

Segment Concentration Profile of Myoglobin Adsorbed to Metal Ion Chelating Lipid Monolayers at the Air–Water Interface by Neutron Reflection

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The interaction of biomacromolecules with dense packed lipid layers is of great interest for biotechnology in the areas of biocompatibility, medicine, sensors, and biomolecular devices. Such interactions are also important for understanding certain biochemical processes such as cell signaling and toxin assault on cells. One important goal is to understand how various types of fundamental interactions and their spatial distribution affect the conformation of an adsorbed protein. Important aspects include the orientation of the adsorbed protein (end-on versus side-on) and single layer versus multilayer adsorption. The orientation of an enzyme, for example, can affect access to an active site and thus activity. Another important question is whether rearrangement of protein structure or even denaturation occurs upon adsorption. Indeed, the protein myoglobin has been shown to undergo a dramatic conformational change in solution under certain conditions,¹ and it is important to know whether significant conformational changes may occur upon adsorption to a substrate as well. Furthermore, it is important to understand changes that occur in the conformation of an adsorbed protein for varying solution conditions.

Much work has been reported regarding protein adsorption to solid surfaces, where methods such as scanning probes, the surface forces apparatus, linear and circular dichroism, Fourier transform infrared spectroscopy, and X-ray and neutron scattering are readily available to probe the density and structure of the adsorbed layer.^{2–5} Much less work has been reported at fluid–fluid interfaces. For biomimetic studies, lipid membranes at fluid–fluid in-

terfaces offer the advantage of greater in-plane mobility of the lipids, thus better mimicking the surface of a cell. Studies of protein adsorption to lipid monolayers at the liquid–air interface have involved mainly surface pressure isotherms, ellipsometry, and fluorescence and Brewster angle microscopies,^{6–11} much of the work focusing on lipid reorganization and protein–protein interactions in the two-dimensional (2-D) plane. Additional information on the dimension normal to the interface would provide further depth into the mode of protein interaction with functionalized films. Information regarding monolayer or multilayer adsorption as well as protein structure (i.e., denaturation, conformational change) would provide finer detail of molecular-level interactions of host–guest complexation systems.

Recently, several studies probing the structure of proteins bound to functionalized lipid films have been reported. Losche et al. demonstrated the power of neutron reflectivity in examining the conformation of streptavidin adsorbed to monolayers of biotin-functionalized lipids and binary lipid mixtures.¹² Most recently, grazing incidence X-ray diffraction has been used to examine 2-D protein crystals formed upon adsorption to lipid monolayers^{13,14} and the effect of the binding of a lung surfactant specific protein SP-B on the structure of palmitic acid monolayers.¹⁵ To our knowledge, all of the studies reporting detailed structural information on proteins bound to fluid membranes thus far have involved proteins which form two-dimensional crystals. Information on amorphous structures of proteins bound to fluid membranes, which encompass most of the biological protein–membrane interactions, is almost nonexistent.

We report here the use of neutron reflection to examine the segment conformation profile of myoglobin adsorbed to a Langmuir monolayer containing a metal ion chelating lipid that targets histidine residues. The metal-chelating lipid is PSIDA, shown in Figure 1, which has an iminodiacetate headgroup for metal ion chelation and a pyrene group on one of the tails to enable fluorescence studies of metal ion^{9,16} and protein^{8,17} adsorption. The use of metal ion coordination to target the adsorption of proteins to lipid membranes has been studied extensively by

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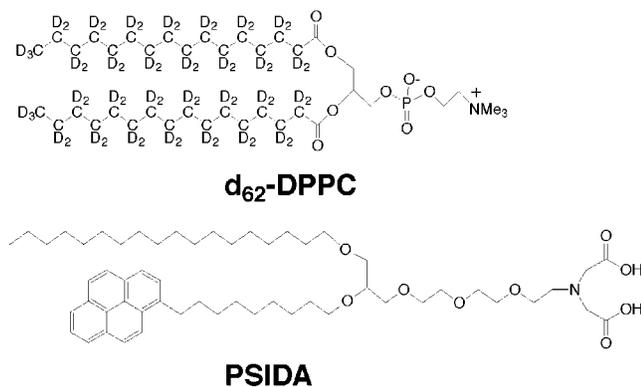


Figure 1. Chemical structures of the lipids used in this work.

others.^{6–10,17–21} This method utilizes coordination interactions between metal ions and naturally occurring histidine units, genetically engineered metal ion binding sites, or polyhistidine units inserted at either the N or C terminus of proteins. This versatile method provides a strategy for selectively targeting proteins for adsorption, for orienting proteins at engineered interfaces, and for 2-D crystallization. Mixtures of metal-chelating lipids with other lipids are being investigated for sensor applications, as protein adsorption can lead to in-plane rearrangements of the lipids detectable by fluorescence techniques.^{8,9} While the adsorption of histidine-containing proteins to such metal ion chelating lipids is well established, the detailed structure of the adsorbed protein layer has not been examined. That is the focus of this work. In this initial study, we focused on the specific case of myoglobin adsorbing to Cu²⁺-loaded PSIDA lipids under a single set of solution conditions.

Due to the different types of interactions that may contribute to protein–membrane complexation and the spatial distribution of the interaction sites on the protein surface, predicting the structure of the adsorbed layer in the present case is much more difficult than for the case of a strong specific interaction that determines a unique binding orientation, such as for streptavidin adsorbing to biotinylated lipids.¹² The distribution of histidine units on the surface of myoglobin allows for several possible orientations for metal ion coordination. Also, in general, ionic and hydrophobic interactions may contribute more significantly than in the case of biotin–streptavidin, where there is a single deep potential well for binding. However, the present experiments were performed at pH = 7.4, which is very close to the isoelectric point of myoglobin (7.2), so we expect minimal effects from electrostatic interactions. Finally, the conditions (nature and distribution of interfacial interactions) for which protein unfolding may occur are not understood.

With neutron reflection, detailed segment concentration profiles of macromolecules at interfaces can be determined noninvasively with excellent spatial resolution (~10 Å) normal to the surface.²² In the present case, a noninvasive probe is important because of the sensitive nature of interfacial interactions in biological systems. Even scanning probes employing nanonewton forces can affect the

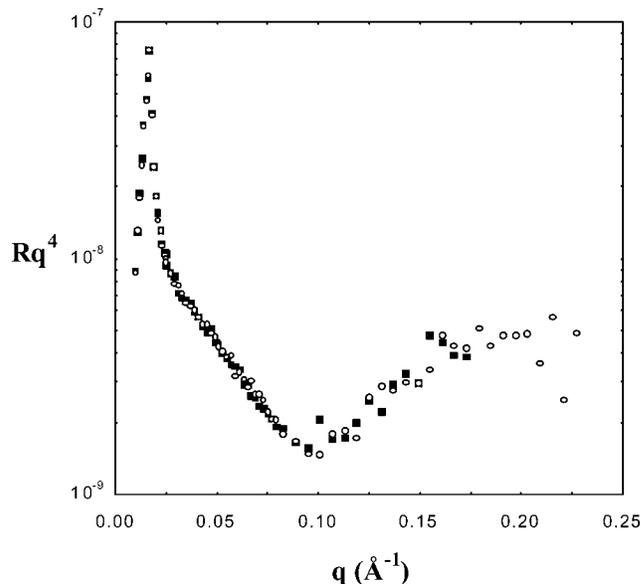


Figure 2. Neutron reflectivity for a 20 mol % PSIDA/*d*-DPPC mixed lipid monolayer on a MOPS buffered D₂O subphase (○). Also shown (■) is the reflectivity after addition of myoglobin to the subphase (but in the absence of Cu²⁺ ions.)

adsorbed layer structure. Regarding other techniques, ellipsometry may be used at the air–liquid interface, but the adsorbed amount and average layer thickness are difficult to decouple in this method. Furthermore, the contrast is weak compared with that obtained with neutron reflection using deuterium labeling. The surface forces apparatus has been used to study proteins adsorbed to supported lipid bilayers, as well as protein adsorption to other solid surfaces.² However, this technique cannot address fluid interfaces, and structural information must be inferred indirectly from the force profiles. In this work, neutron reflection was performed on the NG7 (NIST) and SPEAR (Los Alamos) reflectometers. The technique and analysis methods have been described elsewhere.²³ Complementary X-ray reflection was performed on Beamline X22B (Brookhaven).

The experiment was performed on a Teflon Langmuir trough (KSV) containing a MOPS (4-morpholine propanesulfonic acid) buffered D₂O subphase. The surface tension was monitored with an R&K tensiometer (GmbH, Germany).²⁴ The trough was contained within a closed canister to minimize evaporation. PSIDA was mixed at 20 mol % with *d*₆₂-2-dipalmitoyl-*sn*-glycero-3-phosphocholine (*d*-DPPC) in a chloroform solution and spread onto the subphase to a pressure of ~15 mN/m using a Hamilton microsyringe. Then, following incubation of the film for several minutes, the film was compressed to ~39 mN/m. A peristaltic pump, with inlet and outlet tubes placed at each end of the trough, was used to mix the subphase following the addition of CuCl₂ solution and subsequent addition of myoglobin to the subphase. The subphase was mixed for 1 h at a rate of 4 mL/min after each addition prior to the reflectivity measurements. The trough was maintained at ambient temperature, roughly 20 °C.

Reflectivity for the mixed lipid layer on the buffered subphase is shown in Figure 2. A thickness of 29 ± 3 Å was obtained for the lipid layer, indicated by the position

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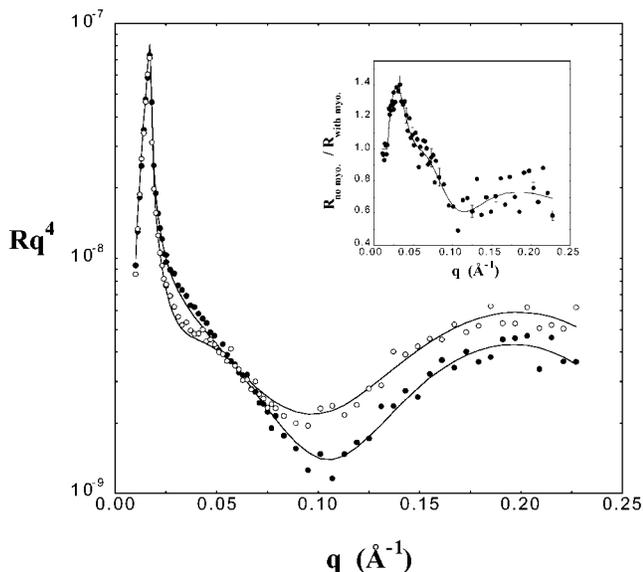


Figure 3. Neutron reflectivity for a 20 mol % PSIDA/*d*-DPPC mixed lipid monolayer on a MOPS buffered D₂O subphase after addition of CuCl₂ solution (○) and again after addition of myoglobin to the subphase and attainment of a quasi-equilibrium (●). The inset corresponds to the ratio of these two curves. Lines through the data correspond to best fits yielding the SLD profiles in Figure 4.

of the minimum in the curve, which is consistent with a value reported previously for bilayers of DPPC on silicon.²⁵ Also shown in Figure 2 is the reflectivity for a control experiment in which myoglobin was introduced into the subphase in the absence of Cu²⁺ ions at a concentration of 5 μM. Without divalent metal ions, little interaction is expected between PSIDA and myoglobin. Indeed, the reflectivity was monitored for a period of 20 h after addition of myoglobin, with no change observed.

Figure 3 shows reflectivity before and after addition of myoglobin for a 20/80 PSIDA/*d*-DPPC monolayer which had previously been loaded with copper ions (10 μM). Upon addition and circulation of myoglobin (5 μM), a significant change in reflectivity was observed. The reflectivity changed as a function of time for several hours. After ~6 h, no further time dependence was observed (monitored for an additional ~6 h). The final curve is shown in Figure 3 along with the reflectivity curve obtained prior to addition of myoglobin.

The inset to Figure 3 shows the ratio of the two curves, which clearly displays the effect due to myoglobin adsorption. There are several features to note in the latter curve, which will be discussed in relation to the best-fit scattering length density (SLD) profiles shown in Figure 4. First, there is a peak centered at $q \sim 0.028 \text{ \AA}^{-1}$, which indicates an adsorbed protein layer with an average thickness of $87 \pm 6 \text{ \AA}$. Second, the peak is followed by a weak shoulder at $q \sim 0.07 \text{ \AA}^{-1}$, which is indicative of the width of the myoglobin/subphase interface. Third, for $q > 0.07 \text{ \AA}^{-1}$ the reflectivity for the system with the adsorbed protein layer falls above that obtained in the absence of myoglobin. This corresponds to a decrease in the roughness of the lipid layer at the air surface upon protein binding. This likely indicates an increased rigidity of the lipid layer upon adsorption of the protein that damps the capillary fluctuations. We note that this effect could also result from a decrease in the SLD of the PSIDA/*d*-DPPC headgroup region, such as would occur if myoglobin

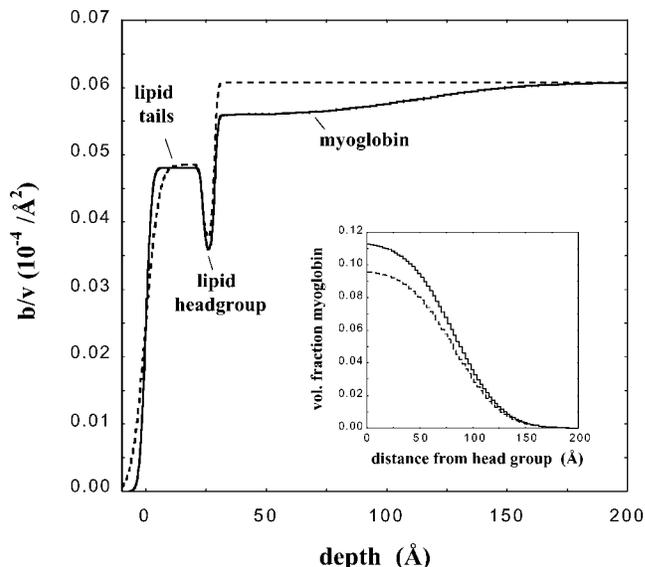


Figure 4. Scattering length density profiles obtained from the reflectivity data in Figure 3. The dashed curve is the SLD profile prior to addition of myoglobin. The solid curve is the SLD profile obtained after addition of myoglobin and attainment of quasi-equilibrium. The inset shows the amino acid concentration profile calculated from the SLD curves. The solid (dashed) line was calculated assuming that all (none) of the labile protons exchanged with deuterons from the solvent.

segments inserted into that region. But we discuss below that this is not supported when X-ray reflectivity data are included in the analysis.

The inset to Figure 4 shows the concentration profile of amino acid segments calculated from the SLD profiles. The SLD profile consisted of three layers describing the lipid tails, the lipid headgroups, and the protein, respectively. The interfaces between all layers were smeared using a Gaussian function. The interfacial function is not required to be a Gaussian, but rather any profile with the same average layer thickness and same interfacial width will be consistent with the data. The segment concentration profile was determined using the calculated SLD of the buffered D₂O subphase and an average SLD value for the myoglobin segments calculated from the atomic composition. In this calculation, we assumed that the ion concentration in the adsorbed myoglobin layer is the same as that of the bulk subphase. Furthermore, exchange of protons from the protein with deuterons from the subphase was also considered. There are 263 labile protons in myoglobin. The two curves in the inset correspond to the cases of complete exchange (solid curve) and no exchange (dashed curve) of labile protons. For complete exchange, which is most likely the case, the integrated adsorbed amount is 9.5 Å or ~0.95 mg/m². The profile extends to ~150 Å with an average thickness of 87 Å.

The thickness and the segment volume fraction of the adsorbed layer can be compared with data for myoglobin in crystalline form. The unit cell dimensions of myoglobin crystals determined by X-ray diffraction are 65 Å × 31 Å × 35 Å. From this, we estimate the segment volume fraction in the crystalline form to be ~0.5.²⁶ This value is much larger than the maximum segment volume fraction in the adsorbed layer of ~0.10. Note also that the average thickness of 87 Å is greater than the maximum dimension of the unit cell of the crystalline form.

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Data from fluorescence measurements performed on Langmuir monolayers and atomic force microscope (AFM) images of supported bilayers strongly suggest that the PSIDA–Cu²⁺ chelated lipids are dispersed in the DPPC matrix.²⁷ At a mole fraction of 0.20, complete dispersion of PSIDA would produce a surface that has a homogeneous affinity for myoglobin on the scale of the protein dimension. If such a surface is present, then the adsorption of a homogeneous layer of protein should result. The values of 0.10 for the segment volume fraction and protein layer thickness of 87 Å can then be interpreted as a monolayer of myoglobin that is highly denatured and extending itself out into the subphase. Small-angle X-ray scattering of myoglobin in aqueous solution has shown that whereas the dimensions of the protein in the native folded state in solution are comparable to those in the crystalline form, the maximum dimension in the unfolded, chain state can be as great as 120 Å.²⁸

An interesting feature of the adsorption is the rather long time scale required to reach a quasi-equilibrium state.²⁹ This time scale is much longer than that for diffusion-limited adsorption. This may suggest gradual rearrangements within the protein layer to accommodate additional chains or a potential barrier to adsorption formed by the presence of already adsorbed chains. The time scale may also be related to lipid rearrangement. Lipid aggregation or deaggregation upon protein binding has been reported to occur on the time scale of 1–4 h for poly-L-histidine binding to mixtures of PSIDA–Cu²⁺ with other lipids.⁸ The long time scale may also suggest the gradual buildup of multilayers. We note that multilayer adsorption and relatively long time scales have been observed for other globular proteins such as cytochrome *c* and concanavalin A adsorbing onto charged mica surfaces using the SFA.²

While determination of the segment volume fraction and dimension of the protein layer is the main focus of this work, another aspect of interest is whether amino acid segments insert into the lipid headgroup or tail regions. Making this determination is severely hampered by the limited q range which is not adequate to directly determine correlations on that length scale. We have attempted to draw inferences from modeling. However, there is significant uncertainty in the model profile in that region. For example, for the reflectivity curve in the

absence of myoglobin, a single 32 Å layer with SLD of $\sim 4.6 \times 10^{-6}$ and roughness at the air surface of 2.8 Å gives equally good agreement with the data as does the two-layer model in Figure 4. However, an acceptable fit to the data in the presence of myoglobin cannot be obtained if the lipid layer is described by a single layer. If a two-layer model for the lipid layer is used for the data without myoglobin, there is a wide range of parameters that give an acceptable fit to the data. To restrict the model profile, we used additional information obtained from X-ray reflectivity data, which extended to $q = 0.7 \text{ \AA}^{-1}$. In that work, no change in the lipid layer was detected upon adsorption of myoglobin. Therefore, in our analysis of the curves in Figure 3, we constrained the fits such that a two-layer model was used for the lipid layer, and comparable SLD in the lipid tail region and comparable overall length of the lipid layer were maintained in the presence and absence of adsorbed myoglobin. From this analysis, we conclude that at most only a small concentration of segments insert into the lipid headgroup region, and the data are also consistent with no insertion of protein segments. We emphasize that the uncertainty in the lipid SLD profile in the absence of myoglobin does not affect the determination of the length scale or average segment density for the adsorbed myoglobin layer but is important in considering whether myoglobin segments insert into the lipid headgroup region.

Thus, myoglobin adsorbs to the chelated Cu²⁺ ions in the lipid monolayer with an average thickness of 87 Å. The average segment volume fraction is lower, and the average thickness larger, than in the crystalline unit cell. This suggests that the protein unfolds to some extent upon adsorption. However, fractional coverage resulting from PSIDA domain formation on a scale below the resolution of the fluorescence and AFM work and the existence of multilayers cannot as yet be definitively ruled out. This work demonstrates the ability of neutron reflectivity studies to yield valuable information regarding the structure of amorphous films of proteins bound to fluid interfaces.

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