AN ABSTRACT OF THE DISSERTATION OF


Abstract approved: [Signature]

In this study, the effect of protein structural stability on adsorption at solid-water interfaces was investigated with in-situ ellipsometry, circular dichroism, and radiiodination methods. Model proteins were used for this purpose, including the wild type bacteriophage T4 lysozyme (T4L), and two synthetic stability mutants of T4L produced by site-directed mutagenesis.

Conformational alterations were recorded upon adsorption to colloidal silica with circular dichroism. Both the rate and extent of secondary structure loss were related to protein thermal stability, with greater loss of secondary structure occurring for less stable variants. A kinetic model for protein adsorption was developed and used to simulate adsorption kinetic data recorded with circular dichroism and ellipsometry. The model allows for two different adsorbed states, characterized by different binding strengths and different occupied areas. The presence of an increasing energy barrier to adsorption was incorporated into the
model by formulating the adsorption rate constants as functions of surface coverage or adsorption time. Numerical analyses were performed using the Levenburg-Marquardt method, and estimated kinetic parameters were consistent with the expected effect of structural stability on adsorption. Protein concentration effects on adsorption were interpreted with reference to an adsorption mechanism allowing for three different adsorbed states. Protein concentration in solution was shown to have a strong effect on the relative amounts of protein present in these three adsorbed states. The effect of a protein's structural stability on its competitive adsorption from binary protein mixtures at hydrophilic glass was studied using T4L variants labeled with $^{125}$I. Less stable variants were more competitive than more stable variants, with the presence of the less stable variant inhibiting the adsorption of the more stable variant. Adsorbed variants of greater stability apparently underwent exchange reactions with less stable variant during the early stage of adsorption.
KINETIC MODELING OF THE ADSORPTION OF STRUCTURAL STABILITY MUTANTS OF BACTERIOPHAGE T4 LYSOZYME AT SOLID-WATER INTERFACES

by

Woo-Kul Lee

A DISSERTATION

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

Minghua Tian and Ahmad Bani-Jaber were involved in data collection and writing manuscripts of chapter 5 and Appendix C, respectively. Dr. Michelle K. Bothwell assisted in the preparation of manuscript of chapter 2, 3, 4, 5, and Appendix B. Dr. Mark A. Daeschel assisted in the preparation of Appendix C. Dr. DeQian Wang was involved in the preparation of Appendix B. Dr. V. Krisdhasima was involved in the development of the FORTRAN program presented in Appendix D.
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This dissertation is dedicated to my Lord and for their love and care for family, to my mother, grandmother, and father, and for their endless love to my mother, brother, and family. For His guidance.
CHAPTER 1. INTRODUCTION

Protein adsorption occurs spontaneously when a protein-containing fluid is allowed to contact a synthetic surface, since protein molecules are surface-active substances. Protein adsorption has attracted much interest due to its relevance to biomedical and biological applications, such as interactions between blood and implanted artificial organs, preparation of synthetic lung surfactants, drug formulation and delivery, and chromatographic purification of bioproducts (Addiego et al., 1992). As a result, protein adsorption has been extensively studied (Andrade, 1985; Horbett and Brash, 1995).

In this work, the effect of protein structural stability on adsorption to solid-water interfaces was investigated using in-situ ellipsometry, circular dichroism, and radiiodination methods. Among several influential factors, molecular stability is considered as a major driving force in protein adsorption.

The dissertation is comprised of five chapters. Chapter 1 is a comprehensive review of the relevant literature, and of the materials and methods used in this study. Chapter 2 describes the conformational changes undergone by protein upon adsorption to colloidal silica as monitored with circular dichroism. This chapter was published in *J. Colloid Interface Sci.* in 1998. A mechanistic approach to
modeling protein adsorption was presented in chapter 3, now in press at *J. Colloid Interface Sci.* In this chapter, adsorption kinetic data recorded in situ with ellipsometry were compared to model simulations. In chapter 4, the effect of protein concentration in solution on adsorption is discussed. Adsorption, rinsing and surfactant-mediated elution were all recorded in situ, with ellipsometry. Chapter 5 describes the competitive adsorption from binary protein mixtures to hydrophilic glass surfaces.

Five appendices are included as well.

Appendix A is a more detailed discussion on the theoretical development of the adsorption model described in chapter 3. In Appendix B, the adsorption behavior of two formulations of recombinant Factor VIII in contact with well-characterized glass vials and administration sets (tubing) are discussed. This work is the result of a project supported by Bayer Corporation, which involved expertise acquired in this research. Appendix C describes the concentration-dependent adsorption kinetics of nisin and β-casein at n-hexadecane-water interfaces, recorded with tensiometry. These data were analyzed with reference to same kind of multi-state mechanisms in the body of the dissertation. Appendix D contains a FORTRAN program developed for the purpose of the calculation of adsorbed mass at a surface using monitored optical properties of reflected laser beam. Appendix E includes MATLAB files to estimate the adsorption kinetic parameters using nonlinear regression method.
1.1. Literature Review

1.1.1. Characteristics of Proteins at Solid-Water Interfaces

Protein molecules are surface-active substances consisting of both polar and nonpolar portions. In many cases polypeptide chains are present in helical or $\beta$-sheet conformations which are stabilized by intramolecular bonds such as disulfide and hydrogen bonds. Tertiary structure is determined by the folding of the polypeptide chains into a more compact structure, maintained by hydrogen bonding, van der Waals forces and disulfide bonds. These features of protein structure determine the differences in surface activity among proteins. Adsorption experiments using plasma proteins clearly show that the surface affinity of an individual protein is very important for characterization of the overall adsorption process of the protein.

The primary molecular properties responsible for the surface activity of proteins are size, charge, structural features, stability, amphiphilicity, and lipophilicity (Magdassi and Kamyshny, 1996). Based on experiments using plasma proteins, Horbett (1980) suggested that the surface affinity of an individual protein is also very important for the characterization of the overall adsorption process of a protein. At the same time, the interaction of the other proteins may have very significant influence on the adsorption of the protein. The interfacial behavior of proteins has also been characterized in terms of electrostatic interactions (Norde et al., 1978; Arai and Norde, 1990), hydrophobicity (Elwing et al., 1987; Malmsten,
1995; Tilton et al., 1991), and molecular properties such as mass and dimensions.
(Wahlgren et al., 1993a). A study on the influence of net charge and charge
location on the adsorption of a protein at both hydrophilic and hydrophobic
surfaces, however, showed that there exists no simple relationship between the net
charge and surface behavior of charge mutants (McGuire et al., 1995c). The
authors suggested that the location of each substitution of selected lysine residues
with other amino acids may be more favorably influential rather than the protein
net charge. Nevertheless, several researchers pointed out the strong influence of
charge and its density and distribution on the surface activity (Magdassi and
Kaymshy, 1996). Experimentally, proteins have frequently been found to exhibit
greater surface activity near their isoelectric point, because of minimization of
electrostatic repulsion between the identically charged adsorbed molecules
(Kinsella, 1982; Horbett and Brash, 1991; Magdassi and Garti, 1991; Morrissey
and Stromberg, 1974, MacRitchie, 1978; Haynes and Norde, 1994). At the same
time, a nonuniform distribution of ionic “patches” on the surface of a protein can
lead to attractive electrostatic interactions between the patches and the surface even
when the “net” charge of the protein is of the same type as that of the surface
(Lesins and Ruckenstein, 1988). Since proteins have many charges and
hydrophobic regions on their surfaces, the electrostatic interactions and the entropic
gains resulting from dehydration have been perceived as important driving forces
(Kondo et al., 1991). van der Waals interactions and hydrogen bonds are also
driving forces. Since the protein structure is flexible, a part of the secondary
structure such as α-helix is affected during the adsorption process (Tian et al., 1998). Thus, the entropic gain resulting from structural changes is an important driving force (Norde et al., 1986). In a study of interactions between cells and substrate, the biological activity of an adsorbed protein layer towards cells cultured on top of that layer depended on the material to which the protein was adsorbed. This information is critical importance for biomedical researchers since the thrombogenic response of blood towards artificial surfaces depends on the protein layer coating the surface (Darst et al. 1986).

### 1.1.2. Concentration-dependence of Adsorption

Although a great deal of knowledge on protein adsorption behavior has been accumulated during the last two decades, one of the questions still remaining is the concentration-dependent adsorption behavior. Adsorbed mass at an interface shows proportional increase with the increase of the bulk concentration. The Langmuirian adsorption theory does not provide a perfect answer since it considers protein adsorption as a reversible process. We need to know how the bulk concentration of protein can affect the overall adsorption process, in terms of adsorbed states and binding strength. At the same time, the correlation between the effect of bulk concentration and other controlling factors such as surface affinity of protein, stability of molecular structure, or surface types should be investigated to provide full understanding on protein adsorption.
Some adsorption studies were carried out using fibrinogen on polyethylene surface (Brynda et al., 1980) and fibronectin on silicone surfaces (Jonsson et al., 1982) at an initially low bulk concentration that was subsequently increased. The final adsorbed mass of a low concentration showed a lower adsorbed mass than that obtained from a high bulk concentration after the direct adsorption onto bare surface. Although some researchers interpret such behavior as complete occupancy of the surface sites by molecules even at low bulk concentration (Brynda et al., 1986), it is more likely that the increase of the energy barrier suppresses further adsorption. Enhanced area occupancy by unfolding protein molecules may also play an important role.

Horbett (1980) observed that hemoglobin adsorbed 0.03 \( \mu \text{g/cm}^2 \) which is far higher than can be expected even at very low concentration levels such as 0.02 mg/ml in plasma out of 61 mg/ml total protein concentration. For hemoglobin, adsorbed mass was higher at hydrophobic surface than hydrophilic surfaces. Horbett also observed that albumin rapidly adsorbed on pEMA/PE surfaces but was displaced by slower adsorbing proteins, such as hemoglobin. The orientation of the bound pentamer of \( \gamma G \) was also found to be concentration dependent. At low surface densities of pentamer, no molecules were dissociated at pH 3.0, but when the concentration of bound pentamer was increased, either one or two monomers were released (Kochwa et al., 1977).

Morrissey (1977) suggested that at low solution concentrations the adsorbed protein has sufficient time and surface area to adopt to its new micro-environment.
by undergoing conformational changes. On the contrary, at high bulk concentration the contact frequency between protein and surface is too high to optimize protein’s interaction with surface. This phenomenological interpretation can be generally applied to the air/water interfaces as well (MacRitchie, 1978).

1.1.3. Energy Barrier to Adsorption

The presence of an energy barrier to adsorption at an interface is due to the film layer formed by adsorbing molecules. Protein molecules adsorbing to a surface already covered by a film of adsorbed protein need more energy than those adsorbing to a bare surface in order to create available surface area for attachment. Passivation of the surface will eventually occur when all binding sites are filled with adsorbed molecules. The change of the adsorption rate coefficient may depend on several factors to which the increase of the energy barrier to adsorption can be attributed. However, under certain conditions, the time variable can be considered more responsible than all other system variables for the change in the system. The dependence of the rate coefficient on time is presumably nonlinear which will be discussed in following section.

The influence of the energy barrier on adsorption has captured the interest of many researchers (Graham and Phillips, 1979; Ward and Tordai, 1946; MacRitchie and Alexander, 1963a and 1963b; Tornberg, 1978; Damodaran and Song, 1988, 1991). Ward and Tordai (1946) brought up the original idea of the existence of an energy barrier to adsorption. They suggested that the deviation
between the experimental and estimated diffusion coefficients originated from this energy barrier to adsorption. Later, the energy barrier was related to the surface pressure change at an interface (MacRitchie and Alexander, 1963b, MacRitchie, 1978). Tian et al. (1998) suggested that the increase in surface coverage leads to an increase in the energy barrier to adsorption, such that adsorption rates may be function of surface coverage. A dependence of adsorption rate constants on surface concentration has been proposed by Guzman et al. (1986) in terms of activation energies for adsorption and desorption. Their model required that adsorption rate constants be at their maximum at the beginning of the adsorption, decreasing as the surface coverage increases.

The interfacial free energy hypothesis proposes that the energy barrier to adsorption increases with time due to the adsorbed protein layers (Andrade et al., 1984). While adsorption is occurring, the energy barrier to adsorption must continuously increase or the interfacial free-energy will decrease to zero due to the film layer formed with adsorbed protein molecules. The adsorption will finally level off when the energy barrier completely suppresses the potential of further adsorption. Other researchers also suggested that the energy barrier to adsorption at an interface gradually increased as adsorption proceeds (Graham and Phillips, 1979; Morrissey, 1977).
1.1.4. Effect of Structural Stability on Protein Adsorption

The binding strength of an adsorbed protein may depend on several factors. The intrinsic nature of an individual protein molecule plays an important role in determining the binding strength of the protein. At the same time, the binding strength of the protein can be affected by any conformational change that allows the protein to establish more binding sites with surface. Therefore, the effect of the molecular stability of a protein cannot be dismissed. Protein folding and denaturation events show that the normal or native state is only marginally stable. The free energy change involved in the transition to a denatured state is only 5 to 14 kcal/mol (Andrade et al., 1984; Pace, 1975; Schulz et al., 1979; Cooper, 1976), which is an energy corresponding to only a few hydrogen bonds per molecule. Adsorption free energies of proteins are normally in the range of 5 to 20 kcal/mol (Brash, 1982; Dillman et al., 1973), thus adsorption-induced "denaturation" is highly probable. Proteins, upon exposure to an altered environment such as contact with a solid surface, will experience conformational changes different from that in bulk solution.

The secondary structure such as α-helix, β-sheet (parallel and antiparallel) and β-turns is stabilized mainly by intrachain hydrogen bonds. Generally the α-helical and antiparallel β-sheet structures are found at the surface of the protein. This has been attributed to the amphiphilic character of these structures originating from a periodicity in the polarity of the polypeptide chain. The parallel β-sheet,
having a more apolar composition of amino acids, is often located in the protein interior. The turns tend to occur at the surface of the protein (Richardson, 1981).

Hunter et al. (1990) reported that lysozyme adsorbs on aqueous-gas interfaces in a side-on configuration at dilute coverage, with a transition to an end-on configuration at more elevated coverage. Although classifying adsorbed protein as side-on or end-on is not exact method of quantifying the configuration of this tightly adsorbed state, the qualitative analysis on the adsorbed states can be deduced from this explanation. In practice, it is impossible to introduce the exact dimension of the denatured protein in state 2. Therefore the assumption that the tightly adsorbed state is in the side-on configuration, and the loosely bound state is in an end-on configuration should be considered as a close approximation. Quantification of the adsorbed states as loosely or tightly bound is necessary in order to be incorporated into the simulation model. However, it has been difficult to measure the portion of tightly bound molecules. The most reasonable direct quantification of this adsorbed mass can be measured after rinsing the adsorbed mass with surfactant-mediated elution using DTAB or SDS.

Many researchers including Dillman & Miller (1973) suggested that protein adsorption takes place in two distinctly different ways. Their speculation was that protein adsorption occurs in two distinct types of states, differentiated in terms of hydrophobic and hydrophilic bonding. Intuitively, the interior hydrophobic residues might expose themselves in order to form hydrophobic interactions with the surface. These processes taking place simultaneously were called Type 1 and Type
2 under physiological environment and were Langmuirian type adsorption, i.e. monolayer adsorption. Type 1 adsorption is considered as being relatively hydrophilic, easily reversible, with a heat of adsorption characteristic of a condensation process. On the other hand, Type 2 adsorption is considered as being tightly bound, hydrophobic, with an endothermic heat of adsorption characteristic of chemical bond formation. It is widely acknowledged that conformational changes of protein molecules take place upon adsorption. This was confirmed in a detailed study on protein structural conformation using circular dichroism (Tian et al., 1998). These studies suggest that the loss of secondary structure of an adsorbed protein is due to noncovalent interactions between protein and surface. Since free energy changes involved in the denaturation of a protein are only 5 to 14 kcal/mole, surface-induced denaturation of a protein is a highly probable process in that the adsorption free energy is around 5 to 20 kcal/mole (Andrade et al., 1984). It is known that the amount of the adsorbed mass of molecules whose conformation has been altered due to the interaction with the surface strongly depends on the contact time with the surface. The effect of thermodynamic structural stability on the conformational change upon adsorption has been studied by several researchers (Rapoza and Horbett, 1990; Wei et al., 1990). McGuire et al. (1995a, b, c) also investigated the effect of structural stability on protein adsorption using wild type bacteriophage T4 lysozyme and its synthetic mutants. Mutants with lower stability were more resistant to surfactant-mediated elution than higher stability mutants.
We can, therefore, reasonably anticipate that the mutants with unstable thermal structure will unfold more readily and bind to the surface more tightly.

Conformational changes in protein molecules that occur during adsorption do not necessarily destroy the biological properties of the adsorbed protein. Puszkin et al. (1975) have found that Lytron-bound actomyosin retains its Ca\(^{++}\) and Mg\(^{++}\) ATPase activity and that components of actomyosin, actin and myosin, may be sequentially bound to Lytron, indicating preservation not only of the enzymatically active site, but also of the attachment site of myosin for actin. Protein adsorbing at solid surfaces undergoes structural change to various extents depending on the structural stability of the individual protein.

Kondo et al. (1991) suggested that the structural adaptability of proteins increased with increasing compressibility, that is, flexibility, of proteins. In particular, the flexible proteins, such as BSA and hemoglobin, undergo major structural changes during adsorption. The magnitude of the structural changes in flexible proteins increased with increasing affinities of proteins to surfaces. The structural changes in BSA (the flexible protein) during adsorption on a silica surface were highly reversible. The adsorption amount of proteins reaches maximum near their isoelectric points, regardless of their structural adaptability.

The elutability of proteins in surfactant solutions can be used to distinguish between several types of adsorbed proteins: those that adsorb strongly to surfaces and do not elute even in high surfactant concentrations, and those that are desorbable at various surfactant concentrations. These types of adsorbed protein
evidently reflect the existence of at least two general adsorbed states. The fraction of the elutable states is quite different, depending on the surface concentrations of protein. With the exception of albumin adsorbed on polyethylene, populations adsorbed from low bulk concentrations show tighter binding to the surface, as indicated by lower elutability levels at high surfactant concentrations and higher surfactant concentrations required to effect elution. Proteins adsorbed from higher bulk concentrations tend to adsorb less tightly. Thus, a lower surfactant concentration is required for elution (Rapoza and Horbett., 1990). Magdassi and Kamysny (1996) stated that the denaturation process was usually irreversible and occurs when the protein is adsorbed at high-energy air/water or oil/water interfaces. Unfolding of adsorbed molecules allows the polypeptide chains to orient with most of the polar groups in the water phase and most of the nonpolar groups towards the air or oil phase.

1.1.5. Irreversibility of Adsorption

It is controversial whether protein adsorption is a reversible or irreversible process. The Langmuirian adsorption model, which assumed that protein adsorption as the reversible process, has been widely used to simulate the adsorption behavior of proteins. However, experimental observation has shown that protein adsorption is more likely an irreversible process. Strict interpretation of the terms “reversibility” and “irreversibility” implies certain contradictions when related to the mechanism of protein adsorption. If adsorption is “irreversible,” we
cannot properly evaluate adsorption isotherms since they demonstrate the dynamic equilibrium that exists between adsorbed proteins and proteins in solution. These contradictions may be avoided if it is assumed that the formation of an irreversibly adsorbed protein layer, the layer tightly bound with the surface under given conditions, represents a dynamic nonequilibrium accompanied by molecular desorption and conformational changes. Formation of a reversibly adsorbed protein layer, the layer loosely bound with the surface under given conditions, is determined by the dynamic equilibrium between the processes of adsorption and desorption. At this stage, molecular desorption is not necessarily followed by denaturation.

Morrissey and Stromberg (1974) reported that protein forms multicontacts with a surface (e.g., 77 contact points for albumin and 703 for fibrinogen adsorbed on silica). Therefore, simultaneous breakage of all the contacts between adsorbed protein and a surface was considered unlikely. The concept of multipoint binding during adsorption might be a significant idea that suggests that adsorption is an irreversible process. Protein inducing rapid surface passivation by way of the irreversible adsorption on hemocompatible biomaterials may reduce the adsorption of other proteins (Brash et al., 1985). This speculation supports our assumption that the passivation effect for a tightly bound protein molecule with a conformationally altered structure is higher than that of a loosely bound protein. Dillman & Miller (1973) considered the reversibly and irreversibly adsorbed molecules as the relative amounts of Type 1 and Type 2, and studied then as a function of the pH,
temperature and nature of the polymer substrate and protein involved. They suggested a solution to the paradox that protein adsorption can either increase or decrease with increasing temperature by proposing that Type 1 adsorption decreases with increasing temperature while Type 2 adsorption increases with increasing temperature.

The rates of desorption are, by nature, much lower than those of adsorption, and in many cases it is impossible to attain the equilibrium state for a desorbing protein (Kamyshny, 1981). In other words, the formation of one or several bonds with the surface increases the probability of adsorption of neighboring sites of the same molecule. On the other hand, the desorption of a protein molecule requiring the simultaneous breakage of a large number of bonds may suggest that equilibrium is not attainable from a kinetic standpoint (James and Augenstein, 1966; Kamyshny, 1982; MacRitchie, 1972). This corresponds to a considerable difference between the activation energies for the adsorption and desorption processes (MacRitchie, 1978; 1993). Haynes and Norde (1994) also considered protein adsorption as a non-equilibrium, irreversible process, whose description should be based on the laws of irreversible thermodynamics.

1.1.6. Competitive Adsorption and Exchange Reaction at Solid-Water Interfaces

Horbett (1980) observed that hemoglobin adsorbed 0.03 μg/cm² on pEMA/PE surfaces which is far higher than can be expected even at very low
concentration levels such as 0.02 mg/ml in plasma out of 61 mg/ml total protein concentration. For hemoglobin, higher adsorbed mass was achieved at hydrophobic surfaces than at hydrophilic surfaces. Horbett also observed that albumin adsorbed more rapidly on pEMA/PE surfaces but was displaced by the more slowly adsorbing proteins, such as hemoglobin. There is some evidence that there might exist no exchange between identical proteins. The adsorption of blood proteins on a solid surface is regarded as one of the first processes in the contact between surface and blood (Baier and Dutton, 1969; Cooper and Peppas, 1982). Hunter et al. (1990) studied exchange reactions by sequential adsorption of the same kind of protein and found that the exchange was negligible under the conditions they used. They initially let radiolabeled protein adsorb onto an air/water interface and then they added either labeled or unlabeled protein with varying concentration ratios. They found there was no evidence that serious exchange reaction occurred. If the solution contains a displacer or other protein whose molecules have an affinity for the adsorbent, any desorbing molecule will be replace by another. Desorption of the molecule is now virtually an exchange process and, as $\Delta_{\text{exchange}} G \ll \Delta_{\text{desorption}} G$.

Several authors (Brash and Samak, 1978; Fraaije, 1987; Brash et al., 1983; Lok et al., 1983; Chan and Brash, 1981; Weathersby et al., 1977) have shown examples of exchange between adsorbed and dissolved protein molecules in systems where desorption upon dilution did not take place.

The difference in the bound strength of an adsorbed protein due to a conformational change is an important factor in the protein adsorption process.
Evaluating the bound strength of an adsorbed protein was achieved by conducting competitive adsorption experiments, and also by measuring the resistance of adsorbed protein against surfactant-mediated elution (McGuire et al., 1995a,b,c). Although homogeneous and heterogeneous exchange reactions are influenced by several factors, eventually the bound strength will characterize the whole processes. In other words, exchange reactions occur when the externally imposed displacement force is strong enough to overcome the bound strength of the adsorbed protein.

1.1.7. Adsorption Issues in Hemocompatibility

The circulatory system must be self-sealing; otherwise continued blood loss from even the smallest injury would be life threatening. Normally, all but the most catastrophic bleeding is rapidly stopped, a process known as hemostasis, through several sequential processes. First, an injury stimulates platelets to adhere to damaged blood vessels and then to each other so as to form a plug that can stop minor bleeding. This association is mediated by von Willebrandt factor, a large (up to $10^4$ kD) multimeric plasma glycoprotein of subunit mass 225 kD. This protein binds to both a specific receptor on the platelet membrane and to the collagen and possibly other components of the subendothelial membrane exposed by vascular injury. Then, as the platelets aggregate, they release several physiologically active substances, including serotonin (5-hydroxytryptamine) and thromboxane A2, that stimulate vasoconstriction, thereby reducing the blood flow to the injury site.
Finally, the aggregating platelets and the damaged tissue initiate blood clotting or coagulation, which is the body’s major defense against blood loss. A blood clot (medically known as a thrombus) forms through the action of a cascade of proteolytic reactions involving the participation of nearly 20 different substances, most of which are liver-synthesized plasma glycoproteins (Voet and Voet, 1995).

Hemocompatibility could be improved by the adsorption of albumin onto the surface. This phenomena can be interpreted in terms of surface affinity with albumin being so tightly bound that other proteins cannot penetrate to form or find the binding sites on the surface. At the same time, the competitiveness of the albumin molecules on hydrophobic surfaces is also very strong so that the replacement or exchange reaction cannot be initiated.

When SRI polyurethane was first exposed to unlabeled protein and then to labeled protein, the prior exposure was always found to decrease the uptake of the labeled protein as compared to that observed for the labeled protein on a bare surface. The greatest blocking effect was obtained with fibrinogen where over 80% of the polymer surface became inaccessible to the labeled fibrinogen. Similarly, IgG blocked the sequential adsorption of labeled IgG by over 50%. Smaller degrees of blocking were observed when nonideal proteins were applied (Kochwa et al., 1977). Endothelium (for reviews, Fishman, 1982; Nossel and Vogel, 1982) is nature’s hemocompatible surface, and the performance of any biomaterial designed to be hemocompatible must be compared with that of endothelium. Endothelium is a monolayer of cells in close apposition, lining all blood vessels and the heart.
(endothelium also lines lymph vessels, but the characteristics of lymph endothelium have been less thoroughly investigated). The total length of vascular endothelium in the human body is several thousand kilometers with a surface area greater than that of a football pitch, the greater part of it lining the microcirculation. In the human lung alone there are more than 10^" endothelial cells to which the blood passing through the lungs in a single circulation is inevitably exposed. This transient exposure to a massive endothelial surface provides optimal conditions for activities such as the metabolism of circulating vasoactive agents (i.e., prostaglandins, amines and adenylates) either via uptake into the endothelial cells or by the actions of ectoenzymes. Intermittent contact with the endothelial surface in microvasculature also gives an opportunity for interactions between endothelial cells and cells in the circulating blood.

1.1.8. Experimental Methods for Evaluating Adsorption

Metal surfaces, in general, have very different properties than low energy surfaces such as polymers. Most metals form an oxide layer spontaneously under normal atmospheric conditions. Being conductors, they have very high dielectric constants (+∞), whereas their oxide layers exhibit intermediate values. The dielectric constants affect the formation of potentials at the metal/water interface, and also influence the van der Waals interaction according to the Lifshitz theory (Israelachvili, 1985). The measurement of contact angle of liquids can be used for the estimation of the surface free energy. The critical surface tension of wettability,
γc, is used for the characterization of low energy solids with low polar contribution to the interfacial energy (Zisman, 1964). Correlations between the critical surface tension, γc, and biocompatibility have been studied by several researchers (Baier, 1970; Lyman et al., 1965). They adopted this method for the determination of γc for a chromium surface treated with chloroform to be hydrophobic, and they obtained a value of about 45 mN/m.

Since Rothen (1945) first introduced the ellipsometer as a tool to measure the film on metal surfaces, ellipsometry has been abundantly used to measure the adsorbed mass of protein films forming on surfaces (McGuire et al., 1995a, b, c). The measurement of optical angles, psi and delta, were used to calculate the change in thickness of protein film layer. The mathematical procedure to calculate the film layer was proposed by several researchers (Cuypers et al., 1983; McCrackin, 1969).

CD spectroscopy can be used to analyze the structural behavior of proteins upon adsorption. The circular dichroism of a protein is the circular dichroism of the amide chromophore, the difference between the extinction coefficients for left and right circularly polarized light. The secondary structure of protein, specifically α-helix structure, can be estimated with considerable accuracy (Johnson, 1990). Walton and Koltisko (1982) used CD for examining the optical activity of proteins eluted from the surface of polydimethyloxane and polyethylene (“bead column test”). Tian et al. (1998) followed the change in circular dichroism spectra of α-helix to estimate the adsorption kinetic rate coefficients of T4 lysozyme variants.
The CD spectra showed that a greater loss of secondary structure occurred among proteins with less stable structures.

In order to monitor the changing quantities of protein on a surface during adsorption from a solution, radiotracer methods have been introduced using radioactive isotopes, such as $^{14}$C or $^{125}$I. Graham and Phillips (1979a,b,c, 1980a,b) investigated the adsorption of $^{14}$C-acetylated lysozyme, $\beta$-casein, and bovine serum albumin at the air/water interface. The monolayer plateau was achieved only by $\beta$-casein. At high bulk concentration, all proteins showed multilayer adsorption. From desorption experiments, they observed the irreversibly adsorbed layer when they replaced the protein containing bulk solution with pure buffer. Hunter et al. (1990) presented the adsorption of reductively methylated chicken egg white lysozyme to the air/water interface. They observed that adsorption occurred in monolayers at low bulk concentrations, but formed multilayers at high bulk concentrations. Intermediate concentrations experienced a change in orientation during adsorption. Their exchange experiments rendered some evidence that lysozyme molecules that have adsorbed in the first layer do not exchange significantly with lysozyme molecules in the bulk solution. Several researchers used radioactive iodine as a tracemarker (van Dulm and Norder, 1983; van Oss et al., 1981; Bornzin and Miller, 1982; Schmitt et al., 1983; Penners et al., 1981; Horbett, 1980, 1981).

Total internal reflection fluorescence (TIRF) can be used to continuously monitor the adsorption of fluorescently labeled protein in a noninvasive and quantitative manner (Buijs and Hlady, 1997). Use of antibody-antigen assays...
involving the adsorption of protein antigen onto a solid surface is seeing widespread use to determine the effect of adsorption on protein conformation. An attractive method for examining the effects of adsorption on protein conformation is the use of antibodies as a conformational probe of adsorbed protein antigen. A study was conducted of the adsorption properties of the protein sperm whale myoglobin (Mb) onto crosslinked polydimethylsiloxane (filler-free silicone rubber) surfaces and the interactions of anti-Mb antibodies with the adsorbed Mb (Darst et al. 1986). Myoglobin was chosen because of the availability of monoclonal antibodies that have been highly characterized with respect to their binding sites on the Mb molecule. They incubated the protein solution reservoirs for several hours with a Mb solution of equal concentration as would be used in the experiment to prevent any loss of protein from the solution by adsorption to the glass wall of the reservoir. It should be noted that the phosphate buffer used in the TIRF experiments contains 0.02% NaN₃ to prevent microbial growth.

1.1.9. Adsorption Modeling and Simulation

Several numerical attempts have been made to characterize the dynamic process of protein adsorption. Modeling approaches can be classified into two major categories: One is when diffusion is the rate controlling process (Wojciechowski and Brash, 1990), and the other is based on a kinetically limited adsorption process (Lundstrom, 1985). It is rather controversial to define the protein adsorption mechanism as a diffusion-controlled or kinetically controlled
process. However, the pathway corresponding to the ultimate goal of describing protein adsorption should be considered as the most appropriate way to go. The Langmuir model or diffusion-based model cannot enumerate this kind of feature. Of course, some statistical or probability concept should be incorporated in order to enhance the applicability of these models. However, a model which can appropriately describe overall characteristics of the adsorption process in which not only the direct adsorption of a single component is considered, but also adsorption in different conformational states, exchange reactions between different types of protein, and competitive adsorption from a mixture (Kipling, 1965). Numerical analysis based on a diffusion-controlled adsorption model did not give satisfactory simulation results on the rear part of the kinetic adsorption data (Young et al., 1988). On the other hand, the kinetically limited adsorption model does not predict the quick initial part of an adsorption.

For the development of an adsorption model, several different approaches have been attempted and a few different adsorption mechanisms have been considered. In most cases, a monolayer adsorption mechanism has been assumed. On the other hand, since some experimental results show that the adsorbed mass exceeds the theoretical maximum adsorbed mass for monolayer coverage, especially adsorption from blood plasma, multilayer adsorption is often considered to be present. Nevertheless, monolayer adsorption has significant meaning in that the adsorption is mainly an interaction between a protein and a surface. The rate coefficients representing the protein-protein interaction should be separately
considered during model build-up when the protein-protein interaction is considered to be significant (Walhgren, 1997). However, an experimental technique that measures only protein-protein interactions has not been clearly introduced yet.

The Langmuirian adsorption model (1918) has been a favorite choice for the simulation of protein adsorption since the model is simple and fits experimental data well. Langmuir considered the surface of a solid to be made up of elementary spaces, each of which could adsorb one gas molecule. He assumed that all the elementary spaces were identical in their affinity for a gas molecule and that the presence of a gas molecule on one space did not affect the properties of neighboring spaces.

The derivation of the Langmuir adsorption isotherm involves five implicit assumptions: (1) the adsorbed gas behaves ideally in the vapor phase, (2) the adsorbed gas is confined to a monomolecular layer, (3) the surface is homogeneous, that is, the affinity of each binding site for gas molecules is the same, and (4) there is no lateral interaction between adsorbate molecules, and (5) the adsorbed gas molecules are localized, that is, they do not move around on the surface. Although the first assumption is good at low pressure, the second one nearly always breaks down as the pressure of the adsorbed gas is increased. As the gas pressure approaches the saturation vapor pressure, the vapor condenses without limit on all surfaces if the contact angle is zero. The third assumption is poor on different gases, since different kinds of binding sites are produced as the binding energy increases with increasing surface coverage. The incorrectness of the fourth
assumption was first shown experimentally when it was found that in certain cases the heat of adsorption may increase with the surface concentration of adsorbed heterogeneity, caused by lateral attractions of adsorbed molecules. The fifth assumption is incorrect because there is evidence that surface films may be mobile. Consequently, adsorption of protein films onto solid surfaces is more complicated than the Langmuir theory may indicate.

A theoretical model based on diffusion-controlled adsorption was tested with experimental data (Young et al., 1988). The very initial adsorption data were fitted well using ideal sink diffusion model. The prediction after the initial period of adsorption, however, showed higher estimates than the experimental data. Aizenbud et al. (1985) also presented an irreversible adsorption model using experimental data produced by Penners et al. (1981). The controlling factor for their model was also substrate diffusion. The model equation was obtained from the simplified equation of continuity considering one dimensional movement of protein molecules. Although their experimental data were reasonably well fitted at very low concentration, \( C_b = 2 \, \mu g/ml \), the deceleration period of adsorption data at the higher concentration, \( C_b = 20 \, \mu g/ml \), were not well fitted. That is because at very low concentrations, the adsorption process presumably can be considered more like a diffusion controlled process, however, at higher concentrations, adsorption may be kinetically controlled. Déjardin and Cottin (1991, 1995) performed adsorption experiments under the flowing solution system using fibrinogen. They introduced a computational model incorporating an apparent kinetic constant. This apparent
kinetic constant was the combination of the adsorption constant at the interface and the kinetic constant directly related to the transport controlled process based on the Lévéque model. The estimation of the kinetic constant will be infinite at the entrance of the slit. They showed that the linear variation of the inverse of the apparent rate constant with the 1/3 power of the distance from the slit entrance gives a reasonable approximation although the approximation of the apparent kinetic constant has certain limitations when describing the early steps of adsorption.

Discussion on the simple kinetic adsorption model is available (Lundström and Elwing, 1990). Beissinger and Leonard (1982) performed binary-component adsorption experiments using γ-globulin and albumin on an artificial quartz surface. The amount of adsorbed protein was determined at 0.5 and 5 minutes respectively it was observed that the adsorbed amounts were nonlinearly proportional to the bulk solution concentration. The analysis was performed with a model based on kinetically controlled adsorption in which the second order irreversible transition from state 1 to state 2 were assumed. Desorption from both state 1 and state 2 was also allowed. The surface concentration, $C_s$, was defined by $\Lambda_5(\theta_1 + \theta_2)$, where $\Lambda_5$ is the surface density of molecules adsorbed in state 1, and $\theta_1$ and $\theta_2$ are the surface area in state 1 and state 2 respectively. The definition of the surface density carries some discrepancy since the surface density for molecules in state 1 and state 2 would be different. The adsorbed mass of a protein in state 2 is less than the protein adsorbing in state 1 (McGuire et al., 1995a, b, c). Based on our study, if we
neglect this effect in the analytical solution of model equations, a simultaneous nonlinear heterogeneous ordinary differential equations was obtained that still simulated the adsorption data fairly well. However, the differentiation between these two distinct adsorbed states, at least two different adsorbed states for the modeling purpose, is indispensable in order to characterize the interfacial behavior of a protein in reality. Three-rate adsorption model (Krisdhasima et al., 1992) and irreversible parallel adsorption model (McGuire et al., 1995a) have been proposed based on the kinetically-controlled adsorption model. Both models allow the multiple adsorbed states, i.e. state 1 and 2, based on binding strength and the degree of denaturation. By doing this, the unfolding process occurring due to the interaction between protein and surface is incorporated into the model. The main goal of this study is to provide a more applicable simulation model consisting of system parameters representing the adsorption characteristics for a protein of interest.

In adsorption modeling, a prediction of the amount of adsorbed protein on a surface is important only in that the simulated results can be related and compared with the adsorbed mass obtained experimentally. On the other hand, the adsorbed states by which the adsorbed molecules can be characterized for the binding strength and the resistance against exchange or displacement by different proteins are another significant factor to be taken into account. The adsorption model can be applied to the competitive adsorption in the presence of dissimilar proteins. At the same time, only the overall adsorbed mass data obtained from the single-
component adsorption experiment can not account for the system where
competitions among the coexisting different proteins exist. In this regard, the
diffusion model cannot be applicable in this kind of situation since diffusion model
just considers the overall adsorption only. Although there is modified adsorption
model based on diffusion and mass transfer mechanisms that incorporates the
kinetic aspect of protein adsorption, some additional statistical or probability
concept should be applied to distinguishing different proteins adsorbing into
different states.

1.1.10. References


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1.2. Materials and Methods

1.2.1. Surface Preparation

For the preparation of hydrophilic surfaces used for collecting ellipsometric adsorption data, the treatment procedure has been previously described by McGuire et al. (1995a,b,c). Silicon (Si) wafers (hyperpure, type N. Phosphorous doped, plane 1-0-0) were purchased from Wacker Siltronic Corporation (Portland, OR). Oxidation of surfaces was carried out in a furnace at 1000 °C (P\textsubscript{O\textsubscript{2}} = 1 atm) for 18 minutes. Surfaces were cut into approximately 1.2 cm x 3 cm plates, using a tungsten pen. Each plate was placed into a test tube, and 10 mL of 1:1:5 of NH\textsubscript{4}OH:H\textsubscript{2}O\textsubscript{2}:H\textsubscript{2}O was added. Then, they were heated to 80 °C in a water bath for 15 minutes. After rinsing with 20 ml DDW (Distilled and Deionized Water), 10 ml of 1:1:5 of HCl:H\textsubscript{2}O\textsubscript{2}:H\textsubscript{2}O was added. After heating to 80 °C for 15 minutes, the silica surfaces were rinsed with 30 ml DDW, and stored in 20 ml of 50% ethanol in order to maintain their hydrophilic properties.

For the preparation of surfaces for radioiodination experiments, the same procedure was used. Circular test glasses were purchased from Fisher (12 mm dia.) and cut in half due to the size limitation of the counting tubes used in the Gamma 5500 counter (Beckman, Fullerton, CA). After treatment, each surface was stored...
in a separate vial filled with 50% ethanol solution. Immediately before use, the glass surfaces were thoroughly rinsed with excess amount of distilled deionized water.

1.2.2. Buffers

All the kinetic data from in-situ ellipsometry were obtained in 0.01 M sodium phosphate buffer. In radiolabeling experiments, we used 0.05 M sodium phosphate buffer (pH 7.5), 0.01 M sodium phosphate buffer saline (pH 7.0), 0.05 EDTA (ethylenediamine tetraacetic acid, Sigma Chemical Co.)-sodium phosphate buffer saline (pH 7.0) and 0.05 M Tris-HCl buffer were used. The adsorption and elution experiments were performed in 0.01 M sodium phosphate buffer, pH 7.0.

1.2.3. Proteins

1.2.3.1. T4 lysozyme and Its Stability Mutants

In this study, we used wild type of T4 lysozyme and its mutants in which isoleucine at position 3 was substituted with cysteine (I3C) or tryptophan (I3W) using site-directed mutagenesis. The thermal stability of these stability mutants were summarized in Table 1.1. Lysozyme is an enzyme destroys bacterial cell walls by hydrolyzing the β(1→4) glycosidic linkages from N-acetylmuramic acid (NAM) to N-acetylglucosamine (NAG) in the alternating NAM–NAG polysaccharide component of cell wall peptidoglycans. T4 lysozyme is a small protein whose
polypeptide chain consists of 164 amino acid residues. The molecular weight is 18,700 (Matthews et al., 1973) with the dimensions of approximately 54Å x 28Å x 24Å (Weaver and Matthews, 1987). The thermal and mechanistical properties of this protein are well-characterized (Tsugita et al., 1968). T4 lysozyme carries an excess of nine positive charges at physiological pH, which makes the protein basic (Sun et al., 1991).

The N-terminus domain of T4 lysozyme consists of residues 1 to 60 forming two α-helices, and β-sheets which exist only on N-terminus. The C-terminus domain consists of residues 80 to 164 which form seven (7) α-helices without β-sheet. Residues 159 to 164 form a distorted α-helix (Weaver and Matthews, 1987a,b). A long strand of α-helix, residues 60 to 80, bridges the two domains and traverses the length of the molecule (Alber and Matthews, 1987). The α-carbon backbone of T4 lysozyme is shown on Fig. 1.1. with α-helices and β-sheets. Unlike hen egg white lysozyme which is internally crosslinked by four disulfide bonds, wild type T4 lysozyme does not have any disulfide bonds.

The isoleucine at position 3 located in the middle of the wild type lysozyme is mostly inaccessible to solvent and plays a major role of the hydrophobic core of the C-terminus (Wozniak et al., 1994). It also helps to link the C- and N-terminal domains and its hydrophobicity may contribute to the stability of the protein. The side chain of Ile 3 contacts the side chains of methionine at position 6, leucine at position 7, cysteine at position 97 and isoleucine at position 100. These residues are buried within the protein interior (Matsumura et al., 1988). T4 lysozyme is known
to reversibly denature by when heated either at acidic or neutral pH (Hawkes et al., 1984; Elwell and Schellman, 1975; Becktel and Baase, 1987). The stability mutants were produced by recombinant DNA technique, specifically site-directed mutagenesis. Several stability mutants were produced by replacing the isoleucine residue at position 3 in wild type of bacteriophage T4 lysozyme with different amino acid residues. The secondary structures of these mutants were known to be similar to wild type (Grutter et al., 1979 and 1983; Tian et al., 1998). The hydrophobicity of the residue replacing the Ile 3 may affect the overall stability of the protein molecule which can be correlated with the role of the residue as the hydrophobic core at position 3. The substitution of Ile 3 for tryptophan showed a decreased stability, because the substitution caused unfavorable steric interactions and resulted in unsatisfied hydrogen bonds.

1.2.3.2. Hen Egg White Lysozyme

Lysozyme is a relatively small secretory enzyme that catalyzes the hydrolysis of specific kinds of polysaccharides comprising the cell walls of bacteria. The secondary structure of hen egg white lysozyme was shown in FIG 1.2. In birds, lysozyme is also an exceptionally abundant protein in egg whites, although its biological function is unclear. In vertebrates, this "glycosidase" is found mainly in biological secretions (such as tears) where it probably serves as an anti-bacterial agent by digesting and weakening the rigid bacterial cell wall, thereby
rendering the bacteria susceptible to osmotic lysis. The effect of lysozyme is similar to the effect of penicillin which also weakens the cell walls of

<table>
<thead>
<tr>
<th>Variants</th>
<th>$\Delta T_m$ (°C)</th>
<th>$\Delta G$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 2.0</td>
<td>pH 6.5</td>
</tr>
<tr>
<td>Cys mutant</td>
<td>4.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Trp mutant</td>
<td>-16.4</td>
<td>-8.0</td>
</tr>
</tbody>
</table>

**Table 1.1** Thermal stability of the T4 lysozyme variants with different amino acid residues at position 3. $\Delta T_m$ is the change in melting temperature of the variant relative to wild type ($T_m = 41.9$ °C at pH 2.0; $T_m = 64.7$ °C at pH 6.5. $\Delta G$ indicates the difference between the free energy of unfolding of the variant and that of wild type at the melting temperature of wild type. (Matsumura et al., 1988).

bacteria by irreversibly inhibiting a transpeptidase enzyme required for crosslinking peptidoglycan macromolecules formed in the biosynthesis of the cell wall (for more information see What the Heck is Penicillin?). Under normal conditions, bacteria grow very rapidly, in some cases doubling more than once an hour. However, when cell wall crosslinking is disrupted, bacteria tend to lyse in hypotonic media as a result of the mechanical weakening of their cell.
FIG 1.1 The secondary structure of the wild type lysozyme from bacteriophage T4. The coil and thread indicate the $\alpha$-helical and $\beta$-sheet structure respectively (from Protein Data Bank, D.R. Rose, Protein identification number: 1LYD, 1989).
FIG 1.2. The secondary structure of hen egg white lysozyme (from Protein Data Bank, K.P. Wilson, B.A. Malcolm, and B.W. Matthews, Protein Identification number: 1HEL, 1992).
1.2.3.3. Recombinant Factor VIII

Antithemophilic factor (Recombinant) is a glycoprotein synthesized by a genetically engineered Chinese Hamster Ovary (CHO) cell line. In culture the CHO cell line secretes recombinant antihemophilic factor (rAHF) into the cell culture medium. The secondary structure of coagulation factor VIII is shown in FIG 1.3. The rAHF is purified from the culture medium utilizing a series of chromatography columns. A key step in the purification process is an immunoaffinity chromatography methodology in which a purification matrix prepared by immobilization of a monoclonal antibody directed to factor VIII is utilized to selectively isolate the rAHF in the medium. The rAHF produced has the same biological effects as Antihemophilic Factor (Human) and structurally has a similar combination of heterogeneous heavy and light chains.

1.2.4. Fermentation and Purification of T4 Lysozyme

Synthetic mutants of T4 lysozyme were produced from transformed cultures of Escherichia coli strain RR1. Individual bacteria strains, carrying the mutant lysozyme expression vectors, were provided by Professor Brian Matthews and his co-workers at the Institute of Molecular Biology, University of Oregon. Expression and purification of T4 lysozyme and its mutants were performed following established procedures (Alber and Matthew, 1987a,b; Muchmore et al., 1989).
1.2.4.1. Fermentation

Cells bearing the desired mutant lysozyme expression vector stored in a –80°C freezer were transferred to 100 ml LB-H broth (2g tryptone, 1g yeast extract, 1g NaCl, 0.2 ml 1N NaOH and 200 ml distilled deionized water (DDW)) with 20 mg ampicillin. The cells were grown at 37°C for 6 to 8 hours, and then transferred to a fermentation reactor. The reactor contained in 4.8 liters of sterilized LB broth (57.6g tryptone, 24.0g yeast extract, 4.8g glucose, 48g NaCl and 4.8 liters DDW) with 400 mg ampicillin and 1.5 ml tributyl phosphate (Sigma Chemical Co., St. Louis, MO). The contents of the reactor (Applikon, Foster City, CA) were agitated at 600 rpm with a speed controller (ADI 1012, Applikon Dependable Instruments, Schiedam, Holland) and a supply of air at 0.8 kg/s. The fermentation was continued until the optical density of the culture at 595 nm (DU 62 Spectrophotometer, Beckman Instruments, Inc.) was between 0.8 and 1.0 (approximately 2 hours).
Once this condition was reached, the temperature of the water bath was lowered to 30 °C. And the agitation and air flow were reduced to 200 rpm and 0.52 kg/s respectively. The induction of the expression of the gene coding for lysozyme was started with 750 mg isopropyl-β-thiogalactoside (IPTG, Sigma Chemical Co., St. Louis, MO) dissolved in 100 ml sterilized distilled deionized water. The fermentation was continued for 110 more minutes followed by the harvesting of the broth into 250 ml polypropylene bottles. FIG 1.4 shows the fermentation of T4 lysozyme.

1.2.4.2. Centrifugation

The first centrifugation was done at 4°C, 13,000 rpm (JA-14 Rotor, Beckman Model J2-MI Centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 25 minutes. Both the supernatant and pellet after the first centrifugation contained the desired protein. The supernatant was centrifuged again at 13000 rpm for 45 minutes, and the pellet from this spin was discarded. The pellets from the first centrifugation were resuspended with 20 ml of 10 mM Tris buffer at pH 7.4. Lysis buffer of pH 6.6 (0.1 M sodium phosphate buffer, 0.2 M NaCl, 10 mM MgCl₂) was added to this solution to make a final volume of 200 ml, and then 2 ml of 0.5 M ethylenediamine tetraacetic acid at pH 8.0 (EDTA, Sigma Chemical Co.) was added to the 200 ml of resuspended lysozyme pellet. This solution was stirred in a cold room (4°C) for 12 hours to release the trapped lysozyme. Then, 0.02 mg of deoxyribonuclease I (DNase-I stored in -20 °C freezer, crude powder from bovine
pancreas, Sigma Chemical Co.) and 2 ml of 1 M MgCl$_2$ were added to each 100 ml of pellet suspension. This mixture was stirred at room temperature for 2 hours, and then centrifuged at 4 °C, 20000 rpm (JA-20 Rotor, Beckman Model J2-MI Centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 35 minutes FIG 1.5. The pellet of this last centrifugation was discarded and the supernatant was combined with that from the second centrifugation.

1.2.4.3. Dialysis and Ion-Exchange Chromatography

About 1100 ml of the supernatant from the centrifugation was dialyzed in 1200 ml plastic fleaskers against about 4 liters DDW with Spectra/Por regenerated cellulose hollow fiber bundles (MWCO 18,000, Spectrum Medical Industries, Inc., Houston, TX) until the conductivity (Cond/TDS, Corning Glass Works, Corning, NY) of the supernatant was less than 2 µS/cm (FIG 1.6). Then the pH of the solution was adjusted to 6.5 – 7.5 with 1 N NaOH or 1 N HCl. The whole dialysis process required approximately 48 hours.

The dialyzed solution was then loaded onto a CM Sepharose cationic exchange chromatography column (CM sepharose CL-6B CCL-100, Sigma Chemical Co.) that has been settled with 300 ml of 50 mM Tris buffer at pH 7.25.

As the dialysate moved through the column, a thick white band of protein was formed on the top of the sepharose FIG 1.7. After all the dialyzed solution had passed through the column, the column was loaded with 200 ml of 50 mM Tris
FIG 1.4 Fermentation of T4 lysozyme

FIG 1.5 Centrifugation of culture medium to separate cell debris from the lysozyme-rich broth.
buffer to remove any yellow colored materials which would interfere with the UV signal during elution of the lysozyme. This step of loading dialyzed solution through the chromatography column took 24 to 36 hours. A salt gradient of 50 mM to 0.3 M NaCl in 50 mM Tris buffer was used to elute the protein from the column into a fraction collector (Frac-100, Pharmacia LKB Biotechnology, Alameda, CA). The optical density of the eluant corresponding to each fraction was recorded on a chart recorder. The fractions containing proteins were transferred into Spectra/Por dialysis membrane tubing with molecular weight cutoff of about 13,000 (MWCO 12-14K, Spectrum Medical Industries, Inc.) for 12 hours of dialysis against 50 mM sodium phosphate buffer (20 times the eluant volume) of pH 5.8. The preparations were stored without further treatment at 4°C. SDS gel electrophoresis showed the presence of only one band. The isolated protein were on the average over 95% pure with the remaining fraction consisting of salts and peptide fragments.

1.2.4.4. Concentration

The dialyzed protein solution from the tubing was concentrated using a SP Sephadex column (SP Sephadex C-50, Sigma Chemical CO.) shown in FIG 1.8. The solution was allowed to pass through the column, and the thick white band of protein was formed on top of the column bed. Then the lysozyme was eluted from
FIG 1.6 Dialysis of the supernatant obtained from centrifugation.

FIG 1.7 Cationic chromatographic separation of protein from dialyzed solution.
the column with 0.1 M sodium phosphate buffer of pH 6.5. The 1 ml of concentrated solution was collected in 1.5 ml vials. The optical density of each fraction was measured at 280 nm with UV spectrophotometer (Model DU-62, Beckman Instruments, Inc., Fullerton, CA) using samples diluted 1:100 (1:10 if the protein was not very concentrated in some vials) with 0.1 M sodium phosphate buffer at pH 6.5. Then the concentration of each fraction was determined by dividing the measured absorbance with the molar absorption coefficient of 1.28 for wild type and all variants or 1.46 for the tryptophan mutant according to Beers’ law.

1.2.5. Ellipsometry

Ellipsometry is a very old technique for studying surfaces and thin films. The name ellipsometer was first introduced by Rothen (1945). Ellipsometry can be applied for measuring surface films ranging from partial monoatomic coverage up to microns. This technique has been used in a variety of fields including microelectronic integrated circuit (IC) fabrication and corrosion science. We used the automated rotating ellipsometer (L-104 SA, Gaertner Scientific Corp., Chicago, IL) shown in FIG 1.9. For measurement purposes, the angle of reflection was set at the same angle of incidence (70°). The light source was a He-Ne laser with a wavelength of 6328 Å. The ellipsometer is an instrument that measures the optical
FIG 1.8 2nd chromatographic separation of dialyzed protein in order to remove excess from the water protein solution.

FIG 1.9 Configuration of ellipsometric measurement of protein adsorption on silicon wafer surfaces.
changes in the state of polarization upon the reflection of chromatic, collimated,
and polarized light. The state of polarization is defined by the phase and amplitude
relationships between the two component plane waves into which the electric field
oscillation is resolved. One wave, designated as p, is in the plane of incidence. The
other, designated as s, is normal to the plane of incidence. If the p and s
components are in phase, the resultant wave is plane polarized. A difference in
phase, other than 180°, corresponds to elliptical polarization. In general, reflection
causes a change in the relative phases of the p and s waves and a change in the ratio
of their amplitudes.

In order to determine the properties of the film on top of a substrate, the
properties of the substrate should be known in priori, specifically the complex
index of refraction, \( \tilde{n}_3 \), which is \( \tilde{n}_3 = n_3 - i k_3 \). \( n_3 \) is the index of refraction and
\( k_3 \) is the extinction coefficient of the substrate and \( i \) is \( \sqrt{-1} \). The values of \( n_3 \) and
\( k_3 \) were determined by using ellipsometry. The medium 1 is the ambient which is
an aqueous solution in our case. A dielectric materials such as glass, nonabsorbing
medium is \( k_3 = 0 \). The subscript 3 was reserved for the designation of a substrate.
For the description of reflection, consider the case where the incident light beam is
reflected from the incident plane surface shown in FIG 1.10.
FIG 1.10 Schematic diagram of the reflection of a light beam.

The plane of incidence consists of both the incident and reflected light beams. The Fresnel reflection coefficient $r$ is defined as the ratio of the amplitude of the reflected wave to that of the incident wave for a single interface which can be given as

$$
r_{13}^p = \frac{\tilde{n}_3 \cos \phi_1 - \tilde{n}_1 \cos \phi_3}{\tilde{n}_3 \cos \phi_1 + \tilde{n}_1 \cos \phi_3} \\
r_{12}^s = \frac{\tilde{n}_1 \cos \phi_1 - \tilde{n}_3 \cos \phi_3}{\tilde{n}_1 \cos \phi_1 + \tilde{n}_3 \cos \phi_3}
$$

[1.1] [1.2]

where the subscripts, 1 and 2, indicate the medium 1 and 2 and the superscripts, $s$ and $p$, indicate the waves perpendicular and parallel to the plane of incidence respectively.
When we have a substrate covered with a thin film shown in FIG 1.11, these coefficients can be obtained in similar pattern.

\[ \text{Index} = n_l \]
\[ \text{Index} = n_i \]
\[ \text{Index} = n_3 \]

**FIG 1.11** Reflection of light beam from a film-covered interface.

For a case where multiple interfaces are involved, the reflected wave consists of the addition of light reflected directly from first interface together with reflected light from all successive lower interfaces. The total reflection coefficients of waves, defined as the ratio of the amplitude of the subsequent reflected wave to the amplitude of the incident wave were derived by Azzam and Bashara (1977) and Heavens (1965) in the form of

\[ R^p = \frac{r_{12}^P + r_{23}^P \exp(-i2\delta)}{1 + r_{12}^P r_{23}^P \exp(-i2\delta)} \]  \[ 1.3 \]

\[ R^s = \frac{r_{12}^s + r_{23}^s \exp(-i2\delta)}{1 + r_{12}^s r_{23}^s \exp(-i2\delta)} \]  \[ 1.4 \]
where the subscripts "12" and "23" indicate that the Fresnel reflection coefficients are for the interface between medium 1 and medium 2 and between medium 2 and medium 3 respectively. \(\delta\) is the film phase thickness in the form of

\[
\delta = 2\pi \left( \frac{d_f}{\lambda} \right) n_2 \cos \phi_2
\]  

[1.5]

where \(d_f\) is the film thickness and \(\lambda\) is the wavelength of the light beam in a vacuum.

The effect of reflection is characterized by the angle, \(\Delta\), defined as the change in phase, and the angle \(\psi\), the arctangent of the factor by which the amplitude ratio changes. Symbolically, if the amplitudes of the incident and reflected beams are designated \(E\) and \(R\), respectively, and the phase angles, \(\beta\),

\[
\Delta = (\beta_p - \beta_s)_{\text{reflected}} - (\beta_p - \beta_s)_{\text{incident}}
\]  

[1.6]

\[
\psi = \arctan \left( \frac{R_p}{R_s} \cdot \frac{E_s}{E_p} \right)
\]  

[1.7]

The change of the angles of \(\Delta\) and \(\psi\) during adsorption were measured using the ellipsometer.

The relationship between \(\Delta\) and \(\psi\) and the properties of a reflecting system are expressed by the Fresnel reflection coefficients. The Fresnel reflection coefficient, \(r\), of an interface is the ratio of the electric field vector, \(R'\), of the reflected wave to that, \(E'\), of the incident wave; in terms of the amplitudes of the
incident and reflected waves \(E\) and \(R\), respectively, and the phase change, \(\beta\), accompanying reflection,

\[
r = R^1/E^1 = (R/E)e^{i\beta}
\]  

[1.8]

The coefficient depends upon the orientation of the wave relative to the plane of incidence, and the reflection of a wave of any polarization is described by the two coefficients, \(r_s\), and \(r_p\), for the component waves. The ratio of these reflection coefficients is

\[
\frac{r_p}{r_s} = \frac{R_p}{R_s} \frac{E_s}{E_p} e^{i(\beta_p - \beta_s)}
\]  

[1.9]

From Eqns [1.6] and [1.7] it follows that

\[
\frac{r_p}{r_s} = \tan \psi e^{i\Delta}
\]  

[1.10]

For an optically isotropic substrate with a clean surface the Fresnel reflection coefficients are

\[
r_{12}^p = \frac{n_1 \cos \phi_2 - n_2 \cos \phi_1}{n_1 \cos \phi_2 + n_2 \cos \phi_1}
\]  

[1.11]

\[
r_{12}^s = \frac{n_1 \cos \phi_1 - n_2 \cos \phi_2}{n_1 \cos \phi_1 + n_2 \cos \phi_2}
\]  

[1.12]

As shown in FIG 1.11, the subscripts 1 and 2 refer to the media bounding the reflecting interface; \(\phi_1\) is the angle of incidence and \(\phi_2\) the angle of refraction.
The complex quantity $\rho$, the complex ratio of the total reflection coefficients is defined as (Azzam and Bashara, 1977)

$$\rho = \frac{R^P}{R^S} = \tan \Psi e^{i\Delta}$$ \hspace{1cm} \text{[1.13]}

The quantities of $\Psi$ and $\Delta$ can be obtained from ellipsometric readings. The Eqn [1.13] can be rewritten as (So and Vedam, 1972)

$$C_1(\exp D)^2 + C_2(\exp D) + C_3 = 0$$ \hspace{1cm} \text{[1.14]}

where $C_1$, $C_2$, and $C_3$ are complex functions of the refractive indices, angles of incidence, $\Delta$ and $\Psi$. Two solutions for $\exp D$ can be obtained at a given value of the coefficients in Eqn [1.14].

$$D = i2\delta = -4\pi n_2 \left( 1 - \left( \frac{n_1 \cos \phi_1}{n_2} \right)^2 \frac{d_f}{\lambda} \right)^{1/2}$$ \hspace{1cm} \text{[1.15]}

The calculated film thickness from the solution is expected to be complex since the coefficients are complex. Since the film thickness is not an imaginary but real quantity, the solution of the Eqn [1.14] yielding a real value is an appropriate answer. Therefore, the Eqn [1.15] is solved by iteration while $n_2$ is being adjusted to the direction that the complex part of film thickness is minimized. The FORTRAN program (OEP) was used to calculate the adsorbed mass of protein at silica wafer surface.
1.2.6. Radioiodination

In this study, we investigated the effect of competitiveness, affinity, and structural stability of proteins on the overall adsorption processes by using synthetic mutants of bacteriophage T4 lysozyme. Radioactive iodine is one of the most commonly used radioactive isotopes for protein labeling in radioimmunoassay (RIA). Both isotopes, $^{125}$I and $^{131}$I, emit gamma radiation, while $^{132}$I emits $\beta$-radiation. Although some of the $\beta$ radiation emitters, such as $^{14}$C and $^3$H, are currently being used as trace markers, counting the radioactivity using these isotopes still requires liquid scintillation techniques. However, the gamma radiation from iodine can be more conveniently and directly monitored from the test tubes than beta radiation by using a commercially available gamma radiation counter. $^{125}$I has been preferable choice in most radioimmunoassays because the 60 day half-life of $^{125}$I is much longer than $^{131}$I which has an 8 day half-life.

There are several methods available for the iodination of proteins, for example, the iodine monochloride method (Samols and Williams, 1961; Horbett, 1981), enzymatic iodination using lactoperoxidase (Thorell, 1972), and the chloramine-T procedures (Hunter and Greenwood, 1962). Greenwood et al., (1963) developed an efficient method for the direct substitution of $^{125}$I into the tyrosyl residues of proteins by using chloramine-T whose chemical structure is shown in FIG 1.12. This method has been the most successful and widely used method during the past several decades (Greenwood, 1971; Kirkham and Hunter, 1971). This method has been further diversified by several researchers (Bolton and Hunter,
1972). Chloramine-T, the sodium salt of the N-monochloro-derivative of p-toluene sulphonamide, occasionally abbreviated as CAT, is universally used as an analytical oxidizing material, and particularly as a reagent for radiolabeling techniques. For the radiolabeling techniques, CAT is used for oxidizing radioactive iodine to diatomic iodine. The oxidized $^{125}$I produces free iodine ($I_2$). These free iodines react with water (hydrolyzed) to form the iodinium species which will be substituted into tyrosyl residues in the protein structure. This reaction occurs from the replacement of a hydrogen by an iodine. Diiodination may possibly be occurring in the presence of an excess amount of iodine. It is known that the -SH groups are oxidized by the iodine during the reaction process (Hughes and Straessle, 1950). The optimum pH for the radioiodination of protein is slightly alkaline, pH 7.5 (Hunter, 1962). The reaction using chloramine-T is performed in extremely small volume ranging between 0.05 and 1.0 ml. Immediately after the substitution of radioactive iodine into the protein structure, the reaction mixture is transferred to a molecular sieve column in order to separate unreacted free iodine from the labeled protein.

$$\text{Na}^+ \left[ \text{H}_3\text{C-} \begin{array}{c} \text{SO}_3^- - \text{N} - \text{Cl} \end{array} \right]^-$$

**FIG 1.12** Chemical structure of chloramine-T.
Few studies are available for the effects of radiolabeling on the protein structure and the adsorption behavior of labeled protein. In some studies, the preferential adsorption of the labeled proteins depended on the surface materials (Grant et al., 1977; van der Scheer et al., 1978). van der Scheer (1983) also states that the labeled proteins show preferential adsorption due to the structural alteration caused by iodination. He pointed out that the structural alteration rather than the presence of the iodine molecule in the protein may contribute to the preferential adsorption. On the other hand, some researchers suggest that there is no preferential adsorption (Brash et al., 1974; Weathersby et al., 1977; Horbett, 1980, 1981). Several researchers have suggested that there is no effect of the presence of a label on the adsorption of proteins onto different surfaces (van Dulm and Norder, 1983; van Oss et al., 1981; Bornzin and Miller, 1982; Schmitt et al., 1983; Penners et al., 1981; Chuang et al., 1978).

The fact that the tracer proteins used in most radioimmunoassays maintain their physiological functionality can be considered as a good indication that the radioiodination does not cause a serious alteration of the chemical or structural properties of proteins when the labeling conditions are properly chosen. van Dulm and Norde (1983) used human plasma albumin labeled with $^{125}\text{I}$ to study sorbent hydrophobicity and charge on the adsorption. They found neither the labeled nor the nonlabeled albumin molecules were preferentially adsorbed.

In the case of protein adsorption at an air/water interface, $^{14}\text{C}$ labeling has been used. Khaiat and Miller (1969) introduced this method when they studied
adsorption isotherm at air/water. Acetylation of the terminal amine and lysine residues is a commonly used method of radiolabeling a protein molecule. A labeled protein may become hydrophobic since labeling removes the charge from the derivatized residues. Numerous studies have been performed using $^{14}$C radiotracer methods up to date (Damodaran and Song, 1991; Hunter et al., 1990). Although they did not observe conformational change due to labeling, some researchers have reported altered conformationals which may be introduced due to chemical changes by the labeling procedure (Chen et al., 1982).

The gel filtration method is used to separate the labeled protein molecules from the free iodine molecules. Gel filtration is commonly accepted since it is simple and rapid to separate solute from mixture, and is so mild that denaturation of labile substances does not occur. This method can be used when the differences in molecular size are sufficient. The obtained results are reproducible most of the time. Approximately 100 % of solute recovery can be achieved, and scale-up to large sample volumes is readily executable. (Reiland, 1971).

Iodinated derivatives of histidine that contain iodine on either C or N of the imidazole ring have been prepared. However, only carbon-bound iodine is stable to sulfite. Li (1944) has measured the rate of iodination of histidine, and he has shown that it is the only other amino acid likely to be substituted under the usual iodinating conditions, although tryptophan may be destroyed. Evidence for the iodination of histidyl residues in proteins is based upon analogy with the free amino acids coupled with the finding of bound iodine in excess of the sites available as
tyrosyl residues. Iodinated histidine has not been isolated from a digest of the iodinated protein. Tyrosyl residues usually iodinate more readily than histidyl, but the difference is not extreme. Thus, in iodinating serum albumin, the less reactive portion of the tyrosyl residues reacted concomitantly with histidyl (Hughes and Straessle, 1950; Masouredis, 1957).

1.2.7. Radioiodination of Model Proteins

Radioactive sodium iodide in NaOH solution was purchased from Amerhsam (Arlington Heights, Illinois). All proteins were labeled by using Chloramine-T as an oxidizing reagent. The reaction was carried out at room temperature (about 25 °C). A 10 mL disposable pipette (after sawing off the top part) was used as a column for the purification of labeled protein. A glass bead of 4 mm diameter was dropped down the bottom of the column. Approx. 2.5 cm of tubing with a clamp was attached to the bottom of the column. The Sephadex solution, prepared one day before use with 0.6 g of Sephadex (G-75-120, Sigma) in 100 mL distilled water, was continuously added to build up the column bed inside the pipette. Iodination was carried out by mixing 10 μL of protein solution (1 mg/mL) with 1 mCi of Na\textsuperscript{125}I followed by adding 10 μL (5μg) of Chloramine-T solution made of 5 mg of chloramine-T in 10 mL 0.05 M PB. Chloramine-T solution was prepared a few minutes before use. The mixture was allowed to react for 60 seconds with finger-flicking agitation. The reaction was stopped by adding 10 μL of sodium metabisulfite solution of concentration 1 mg/mL. The labeled
protein solution was immediately applied to the gel filtration column of cross-linked dextrans (Sephadex) in order to separate the labeled protein from unreacted free iodines. The separated fractions were collected in 30 test tubes. 10 µL aliquots from each fraction after vortex were taken and counted to locate the separated portion of the labeled protein.

1.2.8. Adsorption Kinetic Data

All experiments were performed at room temperature in 0.01 M phosphate buffer unless otherwise stated. Adsorption data were collected with time from both single and competitive adsorption. Hydrophilic glass surfaces were submersed in the test tubes which contained 1 mL of 0.01 M phosphate buffer. When the surface was put into the tubes, the surface was completely submersed in the solution. Protein stock solution was prepared to carry a specific activity of approximately 5 x 10^5 cpm/µg. Adsorption was started by adding 0.5 mL of protein solution to each tube containing a completely submerged surface in 1.0 mL buffer. The solution was mixed by a gentle agitation to reach a homogeneous final concentration in the bulk. After desired contact time, the adsorption was terminated by transferring surfaces to phosphate buffer performing gentle dip-rinsing while minimizing the exposure of surface to the air. The radioactivity of the adsorbed protein was counted after transferring the samples into the empty tube. In all cases, the time-dependent disintegration of the radioactivity was taken into account.
1.2.9. References


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CHAPTER 2

STRUCTURAL STABILITY EFFECTS ON ADSORPTION OF BACTERIOPHAGE T4 LYSOZYME TO COLLOIDAL SILICA

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Abstract

Circular dichroism (CD) spectra were obtained for bacteriophage T4 lysozyme and three of its mutants in the presence and absence of colloidal silica nanoparticles. Mutant lysozymes were produced by substitution of the isoleucine at position 3 with tryptophan, cysteine, and leucine. Each substitution resulted in an altered structural stability, quantified by a difference in free energy of unfolding from the wild type. CD spectra recorded in the absence of colloidal silica agreed with X-ray diffraction data in that the mutants and wild type showed similar secondary structures. CD spectra of protein-nanoparticle complexes recorded after contact for 90 min showed significant differences from those recorded in the absence of nanoparticles, and these differences varied among the proteins. The percentage of α-helix lost in these proteins upon adsorption was also recorded as a function of time by CD. For a 1:2 protein to nanoparticle mixture, the more unstable the protein, the greater the rate and extent of secondary structure loss upon adsorption. These kinetic data were evaluated using a model allowing proteins to exist in two different conformational states at the interface: state 1 molecules retain their native conformation, and state 2 molecules lose a certain amount of secondary structure and occupy more surface area than state 1 molecules. This analysis indicated that proteins of lower thermal stability have a greater tendency to adopt state 2 on silica. Rate constants governing generation of state 1 and state 2 molecules determined by CD were used as initial values of surface coverage-dependent rate constants in order to simulate adsorption kinetics. Comparison of
simulated curves to adsorption data recorded with in situ ellipsometry suggests that protein adsorption may adequately be described with a model allowing for only two functional states.

2.2. Introduction

Circular dichroism (CD) is a popular method for gaining information about protein secondary structure in solution (1), but there are difficulties in distinguishing orientational rearrangement from conformational change during adsorption (2). The introduction of nanoparticles as a solid surface for protein adsorption has made it possible to measure less ambiguously adsorbed protein conformational changes using CD (3).

There are many factors that govern the adsorption process, and it would be useful to learn the specific influences of individual factors. Comparative studies with genetic variants and site-directed mutants of single proteins have particularly demonstrated the importance of structural stability in protein interfacial behavior (4). In this work, bacteriophage T4 lysozyme (T4L) and three single site mutants differing only in thermal stability were selected to study the effects of structural stability on adsorption to silica nanoparticles using CD. Billsten et al. (5) showed that both the rate and the extent of α-helix loss upon adsorption were greater for less stable variants of T4L than for more stable variants. The goals of this research were to compare the CD spectra of T4L variants to a simple adsorption kinetic model allowing for conformationally dissimilar adsorbed states and to measure the
effect of structural stability on adsorption kinetic rate constants governing adsorption into each state. Rate constants determined through analysis of the CD data were then used to simulate adsorption kinetics, and these simulations were compared to T4L adsorption kinetic data recorded with in situ ellipsometry.

2.3. Materials and Methods

2.3.1. T4L Production and Purification

Two mutants of greater stability and one mutant of lower stability than the wild type protein were produced. More stable mutants were made with substitution of the isoleucine at position 3 with cysteine (I3C) and leucine (I3L). In the case of I3C, increased stability is a result of a disulfide link formed with C97; in the case of I3L it is a result of an increase in hydrophobic stabilization in this region of the protein’s interior (6). The less stable mutant was made by substitution with tryptophan (I3W). This causes unfavorable steric interactions, unsatisfied hydrogen bonds, and differences in both van der Waals and hydrophobic interactions in the region of the substitution. The structural stability of each mutant was quantified by $\Delta\Delta G$: the difference between the free energy of unfolding of the mutant protein and that of the wild type at the melting temperature of the wild type. At pH 6.5, $\Delta\Delta G = +1.2 \text{ kcal/mol for I3C, } +0.4 \text{ kcal/mol for I3L, and } -2.8 \text{ kcal/mol for I3W}$ (6).

The production of T4L variants was performed using transformed cultures of Escherichia coli strain RR1. Individual bacteria strains containing the mutant
lysozyme expression vectors selected for this work were provided by Professor
Brian Matthews and co-workers at the Institute of Molecular Biology, University of
Oregon (Eugene, OR) and stored at –80 °C until use. The T4L variants were
prepared exactly as described earlier (4). Preparations were stored without further
treatment at 4 °C and used within 1 week. SDS-gel electrophoresis showed the
presence of only one band (7); the isolated protein were on the average over 95%
pure with the remaining fraction consisting of salts and peptide fragments.

2.3.2. Circular Dichroism

Circular dichroism can be considered to be the absorption spectrum
measured with left circularly polarized light minus the absorption spectrum
measured with right circularly polarized light (8). At a given wavelength, the
difference in absorbance is given by

$$\Delta A = \Delta \varepsilon C l$$

[2.1]

where $\Delta A$ is the difference in sample absorbance of left and right circularly
polarized light, $\varepsilon$ (L/mol·cm) is the molar absorption coefficient, $C$ (mol/L) is
solution concentration, and $l$ (cm) is the pathlength. A simple relationship exists
between the difference in absorbance and ellipticity; Johnson (9) showed that the
molar ellipticity, $[\theta]$ (degree·L/mol·cm) an intrinsic property, is related to $\Delta \varepsilon$ by
\[ \Delta \varepsilon = A \sqrt[6]{3298} \] \[\text{[2.2]}\]

In this work, the wavelength spectra were recorded according to \( \Delta \varepsilon \) on a per amide basis; Eqn [2.2] was used to report results measured in terms of ellipticity.

A good approximation of the \( \alpha \)-helix content of a protein molecule can be obtained from the CD spectrum (\( \Delta \varepsilon \)) at 222 nm (10). The absorption band at this wavelength is mainly due to the presence of \( \alpha \)-helices, and other secondary structures and chromophores do not have an absorption peak at this wavelength. The relationship between \( \alpha \)-helix content and \( \Delta \varepsilon_{222} \) is \% \( \alpha \)-helix = -10 (\( \Delta \varepsilon_{222} \)).

2.3.3. Protein Solutions and Nanoparticle Suspensions

A 0.01 M phosphate buffer (pH 7) was used to dilute all T4L stock solutions to 0.2 mg/mL. Colloidal silica particles (food grade quality) were purchased from EKA-Nobel (Stenumgssund, Sweden). The original stock solution contained \( 5.1 \times 10^{17} \) particles/mL suspended in pH 10.2 alkaline buffer. The particles were 9 nm in diameter and were used without further modification. The same phosphate buffer used to dilute the T4L preparations was used to dilute the stock particle suspensions. The final concentrations of particles were \( 3.2 \times 10^{15} \), \( 6.4 \times 10^{15} \), and \( 12.9 \times 10^{15} \) particles/mL. The pH of the final dilution was adjusted to between 7 and 7.3 with 1 N HCl. These suspensions were prepared in order to supply solid surfaces for protein adsorption and were used within 6 h. Mixing equal volumes of particle suspension and protein solution resulted in solutions with
protein-to-particle ratios of 2:1, 1:1, and 1:2, respectively. The light diffraction of the particles in solution was negligible for these CD spectra measurements.

2.3.4. CD Spectra

CD spectra were recorded using a JASCO J-720 UV (Japan). All experiments were carried out at room temperature, with a rectangular cuvette of 1 mm pathlength. CD spectra were recorded in the absence of nanoparticles after protein-free buffer was used to record the baseline spectrum from 260 to 195 nm; the spectrum was then recorded for the T4L solution. The net protein spectrum from 260 to 195 nm was obtained by subtracting the baseline from the protein solution spectrum.

Suspensions of T4L-nanoparticle complexes were made by combining 100 µL of nanoparticle suspension with the same volume of T4L solution. The resulting suspension was mixed gently for 30 s and then transferred to a cuvette for immediate CD spectral analysis. The spectrum at 222 nm was recorded for 90 min. The CD spectra of T4L-nanoparticle complexes were also recorded from 260 to 195 nm, 90 min after combining the nanoparticles and proteins. A protein-free nanoparticle suspension was used to record the baseline for these experiments.
2.4. Results and Discussion

2.4.1. CD Spectra of Proteins in Nanoparticle-Free Buffer

The CD spectra from 260 to 195 nm for T4L and its mutants are plotted in Fig. 2.1. These spectra revealed no differences among the variants. The \( \alpha \)-helix content of each protein in buffer is shown in Table 2.1. The \( \alpha \)-helix contents determined here were calculated from measurements of ellipticity at 222 nm, but the results are very close to those determined by the CONTIN program (5), which is more accurate and considers the effects of all secondary structure on the spectrum. In particular, Billsten et al. (5) reported average \( \alpha \)-helix contents of 57\% for wild type, 58\% for I3C, and 59\% for I3W. These values are similar to those reported in Table 2.1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average ( \alpha )-helix Content (%)(^a)</th>
<th>Average ( \alpha )-helix Loss after 90 min (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3C</td>
<td>57.0 (0.4)</td>
<td>12</td>
</tr>
<tr>
<td>I3L</td>
<td>60.5 (2.8)</td>
<td>14</td>
</tr>
<tr>
<td>Wild type</td>
<td>58.3 (0.8)</td>
<td>18</td>
</tr>
<tr>
<td>I3W</td>
<td>60.3 (1.5)</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 2.1 Amount of \( \alpha \)-helix present in each T4L variant in solution and the amount of \( \alpha \)-helix lost after adsorption to colloidal silica.
FIG 2.1 CD spectra of T4L variants in the absence of colloidal silica.
2.4.2. CD Spectra of Proteins in the Presence of Nanoparticles

Figure 2.2 shows the CD spectra of T4L and its three mutants after being mixed with nanoparticle suspensions for 90 min. The intensities of the CD spectra at 222 nm for all variants were reduced after adsorption, implying that these proteins had undergone a conformational rearrangement at the silica surface. The $\alpha$-helix loss for each variant when bound to silica is shown in Table 2.1. As expected, I3W lost more $\alpha$-helix than the wild type, which in turn lost more $\alpha$-helix than the more stable mutants.

Three different protein-to-particle ratios (1:1, 1:2, and 2:1) were tested for each protein. Spectra could not be recorded for the 2:1 ratio, because these mixtures were not stable to aggregation and precipitation, the effects of this becoming visible within about 30 min. The silica nanoparticles used here are negatively charged at pH 7.0, while each T4L variant has nine out-of-balance positive charges in solution. T4L adsorption to the surface of the nanoparticles would decrease the repulsion among particles, thus decreasing the stability of the colloidal suspension. In the case of the 1:1 ratio, no denaturation was observed, but results were less reproducible than those recorded with the 1:2 ratio. Moreover, all mixtures prepared at the 1:2 ratio showed a larger change in $\alpha$-helix than those prepared at the 1:1 ratio. Thus, all spectra discussed here refer to suspensions prepared at a T4L:nanoparticle ratio of 1:2.

FIG 2.3 shows the kinetic behavior of the four variants. The change in secondary structure (represented by changes in $\alpha$-helix content) exhibited by each
FIG 2.2 CD spectra of protein-nanoparticle complexes recorded after contact for 90 min.
FIG 2.3 Kinetics of $\alpha$-helix loss among the T4L variants upon adsorption to colloidal silica.
stability mutant differed from that of the wild type. These results are consistent
with the results obtained by Billsten et al. (5). I3W displayed the largest and most
rapid loss of secondary structure. The most stable mutant, I3C, lost the smallest
amount of secondary structure. The wild type protein and I3L behaved in a manner
that was between these extremes. In particular, the rate and extent of the secondary
structure loss decreased as ΔΔG increased. These results are also consistent with
previous experiments involving in situ ellipsometry and surfactant-mediated
elution, ring tensiometry, and the interferometric surface force technique, all
indicating that less stable variants more readily adopt a conformationally altered
state at an interface (4, 11, 12).

2.4.3. Analysis with Reference to a Two-State Irreversible Adsorption
Mechanism

Here we consider that protein molecules adsorb to the nanoparticle surface
into one of two states with first order adsorption rates constants \( k_1 \) for adsorption
into state 1 and \( k_2 \) for adsorption into state 2. We define a state 1 molecule as
retaining its secondary structure, while a state 2 molecule loses a certain amount of
secondary structure. State 1 molecules would thus be less tightly bound than those
in state 2, and a state 2 molecule may occupy a greater interfacial area (\( A_2 \)) than it
would occupy in state 1 (\( A_1 \)). This mechanism was used to interpret the surfactant-
mediated elution of T4 lysozyme stability and charge mutants (4, 13) and showed
that state 2 molecules exhibited greater resistance to elution than did state 1
molecules. The present data were analyzed with reference to the adsorption kinetic model evolving from this mechanism.

As the CD data provide an estimate of the total percentage of secondary structure loss for all molecules in the sample, it is necessary to establish the percentage of secondary structure which is lost for a given state 2 molecule in order to use the two-state mechanism described here. McGuire et al. (4) provided justification from analysis of adsorption kinetic and surfactant-mediated elution data recorded for several I3 mutants at hydrophilic silica that I3W can be considered as adsorbing entirely in state 2. Combining that result with data in Table 2.1 indicates that a state 2 molecule could be defined as one that had lost 29% α-helix. They also determined that the fraction of wild type adsorbed in state 2 was 0.61, while that for I3C was 0.48 (no data were recorded for I3L). Defining a state 2 molecule as one having lost 29% α-helix, data from Table 2.1 indicate that the fraction of protein adsorbed to the nanoparticles in state 2 was 18/29 = 0.62 and 12/29 = 0.41 for wild type and I3C, respectively. This agrees well with the earlier determination. The underprediction in the case of I3C may be related to the assumption of monolayer coverage being required to estimate the fraction of protein adsorbed in state 2; I3C adsorption did not achieve monolayer coverage (4).

CD data were replotted as fractional surface coverage in state 2 vs time for each variant, with a state 2 molecule being defined as one having lost 29% α-helix. The conversion was made by estimating the fraction of T4L adsorbed in state 2 as $(60 - \Delta e_{222}(-10))/29$. Adsorption rate constants $k_1$ and $k_2$ can be estimated by first
solving equations describing the time-dependent fractional surface coverage of protein in each of the two states,

\[
d\theta_1/dt = k_1C(1 - \theta_1 a\theta_2) \tag{2.3}
\]

and

\[
d\theta_2/dt = k_2C(1 - \theta_1 a\theta_2) \tag{2.4}
\]

where \(a = A_2/A_1\), and \(C\) (mg/mL) is protein concentration. The \(\theta_i\) is defined as the amount of protein adsorbed in state \(i\) (mg/m²) divided by the amount of protein constituting a monolayer of state 1 molecules (\(\Gamma_{\text{max}}\), mg/m²), such that at any time the adsorbed mass equals \(\Gamma_{\text{max}} (\theta_1 + \theta_2)\), and when the surface is covered, \(\theta_1 + a\theta_2 = 1\). In the CD experiments, the total surface area for adsorption is very large: treating the nanoparticles as 9-nm spheres, the adsorption area is about 1.64 m²/mL. \(\Gamma_{\text{max}}\) can be approximated as 3.6 mg/m², corresponding to a monolayer of molecules adsorbed end-on (4). Thus, even after adsorption of all protein in the cuvette (initial concentration = 0.10 mg/mL), \(\theta_1 + a\theta_2 << 1\). Our aim in estimating the rate constants was to use them to simulate adsorption kinetics for comparison to experimental data (adsorbed mass vs time) recorded with ellipsometry. The surface area used for adsorption in the ellipsometry experiments was about 1/10,000th of that in the CD experiments, while the protein concentration used was 10 times higher than that in the CD experiments. In order to compare rate constants based on CD data to adsorption kinetics measured with ellipsometry, \(\Gamma_{\text{max}}\) was defined as
0.10 mg/1.64 m², the maximum adsorbed mass attainable in the CD experiments, for the purpose of estimating $k_1$ and $k_2$ for each variant.

The change in protein concentration during adsorption to the nanoparticles would then be given by

$$\frac{dC}{dt} = -0.10\left(\frac{d\theta_1}{dt} + \frac{d\theta_2}{dt}\right)$$  \hspace{1cm} [2.5]

Equations [2.3] through [2.5] do not have an analytical solution. However, it was possible to fit the CD kinetic data numerically to estimate $k_1$ and $k_2$ in each case. These results are plotted in FIG 2.4, and the rate constants are shown in Table 2.2. The results in FIG 2.4 indicate that the model fits the data very well. As expected, $k_2$ and $k_2/k_1$ increase as protein thermal stability decreases. From Eqns [2.3] and [2.4], $k_2/k_1 = \theta_2/\theta_1$ at any time. A decrease in the value of $k_2/k_1$ would indicate that

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_2/k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3C</td>
<td>0.50</td>
<td>0.24</td>
<td>0.48</td>
</tr>
<tr>
<td>I3L</td>
<td>0.17</td>
<td>0.08</td>
<td>0.47</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.19</td>
<td>0.19</td>
<td>1.0</td>
</tr>
<tr>
<td>I3W</td>
<td>0.11</td>
<td>0.90</td>
<td>8.18</td>
</tr>
</tbody>
</table>

*Table 2.2* Values of the adsorption rate constants estimated by analysis of CD kinetic data with Eqns [2.3] through [2.5].
FIG 2.4 Kinetics of generation of state 2 molecules on colloidal silica. The solid line in each case is the regressed fit of \( \theta_2 \), gained by simultaneous solution of Eqns [2.3] through [2.5] for (a) I3C, (b) wild type, and (c) I3W.
FIG 2.4 Continued
FIG 2.4 Continued
less protein is adsorbing in state 2; thus the tendency of the protein to adopt state 2 is increased with a decrease in its stability.

2.4.4. Simulation of Adsorption Kinetics and Comparison to Ellipsometric Data

The CD data of FIGs 2.3 and 2.4 describe the kinetics of generation of state 2 molecules, i.e., \( \theta_2 \) as a function of time, while adsorption kinetic data describe generation of adsorbed mass, corresponding to \( \theta_1 + \theta_2 \) as a function of time. If the concentration of protein is large in a given experiment relative to the adsorbed amount, we can treat the bulk concentration as constant. Solving Eqns \([2.3]\) and \([2.4]\) in that case would yield expressions for \( \theta_1 \) and \( \theta_2 \) as a function of time such that

\[
\theta_1 + \theta_2 = \frac{1 + k_2/k_1}{1 + ak_2/k_1} \left[ 1 - \exp(-k_1C - ak_2C)t \right]
\] \[2.6\]

Parameter \( a \) was set equal to 1.93 (=3.96/2.05), where 2.05 was taken as the specific interfacial area occupied by “side-on” T4L molecules, and 1/3.96 the specific interfacial area occupied by “end-on” molecules (4), and Eqn \([2.6]\) was plotted for the wild type, I3C and I3W using the rate constants recorded in Table 2.2. These simulations are shown in Fig. 2.5, along with actual kinetic data recorded for each variant adsorbing to a planar silica surface, measured by ellipsometry and reported earlier by McGuire et al. (4).
The simulated kinetics differs to some extent from the actual kinetic data. In particular, initially, the actual adsorbed mass increased faster than that predicted. After that, the simulated curves reached their plateaus faster than the actual data. These observations are consistent with an increase in surface coverage, leading to an increase in the energy barrier to adsorption, such that adsorption rates may be functions of surface coverage. A dependence on surface concentration for adsorption rate constants has been proposed by Guzman et al. (14) in terms of activation energies for adsorption and desorption. Their model required that adsorption rate constants be at their maximum at the beginning of the adsorption, decreasing as the surface coverage increases. Our model assumed that the adsorption rate constants were indeed constant, which was probably a fair assumption in the case of the CD data, since the available surface area was so large that any change in rate constants could be considered negligible.

As the CD data were recorded at low surface coverages, and adequately described by constant values of $k_1$ and $k_2$, a second simulation was performed in which the rate constants of Table 2.2 were taken only as initial values, valid only when surface coverage is very low. Rate constants were then allowed to change with surface coverage according to
FIG 2.5 Adsorption kinetic data recorded for each variant adsorbing to a flat silica surface, using in situ ellipsometry (4). The solid line in each case follows Eqn [2.6] for (a) I3C, (b) wild type, and (c) I3W, where $k_1$ and $k_2$ are given in Table 2.2.
FIG 2.5 Continued
FIG 2.5 Continued


\[ k_1 = \alpha_1 \exp(-\beta_1 \theta_{\text{total}}) \quad [2.7] \]

and

\[ k_2 = \alpha_2 \exp(-\beta_2 \theta_{\text{total}}) \quad [2.8] \]

where the \( \alpha_i \) and \( \beta_i \) are positive constants, and \( \alpha_1 \) and \( \alpha_2 \) for each mutant are equal to the values of \( k_1 \) and \( k_2 \), respectively, reported in Table 2.2. These simulations are compared to the adsorption kinetic data in FIG 2.6; values of the \( \alpha_i \) and \( \beta_i \) are also shown for each fit. While the fits are improved, the actual adsorbed mass again increased faster than predicted. It may be that use of Eqns [2.7] and [2.8] is appropriate, but as all protein-nanoparticle mixtures prepared at the 1:2 ratio showed a larger, more rapid change in a-helix than those prepared at the 1:1 ratio, the estimates of \( \alpha_1 \) and \( \alpha_2 \) may be lower than they would be with higher nanoparticle concentrations. Additionally, adsorption to the colloidal silica used in these experiments may not be identical to adsorption on the optically flat silica used in ellipsometry (4). In either event, these results are encouraging as they suggest that protein adsorption may adequately be described with a model allowing for only two functional states. If that is the case, state-dependent, bulk-surface exchange constants could be estimated experimentally for any pair of proteins (15).

Knowledge of a protein’s tendency to exchange with protein adsorbed in a given state, its tendency to attain a given state upon adsorption itself, and its adsorption
FIG 2.6  Comparison of data of FIG 2.5 with Eqn [2.6] for (a) I3C, (b) wild type, and (c) I3W, where $k_1$ and $k_2$ are surface-coverage dependent rate constants, following Eqns [2.7] and [2.8].
FIG 2.6 Continued
FIG 2.6 Continued

\[ \begin{align*}
\alpha_1 &= 0.11 \\
\alpha_2 &= 0.90 \\
\beta_1 &= 1.70 \\
\beta_2 &= 4.00
\end{align*} \]
state-dependent tendency to exchange with incoming protein might be sufficient to quantify its ability when dissolved in a mixture of similarly characterized proteins to compete for surface sites and consequently sufficient to predict the time-
dependent makeup of an adsorbed layer.

2.5. Acknowledgements

We are indebted to Professor Brian Matthews and co-workers of the Institute of Molecular Biology, University of Oregon, for providing the bacterium strains needed for lysozyme production. We are grateful to Professor Curtis Johnson of Oregon State University for use of his instrumentation and expertise in CD and to Peter Billsten of Linköping University. Sweden, for providing insight on experimentation with silica nanoparticles. This material is based in part upon work supported by the National Science Foundation under Grant CTS-9501842 and The Whitaker Foundation.

2.6. References


CHAPTER 3

A MECHANISTIC APPROACH TO MODELING SINGLE PROTEIN ADSORPTION AT SOLID-WATER INTERFACES

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3.1. Abstract

A kinetic model for single component protein adsorption which can be readily extended to adsorption from multi-protein solutions was developed, and used to simulate adsorption of site-directed, structural stability mutants of bacteriophage T4 lysozyme. The model allows for two different adsorbed “states,” distinguished by different binding strengths and different occupied areas. The presence of an increasing energy barrier to adsorption was incorporated into the model by formulating the adsorption rate constants as functions of time. Numerical analysis was performed using the Marquardt method. Estimated model parameters were consistent with the effect of structural stability on adsorption. In particular, kinetic parameters were such that adsorption into the more tightly bound, conformationally-altered state was favored by less stable variants.

Key Words: bacteriophage T4 lysozyme; synthetic stability mutants; kinetic model; two-state adsorption mechanism

3.2. Introduction

Protein behavior at interfaces has been described with reference to electrostatic interaction (1), contact surface hydrophobicity (2-4), and protein properties such as molecular weight and dimensions in solution (5), among others. It is widely acknowledged that conformational changes take place upon adsorption (6). The effect of thermal stability on surface-induced conformational change has
been studied by several researchers (7-9). McGuire et al. (9) investigated the effect of structural stability on adsorption using the wild type and selected synthetic mutants of bacteriophage T4 lysozyme (T4L). They found that mutants with lower stability were more resistant to surfactant-mediated elution than mutants of higher stability. Using circular dichroism, Billsten et al. (10) and Tian et al. (6) found less stable T4L mutants to lose secondary structure at a greater rate and to a greater extent than more stable mutants, when adsorbed to colloidal silica.

While we know much about protein adsorption in a phenomenological sense, a more comprehensive, quantitative understanding of the process is still needed. Attempts to model protein adsorption can be classified into two major categories based upon whether diffusion or kinetics is rate limiting (11-12). A number of investigators have modeled adsorption from single-protein solutions as a mass transfer process. For example, Déjardin and Cottin (13) developed a model incorporating an apparent kinetic constant constructed as a combination of the adsorption constant at the interface and the rate constant related to the transport-controlled process based on the Lévêque model. They were able to describe the adsorption of a flowing solution of fibrinogen with good success. A shortcoming with diffusion models, however, is that they overlook structural alterations undergone by protein upon adsorption, which is relevant to protein function at an interface, and ultimately to competitive adsorption behavior.

Structural changes often constitute an important feature of kinetic models. Lundström and co-workers (12, 14) have constructed models for protein adsorption
isotherms, as well as adsorption kinetics from single- and multi-protein solutions. An essential feature of their mechanistic approach to modeling competitive adsorption is the existence of multiple conformational states, exhibiting different binding strengths, and therefore, different rates of exchange with protein in solution. Competitive adsorption is thus made a function of the rates of adoption of these states, and the state-dependent exchange rates. If adsorption from a single component protein solution could be adequately described by a model allowing for only two conformational states, all rate constants describing adsorption, unfolding and exchange could be measured experimentally for any protein pair. This would allow construction of a predictive model for competitive adsorption for any number of proteins that could be characterized in this way. In this paper, we develop such a model, and test its utility by comparing it to adsorption kinetic data recorded for synthetic structural stability mutants of T4L. In this way, estimated kinetic parameters could be compared to protein structural stability.

3.3. Model Development

For monolayer protein adsorption, occurring irreversibly into two adsorbed states, we can write expressions for the rate of change in fractional surface coverage for proteins adsorbed in state 1 and state 2, as follows:

\[ \frac{d\theta_1}{dt} = k_1 C(1-\theta_1 - a\theta_2) \]  

[3.1a]

and
where $\theta_i$ is the ratio of the adsorbed mass in state $i$, $\Gamma_i$, to the theoretical maximum adsorbed mass in a monolayer, $\Gamma_{\text{max}}$, $k_i$ is the rate coefficient for adsorption into state $i$, $a$ is the ratio of interfacial area occupied by a molecule in state 2 to that of a molecule in state 1, $C$ is the bulk concentration, and $t$ is the adsorption time. When compared with experimental adsorption data, kinetic models often underestimate the adsorbed mass at early times. This is because the adsorption rate constant decreases with time, a result of an increasing energy barrier to adsorption accompanying an increase in adsorbed mass.

A question arises as to the proper functional form to use for the rate constant. For the case of irreversible adsorption described here, the functional representation of the rate constant should allow it to be large initially, approaching zero near monolayer coverage. A simple expression for the nonlinear time-dependence of the rate constant, consistent with the criteria just stated, would be given by:

$$k = \alpha t^{-\beta}$$  \[3.2\]

where $\alpha$ and $\beta$ are variant-specific constants. Eqs. [3.1a] and [3.1b] could then be rewritten as:

$$\frac{d\theta_2}{dt} = k_2 C (1-\theta_1 - a\theta_2)$$  \[3.1b\]

$$\frac{d\theta_1}{dt} = \alpha_1 t^{-\beta_1} C (1-\theta_1 - a\theta_2)$$  \[3.3a\]
\[ \frac{d\theta_2}{dt} = \alpha_2 t^{-\beta_2} C(1 - \theta_1 - a \theta_2) \]  

3.3.1. Estimation of Model Parameters During the Initial Period of Adsorption

Tian et al. (6) measured the circular dichroism spectra of the T4L mutants used here in the presence of colloidal silica. In particular, they measured the kinetics of \( \alpha \)-helix loss, and used that data to provide a measure of generation of state 2 molecules. Upon comparison of their estimate for \( \theta_2 \) as a function of time to experimental adsorption data (the quantity \( \theta_1 + \theta_2 \) recorded as a function of time), it is clear that during the initial period of adsorption, the preponderance of adsorbed molecules exist in state 1. In this case, the adsorption rate during the initial period of protein solution-surface contact would be given by:

\[ \frac{d\theta_1}{dt} = \alpha_1 C t^{-\beta_1} (1 - \theta_1). \]  

Performing logarithmic transformations on both sides of the analytical solution of Eq. [3.4] yields:

\[ \ln(-(\ln(1 - \theta_1))) = \ln\left(\frac{\alpha_1 C}{1 - \beta_1}\right) + (1 - \beta_1)\ln t. \]  

[3.5]
We can therefore fit adsorption kinetic data to Eq. [3.5], in order to estimate parameters $\alpha_i$ and $\beta_i$.

### 3.3.2. Estimation of Parameters During the Entire Adsorption Period

Adsorption parameters were estimated using the Marquardt method for nonlinear least squares minimization (15-17). The forward finite difference method was used to approximate the first-order derivatives of the model function, giving the shortest iteration time:

$$\frac{\partial f_i(k_0, t)}{\partial k_i} \approx \frac{1}{\partial k_0} \left( f(k_0 + \delta k_0, t) - f(k_0, t) \right)$$

where $k$ is a column vector of adsorption parameters, and $f(k, t)$ is the model equation. Equation [3.6] was used at each iteration to calculate each element of the Hessian matrix, $A$, defined as

$$A_{ij} \approx 2 \sum_{m=1}^{t} \left( \frac{\partial f(k, t_m)}{\partial k_i} \frac{\partial f(k, t_m)}{\partial k_j} \right).$$

The Hessian matrix was used to determine the step size for each subsequent calculation of the objective function until the minimization was within an acceptable tolerance, where:

$$\delta k = -A^{-1} \nabla_k \chi^2(k_0).$$
3.4. Results and Discussion

Adsorption kinetic data have been recorded for the wild type, and several structural stability mutants of T4L at derivatized silica surfaces (9). The wild type and two of these mutants were of interest here. In one mutant, the isoleucine at position 3 was replaced with cysteine (I3C), resulting in a protein with $\Delta G_{\text{unfolding}}$ of 1.2 kcal/mol greater than that of the wild type. The other mutant was produced by substitution of I3 with a tryptophan residue (I3W), resulting in a variant with $\Delta G_{\text{unfolding}}$ of 2.8 kcal/mol less than that of wild type. Data recorded during the first two minutes of adsorption of each variant (35-40 measurements in each case) to hydrophilic silica were fit with Eq. [3.5], in order to estimate the kinetic parameters $\alpha_1$ and $\beta_1$. These estimates, along with an indication of the goodness of fit ($R^2$) achieved in each case, are shown in Table 3.1.

Parameter $\alpha_1$ was estimated to be the least for I3W, indicating that its adsorption into state 1 was small relative to the other two variants. This is consistent with I3W being the least stable mutant. In particular, a greater proportion of adsorbed molecules in an I3W layer would exist in the unfolded form (state 2) in comparison to the wild type and I3C (9). The value of $\beta_1$ was found to be greatest for I3W, consistent with its value of $k_1$ decreasing faster than that of the other two variants. The fact that $\alpha_1$ was greatest and $\beta_1$ the least for wild type and not for the more stable I3C variant, is probably due to I3C not achieving monolayer coverage on any surface tested (9). Its initial rate may therefore be slower than that of the wild type, even though it adsorbed mainly into state 1.
Table 3.1 Kinetic parameters $\alpha_l$ and $\beta_l$ estimated for each T4L variant, using initial adsorption data. Coefficients of determination ($R^2$) are shown in parentheses.

Theoretically, this simple analysis assumes that each T4L variant adsorbs only in state 1 during the first two minutes of adsorption. Actually, there may be a concomitant adsorption of molecules into state 2. In any event, the data of Table 5.1 are consistent with the thought that either more stable variants adsorb more readily into state 1, or adsorption into state 2 is a slower process than adsorption into state 1.

The kinetic data for each variant fit to the solution of Eq. [3.3] (i.e. $\theta_1 + \theta_2$) are shown in Figure 3.1. Individual simulations of $\theta_1$ and $\theta_2$ are shown as well. Parameters $\alpha_2$, $\beta_1$ and $\beta_2$ were estimated for each variant, and are listed in Table 3.2. Note that the value of $\alpha_1$, estimated by analysis of the initial adsorption data, was retained, while the value of $\beta_1$ was recalculated in each case. The values of $\alpha_1$ estimated using the initial adsorption data would be more reliable than those of $\beta_1$ estimated using the same data set, as $\beta_1$ is relevant to the decrease in $k_1$ throughout
Figure 3.1 Adsorption kinetic data fit to the solution of Eq. [3.3] (i.e. $\theta_1 + \theta_2$) for:
(a) I3C; (b) wild type; and (c) I3W. Individual simulations of $\theta_1$ and $\theta_2$ are shown for each T4L variant as well.
Figure 3.1 Continued.
Figure 3.1 Continued.
Table 3.2 Kinetic parameters estimated for each T4L variant.

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<tr>
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</tr>
<tr>
<td>$\beta_2$</td>
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</tr>
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</table>

the entire period of adsorption. Parameter $\alpha_1$ on the other hand can be determined with certainty, if adsorption during the initial period occurs in state 1.

Table 3.2 shows that $\alpha_2$ estimated for I3W was greater than that estimated for each of the more stable variants. In addition, the value of $\beta_2$ estimated for I3W was two orders of magnitude smaller than those of the more stable variants. This indicates that relative to the other T4L variants, I3W adsorbs more readily into state 2, with its value of $k_2$ decreasing much more slowly with increasing surface coverage. The fact that the model adequately describes the adsorption kinetics in each case, and that the estimated parameters were found to make sense physically, lends credibility to the mechanistic model described here. In particular, single protein adsorption can be modeled as an irreversible process using a two-state mechanism where molecules in each state occupy different interfacial areas, and in which the rate constants are initially high, and approach zero as monolayer coverage is attained. Since the variant-specific kinetic parameters varied in a
manner consistent with structural stability, the model may eventually be useful as a predictive tool, if relevant protein molecular properties are quantified. Further, this model ought to be extendible to quantifying competition in multi-protein mixtures, if adsorption in such cases is a function of a given protein's tendency to adopt each state (given by its time-dependent values of $k_1$ and $k_2$), and state-dependent exchange constants. Exchange constants are experimentally accessible, and comparisons of competitive adsorption data to the behavior predicted by this two-state mechanism will contribute to the subject of a future report.

3.5. Acknowledgement

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CHAPTER 4

CONCENTRATION EFFECTS ON ADSORPTION OF HEN LYSOZYME AND T4 LYSOZYME VARIANTS TO SILICA

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This research manuscript will be submitted for the possible consideration of publication.
4.1. **Abstract**

The effect of protein concentration on the interfacial behavior of protein was investigated using hen egg white lysozyme and wild type and its stability mutants of bacteriophage T4 lysozyme. The adsorption kinetic data at hydrophilic silica surface were monitored with in-situ ellipsometry. The wild type and stability mutants of T4 lysozyme were produced by site-directed mutagenesis at which isoleucine residue at position 3 was replaced with tryptophan and cysteine amino acid residue, respectively. An adsorption mechanism was introduced allowing protein to adopt three adsorbed states differentiated by binding strength, i.e. weak, moderate, and tight bindings. The binding strength was determined by performing rinse and dodecyltrimethylammonium bromide-mediated elution. The considerable effect of protein concentration on the interfacial behavior of protein was substantial in determining the weakly and moderately bound forms. However, the influence of the protein concentration on the tightly bound state was not so significant within the frame of our study. The formation of the adsorbed states was directly related to the stability of the molecular structure. It is also plausible that the binding strength could be characterized by the nature of the implicit interaction between a protein and a surface according to the comparison between T4’s and hen egg white lysozyme. In general, the stable protein adsorbed more than the less stable protein.
4.2. Introduction

The interfacial behavior of protein has been considered to be affected by several factors and extensive studies have been performed in terms of electrostatic interactions (Norde et al., 1978; Arai and Norde, 1990), hydrophobicity (Elwing et al., 1987; Malmsten, 1995; Tilton et al., 1991), and molecular properties such as mass and dimensions (Wahlgren et al., 1993a), and surface types (Horbett and Weathersby, 1981). A few studies relevant to concentration-dependent adsorption have been performed, and it has been found that there exists a substantial influence of protein concentration on protein adsorption (Wahlgren and Arnebrant, 1992, 1995; Elofsson et al., 1997). Fabrizius-Homan and Cooper (1991) reported that the adsorption of human serum albumin, fibrinogen, and fibronectin at polymeric materials from binary protein mixture was decreased with increasing the concentration of vitronectin. Therefore, the increased concentration of vitronectin had a negative effect on adsorption of other proteins. However, Horbett (1980) observed that the adsorbed mass of hemoglobin reached 0.03 μg/cm² even at very low concentration such as 0.02 μg/cm² in plasma protein although the concentration of all proteins were much higher. At the same time, the adsorbed states at different concentrations have not been clearly quantified to apply improving the understanding of the more complicated systems such as a multicomponent system. Therefore, it is desirable to gain more detailed information on the role of the protein concentration in adsorption. There has not been any attempt to relate the effect of protein concentration on the interfacial behavior in conjunction with the stability of the molecular structure using stability mutants produced by
recombinant DNA technology. In this work, we investigated the correlation between the protein concentration and adsorbed states of the protein. The quantification of the different adsorbed states was made by the ellipsometric adsorption data obtained during rinse and surfactant mediated elution. Use of the stability mutants enabled us to study how the molecular stability of a protein to take part in the characterization of the adsorption.

The model proteins used in this study were hen lysozyme, bacteriophage T4 lysozyme (T4L), and two T4L stability mutants. These stability mutants were produced by substituting isoleucine at position 3 with one amino acid residue, such as cysteine (I3C) or tryptophan (I3W). The produced I3C was more stable than the wild type of T4L due to the added disulfide bond but I3W was less stable than the wild type due to the unfavorable steric interactions and unsatisfied hydrogen bonds. The hen lysozyme (HEW) was chosen since HEW has very close similarity in the enzymatic functionality and the molecular properties, such as size and molecular weight, compared to T4L but has four disulfide bonds inside of the molecule so that the molecular stability can be closely related to that of T4L (Matthews et al., 1981).

4.3. Materials and Methods

4.3.1. Surface Preparation.

The silicon wafers (hyperpure, type N, phosphorous doped, plane 1-0-0) were purchased from Wacker Siltronic Corporation (Portland, OR) and used as a model
surface since they could provide almost perfect reflection. The wafers were cut into rectangular shape of approximately 1 cm x 3 cm. These silica surfaces were baked in furnace for 18 minutes at 1000 °C (P_{O_2} = 1 atm) to deposit an oxide layer of approximately 300 Å on the surface. Then each surface was treated to be hydrophilic following the procedure previously described by McGuire et al. (1995a, b, c). Each silica surface was put into 10 ml of mixture of NH_4OH:H_2O_2:H_2O (1/1/5 volume ratio) and heated for 15 minutes at 80 °C in a water bath. Then the heated surfaces were rinsed with 20 ml of distilled deionized water followed by heating in a 10 ml mixture solution of HCl:H_2O_2:H_2O (1/1/5 volume ratio) at 80 °C in a water bath for 15 minutes. Then, each surface was rinsed with 30 ml of distilled deionized water. All the surfaces after treatment were stored in a 50 % ethanol solution in order to maintain the stability of the surface properties. For the measurement, the surface was rinsed with an excess of distilled deionized water and blown dry with N_2 gas.

4.3.2. Proteins

Hen egg white lysozyme was purchased from Sigma (L-6876) and used without further treatment. The wild type of bacteriophage T4 lysozyme and its stability mutants were produced from transformed cultures of Escherichia coli strain RR1 and purified in our laboratory. Individual bacteria strains containing the mutant lysozyme expression vectors selected for this work were provided by Professor Brian Matthews and co-workers at the Institute of Molecular Biology, University of Oregon (Eugene, OR) and stored at −80 °C until use. The complete description on the
procedures for the expression and purification of T4L was described elsewhere (Alber and Matthews, 1987; Muchmore et al., 1989; McGuire et al., 1995a, b, c). Preparations were stored without further treatment at 4 °C.

The substitution of the isoleucine at position 3 with cysteine (I3C) stabilizes the molecule due to the increased thermal stability resulting from a formation of a disulfide link with cysteine at position 97 (Alber and Matthews, 1987). The less stable mutant was produced by the substitution of isoleucine at position 3 with tryptophan (I3W). This causes unfavorable steric interactions, unsatisfied hydrogen bonds, and differences in both van der Waals and hydrophobic interactions in the region of the substitution. The structural stability of each mutant was quantified by ΔΔG: the difference between the free energy of unfolding of the mutant protein and that of the wild type at the melting temperature of the wild type. At pH 6.5, ΔΔG = +1.2 kcal/mol for I3C and −2.8 kcal/mol for I3W (Matsumura et al., 1988).

4.3.3. Adsorption Kinetics and Surfactant-Mediated Elution

The change of the surface properties due to the formation of protein film at a solid surface was measured in terms of the changes of the optical angles, ψ, and Δ, with in-situ ellipsometry. These angles were analyzed in order to calculate the thickness of the adsorbed layer and subsequently converted into adsorbed mass using the ratio of a molecular weight to molar refractivity, M/A, and specific volume, ν, of a protein by a numerical calculation using the FORTRAN (OEP) program. The values of M/A were 3.825 for I3W, 3.827 for both Wild type and I3C, and 4.19 for HEW
according to Cuypers et al. (1983). The values of $v$ were 0.78 for all T4L variants and 0.712 for HEW. The maximum adsorbed masses for T4’s and HEW were calculated based on the compact monolayer coverage in native conformation as 0.396 μg/cm$^2$ and 0.31 μg/cm$^2$, respectively.

All experiments were performed in 0.01 M phosphate buffer (pH 7) to dilute all T4L stock solutions to the desired concentrations. Each protein used in this study was obtained from the same batch. Initially the quartz cuvette was filled with 4.5 ml of 0.01 M phosphate buffer (pH 7.2) with a magnetic stir bar. The surface was submerged into the cuvette and allowed to be equilibrated with the buffer at least 30 minutes. When the optical readings of the bare surface became steady, 0.5 mL of the diluted protein stock solution was injected into the cuvette while the magnetic stir bar was rotating in a speed approximately 300 rpm. The time interval of each measurement was set at 15 seconds. The contact between protein and surface was allowed for one hour followed by five minutes rinse with about 200 ml of 0.01 M phosphate buffer. Then the surface was monitored for additional 10 minutes. After that, the surface was eluted with dodecyltrimethylammonium bromide (DTAB) for 10 minutes followed by five minutes rinse with 0.01 M phosphate buffer. Then the surface was monitored for additional ten minutes. Five different bulk concentrations for all proteins were used at 0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL. All measurements were collected at room temperature.
4.4. Results and Discussion

The adsorption kinetic data at silica surfaces were shown in FIG 4.1 with 60 min contact time and 5 min of rinse followed by 10 min monitoring for all proteins. It was apparent that the adsorbed rate became faster with increasing protein concentration. The adsorbed mass in relation with the stability of proteins showed clear difference in the interfacial behavior that stable protein, I3C, adsorbed more than unstable proteins. It can be explained in that the unstable protein readily unfolded at surface and occupied more surface area. As a result, fewer molecules could adsorb than stable protein. The adsorption rate became faster with the increase of protein concentration. The amount remaining after rinse was also increased with increasing protein concentration. This result indicates the more population of the adsorbed molecules adopted the more tightly bound states with increasing protein concentration. The molecules removed at rinse were considered to form a weakly bound form to the surface (state 1). The resistance to elution mediated by dodecyltrimethyl-ammonium bromide (DTAB) could be treated as a fair measurement of the population of the adsorbed molecules with stronger binding (McGuire et al., 1995b). Therefore, the molecules remaining after elution can be considered as a tightly bound state (state 3) and those removed by surfactant might be in a moderately bound form (state 2). The adsorbed mass of I3C variant at 1.0 mg/mL obtained in this study was higher than that previously reported (McGuire et al., 1995b; Singla et al., 1996). We are not sure for the reason of this difference. However, the fact that the concentration of the stock solution obtained from fermentation was lower than that used in the previous study
FIG 4.1 Adsorbed mass of each protein as a function of time on hydrophilic surface. (a) I3W, (b) Wild, (c) I3C, and (d) HEW. The contact time was 60 min and rinse for 5 min followed by 10 min monitoring session. Protein concentrations were 1 mg/mL (■), 0.5 mg/mL (★), 0.1 mg/mL (▲), 0.05 mg/mL (+), 0.01 mg/mL (○).
FIG 4.1. Continued
would have an effect to increase the salt concentration in stock solution. Hence, it was possible that the higher salt concentration might promote the I3C adsorption. Nevertheless, the experimental data presented in this paper is corresponding to the hypothesis that a more stable variant may yield the greater adsorbed mass than a less stable variant. It was probably because the structure of the less stable protein was more spread out than the stable protein at the silica surfaces which would reduce the available surface area to a greater extent. The adsorbed amounts of HEW were slightly lower than those reported elsewhere (Wahlgren et al., 1995). This is possibly due to the effect of continuous stirring during their measurements which might force proteins to adsorb more.

From the experiments, we could obtain three different adsorbed masses at each protein concentration, i.e. after a 60 min contact time, after rinse, and after elution. A schematic diagram of an adsorption mechanism incorporating these measurements by allowing protein to adopt three different binding states at surfaces was shown in FIG 4.2. The adsorbed amounts measured after a 60 min contact time, after rinse, and after elution, designated as $\Gamma_{60}$, $\Gamma_{\text{rinse}}$, and $\Gamma_{\text{elution}}$, respectively, for all protein concentrations were plotted in the FIG 4.3. The changes of the amounts of $\Gamma_{60}$ and $\Gamma_{\text{rinse}}$ were proportional. The $\Gamma_{60}$ of I3C was the greatest followed by HEW. Both I3W and the wild type adsorbed less than HEW while $\Gamma_{60}$s of Wild and I3W were very close. The amount remaining after elution, $\Gamma_{\text{elution}}$, seemed to reach plateau value even at low concentration. The adsorbed masses in state 1 and 2, $\Gamma_1$ and $\Gamma_2$, were obtained simply from $\Gamma_{60} - \Gamma_{\text{rinse}}$ and $\Gamma_{\text{rinse}} - \Gamma_{\text{elution}}$, respectively. The $\Gamma_3$ was equivalent to the $\Gamma_{\text{elution}}$. 
FIG 4.2 Schematic diagram describing adsorption mechanism allowing proteins to adopt three different adsorbed states, i.e. loosely, moderately, and tightly bound states.

In all cases, the amount of $I_2$ was greater than other adsorbed states and the $I_1$ was remaining constant. The reason for the dominantly greater amount of $I_2$ in most cases could be explained by the fact that adsorption was driven to the direction of minimizing the free energy at interface which will contribute to the conformational change of the molecule (Kaelble and Moacanin, 1977; Absolom et al., 1987). Hence, it is possible that the more adsorbed molecules tended to unfold their structure rather than sustaining the native form. For 13C, the relative amount of the $I_2$ compared to other adsorbed states was greater than other proteins. This was because the structural rigidity of the protein might restrict the protein from adopting the fully denatured form and proceed to the moderately unfolded form. In this regard, the moderately unfolded conformation would be a more favorable form for 13C compared to other proteins. For $I_3$, all proteins reached plateau values even at low concentration and $I_3$ was less than 0.1 $\mu$g/cm$^2$ which is less than 30% of the compact monolayer coverage. This
FIG 4.3 Adsorbed masses measured at 60 min contact time (■), after rinse (◆), and after elution (▲) for (a) 13W, (b) Wild, (c) 13C, and (d) HEW at all protein concentrations. Solid curves indicate the trend of the change of adsorbed data.
FIG 4.3. Continued
observation may imply that a surface cannot be covered with molecules in state 3 only and that there exists a transitional moment from which the adsorbed molecules can not unfold to reach the fully unfolded form. Once the surface coverage reaches to the transitional coverage, adsorption will occur into other states, i.e. state 1 or 2. The fractional surface coverages of state 3 were calculated and tabulated in the Table 4.1 together with the estimates of the critical surface coverage of state 3. The critical surface coverages were obtained from the average of the measurements at 0.5 and 1.0 mg/ml since the critical surface coverage considered to reach the plateau values above the 0.5 mg/mL. The results showed that the critical surface coverage was inversely proportional to the molecular stability. This trend was presumably due to the fact that proteins with more flexible structure would readily achieve the bound states hard to remove from the surface. It is also worthy to note that the progress to state 3 was also

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</table>

Table 4.1 Surface coverage after surfactant-mediated elution at all bulk protein concentrations and transition surface coverage of all proteins where adsorption transfer from tight binding to loose or moderate bindings.
dependent on the contact time. A study on the elutability was reported that the
elutability of fibrinogen mediated with sodium dodecyl sulfate (SDS) was decreasing
with increasing contact time (Chinn et al., 1992). At the same time, their data showed
that the elutability approached a limiting value with time, which might be considered
to be the critical surface coverage. The molecular structure of HEW was considered to
be more stable than T4’s since HEW had four disulfide bonds inside. Therefore, we
expected the transitional adsorbed mass of HEW should be smaller than that of Wild
or I3W. Interestingly when we compared the estimates of T4’s with HEW, we found
that the transitional adsorbed mass of HEW was greatest among all proteins used in
this study. This is presumably because HEW has a stronger affinity with the silica
surface than T4’s and can form very tight bonds with the surface although the stability
of HEW was greater than T4’s. As Vinaraphong et al. (1995) pointed out, HEW was a
protein forming the most compact monolayer of any protein at surfaces and was bound
to the surfaces more tightly in spite of its high thermal stability. In spite of the
existence of the evolutionary implications and same enzymatic functions of both HEW
and T4 lysozymes (Matthews et al., 1981), the nature of adsorption of these proteins
was different. It was probably because the divergent evolution resulted in
discrepancies of molecular properties. A mathematical description that may explain
the adsorption mechanism into three different adsorbed states can be formulated by
incorporating the existence of the transitional point for the adsorbed state 3. Defining
the maximum adsorbed mass of protein that could be adsorbed in a monolayer as $\Gamma_{\text{max}}$
($\mu g/cm^2$) and $\theta_i$ is a surface coverage of an adsorbed state $i$, defined as $\Gamma_i/\Gamma_{\text{max}}$. 
\[ \frac{d\theta_1}{dt} = k_1C(1 - \theta_1 - \theta_2 - \theta_3) \]  \hspace{1cm} [4.1] \\
\[ \frac{d\theta_2}{dt} = k_2C(1 - \theta_1 - \theta_2 - \theta_3) \]  \hspace{1cm} [4.2] \\
\[ \frac{d\theta_3}{dt} = k_3C(\theta_{\text{critical}} - \theta_3) \]  \hspace{1cm} [4.3]

where \( k_i \) (mg\(^{-1}\)) is a rate coefficient of adsorption to state \( i \). \( C \) (mg/mL) is the protein bulk concentration. \( t \) is time (min). \( \theta_{\text{critical}} \) is the transitional surface coverage of state 3 which is the ratio of the \( \Gamma_3 \) to the \( \Gamma_{\text{max}} \).

The nature of the interaction of protein with a surface can be analyzed from the comparison of the fraction of each adsorbed state in adsorbed molecules. By defining the fraction of each state as \( F_i/F_0 \), the calculated fractions of all adsorbed states were shown in the FIG 4.4. For all the proteins studied, approximately 80% of adsorbed molecules were existing in conformationally rearranged form up to some extent. The greatest amount consisting of the protein layer at the surface was molecules in state 2. This may imply that the conformational alteration is a thermodynamically favorable event as protein interacts with the surface. Above 0.1 mg/mL, the fraction of each adsorbed state became more likely consistent only except \( \text{I3C} \).

The increase of the protein concentration had a negative effect on state 1 so that the relative amount of state 1 in the adsorbed protein became less with increasing protein concentration. No simple implication of the effect of stability on adsorption of state 1 could be obtained. However, \( \text{HEW} \) and \( \text{I3W} \) of which binding strengths were
FIG 4.4 Fraction of each bound state, i.e. weak binding (■), moderate binding (●), and tight binding (▲): (a) I3W, (b) Wild, (c) I3C, and (d) HEW. The fraction was obtained from the ratio of the adsorbed mass of each bound state to the adsorbed mass measured after 60 min of contact time. Adsorbed masses of these adsorbed states were obtained from \( \Gamma_{60} - \Gamma_{\text{rinse}} \), \( \Gamma_{\text{rinse}} - \Gamma_{\text{elution}} \), and \( \Gamma_{\text{elution}} \), respectively.
FIG 4.4 Continued
considered to be stronger than other proteins adsorbed in a similar pattern. Their fraction in state 1 was smaller than Wild type but greater than I3C. Above 0.1 mg/mL, the calculated fraction of state 1 was not significantly affected by the increase of protein concentration. In all cases, the fraction of the moderately bound form was the greatest among all adsorbed states. The effect of protein concentration on the adsorption to the moderately bound form was most pronounced in the case of I3C. The fraction of I3C in state 2 was increased from 0.11 at 0.01 mg/mL to 0.67 at 1.0 mg/mL. For I3C, the adoption of molecules to the moderately bound form was gradual with the change of protein concentration while other proteins were relatively independent on the protein concentration. For the tightly bound form, I3W, the most unstable protein, reached a greater level than other T4’s. HEW lysozyme reached almost the same level as I3W in spite of a relatively stable structure. As pointed out earlier in this paper, it is presumably due to the stronger affinity of HEW toward the silica surface. Only for I3C, the protein concentration affected the tightly bound state. The fraction of state 3 was decreased from 0.60 down to 0.19 with increasing protein concentration. The adsorbed amount of state 3 was directly related with the stability of molecular structure.

4.5. Acknowledgements

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vector required for the production of T4 lysozyme. We also are grateful to Dr. S. Subramanian (Electronic and Computer Engineering, Oregon State University) for helping us to deposit the oxide layer on the silica surfaces.

4.6. References


CHAPTER 5

COMPETITIVE ADSORPTION OF BACTERIOPHAGE T4 LYSOZYME AND TWO STABILITY MUTANTS AT HYDROPHILIC GLASS SURFACES

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This research manuscript will be submitted for the possible consideration of publication.
5.1. **Abstract**

Single component adsorption and competitive adsorption of bacteriophage T4 lysozyme and two stability mutants, labeled with $^{125}$I, were performed on hydrophilic silica. The stability mutants were produced by the site-directed mutagenesis by replacing the isoleucine amino acid residue at position 3 with cysteine or tryptophan. The thermal stability of produced mutants was characterized by the difference between the free energy of unfolding of the mutant protein and that of the wild type at the melting temperature of the wild type, $\Delta \Delta G$: +1.2 kcal/mol for I3C and −2.8 kcal/ml for I3W at pH 6.5. The correlation between of structural stability of proteins and competitive adsorption of protein in the presence of other kind of protein was also studied using labeled protein with radioactive $^{125}$I. The effect of the molecular stability on the adsorption was substantial in the interfacial behavior of protein. The more stable protein showed faster adsorption kinetics at initial period of time. However, the adsorbed mass of the less stable protein achieved the higher adsorbed mass. The presence of the less stable protein had an inhibitory effect on the adsorption of the more stable protein. A cooperative effect of the presence of one protein on the other protein could exist promoting the adsorption of the other protein. The molecular stability was appeared to be a factor determining the organization of the adsorbed protein layer at a surface.
5.2. Introduction

The interfacial behavior of proteins has a variety of applications in biotechnology and biological engineering. The competitive protein adsorption in the presence of dissimilar protein is significant phenomena such as blood compatibility of biomaterials (Horbett and Brash, 1995), bacterial adhesion to teeth, and formation of fouling film in food processing, and coating of medical devices with thin, transparent, and biocompatible films (Tsai et al., 1990) etc. Adsorption process will be affected by several factors such as the hydrophobicity of surface (van Dulm and Norde, 1983), protein concentration in bulk (Brynda et al, 1986; Lee et al., 1999), the stability of molecular structure (Tian et al., 1998), the charge density of a protein (McGuire et al., 1995c). The concentration of the dissimilar protein in a multicomponent system also plays an important role (Fabrizius-Homan and Cooper, 1991).

However, the detailed reason of the difference in adsorption behavior of a protein at multicomponent system has not been well understood. In this study, we investigated the effect of the stability of molecular structure of protein on the competitive and the sequential adsorption.

Several researchers performed the competitive adsorption behavior of protein in mixture. The interaction of one protein with a given surface depends on the presence of dissimilar proteins. Fabrizius-Homan and Cooper (1991) studied the effect of the pressure dissimilar proteins, human serum albumin, fibrinogen, and fibronectin on the adsorption of vitronectin at polymeric biomaterials in binary-component systems. Their result showed that vitronectin adsorbed more in the presence of
albumin than in the presence of fibrinogen or fibronectin. They also reported the increase of concentration of dissimilar protein attenuated the adsorption of vitronectin. The competitive adsorption behavior to be susceptible to the nature of surfaces. Warkentin et al. (1994) observed that the albumin was interrupting the IgG adsorption on hydrophobic surfaces but promoted on hydrophilic surfaces. Adsorption from a binary and ternary mixture of human serum albumin, human IgG, and human fibrinogen on plasma polymer surfaces was studied with total internal reflection fluorescence spectroscopy and ellipsometry (Lassen and Malmsten, 1997). They found human serum albumin and IgG were dominating at the hydrophobic surface but fibrinogen was dominant at the hydrophilic surfaces. Ellipsometry has been widely used to monitor the protein adsorption at surface. Although indirect measurements of the adsorbed mass consisting of mixture of proteins with ellipsometry is possible, it does not give accurate measurements of the adsorbed mass if the layer consists of different types of protein.

Despite of extensive studies on competitive protein adsorption, the exact reason how the interfacial behavior of a protein varies in the presence of a dissimilar protein has not been fully explained. In this paper, we investigated the effect of stability of the molecular structure on the competitive adsorption from mixture using stability variants of T4 lysozyme. The sequential adsorption was performed in order to find the exchange reaction between the adsorbed protein molecule and the oncoming dissimilar protein molecules. The adsorbed mass of protein was measured using labeled protein with $^{125}$I as a tracer.
Radioactive iodine has been one of the most commonly used radioactive isotope for the protein labeling in radioimmunoassys (Bolton and Hunter, 1972). Although there are several methods available for the iodination of proteins, i.e. the iodine monochloride method (Samols and Williams, 1961; Horbett, 1981), and enzymatic iodination using lactoperoxidase (Thorell, 1972), we chose the chloramine-T method because of the simplicity and convenience of the procedure. This method was developed by (Hunter and Greenwood (1962) as an efficient procedure directly to substitute 125I into the tyrosyl residues in the proteins while minimizing the chemical damage on molecular structure (Greenwood et al., 1963; Greenwood, 1971; Kirkham and Hunter, 1971; Bolton and Hunter, 1972).

5.3. Materials and Methods

5.3.1. Buffers

For the radioiodination, we used 0.05 M sodium phosphate buffer (pH 7.5), 0.01 M sodium phosphate buffer saline (pH 7.0), 0.05 EDTA-sodium phosphate buffer saline (pH 7.0) and 0.05 M tris-HCl buffer. The adsorption and elution experiment were performed in 0.01 M sodium phosphate buffer, pH 7.0.
5.3.2. Surface Preparations

Circular test glasses were purchased from Fisher (12 mm dia.; 12-545-80, Pittsburgh, PA). Before treatment of the surfaces, we cut the surfaces into half due to the size limitation of the counting tubes used in the Gamma 5500 counter (Beckman, CA). Each surface was treated to be hydrophilic following the procedure mentioned elsewhere (McGuire et al., 1995a,b,c). Each surface was heated in a glass tube containing the mixture solution of NH$_4$OH:H$_2$O$_2$:H$_2$O (1:1:5 volume ratio) at 80 °C in water bath. After 15 min heating, each surface was rinsed with distilled deionized water and heated in the solution of HCl:H$_2$O$_2$:H$_2$O (1:1:5 volume ratio) for additional 15 min at 80 °C in the water bath. After heating, each surface was rinsed with 30 mL of distilled deionized water and stored in 50 % ethanol solution separately. Prior to use, we rinsed the surfaces with an excess amount of the distilled deionized water.

5.3.3. Proteins

Proteins used in this study were the stability mutants of bacteriophage T4 lysozyme, an endoacetyl-muramidase produced late by T4 phage infection of Escherichia Coli. T4 lysozyme causes the bacteria cell wall lysis along with the release of the progeny phage particles (Tsugita, 1971). Two stability mutants of T4 lysozyme were produced by replacing the isoleucine at position 3 with cysteine or tryptophan and designated as I3C or I3W, respectively. For I3C, due to the disulfide bond formed between the substituted cysteine and cysteine at position 97 (Alber and
Matthews, 1987), the thermal stability of I3C was increased compared to wild type (WLD). The less stable mutant was produced by the substitution of isoleucine at position 3 with tryptophan (I3W) resulting from the unfavorable steric interactions, unsatisfied hydrogen bonds, and differences in hydrophobic interactions in the region of the substitution (Matsumura et al., 1988). Produced protein stock solutions were stored at 4 °C and used without further treatment. The detailed procedure of the fermentation and purification of T4's was summarized in Chapter 3, Materials and Methods.

5.3.4. Adsorption Kinetics

The radioactive sodium iodide in NaOH solution was purchased from Amerhsam (Arlington Heights, Illinois). Chloramine-T was used as an oxidizing reagent when proteins were labeled. All adsorption experiments were performed at room temperature. Adsorption data were collected with time. First, each hydrophilic glass surface was put into test tubes which contain 0.1 mL of 0.01 M phosphate buffer so that the surface was completely submerged under the solution. Protein stock solution was prepared to possess a specific activity of approximately $5 \times 10^5$ cpm/mg. By adding 0.5 mL of the stock solution into each tube followed by gentle agitation, adsorption was started. The adsorption was terminated after desired contact time by transferring the surface to the phosphate buffer for dip-rinsing while minimizing the exposure of the surface to the air. The radioactivity of the adsorbed protein was counted after transferring the rinsed samples into the empty tubes.
5.4. Results and Discussion

The adsorption kinetic data of the single component of stability variants at the hydrophilic glass surface were shown in FIG 5.1. Data showed that the interfacial behavior of the variants was substantially dependent on their molecular stability. The early time adsorption kinetics of the most stable variant, I3C, was fastest and WLD was slowest. The adsorbed mass of both I3W and WLD were gradually increased with increasing contact time while I3C reached the plateau value of adsorbed mass earlier. The approximated plateau values of adsorbed mass of these variants were 0.803, 0.892, and 0.985 µg/cm² for I3C, Wild, and I3W, respectively. These adsorbed masses
of the stability variants were inversely proportional to the degree of the stability of the variants. This result may be explained according to the earlier studies. Tian et al. (1998) reported that the unstable variant underwent the conformational change faster and to a greater extent than the stable protein. At the same time, the greater amount in the adsorbed mass of the unstable protein existed in the tightly bound form which is harder to remove than loosely bound form (Lee et al., 1999). Therefore, the adsorbed mass obtained after rinse could be due to the result of the binding strength and this result was in agreement with the report from these earlier studies.

The interfacial behavior of a protein in the presence of a dissimilar protein differs from that in a single component system due to the competition for the surface area and the exchange reaction between proteins (Horbett, 1980; Tremsina et al., 1998). When one protein possesses the stronger surface affinity, then the protein possibly replaces the dissimilar protein existing on the surface. A mechanism describing the competitive adsorption from a binary protein mixture was depicted in FIG 5.2 allowing proteins to adopt the multiple adsorbed states. The rate of the exchange reaction between two dissimilar proteins was given with the rate coefficient of $S_{AB}$.

The adsorption kinetic data of one variant in the presence of a dissimilar variant were compared with the single component adsorption in FIGs 5.3 through FIG 5.5 for I3C, WLD, and I3W, respectively. According to the FIG 5.3, the presence of either WLD or I3W variant appeared to be inhibitory to the adsorption of the I3C variant. Initially, I3C adsorbed more than its single component adsorption but the
FIG 5.2 A simple mechanism describing the competitive adsorption from binary protein mixture. The more competitive protein was designated in filled circle.

adsorbed mass decreased presumably due to the exchange by I3W or WLD. As shown in FIG 5.4, the adsorption pattern of the WLD was not significantly changed in the presence of either I3W or I3C while the negative effect of the presence of the WLD on the adsorption of the I3C was observed from the FIG 5.3. The adsorbed mass of the

FIG 5.3 Competitive adsorption of I3C labeled with $^{125}$I in the presence of WLD (triangle) or I3W (diamond). The single component adsorption of I3C (square) was included for comparison. In all cases, protein concentrations were 100 µg/ml.
FIG 5.4 Competitive adsorption of WLD labeled with $^{125}$I in the presence of I3W (triangle) or I3C (diamond). The single component adsorption of WLD (square) was included for comparison. All conditions were same as FIG 5.3.

FIG 5.5 Competitive adsorption of I3W labeled with $^{125}$I in the presence of WLD (triangle) or I3C (diamond). The single component adsorption of I3W (square) was included for comparison. All conditions were same as FIG 5.3.
WLD in the presence of I3W was slightly lower than its single component adsorption. The consistent increase of the adsorbed mass of the WLD may give an implication that the exchange reaction by the I3W variant was not taking place. Rather a moderate degree of the competition for adsorption sites between these two variants might exist so that the adsorbed mass was lower than the single component adsorption. The adsorption of I3W variant from binary systems was shown in the FIG 5.5. The I3W variant adsorbed less than the single-component adsorption when WLD was present. Accordingly it was not likely that the exchange reaction took place between I3W and WLD. The adsorption of I3W and WLD was found to be very similar according to the ellipsometric experiments (Lee et al., 1999). Therefore, these two variants might not have a strong inhibitory effect on the adsorption to each other. On the other hand, in the mixture of I3W and I3C variants, the adsorption of the I3W variant was not active at early time. After 30 min, the adsorbed mass of the I3W variant increased quickly and eventually exceeded the adsorbed mass of the single component adsorption of the I3W variant.

Therefore, it is possible that the initial adsorption of I3W was restricted at early time by I3C but reached above that of the single component system. This observation might indicate that the already adsorbed I3C eventually started being replaced. In some instances, surface was dominated by one protein even though other proteins were present. Lassen and Malmsten (1997) used human fibrinogen, IgG, and human serum albumin and found that fibrinogen was dominantly adsorbed on the polymer surface. However, we have not observed this type dominance of one protein
presumably because the proteins used in this study were not too different. However, the obtained results proved that the exchange reaction existed among the proteins.

The competitive adsorption kinetic data from a binary protein mixtures were given in FIGs 5.7, 5.8, and 5.9 representing the binary mixture of I3C + WLD, I3C + I3W, and WLD + I3W, respectively. The adsorption of I3C variant was initially dominating which was corresponding to the fact that the adsorption kinetics of I3C was faster than other variants. However, the adsorbed mass of I3C was receded to end up with a plateau value. This result clearly indicates the occurrence of the exchange reaction by I3W or WLD. Therefore, it is reasonable to conclude that a protein becomes more competitive as the molecular stability becomes less stable. In all cases, the combined adsorbed mass of two proteins was greater than the adsorbed mass of the single component system of an individual variant. This may indicate that either protein was able to competitively adsorb on the surface unless one of dissimilar proteins dominates the adsorption which was observed in some instances (Lassen and Malsten, 1997).

5.5. **Acknowledgement**

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FIG 5.6 Competitive adsorption from a 13C (circle) and WLS (triangle) mixture together with the combined adsorbed mass (filled square).

FIG 5.7 Competitive adsorption from a 13C (circle) and 13W (diamond) mixture together with the combined adsorbed mass (filled square).
FIG 5.8 Competitive adsorption from a WLD (circle) and I3W (diamond) mixture together with the combined adsorbed mass (filled square).

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APPENDIX A

NONLINEAR SIMULATION OF A PARAMETRIC MODEL FOR SINGLE COMPONENT PROTEIN ADSORPTION

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Abstract

A kinetic model for single component protein adsorption which can be readily extended to adsorption from multi-protein solutions was developed, and used to simulate adsorption of site-directed, structural stability mutants of bacteriophage T4 lysozyme. The model allows for two different adsorbed “states”, distinguished by different binding strengths. The presence of an increasing energy barrier to adsorption was incorporated into the model by formulating the adsorption rate constants as functions of time. The model allowed for an infinitely fast initial adsorption rate as well. The numerical analysis was performed using the Marquardt method. For the case where molecules in either adsorbed state were proposed to occupy the same interfacial area, optimized parameters were inconsistent with expectations based on the effect of structural stability on protein adsorption. When the adsorbed states were allowed to occupy different interfacial areas, the estimated parameters were consistent with the effect of structural stability on adsorption. In particular, for the least stable mutant, the estimated parameters were consistent with its great tendency relative to the more stable variants to adsorb in a more unfolded, spread form (state 2). Moreover, the estimated parameters for adsorption into state 2 were directly related to their structural stability for all the mutants examined. Structural stability was not clearly related to a protein’s tendency to adsorb into state 1.
Introduction

The adsorptive behavior of protein can be characterized according to electrostatic interaction (Norde *et al.*, 1978), surface hydrophobicity (Elwing *et al.*, 1987; Malmsten, 1995; Tilton *et al.*, 1991), and molecular properties such as weight or its dimensions in solution (Wahlgren *et al.*, 1993). It is widely acknowledged that conformational changes take place upon adsorption (Tian *et al.*, 1998). Loss of secondary structure is due to noncovalent interaction between the protein and surface. The adsorbed mass of molecules whose conformation has been altered due to the interaction with the surface is strongly dependent on the contact time with the surface.

The effect of thermal stability on surface-induced conformational change has been studied by several researchers (Rapoza and Horbett, 1990; Wei *et al.*, 1990). McGuire *et al.* (1995) investigated the effect of structural stability on adsorption using the wild type and selected synthetic mutants of T4 lysozyme. They found that mutants with lower stability were more resistant to surfactant-mediated elution than mutants of higher stability. We can, therefore, reasonably anticipate that mutants with an unstable structure would unfold more readily and bind to a surface more tightly. Based on these phenomenological findings, the adsorption rate will be affected by the protein layer as it forms on the solid surface.

The interfacial free energy hypothesis states that the energy barrier to adsorption increases with time due to the presence of adsorbed protein layers.
(Andrade et al., 1984). While adsorption occurs, the energy barrier increases or interfacial free energy approaches zero so that adsorption will stop when the energy barrier completely suppresses the potential for further adsorption.

Research on modeling to simulate adsorption behavior has been less substantial than experimental research. Modeling approaches can be classified into two major categories: one is when diffusion is the rate controlling process (Wojciechowski and Brash, 1990) and the other is based on adsorption as a kinetically limited adsorption process (Lundstrom, 1985). A theoretical model based on diffusion-controlled adsorption was tested with experimental data (Young et al., 1988). The initial adsorption data fit well using an ideal sink diffusion model. The prediction after the initial period of adsorption, however, shows higher estimates than the experimental data. Aizenbud et al. (1985) presented an irreversible adsorption model using experimental data recorded by Penners et al. (1981). The controlling factor for their model was also substrate diffusion. The model was obtained from the simplified equation of continuity, considering one dimensional movement of protein molecules. Although their experimental data fit well at low concentration (2 µg/ml), the deceleration period of adsorption data at a higher concentration (20 µg/ml) did not fit well presumably because the adsorption process is influenced by diffusion only at low concentrations. Déjardin and Cottin (1991, 1995) performed adsorption experiments with a flowing solution of fibrinogen. They developed a computational model incorporating an apparent kinetic constant, the combination of the adsorption constant at the interface and the
rate constant related to the transport controlled process based on the Lévêque model.

A shortcoming with the diffusion model is that it overlooks structural alterations undergone by protein upon adsorption, which is related to both competitive adsorption and binding strength. On the other hand, structural changes are often considered in kinetic modeling. Beissinger and Leonard (1982) performed adsorption experiments with binary mixtures of γ-globulin and albumin on quartz. The adsorbed mass was determined at 0.5 and 5 min, and they observed that adsorbed mass was not linearly related to bulk concentration. The analysis was based on a model that incorporated a second order irreversible transition from state 1 to the state 2. Desorption from both state 1 and 2 was allowed as well. The surface concentration was defined as a function of the surface density of molecules adsorbed in state 1, and the surface area occupied by molecules in states 1 and 2. The definition of the surface density carries some discrepancy since the surface density for molecules in state 1 and state 2 would be different. The adsorbed mass of a protein in state 2 is less than protein adsorbing in state 1, which is experimentally observed (McGuire et al., 1995). Based on our study presented in the following section, if we neglect this effect, the analytical solution of model equations, simultaneous nonlinear heterogeneous ordinary differential equations, was obtained and fairly good fitting was available. However, the differentiation between these two distinct adsorbed states, at least two different adsorbed states for the modeling purpose, is indispensable in order to characterize the interfacial
behavior of protein in reality. Three-rate adsorption model (Krisdhasima et al., 1992) and irreversible parallel adsorption model (McGuire et al., 1995) have been proposed based on the kinetically-controlled adsorption model. Both models allow the multiple adsorbed states, i.e. state 1 and 2, based on binding strength and the degree of denaturation. By doing this, the unfolding due to the interaction between protein and surface was incorporated in the model.

**Theoretical Development**

Assuming monolayer protein adsorption, occurring irreversibly into two distinct adsorbed states, we can simply formulate the adsorption mechanism assuming that the adsorption occurs irreversibly into two distinct adsorbed states:

\[
\frac{d\theta_1}{dt} = k_1 C (1 - \theta_1 - \theta_2) \quad [A.1a]
\]

\[
\frac{d\theta_2}{dt} = k_2 C (1 - \theta_1 - \theta_2) \quad [A.1b]
\]

where \( \theta_i \) is the ratio of the actual adsorbed mass in state \( i \), \( \Gamma_i \), to the theoretical maximum adsorbed mass in a monolayer, \( \Gamma_{max} \). \( k_i \) is the rate coefficient for adsorption into state \( i \). \( C \) is the bulk concentration. \( t \) is the contact time between protein and surface. In this model, the adsorbed mass in state 2 was allowed to reach the theoretical maximum monolayer coverage. As we observed from kinetic adsorption data, the initial adsorption rate is infinitely fast so that the derived model function cannot simulate the experimental data properly. The adsorption rate
constant will decrease with time, a result of an increasing energy barrier accompanying an increase in adsorbed mass.

Therefore, we performed an analysis to find the proper form of function in order to determine the observed rate constant. For this purpose, the following analytical solution of the model functions can be obtained after adding Eqns. [A.1a] and [A.1b]:

\[ \theta = 1 - \exp(-kCt) \]

[A.2]

where \( \theta \) is the overall surface coverage at time \( t \) defined as the sum of the surface coverage in state 1 and 2, \( \theta_1 + \theta_2 \). \( k \) is the sum of each rate coefficient, \( k_1 + k_2 \).

Since we have the kinetic experimental data, we can calculate the variation of the rate constant as a function of time using Eqn. [A.2]. The plot of the obtained variation of the rate constant against time showed the nonlinear dependence of the rate constant in time. The initial adsorption is infinitely fast and gradually level off until the surface area is depleted so that no more adsorption can occur. The schematic diagram for the depletion process of the surface area was shown on the FIG A.1. Initially, the bare surface provides extremely favorable environments for adsorption, which can be represented with the infinitely fast adsorption rate momentarily. With time, the surface activity for adsorption will rapidly decrease due to the development of all kind of unfavorable environments for adsorption such as energy barrier, depletion of available surface area, etc. When the surface does
not have activity any more, the adsorption will level off. According to this postulation, we can imagine the observed rate constant should be different from the very initial moment. This behavior was well explained when the observed adsorption rate constant was expressed in the power function of time when it was applied to the simulation of the adsorption data. The nonlinear dependence of the adsorption rate constant on time was proposed in the form of:
where \( k \) is the observed adsorption coefficient. \( \alpha \) indicates the parameter representing the early stage of adsorption. The parameter, \( \beta \), describes the effect of the energy barrier to adsorption or the decrease of the surface activity for adsorption due to the protein film formed on the surface or the increase of the energy barrier to adsorption. For the case where two adsorbed states are allowed, then the observed rate constant for each state can be given in the form of:

\[
k_i = \alpha_i t^{-\beta_i}
\]

where the subscript \( i \) indicates the adsorbed state. Accordingly, the model functions, [A.1a] and [A.1b] can be modified as:

\[
\frac{d\theta_1}{dt} = \alpha_1 t^{-\beta_1} C(1-\theta_1-\theta_2)
\]

\[
\frac{d\theta_2}{dt} = \alpha_2 t^{-\beta_2} C(1-\theta_1-\theta_2)
\]

The analytical solution for the Eqns. [A.5a] and [A.5b] is

\[
\theta = 1 - \exp \left[ \frac{\alpha_1}{1-\beta_1} t^{1-\beta_1} + \frac{\alpha_2}{1-\beta_2} t^{1-\beta_2} \right].
\]

The irreversible parallel adsorption model proposed by McGuire et al. (1995a) incorporates the difference in the surface coverage of the adsorbed states given below:
\[ \frac{d\theta_1}{dt} = k_1 C (1 - \theta_1 - a \theta_2) \]  
\[ \frac{d\theta_2}{dt} = k_2 C (1 - \theta_1 - a \theta_2) \]  

The irreversibility of adsorption was assumed based on the fact that the breakage of the multi-binding sites of an adsorbed molecule at once may be unfavorable.

Previous investigation showed that the simulation using the model function, Eqns. [A.7a] and [A.7b], showed some limitation for the prediction of overall adsorption data. In order to improve the applicability of this model, introducing the observed rate constants, Eqn [A.4], into the Eqns [A.7a] and [A.7b] yields:

\[ \frac{d\theta_1}{dt} = \alpha_1 C t^{-\beta_1} (1 - \theta_1 - a \theta_2) \]  
\[ \frac{d\theta_2}{dt} = \alpha_2 C t^{-\beta_2} (1 - \theta_1 - a \theta_2) \]  

**Estimation of Model Parameters During Initial Period of Adsorption**

For a parametric model, it is more desirable to reduce uncertainty of the estimation of parameters if we can estimate the parameters measurable separately. Since we have seen that adsorption takes place mostly in state 1 during the initial period of adsorption (Tian et al., 1998), it is reasonable to estimate the parameters
which represent the adsorption into state 1. This adsorption process can be given as:

\[
\frac{d\theta_1}{dt} = \alpha_i C r^{-\beta_1} (1 - \theta_1)
\]  

[A.9]

Linearization of variables can be achieved by taking logarithmic transformations for both sides of the analytical solution of Eqn [A.9] as follow:

\[
\ln(-\ln(1 - \theta_1)) = \ln\left(\frac{\alpha_i C}{1 - \beta_1}\right) + (1 - \beta_1)\ln t
\]  

[A.10]

Using this relation, we can estimate the adsorption parameters of state 1, \(\alpha_i\) and \(\beta_i\), using the initial adsorption data. The result of linear regression to estimate the parameters, \(\alpha_i\) and \(\beta_i\), with Eqn [A.10] will be discussed in the following chapter in more detail.

**Estimation of Parameters using Nonlinear Least Squares Method.**

In this study the adsorption parameters are estimated using the Marquardt method to perform nonlinear least squares minimization (Levenberg, 1944; Marquardt, 1963, 1970). The computational algorithm employs the minimization of the Chi-squares which is defined as

\[
\chi^2(\hat{k}) = \sum_{i=1}^{N} \left[y_i - f(\hat{k}, t_i)\right]^2
\]  

[A.11a]
or

\[ \chi^2(k) = \| y - f(k) \|^2 \]  \hspace{1cm} [A.11b]

where \( y_i \) is the \( i \)th data point. \( \tilde{k} \) is a column vector of adsorption parameters.

\( f(\tilde{k}, \tilde{t}) \) is the model equation.

The optimized adsorption parameters are determined when the global minimum of Chi-squares is calculated. The approximation of the Chi-squares in an expanded form up to quadratic terms using Taylor expansion was given as:

\[ \chi^2(\tilde{k}) = \chi^2(\tilde{k}_0) - \delta \tilde{k}^T \nabla_k \chi^2(\tilde{k}_0) + \frac{1}{2} \delta \tilde{k}^T \cdot A \cdot \delta \tilde{k} \]  \hspace{1cm} [A.12]

where, \( \delta \tilde{k} = \tilde{k} - \tilde{k}_0 \) and \( A \) is the Hessian matrix defined as a set of second-order derivatives of Chi-squares with respect to each parameter, \( \partial^2(\chi^2) / \partial k_i \partial k_j \).

The step size for the iterative calculation is determined using the gradient of Chi-squares expanded in Taylor series.

\[ \nabla_k \chi^2(\tilde{k}) = \nabla_k \chi^2(\tilde{k}_0) + A \cdot \delta \tilde{k} + \ldots \]  \hspace{1cm} [A.13]

The elements of the gradient of the Chi-squares with respect to each parameter has been calculated using the relation shown below:

\[ \frac{\partial \chi^2}{\partial k_i} = -2 \sum_{i=1}^{N} (y_i - f(\tilde{k}, \tilde{t})) \left( \frac{\partial f(\tilde{k}, \tilde{t})}{\partial k_i} \right) \]  \hspace{1cm} [A.14]
We chose the forward finite difference method to approximate the first-order derivatives of the model function which gave us the shortest iteration time. The forward finite difference is defined in the form of

\[ \frac{\partial f_i(k_0)}{\partial k_i} \approx \frac{1}{\delta} \left( f(k_i + \delta; k_0) - f(k_0) \right) \]  

[A.15]

where \( \frac{\partial f_i(k_0)}{\partial k_i} \) is the first-order derivatives of model function with respect to \( i \)th adsorption parameter, \( k_i \), evaluated at the point, \( k_0 \). The obtained gradient of Chi-squares using the approximation from Eqn [A.15] at each iteration is further used to calculate the each element of the Hessian matrix, \( A \), which is defined as

\[ A_{ij} = 2 \sum_{m=1}^{n} \left[ \frac{\partial f(k, x_m)}{\partial k_i} \frac{\partial f(k, x_m)}{\partial k_j} \right] \]  

[A.16]

Since our model equation does not have an analytical solution, each derivative has been calculated iterative manner. The obtained Hessian matrix was used to determine the step size for the next calculation in iteration loop until the minimization reaches acceptable tolerance.

\[ \delta k = - A^{-1} \nabla_k \chi^2(k_0) \]  

[A.17]

The simulation was accomplished successfully and the obtained results were consistent.
Results and Discussion

Single component protein adsorption data using bacteriophage T4 lysozyme and synthetic mutants, Ile 3 → Cys and Ile 3 → Trp mutants were recorded at the bulk concentration of 1.0 mg/ml at hydrophilic silica surfaces (McGuire et al., 1995). In-situ ellipsometry was used to record the data. It is known that Ile 3 → Cys mutant has a more stable structure and Ile 3 → Trp mutant has a less stable structure than that of the wild type T4 lysozyme (ΔΔG_{Ile 3 → Cys} = 1.2; ΔΔG_{wild} = 0.0; ΔΔG_{Ile 3 → Trp} = -2.8 kcal/mol) (Matsumura et al., 1988). The greatest adsorbed amount was achieved by wild type. The Ile 3 → Trp mutant adsorbed less than the others which had been attributed to a faster conformational change.

The Eqn [A.2] was solved for k to be a function of time and surface coverage. Since both surface coverage and time were available we could obtain the values for the rate coefficient, k. The calculated results of the adsorption rate constant were plotted in the FIG A.2. The variation of the rate constant for all the proteins was most well fitted with the form of Eqn [A.3]. The fitted result showed α and β for Ile 3 → Cys and Ile 3 → Trp to be same as 0.45 and 0.80 respectively. The estimates for the wild type were 0.55 and 0.72 respectively. The estimate of each parameter does not have a significant meaning at this stage. However, the preliminary recognition for the probable formalism of the adsorption rate coefficient was considerable. This formalism for the apparent rate constant in the
FIG A.2 Plots for the calculated apparent adsorption rate constant against time using an adsorption model assuming a single adsorbed state: (a) Ile 3 → Cys mutant, (b) Wild type, and (c) Ile 3 → Trp mutant.
form of power function of time will be applied for the further analysis of the adsorption process.

**Adsorption Model Where Adsorbed Mass in State 2 Can Reach the Theoretical Maximum Adsorbed Mass.**

The model function, Eqns [A.1a] and [A.1b], were formulated assuming that the adsorbed mass in both states can reach the theoretical adsorbed mass. Since the rate coefficient was assumed to be a function of time, the Eqn [A.4] was substituted into the model functions resulting in the Eqn [A.5a] and [A.5b]. The analytical solution for this simultaneous differential equations was obtained given
in the Eqn [A.6]. Using this analytical solution of the model functions, the unknown parameters were estimated and tabulated in the Table A.1. The result

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Parameters</th>
<th>Parameters</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$</td>
<td>$\alpha_2$</td>
<td>$\beta_1$</td>
</tr>
<tr>
<td>Ile 3 $\rightarrow$ Cys</td>
<td>0.0996</td>
<td>0.0146</td>
<td>0.7310</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.0844</td>
<td>0.0531</td>
<td>0.7649</td>
</tr>
<tr>
<td>Ile 3 $\rightarrow$ Trp</td>
<td>0.0848</td>
<td>0.0136</td>
<td>0.7894</td>
</tr>
</tbody>
</table>

**Table A.1** The estimates of unknown parameters using the analytical form of model function, Eq. [A.6].

shows that the parameters, $\beta_1$ and $\beta_2$, were estimated almost equal for the effect of the adsorption of an individual protein on the decrease of the surface activity. This result is corresponding to the assumption that the adsorbed mass in each state can reach the theoretical maximum adsorbed mass since the surface activity is affected solely by the adsorbed mass not by the adsorbed state. The estimates for the parameters, $\alpha_1$ and $\alpha_2$, do not give a comprehensive explanation on the adsorption behavior, particularly for the multiple adsorbed states since the estimate of $\alpha_2$ for Ile 3 $\rightarrow$ Trp is lowest compared to the other proteins which is not corresponding to the experimental observation. The resultant plots according to this assumption were plotted in FIG A.3. Although it is not so practical approach, it can be applied to the
cases where the structural configuration of a protein is not available so that only the prediction of the overall adsorption is necessary.

**Model that Adsorbed Mass in State 2 Cannot Reach the Theoretical Maximum Adsorbed Mass.**

As we already mentioned at previous section, the model used in this analysis is assuming that the adsorbed mass in state 2 can reach the theoretical maximum adsorbed mass which may not be exactly the case in reality. In adsorption

![Graph](image)

**FIG A.3** Simulation using the Eq. [A.6] applying the assumption that the adsorbed mass in the unfolded state can reach the theoretical maximum monolayer adsorbed mass: (a) Ile 3 → Cys mutant, (b) Wild type, and (c) Ile 3 → Trp mutant.
FIG A.3 Continued.
modeling, there have been no studies that provide the analysis on the adsorbed mass in different adsorbed states with experimental data except Tian et al. (1998). Since the exchange reaction between adsorbed molecules and adsorbing molecules may occur dependent on the adsorbed states in multi-component protein adsorption system the consideration on the adsorbed states in model will be critical. Therefore, we further the simulation analysis for the case which includes the effect of the adsorbed state on the adsorbed mass. The surface area occupied by single protein molecule on a surface will be different for different adsorbed states, i.e., native or unfolded state. The unfolded state can be imagined more like squeezed toward surface to form multiple binding sites with surface and expanded to occupy more surface area compared with the molecule in state 1, i.e., a native form. Therefore, the thickness of the unfolded protein will be thinner and the occupied surface area will be greater than the native form. The difference of the surface area occupied by a single protein can be directly related to the adsorbed mass which will be determined by the number of molecules adsorbed on unit surface area. Even though the experimental procedure to classify the absolute extent of the denaturation of a protein molecule is not available so far, it is indispensable to incorporate the effect of the denaturation on the adsorption process in order to achieve the more realistic simulation model. For the modeling purpose, the proportionality of the difference of the adsorbed mass for the adsorbed states, a, was approximated with the ratio of the surface area occupied by a single molecule adsorbed in state 2 to 1 (McGuire et al., 1995).
The initial part of the adsorption is infinitely fast than the rest of time span of adsorption except very low bulk concentration in which diffusion is possibly the rate controlling factor. For the concentration used in this study, however, the adsorption will be kinetically controlled process since it may not be plausible that the possibility of the concentration depletion near the surface is not so high due to the abundance of protein. It has been hard to predict the overall adsorption process using previously proposed adsorption models, Eqns [A.1a] and [A.1b] or Eqns [A.7a] and [A.7b].

In order to estimate the parameters for apparent rate constant for state 1, we assumed the adsorption occurs mostly in state 1 during the initial period of adsorption. Eqn [A.10] was obtained by taking logarithm of Eqn [A.9]. The initial part of kinetic adsorption data up to 2 minutes were pulled and analyzed in conjunction with Eqn [A.10]. The result of the regression of the pulled data demonstrates excellent fitting. The statistical analysis on this fitting showed the reliability of the model with respect to the experimental data was compelling for all cases. The plots were shown in FIGs. A.4a, b, and c for Ile 3 → Cys, wild type and Ile 3 → Trp, respectively. The estimates of the parameters, $\alpha_i$ and $\beta_i$, after regression were tabulated on Table A.2.
Table A.2 The result of linear regression on Ile 3 → Cys mutant, wild type and Ile 3 → Trp mutant for the estimate the adsorption rate parameters, $\alpha_i$ and $\beta_i$ according to Eqn [A.10].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ile 3 → Cys</th>
<th>Wild type</th>
<th>Ile 3 → Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$</td>
<td>0.1107</td>
<td>0.1744</td>
<td>0.0723</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>0.7469</td>
<td>0.7143</td>
<td>0.8472</td>
</tr>
</tbody>
</table>

FIG A.4 Results of the linear regression using the Eq. [A.10] for (a) Ile 3 → Cys mutant, (b) Wild type, and (c) Ile 3 → Trp mutant.
FIG A.4 Continued.

(b) $y = 0.2857x - 0.4934$
$R^2 = 0.9493$

(c) $y = 0.1528x - 0.7479$
$R^2 = 0.923$
The estimated $\alpha_i$ for Ile 3 $\rightarrow$ Trp mutant was the least compared with other proteins indicating that the adsorption rate into state 1 of the Ile 3 $\rightarrow$ Trp mutant is by far less than others. This result agrees with the fact that the structural stability of Ile 3 $\rightarrow$ Trp mutant is least stable among all the proteins used in this study so that greater portion of adsorbed protein molecules will exist in the unfolded form, state 2. The estimates of wild type and Ile 3 $\rightarrow$ Cys mutant are consistently greater than Ile 3 $\rightarrow$ Trp mutant implying that the adsorption into the native form, i.e. in state 1, dominates in the cases of these proteins. The total adsorbed mass of the wild type was the greatest among all the proteins. The estimate of rate coefficients for wild type also shows fastest rate, which is corresponding to the fact that the wild type has highest surface affinity. Therefore, the estimated value for Ile 3 $\rightarrow$ Cys mutant, $\alpha_i$, could be smaller than that of wild type although it has more stable structural conformation than wild type. For the estimate of the parameter, $\beta_i$, Ile 3 $\rightarrow$ Trp mutant shows the fastest decrease of the rate coefficient based on the formalism of the rate coefficient, Eqn [A.3], which may indicate that the Ile 3 $\rightarrow$ Trp mutant cause the most stronger effect on the decrease of the surface activity. We postulated that the effect of the molecules in denatured form on the decrease of the surface activity is greater than that in native form. This effect can be seen from the comparison between adsorption of wild type or Ile 3 $\rightarrow$ Cys and Ile 3 $\rightarrow$ Trp mutants. Ile 3 $\rightarrow$ Trp mutant known to readily unfold on the surface reaches lower adsorbed mass than the wild type, which implies that the effect of Ile 3 $\rightarrow$ Trp mutant adsorption on the suppression on further adsorption is much greater than
other proteins. For the estimate of the parameter, $\beta_i$, Ile 3 $\rightarrow$ Trp mutant shows the fastest decrease of the rate coefficient since the adsorption to state 1 is considerably small compared with other proteins. For the wild type and Ile 3 $\rightarrow$ Cys mutant, the estimates show that the wild type adsorbs more vigorously than Ile 3 $\rightarrow$ Cys for longer time.

The plots shown on FIGs A.5a, b and c are the result of simulation using the analytical solution of the Eqn [A.9] with the parameters predetermined from Eqn [A.10] considering all the proteins in state 1. For Ile 3 $\rightarrow$ Cys mutant, the assumption that all molecules adsorb in state 1 may be valid since Ile 3 $\rightarrow$ Cys mutant is most stable protein. Therefore, the simulation curve shown on FIG A.5a corresponds with the experimental data. For the wild type which is less stable than Ile 3 $\rightarrow$ Cys mutant, the more portion of the adsorbed molecules will exist in state 2 which will give smaller adsorbed mass than that of state 1. Therefore, prediction using one adsorbed state may predict higher value than the real data which was shown on FIG A.5b. For Ile 3 $\rightarrow$ Trp mutant, it was observed that the greater portion of the adsorbed molecules transform into the denatured form, i.e. state 2. Since the adsorption of Ile 3 $\rightarrow$ Trp mutant to state 1 is much lower than state 2, the simulation on overall data using parameter estimated from the initial data will predict less adsorbed mass in overall process. The FIG A.5c shows this effect.

The parameter, $\beta_i$, estimated at the previous step was the estimate under the assumption that adsorption takes place into only one adsorbed state. As we already discussed, however, this is not exactly the case since adsorption occurs in multiple
FIG A.5 Simulation plots using the pre-estimated parameters in Eq. [A.9] with the same assumption applied in the FIG A.3: (a) Ile 3 → Cys mutant, (b) Wild type, and (c) Ile 3 → Trp mutant.
FIG A.5 Continued.

states. Therefore, the estimate of the parameter, $\beta_i$, is not exactly representing the suppression of surface activity to adsorption although the parameter, $\alpha_i$, can be considered as the adsorption parameter into state 1. Hence, we used the estimate of $\alpha_i$ to proceed the nonlinear estimation of the unknown parameters at following section. The simulation results are shown on the FIG A.6a, b, and c. The Ile 3 $\rightarrow$ Trp mutant shows the most amount of adsorbed mass in state 2. For the wild type and Ile 3 $\rightarrow$ Cys mutant, since wild type adsorb faster than Ile 3 $\rightarrow$ Cys into state 1, the surface cover of wild type in state 1 is greater than that of Ile 3 $\rightarrow$ Cys mutant.
FIG A.6 Final simulation plots using nonlinear least squares method using Eqs. [A.8a] and [A.8b]. The surface coverages in both states were plotted together with the overall surface coverage: (a) Ile 3 → Cys mutant, (b) Wild type, and (c) Ile 3 → Trp mutant.
The optimized parameters for $\alpha_2$, $\beta_1$, and $\beta_2$ were estimated by performing minimization of nonlinear least squares method using the Eqn [A.4] and [A.5]. The magnitude of $\beta_1$ and $\beta_2$ may indicate the effect of the adsorbed protein molecules on the change of energy barrier to adsorption for newly adsorbing protein molecules. The degree of interference of the adsorbed protein molecules against newly adsorbing protein molecules is inversely proportional to the magnitude of these parameters. Therefore, we can interpret that the greater interference by the already adsorbed molecules toward newly adsorbing molecules the smaller the magnitude of these parameters is.
The optimized estimates of these parameters are tabulated on A.3 together with the preestimated adsorption parameter, $\alpha_l$. The estimate of $\beta_l$ becomes greater dependent on the surface affinity of each protein. The greater the magnitude of this parameter indicates that the less interference of the adsorbed molecules in state 1 so that more adsorbed mass can be achieved. At the same time, this parameter seems directly proportional with the adsorbed mass according to the simulation result. For the $\beta_l$'s, the magnitude of the estimate of each protein is in the order of structural stability, i.e. Ile 3 $\rightarrow$ Cys$>$ wild type$>$ Ile 3 $\rightarrow$ Trp. Hence the greater quantity of the parameter, $\beta_2$, may indicate the less effect of the adsorbed molecules on the energy barrier to adsorption. As shown on the Table A.3, Ile 3 $\rightarrow$ Trp mutant shows the smallest parameter which corresponds to the foregoing explanation. The adsorbed mass, equivalently surface coverage, in state 2 estimated from simulation shows

<table>
<thead>
<tr>
<th>Protein</th>
<th>Adsorption Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$</td>
</tr>
<tr>
<td>Ile 3 $\rightarrow$ Cys</td>
<td>0.1107</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.1744</td>
</tr>
<tr>
<td>Ile 3 $\rightarrow$ Trp</td>
<td>0.0723</td>
</tr>
</tbody>
</table>

Table A.3 The estimated parameters for Ile 3 $\rightarrow$ Cys mutant, wild type, and Ile 3 $\rightarrow$ Trp mutant according to Eqns [A.8a] and [A.8b] with the nonlinear least squares method.
approximately same trend as the adsorbed mass on the surface after surfactant mediated elution in experiments where Ile 3 → Trp mutant adsorbed in state 2 more than other proteins (McGuire et. al., 1995).

Conclusion

For the simulation of the multi-component adsorption system, we have to find an accurate model which can be used to describe the single component adsorption system. Since the protein layer formed on the surface is considered to contribute to the decrease of the surface activity for adsorption, we have incorporated this effect as a power function of time. Analysis with the formalism for the apparent adsorption rate constant demonstrated the excellent agreement with the experimental data. The structural stability of protein molecules was also found to be significant on the adsorption behavior of the protein at solid/water interface. Two adsorption models were investigated for the development of the simulation model on the single component of protein adsorption at solid/water interface. The model which assumes that adsorption into state 2 can reach the theoretical maximum adsorbed mass showed the estimated parameters do not properly interpret the adsorptive behavior occurring into two distinct adsorbed states, i.e., folded and unfolded states. For the model in which the maximum adsorbed mass in state 2, unfolded state, is less than the theoretical maximum adsorbed mass by a factor of a, very accurate estimation of the parameters was obtained from the
nonlinear least squares method. The estimated parameters were consistent with the
effect of the structural stability of proteins on adsorption.

Acknowledgement

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APPENDIX B

ADSORPTION OF RECOMBINANT COAGULATION FACTOR VIII ON SOLID SURFACES

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A Bayer Corporation Internal Report
Abstract

Adsorption of recombinant coagulation factor VIII (rFVIII) on derivatized glass and plastic surfaces supplied by Bayer Corporation was studied using a radioiodination method. The amount of rFVIII adsorbed on surfaces was measured using radioiodination method. Surfaces 2 (2% silicone solution), 9 (silicon oxide by PE-CVD), and 14 (reference surface) showed relatively small quantities of adsorbed masses less than 2% loss of a single dose of rFVIII in a vial. Surface 17 (plastic syringe) showed significantly high quantity (0.828 µg/cm²) compared to other glass surfaces. This quantity represents approximately 10 % loss of rFVIII if the syringe is used for administration. The adsorbed amounts on plastic tubings were relatively similar in all cases yielding expected loss of rFVIII ranging from 4.5 to 5.9% of single dose.

Introduction

When biomolecules are allowed to contact with solid surfaces, adsorption occurs spontaneously since entropic gain through adsorption onto surface is thermodynamically favorable. The intensive research on the physicochemical process of adsorption has been carried out due to application to severe problems in a variety of industrial situations (Sawyer, P. N., 1983; Addiego et al., 1992).

The modern genetic technology makes enormously improved treatment possible for the chronicle disease difficult to heal. Disease such as hemophilia A,
deficiency of coagulation factor VIII in patients, can be treated with a small amount of genetically synthesized drugs. In the cascade of blood clotting, fibrin is derived from fibrinogen by a series of proteolytic cleavages that will lead blood clot. In this process, coagulation factor VIII is a protein required to activate factor X in the intrinsic pathway by factor IX (Christmas factor) in the cascade in blood clotting. The gene for this protein has been cloned and expressed in bacteria, and allows treatment of hemophiliacs.

Research on adsorption of expensive genetically synthesized medicine should be carried out in order to protect the loss of the medicine due to interaction with solid surfaces. Although studies on plasma proteins are available (Morriessey, 1977; Tsuruta, 1996), studies on factor VIII are not available. The loss of medicine due to adsorption may cause a great deal of problems in both economical and therapeutic aspects. In this study, investigation on the adsorption of the recombinant factor VIII on solid surfaces which were chemically treated to prevent the adsorption of recombinant factor VIII was performed. The effect of Tween 80 on factor VIII adsorption was investigated using two different Tween 80 concentrations.
Materials and Method

Surfaces and Reaction Cells

The surfaces used in this study were prepared from glass vials, a plastic syringe, and plastic tubing from administration sets provided by Bayer Corp. (Berkley, CA). These samples are listed in Table 8.1 with brief description of chemical treatment. The reaction compartments were prepared of dimensions 1.5 x 2.7 x 2.5 cm where protein solution was injected. Glass vials were cut into the rectangular shape of dimension approximately 0.7 x 3 cm and then attached to the compartments. The reaction cells and other materials were shown in FIG B.1.

FIG B.1 Reaction cells used in the rFVIII adsorption experiments.
<table>
<thead>
<tr>
<th>No.</th>
<th>Suppliers</th>
<th>Surface Treatment</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kimble</td>
<td>1% silicone solution</td>
<td>Emulsion</td>
</tr>
<tr>
<td>2</td>
<td>Kimble</td>
<td>2% silicone solution</td>
<td>Emulsion</td>
</tr>
<tr>
<td>3</td>
<td>Kimble</td>
<td>4% silicone solution</td>
<td>Emulsion</td>
</tr>
<tr>
<td>4</td>
<td>Kimble</td>
<td>Uncoted references (USP/EP Type I)</td>
<td>Untreated as reference</td>
</tr>
<tr>
<td>5</td>
<td>Kimble</td>
<td>&quot;EP&quot; 62121-U10</td>
<td>&quot;Quartz&quot; coated</td>
</tr>
<tr>
<td>6</td>
<td>Schott</td>
<td>&quot;EP&quot; 52121-T10</td>
<td>Standard glass, Wettability: Variable with time</td>
</tr>
<tr>
<td>7</td>
<td>Schott</td>
<td>Titanium Oxide (TiO2) by PE-CVD (6R0197/00)</td>
<td>Dense, nonporous TiO2 (no extractables), Wettability: hydrophobic/hydrophilic</td>
</tr>
<tr>
<td>8</td>
<td>Schott</td>
<td>Carbon-rich SiHCO by PE-CVD (Schott Type I Plus) (6R0197/03)</td>
<td>Porous (negligible barrier to extractables), Wettability: hydrophobic</td>
</tr>
<tr>
<td>9</td>
<td>Schott</td>
<td>Silicon Oxide (SiO2) by PE-CVD (Schott Type I Plus) (6R0197/03)</td>
<td>Dense, nonporous SiO2 (no extractables), Wettability: hydrophobic/hydrophilic</td>
</tr>
<tr>
<td>10</td>
<td>Schott</td>
<td>SiHCO by PE-SVD (6R0197/04)</td>
<td>Dense, nonporous SiO2 w/ hydrocarbon, Wettability: slight hydrophilic</td>
</tr>
<tr>
<td>11</td>
<td>Schott</td>
<td>Hydrophilic SiO2 by PE-CVD (6R0197/05)</td>
<td>Dense, nonporous SiO2 w/ nitrogen, Wettability: slightly hydrophilic</td>
</tr>
<tr>
<td>12</td>
<td>Schott</td>
<td>SiHCO over Type I plus by PE-CVD (surface 5 on top of surface 4) (6R0197/06)</td>
<td>Full barrier properties of surface 4, Wettability: hydrophobic (same as surface 5)</td>
</tr>
<tr>
<td>13</td>
<td>Schott</td>
<td>Gas phase passivation similar to silanization (6R0197/07)</td>
<td>Porous (negligible barrier to extractables), Wettability: hydrophobic</td>
</tr>
<tr>
<td>14</td>
<td>Bayer</td>
<td>Current KG-2 vials</td>
<td>Vial containing rFVIII solution</td>
</tr>
<tr>
<td>15</td>
<td>Baxter</td>
<td>Unknown treatment, rFVIII formulation with albumin</td>
<td>Unknown treatment: Baxter vial; Tween included</td>
</tr>
<tr>
<td>16</td>
<td>Bayer</td>
<td>Current KG-2 vials</td>
<td>Vial containing rFVIII solution</td>
</tr>
<tr>
<td>17</td>
<td>Bayer</td>
<td>Plastic syringe</td>
<td>Plastic</td>
</tr>
</tbody>
</table>

**Table B.1** Types of vials and plastic syringe tested in this study.

**Buffers and Column Preparation**

For the iodination, we used 0.05 M sodium phosphate buffer (pH 7.5).

Approximately 0.6 g of Sephadex (G-75-120, Sigma) was put into the column after incubation for 1 day in 100 ml of double distilled and deionized water. 0.05 M tris-HCl buffer was used to rinse and settle the column.
Proteins

Genetically engineered recombinant factor VIII (rFVIII) was obtained in two formulations, prepared by Bayer Corp. and Baxter Corp (Glendale, CA). The amount contained in one vial was 62.5 µg for Bayer and 251 IU (International Unit) for Baxter. Factor VIII is a large plasma glycoprotein with a molecular weight of about 280,000 (Hoyer and Trabold, 1981). Factor VIII has been purified from several sources such as human, porcine, and bovine plasmas (Vehar and Davie, 1980; Fass et al., 1982; Fulcher and Zimmerman, 1982; Rotblat et al., 1985). Preparations of plasma factor VIII contain polypeptides with varying molecular weights of 90,000 – 190,000, noncovalently associated with a peptide of molecular weight approximately 76,000. Factor VIII is subject to proteolytic processing by factor X and activated protein C (Vehar and Davie, 1980; Kisel et al., 1977; Eaton et al., 1986). The expression of recombinant factor VIII in mammalian cell lines has permitted the use of site-directed mutagenesis as a tool for studying the relative importance of the various cleavage sites and regions within the factor VIII molecule (Tool et al., 1986; Eaton et al., 1986). The concentration of Tween 80 in each vial was 5 ppm at the dilution of the formulation with designated volume of diluent (2.5 mL for Bayer and 1.5 µg Tween 80 per IU rFVIII for Baxter.)
Iodination of the recombinant Factor VIII

The rFVIII was labeled with radioactive iodine using the chloramine-T method (Hunter and Greenwood, 1962). Radioactive sodium iodide in NaOH solution was purchased from Amerhsam (Arlington Heights, Illinois). Chloramine-T was used as an oxidizing reagent of which chemical structure was shown in the FIG. 3.11, Chapter 3. Iodination reaction was carried out by mixing 20 µL of rFVIII solutions (156.25 µg/mL for Bayer and 251 IU/mL for Baxter) with 1 mCi of Na\textsuperscript{125}I followed by adding 10 µL of chloramine-T solution for 2 min. The reaction was stopped by adding 10 µL of sodium metabisulfite solution of concentration. Both solutions of chloramine-T and sodium-metabisulfite were prepared few minutes before iodination. Labeled protein was separated from the free iodine molecules using the gel filtration column of cross-linked dextran (Sephadex). The fractions were collected in 30 test tubes.

Adsorption of rFVIII

The reaction compartments were rinsed with excess amount of distilled and deionized water and blown dry with nitrogen gas before experiments. The rFVIII solution was prepared so that the radioactivity of the solution became approximately 500 cpm/µg of rFVIII. The 0.8 ml of prepared solution was injected to the reaction compartments and allowed rFVIII solution to contact with surface for 24 hours. After desired contact time, the surfaces were put into test tubes after
removing protein solution. For the tubing test, the tubings were filled with rFVIII solution. After 24 hours, tubings were blown empty and cut into pieces of length 2 cm. These segments of tubings were put into tubes to measure the radioactivity emitted from adsorbed rFVIII. The radioactivity of the adsorbed rFVIII was counted using gamma counter (Beckmann, CA) and then converted into adsorbed mass of the protein. For all the surfaces, experiments were performed three times. The background and time-dependent disintegration of the radioactivity were taken into account in all cases.

**Results and Discussion**

The iodination results were shown in the FIG B.2 and B.3 for both recombinant factor VIIIs manufactured by Bayer and Baxter. At the elution of labeled rFVIII, two distinct peaks representing labeled rFVIII and free iodine respectively were obtained. The amount of labeled rFVIII of Bayer was very small compared to Baxter presumably due to the low concentration of rFVIII used for iodination. It was also possible the albumin included in the formulation of Baxter could be iodinated.

**Adsorption on Glass vials and Plastic Syringe**

The adsorption results on glass and plastic surfaces were plotted in the FIG B.4 The numbering of surfaces was same as given in Table 8.1. Surface 16 was
FIG B.2 Collection of fractions of labeled rFVIII of Baxter separated from free iodine molecules.
FIG B.3 Collection of fractions of labeled rFVIII of Bayer separated from free iodine molecules. The small box left upper inside of plot is the enlargement of the peak of the labeled rFVIII.
FIG B.4 Adsorbed mass of recombinant factor VIII on each surfaces. The surface numbering was same as shown in the Table 8.1.
same as surface 14 but used for Tween 80 concentration of 80 ppm. Surface 17 represents the surface of plastic syringe.

The surfaces, 2 (treated in 2% silicone), 9 (coated with silicon oxide by PE-CVD), and 14 (control surface) showed relatively small adsorbed mass. The obtained adsorbed masses were 0.189, 0.203, and 0.233 μg/cm² respectively. The highest adsorbed mass (0.993 μg/cm²) was obtained at the surface (titanium oxide) among glass surfaces. Adsorbed mass on surface 15 (Baxter) was 0.065 μg/cm² which was the lowest. Surfaces 7 (titanium oxide) and 8 (carbon-rich SiH2O) showed greater amount of adsorbed mass. The result from surface 15 may imply that either surface or formulation was prepared to prevent adsorption in that both albumin and rFVIII were surface-active substance but did not adsorb on the surface. The greatest adsorbed amount of rFVIII was 0.828 μg/cm² obtained from the surface 17 (plastic syringe). According to the results shown in the FIG 8.3. The expected amounts of the loss of rFVIII in the case that the formulation was used in practice for administration to patients were estimated and shown in the Table B.2. The values included in parenthesis indicates the standard deviation of the estimate. All the estimates for expected loss of rFVIII were calculated based on the surface area contacting rFVIII solution at the dilution with 2.5 mL diluent. The loss of rFVIII due to adsorption on vial surface was estimated ranging from 1.5% up 8%. For plastic syringe, approximately 11% of a single dose could be lost, which was significantly high.
### Table B.2

The amounts of the expected loss of rFVIII when the formulation was used with the actual volume of diluent for the administration to patients incorporating the geometric aspect of vials and syringe.

<table>
<thead>
<tr>
<th>Vials</th>
<th>Expected loss of rFVIII (µg)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg (S.D.)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.845 (0.867)</td>
<td>Kimble</td>
</tr>
<tr>
<td>2</td>
<td>0.944 (0.652)</td>
<td>Kimble</td>
</tr>
<tr>
<td>3</td>
<td>1.936 (1.114)</td>
<td>Kimble</td>
</tr>
<tr>
<td>4</td>
<td>2.225 (0.502)</td>
<td>Kimble</td>
</tr>
<tr>
<td>5</td>
<td>3.019 (0.572)</td>
<td>Kimble</td>
</tr>
<tr>
<td>6</td>
<td>3.140 (0.396)</td>
<td>Schott</td>
</tr>
<tr>
<td>7</td>
<td>4.964 (0.583)</td>
<td>Schott</td>
</tr>
<tr>
<td>8</td>
<td>3.925 (1.235)</td>
<td>Schott</td>
</tr>
<tr>
<td>9</td>
<td>1.016 (0.494)</td>
<td>Schott</td>
</tr>
<tr>
<td>10</td>
<td>2.234 (0.566)</td>
<td>Schott</td>
</tr>
<tr>
<td>11</td>
<td>2.355 (0.738)</td>
<td>Schott</td>
</tr>
<tr>
<td>12</td>
<td>3.302 (0.715)</td>
<td>Schott</td>
</tr>
<tr>
<td>13</td>
<td>2.701 (0.965)</td>
<td>Schott</td>
</tr>
<tr>
<td>14</td>
<td>1.164 (0.430)</td>
<td>Bayer</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>Baxter vial</td>
</tr>
<tr>
<td>16</td>
<td>1.046 (0.452)</td>
<td>Bayer</td>
</tr>
<tr>
<td>17</td>
<td>6.621 (0.464)</td>
<td>Syringe</td>
</tr>
</tbody>
</table>

The surfaces 14 and 16 prepared from vials which contained rFVIII formulation were thoroughly rinsed with excess amount of distilled and deionized water and blown dry with high nitrogen gas, but the effect of the previous contact of the surface to the formulation on the adsorption of rFVIII should not be dismissed. This concern applies to the vials from Baxter as well. At the same time, it should be pointed out that the expected loss of rFVIII due to adsorption shown in
the Table B.2 will be greater in practice since the solution may contact with greater surface area if it is shaken to dissolve the content in the vial.

Tubings

The radioactivity of adsorbed rVIII inside surface of tubings was measured. Table 8.3 shows the adsorbed amount and the expected loss of rFVIII for each tubing. There was not significant difference among tubings except the tubing

<table>
<thead>
<tr>
<th>Tubings</th>
<th>Adsorbed mass (µg/cm²)</th>
<th>Expected loss of rFVIII (µg)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.122 (0.031)</td>
<td>3.531 (0.908)</td>
<td>Bayer USA</td>
</tr>
<tr>
<td>B</td>
<td>0.118 (0.006)</td>
<td>3.544 (0.191)</td>
<td>Bayer Europe</td>
</tr>
<tr>
<td>C</td>
<td>0.096 (0.030)</td>
<td>2.868 (0.905)</td>
<td>Terumo</td>
</tr>
<tr>
<td>D</td>
<td>0.124 (0.012)</td>
<td>3.672 (0.363)</td>
<td>Nipro</td>
</tr>
</tbody>
</table>

Table B.3 The adsorbed amounts of rFVIII on the inside surface of tubings. The measured values were the quantity per unit length of the tubing.

manufactured by Terumo. The adsorbed mass of rFVIII on tubing from Terumo was the least at the value of 2.868 µg/cm. The expected loss at administration was calculated according to the total length of tubing. The expected loss was ranging from 4.5 to 5.9 % of a single dose. According to the experimental results, the worst combination of administration sets may cause 15.257 µg of the loss of rFVIII. The
The best combination can be 11.168 μg. However, it should be pointed out that almost 44% to 60% of total loss was attributed to adsorption on plastic syringe.

**Tween 80 Study**

The effect of Tween 80 on adsorption of rFVIII on the adsorption of rFVIII was studied using two different bulk concentrations of Tween 80. The concentration of Tween 80 in the original protein solution was 5 ppm. The concentration of the other solution was 80 ppm. The glass surfaces were same type as the surface 14. The adsorbed amounts of rFVIII measured from both concentrations of 5 ppm and 80 ppm were 0.233 and 0.221 μg/cm² respectively. Therefore, the concentration range used in this study appeared not to affect the adsorption of rFVIII. However, we are not sure whether use of higher concentration of Tween 80 can reduce the adsorption of rFVIII.

**Acknowledgement**

We are grateful to Professor F. Stormshak of Department of Animal Science, Oregon State University for helping us to perform radioiodination experiments in his lab.
References


APPENDIX C

INTERFACIAL TENSION KINETICS OF NISIN AND β-CASEIN
AT AN OIL-WATER INTERFACE

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Abstract

The concentration- and time-dependence of interfacial pressure of nisin and β-casein at an n-hexadecane-water interface were evaluated using DuNoüy tensiometry. The two emulsifiers attained interfacial saturation at a bulk concentration of about 0.1 mg/ml, the reduction of the interfacial tension by nisin at that concentration being about equivalent to that of β-casein. The time dependence of interfacial tension recorded for each protein was described using two kinetic models. In the first, the reduction of interfacial tension with time was considered to be a result of molecular penetration into the interface followed by rearrangement. Nisin exhibited more rapid penetration and rearrangement at the interface than did β-casein. The second model allowed for the parallel, irreversible adsorption of protein into each of two states from solution, where state 2 molecules occupy a greater interfacial area and are more tightly bound than state 1 molecules. The extent of adsorption in state 1 and state 2 was determined to be highly concentration dependent for each protein; adsorption occurs mostly in state 1 at high concentration and mostly in state 2 at low concentrations.

Introduction

Proteins adsorb at air-water and oil-water interfaces, decreasing interfacial energy. The rate and extent of this decrease depend on many factors, including the size, charge, and flexibility of the adsorbing protein (1). Fluid interfaces differ from
solid interfaces in allowing adsorbate molecules greater mobility at the interface and greater penetration into the nonaqueous phase (2). Thus, studying protein adsorption at gas-liquid and liquid-liquid interfaces is important for proper understanding of the ability of proteins to stabilize emulsions and foams in a variety of applications (2-7). Graham and Philips (1) suggested that, native molecules must first penetrate the air-water or oil-water interfaces, then unfold and rearrange for optimal packing. They also stated that adsorption is controlled by diffusion, a function of the size of the molecule. Thus, at low surface coverage, every protein molecule that arrives at the interface adsorbs spontaneously. Eventually, a steady state is achieved when the interface is saturated, and all of the molecules have rearranged to their preferred orientation (1,3).

The adsorption of proteins at air-water interface was described by a model that allows for tight adsorption of a first layer and loose packing of a second layer (4); in the first layer, protein adsorbs in different conformations with different occupied areas per adsorbed molecule, dependant upon surface concentration. Cho et al. (5) reported that the native and alkylated derivatives of bovine serum albumin occupy greater area at the air-water interface than that corresponding to molecular dimensions. They also found that the rate of surface pressure increase for these proteins was higher for higher bulk concentration at low times. In the classical 2-state theory of the globular protein unfolding transition (2), there are two protein structures: native and highly disordered. However, it has now been established that an intermediate conformation can be present, termed the “molten globule”, defined
as a protein with a native-like secondary structure but disordered (unfolded) tertiary structure (2). It was found that α-lactalbumin in the molten globule state (produced in the presence of EDTA) reduced the surface tension at the air-water and n-tetradecane-water interfaces more rapidly and to a lower level than the native protein (2). For globular proteins at the air-water interface, Farooq and Narsimhan (6) proposed that adsorbed segments are present in the form of “trains”. They concluded that the degree of unfolding of bovine serum albumin upon adsorption was greater than that of lysozyme, as they found that the number of segments per molecule increased linearly with the increase of surface concentration for bovine serum albumin, and was independent of surface concentration for lysozyme. Other studies suggested that protein molecules undergo conformational change during the adsorption process due to the interaction with the surface or during overcoming energy barrier to adsorption (8,9). In particular, free energy change of a protein molecule for conformational change to a denatured form, 5 to 14 kcal/mol, is comparable to the free energy for adsorption, 5 to 20 kcal/mol. Thus, the conformational change during adsorption is highly probable.

β-casein is a single chained, fibrous protein of molecular weight 24,000 that has no disulfide bonds (7). 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 nonpolar and very hydrophobic, making β-casein distinctly amphiphilic (7). Hunter et al. (7) modeled the adsorption of β-casein at the air-water interface using Langmuirian kinetics,
defining an adsorption activation energy that depends on surface coverage. They found the isotherm exhibited two plateaus in surface coverage. The isotherm was interpreted as indicating adsorption of a saturated layer of \( \beta \)-casein at low concentration followed by molecular reorientation and continued adsorption until the surface is once again saturated.

Nisin is a polypeptide (3510 Da) consisting of 34 amino acid residues (10). As far as the amino acid sequence is concerned, nisin possesses an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus (10). Nisin can withstand activity loss during thermal processing and exposure to acidic environments and pressure extremes (11). These characteristics and others, such as non-toxicity, high surface activity and antimicrobial activity (12), make it an attractive candidate for use as an emulsifier in food and pharmaceutical emulsions. The efficacy of nisin in emulsifying an oil in water was evaluated (13) and found to be significant compared to \( \beta \)-casein and Tween® 80. The interfacial behavior of nisin and \( \beta \)-casein at hydrophilic and hydrophobic solid surfaces has been investigated using ellipsometry (14,15). Each of the proteins, when dissolved in single-protein solution, more favorably adsorbed at hydrophilic than at hydrophobic surfaces.

In this work, we evaluated the interfacial tension kinetics of nisin and \( \beta \)-casein at n-hexadecane-water interface using DuNoüy tensiometer. In order to account for the effect of conformational change during adsorption, the interfacial
tension kinetic data of each protein were analyzed with reference to a two state mechanism that allows for protein to adsorb in structurally dissimilar forms.

**Materials and Methods**

Pure nisin (about $5.0 \times 10^7$ IU/g) was obtained from Aplin and Barrett Ltd. (Dorset, U. K.). n-Hexadecane (Lot No. 105H3530) and β-casein (Lot No. 25H9550) were purchased from Sigma Chemical Co. (St Louis, MO). Monobasic sodium phosphate monohydrate (Lot No. 77892KLJP), dibasic sodium phosphate heptahydrate (Lot No. 7914KJKA) and citric acid monohydrate (Lot No. 062777KMBX) were obtained from Mallinckrodt Specialty Chemical Co. (Paris, Kentucky). Sodium citrate (Lot No. 402346) was from J.T. T. Baker (Philipsburg, NJ).

**Solution preparation.**

Nisin was dissolved in 0.01 M sodium phosphate monobasic (pH 4.5), to assure complete solubilization. A suitable volume of sodium phosphate dibasic (pH 9.1, 0.01 M) was added to the solubilized nisin to bring the pH 7.4. The same buffer was used to prepare β-casein solutions.
Interfacial tension measurement.

A DuNoüy ring tensiometer (Model No. 70535, CSC Scientific Co., Inc., Fairfax, VA) was used to measure interfacial tension at the n-hexadecane-water interface. Immediately after gentle stirring for 45 s, 20 ml of protein solution at a concentration in the range of $1 \times 10^{-6}$ to 1 mg/ml, was placed in a beaker (5 cm dia). The ring was immersed about 10 mm below the surface of the protein solution, and this was followed by the addition of 20 ml n-hexadecane to the surface of the solution. The position (height) of the beaker was adjusted until the ring was in the interface and the apparent interfacial tension was measured (16). To account for the force needed to support the weight of the liquid clinging to the ring at the break point, the apparent interfacial tension was multiplied by a correction factor to get the true interfacial tension. All measurements were performed at room temperature (23-25 °C). Before each measurement, the ring was cleaned by rinsing in benzene followed by rinsing in methylethylketone, and then heating in the oxidizing portion of the flame of an alcohol burner.

Interfacial pressure

Interfacial pressure, $\Pi$ (mN/m), is the reduction of interfacial tension by a surfactant. The average of three interfacial tension readings between phosphate buffer (pH 7.4) and n-hexadecane was 52.4 mN/m. The interfacial pressure was
obtained by subtracting the interfacial tension in the presence of a surfactant from this value.

**Results and Discussion**

**Effect of concentration on steady-state interfacial pressure**

The concentration dependence of $\Pi$ for nisin and $\beta$-casein is shown in Figure C.1. Each protein showed an increase in $\Pi$ with increasing bulk concentration. The maximum reduction in interfacial tension was 32.2 and 30.4 mN/m for $\beta$-casein and nisin, respectively.

**FIG C.1** Effect of concentration of nisin (filled squares) and $\beta$-casein (open squares) on interfacial pressure (concentration is plotted on semilog scale).
Time dependence of surface pressure: empirical analysis

Figure C.2 shows the time dependence of $\Pi$ for nisin and $\beta$-casein (solid lines for the higher concentration data represent fits to a model that will be discussed in the next section). The three emulsifiers attained a steady state interfacial pressure, at all concentrations, after about 2 hours.

For single protein solutions of $\beta$-casein, BSA and lysozyme, Graham and Philips (1) suggested that the rate of change of interfacial pressure can be defined with reference to two kinetic regions, each characterized by a first-order rate constant. The first region, the adsorption period, is one in which both adsorbed mass and interfacial pressure are observed to increase. The other region, the rearrangement period, is characterized by attainment of a plateau in adsorbed mass while the interfacial pressure continues to increase. The rates of adsorption and conformational change can be represented by the first order equation:

$$\ln \left( \frac{\Pi_\infty - \Pi_t}{\Pi_\infty - \Pi_0} \right) = -kt, \quad [C.1]$$

where $\Pi_\infty$, $\Pi_t$ and $\Pi_0$ are the surface pressure values at steady state, at any time $t$, and at $t = 0$, respectively, and $k$ is a first-order rate constant.

Fitting the interfacial kinetic data for nisin and $\beta$-casein at 0.1 mg/ml according to Eqn [C.1] yielded two linear segments in each case. The slope of the first linear segment was considered as an adsorption rate constant, while the slope of the second was interpreted as a rearrangement rate constant. Compared to 0.183 and 0.003 for $\beta$-casein, nisin had an adsorption rate constant and a rearrangement
FIG C.2 Time dependence of interfacial pressure as a function of concentration for (a) nisin and (b) β-casein. The protein concentrations used were 0.000001 (circle), 0.00001 (diamond), 0.0001 (square), 0.001 (triangle), 0.01 (asterisk), 0.1 (cross), 0.5 (plus), 1 (minus), respectively.
rate constant of 0.736 and 0.012, respectively. The fast adsorption and rearrangement of nisin might be due to its small size and lack of tertiary structure.

Analysis with reference to a kinetic model

Wang and McGuire (17) applied a kinetic model to describe the spreading pressure of T4 lysozyme solutions, allowing protein to be adsorbed in structurally and functionally dissimilar states. State 2 molecules are unfolded to some extent and more tightly bound to the surface (i.e., they reduce surface tension more per unit area than those in state 1); also the area occupied by a state 2 molecule (A2) is larger than that occupied by a state 1 molecule (A1). The same adsorption mechanism can be applied to protein adsorption at an oil-water interface. Let rate constants k1 and k2 describe adsorption into states 1 and 2, respectively. Equations describing the time-dependent fractional surface coverage of protein in each of the two states (θ1 and θ2) are

\[
θ_1 = \frac{1}{1 + a k_2 / k_1} \left[ 1 - \exp(-k_1 C - a k_2 C t) \right] \tag{C.2}
\]

and

\[
θ_2 = \frac{(k_2 / k_1)(1 + a k_2 / k_1)}{1 - \exp(-k_1 C - a k_2 C t)} \tag{C.3}
\]

where a is \(A_2 / A_1\), and C (mg/ml) is the bulk protein concentration.

\(\Pi_1\) and \(\Pi_2\) are defined as the interfacial pressure when the interface is covered entirely by state 1 and state 2, respectively, and b is \(\Pi_1/\Pi_2\). The maximum
interfacial pressure measurable would correspond to a monolayer of state 2
molecules; i.e., \( \Pi_{\text{max}} = \Pi_{\text{2}} \geq \Pi_{\text{1}} \). \( \theta_1 \) and \( \theta_2 \) were defined such that when the surface is covered,

\[
\theta_1 + a\theta_2 = 1. \tag{C.4}
\]

The interfacial pressure at any time is therefore given by

\[
\Pi = \Pi_{\text{max}} \left( b\theta_1 + a\theta_2 \right). \tag{C.5}
\]

A model for spreading pressure at any time can be obtained by substituting Eqns [C.2] and [C.3] into Eqn [C.5]:

\[
\Pi = \Pi_{\text{max}} \left[ (b + ak_2 / k_1) / (1 + ak_2 / k_1) \right] \times \left[ 1 - \exp(-k_1C - ak_2C)t \right]. \tag{C.6}
\]

In order to solve for \( k_1C \) and \( k_2C \) in Eqn [C.6], the values of \( a, \Pi_{\text{max}}, \) and \( b \) should be known or approximated. If we assume as a first approximation that adsorption in states 1 or 2, while occupying different interfacial areas, would result in the same interfacial tension reduction, Eqns [C.5] and [C.6] become

\[
\Pi = \Pi_{\text{max}} \left( \theta_1 + \theta_2 \right), \tag{C.7}
\]

and

\[
\Pi = \Pi_{\text{max}} \left[ (1 + k_2 / k_1) / (1 + ak_2 / k_1) \right] \times \left[ 1 - \exp(-k_1C - ak_2C)t \right], \tag{C.8}
\]

respectively.
Based on molecular dimensions, $20 \times 20 \times 50 \, \text{Å}$ for nisin (10) and $14.6 \times 14.6 \times 175 \, \text{Å}$ for $\beta$-casein (7), the parameters $A_1$ and $A_2$ can be estimated as the interfacial area occupied by adsorbed “end on” and “side on” molecules, respectively, such that $a$ is 2.50 for nisin and 11.99 for $\beta$-casein. The maximum measured reductions in interfacial tension (32.2 for $\beta$-casein and 30.4 for nisin) provide an estimate for the theoretical $\Pi_{max}$.

The values of $k_1 C$ and $k_2 C$ for nisin and $\beta$-casein were estimated according to Eqn [C.8] and are listed in Table C.1. In general, we can see that the rate of adsorption in state 1 decreases with decreasing concentration, while the rate of adsorption in state 2 increases with decreasing concentration. At high bulk concentration

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Nisin $k_1 C^a$</th>
<th>Nisin $k_2 C^a$</th>
<th>$\beta$-Casein $k_1 C^a$</th>
<th>$\beta$-Casein $k_2 C^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50.952</td>
<td>0.687</td>
<td>38.019</td>
<td>0.060</td>
</tr>
<tr>
<td>0.5</td>
<td>50.001</td>
<td>2.164</td>
<td>33.378</td>
<td>0.057</td>
</tr>
<tr>
<td>0.1</td>
<td>50.591</td>
<td>0.405</td>
<td>31.197</td>
<td>0.069</td>
</tr>
<tr>
<td>0.01</td>
<td>41.991</td>
<td>5.947</td>
<td>28.477</td>
<td>0.263</td>
</tr>
<tr>
<td>0.001</td>
<td>6.388</td>
<td>9.340</td>
<td>11.290</td>
<td>0.645</td>
</tr>
<tr>
<td>0.0001</td>
<td>-</td>
<td>1.972</td>
<td>2.518</td>
<td>0.413</td>
</tr>
<tr>
<td>0.00001</td>
<td>-</td>
<td>1.518</td>
<td>1.984</td>
<td>0.810</td>
</tr>
<tr>
<td>0.000001</td>
<td>-</td>
<td>1.011</td>
<td>0.755</td>
<td>0.410</td>
</tr>
</tbody>
</table>

$a$ ml/mg.min

* negative value was obtained

**Table C.1** Rate constants $k_1 C$ and $k_2 C$, based on a calculated from molecular dimensions and fitting interfacial pressure kinetic data to Eqn [C.8].
concentration, the ratio of the interfacial area to the total number of molecules available for adsorption is very small, and saturation of the interface is attained in a very short period of time. This suggests that the sub-layer would be crowded, with only little uncovered interface being available as adsorption progresses. Alternatively, the possibility for a protein molecule to extend its conformation and be adsorbed in state 2 would be maximized in a dilute solution.

An experimentally determined "a" may allow for more accurate estimation of adsorption in states 1 and 2 using Eqn [C.8] than what was estimated using the molecular dimensions. The isotherms of the steady-state interfacial pressure versus the bulk concentration (Fig. C.1) appear to be sigmoidal with upper and lower critical concentrations. The interfacial pressure does not change significantly below the lower critical concentration, probably because adsorption occurs only in state 2. The lower critical concentrations were selected as $1 \times 10^{-4}$ mg/ml for nisin and $1 \times 10^{-5}$ mg/ml for β-casein. We assume that adsorption below these critical concentrations occurs in state 2 only, such that

$$\frac{d\theta_2}{dt} = k_2 C[1 - a\theta_2], \quad [C.9]$$

or

$$\Pi = \Pi_{max} / a[1 - \exp(-k_2/a)Ct]. \quad [C.10]$$

Accordingly, a values obtained from Eqn [C.10] at the lower critical concentration (4.18 for β-casein and 3.72 for nisin) were used in estimating $k_1C$ and $k_2C$ for the two proteins according to Eqn [C.8] at concentrations higher than the lower critical
concentration. The interfacial tension kinetic data, along with their fit to Eqn [C.8], are shown in Figure C.1. The plots of \( k_1C \) and \( k_2C \) versus log concentration for both proteins (Figs. C.3 and C.4) are consistent with the tendency for more adsorption in state 2 with the decrease in concentration. Figures C.3 and C.4 were also consistent with the thought that \( k_1 \) and \( k_2 \) decrease with increasing concentration. Guzman et al. (4) modeled the dependence on surface concentration for adsorption rate constants in terms of activation energies for adsorption and desorption. Accordingly, \( k_1 \) and \( k_2 \) might be best represented in the form \( k_i = k_{i0} \exp(-E_{ai}/RT) \), where \( E_{ai} \), the activation energy for adsorption, is considered to be surface-coverage dependent. The results in our study suggest that the activation energy for protein adsorption increase with increasing surface concentration of nisin and \( \beta \)-casein; consequently, the ease with which a protein molecule adsorbs should decrease with increasing surface concentration. Hunter et al. (7) found that for \( \beta \)-casein, at low concentrations (<10\(^{-3}\) mg/ml), adsorption at the air-water interface was "cooperative," becoming easier as the surface coverage increased, while at higher concentrations (>10\(^{-3}\) mg/ml), adsorption became more difficult as the surface coverage increased.

At steady state, Eqn [C.6] becomes

\[
\Pi_{st} = \Pi_{\text{max}} \left[ \frac{(b + ak_2/k_1)}{(1 + ak_2/k_1)} \right]. \tag{C.11}
\]

Under some conditions, values of \( k_1C \) and \( k_2C \) estimated from Eqn [C.11] might
FIG C.3 Adsorption rates $k_1C$ (filled square) and $k_2C$ (open square) (ml/mg.min) for nisin as a function of concentration based on fitting interfacial pressure kinetic data to Eq. [C.8].

FIG C.4 Adsorption rates $k_1C$ (filled square) and $k_2C$ (open square) (ml/mg.min) for β-casein as a function of concentration based on fitting interfacial pressure.
not be very different from what can be estimated using Eqn [C.6]. This would be particularly true at high concentrations, at which b is expected to be close to 1 and \( k_2/k_1 \) is very small. Accordingly, b can be roughly estimated by substituting \( k_1C \) and \( k_2C \) obtained from Eqn [C.8] into Eqn [C.11]. Values of b obtained in this way for nisin and \( \beta \)-casein are listed in Table C.2. Negative b-values were obtained for

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Nisin</th>
<th>( \beta )-Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>0.5</td>
<td>0.985</td>
<td>0.994</td>
</tr>
<tr>
<td>0.1</td>
<td>0.944</td>
<td>0.998</td>
</tr>
<tr>
<td>0.01</td>
<td>0.808</td>
<td>0.913</td>
</tr>
<tr>
<td>0.001</td>
<td>-</td>
<td>0.360</td>
</tr>
<tr>
<td>0.0001</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- negative value was obtained.

Table C.2  Values of parameter b, based on Eqn [C.11].

the fitted data of each protein at the lowest concentration. This might be due to shortcomings in the assumptions that allowed for estimation of adsorption rate constants. The areas of state 1 and 2 might not be constant with changing concentration. In addition, the two state mechanistic model might not work well at very low concentrations, due to the fact that adsorption from highly dilute solutions can be a transport-limited process; i.e., the rate of diffusion to the surface is slower than the rate of protein binding to the surface (17). Moreover, protein adsorption at
such low concentrations might not result in complete coverage of the interface at steady state. In any event, modeling of protein adsorption in two states can allow for more quantitative, useful characterization of the effect of protein conformational changes at interfaces and most importantly, the approach can be extended to the problem of sequential and competitive adsorption in protein mixtures.

References


APPENDIX D

FORTRAN PROGRAM FOR THE CONVERSION OF THE ELLIPSOMETRIC DATA TO ADSORBED MASS

Lee, Woo-Kul, Viwat Krisdhasima, and Joseph McGuire

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This OEP FORTRAN program was developed to convert the measurements of the optical properties, psi and delta, obtained from ellipsometry to the adsorbed mass on the surface.
CALCULATE SUBSTRATE REFRACTIVE INDEX

TC = TAN(PSIS)*COS(DELS)
TS = TAN(PSIS)*SIN(DELS)
RHOS = CMPLX(TC,TS)
NS = N1*TAN(PHI(1))*CSQRT(1.-4.*RHOS*(SIN(PHI(1))**2.)
# /((RHOS+1.)**2.))
WRITE(*,*) TC = , TC
WRITE(*,*) TS = , TS
WRITE(*,*) 'RHOS = ', RHOS
WRITE(*,*) 'NS = ', NS
WRITE(*,51)
51 FORMAT(//)
WRITE(*,*)' SURFACE REFRACTIVE INDEX = ',NS
GOTO 220
100 WRITE(*,110)
110 FORMAT (//,5X,' ENTER FILM REFRACTIVE INDEX RANGE (LOWER,UPPER)')
READ(*,*) (N(I), 1=1,2)
GOTO 122
120 N(1) = F1
     N(2) = F2
122 ER = ER1
ITER1 = 1
ITER2 = 1
125 DO 130 I=1,2
     N2 = N(I)
     CALL REFRAC
     CALL THICK
     D2(I,1) = D21
     D2(I,2) = D22
130 CONTINUE
DO 140 J=1,2
     CALL SEARCH(*170,*140,*150,*160)
140 CONTINUE
150 CALL REFRAC
     CALL THICK
     IF (CODE.EQ.1.) THEN
         D211 = D2(1,1)
         D212 = D2(2,1)
     ELSEIF (CODE.EQ.2.) THEN
         D211 = D2(1,2)
         D212 = D2(2,2)
     ELSEIF (CODE.EQ.3.) THEN
         D211 = D2(1,1)
         D212 = D2(2,2)
     ELSE
         D211 = D2(1,2)
         D212 = D2(2,1)
     ENDIF
C CHECK NUMBER OF ITERATIONS
ITER1 = ITER1 + 1
C
IF (ITER1.GT.500) THEN
WRITE(*,*)' NUMBER OF ITERATIONS #1 EXCEEDED 200'
GOTO 220
ENDIF

CALL COMPR1

GOTO 125
160 GOTO 100

170 CALL REFRAC
CALL THICK

IF (AIMAG(D21).LT.AIMAG(D22)) THEN
   D2R = REAL(D21)
   D2A = AIMAG(D21)
ELSE
   D2R = REAL(D22)
   D2A = AIMAG(D22)
ENDIF

C CHECK NUMBER OF ITERATIONS
ITER2 = ITER2 + 1
IF(ITER2.GT.1000) THEN
WRITE(*,*)' NUMBER OF ITERATIONS #2 EXCEEDED 1000'
GOTO 220
ENDIF

CALL COMPR2(DPSI,DDEL,*180,*210)

180 IF (D2R.LE.0.) THEN
WRITE(*,*)' NEGATIVE THICKNESS, PROGRAM TERMINATED'
GOTO 220
ENDIF
PSI = PSI/0.0174532925
DEL = DEL/0.0174532925
KOUNT1 = KOUNT1 + 1
C
WRITE(*,185)
C 185 FORMAT(/,4X,PSI',6X,DEL',5X,'N2',12X,THK',7X,ERR')
C
WRITE(*,190) PSI,DEL,N2,D2R,D2A
D2RM(KOUNT1) = D2R
N2M(KOUNT1) = N2
C
190 FORMAT(2X,F7.3,2X,F7.3,2X,F8.5,2X,F10.3,2X,F7.5)
GOTO 265
210 GOTO 125

220 WRITE(*,230)
230 FORMAT(/,
   #5X, Select....,/#,
   #5X,'1) CALCULATE THICKNESS AND REFRACTIVE INDEX',/,
#5X, 2) CALCULATE ADSORBED MASS',
#5X, 3) CREATE KINETIC PLOT',
#5X, 4) CALCULATE PROTEIN ADSORPTION MODEL PARAMETERS',
#5X, 5) CHANGE SURFACE PROPERTIES',
#5X, 6) CHANGE PROTEIN AND SURFACE PROPERTIES',
#5X, 7) CREATE DATA FILE',
#5X, 8) EXIT PROGRAM',(/)

READ(*,*) Z1
IF (Z1.EQ.1) GOTO 240
IF (Z1.EQ.2) CALL MASS(FNAMO,N2M,D2RM,TBG,TINT,AM,V,KOUNT1)
IF (Z1.EQ.3) CALL GRAPH(FNAMO)
IF (Z1.EQ.4) CALL FITMOD(FNAMO)
IF (Z1.EQ.5) GOTO 40
IF (Z1.EQ.6) GOTO 22
IF (Z1.EQ.7) CALL OEPDAT
IF (Z1.EQ.8) GOTO 300
GOTO 220
240 KOUNT1 = 0.
   WRITE(*,5)
   5 FORMAT(5X,' ENTER INPUT FILENAME [D:\FILENAME]'
   READ(*,250) FNAMI
   FNAMI = FANMLGSA
   WRITE(*,10)
   10 FORMAT(5X,' ENTER TIME BEGINNING [min]')
   READ(*,*) TBG
   WRITE(*,15)
   15 FORMAT(5X,' ENTER TIME INTERVAL [min]')
   READ(*,*) TINT
   WRITE(*,20)
   20 FORMAT(5X,' ENTER OUTPUT FILENAME [D:\FILENAME]'
   READ(*,250) FNAMO
   FNAMO = FNAMO.PRN
   250 FORMAT (A)
   OPEN(5,FILE=FNAMI,STATUS='OLD',ACCESS='SEQUENTIAL',
   # FORM='FORMATTED')
   C WRITE(*,260)
   C 260 FORMAT (//,5X,' ENTER FILM REFRACTIVE INDEX RANGE (LOWER,UPPER)')
   C READ(*,*) (N(I), I=1,2)
   N(1) = N1+0.00001
   N(2) = REAL(NS)-0.00001
   Fl = N(1)
   F2 = N(2)
   265 READ(5,270,END=280) PSI,DEL
   270 FORMAT(11X,F7.2,4X,F6.2)
   IF(PSI.LT.0.1) PSI=PSI*100.
   IF(DEL.LT.10.) DEL=DEL*100.
   PSI = PSI*0.0174532925
   DEL = DEL*0.0174532925
   GOTO 120
   280 REWIND 5
   CLOSE(5,STATUS='KEEP')
   GOTO 220
C
300 WRITE(*,*)' EXIT OEP'
   CALL REVIDEO()
STOP
END

C

SUBROUTINE REFRAC

C This subroutine is to be called to determine the Fresnel reflection
C coefficients, the total reflection coefficient and the ratio of
C parallel to normal reflection coefficients (rho).
C
C --> CALCULATE REFRACTION ANGLE
C
REAL CP1, CP2, N1, PSI, DEL, LUMP, ER, D2R, N
# , CODE, PHI, N2
COMPLEX RP12, RP23, RN12, RN23, RHO, NS, D21, D22, TRP, TRN
# , X, Y, D2, D211, D212, CP3
COMPLEX CDEL
COMMON/ALL/N2, RP12, RP23, RN12, RN23, CP1, CP2, RHO, N1, NS
# , D21, D22, D211, D212, PSI, DEL, LUMP, TRP, TRN, X(2), Y, D2(2,2)
# , ER, J, D2R, N(2), CODE, PHI(2)
C
CP1 = COS(PHI(1))
CP2 = SQRT(1-(N1/N2*SIN(PHI(1)))**2.)
CP3 = CSQRT(1-(N1/NS*SIN(PHI(1)))**2.)
C
CALCULATE REFRACTION COEFFICIENTS
RP12 = (N2*CP1-N1*CP2)/(N2*CP1+N1*CP2)
RP23 = (NS*CP2-N2*CP3)/(NS*CP2+N2*CP3)
RN12 = (N1*CP1-N2*CP2)/(N1*CP1+N2*CP2)
RN23 = (N2*CP2-NS*CP3)/(N2*CP2+NS*CP3)
C
CALCULATE RHO
CDEL = CMPLX(0, DEL)
RHO = TAN(PSI)*CEXP(CDEL)
RETURN
END

C

SUBROUTINE THICK

C This subroutine is called to solve the equation AX^2+BX+C=0,
C yielding two values of film thickness for a given refractive
C index.
C
REAL CP1, CP2, N1, PSI, DEL, LUMP, ER, D2R, N
# , CODE, PHI, N2
REAL S
COMPLEX RP12,RP23,RN12,RN23,RHO,NS,D21,D22,TRP,TRN
# , X,Y,D2,D21,D211,D2I2
COMMON E,B,C,YY,T,LX1,LX2
COMMON/ALL/N2,RP12,RP23,RN12,RN23,CP1,CP2,RHO,N1,NS
# , D21,D22,D211,D2I2,PSI,DEL,LUMP,TRP,TRN,X(2),Y,D2(2,2)
# , ER,J,D2R,N(2),CODE,PHI(2)
C
CALCULATE THE TWO SOLUTIONS OF FILM THICKNESS
C
E = RHO*RP12*RP23*RN23-RN12*RN23*RP23
B = RHO*(RN23+RP12*RP23*RN12)-(RP23+RP12*RN12*RN23)
C = RHO*RN12-RP12
C
T = CSQRT(B**2.-4.*E*C)
X(1) = (-B+T)/(2.*E)
X(2) = (-B-T)/(2.*E)
LX1 = CLOG(X(1))
LX2 = CLOG(X(2))
S = (-4.*22./7.*N2*CP2)
YY = CMPLX(O.,S)
D21 = LUMP*LX1/YY
D22 = LUMP*LX2/YY
RETURN
END
C
SUBROUTINE SEARCH(*,*,*,*)
C
REAL CP1,CP2,N1,PSI,DEL,LUMP,ER,D2R,N
# , CODE, PHI, N2
REAL DP,DQ
COMMON/ALL/N2,RP12,RP23,RN12,RN23,RHO,NS,D21,D22,TRP,TRN
# , X,Y,D2,D21,D211,D2I2
REAL DP,DQ
DP = AIMAG(D2(1,J))
DQ = AIMAG(D2(2,J))
IF (ABS(DP).LT.ER) THEN
    N2 = N(1)
GOTO 1300
ELSEIF (ABS(DQ).LT.ER) THEN
   N2 = N(2)
   GOTO 1300
ELSEIF((DP.LT.0..AND.DQ.GT.0.).OR.(DP.GT.0..AND.DQ.LT.0.)) THEN
   GOTO 900
ELSEIF (J.EQ.1) THEN
   GOTO 1400
ELSE
   GOTO 930
ENDIF
900 IF (J.EQ.1) THEN
   CODE = 1.
   GOTO 1200
ELSE
   CODE = 2.
   GOTO 1200
ENDIF
930 DP = AIMAG(D2(1,1))
   DQ = AIMAG(D2(2,2))
   IF ((DP.LT.0..AND.DQ.GT.0.).OR.(DP.GT.0..AND.DQ.LT.0.)) THEN
      CODE = 3.
      GOTO 1200
   ELSE
      GOTO 950
   ENDIF
950 DP = AIMAG(D2(1,2))
   DQ = AIMAG(D2(2,1))
   IF ((DP.LT.0..AND.DQ.GT.0.).OR.(DP.GT.0..AND.DQ.LT.0.)) THEN
      CODE = 4.
      GOTO 1200
   ELSE
      GOTO 1000
   ENDIF
1000 WRITE(*,1100)
1100 FORMAT (5X,'SOLUTION OUT OF RANGE')
   GOTO 1600
1200 N2 = (N(1)+N(2))/2.
   GOTO 1500
1300 RETURN 1
1400 RETURN 2
1500 RETURN 3
1600 RETURN 4
END

C
C===============================================================================
SUBROUTINE COMPR1
C===============================================================================
C This subroutine is called to replace either the lower- or upper-
C bound refractive index based on the criteria.
C===============================================================================
C
REAL CP1,CP2,N1,PSI,DEL,LUMP,ER,D2R,N
C

C

DM1 = AIMAG(D2I1)
DM2 = AIMAG(D2I2)
DN1 = AIMAG(D21)
DN2 = AIMAG(D22)

C

IF (ABS(DN1).GT.ABS(DN2)) THEN
  DT = DN2
ELSE
  DT = DN1
ENDIF

SUBROUTINE COMPR2(DPSI,DDEL,*,*)

 This subroutine 'back calculates' the value of psi and delta
 based on the refractive index satisfying the first end criterion.
 The calculated psi and delta values are compared with the
 experimental values to establish whether the second end criterion
 is satisfied. If it is, the subroutine returns the final film
 thickness and refractive index to the main programa. If the second
 end criterion is not satisfied, the first end criterion will be
 updated by decreasing the maximum allowable value of the imaginary
 part of film thickness in order to 'tighten' this end criterion for
 the next iteration.

REAL CP1,CP2,N1,PSI,DEL,LUMP,ER,D2R,N
#CODE,PHI,N2
COMPLEX RP12,RP23,RN12,RN23,RHO,NS,D21,D22,TRP,TRN
#X,Y,D2,D2I1,D2I2
REAL DM1,DM2,DN1,DN2,DT
COMMON/ALL/N2,RP12,RP23,RN12,RN23,CP1,CP2,RHO,N1,NS
#D21,D22,D2I1,D2I2,PSI,DEL,LUMP,TRP,TRN,X(2),Y,D2(2,2)
#ER,J,D2R,N(2),CODE,PHI(2)

C

C

RETURN
END
Q = -4.*22./7.*N2*CP2*D2R/LUMP
Y = CMPLX(0.,Q)
TRP = (RP12+RP23*CEXP(Y))/(1+RP12*RP23*CEXP(Y))
TRN = (RN12+RN23*CEXP(Y))/(1+RN12*RN23*CEXP(Y))
RHOI = TRP/TRN
TPSI = CABS(TRP)/CABS(TRN)
PSIP = ATAN(TPSI)
EDEL = RHOI/TPSI
JDEL = CLOG(EDEL)
DPSI = ABS(PSI-PSIP)
DDEL = ABS(DEL-DELP)
IF (DPSI.LT.ER2) THEN
  GOTO 2000
ELSE
  GOTO 2200
ENDIF
2000 IF ((DPSI.LT.ER2).AND.(DDEL.LT.ER2)) THEN
  GOTO 2300
ELSE
  ER = ER - 0.1*ER
  GOTO 2400
ENDIF
2200 ER = ER - 0.1*ER
2300 RETURN 1
2400 RETURN 2
END

SUBROUTINE OEPDAT
OEPDAT: GENERATE DATA FILE FOR OEP PROGRAM

REAL PSI,DEL
CHARACTER*64 DNAME
WRITE(*,*) ' ENTER DATA FILENAME [DAFILENAME.GSA]' READ(*,5) DNAME
5 FORMAT (A)
OPEN(7,FILE=DNAME,STATUS='NEW',ACCESS='SEQUENTIAL',
#FORM= FORMA ITEM
I = 1
WRITE(*,10)DNAME
10 FORMAT(/,5X,' GENERATE DATA FILE: ',A,/) WRITE(*,20)
20 FORMAT(5X,' ENTER "0,0" TO QUIT',/)
C

C SUBROUTINE MASS(DNAMO,RN,TK,TBG,TINT,AM,V,KOUNT1)
C
C MASS PROGRAM: CALCULATED MASS ADSORBED ON THE SURFACE
C
C
REAL RN(1000),TK(1000),PM(1000),DN(1000),TIME(1000)
CHARACTER*64 DNAMO
OPEN(9,FILE=DNAMO,STATUS='UNKNOWN',ACCESS='APPEND',
+ FORM=FORMATTED)
C
TIME(1) = TBG
DO 5 I=2,KOUNT1
   TIME(I) = TIME(I-1)+TINT
5 CONTINUE
WRITE(*,7)
7 FORMAT(/,5X,' ENTER MAXIMUM ALLOWABLE OF REFRACTIVE INDEX')
READ(*,*) XALLW
10 WRITE(*,20)
20 FORMAT(/,5X,' 1) DRY FILM',/
+,5X,2) FILM IN BUFFER SOLUTION',/
+,5X,3) EXIT TO MAIN MENU',/
+,2X, SELECT...'/)
READ(*,*) JJ
IF(JJ.EQ.1) GO TO 30
IF(JJ.EQ.2) GO TO 70
IF(JJ.EQ.3) GO TO 150
WRITE(*,*) ' ENTER AGAIN'
GO TO 10
30 DO 40 I=1,KOUNT1
   IF(RN(I).GT.XALLW) GOTO 40
   PM(I) = 0.1*AM*TK(I)*((RN(I)**2.-1.)/(RN(I)**2.+2.))
C
WRITE(*,62)
C 62 FORMAT(8X,TIME',13X,MASS')
C WRITE(*,65)TIME(I),PM(I)
  WRITE(9,65)TIME(I),PM(I)
65 FORMAT(5X,F7.2,2X,F15.5)
40 CONTINUE
GOTO 150
70 BN = 1.333
DO 100 I=1,KOUNT1
  IF(RN(I).GT.XALLW) GOTO 100
  FN = (RN(I)+BN)/((RN(I)**2.+2.)*(BN**2.+2.))
  DN(I) = 1./AM - V*((BN**2.-1.)/(BN**2.+2.))
  PM(I) = 0.3*TK(I)*FN*(RN(I)-BN)/DN(I)
C WRITE(*,130)
C 130 FORMAT(8X,TIME',13X,MASS')
C WRITE(*,140)TIME(I),PM(I)
  WRITE(9,140)TIME(I),PM(I)
140 FORMAT(5X,F7.2,2X,F15.5)
100 CONTINUE
150 CLOSE(9,STATUS='KEEP')
RETURN
END
C
C================================================================================================
SUBROUTINE fitmod(FNAMI)
C================================================================================================
C REAL x(1000),y(1000),sig(1000),var(2)
CHARACTER*64 FNAMI
OPEN (9,FILE=FNAMI,STATUS='OLD',ACCESS='SEQUENTIAL',
#FORM='FORMATTED')
REWIND 9
k = 0
DO 1, i=1,1000
  x(i) = 0.
  y(i) = 0.
1 continue
DO 5, i=1,1000
  READ(9,10,END=15) x(i),y(i)
10 FORMAT(5X,F7.2,2X,F15.5)
k = k + 1
5 continue
15 ssy = 0.
sy = 0.
l = k
DO 3 m = 1,2
  DO 7 i=1-9,l
    ssy = (y(i)**2.)+ssy
    sy = y(i) + sy
7 continue
  var(m) = (ssy-((sy**2.)/10.))/9.
  ssy = 0.
sy = 0.
l = INT(l/2)
3 continue
\[
\text{sd} = \sqrt{\frac{\text{var}(1) + \text{var}(2)}{2.}}
\]
do 20 \ j = 1, k
\hspace{1cm} \text{sig}(j) = \text{sd}
20 \text{continue}

CALL nonlin(x,y,sig,k)
REWIND 9
CLOSE (9,STATUS='KEEP')
END

SUBROUTINE nonlin(x,y,sig,npt)
C driver for routine mrqmin
INTEGER NPT,MA
PARAMETER(MA=4)
INTEGER i,ia(MA),itst,k,mfit
REAL alamda,chisq,funcs,ochisq,x(NPT),y(NPT),sig(NPT),
* a(MA),covar(MA,MA),alpha(MA,MA),gues(MA)
EXTERNAL funcs
C Guessing value of the fitted parameters
DATA gues/-0.05,-0.1,-0.01,-1.0/
mfit=MA
do 13 i=1,mfit
\hspace{1cm} ia(i)=1
13 continue
do 14 i=1,MA
\hspace{1cm} a(i)=gues(i)
14 continue
alamda=-1
CALL mrqmin(x,y,sig,NPT,a,ia,MA,covar,alpha,
* MA,chisq,funcs,alamda)
k=1
\hspace{1cm} itst=0
\hspace{1cm} 1 \text{write}(*,'(1/x,a,i2,t18,a,f10.4,t43,a,e9.2))') Iteration 
\hspace{1cm} '* Chi-squared:',chisq,'ALAMDA:','alamda
\hspace{1cm} write(*,'(1,x,t5,a,t13,a,t21,a,t29,a))' a(1),
\hspace{1cm} 'a(2)',y(1)',y(2)
\hspace{1cm} write(*,'(1,x,6f8.4))') (a(i),i=1,4)
\hspace{1cm} k=k+1
\hspace{1cm} ochisq=chisq
\hspace{1cm} CALL mrqmin(x,y,sig,NPT,a,ia,MA,covar,alpha,
\hspace{1cm} * MA,chisq,funcs,alamda)
\hspace{1cm} if (chisq.gt.ochisq) then
\hspace{1cm} \hspace{1cm} itst=0
\hspace{1cm} else if (abs(ochisq-chisq).lt.0.0001) then
\hspace{1cm} \hspace{1cm} itst=itst+1
\hspace{1cm} endif
\hspace{1cm} if (itst.lt.4) then
\hspace{1cm} \hspace{1cm} goto 1
\hspace{1cm} endif
alamda=0.0
CALL mrqmin(x,y,sig,NPT,a,ia,MA,covar,alpha,
* MA,chisq,funcs,alamda)
\hspace{1cm} write(*,'*') 'Uncertainties:'
\hspace{1cm} write(*,'(1,x,6f8.4))') (sqrt(covar(i,i)),i=1,4)
PAUSE 'Press <ENTER> to return to the main menu'
RETURN
END

SUBROUTINE mrqmin(x,y,sig,ndata,a,ia,ma,covar,alpha,nca,chisq,
* 
  funcs,alamda)

INTEGER ma,nca,ndata,ia(ma),MMAX
REAL alamda,chisq,funcsa(ma),alpha(nca,nca),covar(nca,nca),
* sig(ndata),x(ndata),y(ndata)
PARAMETER (MMAX=20)

USES covsrt,gaussj,mrqcof

INTEGER j,k,l,m,mfit
REAL ochisq,atry(MMAX),beta(MMAX),da(MMAX)
SAVE ochisq,atry,beta,da,mfit
if(alamda.lt.0.)then
  mfit=0
  do 11 j=1,ma
    if (ia(j).ne.0) mfit=mfit+1
  11 continue
  alamda=0.001
  call mrqcof(x,y,sig,ndata,a,ia,ma,alpha,beta,nca,chisq)
  ochisq=chisq
  do 12 j=1,ma
    atry(j)=a(j)
  12 continue
endif
j=0
do 14 l=1,ma
  if(ia(l).ne.0) then
    j=j+1
    k=0
    do 13 m=1,ma
      if(ia(m).ne.0) then
        k=k+1
        covar(j,k)=alpha(j,k)
      endif
    13 continue
    covar(j,j)=alpha(j,j)*(1.+alamda)
    da(j)=beta(j)
  endif
14 continue
  call gaussj(covar,mfit,nca,da,1,1)
if(alamda.eq.0.)then
  call covsrt(covar,nca,ma,ia,mfit)
  return
endif
j=0
do 15 l=1,ma
if (ia(l).ne.0) then
  j=j+1
  atry(l)=a(l)+da(j)
endif
15 continue

call mrqcof(x,y,sig,ndata,atry,ia,ma,covar,da,nca,chisq)
if(chisq.lt.ochisq)then
  alamda=0.1*alamda
  ochisq=chisq
  j=0
  do 17 l=1,ma
    if (ia(l).ne.0) then
      j=j+1
      k=0
      do 16 m=1,ma
        if (ia(m).ne.0) then
          k=k+1
          alpha(j,k)=covar(j,k)
        endif
      16 continue
      beta(j)=da(j)
      a(l)=atry(l)
    endif
  17 continue
else
  alamda=10.*alamda
  chisq=ochisq
endif
return
END
call funcs(x(i),a,ymod,dyda,ma)
sig2i=1./(sig(i)*sig(i))
dy=y(i)-ymod
j=0
do 15 i=1,ma
   if(ia(l).ne.0) then
      j=j+1
      wt=dyda(l)*sig2i
      k=0
do 14 m=1,1
         if(ia(m).ne.0) then
            k=k+1
            alpha(j,k)=alpha(j,k)+wt*dyda(m)
         endif
      continue
   beta(j)=beta(j)+dy*wt
   endif
15 continue
chisq=chisq+dy*dy*sig2i
16 continue
do 18 j=2,mfit
do 17 k=1,j-1
   alpha(k,j)=alpha(j,k)
17 continue
18 continue
return
END

C=================================================================================================

SUBROUTINE covsrt(covar,npc,ma,ia,mfit)
C=================================================================================================
C
INTEGER ma,mfit,npc,ia(ma)
REAL covar(npc,npc)
INTEGER i,j,k
REAL swap
do 12 i=mfit+1,ma
   do 11 j=1,i
      covar(i,j)=0.
covar(j,i)=0.
11 continue
do 12 continue
k=mfit
do 15 j=ma,1,-1
   if(ia(j).ne.0) then
      do 13 i=1,ma
         swap=covar(i,k)
covar(i,k)=covar(i,j)
covar(i,j)=swap
13 continue
   do 14 i=1,ma
      swap=covar(k,i)
covar(k,i)=covar(j,i)
14 continue
covar(j,i)=swap
14 continue
  k=k-1
endif
15 continue
return
END

C
C==============================================================================

SUBROUTINE gaussj(a,n,np,b,m,mp)
C==============================================================================

INTEGER m,mp,n,np,NMAX
REAL a(np,np),b(np,mp)
PARAMETER (NMAX=50)
INTEGER i,icol,irow,j,k,l,l1,indxc(NMAX),indxr(NMAX),ipiv(NMAX)
REAL big,dum,pivinv

do 11 j=1,n
  ipiv(j)=0
11 continue

do 22 i=1,n
  big=0.
  do 13 j=1,n
    if(ipiv(j).ne.1)then
      do 12 k=1,n
        if (ipiv(k).eq.0) then
          if (abs(a(j,k)).ge.big)then
            big=abs(a(j,k))
            irow=j
            icol=k
          endif
          else if (ipiv(k).gt.1) then
            pause 'singular matrix in gaussj'
          endif
        endif
      12 continue
    endif
  13 continue
  ipiv(icol)=ipiv(icol)+1
  if (irow.ne.icol) then
    do 141 =1,n
      dum=a(irow,l)
      a(irow,l)= a(icol,1)
      a(icol,1) =dum
  141 continue
    do 151 =1,m
      dum=b(irow,l)
      b(irow,l)=b(icol,1)
      b(icol,1) =dum
  151 continue
  endif
  indxr(i)=irow
  indxc(i)=icol
  if (a(icol,icol).eq.0.) pause 'singular matrix in gaussj'
endf
259
pivinv = 1./a(icol,icol)
a(icol,icol) = 1.
do 16 i = 1,n
   a(icol,1) = a(icol,1)*pivinv
16 continue
do 17 i = 1,m
   b(icol,1) = b(icol,1)*pivinv
17 continue
do 21 i = 1,n
   if(icol .ne. icol) then
      dum = a(icol,icol)
      a(icol,icol) = 0.
do 18 i = 1,n
      a(i,1) = a(i,1) - a(icol,1)*dum
18 continue
do 19 i = 1,m
   b(i,1) = b(i,1) - b(icol,1)*dum
19 continue
endif
21 continue
22 continue
do 24 i = n,1,-1
   if(indxr(1) .ne. indxc(1)) then
      do 23 k = 1,n
         dum = a(k,indxr(1))
         a(k,indxr(1)) = a(k,indxc(1))
         a(k,indxc(1)) = dum
23 continue
endif
24 continue
return
END

SUBROUTINE funcs(x,a,y,dyda,na)
INTEGER na
REAL x,y,a(na),dyda(na)
y = -(a(1)+a(2))+(a(1)*exp(a(3)*x))+(a(2)*exp(a(4)*x))
dyda(1) = -1+exp(a(3)*x)
dyda(2) = -1+exp(a(4)*x)
dyda(3) = a(1)*x*exp(a(3)*x)
dyda(4) = a(2)*x*exp(a(4)*x)
return
end

CC GRAPH - This subroutine sets axis parameters
C
C
SUBROUTINE graph(FNAMO)
C
C
INCLUDE 'FGRAPH.FD'
C

INTEGER*2 xwidth, yheight, cols, rows, status

DOUBLE PRECISION XMAX, XMIN, DFFX, YMAX, YMIN, DFFY, DX(1000), DY(1000)
* ZX, ZY, YINT, XINT

CHARACTER*64 FNAMO

RECORD /videoconfig/ screen

RECORD /wxycoord/ wxy

COMMON screen

COMMON SIZE/ZX, ZY, DFFX, DFFY

CALL clearscreen( $GCLEARSCREEN )

xwidth = screen.numxpixels
yheight = screen.numypixels
cols = screen.numtextcols
rows = screen.numtextrows

CALL setviewport(0, 0, xwidth-1, yheight-1)
CALL settextwindow(1, 1, rows, cols)
CALL ginfo (XMAX, XMIN, YMAX, YMIN, DX, DY, K, YINT, XINT, FNAMO)

DFFX = XMAX-XMIN
DFFY = YMAX-YMIN
ZX = DFFX/1000.
ZY = DFFY/1000.
status = setwindow(.TRUE., XMIN-DFFX/5., YMAX+DFFY/4.,
* XMAX+DFFX/5., YMIN-DFFY/4.)
status = setbcolor($BLUE)
status = setcolor(7)
status = rectangle_W($GFILLINTERIOR, XMIN-2.*ZX, YMAX+2.*ZY,
* XMAX+2.*ZX, YMIN-2.*ZY)
J = INT(YINT-1.)
DO 10 I=1, J
   II = DBLE(I)
   status = setcolor(15)
   CALL moveto_w(XMIN, YMIN+II*DFFY/YINT, WXY)
   CALL setlinestyle( #8888 )
   status = lineto_w(XMAX, YMIN+II*DFFY/YINT)
10 CONTINUE
JJ = INT(XINT-1.)
DO 20 I=1, JJ
   III = DBLE(I)
   status = setcolor(15)
   CALL moveto_w(XMIN+III*DFFX/XINT, YMIN, WXY)
   CALL setlinestyle( #FFFF )
   status = lineto_w(XMIN+III*DFFX/XINT, YMIN+DFFY/50.)
20 CONTINUE

CALL setlinestyle( #FFFF)

status = setcolor(12)
status = rectangle_W( $GBORDER, XMIN-2.*ZX, YMAX+2.*ZY,
* XMAX+2.*ZX, YMIN-2.*ZY)
CALL pdata(FNAMO)

C READ (*,*) ! Wait for ENTER key to be pressed
CALL clearscreern($GCLEARSCREEN)
status = setbkcolor($BLUE)
END

C This subroutine plots the data.
C
SUBROUTINE pdata(FNAMO)

INCLUDE 'FGRAFH.FD'

INTEGER*2 status
CHARACTER*9 str1, str2, str3, str4
CHARACTER*64 FNAMO
DOUBLE PRECISION ZX, ZY, XMAX, XMIN, YMAX, YMIN, DX(1000)
*, DY(1000), DFFX, DFFY, YINT, XINT
RECORD /videoconfig/ screen
RECORD /wxycoord/ wxy
RECORD /rccoord/ curpos
COMMON screen
COMMON/SIZE/ZX, ZY, DFFX, DFFY
C Print text on the screen

CALL ginfo(XMAX, XMIN, YMAX, YMIN, DX, DY, K, YINT, XINT, FNAMO)
CALL settextposition(4, 5, curpos)

status = settextcolor(14)
CALL outtext(‘mass’)
WRITE (str1, '(E9.2)') YMAX
CALL settextposition(6, 2, curpos)
CALL outtext(str1)
WRITE (str3, '(E9.2)') YMIN
CALL settextposition(25, 2, curpos)
CALL outtext(str3)
CALL settextposition(28, 38, curpos)
status = settextcolor(11)
CALL outtext(‘time’)
WRITE (str2, '(E9.2)') XMAX
CALL settextposition(27, 65, curpos)
CALL outtext(str2)
WRITE (str4, '(E9.2)') XMIN
CALL settextposition(27, 8, curpos)
CALL outtext(str4)
status = settextcolor(15)
CALL settextposition(4, 23, curpos)
CALL outtext(‘PROTEIN ADSORPTION KINETIC PLOT’)
status = settextcolor(15)
CALL settextposition(30, 50, curpos)
CALL outtext('Press <Enter> to continue')

Plot the points.

DO 10 I=1,K
   STATUS = SETCOLOR(4)
   CALL MOVETO_W(DX(I),DY(I),WXY)
   STATUS = RECTANGLE_W($GBORDER,DX(I)-5*ZX,DY(I)+5*ZY
   * ,DX(I)+5*ZX,DY(I)-5*ZY)
10 CONTINUE

END

This subroutine generates data

SUBROUTINE ginfo(AA,BB,CC,DD,DX,DY,K,YINT,XINT,FMAMO)

DOUBLE PRECISION A,B,C,D,DX(1000),DY(1000),AA,BB,CC,DD,XINT
CHARACTER*64 FMAMO
OPEN(9,FILE=FMAMO,STATUS='OLD',ACCESS='SEQUENTIAL',
#FORM='FORMATTED')
K = 0
A = 0.
B = 1.0E+20
C = 0.
D = 1.0E+20
DO 1,1= 1,1000
   READ(9,2,END=5) DX(I),DY(I)
2 FORMAT(5X,D7.2,2X,D15.5)
K = K+1
1 CONTINUE
5 REWIND 9
CLOSE (9,STATUS='KEEP')
DO 20 I=1,K
   C = DMAX1(C,DY(I))
   A = DMAX1(A,DX(I))
20 CONTINUE
6 DO 30 I=1,K
   D = DMIN1(D,DY(I))
   B = DMIN1(B,DX(I))
30 CONTINUE
CALL AXIS(C,D,AA,BB,XINT)
CALL AXIS(A,B,CC,DD,YINT)
RETURN
END

SUBROUTINE AXIS(YMAX,YMIN,YMXL,YMN1,YINT)
DOUBLE PRECISION YMAX, YMXI, YMNI, AA, Y(10,10), XINT, YN,
* YINT, YINL
XINT = 6.
DO 20 I=1, 5
   IF (DLOG10((YMAX - YMIN)/6.).GE.0.0) THEN
      AA = INT(DLOG10((YMAX - YMIN)/6.))
      IF (AA.GE.0.0) AA = AA + 1.
   ELSE
      AA = INT(DLOG10((YMAX - YMIN)/6.))
      IF (AA.GT.0.0) AA = AA + 1.
   ENDIF
   IF (((YMAX - YMIN)/XINT).LE.((10.**AA)/10.)) .OR.
      * (ABS((YMAX - YMIN)/XINT-(10.**AA)/10.).LT.0.0000001)) THEN
      Y(I,2) = (10.**AA)/10.
      ELSEIF (((YMAX - YMIN)/XINT).LE.((10.**AA)/5.)) .OR.
         * (ABS((YMAX - YMIN)/XINT-(10.**AA)/5.).LT.0.0000001)) THEN
      Y(I,2) = (10.**AA)/5.
      ELSEIF (((YMAX - YMIN)/XINT).LE.((10.**AA)/2.5)) .OR.
         * (ABS((YMAX - YMIN)/XINT-(10.**AA)/2.5.).LT.0.0000001)) THEN
      Y(I,2) = (10.**AA)/2.5
      ELSEIF (((YMAX - YMIN)/XINT).LE.((10.**AA)/2.)) .OR.
         * (ABS((YMAX - YMIN)/XINT-(10.**AA)/2.).LT.0.0000001)) THEN
      Y(I,2) = (10.**AA)/2.
   ELSE
      Y(I,2) = 10.**AA
   ENDIF
   Y(I,1) = (Y(I,2)*XINT+(ANINT(YMIN/Y(I,2))*Y(I,2)))-YMAX
   Y(I,3) = XINT
   XINT = XINT + 1.
20 CONTINUE
YN = 10.E20
DO 30 I=1, 5
   IF (Y(I,1).LE.YN) THEN
      YN = Y(I,1)
      YINL = Y(I,2)
      YINT = Y(I,3)
   ENDIF
30 CONTINUE
YMXI = YINT*YINL+(ANINT(YMIN/YINL)*YINL)
IF (YMXI.LT.YMAX) THEN
   YMXI = YMXI+YINL
   YINT = YINT + 1.
ELSEF
   YMNI = YMXI-YINL*YINT
   IF (YMNI.GT.YMIN) THEN
      YMNI = YMNI-YINL
      YINT = YINT + 1.
   ENDIF
RETURN
END
SUBROUTINE title()

C===========================================================================
C
C INCLUDE 'FGRAPH.FD'

INTEGER*2 xwidth, yheight, cols, rows, status
RECORD /videoconfig/ screen
RECORD /wxycoord/ wxy
RECORD /rccoord/ curpos
COMMON screen

CALL clearscreen( $GCLEARSCREEN )
xwidth = screen.numx pixels
yheight = screen.numy pixels
cols = screen.numtextcols
rows = screen.numtextrows

CALL setviewport( 0, 0, xwidth-1, yheight-1 )
CALL settextwindow( 1, 1, rows, cols )
status = setwindow(.TRUE.,0.,5.,5.,0.)
CALL settextposition(9,23,curpos)
status = setbkcolor($LIGHTRED)
status = settextcolor(15)
CALL outtext('ONE FILM MODEL ELLIPSOMETRY PROGRAM')
CALL settextposition(11,30,curpos)
status = settextcolor(9)
CALL outtext('by Viwat Krisdhasima')
CALL settextposition(12,30,curpos)
status = settextcolor(9)
CALL outtext('Oregon State University')
CALL settextposition(27,45,curpos)
status = settextcolor(15)
CALL outtext('Press <ENTER> to continue')

READ (*,*) ! Wait for ENTER key to be pressed
CALL clearscreen( $GCLEARSCREEN )
status = setbkcolor($BLUE)
END

C===========================================================================

SUBROUTINE REVIDEO()

C===========================================================================

C INCLUDE 'FGRAPH.FD'

INTEGER*2 xwidth, yheight, cols, rows, status
RECORD /videoconfig/ screen
RECORD /wxycoord/ wxy
COMMON screen

CALL clearscreeln( $GCLEARSREEN )
xwidth = screen.numxpixels
yheight = screen.numypixels
cols = screen.numtextcols
rows = screen.numtextrows

CALL setviewport( 0, 0, xwidth-1, yheight-1 )
CALL settextwindow( 1, 1, rows, cols )
status = setvideomode( $DEFAULTMODE )
end

SUBROUTINE surprop

CHARACTER*64 FNSUR
WRITE(*,*)' ENTER BARE SURFACE FILENAME [D:\FILENAME.GSA]'WRITE(*,*)' (Enter "N", if no data file)'
READ(*,10) FNSUR
IF((ENSUR.EQ.'N').OR.(FNSUR.EQ.'n')) GOTO 200
10 FORMAT(A)
OPEN(10,FILE=ENSUR,STATUS='OLD',ACCESS='SEQUENTIAL', #FORM= 'FORMATTED')
SK = 0.
SUMP = 0.
SUMD = 0.
FMNPSI = 1.0E+20
DO 201 =1,200
   READ(10,30,END=100) PSI,DEL
30 FORMAT(11X,F7.2,4X,F6.2)
   IF(PSI.LT.0.1) PSI=PSI*100.
   IF(DEL.LT.10.) DEL=DEL*100.
   SUMP = SUMP + PSI
   SUMD = SUMD + DEL
   SK = SK + 1.
   FMNPSI = MIN(FMNPSI,PSI)
20 CONTINUE
100 REWIND 10
CLOSE (10,STATUS='KEEP')
AVGPSI = SUMP/SK
AVGDEL = SUMD/SK
WRITE(*,120) AVGPSI,FMNPSI
120 FORMAT(//,10X,'SURFACE PSI = ',F6.3,3X,'(min = ',F6.3,)WRITE(*,130) AVGDEL
130 FORMAT(10X,'SURFACE DELTA = ',F8.3,//)
200 RETURN
END
C
C=====================================================================
CC GRAPH.FOR: DRAW XY GRAPH
C=====================================================================
C
INCLUDE 'FGRAPH.FI'
INCLUDE 'FGRAPH.FD'

LOGICAL fourcolors
EXTERNAL fourcolors

IF( fourcolors() ) THEN
   CALL TITLE()
   CALL OEP()
ELSE
   WRITE (*,*) 'This program requires a CGA, EGA, or',
   'VGA graphics card.'
END IF
END

C Additional functions defined below

CC FOURCOLORS - Function to enter graphics mode for REALG.

LOGICAL FUNCTION fourcolors()

INCLUDE 'FGRAPH.FD'

INTEGER*2 dummy
RECORD /videoconfig/ screen
COMMON screen

C Set to maximum number of available colors.
C
CALL getvideoconfig( screen )
SELECT CASE( screen.adapter )
   CASE( $CGA, $OCGA )
      dummy = setvideomode( $MRES4COLOR )
   CASE( $EGA, $OEGA )
      dummy = setvideomode( $ERESCOLOR )
   CASE( $VGA, $OVGA )
      dummy = setvideomode( $VRES16COLOR )
   CASE DEFAULT
      dummy = 0
END SELECT

CALL getvideoconfig( screen )
fourcolors = .TRUE.
IF( dummy .EQ. 0 ) fourcolors = .FALSE.
END
APPENDIX E

MATLAB PROGRAM TO ESTIMATE THE ADSORPTION PARAMETERS USING NONLINEAR LEAST SQUARES METHOD

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This program was developed for the determination of the adsorption parameters using nonlinear least squares method.

```
clear; clc; clf; clear memory;

global KR KR1 option KR2 tf1 tf2 t_int1 t_int2
global dt dq q_tot t_data q_data q1 q2 Ca t_interval nk nq q_new q_interm

kapa = 1.e-2; aa = 10; itmax = 5; Ca = 1.0; tol = 1.e-4; dy = 0.1;

% The following text can be used if you want to enter the data
% filename from keyboard. The data filename can be input from keyboard
% using the following text.

file = input('Enter data filename without extension : ', 's');
filename = [file '.dat'];
eval = ([load ' filename]);
data = eval(file);
dt = data(:,1)/60;
dq = data(:,2)/3.96;
disp('Enter the preestimated rate coefficients for alphal and beta 1')
KR1 = input('Enter the preestimated rate coefficients')
disp('Enter the initial guess of the rate coefficients you want to estimate')
KR0 = input('Enter the initial guess of rate coefficients')

leeint

diff_chi = 1; err = tol +1; KR = KR0; it=0;

nk = length(KR); nq = length(q_tot); E = eye(nk);

leeode

q_initial = q_tot;
chi = sum( (q_data - q_initial).^2 ) / dy^2;
```
while ( abs(diff_chi) > tol ) & ( itmax > it )

leeode;

q_old = q_tot;

grad = feval('leegrad',KR);
csqr_grad = -2*grad*(q_data - q_old)/dy^2;
hess = 2*csqr_grad*csqr_grad*((1+kapa)*E)/dy^2;
delta_k = -hess/csqr_grad;

k_interm = KR + delta_k; KR_old = KR; KR = k_interm;

leeode;

q_new = q_tot; % ss = sum( (q_data-q_new).^2 );
diff_chi = sum( (q_data-q_new).^2 )/ dy^2 - chi;

    if diff_chi >= 0;
        kapa = 10*kapa;
    else
        kapa = kapa/10;
    end

%-----------------------------------------
    it_speed = it_speed + 1;

    if it_speed < 6
        kapa = kapa/10;
        KR = KR_old + delta_k;
        chi = chi + diff_chi;
    else
        kapa = kapa*10;
        KR = KR_old + delta_k;
        chi = chi + diff_chi;
        it_speed = 0;
    end

end

%-----------------------------------------
% kapa = kapa/10;
% KR = KR_old + delta_k;
% chi = chi + diff_chi;
% end

it = it+1;
fprintf('Iteration: %4.0f Chi-sqr: %5.8e \n', it, chi)

cha_chi(it,l) = chi; cchi = cha_chi; d_chi(it) = diff_chi; dchi = d_chi;
KR_est(it,:) = k_interm; KR_all = KR_est;
end

disp('estimated rate constants are = : ')
disp(KR)

q_tot = q1+q2;

plot(dt,dq, 'ro') % plot with experimental data
hold on
plot(t_data,q1,'g-', t_data,q2,'b-', t_data,q_tot,'w-');
axis([x_start x_end y_start y_end])

%-----------------------------------------------------------------------------
% leedo_de.m
%-----------------------------------------------------------------------------

global delta_k q_tot q1 q2 q3 tf tf1 tf2 tint1 tint2 t_int t_interval t_data x

global x_start x_end q_initial

t0 = x_start; tf = x_end; q0 = y_initial

[t, q] = ode45('lzm1', t0, tf, q0);
q1 = spline(t, q(:,1), t_data); q2 = spline(t, q(:,2), t_data);

% For new version:
% tspan = [0; 30];
% q0 = [0; 0];
% [t, q] = ode45('lzm2', tspan, q0);

% t_interval = [0:t_int:tf-t_int]';
% q1 = spline(t, q(:,1), t_interval);
% q2 = spline(t, q(:,2), t_interval);

q_tot=q1 + q2;

%-----------------------------------------------------------------------------
% leegrad.m
%-----------------------------------------------------------------------------
function grad = leegrad(KR)

global KR q_tot nk nq q_new q_interm

grad = zeros(nq, nk);

for j=1:nk
    kk = KR; k_1 = KR;
    dsj = max(sqrt(eps)*abs(KR(j)),100*eps);
    dsj = 10000*eps; kk(j) = kk(j) + dsj;
    KR=kk; q_interm = q_tot;
    leecode;
    q_new = q_tot;
    grad(:,j) = (q_new - q_interm). /dsj;
    KR = k_1;
    delta_k = del_old;
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% leemtx.m
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

function dff = leemtx(KR)

global KR q_tot nk nq

dff = zeros(nq, nk)

for j=1:nk
    kk = KR;
    k_1 = KR;
    dsj = max(sqrt(eps)*abs(KR(j)),100*eps);
    dsj = KR(j)*sqrt(eps)
    dsj = 0.001
    kk(j) = kk(j) + dsj;
    KR=kk
    q_interm = q_tot
leeode;

q_new = q_tot;
% dff(:,j) = (q_new - q_interm);
dff(:,j) = (q_new - q_interm)/dsj;
KR = k_l
end

%-------------------------------------------------------------
% leeint.m
%-------------------------------------------------------------

global option dt dq t_data q_data tf1 tf2 tf t_int t_int1 t_int2

N_total = length(dt); t_int1 = 0.02; t_int2 = 0.5; tf1 = 5; tf2 = dt(N_total);
dtt = 0; i = 0;
while dtt <= tf1
    i = 1 + i; dtl(i,1) = dt(i); dql(i,1) = dq(i); dtt = dt(i);
end
    j = i+1; k = 0;
while j <= N_total
    k = k + 1; dt2(k,1) = dt(j); dq2(k,1) = dq(j); j = j + 1;
end

N1 = length(dt1); N2 = length(dt2); no_intl = fix(tf1/t_int1);
no_int2 = fix(((tf2-tf1)/t_int2); interval1 = 0;

for i = 1:1: no_intl
    te1(i) = interval1;

for j = 1:1:N1-1
    if te1(i) == dt1(j)
        y_init1(i) = dql(j); break;
    elseif te1(i) > dt1(j)
        if te1(i) < dt1(j+1)
            y_init1(i) = dql(j)+(te1(i)-dt1(j))*(dql(j+1)-dql(j))/((dt1(j+1)-dt1(j)));
        end
    end
end
interval1 = interval1 + t_int1;
end

t_data1 = te1(:,); q_data1 = y_init1(:,);

%te2 = zeros(no_int1+no_int2,1);
%dq2 = zeros(no_int1+no_int2,1);

interval2 = interval1 + t_int2;
ii = no_int1;

for i = 1:1: no_int2
  %   ii = ii + 1;
  te2(i) = interval2;
  for j = 1:1:N2-1
    if te2(i) == dt2(j)
      y_init2(i) = dq2(j); break;
    elseif te2(i) > dt2(j)
      if te2(i) < dt2(j+1)
        y_init2(i) = dq2(j)+(te2(i)-dt2(j))*(dq2(j+1)-dq2(j)) ... 
          /(dt2(j+1)-dt2(j));
      end
    end
  end
  interval2 = interval2 + t_int2;
end

t_data2 = te2(:,); q_data2 = y_init2(:,); t_data = [t_data1; t_data2];
q_data = [q_data1; q_data2];

% plot(dt,dq, 'ro') % plot with experimental data
% plot( t_data, q_data, 'go') % plot w/ interpolated data
% hold on

%------------------------------------------------------------------------------
% lzm
%------------------------------------------------------------------------------

function q_caln = lzm(t, q);

global KR Ca KR1

q_caln(1) = KR1*Ca*((t+eps)^(-KR(1)))*(1-q(1)-1.93*q(2));
function q_caln = lzm1(t, q);

global KR Ca KR1 KR2

% This ODE is used for the case when a_1 is pre-estimated and fixed in the
% program. a_2, b_1 and b_2 will be estimated using LEE

q_caln(1) = KR(1)*Ca*((t+eps)^(-KR(2)))*(1-q(1)-1.93*q(2));
q_caln(2) = KR(3)*Ca*((t+eps)^(-KR(4)))*(1-q(1)-1.93*q(2));

function q_caln = lzm2(t, q);

global KR Ca KR1 KR2

% This ODE is used for the case when a_1 is pre-estimated and fixed in the
% program. a_2, b_1 and b_2 will be estimated using LEE

q_caln(1) = KR(1)*Ca*((t+eps)^(-KR(2)))*(1-q(1)-1.93*q(2));
q_caln(2) = KR(3)*Ca*((t+eps)^(-KR(4)))*(1-q(1)-1.93*q(2));

function q_caln = lzm3(t, q);

global KR Ca KR1 KR2

% This ODE is used for the case when a_1 is pre-estimated and fixed in the
% program. a_2, b_1 and b_2 will be estimated using LEE

q_caln(1) = KR(1)*Ca*((t+eps)^(-KR(2)))*(1-q(1)-1.93*q(2));
q_caln(2) = KR(3)*Ca*((t+eps)^(-KR(4)))*(1-q(1)-1.93*q(2));
function q_caln = lzm3(t, q);

global KR Ca KR1

q_caln(1) = KR(1)*Ca*((t+eps)^(-KR(2)))*(1-q(1)-q(2));
q_caln(2) = KR(3)*Ca*((t+eps)^(-KR(4)))*(1-q(1)-q(2));

%q_caln(1) = KR(1)*Ca*(exp(-KR(2)*t))*(1-q(1)-1.93*q(2));
%q_caln(2) = KR(3)*Ca*(exp(-KR(4)*t))*(1-q(1)-1.93*q(2));

%-----------------------------------------------
% lzm4
%-----------------------------------------------

function q_caln = lzm4(t, q);

global KR Ca

q_caln(1) = KR(1)*Ca*(1-q(1)-1.93*q(2));
q_caln(2) = KR(2)*Ca*(exp(-KR(3)*t))*(1-q(1)-1.93*q(2));
% q_caln(2) = KR(3)*Ca*(1-q(1)-1.93*q(2));