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Acetylcholinesterase Complexation with Acetylthiocholine or Organophosphate at the Air/Aqueous Interface: AFM and UV−Vis Studies

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The hydrolysis reaction of acetylthiocholine catalyzed by the enzyme, acetylcholinesterase (AChE), was studied at the air/aqueous interface by spreading the enzyme as a monolayer and dissolving the substrate in the subphase. The reaction progress was monitored by time-dependent UV−vis, and the topography of the Langmuir−Blodgett films was determined by tapping mode atomic force microscopy (TMAFM). For a better understanding of the complex formation mechanism between AChE and its substrate, acetylthiocholine, the AChE monolayer was prepared and examined with TMAFM in two steps. The monolayer was first compressed on the substrate-free buffered subphase. Once a surface pressure of 25 mN/m was reached, the acetylthiocholine was injected into the subphase. The TMAFM images of a transferred monolayer, 6 min after the injection, show the presence of an acetylcholinesterase−acetylthiocholine complex and a homogeneous monolayer composition. However, the images of a second transferred monolayer at the same surface pressure, but 15 min after the injection, indicate the formation of a mixed monolayer due to the presence of both the enzyme−substrate complex and the free enzyme. Compression of the AChE monolayer on a substrate subphase indicates that the hydrolysis reaction took place at the interface and ended before a surface pressure of 25 mN/m was reached. Therefore, the topography of a monolayer prepared on a subphase containing the substrate resulted in a heterogeneous surface structure due to the presence of free enzymes and reaction products. UV−vis data confirmed the observations deduced from the TMAFM images. Furthermore, the effect of the organophosphate, paraoxon, on the enzyme was studied at the air/aqueous and the air/solid interfaces. The structural conformation of the enzyme is altered significantly by the presence of the inhibitor. Large domains were observed rather than an organized acetylcholinesterase monolayer, and the spectroscopic properties indicate that the interaction between the acetylcholinesterase and the paraoxon took place at the air/aqueous interface.

Introduction

Acetylcholinesterase (AChE) is an intrinsic membrane-bound enzyme that is essential for nerve tissue. This enzyme is responsible for the rapid hydrolysis of the cationic neurotransmitter acetylcholine after its release at cholinergic synapses.[1−5] Acetylcholine reacts with a specific residue at the active site of AChE to form a covalent acetyl−enzyme intermediate,[6−8] and choline is released to prevent re-excitation. The hydrolysis reaction by AChE consists of a nucleophilic attack of the acetylcholine by the Ser 200 which is activated by the imidazole group of a histidine, generating an acyl−enzyme. The acetyl−enzyme intermediate then reacts with water to form acetate and regenerates the free enzyme. An irreversible inhibition of this enzyme results in a constant excitation of the nervous system and muscle tissues which ultimately leads to death.[8]

AChE is a target site for a variety of commonly used insecticides in widespread use and nerve gases. Organophosphorus (OP) compounds such as paraoxon and parathion are potent inhibitors of its activity.[9−11] These compounds have a broad commercial market as insecticides because of their superior effect on parasites and their low persistence in the environment. However, their major mode of action has been ascribed to their ability to cause an irreversible inhibition of mammalian AChE, thereby acting as nerve agents.[8,11−16] The organophosphates can form stable complexes with cholinesterases and can

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prevent enzyme function by phosphorylation. Thus, inhibition of AChE by these compounds proceeds by reversible binding followed by irreversible blocking of the active site serine. The key step involves the nucleophilic attack to produce very stable covalent phosphoryl−enzyme complexes.[11] The hydrolysis of these complexes does not occur or takes place at a very slow rate in contrast with the acetyl−enzyme intermediate and causes an excessive over excitation of the cholinergic nervous system. The high reactivity of AChE toward organophosphorus inhibitors is due to the unique architecture of its active center.[17,18] Two main subsites have been distinguished: the esteratic site, which contains two active groups, a basic and an acidic group, and the anionic subsite with a negative charge, which interacts with the quaternary group of the choline moiety of acetylcholine. The hydrolysis reaction by AChE consists of a nucleophilic attack with either the quaternary ammonium of the acetylcholine choline moiety or with the phosphoryl moiety of the organophosphates inhibitors or other competitive inhibitors such as edrophonium or *N*-methylacridinium. As these organophosphorus inhibitors are very toxic to human and animals, the development of sensitive, rapid, and reliable detection of these compounds is very important for the protection of the environment and the human health. To develop a suitable system, an understanding of the molecular interaction between OP compounds and the enzyme is necessary.

In the present paper, we focus our study on the behavior of the pure enzyme, the enzyme−substrate, and the enzyme−inhibitor complexes formation in organized media that mimic the in vivo systems. The technique of Langmuir films and Langmuir−Blodgett films are one of such a model system. The purpose of the present work is to investigate the molecular interactions between the AChE and the substrate, acetylthiocholine (ATCh), and AChE and the inhibitor, paraoxon, at the air/aqueous interface. The progress of the hydrolysis reaction of acetylthiocholine catalyzed by AChE was followed at the air/aqueous interface by spreading the enzyme as a monolayer and dissolving the substrate in the subphase. The UV−vis spectroscopy was used to record, directly at the air/aqueous interface, the increase in the absorption intensity due to the increase in the concentration of the reaction products. Then, the topography of the pure enzyme, and the complexes AChE−ATCh, and AChE−paraoxon were investigated by transferring the film onto highly oriented pyrolytic graphite (HOPG) for the TMAFM study.

Material and Methods

Acetylcholinesterase (EC 3.1.1.7: V−S from electric eel), acetylthiocholine iodide (ATChI), and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The enzyme solutions were prepared freshly on the day of experiments at 1 mg/mL of buffer solution (0.1 KH_2PO_4, 0.1 M NaOH, pH 6.5). The weight of the solid enzyme was determined using a vacuum electrobalance system (ATI-Cahn C-2000, ATI MATTSON, Unicam & Cahn, Madison, WI). The organophosphate, diethyl *p*-nitrophenyl phosphate (paraoxon), was obtained from Chem Service (Chem Service Inc., West Chester, PA). The ATChI was prepared at two different concentrations (i.e., 2.50×10^{-3} and 1.72×10^{-3} M) in a buffer solution (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 8). The concentration of DTNB is 10^{-4} M, and the paraoxon was prepared at a concentration of 9×10^{-5} M in a buffered solution (0.01 M KCl, 0.1 M KH_2PO_4, 0.1 M NaOH, pH 6.5).

All the experiments were conducted in a clean room (class 1000) where temperature (20 ± 1 °C) and humidity (50 ± 1%) were controlled. Two kinds of Langmuir troughs were used in this work. The trough used for the Langmuir−Blodgett (LB) film experiments is a homemade double trough with dimensions of $0.6 \times 15 \times 55$ cm^3. The Langmuir trough KSV 2500 was used for the rest of the experiments. The trough is made up of Teflon and has dimensions of 0.45×0.15 cm^2. A UV−vis spectrophotometer (Hewlett-Packard Co., model 8452A diode array spectrophotometer, Wilmington, DE) was slid over the KSV trough to measure the absorption spectra of the monolayer at the air/aqueous interface. The water used for the preparation of the subphase was purified by a Modulab 2020 water purification system (Continental Water Systems Corp., San Antonio, TX). The specific resistivity and the surface tension of the pure water are 18 MΩ·cm and 72.6 mN/m at 20 ± 1 °C, respectively.

Experiments were first conducted to investigate the structure of the pure enzyme monolayer and the enzyme complexed with either the substrate or the inhibitor. The pure AChE monolayer was deposited at 25 mN/m onto HOPG and scanned with TMAFM. For the investigation of the complex formation of AChE with its substrate, ATChI, at the interface, two methods have been used. First, the enzyme was spread on the buffered subphase (pH 6.5), compressed to 25 mN/m, and held constant at this surface pressure. Then, the ATChI was injected beneath the AChE monolayer, giving a final concentration of 2.5×10^{-5} M. The deposition of the monolayer onto HOPG was made at two different times (6 and 15 min) after the injection of ATChI in the subphase. In another experiment, the AChE monolayer was spread on a subphase containing the ATChI at a concentration of 1.70×10^{-3} M and compressed to 25 mN/m and held at this surface pressure. The AChE monolayer prepared in these conditions was then transferred on HOPG using the Langmuir−Blodgett technique for the TMAFM measurements.

The UV−vis spectra were directly recorded at the air/aqueous interface to follow the progress of the hydrolysis reaction by measuring the absorption of the reaction product. In fact, the product of the reaction, thiocholine, reacts with dithio-bis-(nitrobenzoate) ion to give the anion thio-bis(nitrobenzoic acid) which produces a yellow color. An increase in the absorption intensity at 410 nm indicates an increase in the amount of the reaction product. First, the monolayer was compressed on a subphase free of ATChI and at 25 mN/m, and then the ATChI and DTNB were injected beneath the monolayer (the final concentration of ATChI and DTNB are, respectively, 2.50×10^{-5} and 5×10^{-5} M). Then, the absorption spectra were recorded versus time. In the second method, the AChE monolayer was compressed on a subphase containing the ATChI (1.72×10^{-3} M) and the DTNB (10^{-4} M) and the absorption spectra were taken during the compression of the monolayer.

The AChE monolayer was also compressed on a subphase containing the paraoxon and transferred at 15 mN/m onto HOPG for the TMAFM measurements. The absorption spectra were recorded at the air/aqueous interface at different surface pressures in the presence of paraoxon at a concentration of 9×10^{-5} M.

The TMAFM experiments were performed with a multimode AFM system. A sharpened Si_3N_4 tip attached to a triangular cantilever is used in these experiments. The force was adjusted to the minimum value measured by the system.

Results and Discussion

Enzyme−Substrate Interaction. A comparison between the surface pressure−area isotherms of AChE compressed with or without the substrate in the subphase is illustrated in Figure 1. The apparent limiting molecular area of the AChE extrapolated to zero surface pressure in the linear part of the isotherm in the presence of ATChI is 14 000 Å^2, whereas it is 11 000 Å^2 in its absence.[19] The collapse surface pressure of the AChE monolayers are 30 and 35 mN/m in the presence and the absence of the ATChI in the subphase, respectively. An increase of 3000 Å^2 in

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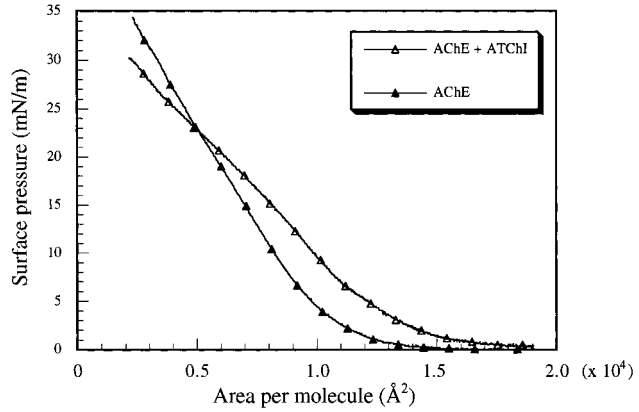

Figure 1. Surface pressure−area isotherms of the AChE monolayer compressed at the air/aqueous interface in the presence and in the absence of acetylthiocholine in the subphase.

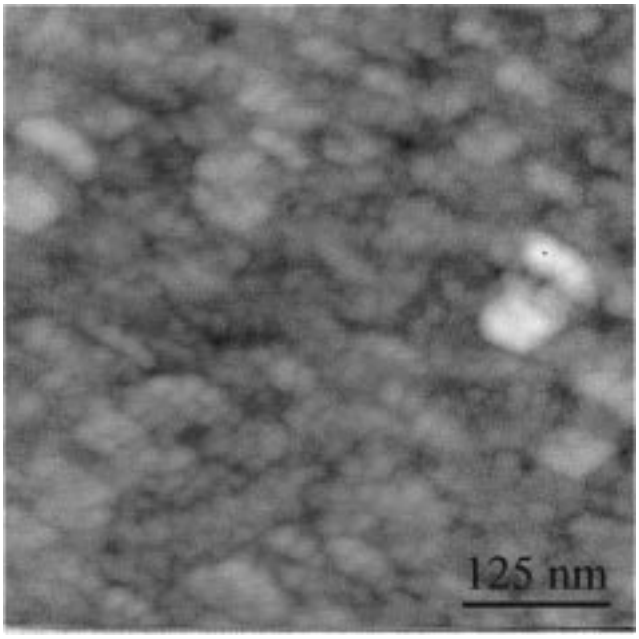

Figure 2. TMAFM image of the AChE monolayer transferred at a surface pressure of 25 mN/m onto HOPG. The image size and the scan rate are, respectively, 500 × 500 nm and 2.4 Hz.

the limiting molecular area is observed when the ATChI is added to the subphase and is due to the interaction between the substrate and the enzyme at the air/aqueous interface.

The complex enzyme−substrate formed at the air/aqueous interface is transferred onto HOPG at 25 mN/m and examined with TMAFM. These results are compared to the pure AChE monolayer. The image presented in Figure 2 (500 × 500 nm) illustrates the surface of the AChE monolayer prepared on a buffered subphase free of ATChI and transferred at 25 mN/m. The TMAFM image shows that the enzymes are well-resolved and their shape tends to be ellipsoidal. Size measurements indicate that AChE can exist in the monomer, dimer, and tetramer forms. This confirms our results reported earlier[20] on the structure of the AChE LB films examined by the atomic force microscope in contact mode (AFM). The AFM results were in agreement with the X-ray data[21] and indicated that AChE has an ellipsoidal shape. Size measurement of the AChE molecules (Figure 2) confirms the abundance of the monomer and tetramer forms.

To better understand the mechanism of complex formation between AChE and ATChI, another AChE monolayer was prepared and examined in two steps. The monolayer was first compressed on the ATChI-free buffered subphase. Once a surface pressure of 25 mN/m was reached, the ATChI was injected into the subphase. The monolayer is transferred on graphite 6 min after the injection. The TMAFM image of Figure 3 shows the topography of the AChE monolayer prepared as described above. The image of Figure 3a (500 × 500 nm) shows a uniform distribution of the particles which have a well-defined shape and are oriented at random. However, the dimensions of these particles are much larger than the AChE tetramer. The distribution of their size is uniform and can be grouped into one category where the average size is 30 × 35 × 3 nm.

Using the same monolayer prepared previously at the air/aqueous interface, a second AChE monolayer sample was transferred 15 min after the injection of the ATChI. The structure of this monolayer is shown in Figure 3b (500 × 500 nm). At first glance, a large difference between the particles shown in images 3a and 3b can be noticed. In the case of Figure 3b, two different categories of particles are seen (i.e., large (28 × 33 × 3 nm) and small (9 × 10 × 2 nm)). It should be mentioned that the large particles are similar to the one in Figure 3a, whereas the small ones are close to the dimension of an AChE monomer.

For a better understanding of the TMAFM results, the progress of the hydrolysis reaction of ATChI catalyzed by AChE was followed at the air/aqueous interface by measuring the increase in the intensity of the absorption during the compression of the monolayer. The absorption spectra were recorded on a monolayer prepared within the same conditions used above for the TMAFM studies. The ATChI (2.5×10^{-5} M) and the DTNB (5×10^{-5} M) were injected beneath the AChE monolayer at 25 mN/m and spectra were recorded versus time. Two absorption bands are observed in the spectra shown in Figure 4. The absorption band located around 325 nm is due to the DTNB absorption. The same absorption band of DTNB is observed when the spectrum is recorded in solution. We should mention that the observed band (around 325 nm) is due to the DTNB compound before its reaction with the thiol groups of the reaction product. One minute after the injection of ATChI and DTNB, only absorption bands around 325 nm due to the presence of high concentrations of DTNB are observed. A new absorption band which appeared 5 min later is around 410 nm and is due to the reaction product absorption after reacting with the 5,5-dithio-bis(2-nitrobenzoate) ion that produces the yellow color (Figure 4). The increase in concentration of the reaction product with time results in an increase in the absorbance at 410 nm and a decrease in the one of the DTNB (325 nm). This indicates that the progress of the ATChI hydrolysis reaction can be followed at the air/aqueous interface by spreading the enzyme as a monolayer and dissolving the substrate into the subphase. The absorption spectra of the monolayer was also recorded in the case where the ATChI is injected beneath the AChE monolayer without the presence of DTNB in order to confirm the decrease in the substrate concentration which was illustrated by a decrease in the absorbance during the hydrolysis.

The UV−vis absorption data are in agreement with the TMAFM results. In fact, the observed absorption spectra (Figure 4) show that 15 min after the ATChI injection beneath the AChE monolayer there is presence of a

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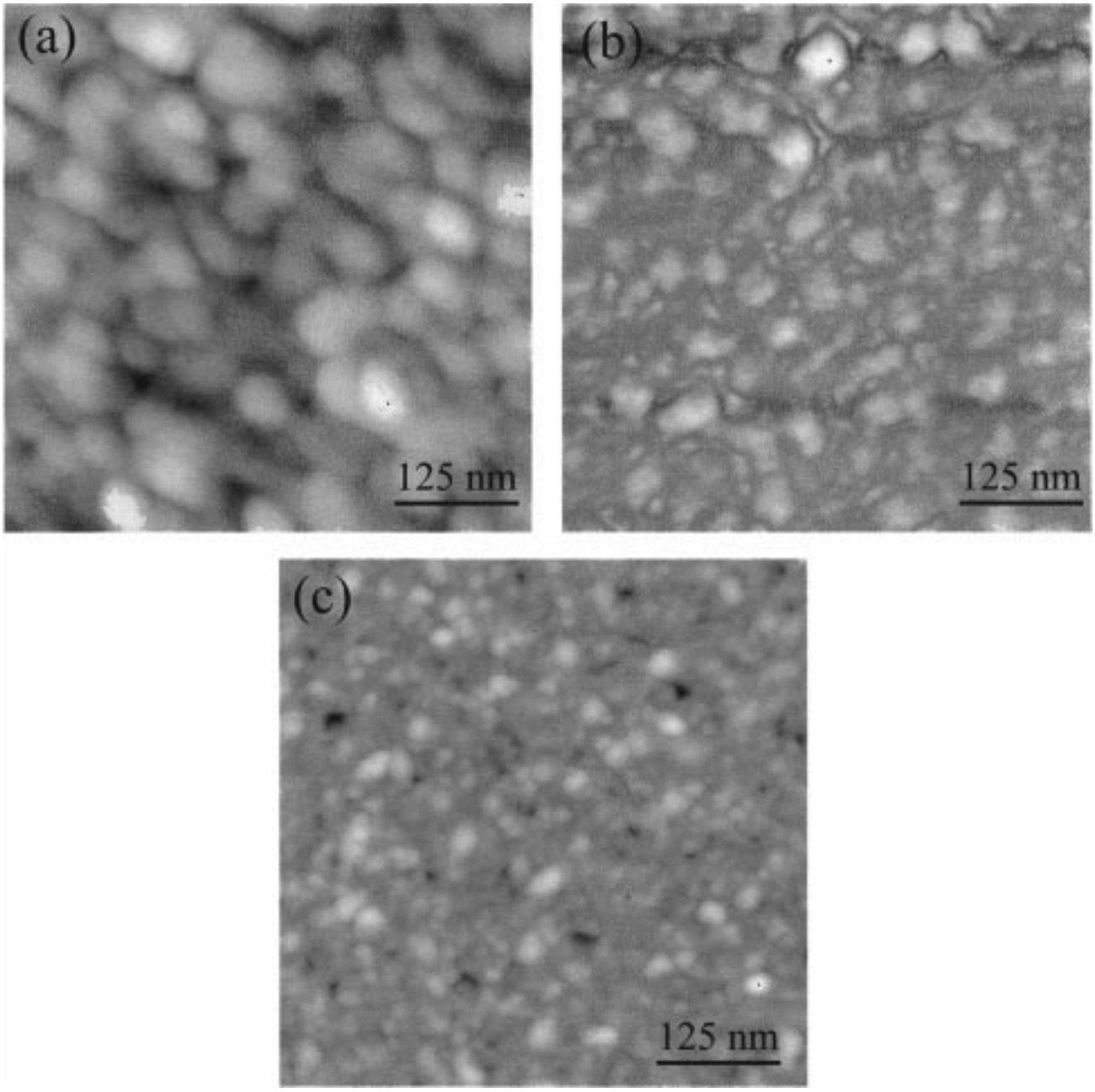

Figure 3. TMAFM images of the AChE monolayer transferred at a surface pressure of 25 mN/m onto HOPG. The ATChI is injected beneath the AChE monolayer at 25 mN/m, and then the monolayer was transferred at two different times: 6 min (a); 15 min (b). (c) The monolayer is compressed on a subphase containing the ATChI. The image sizes are for (a), (b), and (c) 500 × 500 nm. The scan rate is (a) 2.00 Hz, (b) 2.5 Hz, and (c) 3 Hz.

mixture of AChE particles and reaction products. The same absorption spectra of the AChE monolayer reported earlier[19] is obtained. Also, a certain amount of ATChI might still be present. Therefore, the UV−vis data confirm the TMAFM image results shown in Figure 3c,d where the analysis of the monolayer composition leads one to conclude that the monolayer is composed of a mixture of AChE, ATChI, and the product, thiocholine, which are present in different proportions in the film.

The TMAFM images of the AChE monolayer prepared on a subphase containing ATChI molecules at a concentration of 1.72×10^{-3} M are presented in Figure 3c (500 × 500 nm). The image shows that the monolayer is heterogeneous. The particles can be grouped in two different categories. The small ones are the most abundant in the monolayer and their dimensions vary between 8 × 9 × 2.5 and 13 × 18 × 3 nm. The large particles are 22 × 35 × 3 nm and only a few of them are present in the monolayer. The later dimensions are much larger than the AChE tetramer and are similar to the one observed in Figure 3a. Therefore, the monolayer transferred in these conditions is a mixture of the AChE monomer and tetramer and complexes of the enzymes with the reaction products. In fact, by compressing the enzyme on the subphase containing the ATChI, we found that the hydrolysis reaction took place at the interface and ended before the collapse was reached. The enzyme was then liberated into the subphase at the end of the hydrolysis reaction and adsorbed at the air/aqueous interface to form a monolayer. These images of the AChE monolayer differ significantly from the imaged monolayer obtained when the ATChI is added to the subphase at 25 mN/m. As it is shown in images 3a and 3b, the particles are very large compared to the AChE molecules determined earlier,[20]

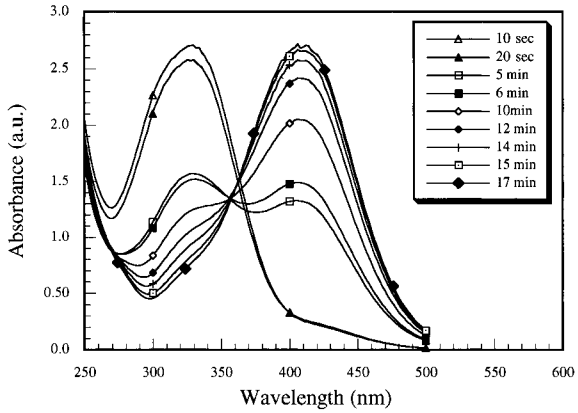

Figure 4. Absorption spectra of the monolayer recorded at the air/aqueous interface after the injection of the ATChI and the DTNB beneath the AChE monolayer. The spectra were recorded versus time.

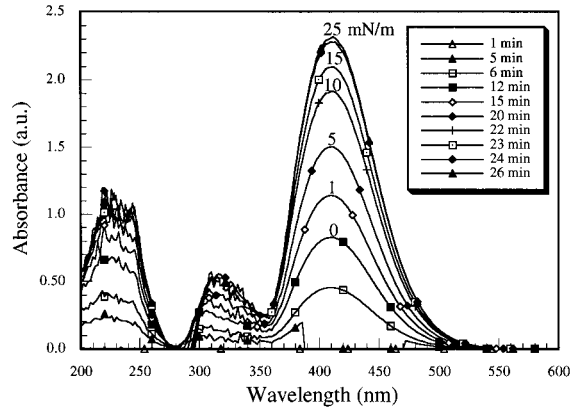

Figure 5. Absorption spectra of the monolayer compressed on a subphase containing the ATChI and DTNB. The spectra were recorded during the AChE monolayer compression.

and the most abundant particles shown in image 3c correspond to the AChE molecules as determined by AFM.

One minute after spreading AChE on the subphase containing ATChI (1.72×10^{-3} M) and DTNB (10^{-4} M), the spectroscopic properties of the monolayer show that no absorption was observed at the interface while the monolayer was compressed (Figure 5). Six minutes after the compression (zero surface pressure), the absorption band due to the reaction product, thiocholine, with the 5,5-dithio-bis(2-nitrobenzoate) ion, appeared around 410 nm with a low absorbance. As the monolayer was compressed, the surface pressure and the absorbance at 410 nm increased. At a surface pressure of 25 mN/m, the monolayer showed maximum absorption at the air/aqueous interface and a saturation of the absorption maximum was observed. Thus, as the monolayer was compressed, the surface concentration of the enzyme increased and induced ATChI hydrolysis at the interface. The product concentration increased and produced an increase in the absorbance of the product at 410 nm. The enzyme was then liberated to the subphase and adsorbed at the interface to form a monolayer. The abundance of the AChE molecules at the interface after the hydrolysis reaction ended is shown in Figure 3c. Two other absorption bands around 230 and 325 nm are also seen in Figure 5 and are attributed to the absorption of ATChI and DTNB molecules, respectively. The same absorption bands of these compounds were observed in solution. In fact, during the compression of the monolayer, the concentration of these compounds increased and their absorption was detected, but the absorbance was lower than the product. These absorption properties are in agreement with the TMAFM results (Figure 3c) based on the fact that there was a mixed monolayer when the reaction ended. Hence, during the transfer of the AChE monolayer on the HOPG, the deposition of the reaction products liberated in the bulk medium might also have occurred during the dipping of graphite. This leads to the formation and deposition of a mixed monolayer. However, the size measurements of the particles indicate that the enzyme molecules are the most abundant population in the monolayer.

The AChE catalysis occurs via formation of an acyl−enzyme complex[6−8] that involves nucleophilic elements. In the TMAFM image (Figure 3a), the large size of the enzyme particles might be explained by a change in the AChE conformation that occurred during the binding of the acetylthiocholine to the enzyme. In fact, it is well-known that binding of either substrates or inhibitors causes conformational changes.[8,22] The deacylation process did not take place and the AChE was in the acylation form. The dimension of the particles suggests that the transfer of the monolayer on the HOPG was made during the elementary step of the AChE−ATChI complex formation. Thus, injection of ATChI beneath the AChE monolayer at 25 mN/m leads to the formation of a complex enzyme−substrate at the interface. In these conditions, the deposition of the LB film is made before the hydrolysis reaction was terminated. On the other hand, the observed dimensions in Figure 3a are very large and correspond to twice the size of an AChE tetramer. However, the maximum opening of the site gorge of the AChE was determined earlier[24] by molecular dynamic simulations and shown to correspond to the size of its substrate, acetylcholine. Therefore, we believe that the oversized lateral dimensions of the particles might be due to slight approaches of the AChE tetramers induced during the substrate binding.

On the other hand, the mixed monolayer observed in Figure 3b corresponds to a step of the reaction where both acylation and deacylation mechanisms of the enzymes might have occurred. This explains the abundance of both free enzymes, which are liberated to the subphase and adsorbed at the interface, and enzymes in the acylation form. Also, a couple of large particles are seen in the TMAFM images that can be attributed to the presence of the complex AChE−ATChI where the substrate is still bound to the enzyme. The composition of the monolayer shown in Figure 4 suggests that the AChE deposition monolayer was made after the hydrolysis reaction ended, and free AChE molecules and reaction products, thiocholine, were liberated to the subphase. By compressing the AChE monolayer on a subphase containing ATChI, the acylation and deacylation mechanism of the complex formation (AChE−ATChI) occurred before a surface pressure of 25 mN/m was reached. On the other hand, the absorption spectra results are in agreement with the TMAFM data.

These data show that the hydrolysis of ATChI by the enzyme occurred by spreading the AChE as a monolayer so that the substrate could dissolve into the subphase. This system better represents the situation in cell membranes where the enzyme is immobilized at the intracellular surface. However, the diffusion phenomena

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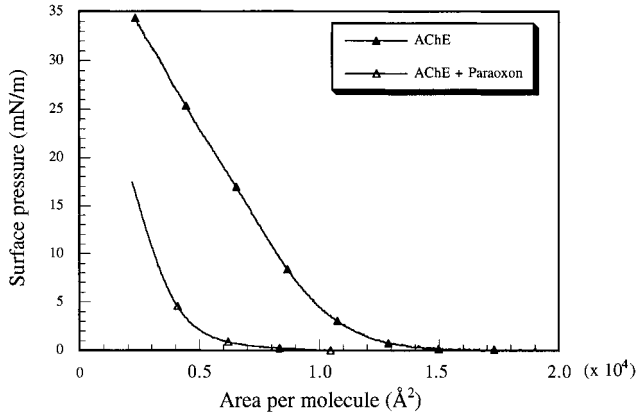

Figure 6. Surface pressure−area isotherms of the AChE monolayer compressed at the air/aqueous interface in the presence and in the absence of paraoxon in the subphase.

of the molecules at the air/aqueous interface need to be studied further. Therefore, any comparison of these results with the hydrolysis reaction rate determined in solution should be considered in a careful manner.

Enzyme−Organophosphate Interaction. The effect of the organophosphate, paraoxon, on AChE was studied at the air/aqueous interface by spreading the enzyme on a dissolved-paraoxon subphase at a concentration of 9×10^{-5} M. The surface pressure−area isotherm of the AChE monolayer compressed in the absence and in the presence of the paraoxon in the subphase is shown in Figure 6. The isotherm of the AChE monolayer in the presence of the paraoxon is significantly different from that of the one in its absence. The limiting molecular areas correspond to 11 000 and 4500 Å^2 in the absence and in the presence of the paraoxon, respectively. The collapse surface pressure is not higher than 16 mN/m in the presence of the organophosphate. The surface potential−area isotherm of the AChE monolayer was also investigated and showed the same behavior of the paraoxon on the enzyme monolayer. No orientation of the molecular dipoles could be deducted from the surface potential isotherm. The absorption properties of the AChE monolayer in the presence of the paraoxon were studied as well. An absorption band was observed around 280 nm and was attributed to the paraoxon molecules. In fact, at the collapse surface pressure, the concentration of the paraoxon becomes important at the interface, resulting in the observed absorption spectrum.

The TMAFM data of the AChE monolayer prepared in the presence of paraoxon are presented in Figure 7 (500 × 500 nm). The structure of the AChE monolayer is altered significantly by the presence of the paraoxon in the subphase. As it is seen in Figure 7, the structure of the monolayer is completely different from the one obtained in the absence of the inhibitor (Figure 2). Large domains are formed and occupy most of the graphite steps. No sign of the particle distribution was noticed. Their lateral dimensions vary between 85−160 and 112−250 nm and their average height is 1 nm. A few particles dispersed on the surface of the graphite are also observed, as shown in Figure 7. However, it is rather difficult to point out the nature of these particles. The TMAFM results and the UV−vis absorption properties indicate that there is an interaction of the paraoxon with the AChE at the air/aqueous interface.

As it is evident from our results on Langmuir films of AChE in the presence and in the absence of paraoxon in the subphase, there is a decrease of 6500 Å^2 in the limiting molecular area. The change in the surface pressure−area isotherm of the AChE monolayer suggests the binding of the paraoxon to the enzyme molecule, causing a degradation and dissolution of the enzyme into the subphase. The TMAFM images indicate that the configuration of the enzyme was completely modified in the presence of the paraoxon and the ellipsoidal shape of AChE disappeared. In fact, the organophosphate causes an irreversible inhibition of AChE by reacting with the catalytic serine hydroxyl in the active site, resulting in a stable tetrahedral configuration of the inhibitor.[11,23] This results in an irreversible change in the enzyme conformation. The inhibition occurs via formation of an AChE−paraoxon Michaelis−Menton complex in the active center.[23] These complexes are very stable and the process of the dephosphorylation mechanism does not occur or takes place very slowly.[25]

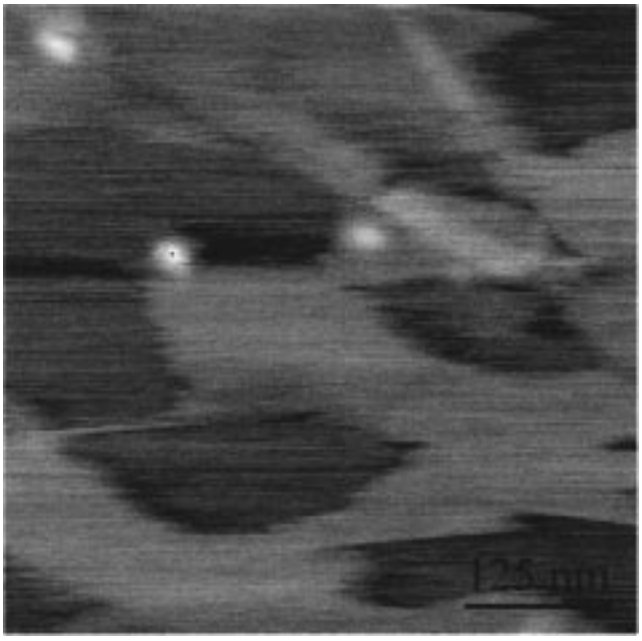

Figure 7. TMAFM image of the AChE monolayer transferred at a surface pressure of 15 mN/m onto HOPG. The monolayer is compressed on a subphase containing the paraoxon. The image size and the scan rate are, respectively, 500 × 500 nm and 2.0 Hz.

Conclusion

In this paper, the hydrolysis reaction of the acetylthiocholine catalyzed by acetylcholinesterase was studied at the air/aqueous interface by spreading the enzyme as a monolayer and dissolving the substrate in the subphase. Using the TMAFM technique, we were able to see different elementary steps of the hydrolysis reaction. First, the binding of the acetylthiocholine to the enzyme to form a complex was observed when the monolayer was transferred 6 min after the injection of acetylthiocholine beneath the AChE monolayer. Then, the scanning of the surface structure of a second transferred monolayer 15 min later indicated the formation of a mixed monolayer where there was presence of both an acylated and deacylated enzyme. Furthermore, the TMAFM images of a transferred monolayer at the end of the reaction showed a heterogeneous surface due to the abundance of the enzyme molecules that were liberated to the subphase and adsorbed at the interface to form a monolayer. Also, reaction products

(25) Walsh, C. *Enzymatic Reaction Mechanisms*; Freeman: San Francisco, CA, 1979.

might have been transferred during the deposition process. The UV−vis results confirmed the TMAFM data.

The interaction of the paraoxon with AChE was also investigated at the air/aqueous interface and the results indicate that the enzyme was irreversibly inhibited and its ellipsoidal shape was completely modified in the presence of the paraoxon.

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