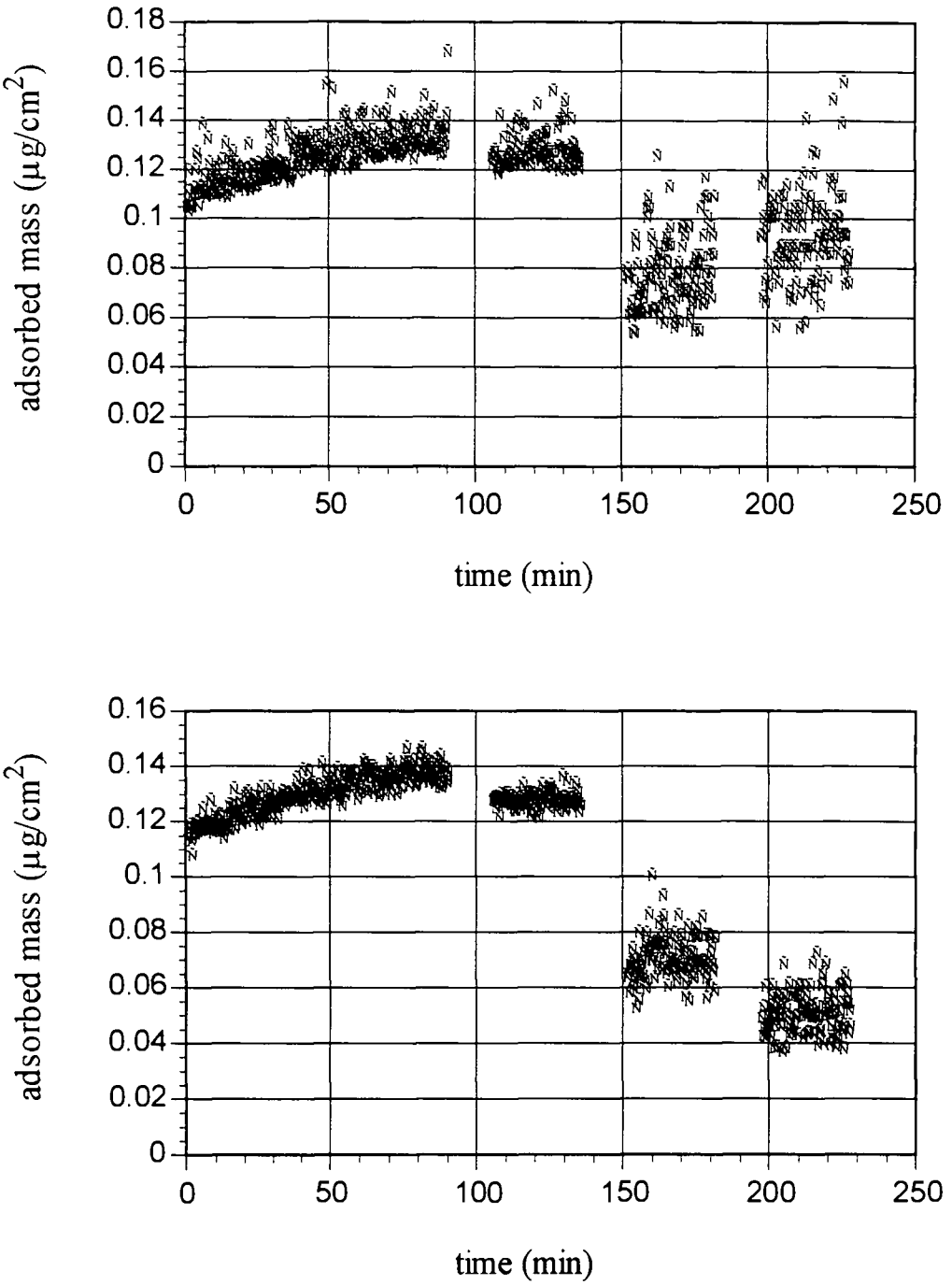

Figure 4.15 Representative plots of the change in adsorbed mass of lysozyme on hydrophobic (top) and hydrophilic (bottom) surfaces using SDS as surfactant.

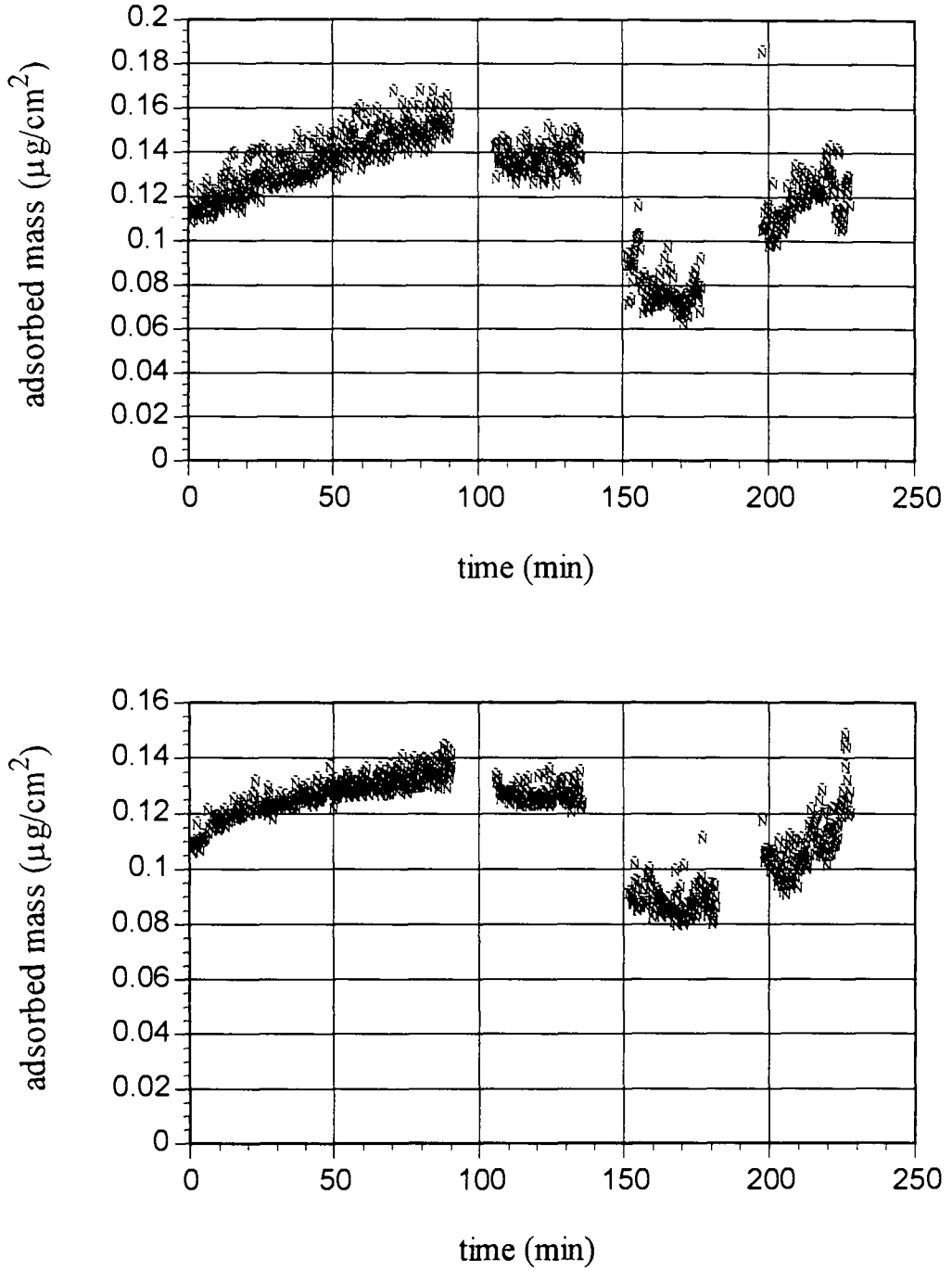

Figure 4.16 Representative plots of the change in adsorbed mass of lysozyme on hydrophobic (top) and hydrophilic (bottom) surfaces using DTAB surfactant.

$$\theta_1 = \theta_{1,ti} \exp(-s_1 t) \quad (4.4)$$

and

$$\theta_2 = \theta_{1,ti} [1-\exp(-s_1 t)], \quad (4.5)$$

where $\theta_{1,ti}$ is the value of θ_1 at $t = t_i$ (the initiation of adsorbed protein contact with clean buffer). At $t = t_s$ surfactant is introduced, and

$$d\theta_1/dt = s_1\theta_1 + k_s\theta_1, \quad (4.6)$$

where k_s is a rate constant describing removal of adsorbed protein by surfactant. After a sufficiently long time, the ratio of the fractional surface coverage of nonremovable protein to that of total protein present upon incubation in buffer would be:

$$\theta_2/\theta_{1,ti} = 1 - [k_s/(s_1+k_s)] \exp(-s_1 t_s) \quad (4.7)$$

The values of $\theta_2/\theta_{1,ti}$ listed in Table 4.2 for each protein were approximated by the ratio of the average value of adsorbed mass after contact with surfactant for 30 min less the associated adsorbed mass of surfactant, and the average value of adsorbed mass after the first 30 min incubation with buffer. Optical properties associated with the adsorbed mass of surfactant for each surface were measured using ellipsometry. The adsorbed mass of surfactant associated with each adsorbed protein was then estimated using the ratio of molecular weight to molar refractivity (M/A) and partial specific volume (ν) specific to the adsorbed protein in each case.

Table 4.2 The fraction of protein not removable by surfactant at each surface.

Protein	Surfactant	Surface	$\theta_2/\theta_{1,ti}$
BSA	SDS	hydrophobic	0.507
BSA	SDS	hydrophilic	0.184
β-lactoglobulin	SDS	hydrophobic	0.079
β-lactoglobulin	SDS	hydrophilic	0.139
α-lactalbumin	SDS	hydrophobic	0.169
α-lactalbumin	SDS	hydrophilic	0.167
β-casein	SDS	hydrophobic	0.256
β-casein	SDS	hydrophilic	0.299
lysozyme	SDS	hydrophobic	0.218
lysozyme	SDS	hydrophilic	0.251
BSA	DTAB	hydrophobic	0.000
BSA	DTAB	hydrophilic	0.442
β-lactoglobulin	DTAB	hydrophobic	0.154
β-lactoglobulin	DTAB	hydrophilic	0.273
α-lactalbumin	DTAB	hydrophobic	0.246
α-lactalbumin	DTAB	hydrophilic	0.114
β-casein	DTAB	hydrophobic	0.323
β-casein	DTAB	hydrophilic	0.239
lysozyme	DTAB	hydrophobic	0.140
lysozyme	DTAB	hydrophilic	0.092

Values of the adsorbed mass of surfactant apparently associated with each protein film at hydrophobic and hydrophilic surfaces are listed in Table 4.3. It is evident that SDS adsorbed more at hydrophobic than at hydrophilic surfaces, while DTAB adsorbed more at hydrophilic surfaces. This finding regarding both surfactants is consistent with their adsorption behavior at silanized and unsilanized silica surfaces reported earlier (Wahlgren *et al.*, 1993a). Figs. 4.2 to 4.6 show that the adsorbed mass of protein is about 60 to 90% of its final value within the first few minutes of adsorption, and after about 90 min of protein contact, the first derivative of adsorbed mass with respect to time approaches

zero. Thus, it is reasonable to assume that the magnitude of k_1 is much larger than that of s_1, and the protein adsorbed after 90 min is mostly in the removable form.

Table 4.3 Values of the adsorbed mass of each surfactant corresponding to M/A and ν calculated for the adsorbed protein at each surface.

Protein	Surfactant	Surface	Adsorbed mass of surfactant ($\mu g/cm^2$)
BSA	SDS	hydrophobic	0.0440
BSA	SDS	hydrophilic	0.0379
β-lactoglobulin	SDS	hydrophobic	0.0447
β-lactoglobulin	SDS	hydrophilic	0.0384
α-lactalbumin	SDS	hydrophobic	0.0438
α-lactalbumin	SDS	hydrophilic	0.0377
β-casein	SDS	hydrophobic	0.0450
β-casein	SDS	hydrophilic	0.0387
lysozyme	SDS	hydrophobic	0.0469
lysozyme	SDS	hydrophilic	0.0404
BSA	DTAB	hydrophobic	0.0590
BSA	DTAB	hydrophilic	0.0692
β-lactoglobulin	DTAB	hydrophobic	0.0599
β-lactoglobulin	DTAB	hydrophilic	0.0703
α-lactalbumin	DTAB	hydrophobic	0.0587
α-lactalbumin	DTAB	hydrophilic	0.0688
β-casein	DTAB	hydrophobic	0.0603
β-casein	DTAB	hydrophilic	0.0707
lysozyme	DTAB	hydrophobic	0.0629
lysozyme	DTAB	hydrophilic	0.0738

For the approximation of the mass of protein adsorbed in state 2, theoretically, the adsorbed mass remaining after rinsing away the surfactant would provide the best estimate (Wahlgren and Arnebrant, 1991). However, in the present tests, after the final rinse the total adsorbed mass was often observed to increase. This behavior could in part

be explained by destabilization of surfactant micelles upon dilution, or a faster decrease in the surface activity of surfactant than of protein or of protein-surfactant complexes, leading to re-adsorption of protein (or complexes) to the surface (Wahlgren *et al.*, 1992). Under these circumstances, then, the best estimate of the adsorbed mass corresponding to θ_2 was taken as the value of adsorbed mass determined after surfactant addition, minus the adsorbed mass of pure surfactant calculated for each adsorbed protein as shown in table 4.3.

In the previous experiments, described in Appendix A, the SDS-mediated removal of BSA, β-lactoglobulin, β-casein, and α-lactalbumin from hydrophobic surfaces was assumed to proceed purely by a displacement mechanism; *i.e.*, one where the adsorbed protein is displaced by incoming surfactant. In that case, removal of adsorbed protein was effected by SDS adsorption to the surface, while SDS adsorption to adsorbed protein followed by solubilization was considered of secondary importance. With that, k_s was taken as independent of the type of protein being displaced. Thus, the quantity $\theta_2/\theta_{1,ti}$ in those tests was considered a function of s_1 alone. The rationale leading to the assumption that removal of the globular milk proteins from the hydrophobic surface went according to a displacement mechanism is fairly applied to removal of lysozyme at the same surface (Wahlgren and Arnebrant, 1991). In that case, the relative ranking of $\theta_2/\theta_{1,ti}$ values pertaining to SDS-mediated removal of each protein from hydrophobic silica, shown in Table 4.4, would represent the relative ranking of s_1 associated with adsorption of each protein as well. The relationship between molecular properties of each milk protein and its relative value of s_1 was found to be physically reasonable, particularly with reference to

each protein's flexibility and stability. The high thermal stability of lysozyme would consequently lead to prediction of a low ranking of s_1, as opposed to the intermediate ranking seen in Table 4.4.

Table 4.4 Ranking of the magnitudes of the fraction of each protein not removable by SDS at hydrophobic and hydrophilic surfaces.

Protein	Surface	Ranking $\theta_2/\theta_{1,ti}$
BSA	hydrophobic	1
β-lactoglobulin	hydrophobic	5
α-lactalbumin	hydrophobic	4
β-casein	hydrophobic	2
lysozyme	hydrophobic	3
BSA	hydrophilic	3
β-lactoglobulin	hydrophilic	5
α-lactalbumin	hydrophilic	4
β-casein	hydrophilic	1
lysozyme	hydrophilic	2

This could imply that some structural alteration is undergone by adsorbed lysozyme in spite of its high stability, yielding a more tightly adsorbed form than that exhibited by α-lactalbumin and β-lactoglobulin. Alternatively, and with regard to its positive net charge, displacement may not be the sole mechanism of removal in this case. Solubilization might be involved as well, and electrostatic considerations alone would lead to the conclusion that lysozyme is the protein most difficult to solubilize; i.e., most difficult to convert to a protein-SDS complex of high negative charge.

Table 4.4 shows the $\theta_2/\theta_{1,ti}$ ranking for each protein at hydrophilic surfaces as well. Removal of the proteins by SDS from hydrophilic surfaces gave magnitudes of $\theta_2/\theta_{1,ti}$ as shown in table 4.2 similar to those measured at hydrophobic surfaces, with the exception of BSA. This suggests that the mechanism of removal of BSA was likely not preserved in moving from the hydrophobic to the hydrophilic surface. In this case, solubilization of adsorbed BSA, where SDS binds to BSA, followed by desorption of a very hydrophilic BSA-SDS complex, may have taken place in addition to displacement. In any event, it is not likely that such a substantial difference in $\theta_2/\theta_{1,ti}$ between hydrophobic and hydrophilic surfaces is due solely to a decrease in s_1, because of the similarity in kinetic behavior characterized by s_1k_1C observed for BSA at hydrophobic and hydrophilic surfaces.

If the removal of protein by SDS at the hydrophobic surface were to take place by the same mechanism as that at the hydrophilic surface, the ranking of s_1 would be expected to be the same at each surface. Thus, with the exception of BSA, it is probably reasonable to use the ranking of s_1 at hydrophobic surfaces to help describe the DTAB-mediated elutability of the proteins at each surface.

It is not likely that removal of proteins by DTAB took place according to the displacement mechanism alone on each surface. The ranking of $\theta_2/\theta_{1,ti}$, shown in Table 4.5, on non-removable fractions of protein at hydrophobic and hydrophilic surfaces is quite different from that of Table 4.4. According to the model, Eq. (4.7), this behavior suggests a difference in the magnitude of k_s among the proteins.

Table 4.5 Ranking of the magnitudes of the fraction of each protein not removable by DTAB at hydrophobic and hydrophilic surfaces.

Protein	Surface	Ranking $\theta_2/\theta_{1,ti}$
BSA	hydrophobic	5
β-lactoglobulin	hydrophobic	3
α-lactalbumin	hydrophobic	2
β-casein	hydrophobic	1
lysozyme	hydrophobic	4
BSA	hydrophilic	1
β-lactoglobulin	hydrophilic	2
α-lactalbumin	hydrophilic	4
β-casein	hydrophilic	3
lysozyme	hydrophilic	5

BSA was completely removed by DTAB from the hydrophobic surface while only about 56% was removed at the hydrophilic surface. This result is consistent with an enhanced electrostatic attraction between a BSA-DTAB complex, which contains less negative charge than native BSA, and the hydrophilic surface. Similarly, when BSA was removed by SDS, an increase in the negative charge of a BSA-SDS complex may have contributed to enhanced electrostatic repulsion at the hydrophilic surface. A higher degree of DTAB-mediated elutability of BSA from the hydrophobic silica surface (62%) than from the hydrophilic silica surface (12%) was also observed by Wahlgren *et al.* (1993a).

The magnitude of $\theta_2/\theta_{1,ti}$ measured for the removal of β-lactoglobulin by DTAB at the hydrophilic surface is 77% higher than at the hydrophobic surface. This behavior is quite similar to what occured with the removal of BSA by DTAB. The formation of a β-

lactoglobulin-DTAB complex may increase the electrostatic attraction between the complex and the hydrophilic surface. However, the removal of β-lactoglobulin by DTAB at hydrophobic and hydrophilic surfaces follows the same behavior as that of SDS. Thus, the dominant removal mechanism by these two surfactants should be different. Since the removal of β-lactoglobulin at each surface by SDS was apparently dominated by a displacement mechanism, the removal of the protein by DTAB might have been dominated by a solubization mechanism. The results here may at first seem to contradict results reported by Wahlgren, et al. (1993) which indicated complete removal of β-lactoglobulin at unsilanized, hydrophilic silica surfaces before introducing DTAB. It is important to note however that the hydrophilic surface used in this study was silanized, and hydrophobic interaction would contribute to binding of β-lactoglobulin to the hydrophilic surface.

The removal of α-lactalbumin by DTAB was measured to be higher at the hydrophilic surface than at the hydrophobic surface. Although the results presented here do not agree with those of Wahlgren *et al.* (1993a), the discrepancy may be due to susceptibility of the elutability of α-lactalbumin to flow pattern; the present experiments were performed under static conditions. With the flow system used by Wahlgren *et al.*, loosely bound protein would be mostly desorbed during the first rinsing. In any event, the present results imply that solubilization did not dominate the removal of α-lactalbumin by DTAB. If solubilization was the dominant mechanism, the degree of removal would have been lower at the hydrophilic surface.

DTAB removed a larger fraction of β-casein at the hydrophilic surface, while SDS removed a larger fraction at the hydrophobic surface. As described earlier, SDS also adsorbed to a greater extent to the hydrophobic surface while DTAB adsorbed to a greater extent to the hydrophilic surface. If the displacement mechanism is applied, SDS should remove more of the protein at the hydrophobic surface and DTAB should remove more protein at the hydrophilic surface. In the case of β-casein, the results are consistent with the above hypothesis. Thus, the removal of adsorbed β-casein in each case should take place mainly according to the displacement mechanism.

In the case of lysozyme, DTAB removed a larger fraction at the hydrophilic surface while SDS removed a larger fraction at the hydrophobic surface. But, the fraction of lysozyme removed by DTAB was higher than that removed by SDS on each type of surface, the difference being more pronounced at the hydrophilic surface. If the solubilization mechanism is applied, due to the electrostatic nature of lysozyme-DTAB complex, it should be rather difficult to remove a lysozyme-DTAB complex from the hydrophilic surface. However, such behavior was not observed in the present experiments. Thus, the mechanism that dominates the removal of lysozyme by DTAB on each surface may be one of displacement. If so, the degree of removal would largely depend on s_1 of the proteins.

If s_1 of protein A is larger than that of protein B, but $\theta_2/\theta_{1,ti}$ for protein A is less than that for protein B, then removal of protein A should be characterized by a larger value of k_s than would removal of B. With these data, a partial, ranking of k_s among the proteins for each surface can be obtained and this is shown in Table 4.6.

Table 4.6 Comparison of k_s for proteins removed by DTAB at hydrophobic and hydrophilic surfaces.

Hydrophobic Surface	Hydrophilic Surface
$k_{s,\,BSA} > k_{s,\,\beta\text{-lg}},\ k_{s,\,\alpha\text{-lac}},\ k_{s,\,\beta\text{-cas}},\ k_{s,\,lys}$	$k_{s,\,\beta\text{-lg}} < k_{s,\,lys},\ k_{s,\,\beta\text{-cas}},\ k_{s,\,\alpha\text{-lac}}$
$k_{s,\,lys} > k_{s,\,\beta\text{-lg}},\ k_{s,\,\alpha\text{-lac}}$	$k_{s,\,\alpha\text{-lac}} < k_{s,\,lys}$

When solubilization plays a major role in the removal of protein, the value of k_s among the proteins would change considerably. Removal of proteins by DTAB was most clearly characterized by a change in k_s, with the exception of the removal of BSA by SDS where k_s apparently changed with surface hydrophobicity.

At the hydrophobic surface, BSA removal was associated with the highest k_s ranking. The high rank of k_s may be due to BSA being a relatively large molecule with a high negative charge favoring binding with DTAB: up to 160 mol/mol of BSA (Takeda, *et al.*, 1992).

With these data, there is no way to distinguish the rank of k_s for β-casein and lysozyme. One might hypothesize that both proteins were removed mainly according to a displacement mechanism, with no significantly different value of k_s among proteins. However, there is no way to verify this hypothesis, although it is also supported by their SDS and DTAB-mediated elution from each surface. β-Lactoglobulin and α-lactalbumin

exhibited relatively low values of k_s at both hydrophobic and hydrophilic surfaces. The s_1 ranking of each proteins is also the lowest among the proteins used in this study. Thus, the relatively high elutability observed for these two proteins on each surface may have been reasonably expected.

In summary, the simple elutability model, Eq. (4.7), allows each rate constant to be discussed in terms of protein and surface properties. Although the major steps in the adsorption mechanism may have been taken into account, there are undoubtedly other steps that play an important role in some cases. If removal of a protein by a surfactant can be considered as taking place according to the mechanisms and associated model described here, one should expect the following results:

s_1	k_s	elutability
low	low	moderate to high
high	low	low
low	high	high
high	high	moderate to high

In this study, the SDS and DTAB-mediated elution of the five proteins at both hydrophobic and hydrophilic surfaces agrees quite well with results qualitatively predicted by the model, with reference to s_1 and k_s influences on elution. But it is important to note that removal of adsorbed protein by a surfactant is only an indirect method to determine how tightly the protein binds to the surface, and anomalous results might be encountered if the removal mechanism is not one of simple displacement. In general, the removal of protein by surfactant would not likely occur according to either a displacement or a solubilization mechanism. Both mechanisms would likely take place in every instance, although one may dominate in a specific case.

5. CONCLUSIONS

The values of $\theta_2/\theta_{1,ti}$ of the adsorbed protein removed by SDS at the hydrophobic surface were used to determine the ranking of s_1 for milk proteins and lysozyme. The relationship between molecular properties of each milk protein and its relative value of s_1 was found to be physically reasonable, particularly with reference to each protein's flexibility and stability. A higher ranking of s_1 for lysozyme than one would expect from its high thermal stability could imply that some structural alteration was undergone during of after adsorption.

Adsorbed proteins were removed by SDS from each surface mainly according to the displacement mechanism except for the case of BSA at the hydrophilic surface, where solubilization of adsorbed BSA, in addition to displacement, may have played an important role.

Removal of the proteins by DTAB does not likely take place according to the displacement mechanism alone. Protein and surfactant apparently form a complex that may change the electrostatic interaction between the complex protein and surface, or may induce structural alteration of the adsorbed protein.

With the comparison of the ranking of s_1 and $\theta_2/\theta_{1,ti}$, a partial ranking of k_s among the proteins removed by DTAB for each surface was obtained. Removal of proteins by DTAB was likely accompanied by a change in k_s.

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APPENDICES

APPENDIX A

Adsorption Kinetics and Elutability of α-Lactalbumin, β-Casein, β-Lactoglobulin, and Bovine Serum Albumin at Hydrophobic and Hydrophilic Interfaces

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Adsorption kinetic data recorded for α-lactalbumin, β-casein, β-lactoglobulin, and bovine serum albumin at silanized silica surfaces of low and high hydrophobicity, along with the surfactant-mediated elutability of each from hydrophobic silica, were interpreted with reference to a simple kinetic model for protein adsorption. The model includes an initial, reversible adsorption step, followed by a surface-induced conformational change yielding an irreversibly adsorbed form. The single-component adsorption kinetic data enabled estimation of the product of rate constants defining protein arrival, and conversion to an irreversibly adsorbed state, thus providing an index of relative adsorption affinity from single-component solutions. Elutability of each protein from hydrophobic silica with sodium dodecylsulfate, interpreted with reference to the same model, allowed further resolution of the single-component affinity data. In particular, the rate constants defining surface-induced unfolding for these proteins could be ranked in order of magnitude. The relative magnitudes of rate constants defining initial arrival and unfolding were found to be consistent with molecular properties shown to affect the surface activity of each protein.

INTRODUCTION

Some of the most relevant contributions to our present understanding of molecular properties that influence protein adsorption affinity have come from comparative studies of protein adsorption, in which selected similar proteins (1–3) or genetic variants of a single protein (4, 5) were studied. We know that a number of factors affect protein surface activity, but protein stability has received much attention, along with charge, hydrophobicity, and flexibility. In earlier work (6), we used *in situ* ellipsometry to measure the adsorption kinetics exhibited by β-lactoglobulin at silanized silica surfaces of varying hydrophobicity. The kinetic data were compared to the kinetic behavior predicted by a simple model for protein adsorption. The model described the data well in all cases, enabling interpretation of the kinetic data in terms of silica surface hydrophobicity influences on rate constants affecting protein attachment and unfolding at the interface. Both experimental and simulation results suggested that the product of rate constants defining protein arrival, and the conversion to an irreversibly adsorbed state, increase with increasing surface hydrophobicity. Thus, the product of those rate constants provided an index of relative adsorption affinity in those tests. In this paper, adsorption kinetic data recorded for the milk proteins α-lactalbumin (α-lac), β-casein, β-lactoglobulin (β-lg), and bovine serum albumin (BSA) at silanized silica surfaces of low and high hydrophobicity, along with their surfactant-mediated elutability from hydrophobic silica, are interpreted with reference to the same model.

For a set of similar, "single domain" proteins, a protein's initial, reversible attachment to a surface could be hypothesized as being most affected by solid surface and protein "surface" properties, while unfolding as being affected by solid surface properties and protein stability. Serious study of these molecular properties' influences on adsorption would require sets of very similar proteins, differing only in some controlled property; we are currently isolating synthetic, charge, and stability mutants of bacteriophage T4 lysozyme to measure adsorption kinetics with reference to the present model. That work would allow absolute identification of protein charge and stability influences on arrival and unfolding rate constants at solid–water interfaces and provide a basis for modeling competitive adsorption. We show here, however, that interpretation of adsorption and elutability experiments with reference to the present model can provide information on the relative rates of initial adsorption, and of surface-induced unfolding, among well-characterized proteins. In any event, the surface activity exhibited by α-lac, β-casein, β-lg, and BSA is of immediate industrial significance. Problems associated with their role in fouling of

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membrane and heat-exchange surfaces, and as possible mediators of microbial and spore adhesion, have been reported (7, 8). Also, preadsorption of bactericidal proteins as a barrier to bacterial adhesion to fluid-milk contact surfaces was recently reported (9). The major problem affecting the long-term stability of such surface preparations is displacement of the preadsorbed proteins by incoming milk serum proteins of higher affinity.

MATERIALS AND METHODS

Proteins

α-lac, β-casein, β-lg, and BSA from bovine milk (Sigma Chemical Co., St. Louis, MO) were independently weighed (Mettler Model AE 240, Mettler Instrument Corp., Hightstown, NJ) and dissolved in a phosphate buffer solution. Each protein solution was stirred for 20 min, then used after dilution to the molar equivalent of 1.000 mg/ml β-lg (27.22 μM). Buffer solutions (pH 7.00) were prepared by mixing a solution of 0.01 M sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) and 0.01 M sodium phosphate dibasic (Na_2HPO_4). Sodium azide (NaN_3), used as an antimicrobial agent, was also added to the solutions at a concentration of 0.02% (mass per volume) prior to mixing. Both buffer and protein solutions were filtered (0.22 or 0.45 μm type GV, Millipore Corp., Bedford, MA) prior to use. Much is known about the chemistry of α-lac, β-casein, β-lg, and BSA; properties of each molecule relevant to surface activity have been listed or otherwise described elsewhere (10, 11).

Surface Preparation

All surfaces were prepared from a single type of silicon (Si) wafer (hyperpure, type N, phosphorus doped, plane 1-0-0, Wacker Siltronic Corp., Portland, OR). First, the Si wafers were cut into small plates of approximately 1 × 2 cm using a tungsten pen. They were subsequently treated to exhibit hydrophilic or hydrophobic surfaces.

The silanizations were modified slightly from the method described by Jönsson *et al.* (12). Each small Si plate was placed into a test tube and 5 ml of the mixture NH_4OH:H_2O_2:H_2O (1:1:5) was added to the tube which was then heated to 80°C in a water bath for 15 min. The Si plates were then rinsed with 20 ml distilled–deionized water (Corning Megapure System, Corning, NY) and immersed in 5 ml HCl:H_2O_2:H_2O (1:1:5) for 15 min at 80°C. Each plate was then rinsed with 30 ml distilled–deionized water and stored in 20 ml of a 50% ethanol/water solution. Each hydrophilic Si plate was rinsed with 40 ml distilled–deionized water, dried with N_2, and stored in a desiccator for 24 h. Dried, hydrophilic Si plates were then immersed in a stirred solution of dichlorodimethylsilane (DDS, Aldrich Chemical Co., Inc., Milwaukee, WI) in xylene for 1 h. The degree of silanization was controlled by the concentration of DDS. The concentrations used in this work were 0.010% DDS (for "hydrophilic" surfaces) and 0.100% DDS (for "hydrophobic" surfaces) in xylene. Finally, the silanized silica surfaces were sequentially rinsed in 100 ml xylene, acetone, and ethanol. The plates were dried with N_2 and then kept in a desiccator, and their relative hydrophobicities were verified by contact angle analysis.

Adsorption Kinetics

These methods were described earlier (6). The kinetic data were monitored *in situ*, with ellipsometry (Model L104 SA, Gaertner Scientific Corp., Chicago, IL). Silanized, bare surfaces were placed into a fused quartz trapezoid cuvette (Hellma Cells, Germany). The cuvette has a volume of about 35 ml; its fused quartz windows were placed perpendicular to the incident and reflected beams (angle of incidence = 70°). The ellipsometer sample stage was then adjusted to obtain a maximum in reflected light intensity. Filtered buffer solution (30 ml) was then injected into the cuvette. The surface was left to equilibrate with the buffer for 30 min. Fine adjustments of the stage were conducted in parallel with ellipsometric measurements of bare surface optical constants Ψ_S and Δ_S until steady values were obtained. Final measurements of bare surface properties were then recorded. The buffer solution was carefully removed from the cuvette and replaced with 30 ml filtered protein solution. The values of Ψ and Δ were ellipsometrically measured and recorded every 30 s for 8 h under static conditions, i.e., no stirring and no flow. Recorded values of Ψ and Δ were stored on a floppy disk. A computer program based on McCrackin's calculation procedure (13) was used to import the data from the disk and determine the refractive index and thickness corresponding to each pair of Ψ and Δ, which were then used to calculate the adsorbed mass of protein according to the Lorentz–Lorenz relationship (14). The required molecular weight:molar refractivity ratios (M/A) were calculated to be 3.816 g/ml for α-lac, 3.8140 g/ml for β-casein, 3.796 g/ml for β-lg, and 3.837 g/ml for BSA (15). The partial specific volumes (v) for each protein are 0.733 ml/g for α-lac, 0.748 ml/g for β-casein, 0.751 ml/g for β-lg, and 0.729 ml/g for BSA. At least three replicate kinetic tests were performed with each protein at each type of surface.

SDS Elutability

These tests were performed to provide an independent, albeit indirect, indication of binding strength among the proteins for comparison with our interpretation of the kinetic data. In each test, a hydrophobic silica sample was placed in the trapezoid cuvette, modified to have flow-through capability. Optical properties were recorded as constant after a 30-min equilibration in 30 ml buffer. Twenty-five ml of the original buffer was then pumped out of the cuvette, at which time protein solution (27.22 μM) was passed through

the cuvette at the rate of 10 ml/min for 6 min. The volume of protein solution was then allowed to reach 30 ml, and contact was maintained for 90 min. Rinsing proceeded by first removing 25 ml of the protein solution, then flowing buffer through the cuvette at the rate of 10 ml/min for 15 min. The volume of buffer was then allowed to reach 30 ml and adsorbed protein contact with buffer was maintained for an additional 30 min. Twenty-five ml of this incubation buffer was then pumped out of the cuvette, after which time sodium dodecylsulphate (SDS; 3% wt/vol) was introduced at the rate of 10 ml/min for 6 min. The volume of SDS solution was then allowed to reach 30 ml and contact was maintained for an additional 30 min. Twenty-five ml of the SDS solution was then pumped out of the cuvette, after which time buffer was introduced at the rate of 10 ml/min for 15 min. The volume of buffer was then allowed to reach 30 ml, and contact was maintained for an additional 30 min. Throughout each test, Ψ and Δ were recorded every 15 s.

RESULTS AND DISCUSSION

Adsorption Kinetics

Lundström (16) developed a model for reversible protein adsorption based on a mechanism allowing for protein to be adsorbed in both a "native" and a "denatured" state. We recently used a very similar mechanism, but for irreversible protein adsorption, consisting of two steps (6). In step 1, corresponding to short contact time, the protein molecule reversibly adsorbs to the surface, with its adopted surface conformation closely approximating its native form. In step 2, a surface-induced conformational change takes place in which the reversibly adsorbed molecule is changed to an irreversibly adsorbed form. A schematic of the two-step mechanism is shown in Fig. 1 (6). Solving equations describing the time-dependent fractional-surface coverage of protein in each of the two states, one reversibly adsorbed (θ_1) and one irreversibly adsorbed (θ_2), yielded an expression for total surface coverage (θ) as a function of time (6),

$$\theta = \theta_1 + \theta_2 = A_1\exp(-r_1 t) + A_2\exp(-r_2 t) + A_3, \quad [1]$$

where A_1, A_2, and A_3 are constants, the roots (r_1 and r_2) are known functions of the three rate constants defined in Fig. 1, and t is time. An expression for total adsorbed mass as a function of time was obtained from Eq. [1] as

$$\Gamma = a_1\exp(-r_1 t) + a_2\exp(-r_2 t) + a_3. \quad [2]$$

The parameters a_1, a_2, and a_3 are the products of Γ_{max}, the "equilibrium" adsorbed mass, with A_1, A_2, and A_3, respectively.

A representative plot of the kinetic data is shown in Fig. 2 for each protein on each type of surface. The pattern of the kinetic data agrees very well with the model of Eq. [2]. Nonlinear regression performed on adsorption kinetic data fit to Eq. [2] would yield estimates of the parameters a_1, a_2, a_3, r_1, and r_2. Parameters r_1 and r_2 are known functions of k_1, k_{-1}, and s_1. In particular (6),

$$(r_1 + r_2) = (k_1 C + k_{-1} + s_1) \quad [3]$$

and

$$r_1 r_2 = s_1 k_1 C, \quad [4]$$

where C is protein concentration. Table 1 lists averaged values of r_1 and r_2 for each protein–surface contact. Due to its smaller magnitude, r_1 affects the rate of increase in adsorbed mass for a longer period than does r_2: r_2 affects Γ only at very early times, as $\exp(-r_2 t)$ rapidly approaches zero as time increases. Based on Eq. [2], with negative a_1 and a_2, it would take longer for Γ to reach a plateau as the magnitude of r_1 decreases. Parameter r_1 was determined to be lower at hydrophilic surfaces for α-lac and β-lg, but β-casein and BSA kinetics showed a lower magnitude of r_1 at hydrophobic surfaces. Krisdhasima *et al.* (6) found, for β-lg adsorption kinetics measured at five surfaces prepared with different DDS

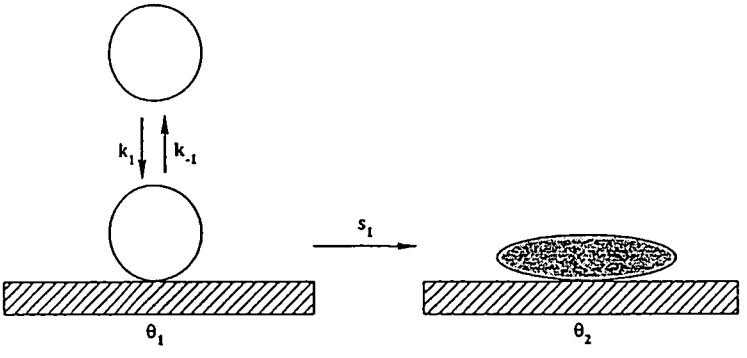

FIG. 1. A simple, two-step mechanism for protein adsorption (from Ref. (6)).

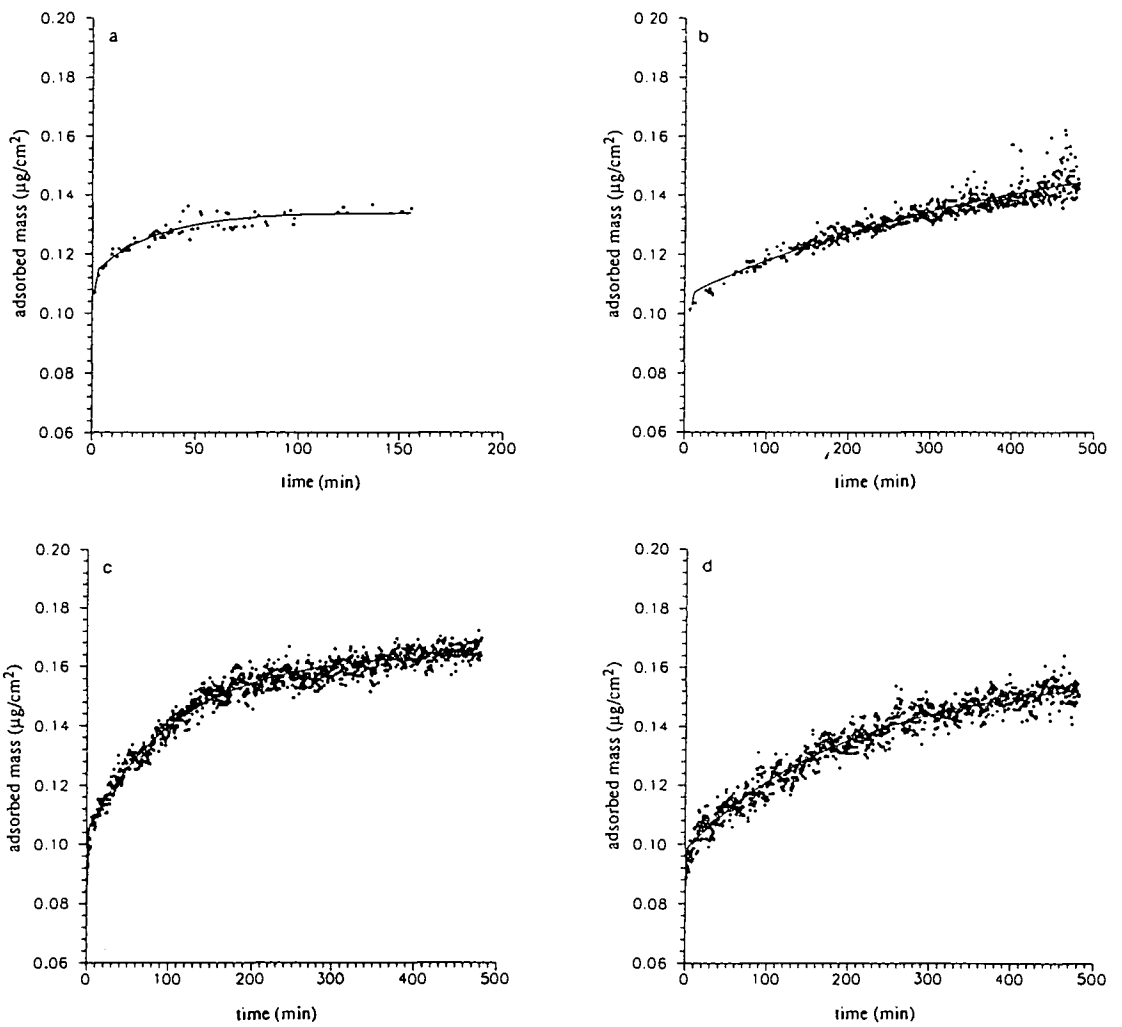

FIG. 2. Representative kinetic data recorded for each protein on hydrophobic and hydrophilic surfaces: α-lac contacted with a (a) hydrophobic and (b) hydrophilic surface; β-lg contacted with a (c) hydrophobic and (d) hydrophilic surface; BSA contacted with a (e) hydrophobic and (f) hydrophilic surface; and β-casein contacted with a (g) hydrophobic and (h) hydrophilic surface. The line drawn through the data on each plot follows Eq. [2].

concentrations, that the magnitude of r_1 increased with surface hydrophobicity while the magnitude of r_2 decreased. As shown in that work as well as in Table 1, however, r_2 is two or three orders of magnitude greater than r_1, and r_2 was not obviously observed to affect the initial adsorption rate as a function of surface hydrophobicity in our tests.

The globular proteins. Due to the disparity in their magnitudes, parameters r_1 and r_2 would be most usefully interpreted in terms of the three rate constants with reference to Eq. [4]. The individual rate constants cannot be measured absolutely with the present experimental system, but it is at first tempting to suggest that the value of s_1k_1C provides an index of relative adsorption affinity. Data of Krisdhasima *et al.* (6) are consistent with that thinking, where s_1k_1C was observed to increase with increasing surface hydrophobicity for β-lg. Data shown in Table 1 would indicate that all proteins but β-casein showed higher affinity for hydrophobic surfaces. Regarding α-lac, β-lg, and BSA, this finding is consistent with adsorption of these globular proteins being entropically driven. Also, a repulsive force would be present at the hydrophilic surfaces, as they carry a net negative charge in these experiments. A lower degree of electrostatic repulsion would be expected at hydrophobic surfaces. This may con-

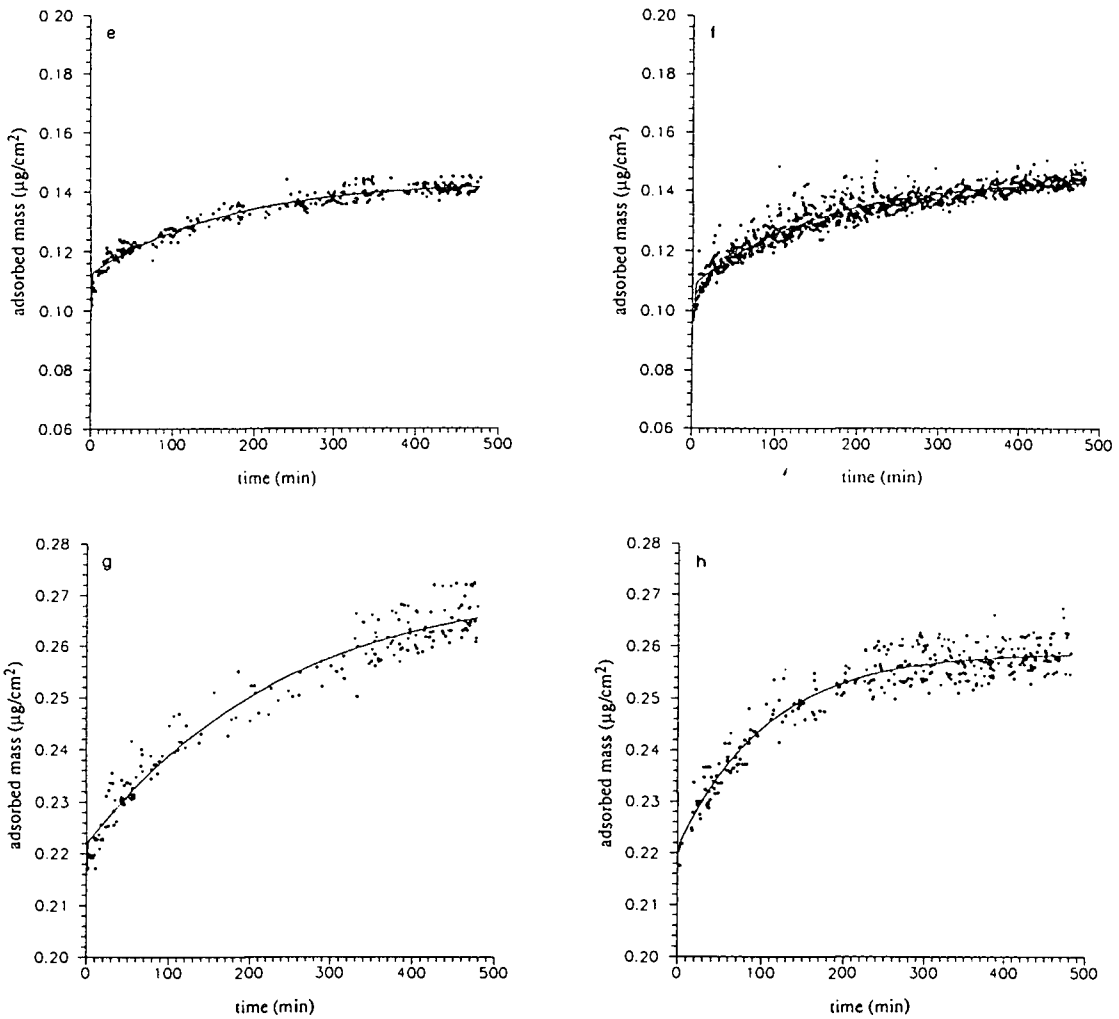

FIG. 2—*Continued*

tribute to the reason that $s_1 k_1 C$ increases with surface hydrophobicity.

Figure 3 shows a representative comparison between the pattern of adsorption kinetics recorded at hydrophobic and hydrophilic surfaces for each protein. Globular proteins α-lac and β-lg clearly show a more rapid approach to the plateau on hydrophobic surfaces. BSA adsorption kinetics were observed to be quite similar on each type of surface, although the initial slope was consistently greater at hydrophobic surfaces. This finding regarding BSA is consistent with differences observed among its adsorption isotherms measured on hydrophilic and hydrophobic silica. Whereas α-lac and β-lg adsorption isotherms each show a relatively large increase with surface hydrophobicity, BSA adsorption isotherms on hydrophilic and hydrophobic silica are similar to each other (17).

Qualitatively, the relative affinities we recorded for adsorption of the globular proteins to hydrophobic surfaces agree with what would be expected based on a kinetic model for globular protein adsorption at air–water interfaces recently reported by Narsimhan and Uraizee (18). In particular, they treated protein adsorption at the air–water interface as resulting from one-dimensional, unsteady-state diffusion in a potential field. The potential field was defined in that case to be due to contributions from a hydrophobic interaction energy, the work required to "clear" area at the interface sufficient for adsorption, and electrostatic interactions. Their model predicted a lower energy barrier to adsorption

TABLE I

Averaged Values of r_1 and r_2 for Each Protein–Surface Contact, and the Corresponding Value of s_1k_1C Calculated According to Eq. [4]

Protein	Silane (%)	r_1 ($\times 10^3$)	r_2	s_1k_1C ($\times 10^2$)
α-Lac	0.010	2.34	0.426	0.100
	0.100	23.9	3.03	7.25
β-Lg	0.010	5.80	2.09	1.21
	0.100	9.20	1.98	1.82
BSA	0.010	7.70	1.19	0.917
	0.100	5.18	2.36	1.22
β-Casein	0.010	9.94	2.04	2.03
	0.100	5.10	3.27	1.67

for proteins of larger "surface" hydrophobicity, smaller size, and prolate orientation. Table I indicates that α-lac exhibited a much higher affinity than β-lg and BSA at hydrophobic surfaces. α-Lac was the smallest globular protein studied; moreover, the α-lac preparation we used consisted of a more hydrophobic molecule than that which would be naturally encountered in milk. α-Lac binds calcium (1 mol α-lac:2 mol Ca^{2+}) that serves to stabilize its structure (19). The calcium-free state yields a more hydrophobic form of the molecule, and the α-lac used in this work contained less than 0.3 mol Ca^{2+} per mol α-lac. Moreover, the adsorption affinity calculated for α-lac relative to affinities calculated for β-lg and BSA at hydrophobic surfaces is in qualitative agreement with the concentration-dependent, air–water surface tension data we recorded earlier (11). In particular, equal molar solutions of α-lac + BSA, α-lac + β-lg, and α-lac + β-lg + BSA yielded data similar to those of single-component α-lac solutions. The air–water interface is quite different from a solid–water interface, but hydrophobic forces dominate adsorption at both the air–water and silanized silica–water interfaces, and this qualitative comparison is probably a fair one.

It is important to note the relatively large difference in s_1k_1C recorded for α-lac at each type of surface. We encountered much difficulty in fitting kinetic data recorded for α-lac on hydrophilic surfaces to Eq. [2]. Data were normally of wider scatter than that shown for the relevant plot in Fig. 2, with plateau values around 0.07 $\mu g/cm^2$. None of the other kinetic experiments, including those involving α-lac adsorption at hydrophobic surfaces, presented such a problem. In any event, both the tendency of this particular α-lac preparation to exhibit extraordinarily high surface activity at an apolar interface, along with the well-documented resiliency of the molecule in a hydrophilic environment (20), i.e., regarding its ability to renature after thermal denaturation, are consistent with the data of Table I.

Although the relative affinities recorded for the globular proteins are consistent with expectations based on protein size, Table I shows that values of s_1k_1C are somewhat similar for β-lg and BSA, even though the BSA molecule is nearly twice as large. It is becoming increasingly evident that proteins of lower stability are more surface active at apolar interfaces than are proteins of higher stability, taking other relevant molecular properties as more or less similar (1, 2, 5, 21). α-Lac is decidedly less stable than either β-lg or BSA (17) and shows a much higher affinity for the hydrophobic surface (Table I). But BSA is a less thermally stable, more flexible protein than β-lg, and those properties may compensate somewhat for its large size.

β-Casein. β-Casein adsorption kinetics were dissimilar to data recorded for the globular proteins. In particular, the adsorbed mass of β-casein on each type of surface was substantially greater than corresponding values for the other proteins, and a higher affinity was consistently recorded for β-casein adsorption to hydrophilic surfaces relative to hydrophobic surfaces.

The β-casein molecule consists of 209 amino acid residues and has a molecular weight of 23,980 Da (22). The molecule is a single chain with five phosphoserine residues. The N-terminal portion (residues 1–43) contains all five phosphoserine residues and carries essentially all of the protein's net charge. The remainder of the molecule is very hydrophobic, particularly the region of residues 136–209. β-Casein is thus a linear amphiphile, and its structure is largely unordered.

β-Casein undergoes endothermic aggregation at temperatures above 4°C. Consequently, adsorption did not occur from a solution of β-casein monomers, and the higher values of adsorbed mass are not surprising. Data from Arnebrant and Nylander (23) show that after 60 min of surface contact with single-component protein solutions (phosphate-buffered saline, pH 7.0), the adsorbed mass of κ-casein on hydrophilic chromium surfaces was about three times greater than that of β-lg to the same surfaces, while κ-casein adsorption to hydrophobic chromium surfaces was about twice as great. Moreover, faster kinetics were observed at hydrophilic interfaces. κ-Casein is also a flexible amphiphile that when isolated from bovine milk consists of a mixture of polymers. Regarding the affinity data of Table I, destabilization of hydrophobic bonds in the β-casein aggregates may have contributed to the slower kinetics observed at hydrophobic interfaces.

Elutability of Adsorbed Protein

The relative adsorption affinities just discussed are lumped parameters. Even if our physical interpretation of the product of r_1 and r_2 is valid, a high affinity value may simply imply fast kinetics while offering no insight into that protein's competitive adsorption behavior; i.e., an infinite number of possible values for k_1 and s_1 would be consistent with any one "affinity" value. An independent, though still indirect, indication of relative binding strength among the proteins

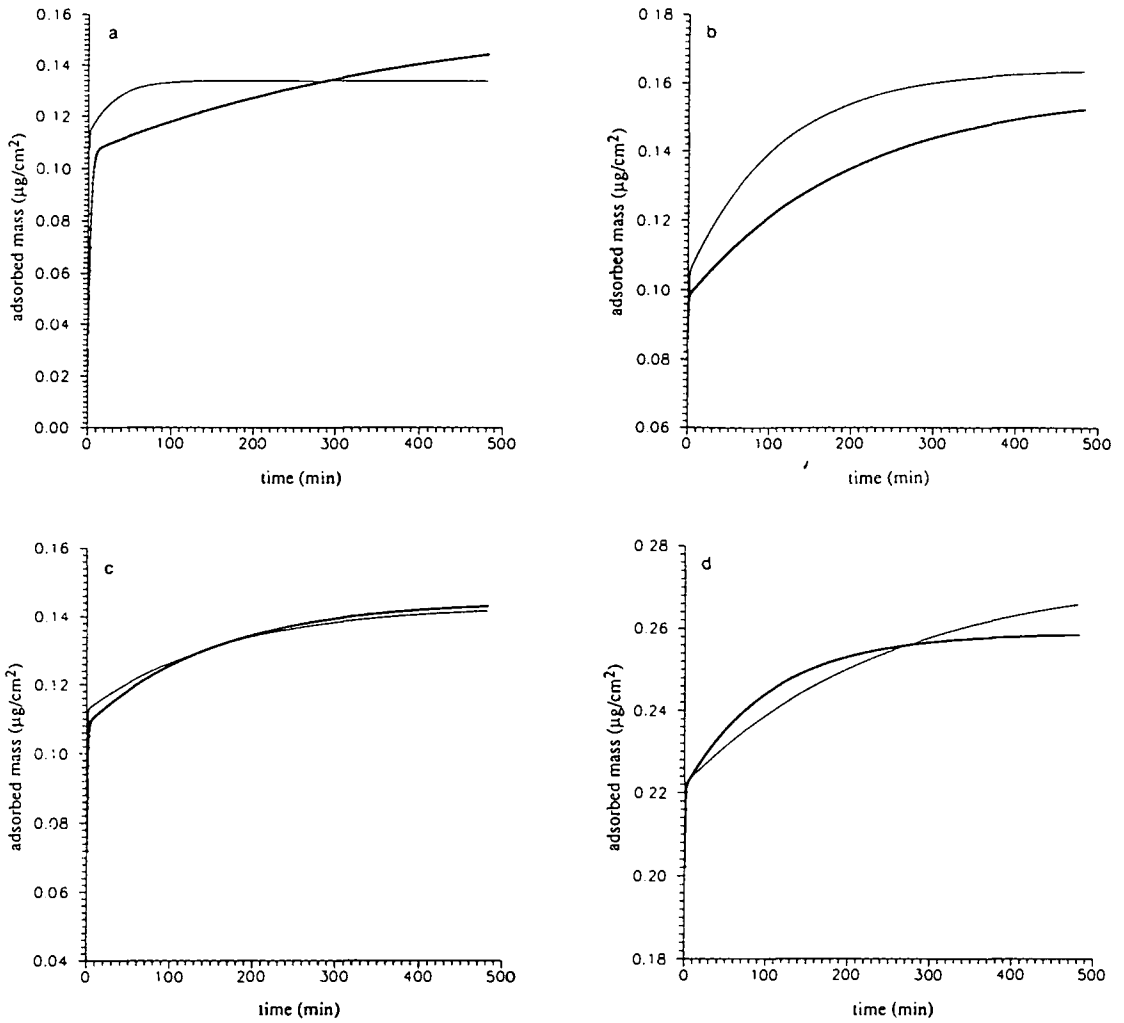

FIG. 3. Comparison of adsorption kinetics (Eq. [2]) recorded for each protein at hydrophobic (light lines) and hydrophilic (heavy lines) surfaces: (a) α-lac. (b) β-lg. (c) BSA. and (d) β-casein.

was gained by examination of the SDS-mediated elutability of each protein.

Representative data illustrating the change in adsorbed mass recorded during 90 min of contact with protein, a 45-min rinse, and 30 min contact with SDS, followed by a final 45-min rinse are shown in Fig. 4 for each protein in contact with a hydrophobic surface. Although elutability of proteins from solid surfaces is sensitive to protein and surface type, and to protein concentration and contact time (24), data on the SDS elutability of β-lg and lysozyme at hydrophobic silica are consistent with a displacement mechanism (25). In any case, incomplete removal of protein can be attributed to the presence of multiple states of adsorbed protein; i.e., some of the protein is apparently bound through a number of noncovalent contacts sufficient to render it nondisplaceable under the selected experimental conditions.

With reference to their model for bulk–surface exchange reactions at an interface involving two different types of protein, Lundström and Elwing (26) considered an experimental situation in which adsorption to a solid surface is allowed to occur from a single-component protein solution, followed by incubation in buffer, after which time a second, dissimilar protein is added. They showed that under certain experimental conditions, their model would lead to an experimentally verifiable expression, which relates the fraction of originally adsorbed protein that is nondisplaceable to rate

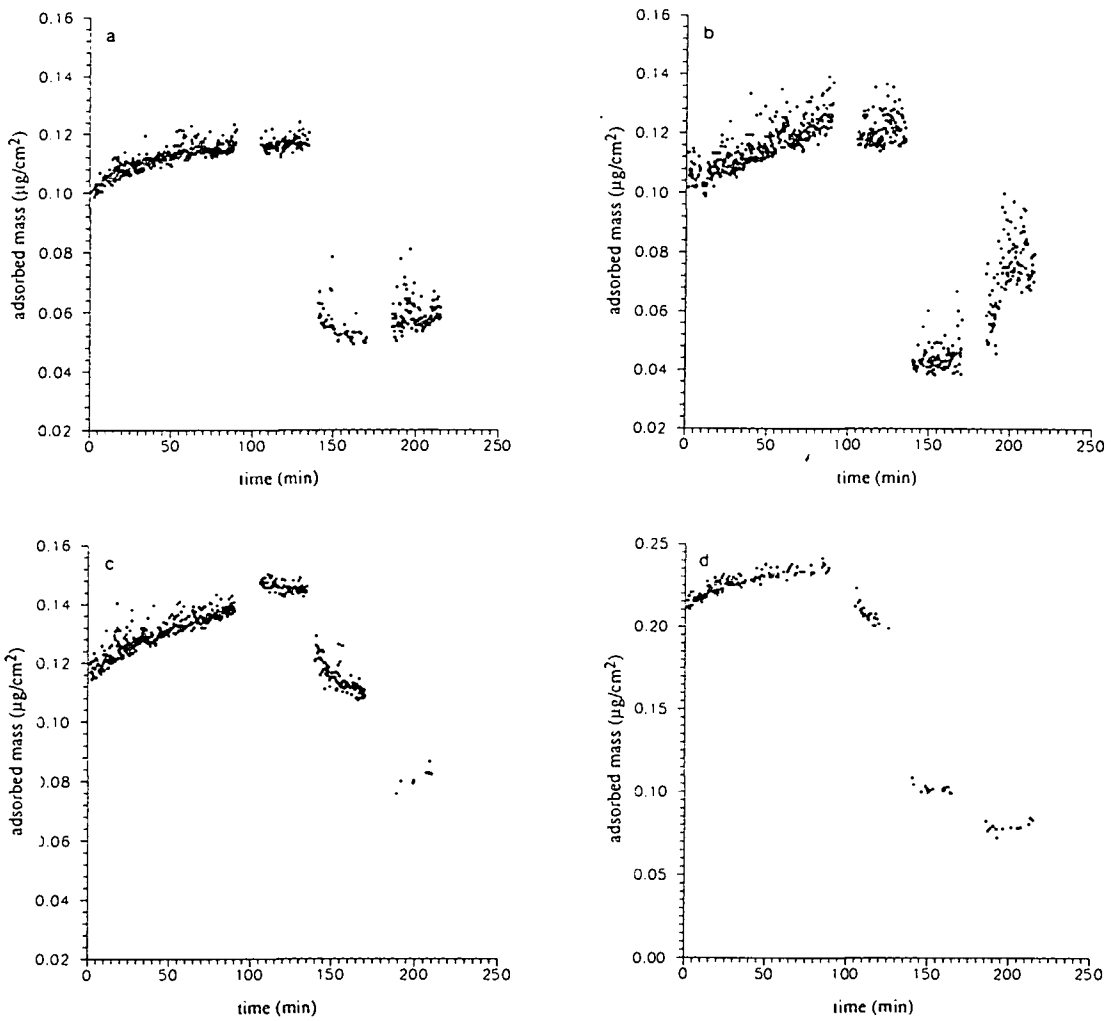

FIG. 4. Representative plots illustrating the change in adsorbed mass recorded on hydrophobic silica during 90 min contact with protein, a 45-min rinse, 30 min contact with SDS, and a final 45-min rinse: (a) α-lac, (b) β-lg, (c) BSA, and (d) β-casein.

constants governing conversion of the originally adsorbed protein to an irreversibly adsorbed form, and an exchange of adsorbed protein by the dissimilar protein introduced to the solution. We can make use of that development by adapting it to the present situation (surfactant-mediated displacement) using the present model. In our tests, protein is contacted with a surface for 90 min, then incubated in buffer for 45 min. We refer to θ_1 as the fractional surface coverage of displaceable protein and θ_2 as the fractional surface coverage that is nondisplaceable, and assume that s_1 is small compared to k_1 and that after 90 min of contact with protein, the surface is mainly covered by displaceable protein (6). During incubation in buffer, and in the absence of spontaneous desorption, $-d\theta_1/dt = s_1\theta_1$, leading to

$$\theta_1 = \theta_{1,t_i}\exp(-s_1 t), \qquad [5]$$

and consequently

$$\theta_2 = \theta_{1,t_i}[1 - \exp(-s_1 t)], \qquad [6]$$

where θ_{1,t_i} is the value of θ_1 at $t = t_i$ (the initiation of adsorbed protein contact with clean buffer). At $t = t_s$, SDS is intro-

duced and

$$-d\theta_1/dt = s_1\theta_1 + k_s\theta_1, \qquad [7]$$

where k_s is a rate constant describing exchange of displaceable protein by surfactant. Lundström and Elwing (26) defined the exchange rates as dependent on displacer (protein) concentration as well. In the present case, the concentration of SDS used is probably much higher than that where concentration effects would be measurable, so k_s is taken as a concentration-independent displacement rate constant. Moreover, if SDS-mediated desorption is assumed to proceed purely by a displacement mechanism, i.e., effected by SDS adsorption to the surface, while SDS adsorption to adsorbed protein is of secondary importance, k_s can be taken as independent of the type of protein being displaced. Equation [7] leads, for $t > t_s$, to

$$\theta_1 = \theta_{1,I_1}\exp(-s_1t_s)\cdot\exp[-(s_1 + k_s)(t - t_s)]. \qquad [8]$$

and since only that fraction of θ_1 molecules depleted by conversion according to rate constant s_1 contributes to formation of irreversibly adsorbed protein, the consequence of Eqs. [7] and [8] is that

$$\theta_2 = \theta_{1,I_1}\exp(-s_1t_s)\cdot\{1 - \exp[-(s_1 + k_s)(t - t_s)]\}$$
$$\times s_1/(s_1 + k_s) + \theta_{1,I_1}[1 - \exp(-s_1t_s)]. \qquad [9]$$

After a sufficiently long time, the fraction of nondisplaceable protein molecules would be, according to Eq. [9],

$$\theta_2/\theta_{1,I_1} = 1 - [k_s/(s_1 + k_s)]\cdot\exp(-s_1t_s). \qquad [10]$$

It should be instructive to interpret the data of Fig. 4 with reference to Eq. [10]. The left side of Eq. [10] is calculable (as $\Gamma_2/\Gamma_{1,I_1}$), assuming all protein bound to the surface was in state 1 upon initial contact with buffer. Since we assumed that elution takes place according to a displacement mechanism in these tests, we calculate Γ_2 as the adsorbed mass after 30 min contact with SDS, minus an amount corresponding to the mass of SDS that would be adsorbed from a single component, 3% wt/vol solution to these hydrophobic silica surfaces. We ellipsometrically determined that amount to be 0.044 μg/cm^2, using M/A = 3.68 g/ml and v = 0.85 ml/g for SDS (27). But since ellipsometrically determined values of adsorbed mass in Fig. 4 are based on protein-specific values of M/A and v, Γ_2 was estimated based on effective values of adsorbed mass of SDS calculated to be 0.038 μg/cm^2 for α-lac and 0.039 μg/cm^2 for the remaining proteins. For a given protein, the left side of Eq. [10] provides a measure of adsorbed protein binding strength (resistance to surfactant-mediated displacement); it increases with increasing value of s_1 associated with the adsorption. In that case, Eq. [10] dictates that for two proteins, the quantity (binding strength of protein A)/(binding strength of protein B) increases with the product of $\exp(s_{1,A} - s_{1,B})$ and $(s_{1,A} + k_s)/(s_{1,B} + k_s)$. If it is fair to approximate k_s as protein independent, differences in binding strength among proteins can be considered a function of s_1 alone in these tests.

The quantity $\theta_2/\theta_{1,I_1}$ is listed for each protein in Table 2, along with the consequent ranking of s_1 among the proteins, 1 corresponding to the highest value of s_1 and 4 to the lowest. Values of s_1k_1C from Table 1 can then be used to approximately rank the relative values of k_1 among the proteins and that ranking is listed in Table 2 as well.

Concerning the globular proteins, the rankings shown for s_1 and k_1 are consistent with what we would expect. BSA is a relatively large protein with three similarly sized domains arranged in series. One (end) domain is neutral at pH 7, while the other two each carry a high negative charge. It is reasonable to expect that BSA would require some finite time, once in the interface, to orient itself "end on" with its neutral domain adjacent to the surface. As adsorption proceeds, increased electrostatic repulsion among nearest neighbors would slow establishment of an incoming protein's first noncovalent contact with the surface. Once adsorbed, the flexible, amphiphilic character of the neutral domain α-helices would allow for increased contact with the surface and would lower interfacial energy. The α-lac molecule is small, flexible, and, in our tests, extraordinarily hydrophobic. Each of those properties would facilitate α-lac adsorption, even in a crowded interface. Although the native conformation of α-lac is not very stable, its resiliency with regard to unfolding and refolding is consistent with its relatively low value of s_1. β-Lg is the most stable and least flexible of the three globular proteins. It is not surprising that for the short contact times of our experiments, β-lg adsorption was apparently governed largely by k_1. Qualitatively, these findings are in agreement with recent work by Wahlgren *et al.* (28) where the elutability of six different globular proteins from hydrophilic and hydrophobic silica surfaces was investigated, using the cationic surfactant dodecyltrimethyl-

TABLE 2

Averaged Values of $\theta_2/\theta_{1,I_1}$ Calculated for Each Protein Contacted with Hydrophobic Silica (0.100% DDS), and the Consequent Ranking of s_1 According to Eq. [10], and of k_1, with Reference to Data in Table 1

Protein	$\theta_2/\theta_{1,I_1}$ ($\times 10^2$)	Ranking	
		s_1	k_1
α-Lac	16.9	3	1
β-Lg	7.90	4	2
BSA	50.7	1	4
β-Cascin	25.6	2	3

ammonium bromide. Certainly, the general mechanism of globular protein elutability would change with surfactant charge, but with regard to α-lac, β-lg, and BSA, after 30 min contact with hydrophobic silica followed by rinsing, incubation in buffer, and contact with surfactant followed by rinsing as before, BSA was reported as the least elutable. They indicated this result may have been due, in part, to larger proteins having the capacity of making more points of contact with the surface. In general, they found that both molecular size and shell hydrophobicity influenced elutability at hydrophobic silica. β-Casein is not globular, does not exist as a solution of monomers or small, uniform polymers, and its adsorption cannot be fairly compared directly with globular protein adsorption. But its low value of k_1 relative to s_1 is consistent with the fact that destabilization of β-casein aggregates contributes to a slow initial adsorption step.

SUMMARY

Adsorption kinetic data recorded for α-lac, β-casein, β-lg, and BSA at silanized silica surfaces of low and high hydrophobicity, along with the surfactant-mediated elutability of each from hydrophobic silica, were interpreted with reference to the mechanism of Fig. 1. Elutability of each protein from hydrophobic silica with SDS allowed further resolution of the single-component affinity data of Table 1. In particular, the rate constants defining surface-induced unfolding for these proteins could be ranked in order of their magnitude. The relative rankings of rate constants defining initial arrival and unfolding were found to be consistent with molecular properties known to affect the surface activity of each protein. These kinds of tests are perhaps most useful in that they allow construction of hypotheses and design of other experiments to better predict the course of competitive adsorption (e.g., protein-mediated displacement of adsorbed, dissimilar protein in a two-component mixture). In any case, our results support the notion that use of the simple mechanism of Fig. 1 to interpret experiments in terms of relative rates of arrival and unfolding is physically realistic.

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APPENDIX B

Thermal Transition Properties for Globular Proteins

The thermal transitions properties of the globular proteins used in this study were evalutated with differential scanning calorimetry (DSC).

Protein	First peak		
	T_{onset1} (°C)	T_{max1} (°C)	ΔH_1 (J/g)
α-lactalbumin[a]	32.2	39.5	13.0
β-lactoglobulin[a]	64.0	70.9	16.3
BSA[a]	54.4	61.2	12.1
Hen lysozyme[b]	72.0	76.2	19.1

Protein	Second peak		
	T_{onset1} (°C)	T_{max1} (°C)	ΔH_1 (J/g)
α-lactalbumin[a]	-	-	-
β-lactoglobulin[a]	120	127	8.63
BSA[a]	109	120	3.89
Hen lysozyme[b]	112.2	117.6	6.85

[a] Suttiprasit, P. and McGuire, J. (1992)

[b] Krisdhasima, V. (1994)