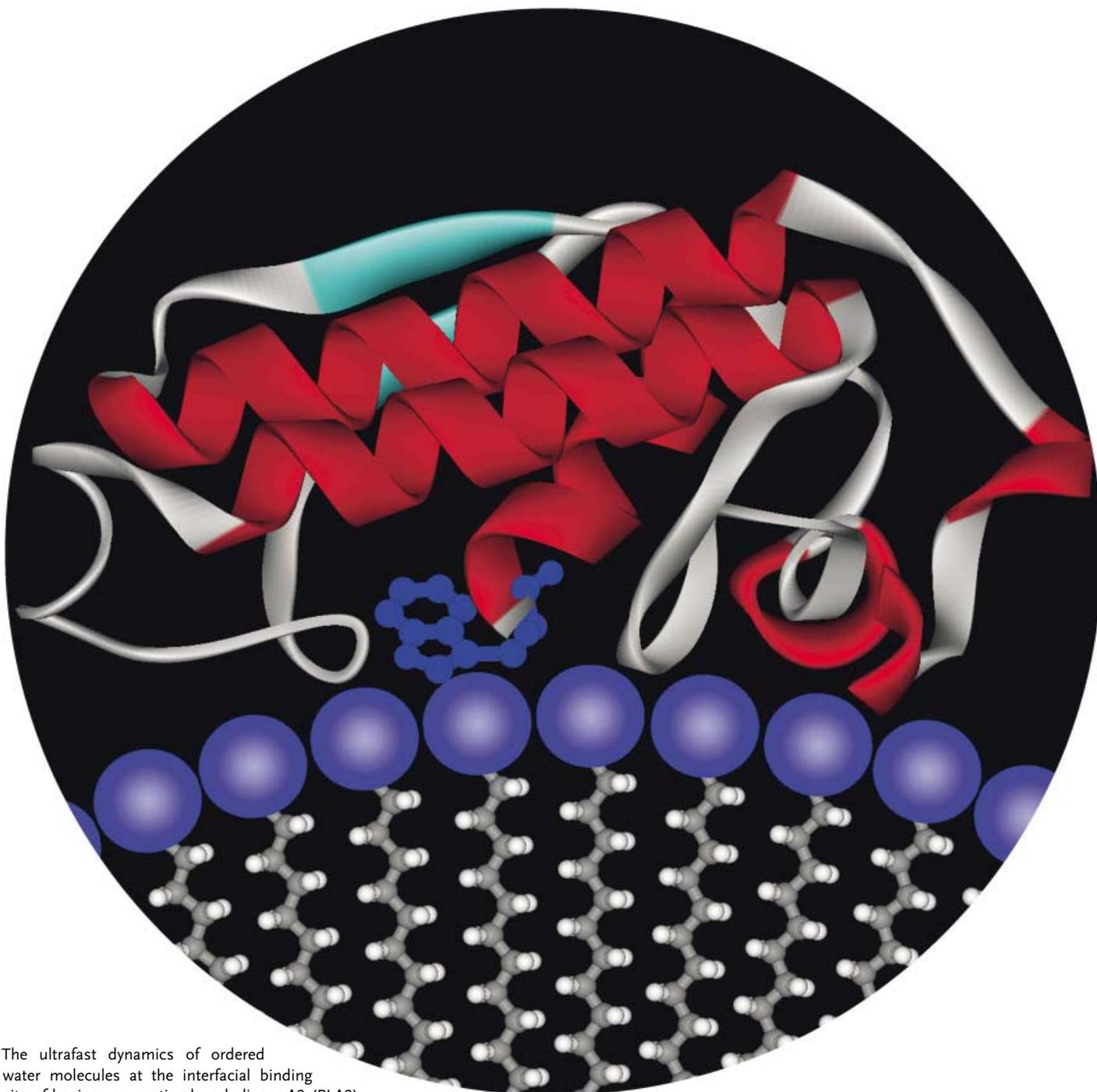# *Communications*

The ultrafast dynamics of ordered water molecules at the interfacial binding site of bovine pancreatic phospholipase A2 (PLA2) have been investigated. The timescales of hydration were established by selectively exciting the tryptophan residue at the substrate-recognition site and probing the motion of water molecules by rotational and translational diffusion. The dynamic role of water as a "lubricant" and in binding is presented by A. H. Zewail et al. on the following pages.

Communications

Dynamics of Ordered Water in Interfacial Enzyme Recognition: Bovine Pancreatic Phospholipase A$_2$**

Liang Zhao, Samir Kumar Pal, Tianbing Xia, and Ahmed H. Zewail*

The bovine pancreatic phospholipase A$_2$ (PLA2) protein consists of a single polypeptide chain with 123 residues. It belongs to a family of calcium-dependent lipolytic enzymes that hydrolyze the 2-acyl ester bond of phospholipids to release fatty acids and lysophospholipids. These proteins are unique prototypes in interfacial enzymology.[1,2] Two structural features of PLA2 are of functional interest: the catalytic site with a conserved Asp–His pair and Ca^{2+} as obligatory cofactor, and the interfacial binding region (i-face, ≈ 1700 Å^2), which determines the interface preference for the membrane-binding equilibrium (Figure 1). The surface wall of the interfacial binding site is covered with hydrophobic and some polar residues.[2]

It is known that PLA2 proteins are activated by aggregated substrates in the form of micelles or vesicles.[3] The mechanism behind the phenomenon of interfacial activation, however, has been a subject of debate, and two models have been proposed.[2] In the "enzyme model", it has been suggested that the conformational changes of the enzyme upon binding play a role in interfacial activation. Conformational changes have been observed by NMR spectroscopy.[4,5] In reference [5], it was demonstrated that PLA2 is not denatured by the anionic surfactant sodium dodecyl sulfate (SDS) at a much higher concentration (50 mM) than its critical micellar concentration (≈ 1 mM)—protein–micelle complexes are formed. The study also indicated that when bound to the micelle, the overall protein structure remains intact, although a more-ordered helical conformation is observed at the N-terminus of the protein. In the "substrate model", the activation was attributed to the orientation, conformation, or hydration sites of the phospholipid molecules in the aggregates, facilitating substrate diffusion from the interfacial binding surface to the catalytic site.[6]

Hydration of these biomolecules must be important for the stability of the structure and the function.[7–10] As discussed in a series of publications from our group, ordered water has a unique role in the dynamics of protein[11–13] and DNA[14] recognition. The PLA2 system serves as a model protein–substrate complex to study the role of intrinsic
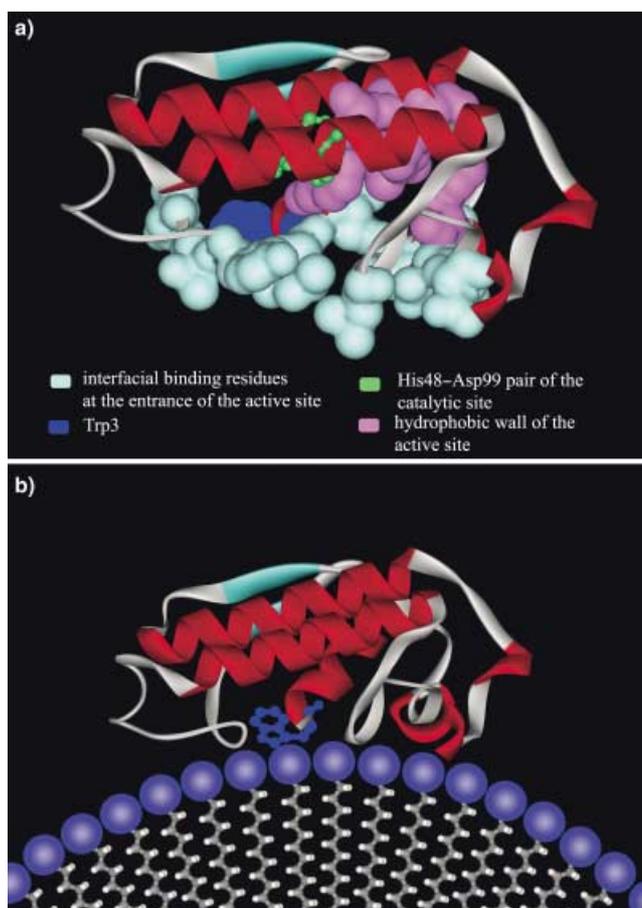

Figure 1. a) X-ray crystal structure of bovine pancreatic phospholipase A$_2$ (PLA2). Trp3 is part of the interfacial binding site and the catalytic site is away (15 Å) from the interface.[2] The assignment of different groups of amino acid residues follows reference [16]. The structure was downloaded from Protein Data Bank (ID code 3BP2). b) A simplified model of protein–micelle complex, indicating the location of Trp3 of the protein at the interface.

interfacial hydration dynamics in enzyme activity. A high-resolution X-ray crystallographic investigation[15] of bovine PLA2 showed that at least one tightly bound water molecule is crucial during catalysis. However, most of the water molecules are located 2.5–3.0 Å away from the protein surface and form hydrogen bonds to the protein atoms.[15] A single tryptophan at position 3 (Trp3) is part of the interfacial binding site[16] and is significantly exposed to the solvent environment.[17] Upon binding to the SDS micelle, the Trp3 residue is sandwiched at the interface and partially protected from bulk solvent.

Herein we present our first studies on the hydration dynamics at the binding site of PLA2, for free protein and for the complex (Figure 1). We observed dynamically ordered water at the interface and we were able to determine the role of substrate hydration from the distinct timescales of the complex. We also determined the rigidity of Trp in this interface region. The importance of these results to the recognition and activity of the enzyme is discussed.

Because of its unique location in the protein, Trp3 serves as an excellent intrinsic probe to study the hydration

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dynamics at the interfacial binding site. After the indole chromophore of tryptophan is excited with a femtosecond pulse, a large dipole moment is created and the surrounding water molecules must reorganize to reach the new equilibrated state. This process is manifested as the dynamic shift of the fluorescence spectrum (Stokes shift). The hydration process can therefore be followed in real time by recording the transient at different wavelengths—on the blue side one observes a decay, whereas on the red side a rise replaces the decay. The shift of the fluorescence peak is then used to construct the hydration correlation function, $C(t) = [\tilde{\nu}(0) - \tilde{\nu}(t)]/[\tilde{\nu}(0) - \tilde{\nu}(\infty)]$, in which $\tilde{\nu}(t)$ is the spectrum maximum position at a given time (e.g., see reference [11]). A study of the time-dependent behavior of $C(t)$ thus provides direct information on local solvation dynamics around the tryptophan probe.

The steady-state fluorescence spectra of PLA2 in water and with the micelle are shown in Figure 2 A. The tryptophan emission maximum in the complex (339 nm) was blue shifted

by 9 nm relative to that on the protein surface (348 nm), indicating a more hydrophobic environment upon micelle binding. The emission of free tryptophan in bulk water is shown for comparison. The absorption peak of tryptophan remains nearly the same in all cases; one complete absorption spectrum of the protein–micelle complex is shown. Note that as our excitation wavelength 297 nm is close to the apparent 0–0 transition (295 nm)[18] of tryptophan, the excess vibrational energy is relatively insignificant.

To investigate the nature of hydration dynamics at the protein surface and in the interface between the protein and substrate micelle, we obtained fluorescence upconversion femtosecond transients when using the technique described previously.[11] Representative fs-resolved transients for PLA2 and PLA2–SDS complex in water are shown in Figure 2 B. Fourteen transients at different wavelengths covering the blue and red sides were obtained. The constructed hydration correlation function, $C(t)$,[11, 13, 19] for the PLA2 surface and PLA2–SDS interface is shown in Figure 3. For PLA2 in water,

Figure 3. Comparison of the hydration correlation function, $C(t)$, for the protein surface and protein–micelle complex. We also include the result from tryptophan in bulk water for comparison. Inset: Time-resolved anisotropy, $r(t)$, of the PLA2–SDS complex.
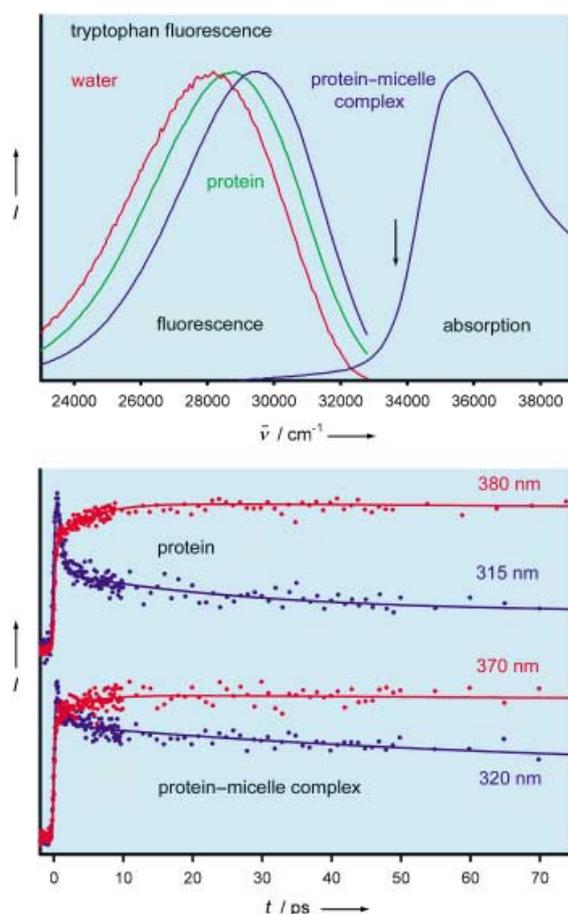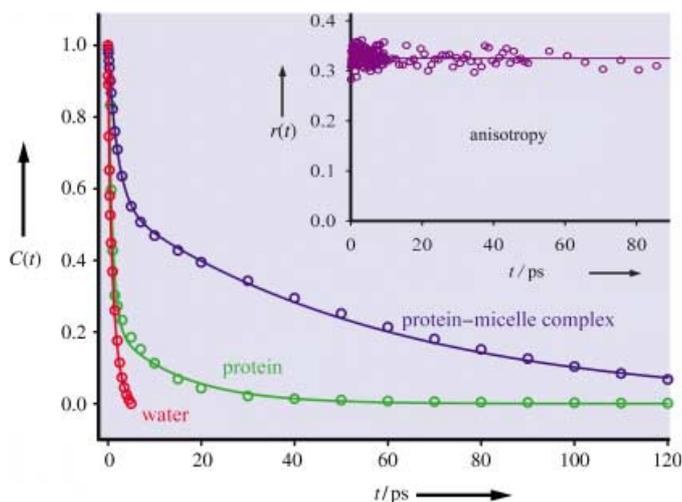

Figure 2. a) Normalized steady-state fluorescence spectra of Trp3 at the protein surface (green) and for the protein–micelle complex (blue). The fluorescence spectrum of free tryptophan in bulk water is shown (red) for comparison. The absorption spectrum for protein-micelle complex is also shown; the arrow indicates excitation (297 nm) (see text). B) Representative femtosecond-resolved fluorescence upconversion transients for Trp3 at the protein surface and in the protein–micelle complex. The excitation wavelength was 297 nm. Fourteen such transients were recorded (see text).

$C(t)$ is the sum of two exponentials with time constants 0.9 ps (78%) and 14.6 ps (22%), indicating that relaxation in the hydration shell of PLA2 occurs through two types of water trajectories: bulk type (0.9 ps) and surface-bound type (14.6 ps); any sub-100-fs components in these dynamics are unresolved. For the free enzyme, the results are consistent with previous hydration times for other proteins.[11–13] For the PLA2–SDS complex, $C(t)$ gives two well-separated timescales: 2.0 ps (44%) and 59 ps (56%). The contribution of the faster component to the total spectral shift significantly decreased from 78% to 44%. Remarkably, the increase in hydration time from ≈15 ps to 59 ps parallels the slowness observed for water at the surface of cationic CTAB micelle.[19]

To study the motion of the probe tryptophan, we measured the time-resolved anisotropy decay, $r(t)$, at 350 nm. For both PLA2 and PLA2–SDS complex, $r(t)$ is

persistent up to 200 ps (Figure 3, inset) except for a small (10%) 40-ps decay component for PLA2. These results indicate that our probe Trp3 is mostly restricted in motion during the timescale of surface hydration.

The bimodality observed in the hydration at the protein surface is understood, as dynamic processes between surface and bulk water lead to the formation of an ordered layer with a thickness in the nanometer range.[20] At the surface of PLA2, the exposure of the Trp3 to the aqueous environment is apparent from the dominance (78%) of bulk-type hydration (0.9 ps), but the presence of the 14.6 ps (22%) component must reflect the contribution of the layered water. Molecular dynamics simulation studies,[17] which have considered solvation at the surface of PLA2 as manifested in the spectral shifts by both water and residues of the protein (internal Stark effect), showed that $\approx 95\%$ of the shift is due to water. The domination of the bulk-type water at the surface interfacial binding site—in the absence of substrate micelle—affords flexibility and efficiency for the recognition process. Upon complexation with the micelle, the hydration dynamics at the interface become fourfold slower (59 ps), replacing a major portion of water with those ordered on the substrate micelle (typically 60 ps).[19] However, to maintain equilibrium, a fraction of mobile (bulk type) water is still present.

Generally the PLA2 binding to the substrate surface is diffusion-controlled, with subpicomolar to micromolar dissociation constants, depending on the exact nature of the aggregates and the source of the enzymes.[21,22] Once bound to the interface, the dissociation of the enzyme is generally very slow relative to the timescale of the catalytic turnover. In a complete characterization of the interfacial catalysis by bovine pancreatic PLA2, analysis of the integrated Michaelis–Menten reaction progress was carried out for the hydrolysis of a 1,2-dimyristoyl-sn-glycero-3-phosphomethanol vesicle in a highly "scooting" mode.[22] In this particular system, the enzyme binds to the vesicle with subpicomolar affinity ($\Delta G^\circ \approx 15$ kcal mol^{-1}). On the vesicle, the enzyme-substrate association occurs on a millisecond timescale, with subsequent catalytic turnover within a few milliseconds. Figure 4 presents a simplified picture of kinetic pathway of the enzyme PLA2 and associated timescales.

In the PLA2–SDS complex system, it has been found that the binding releases almost 40 kcal mol^{-1} of heat (ΔH°), consistent with high-affinity binding.[5] The lateral movement of the enzyme on the surface should be facilitated by the presence of water molecules, with fast mobility (ps) at the interface, which act as lubricant. For the enzymatic reaction to take place in a biologically relevant enzyme–membrane system, individual substrate lipid molecules must be transported at least 15 Å away from its position within the membrane to reach the catalytic center.[2] The role of the water molecules is important in this process.[10,23] The more-rigidly ordered water molecules characterized by their slower dynamics of ≈ 60 ps would maintain a network of hydrogen bonds that stabilizes the interfacial binding and the structure of the transporting channel, hence facilitating the movement to the catalytic site. In this case, the coincidence of the hydration time at the interface with that of micelle substrate suggests the importance of the "substrate model". Moreover,
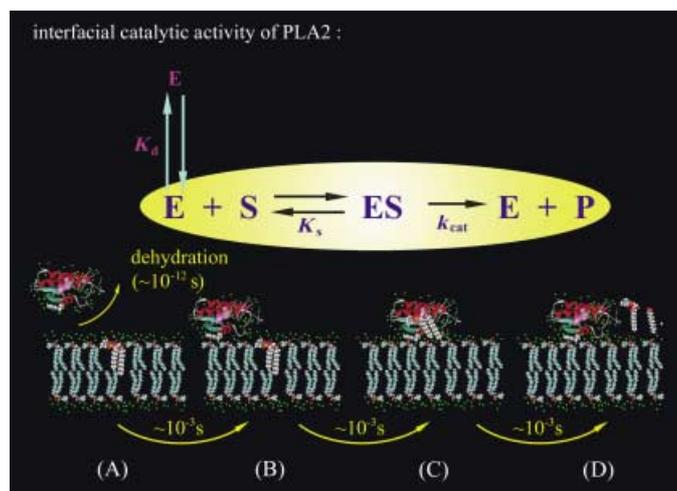

Figure 4. Reaction pathways in the catalytic activity of the protein PLA2. K_d indicates the equilibrium dissociation constant of the enzyme to the membrane. K_s and k_{cat} denote the equilibrium dissociation of the enzyme–substrate complex and catalytic rate constant (see text). Schematic representation of the catalytic steps of the enzyme: A) The enzyme and the host that contains the substrate. B) The complex of the enzyme with the host. C) The complex of the enzyme with the membrane-bound substrate. D) The products. The timescales represent an estimate for the different processes involved.[2]

the hindered motion of Trp3 upon micelle binding, from the anisotropy, indicates that structural rigidity is important for substrate recognition of the enzyme.

Experimental Section

Fluorescence upconversion: A detailed experimental setup for the fs-resolved fluorescence upconversion technique can be found elsewhere.[11] The tryptophan residue was excited at 297 nm with a pulse energy of 250–300 nJ. A total of 14 transients were taken for the free PLA2 and PLA2–SDS complex system at various wavelengths.

Samples: Ultrapure tris (ICN Biomedicals, Ohio), calcium chloride (Mallinckrodt, Kentucky), hydrochloric acid (J. T. Baker, New Jersey), sodium dodecyl sulfate (SDS, Fluka BioChemika, $\geqslant 99\%$), L-α-phosphatidylcholine (Sigma), and PLA2 (Sigma) were purchased and used without further purification. The purity of PLA2 was checked by several techniques, including MALDI and ESI mass spectrometry and tryptophan fluorescence spectrum. The biological function of the enzyme was confirmed by enzymatic assays with L-α-phosphatidylcholine as substrate following the titrametric protocol utilized by Sigma-Aldrich Corporation.

The protein solution (357 μM) was made by dissolving the PLA2 sample (lyophilized powder, approximately 20% protein in tris buffer salt) in water (Nanopure Infinity Ultrapure Water System, Barnstead/Thermolyne), followed by exhaustive dialysis against a pH 7.5 buffer solution (50 mM Tris/5 mM CaCl$_2$) in the molecular porous membrane tubing (Spectrum Laboratories, Inc.). To make the protein–SDS micelle complex, SDS is dissolved in the dialyzed PLA2 solution described above to a concentration of ≈ 50 mM.

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