

Effect of pH on acetylcholinesterase Langmuir and Langmuir–Blodgett films studied by surface potential and atomic force microscopy

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Abstract

Surface potential measurements have been used to determine the dipole interactions of the acetylcholinesterase (AChE) monolayer at the air/aqueous interface. The results show that it is the ionogenic groups of the enzyme that cause significant changes in the surface potential of the monolayer as the pH of the sub-phase is changed. The atomic force microscope (AFM) images of AChE Langmuir–Blodgett (LB) films indicate that at low pH, an unfolding of the enzyme occurs which leads to formation of large domains at the air/aqueous interface rather than an organized AChE monolayer. Furthermore, the AFM images of the AChE LB film prepared at basic pH show that the enzyme forms aggregates. © 1998 Elsevier Science S.A. All rights reserved

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1. Introduction

Acetylcholinesterase (AChE) is an essential enzyme for nerve tissue. Its principal biological role is to terminate the synaptic signal by catalyzing the hydrolysis reaction of the neurotransmitter, acetylcholine, after its release at cholinergic synapses [1–3]. AChE is considered as one of the fastest known enzymes with a high turnover number [4,5]. The enzyme monomer is an α/β protein and consists of a large central mixed β -sheet surrounded by 15 α -helices [6]. AChE exists in two major forms: asymmetric and globular. The asymmetric form is characterized by the presence of collagen-like tail, while the globular forms are heterogeneous. There are amphiphilic and non-amphiphilic globular forms on the basis of their capacity to associate with micelles [7–9].

The three-dimensional structure of this enzyme has been determined by X-ray analysis [10] and shows that it has an ellipsoidal form with dimensions $175\ 000\ \text{\AA}^3$. Recently, the surface topography of AChE Langmuir–Blodgett (LB) films deposited onto graphite have been investigated using atomic force microscopy (AFM) [11]. The AFM results are in agreement with the X-ray data and indicate that AChE

has an ellipsoidal shape. Size measurements of the individual enzyme molecules determined by the AFM indicate that AChE exists in monomer and tetramer forms whose dimensions are comparable to the crystallographic data. In previous papers [11,12], the interfacial parameters of AChE at the air/aqueous interface have been reported. The enzyme forms a highly stable monolayer in the presence of an electrolyte in the sub-phase (pH 6.5). This great stability was attributed to its mixed polar and non-polar nature and to its high molecular weight. The ionic strength of the sub-phase did not have any significant influence on the formation of the AChE monolayer while the pH values had significant effect on the limiting molecular area and the surface pressure of the monolayer. We have previously reported that in an acidic medium (pH = 3.0 and 4.0), larger molecular areas and higher surface pressures were obtained than in a basic medium (pH = 7.0 and 8.0) [12]. Therefore, the pH has a substantial influence on the adsorption and the stability of the enzyme.

Knowing that the dependence on the pH of the reaction medium is one of the fundamental characteristics of enzyme catalysis, the purpose of the present paper is to study the effect of the pH on the AChE monolayer at the air/aqueous and LB films at the air/solid interfaces. In this work, surface potential isotherms of the AChE monolayer and the surface topography of the LB films of this enzyme are investigated

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as function of the sub-phase pH. The variations of the surface potential with pH of the protein monolayers have been demonstrated earlier [13] and showed that it is the contribution of the ionogenic side chains, containing the groups $-\text{CO}_2\text{H}$, $-\text{NH}_2$, that is pH dependent while the non-ionogenic and the non-polar side chains are independent of the pH variation of the sub-phase. The study of the structure of AChE at the air/solid interface along with the surface potential will contribute to an understanding of the state of the ionization of the AChE monolayer and the topography of the LB film at the air/aqueous and the air/solid interfaces, respectively.

2. Materials and methods

Surface potential and surface pressure were measured simultaneously in the presence of an electrolyte in the sub-phase (10 mM KCl). All the experiments were conducted in a clean room (Class 1000) where temperature ($20 \pm 1^\circ\text{C}$) and humidity ($50 \pm 1\%$) are controlled. The Langmuir trough used for the surface potential experiments was home-made and its dimensions are $0.6 \times 12 \times 100 \text{ cm}^3$. Two symmetrically movable barriers, computer controlled, were used to regulate the surface area. The surface pressure and the surface potential are measured using the Wilhelmy method and the ionizing electrode method, respectively. The LB films were prepared using a home-made double trough with dimensions $0.6 \times 15 \times 55 \text{ cm}^3$. The AChE monolayer was deposited onto a freshly cleaved graphite substrate, highly oriented pyrolytic graphite (HOPG) at different sub-phase pH. The AFM experiments were performed with a multimode AFM system. Sharped Si_3N_4 tip attached to a triangular cantilever is used in these experiments. The force was adjusted to the minimum value (0.5 nN) measured by the system.

Acetylcholinesterase (EC 3.1.1.7: V-S from electric eel) was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. The enzyme solutions were prepared freshly at the day of experiments at 1 mg/ml of buffer solution (0.1 M KH_2PO_4 , 0.1 M NaOH, pH = 6.5). The solid enzyme was weighed using a vacuum electrobalance system (ATI-Cahn-2000, ATI Mattson, Unicam and Cahn, Madison, WI). The spreaded volume of the AChE solution was 100 μl for the surface potential measurements and 150 μl for the LB films preparation. The compression rate was set up at 5 $\text{\AA}^2/\text{molecule}$ per min for all the experiments. The experiments were carried out at different sub-phase pH in order to study the behavior of AChE monolayer in acidic and basic medium: pH 3.0 and 4.0 (0.1 M KH phthalate and 0.1 M HCl were used as buffer), 5.3 (0.1 M KH phthalate and 0.1 M NaOH), 7.0 (0.1 M KH_2PO_4 and 0.1 M NaOH), 8.0 (0.1 M Tris and 0.1 M HCl) and 10.0, 11.0 and 12.0 (0.1 M glycine and 0.1 M NaOH). The water was purified by a Modulab 2020 water purification system (Continental Water Systems Corporation, San Antonio, TX) and

has a specific resistivity of 18 M Ω cm and a surface tension of 72.6 mN/m at $20 \pm 1^\circ\text{C}$.

3. Results and discussion

The surface pressure-area isotherms of the AChE monolayer compressed at the air/aqueous interface using different pH in the sub-phase have already been reported in previous work [12]. The sub-phase pH has a significant influence on the AChE monolayer. Surface potential values (ΔV_{max}) at the collapse surface pressures are plotted against the pH of the sub-phase (Fig. 1). The AChE monolayer is insoluble over the whole range of the investigated pH at the ionic strength of 0.01 M KCl. The behavior of the surface potential differs significantly when the monolayer is compressed on an acidic or a basic medium. As it can be seen from Fig. 1, significant changes in the surface potential values occur in the pH range tested. ΔV_{max} varies from 400 mV at pH 3.0 to 180 mV at pH 12.0. This decrease is due to the ionization of the AChE monolayer at the air/aqueous interface. When the AChE monolayer is compressed to the collapse surface pressure, the polypeptide backbone lies at the interface where the hydrophobic side chains are directed away from the surface and the polar side chains are oriented towards the aqueous sub-phase. The non-polar side chains are considered to be independent of the variations of the surface potential whereas the polar groups, mainly $-\text{CO}_2\text{H}$ and $-\text{NH}_2$ contribute to the variations with pH of the sub-phase. As it is shown in Fig. 1, significant changes in ΔV_{max} occur in the pH ranges 3.0–7.0 and 10.0–12.0. At low pH (3.0 and 4.0), the carboxylic acid is undissociated and the amine groups RNH_2 are completely ionized causing the AChE monolayer to acquire an overall positive charge. From pH 4.0 to 7.0, the formation of carboxylate ions in this region causes a significant decrease in ΔV_{max} , making the surface negatively charged. From pH 7.0 to 10.0, the ΔV_{max} remains almost constant. Then, the neutralization of the amino-groups

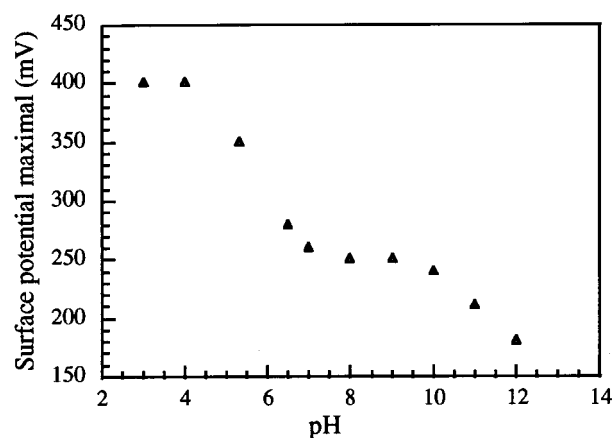


Fig. 1. Surface potential at collapse versus pH for AChE monolayer compressed at the air/aqueous interface in the presence of 0.01 M KCl in the sub-phase.

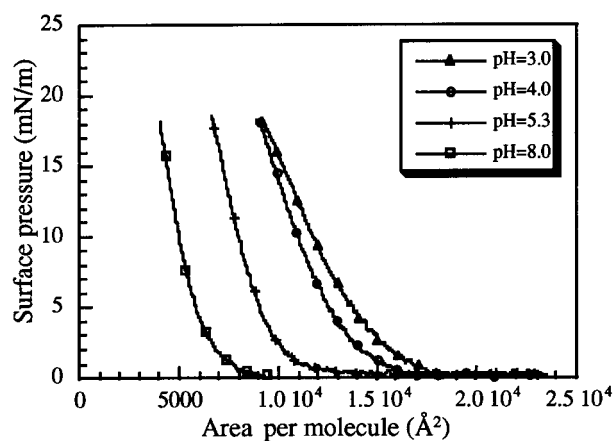


Fig. 2. Surface pressure-area isotherms of AChE monolayer at different pH. The isotherms are shown up to a surface pressure of 18 mN/m which is the one used for deposition of the LB films.

causes further decrease in ΔV_{\max} . During this neutralization, the protons are expelled from the monolayer to the bulk sub-phase causing a decrease to the surface potential. The ionization constants of the AChE monolayer can be derived from Fig. 1. The estimated pKa values are 5.5 and 11.0 for $-\text{COOH}$ and $-\text{NH}_2$ groups, respectively, at the air/aqueous interface.

The surface pressure-area isotherms of AChE monolayer at different pH are shown in Fig. 2. At pH 3.0 and 4.0, larger molecular areas than at pH 5.3 and 8.0 are obtained. The collapse of surface pressures are situated at 35 mN/m for pH 3.0 and 4.0 and around 20–22 mN/m for pH 5.3 and 8.0 as it was reported earlier [12]. Therefore, the AChE LB films examined with the AFM are transferred at 18 mN/m, a surface pressure for deposition below the collapse surface pressure. The surface topography of the LB films prepared at pH 3.0 is shown in Fig. 3a ($1 \times 1 \mu\text{m}^2$). Large domains are formed on top of graphite steps. The lateral dimensions of the separated domains are 75–100 \times 130–350 nm whereas their height varies between 1 and 1.5 nm. The domain height is about 10 times larger than the height of the graphite step. On the other hand, the lateral dimension of the AChE particles is between 16 and 18 nm. No organized structure of the film was noticed when the domains were examined at the molecular resolution.

The AFM image of the AChE LB film prepared at pH 4.0 (Fig. 3b: $1 \times 1 \mu\text{m}^2$) shows a different surface topography. In this case, the domains are much wider and they occupy most of the graphite surface. The dispersed particles in this image are wider (~20–27 nm) than their counter part in the previous image (Fig. 3a). However, at pH 5.3 (isoelectric point of AChE), the LB film appears with a different structure (Fig. 3c: $1 \times 1 \mu\text{m}^2$). The AChE particles are more abundant and their lateral dimensions are 25–40 nm. No domain formation has been observed in these conditions. At higher pH (8.0), the enzyme forms compact LB films. The AFM images in Fig. 4a ($1 \times 1 \mu\text{m}^2$) and 4b ($1 \times 1 \mu\text{m}^2$) show that the constituent of the LB films can be grouped into two categories, the small (~16 nm) and the large (~40

nm) sized particles. This result can be compared to the one obtained at pH 5.3. We should mention that the lateral

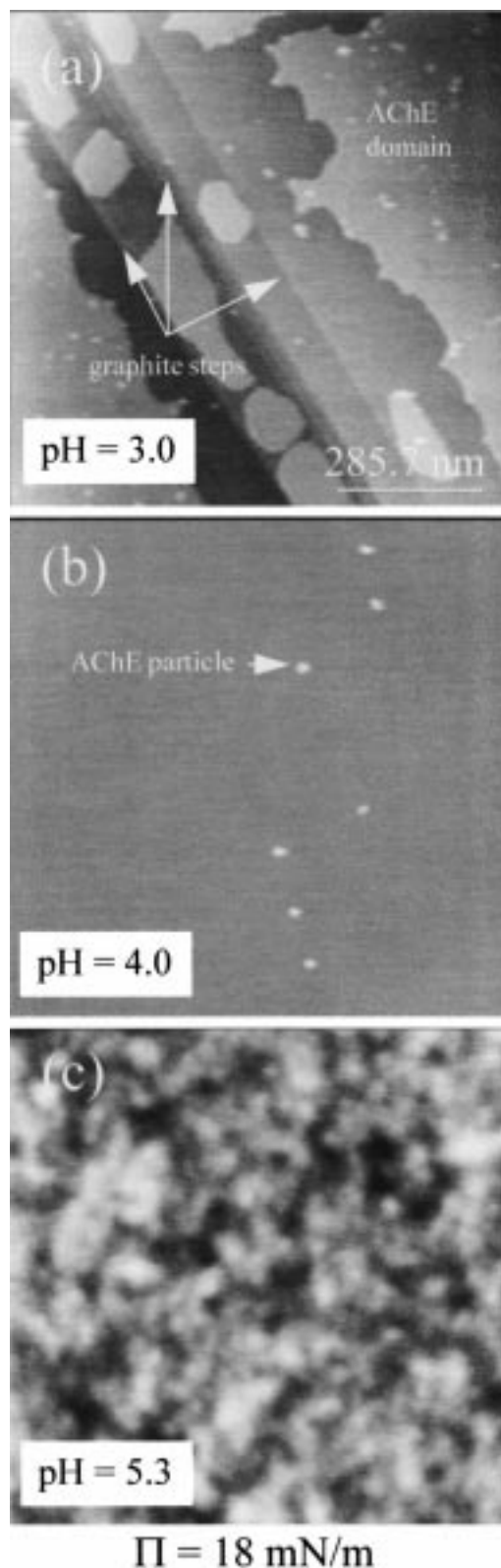


Fig. 3. AFM images of AChE LB film prepared at pH 3.0, 4.0 and 5.0 and deposited at a surface pressure of 18 mN/m. The image size and scan rate are respectively $1 \times 1 \mu\text{m}^2$ and 2 Hz.

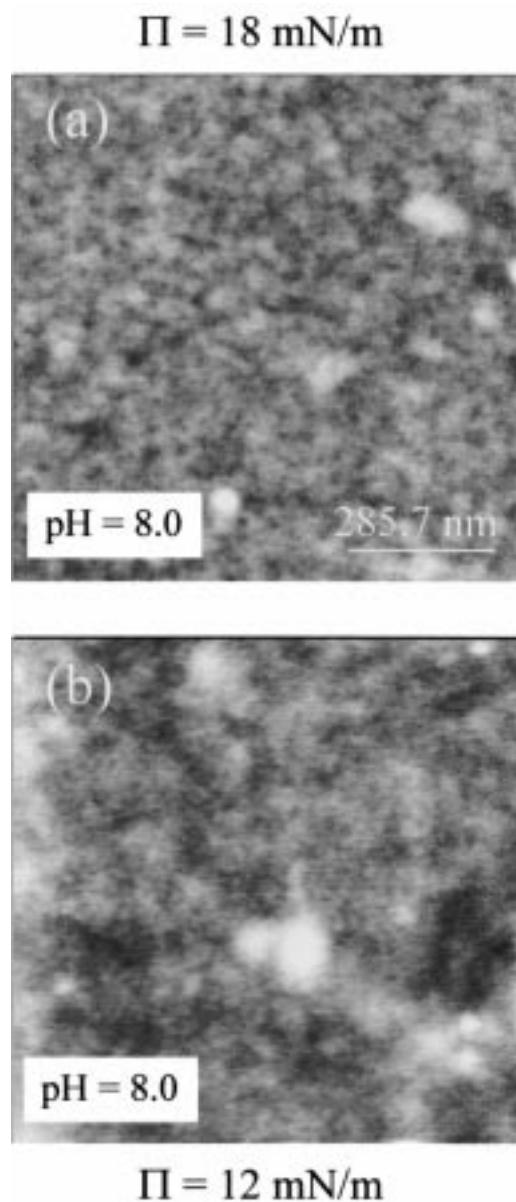


Fig. 4. AFM images of AChE LB film prepared at pH 8.0 and deposited at two surface pressures: 18 and 12 mN/m. The image size and scan rate are, respectively, $1 \times 1 \mu\text{m}^2$ and 2 Hz.

dimensions estimated above are altered in a certain way by the tip broadening effect. Therefore, any comparison of these data with the X-ray should be considered in a careful manner.

As it can be seen from Figs. 3 and 4, the structure of the LB film is significantly altered by the changes of the pH subphase. The surface topographies of the AChE LB films prepared at pH 3.0 and 4.0 are completely different from the ones prepared at pH 5.3 and 8.0. At low pH (3.0 and 4.0), the

formation of wide domains results in large limiting molecular areas of the AChE molecules ($14\,500 \text{ \AA}^2$ at pH 3.0 and $13\,500 \text{ \AA}^2$ at pH 4.0) (Fig. 2). These values are larger than the one obtained at pH 6.5 where the enzyme monolayer was shown to be stable and more organized [12]. The enzyme has probably changed its conformation from a compact globular form to an unfolded two-dimensional conformation at the interface. This unfolding leads to an insolubility of the enzymes, causing their denaturation at the interface. At pH 8.0, the AChE LB film structure is more compact and no domains have been detected. However, a large amount of the enzymes have been dissolved since the limiting molecular area is smaller [12]. This leads us to conclude that there is formation of an agglomerate of enzymes rather than an organized monolayer at the air/aqueous interface. The AFM results showed that the effect of the pH on the structure of the LB film can be associated with changes in the enzyme conformations. However, it is rather difficult to evaluate these changes in terms of dimensions for a particular enzyme since the AFM is limited to 3 nm lateral resolution.

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