Polarization-Modulated FT-IR Spectroscopy Studies of Acetylcholinesterase Secondary Structure at the Air−Water Interface

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Abstract: The vibrational spectrum of acetylcholinesterase (AChE) at the air−water interface in its free form and bound to either its substrate, acetylthiocholine, or organophosphorus (OP) inhibitor has been studied by polarization modulation infrared reflection absorption spectroscopy (PMIRRAS). The shape and position of the amide I band was used to gauge the surface orientation of α-helices and β-sheets. The measured secondary structure content indicated that the enzyme did not unfold for the surface pressures used (0−30 mN/m). At low surface pressures, a strong amide I band indicated that the average tilt axis of the helices was aligned parallel to the air−water interface. Upon further compression, the α-helix component was significantly reduced, because the tilt axis of the helix relative to the water surface achieved a perpendicular orientation. PMIRRAS was also used to investigate the effect of phospholipids on molecular organization and orientation of AChE at the air−water interface. The enzyme was found to be fully inserted into the lipidic film during compression. The hydrolysis and inhibition were studied at the air−water interface. Band frequencies associated with acetylthiocholine binding to the enzyme active site and formation of the reaction products were observed. The OP inhibitor, paraoxon, was observed to unfold the enzyme at the air−water interface, because only high-frequency components associated with the extended conformation were observed upon compression. The secondary structure of the AChE was reestablished 30 min after a reactivator, trimethyl bis-(4-formylpyridinium bromide) dioxime, was injected beneath the paraoxon-inhibited AChE. For the first time, an in situ study of the protein conformation is reported using the PMIRRAS technique, and direct supporting evidence that the enzyme did not lose its native secondary structure upon spreading at the air−water interface is provided.

Introduction

Adsorption of proteins or enzymes at interfaces is recognized to be a critical event in biosensor development and biotechnology. However, many of the fundamental mechanisms of protein adsorption, such as conformation and orientation, remain only partly understood, and direct evidence of their orientational arrangement at interfaces is scarce.[1,2]

Acetylcholinesterase (AChE) is a serine hydrolase that is responsible for rapid hydrolysis of the neurotransmitter acetylcholine after its release at cholinergic synapses.[3,4] AChE is the primary target for organophosphorus (OP) compounds,[5−9] which cause irreversible inhibition of its active site.[10−14] An AChE monomer has an ellipsoidal shape and consists of 12 β-sheets surrounded by 14 α-helices.[15] The infrared (IR) spectrum of the AChE native structure was reported earlier,[16] and the relative content of different secondary structure components was estimated to be 34−36% for α-helices, 19−25% for β-sheets, and 15−16% for turns.[16] Secondary structure studies of proteins in aqueous solution are complicated by the strong absorption of water near the amide I and II regions of interest. High concentrations of proteins and deuterium oxide (D_2O) as solvent are required to overcome this difficulty. Nevertheless, band shifting occurs because of D_2O,[17] and causes uncertainty in band assignments. Until recently, conformation changes were investigated after proteins were removed from the air−water[18] or the solid−water interfaces[19,20] using attenuated total reflection

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spectroscopy. Obviously, determination of the protein conformation is not directly measured at the interface. To directly measure the conformational changes of proteins at interfaces, a new technique known as polarization modulation infrared reflection absorption spectroscopy (PMIRRAS) has been developed.[21−24] The use of Fourier-transform (FT)-IR spectroscopy at the air−water interface is possible within the entire mid-IR range after a polarization modulation. This technique has been proven to be insensitive to the strong IR absorption of water vapor, and only important bands arising from the monolayer are observed.[22] Moreover, PMIRRAS allows the extraction of information about orientation and conformation of monolayer molecules. The PMIRRAS method opens new exciting possibilities for studying the organization and conformation of biological systems in situ.

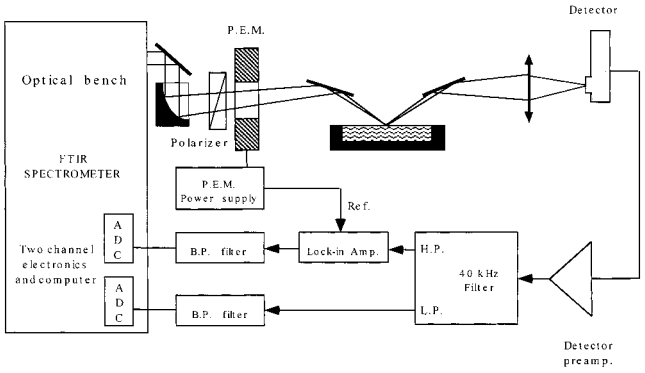
In the present work, we report for the first time a study of AChE secondary structure directly at the air−water interface using in situ IR spectroscopy. The stability of AChE monolayer at the air−water interface was demonstrated earlier.[25] On the basis of spectral features of amide I and II bands, we characterize the orientation of α-helix and β-sheet components relative to the surface as a function of surface pressure. In the second part of this work, we present results of an air−water interface interaction between phospholipids and AChE. AChE is adsorbed on phospholipid monolayers, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoyl phosphatidic acid (DPPA), used as model membranes to mimic the organization of lipids in biological membranes. Finally, the effect of the enzyme binding to either acetylthiocholine or OP inhibitor, paraoxon, is investigated.

Experimental Section

AChE (EC 3.1.1.7: V−S from electric eel), acetylthiocholine iodide (ATChI), [trimethyl bis-(4-formylpyridinium bromide) dioxime] TMB-4, DPPC (99%), and DPPA (99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Diethyl *p*-nitrophenyl phosphate (paraoxon, 99%) was obtained from Chem. Service, Westchester, PA. All chemicals were used as purchased without further purification. The enzyme solutions were prepared freshly on the day of experiments at 1 mg/mL of buffered solution (0.1 M KH_2PO_4, 0.1 M NaOH, pH 6.5). The paraoxon concentrations were 4×10^{-4} and 4×10^{-5} M. Concentrations of ATChI and TMB-4 were 10^{-2} and 10^{-3} M, respectively.

A Nima Langmuir trough (Nima Technology, Coventry, UK) made up of Teflon and with dimensions of 30×10 cm^2 was used. The surface pressure was measured with a Nima Wilhelmy plate. The water used for the preparation of the subphase was purified by a water purification system (Milli-Q from Millipore), and it has a specific resistivity of 18 MΩ·cm and constant temperature of 20 ± 1 °C. PMIRRAS spectra were recorded on a Nicolet 740 spectrometer (Madison, WI) equipped with a photovoltaic detector, HgCdTe (Sat., Poitier, France), cooled with liquid nitrogen (Scheme 1).[22] The incident IR beam was polarized by a ZnSe polarizer and modulated by a ZnSe photoelastic modulator (Hinds type III, Portland, OR) between parallel (p) and perpendicular (s) polarizations to the plane of incidence. The modulation frequency is set up at 1666 cm^{-1} and 400 scans were collected for each spectrum.

Scheme 1. Schematic Diagram of Optical PMIRRAS Setup and of Two-Channel Electronic Processing



The optimal angle of incidence for detection was 75° relative to the interface normal.

Experiments were conducted to assess conformational changes of AChE during the compression. Two initial spreading surface pressures (0 and 5 mN/m) were used to determine the effect of spreading on the degree of unfolding at the air−water interface. An initial surface pressure of 5 mN m^{-1} was obtained by increasing the number of molecules spread at the interface and decreasing the trough surface area. PMIRRAS spectra were collected during compression and decompression of the AChE monolayer. The enzyme−phospholipid interaction was studied by spreading the DPPC or DPPA as a monolayer and injecting a solution of AChE into the subphase. Similarly, the AChE solution was spread on a subphase containing the substrate, ATChI, and PMIRRAS spectra were collected before and during the compression to follow the hydrolysis reaction at the air−water interface. Molecular interaction between the AChE and paraoxon was investigated by dissolving the inhibitor in the subphase and spreading the enzyme as a monolayer. PMIRRAS spectra were recorded during the compression. Reactivation of paraoxon-inhibited AChE was carried out with TMB-4. A solution of dioxime (10^{-3} M) was injected beneath the inhibited AChE and PMIRRAS was used to monitor the spectra as a function of time.

Deconvolutions were performed using PeakSolve 1.01 (Galactic Industry Corp., Salem, NH) to estimate quantitatively the spectral parameters of overlapping components shown in the PMIRRAS spectra. Band positions and bandwidths were fixed. The positions of overlapping peaks have been identified and followed by iterative band fitting to the original spectra.

Results and Discussion

1. Secondary Structure of AChE at the Air−Water Interface. PMIRRAS spectra collected during compression of the AChE monolayer are shown in Figure 1. Band positions and proposed assignments are listed in Table 1. Careful examination of the amide I band (1700−1600 cm^{-1}) revealed three overlapping resonances with two strong absorptions at 1655 and 1630 cm^{-1} and a weaker one at 1696 cm^{-1}. The amide II band (1600−1500 cm^{-1}) was centered at 1535 cm^{-1}. Frequency associated with C−H side-chain vibrations was observed at 1450 cm^{-1}. On the basis of experimental[26−28] and theoretical calculations,[29−32] the 1655 cm^{-1} frequency was assigned to the α-helical conformation, whereas 1630 cm^{-1} was associated with the antiparallel pleated sheet conformation (β-

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Table 1. PMIRRAS Frequency Bands and Proposed Structural Assignments to AChE Secondary Structure

frequency band positions (cm^{-1})	proposed assignment of the amide I to secondary structure	
	spreading at nil surface pressure	spreading at an initial surface pressure of 5 mN/m
1696	antiparallel pleated sheet or turn	antiparallel pleated sheet
1655	α-helix (short population)	α-helix (short population)
1647	α-helix (longer population)	
1640		unordered structure
1630	β-sheet	β-sheet

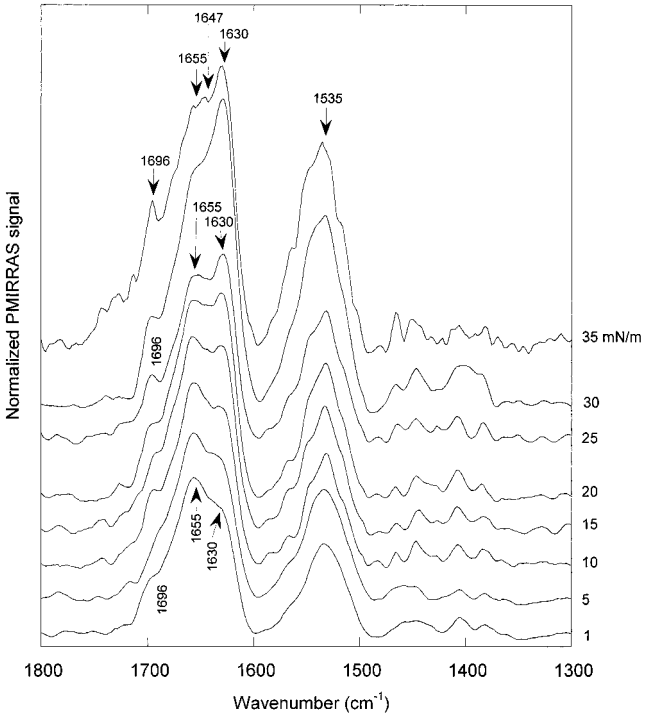

Figure 1. Normalized PMIRRAS spectra of AChE monolayer collected at the air−water interface at different surface pressures. The AChE solution was spread at zero surface pressure.

structure). Our data agree with the IR spectrum of AChE native structure obtained in solution.[16] At low surface pressures (5 and 10 mN/m), α-helical structure represented the strongest component, whereas the β-sheet structure appeared only as a shoulder. The higher frequency 1696 cm^{-1} has been recognized to be associated with the amide I vibration of the antiparallel pleated sheet.[31−33] Further compression led to formation of a new band at 1647 cm^{-1} (Figure 1) associated with a second class of α-helices.[34] The X-ray study[15] showed that an AChE monomer consists of 14 α-helices, varying from 7 to 35 residues per helix in length. Short α-helices with 14 or fewer residues exhibited higher frequencies (1655 cm^{-1}), and lower (1647 cm^{-1}) for longer α-helices (15 or more residues). In this work, the two α-helix classes were distinguished at higher surface pressure (35 mN/m) using the PMIRRAS technique. This phenomenon was predicted earlier[34] and observed experimentally[16] in solution.

PMIRRAS spectra collected after spreading of AChE at a surface pressure of 5 mN/m are shown in Figure 2a. Band positions and proposed assignments are given in Table 1. The spectra are structurally similar to that obtained from zero surface pressure (Figure 1). The AChE monolayer was decompressed from 25 to 5 mN/m (Figure 2b). The band at 1655 cm^{-1} reappeared with a well-defined form, at 20 mN/m, whereas the 1630 cm^{-1} band remained strong. Upon further decompression, both the α-helix and β-sheet bands were prominent. PMIRRAS spectra collected after the monolayer was recompressed to 25 mN/m exhibited bands associated with the α-helix and the β-sheet conformations, whereas the 1640 cm^{-1} frequency disappeared. In all PMIRRAS spectra, at low surface pressures (1−15 mN/m), the α-helix was dominant, whereas the β-sheet structure was less significant. At high surface pressures (20−35 mN/m), the β-sheet became much more pronounced, whereas the α-helix was shown only as a shoulder. A change in the orientation of the AChE secondary structure components occurred during the compression. In fact, at low surface pressure, the α-helices were lying parallel to the water surface and resulted in a strong amide I band, whereas the β-sheet conformation was seen only as a weak shoulder. This agrees with computer simulations.[24]

Quantitative analysis showed that the α-helix orientation relative to the water surface had a relatively constant amide I to amide II ratio, but the integrated intensity ratio of the α-helix to the β-sheet [Ic(α)/ Ic(β)] was significantly altered (Table 2). The average orientation of the α-helix and the β-sheet relative to the water surface during the compression was estimated on the basis of their integrated intensities that are proportional to the orientational factor (Table 2). As the monolayer was compressed, the tilt axis of the helix had the tendency to be oriented perpendicular to the water surface, resulting in a linear decrease of the integrated intensity. This is in agreement with surface potential and surface dipole moment of the AChE monolayer data reported recently.[35] The same qualitative trend was observed in the case of β-sheet conformation. At the end of the compression, both conformations decreased in their intensities and resulted in a significant decrease in the integrated intensity of the amide I band (Figure S1). However, this decrease does not imply necessarily a decrease in the α-helix and β-sheet content, but it is rather due to a reorientation of the α-helix conformation upon compression. A decrease in the integrated intensities of the spectral parameters resulted from spreading at high surface pressures (Figure S2a). However, the decrease was not as significant as in the first case, in which the intensities of the helices decreased by a factor of 5; this factor is only 1.5 in the second case. Decompression of the monolayer showed that this phenomenon was reversible (Figure S2b). The percentages of the α-helix and the β-sheet (Table 2) were estimated at different surface pressures using the area absorptivities (integrated intensities) of each conformation and expressing them as a fraction of the total amide I band area. However, as PMIRRAS is sensitive to the secondary structure orientation, these data are only indications of relative proportions and changes occurring in the films and should not be interpreted as exact values.

2. Temperature Behavior of the AChE Secondary Structure. The thermally induced denaturation of the enzyme

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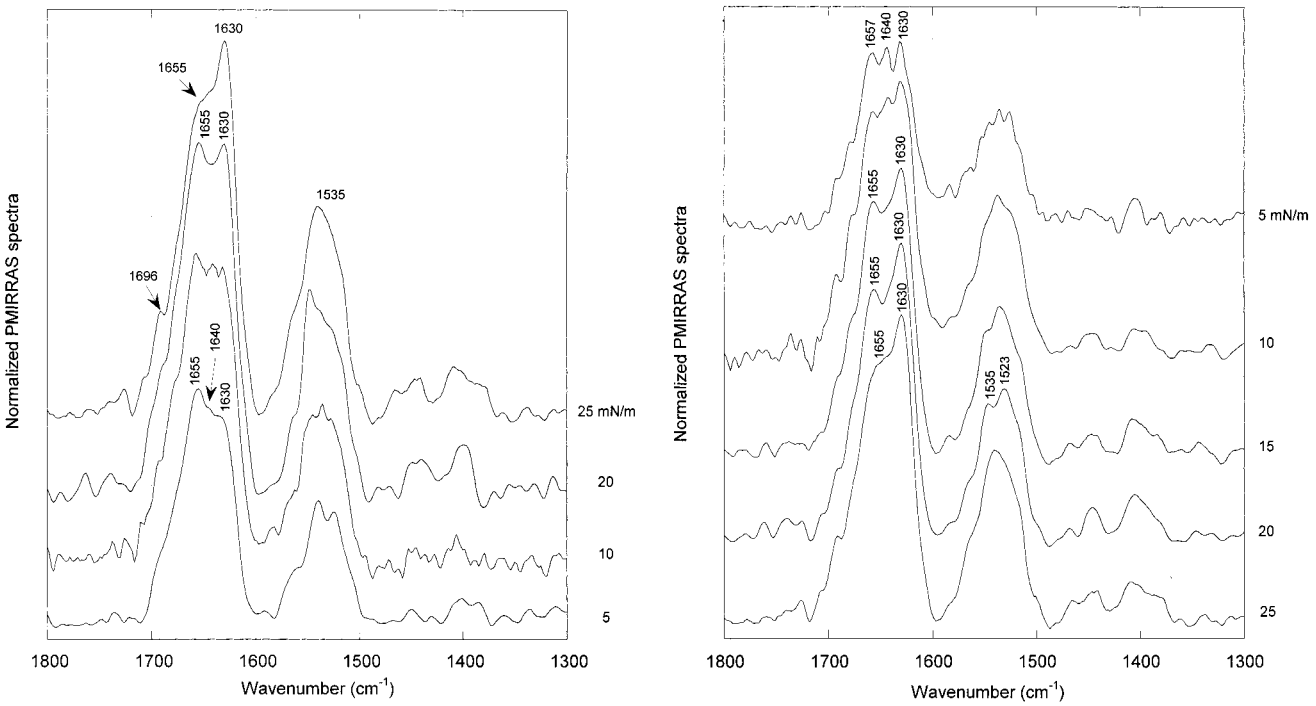


Figure 2. (Left) Normalized PMIRRAS spectra of AChE monolayer collected during the compression at the air−water interface at different surface pressures. The AChE solution was spread at an initial surface pressure of 5 mN/m. (Right) Normalized PMIRRAS spectra of AChE monolayer collected during the decompression at the air−water interface at different surface pressures. The AChE solution was spread at a surface pressure of 5 mN/m.

Table 2. Quantitative Estimation of Secondary Structure at Different Surface Pressures

surface pressure (mN/m)	amide I	amide II	amide I/amide II	Ic (α-helix)/ Ic (β-sheet)	α-helix content (%)	β-sheet content (%)
5	13.43	5.40	2.50	2.00	44	38
10	14.35	4.00	3.52	1.20	36	31
15	14.70	4.43	3.10	1.40	39	27
20	16.50	5.23	3.14	1.00	33	33
25	15.60	5.00	3.06	0.99	30	30
30	18.35	7.50	2.44	0.76	30	39
35	20.80	10	2.07	0.75	29	31

monolayer was examined at 32 °C at the air−water interface. At this temperature, the frequency band associated with the α-helical structure disappeared and unfolding of the enzyme occurred. This is illustrated as a strong band around 1626 cm^{-1} at high surface pressures (Figure 3), whereas the α-helix component appeared as a weak shoulder only at 5 mN/m and disappeared upon further compression. The calculated content of the β-sheet structure increased from 64% at 5 mN/m to 75% at 25 mN/m. The amide I to amide II intensity ratio was 0.92 and 0.84 at surface pressures of 5 and 25 mN/m, respectively. On the other hand, this ratio was in the range 2.5−3.0 at the same surface pressures at 20 °C (Table 2). The presence of a strong absorption band at 1626 cm^{-1} and the low intensity ratio were consistent with the presence of a large content of β-sheet structure.

Comparing these PMIRRAS spectra with the ones obtained at 20 °C (Figure 1), it is obvious that denaturation of the enzyme was induced upon compression at 32 °C. Indeed, a large content of β-sheet was obtained at high temperature, whereas the enzyme retained mostly its native configuration at 20 °C. Recently, a temperature behavior of AChE secondary structure using D_2O as buffer showed that denaturation of the enzyme occurred at 55 °C,[16] and thermal inactivation of AChE above 32 °C.[16] However, it is rather difficult to compare these data with the ones obtained at the air−water interface because high temperature affects the monolayer structure as well.

3. PMIRRAS Study of Phospholipids and Phospholipid−Enzyme Interactions. DPPC and DPPA Monolayers. Normalized PMIRRAS spectra of pure DPPC collected at different surface pressures are shown in Figure 4. The PMIRRAS signal increased as the surface pressure increased. However, the shape and position of the bands remained unchanged. At low surface pressure (10 mN/m), the spectra exhibited six strong bands at 1728, 1468, 1228, 1087, 1058, and 969 cm^{-1}. At higher surface pressures (30 and 40 mN/m), the band position 1728 cm^{-1} shifted to 1735 cm^{-1}. This frequency shift indicates that at low surface pressures, the ester bonds interacted with the water subphase through hydrogen bonding. Upon further compression, there are weak interactions of hydrogen bonding with water molecules and the frequency component associated with the stretching C═O ester bond shifted to 1735 cm^{-1}. Moreover, the high intensity of this band indicates that the orientation of the carbonyl group is in the plane of the film. On the other hand, the sharp band seen at 1468 cm^{-1} is associated with CH_2 bending mode. Its high intensity indicates a high organization and cooperative orientation of the long alkyl chains at the interface, in contrast to the reported DMPC data, where a hydrocarbon chain disorder was observed.[23] Asymmetric and

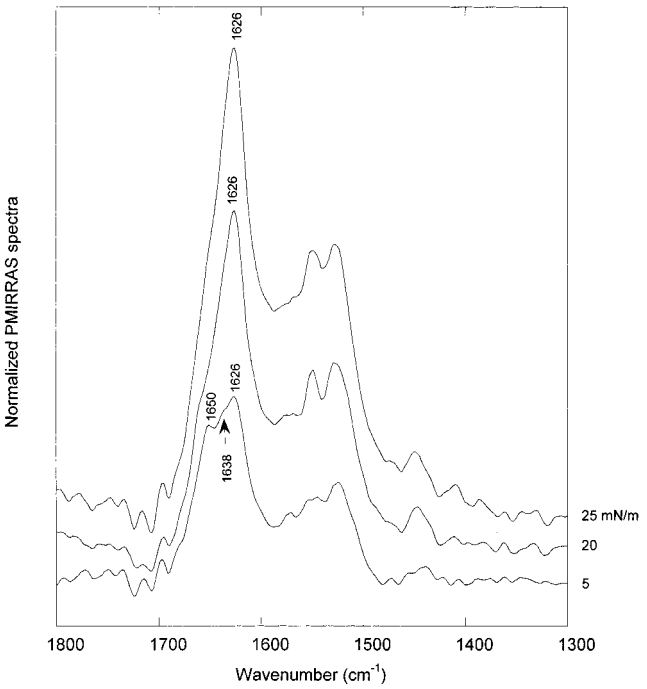


Figure 3. Normalized PMIRRAS spectra of AChE monolayer collected at the air−water interface at different surface pressures. The AChE solution was spread at zero surface pressure on a subphase at temperature of 32 °C.

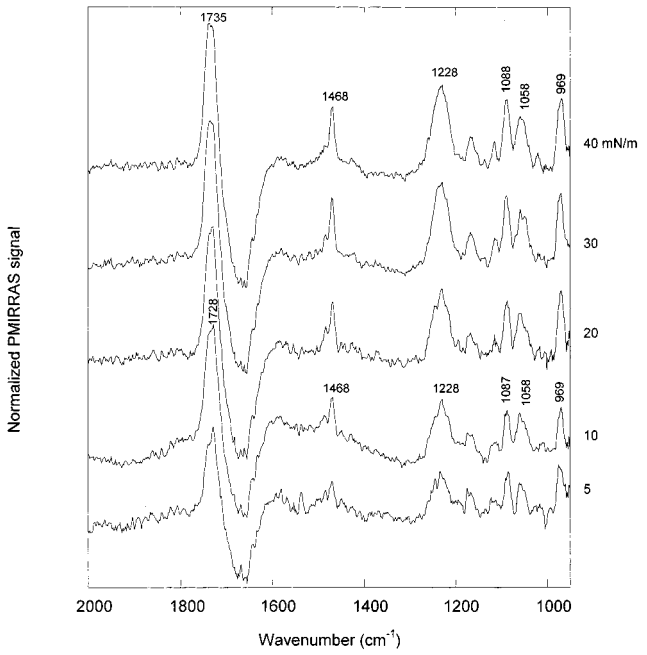


Figure 4. Normalized PMIRRAS spectra of DPPC monolayer collected at the air−water interface at different surface pressures.

symmetric P═O stretching vibrations were observed at 1228 and 1087 cm^{-1}, respectively. Similarly, frequency shifts between low and high surface pressures were observed for the PO_2− (Figure 4), which indicate the presence of hydrogen bonding between the phosphate groups and water molecules at low surface pressures. The C−O−P stretching vibrations were observed at 1058 cm^{-1} and the asymmetric stretching modes of the trimethylammonium group $N^+(CH_3)_3$ was seen as a sharp band at 969 cm^{-1}. The upward position of this band suggests that the orientation of the transition moment of the C−N stretching vibration was parallel to the water interface.

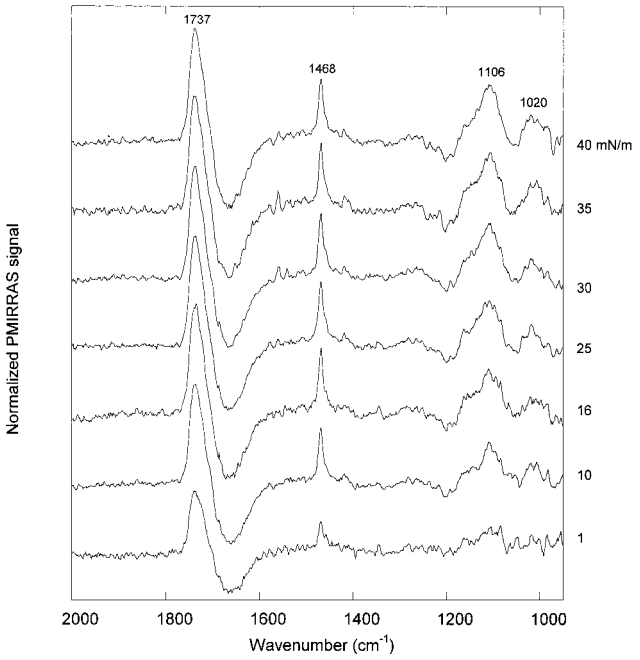


Figure 5. Normalized PMIRRAS spectra of DPPA monolayer collected at the air−water interface at different surface pressures.

Normalized PMIRRAS spectra of DPPA are shown in Figure 5. The PMIRRAS signal increased as the surface pressure increased. Similarly, the stretching vibrations of the ester carbonyl group were exhibited as a strong band at 1737 cm^{-1} and the upward orientation of this band indicates that the transition moment of these vibrations was in the surface plane. The CH_2 bending mode was shown as a sharp band at 1468 cm^{-1} and broad bands were observed at 1106 and 1020 cm^{-1} and were associated with the symmetric P═O and C−O−P vibrations, respectively.

Phospholipid−AChE Interaction. The interaction between DPPC and the enzyme was studied at the air−water interface by spreading DPPC as a monolayer and injecting AChE into the subphase. Normalized PMIRRAS spectra of the mixed film collected at a surface pressure of 15 mN/m are shown in Figure 6. The spectra exhibited bands associated with the presence of both the phospholipid and the enzyme at the air−water interface. Bands assigned to the ester carbonyl group C═O, CH_2 bending mode, asymmetric P═O, symmetric P═O, C−O−P, and asymmetric C−N stretching vibrations were observed at 1733, 1468, 1227, 1085, 1055, and 972 cm^{-1}, respectively. Moreover, the amide I and II bands were observed at the expected positions. The main bands of the transition moments were being oriented upward relative to the baseline (Figure 6). This indicates that their transition moments were in the surface plane. The β-sheet strand is seen at 1630 cm^{-1}, whereas the α-helix was shown as a weak shoulder around 1655 cm^{-1}. The amide II band was shown as a strong positive band centered at 1535 cm^{-1}. The presence of bands associated with the phospholipid and the AChE indicates that the enzyme was incorporated into the lipidic film at the interface. The position of the amide I and II bands obtained in the mixed lipid−enzyme monolayer indicates that no changes occurred in the AChE secondary structure in comparison with the PMIRRAS spectra of the pure AChE monolayer (Figure 1). Furthermore, similar orientation of the α-helix and β-sheet components was observed in the presence of a lipidic film at the interface.

PMIRRAS spectra of the DPPA-AChE interaction (Figure 7) showed similar spectral features to those of the DPPC-AChE

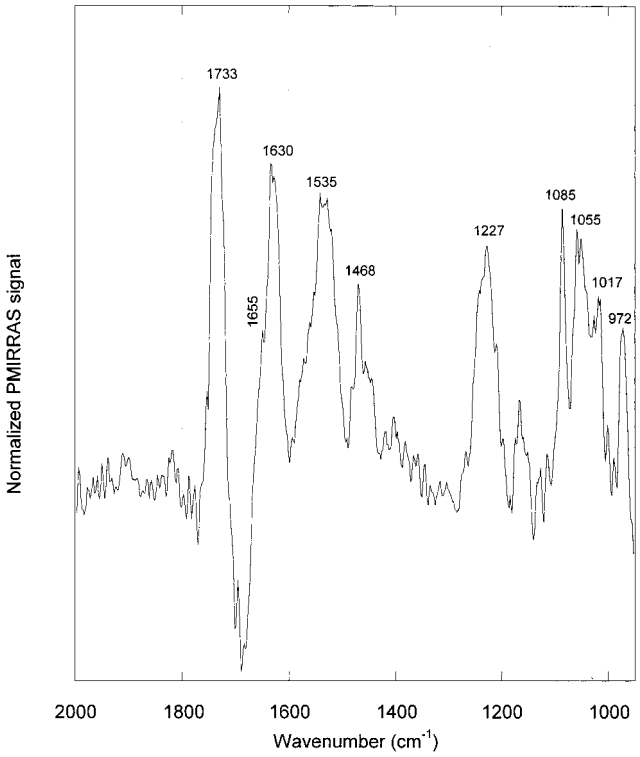


Figure 6. Normalized PMIRRAS spectra of mixed AChE-DPPC film at the air−water interface at a surface pressure of 15 mN/m.

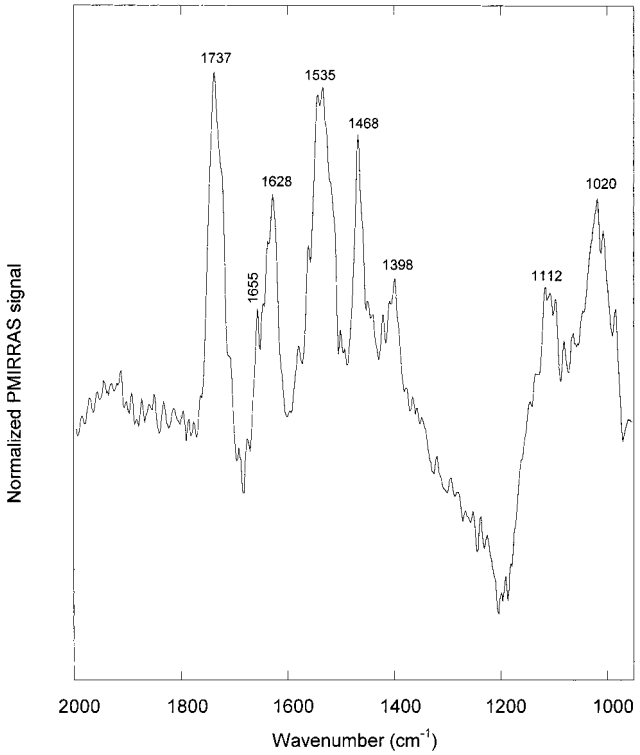


Figure 7. Normalized PMIRRAS spectra of mixed AChE-DPPA film at the air−water interface at a surface pressure of 15 mN/m.

interaction. Bands associated with the ester bond, the CH_2 bending, the symmetric P═O, and C−O−P vibrations are observed around 1737, 1468, 1112, and 1020 cm^{-1}, respectively. The β-sheet was seen at 1628 cm^{-1} and the α-helix was shown only as a weak shoulder at 1655 cm^{-1}. On the other hand, the amide II band was centered at 1535 cm^{-1}. From these results, the PMIRRAS technique was proven to be very sensitive to

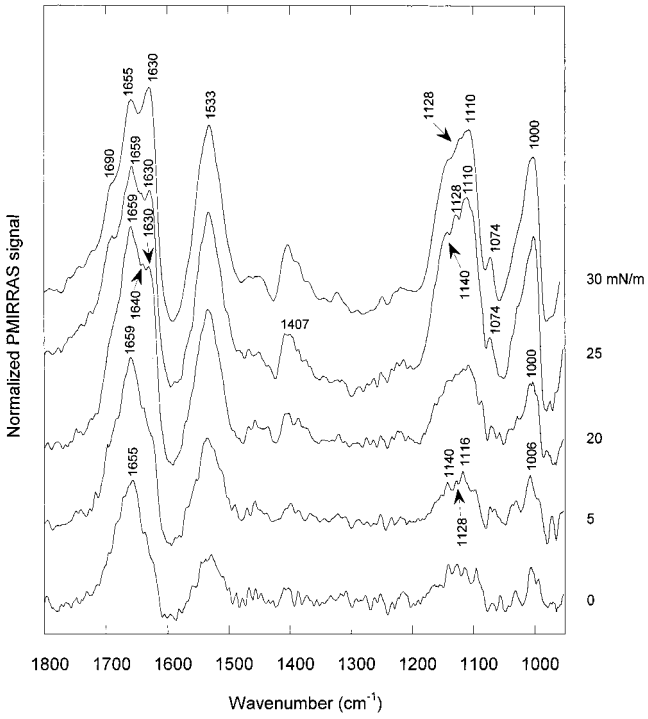


Figure 8. Normalized PMIRRAS spectra of AChE monolayer collected at the air−water interface at different surface pressures. The AChE was spread zero surface pressure and the monolayer was compressed on a subphase containing the acetylthiocholine iodide.

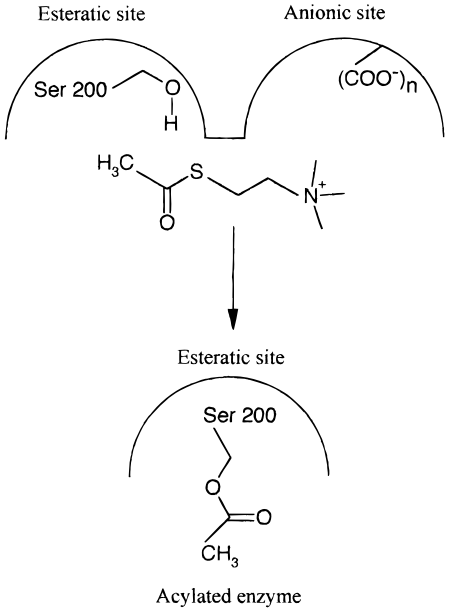
measure the vibrational modes of mixed phospholipid−enzyme monolayer at the interface. These data are the first obtained by IR spectroscopy at the air−water interface and showed that the secondary structure and orientation at the interface were not modified when the enzyme was inserted into a lipidic film. The same phenomenon was observed with synthetic peptides at the air−water interface.[24] The in situ study of secondary structure and orientation of proteins within a lipid matrix is of considerable interest in understanding the molecular organization of biological membranes.

4. AChE−Substrate Interaction at the Air−Water Interface. The enzyme−substrate interaction was studied at the air−water interface by spreading the enzyme as a monolayer and dissolving the substrate, ATChI, into the subphase. Temperature and pH of the subphase were 20 °C and 8, respectively. PMIRRAS spectra were collected at different surface pressures (Figure 8). At zero surface pressure, only an α-helix component was seen at 1655 cm^{-1}. At 5 mN/m, the α-helix shifted to higher frequency (1659 cm^{-1}). These frequency shifts were caused by changes in the AChE conformation, which are reversible and necessary to position the substrate for subsequent intramolecular interactions. Compression to higher surface pressures (30 mN/m) resulted with both the α-helix and the β-sheet structures at 1655 and 1630 cm^{-1}, respectively. The band at 1407 cm^{-1} (Figure 8) is associated with the stretching vibrations of C−N of the choline moiety. The broad band seen at 1160−950 cm^{-1} is mainly attributed to the symmetric stretching vibrations of C−O−C, and this indicates that the enzyme was in the acylation form (Scheme 2). The C−C vibrations occurred in this spectral region and made the band stronger. On the other hand, the frequency bands 1074 and 1128 cm^{-1}, seen only at high surface pressure (25 mN/m), were assigned to vibrational modes of the reaction products, thiocholine iodide. In fact, bands in the same region (1080 and 1130 cm^{-1}) were observed in the IR spectrum of the choline iodide. PMIRRAS data indicated the presence

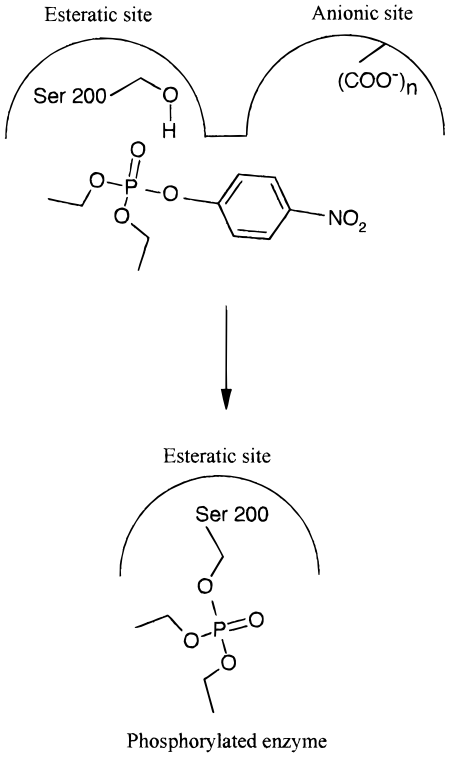
Table 3. PMIRRAS Frequency Band Positions and Their Proposed Structural Assignments to Paraoxon-inhibited AChE

frequency bands (cm^{-1})	paraoxon concentration = 4×10^{-4} M			paraoxon concentration = 4×10^{-5} M	
	1 mN/m	3 mN/m	5 mN/m (collapse surface pressure)	1 mN/m	10 mN/m
1708, 1700		carbonyl C═O stretching	carbonyl C═O stretching (very strong)		carbonyl C═O stretching
1684, 1670	extended configuration	extended configuration		extended configuration	extended configuration
1660	unordered structure	unordered structure			
1085	P═O symmetric stretching	P═O symmetric stretching	P═O symmetric stretching	P═O symmetric stretching	P═O symmetric stretching
1025		P−O−C stretching	P−O−C stretching	P−O−C stretching	P−O−C stretching
1006	P−O−phenyl stretching			P−O−phenyl stretching	

Scheme 2. Diagram of AChE Active Site When Enzyme Is in Acylation Form



Scheme 3. Scheme Showing Phosphorylation of Enzyme Active Site



of both the substrate and the reaction product at the interface 10 min after AChE spreading. This agrees with the atomic force microscopy (AFM) images and the UV−Vis results reported recently.[35]

5. AChE−Inhibitor Interaction. ***Paraoxon.*** Molecular interaction of AChE with paraoxon was studied at the air−water interface by dissolving the paraoxon in the subphase at a concentration of 4×10^{-4} M and spreading the enzyme as a monolayer. At first glance, the AChE secondary structure was destroyed upon the paraoxon binding to the enzyme, because the obtained spectra did not have the shape and the position of the amide I and II bands. Band positions and proposed assignments are shown in Table 3. The amide I band position shifted to higher frequency components and the amide II band was significantly altered and vanished at the collapse surface pressure (Table 3). The frequencies 1684 and 1670 cm^{-1} are associated with the extended configuration of the enzyme, whereas the 1660 cm^{-1} band arose from unordered structure caused by inhibitor binding. Because the frequency associated with α-helices disappeared and only high frequencies were observed, the enzyme was interpreted to be completely unfolded. The band at 1684 cm^{-1} was displaced to higher frequency (1708 cm^{-1}), and shifted to 1700 cm^{-1} at the collapse surface pressure. Thus, the binding of the inhibitor to the AChE active site resulted in a completely unfolded AChE by causing disruption of the intramolecular hydrogen bonding. Therefore, the double-bond character of the carbonyl group had a tendency to increase and resulted in an increase of the stretching frequency. Thus, at the collapse surface pressure, the presence of completely unfolded AChE at the interface results in a strong band around 1700 cm^{-1}. This agrees with AFM results reported recently, where an unfolding of AChE was observed when the enzyme was compressed on a subphase containing the paraoxon.[35]

Moreover, a strong 1006 cm^{-1} at 1 mN/m band was shifted to 1025 cm^{-1} upon further compression. This band arises from P−O−phenyl stretching vibrations of the inhibitor. Therefore, at this surface pressure, there was formation of an enzyme−inhibitor complex at the interface. The leaving group (phenyl group) of the inhibitor was still partially associated with the complex. At a surface pressure of 3 mN/m, a strong band appeared at 1025 cm^{-1} and was assigned to the asymmetric stretching vibration of the P−O−C group after the inhibitor binding to the enzyme (Scheme 3). Furthermore, at the collapse surface pressure, P═O symmetric stretching vibration was seen at frequency 1085 cm^{-1}. The presence of strong bands associated with both the P═O and the P−O−C stretching vibrations indicates that the enzyme was phosphorylated at the interface.

Table 4. PMIRRAS Frequency Bands Position and Their Proposed Structural Assignments to Paraoxon-Inhibited AChE and Reactivated Inhibited AChE

frequency band positions (cm^{-1})	proposed assignment of amide I to secondary structure	
	inhibited AChE	reactivated inhibited-AChE
1656		α-helix
1644	unordered structure	
1640		unordered structure
1628		β-sheet
1626	β-sheet	

The effect of a lower inhibitor concentration (4×10^{-5} M) was investigated and the PMIRRAS data showed the same general spectral features as the high concentration (Table 3). During the compression and at zero surface pressure, frequency bands are observed around 1085 and 1006 cm^{-1}. These frequency bands are associated with the P═O and P−O−phenyl symmetrical stretching modes. Moreover, the presence of completely unfolded enzymes at the interface resulted in the strong band around 1700 cm^{-1}.

Paraoxon had a significant effect on the AChE conformation. Inhibition occurs by nucleophilic attack of the catalytic serine hydroxyl, resulting in a stable tetrahedral configuration that mimics the tetrahedral intermediate during the acylation process.[9] Unlike the deacylation process, the dephosphorylation of the AChE takes place at a very slow rate. These data are in agreement with UV−Vis and AFM results where large domains were observed at the interface, rather than an organized AChE monolayer.[35]

6. Reactivation of Paraoxon-Inhibited AChE. Paraoxon inhibited AChE was reactivated by injecting the nucleophilic agent TMB-4 beneath the inhibited AChE monolayer. The PMIRRAS data are shown in Table 4. The α-helix and the β-sheet components of the amide I band were observed at 1656 and 1628 cm^{-1}, respectively. From the PMIRRAS data, the AChE secondary structure was reestablished 30 min after the dioxime injection following the inhibition by paraoxon. Spontaneous reactivation of the AChE after inhibition by paraoxon was reported earlier, where 100% of the enzyme activity was restored using dioxime as a reactivator.[36−39] However, no data were reported concerning the changes in the enzyme conformation. For the first time, we report in this paper the changes in the enzyme secondary structure after a reactivation following the paraoxon enzyme inhibition. In fact, the reactivation mechanism occurred via a phosphorylation of dioxime. Therefore, dephosphorylation occurred and the enzymes were liberated into the subphase.

Conclusion

The first complete characterization of an enzyme secondary structure when adsorbed at the air−water interface is reported. PMIRRAS was proven to be a sensitive technique to measure the vibration modes of AChE α-helices and β-sheets as a monolayer. The content of the amide I was evaluated qualitatively and quantitatively at different surface pressures and molecular areas, and indicated that the enzyme did not unfold when spread as a monolayer. The orientation of the α-helix and β-sheet components at the air−water interface changed upon compression and decompression of the AChE monolayer. Molecular interaction between AChE and the paraoxon inhibitor caused drastic and irreversible changes of the AChE secondary structure. Refolding of the paraoxon-inhibited AChE was demonstrated by using the PMIRRAS technique.

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Supporting Information Available: Figures showing orientation of amide I components at the air−water interface as a function of surface area during AChE monolayer compression at different surface pressures (PDF). This material is availabe free of charge via the Internet at http://pubs.acs.org.

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