Urease and Hexadecylamine-Urease Films at the Air-Water Interface: An X-Ray Reflection and Grazing Incidence X-Ray Diffraction Study

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ABSTRACT We report the results of surface x-ray scattering measurements performed on urease and hexadecylamine-urease films at the air-aqueous solution interface. It is demonstrated that although hexadecylamine does not form a stable monolayer on the pure aqueous surface, it does self-assemble into a stable, well-organized structure when spread on top of a urease film at the air-water interface. It is also likely that protein and hexadecylamine domains coexist at the interface.

INTRODUCTION

The structure and properties of Langmuir monolayers of long-chain amphiphile molecules at the air-water interface have been studied extensively for the last decade (Als-Nielsen et al., 1994). By combining the results of experimental and theoretical studies, it has been possible to generate a consensus view of the character of the generic phase diagram and of the packing patterns in the several condensed phases that monolayers of these molecules exhibit (Kenn et al., 1991; Bommarito et al., 1996; Kuzmenko et al., 1998).

Much less is known about the structures of Langmuir monolayers of proteins at the air-water interface, or about the structures of monolayers of amphiphile-protein complexes. The structures and properties of monolayers with embedded immobilized enzymes are of great interest because of their potential biosensor applications (Moriizumi, 1988; Okahata et al., 1989; Sun et al., 1991; Dubreuil et al., 1996). Biosensors use biological molecules, mainly enzymes, as recognition elements and can be constructed by immobilization of enzymes on transducers such as amperometric or potentiometric electrodes or field effect transistors (Moriizumi, 1988). To date, most of the studies of these systems have focused attention on practical issues associated with their preparation and overall performance characterization; much less effort has been devoted to direct determination of the structure of enzyme-immobilized films.

Indeed, to date only monolayers of the complexes of biotin-functionalized lipids with streptavidin have been studied in detail with structure-sensitive tools such as grazing incidence x-ray diffraction and neutron reflection (Vaknin et al., 1991; Lösche et al., 1993; Haas et al., 1995). The streptavidin-biotin complex has an exceptionally large binding constant, which makes it a model molecular recognition system (Bayer and Wilchek, 1980; Weber et al., 1989; Blankenburg et al., 1989).

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Another system of interest consists of urease adsorbed onto amine monolayers at the air-water interface; it has been studied by Arisawa et al. (Arisawa et al., 1992; Arisawa and Yamamoto, 1992) in the course of construction of a urea sensor. To date, the extant studies report the relationship between the concentration of enzyme dissolved in the subphase and the quantity of enzyme adsorbed onto the monolayer at the air-water interface, which was determined from surface pressure-area isotherm data and UV-IR spectroscopic data.

In this paper we report x-ray reflection (XR) and grazing incidence x-ray diffraction (GIXD) studies of the structure of the hexadecylamine-urease monolayer at the air-water interface.

MATERIALS AND METHODS

Urease (type VII) from jack beans (Sigma Chemicals) and hexadecylamine (Aldrich) were used without further purification. All other chemical reagents (glucose, 2-(*N*-morpholino)ethanesulfonic acid, glutaraldehyde, NaCl, and $CdSO_4$, all from Sigma Chemicals) were of the highest obtainable quality. In all experiments Millipore purified water was used.

The aqueous subphase developed by Yoshimura et al. for growing two-dimensional crystals of the proteins ferritin (Yoshimura et al., 1994) and apoferritin (Scheybani et al., 1996) at the air-water interface was used as the subphase for spreading the urease films. This subphase, which consists of 2% glucose (w/v), 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 150 mM NaCl, and 10 mM $CdSO_4$ (pH 5.8), has a higher density and surface tension than does the protein solution. Three milliliters of aqueous urease solution (4.2 mg/ml) was deposited on a glass rod contacting the liquid surface (Trurnit, 1960) in a fashion such that it became evenly distributed into a thin layer before contacting the meniscus of the liquid surface-rod interface. Immediately after deposition of the urease, the surface pressure rose to 25 mN/m, indicating adsorption of the protein at the interface. To prevent unfolding of the protein at the interface, glutaraldehyde (3 ml, 50% w/v) was injected beneath the surface shortly after the protein film was spread. Then, 30 min later, 40 μl of a 0.35 mg/ml solution of hexadecylamine ($C_{16}H_{33}NH_2$) in chloroform was spread on top of the protein film.

Our x-ray scattering experiments were carried out at beamline X19C of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, using a custom-built liquid surface diffractometer (Schlossman et al., 1997) with an incident wavelength of $\lambda = 1.54$ Å. A sealed and thermostatted Langmuir trough equipped with a Wilhelmy balance was supported on a vibration isolation stage on the diffractometer. Compression of the monolayer was achieved by moving a motorized Teflon ribbon, which confines the monolayer to one side of the trough. Both the trough

and the ribbon assembly were enclosed in a temperature-controlled aluminum housing with a Kapton window.

The monochromatic x-ray beam was deflected toward the water surface via Bragg reflection from a Si (111) crystal. The angle of incidence for GIXD measurements was chosen to be $0.87\alpha_c$, where $\alpha_c = 0.138°$ is the critical angle for total external reflection of x-rays from the pure water surface. The x-rays were collimated with a set of four-jaw slits. These slits were always set to optimize the resolution in the scattering plane while increasing the signal by lowering the resolution out of the scattering plane; this protocol for the slit settings resulted in somewhat different slit configurations for the GIXD and XR measurements. A beam monitor before the sample provided data for the normalization of the incident beam intensity. The scattered beam intensity was measured with a NaI scintillation detector. Calibrated attenuators between the sample and the detector were inserted and removed as necessary during the course of the XR scan to utilize only the linear regime of the detector. All x-ray scattering measurements were performed at 5°C.

GRAZING INCIDENCE X-RAY DIFFRACTION MEASUREMENTS

Grazing incidence x-ray diffraction measurements are made with the x-ray momentum transfer in or close to the plane of the air-water interface. The analysis of the Bragg peaks observed with this geometry is similar to that of a conventional 3D powder pattern; the reflections can be indexed by two Miller indices, hk. Their angular positions $2\theta_{hk}$, corresponding to $q_{hk} = (4\pi/\lambda)\sin\theta_{hk}$, yield the repeat distances $d_{hk} = 2\pi/q_{hk}$ for the 2D lattice structure.

The GIXD spectra of uncompressed and compressed HDA-urease films yield essentially the same results. The spectrum consists of four peaks (Fig. 1), at $q_{xy} = 1.20$ Å^{-1}, 1.69 Å^{-1}, 1.71 Å^{-1}, and 2.39 Å^{-1}, indexed as {1,0}, {1,1 + 1,$\bar{1}$}, {02}, and {20}. These peaks are characteristic of diffraction from a 2D array with a rectangular unit cell of dimensions $a = 5.21$ Å and $b = 7.34$ Å. The unit cell dimensions are indicative of a herringbone arrangement of the hydrocarbon chains related by glide symmetry, a packing that is often described as the orthogonal O⊥ motif (Kitaigorodski, 1961; Small, 1986; Kuzmenko et al., 1998). The intensity profiles of the observed Bragg rods peak near $q_z = 0$ Å^{-1} (Fig. 2), indicating (Als-Nielsen and Kjaer, 1989; Kjaer, 1994) that the amine hydrocarbon chains are aligned along the normal to the aqueous surface. The fully extended length of a hexadecylamine molecule is ~21.8 Å. The approximate thickness, L, of the 2D crystalline film may be deduced from the Scherrer (Guinier, 1968) formula, $L = 0.9 \times 2\pi/\mathrm{FWHM}(q_z)$, where $\mathrm{FWHM}(q_z)$ is the full width at half-maximum of the Bragg rod. We find that the average FWHM of the Bragg rods is ~0.24 Å^{-1}, which corresponds to $L = 23.6$ Å. The deviation between this value and the hexadecylamine chain length can be explained if there is a single water molecule bound to each of the NH_3^+ amine headgroups. The average $\mathrm{FWHM}(q_{xy})$ of the observed GIXD peaks is 0.027 Å^{-1}, corresponding to a correlation length in the monolayer of $\xi \approx 230$ Å.

Any 2D crystal lattice formed by protein molecules should have a large spacing, on the order of a linear dimension of the protein molecule. Therefore, the first-order diffraction peaks from such a lattice should be observed at very small q_{xy}. We have scanned the entire range of q_{xy} down to 0.07 Å^{-1} but have not seen any diffraction peaks that could be attributed to the existence of a 2D crystalline lattice. This negative finding does not necessarily mean that the protein layer is not ordered. At small q_{xy} the measurements of the GIXD spectrum take place very close to the incident x-ray beam, where the background radiation level is much higher than that at large q_{xy}. It is possible that the diffraction peaks corresponding to a 2D crystalline lattice of urease molecules could be concealed by the background radiation.

The GIXD spectra of both uncompressed and compressed hexadecylamine on pure water are consistent with the absence of an ordered phase at the interface. However, when hexadecylamine was spread on 2% (w/v) aqueous glucose solution ($\Pi = 20$ mN/m), a single diffraction peak was

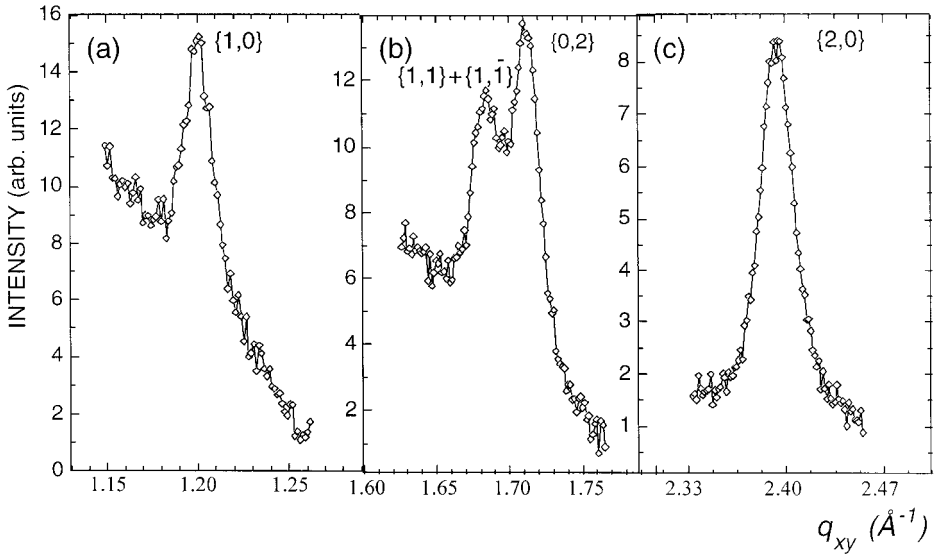

FIGURE 1 GIXD intensity pattern of uncompressed HDA-urease monolayer at the air-water interface. The {h,k} indices of the reflections are indicated.

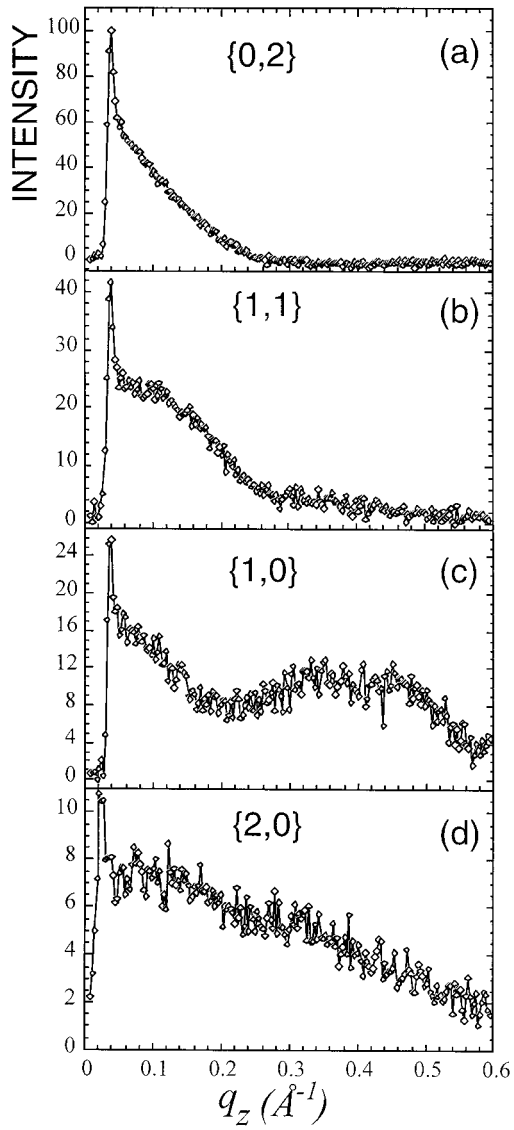

FIGURE 2 Measured Bragg rod intensity profiles along the vertical scattering vector q_z of the {0,2}, {1,1 + 1,$\bar{1}$}, {1,0}, and {2,0} reflections of uncompressed HDA-urease monolayer.

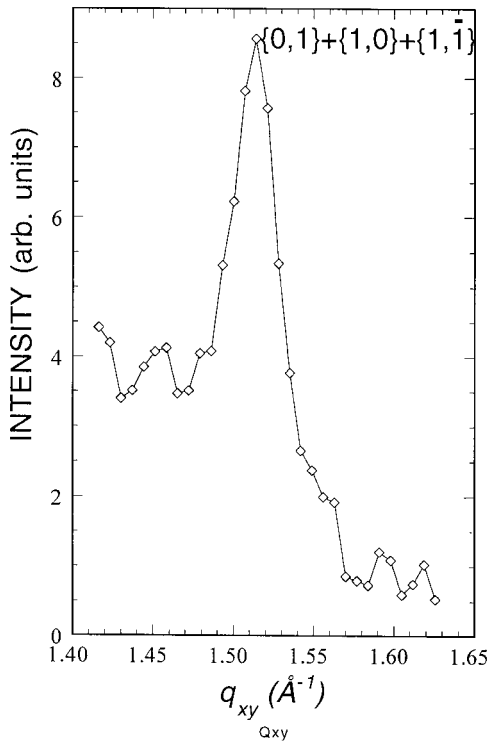

FIGURE 3 GIXD intensity pattern of compressed HDA monolayer over 2% (w/v) glucose solution at the air-water interface.

detected at $q_{xy} = 1.52$ Å^{-1} ($d = 4.13$ Å) (Fig. 3). In our experiments, the hexadecylamine monolayer was unstable; during the course of the GIXD measurements the surface pressure decreased continuously, and ~2 h after initial compression to $\Pi = 20$ mN/m, the surface pressure had dropped almost to zero. This behavior is consistent with previous observations of the behavior of Langmuir monolayers of alkyl chain amines on a neutral subphase (Adam, 1930). The single peak in the diffraction pattern of the monolayer is consistent with close-packed alkyl chains in a hexagonal lattice monolayer; this peak is indexed as {10} + {01} + {1,$\bar{1}$} (Als-Nielsen et al., 1994). We infer that the structure of a hexadecylamine monolayer spread on top of a urease film is quite different from that in the absence of urease in the subphase.

SPECULAR X-RAY REFLECTIVITY MEASUREMENTS

Specular x-ray reflection measurements record the intensity of those x-rays scattered from the air-water interface with momentum transfer strictly perpendicular to the interface. The structure factor associated with the XR profile is determined by the Fourier transform of the gradient of the electron density perpendicular to the interface. The data cannot in general be uniquely inverted to yield the electron density profile. As have many others (Kjaer et al., 1989), we have fit our XR data to a model of the interface consisting of a stack of uniform slabs, each with a different electron density ρ_i and thickness L_i. The effect of capillary waves on the density distribution in the interface is modeled by a single Gaussian roughness, σ, for all of the interfaces.

The x-ray reflectivity of a pure urease film (Fig. 4, *diamonds*) deposited on the meniscus of a subphase contacting a glass rod (Trurnit, 1960) was best fitted with a one-slab model yielding a 8.8-Å-thick layer of normalized electron density $\rho_1/\rho_s = 0.75$. As has recently been determined by small-angle x-ray scattering in aqueous solution (Hirai et al., 1993), the radius of gyration of urease is ~48 Å. The urease molecule dimensions determined by small-angle x-ray scattering in aqueous solution (Hirai et al., 1993) suggest that it is present as a hexamer of six cylindrical subunits of 28.7 Å radius and 44.4 Å height. Therefore, our x-ray reflectivity result suggests the presence of unfolded protein at the air-subphase interface.

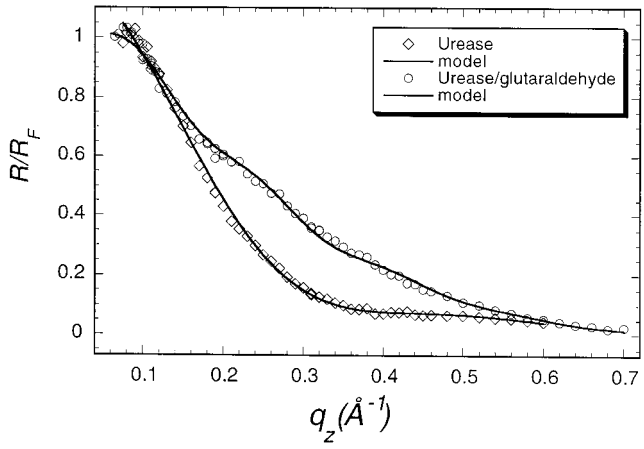

FIGURE 4 Measured (*points*) and calculated (*solid line*) x-ray specular reflectivities from urease layer at the air-aqueous interface. *Diamonds*: pure urease; *circles*: in situ covalently cross-linked urease.

To decrease the extent of unfolding of the protein molecules at the interface, the urease film was cross-linked by injecting 3 ml of glutaraldehyde (50% w/v) beneath the film shortly after spreading. X-ray reflectivity measurements (Fig. 4, *circles*) were made 30 min after the glutaraldehyde was injected. The data were best fitted using a two-slab model with a first box thickness $L_1 = 27 \pm 5$ Å and electron density $\rho_1/\rho_s = 1.02$ and a second box thickness $L_2 = 10 \pm 0.5$ Å and electron density $\rho_2/\rho_s = 0.96$. This fitting implies that the total thickness of the urease film is 37 ± 5 Å, which is within the margin of uncertainty of the value expected if the urease molecules are arranged with the monomer cylinder axis oriented along the normal to the interface.

The x-ray reflectivity of compressed HDA-urease films (Fig. 5, *diamonds*) was measured under the same conditions as used for the GIXD measurements. The best fit obtained with a two-slab model (Fig. 5, *dashed line*, Table 1) does not agree well with data; a three-slab representation gives a much better fit to the measured reflectivity data (Fig. 5, *solid line*). The thickness of the first slab is consistent with the existence of a 26 ± 2 Å urease layer (with normalized electron density $\rho_1/\rho_s = 1.03$) bound to the hexadecylamine monolayer. The amine is modeled with two slabs, one 10 ± 3 Å thick and the other 11.3 ± 0.5 Å thick, with $\rho_2/\rho_s = 0.98$ and $\rho_3/\rho_s = 0.85$, respectively. The total thickness of the hexadecylamine layer is then 21.5 ± 3 Å, which is consistent with fully extended chains aligned along the normal to the water surface. This result is in agreement with the 23.6-Å hexadecylamine layer thickness obtained from Bragg rod measurements (see above). Moreover, the urease monolayer thickness reproduces that obtained for a pure cross-linked urease film. Although most of the GIXD and XR studies of films of fatty acids, amines, or alcohols at the air-water interface (Als-Nielsen et al., 1994; Jacquemain et al., 1992; Gidalevitz et al., 1994, 1996) model the monolayer as two slabs (headgroup and hydrocarbon chain) of uniform density, we were unable to fit our reflectivity data with this model. (The normalized electron density of a protein film at the air-water interface varies from 1 to 1.45, depending on the surface pressure and the nature of the protein molecules.)

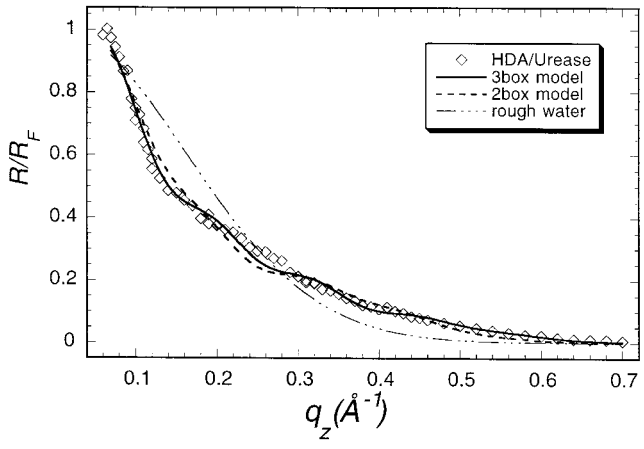

FIGURE 5 Specular x-ray reflectivity data (*diamonds*) from uncompressed hexadecylamine monolayer over cross-linked urease. *Solid line*: Best fit, three-box model. *Dashed line*: Best fit, two-box model. *Dash-dotted line*: Curve representing air-subphase interface in the absence of monolayer. Measured (*diamonds*) and calculated (*solid line*) x-ray specular reflectivities from uncompressed HDA-urease monolayer at the air-water interface.

DISCUSSION

We now examine two possible models for the HDA-urease film organization at the air-water interface. In the first model, the hexadecylamine molecules spread over the already established layer of urease molecules. Then, via interaction of the NH_3^+ amine headgroups with the amino acid residues of the protein molecules, a herringbone 5.21×7.34 Å^2 structure is stabilized. The second model implies that a hexadecylamine monolayer self-organizes in the regions of the water-air interface between islands of the urease monolayer. In this case the spreading of the hexadecylamine is restricted by the limited area of urease-free interface, hence the surface pressure rises when the hexadecylamine is added. The latter model is consistent with the fit of the XR data to a two-slab representation of the amphiphile chain domain. The jack bean urease molecule exists in solution as hexamers of identical subunits (Takishima et al., 1988). We propose that that the urease molecules at the air-water interface are arranged with the monomer cylinder axis oriented along the normal to the interface and with the protein molecule partly in solution and partly in air (Fig. 6). We expect that the electron density profile along the normal the to interface that is characteristic of this model will have three regions with different electron densities. The first region corresponds to the lower part of the urease film (inside the subphase), the second to the combined hexadecylamine film and the upper part of the urease film (outside the subphase), and the third to the hydrocarbon

TABLE 1 Fitted parameters for slab model of electron density corresponding to urease and hexadecylamine/urease x-ray reflectivity

System	No. of slabs	ρ_1/ρ_s	L_1 (Å)	ρ_2/ρ_s	L_2 (Å)	ρ_3/ρ_s	L_3 (Å)	σ
Urease	1	0.75	8.8 ± 0.2	—	—	—	—	2.9
Urease cross-linked	2	1.02	27 ± 5	0.96	10 ± 0.5	—	—	2.9
HDA/urease cross-linked two-box	1	1.02	35 ± 5	0.88	13.5 ± 0.2	—	—	3.5
HDA/urease cross-linked three-box	3	1.03	26 ± 5	0.98	10 ± 5	0.85	11 ± 0.2	3.3

ρ_s is the electron density of the subphase. ρ_1, ρ_2, ρ_3 and L_1, L_2, L_3 are electron densities and thicknesses of the first, second, and third slabs, respectively. σ represents the surface roughness parameter.

tails of the amphiphile molecules. This model is consistent with the three-slab fit to the XR data, where $\rho_1/\rho_s = 1.03$ corresponds to the partly occupied protein layer inside the subphase, $\rho_2/\rho_s = 0.98$ matches the average electron density of the amphiphile tails (with a typical value of 0.95 for close-packed monolayers at the air-water interface) and the electron density of the upper part of the urease monolayer, and $\rho_3/\rho_s = 0.86$ corresponds to the electron density of a partially occupied layer of alkyl chains. (Note that we cannot differentiate between the amine headgroup and the

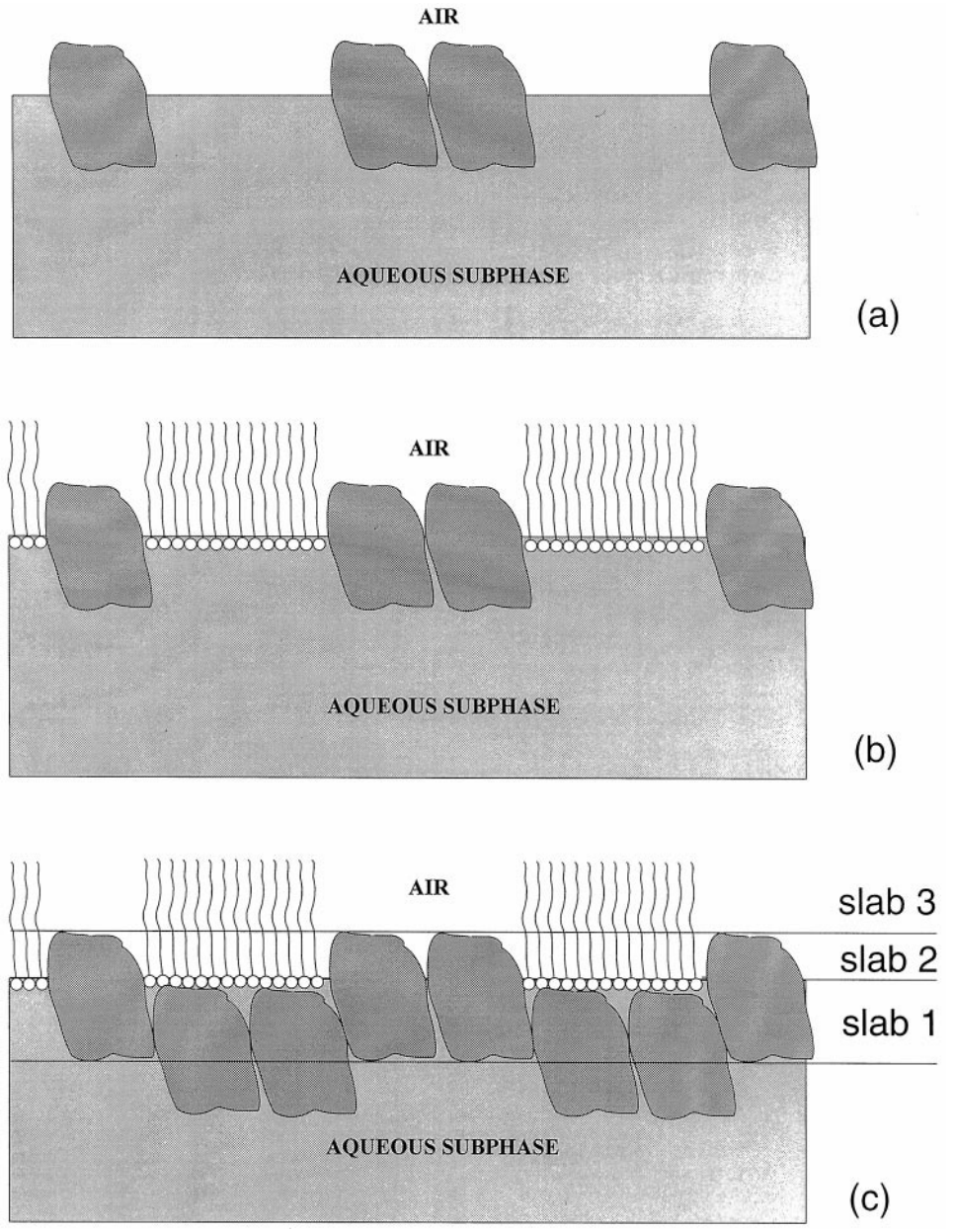

FIGURE 6 The proposed arrangement of the hexadecylamine and the urease enzyme at the air-water interface. The three regions of the film (boxes 1–3) correspond to the three-box model of the reflectivity data.

hydrocarbon chain because they have essentially the same electron density.)

In conclusion, our grazing incidence x-ray diffraction and reflectivity data demonstrate that hexadecylamine, when spread on top of a urease film at the air-water interface, forms a stable, well-organized structure. X-ray reflectivity data show that the urease film in the absence of fatty amine molecules unfolds at the interface to a 9-Å-thick layer. However, with the cross-linking agent glutaraldehyde added to the subphase, the urease molecules most likely remain intact at the interface or unfold only partially. It is also likely that protein and hexadecylamine domains coexist at the interface.

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