Surface Fluorescence Resonance Energy Transfer Studies on Interfacial Adsorption of *Thermomyces* (*Humicola*) *lanuginosa* Lipase, Using Monomolecular Films of *cis*-Parinaric Acid

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ABSTRACT: The fluorescence resonance energy transfer (FRET) technique was adapted to study the process whereby lipase is adsorbed to monomolecular lipid films spread at the air–water interface. When *cis*-parinaric acid (*cis*-PnA) was spread over an aqueous subphase before the injection of sodium taurodeoxycholate (NaTDC) and *Thermomyces lanuginosa* lipase (TLL), no FRET was observed. Under these conditions, no adsorption of TLL was detected using an ELISA. In contrast, FRET occurred when *cis*-PnA was spread over an aqueous subphase containing NaTDC and TLL. The FRET signals observed were attributed to the interactions between the adsorbed TLL and the *cis*-PnA monomolecular films. Comparisons between the fluorescence emission spectra corresponding to the bulk phase and the aspirated film, in the presence and absence of TLL, showed that *cis*-PnA was undetectable in the bulk phase. We concluded that the FRET originated from the interface and not from the bulk phase. Using surface FRET, we estimated that the surface excess of the catalytically inactive mutant, TLL(S146A), was 1.6 higher than that present in the wild-type TLL. This finding is in agreement with independent measurements of the surface excess of TLL and TLL(S146A) on monomolecular films of *cis*-PnA. © 2002 Wiley Periodicals, Inc. Biopolymers (Biospectroscopy) 65: 121–128, 2002

Keywords: fluorescence resonance energy transfer; *Thermomyces lanuginosa* lipase; *cis*-parinaric acid; monomolecular films; surface fluorescence resonance energy transfer

INTRODUCTION

In the field of heterogeneous biocatalysis, a large proportion of the studies published so far deal with lipolysis. Verger and de Haas[1] proposed a kinetic model based on data obtained with a fully automated barostat apparatus to measure the lipolytic reaction rates at air–water interfaces, using medium-chain lipid monomolecular films.

However, the interfacial excess of lipase remained largely undetermined. Several methods have thus been developed to assess the surface excess, which is directly responsible for the cata-

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lytic reaction.[2–6] Fluorescence techniques have been used successfully to study the binding behavior of *Thermomyces lanuginosa* lipase (TLL) to small and large anionic unilamellar vesicles of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) and to vesicles of zwitterionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine.[7] The latter authors concluded that TLL binds with a similar affinity to all types of phospholipid vesicles, but it adopts a catalytically active conformation only in small unilamellar vesicles of POPG. These findings are in agreement with data showing that the lid region is directly involved in the binding of TLL to micelles of the pentaoxyethylene octyl ether (C_8E_5) detergent.[8] In addition, molecular dynamics simulations[9] have indicated that the replacement of the active site serine (S146 to A) may lead to conformational alterations in TLL. Yapoudjian et al.[10] studied the interactions between TLL (and its mutants) with mixed micelles of *cis*-parinaric acid (*cis*-PnA)/sodium taurodeoxycholate (NaTDC), using fluorescence resonance energy transfer (FRET) and crystallographic procedures. The authors pointed out the apparently higher affinity of the inactive mutant with the active site serine 146 mutated to alanine [TLL(S146A)] to mixed micelles of *cis*-PnA/NaTDC as compared to the wild-type TLL. The authors noted that TLL(S146A) had an apparently higher affinity for mixed micelles of *cis*-PnA/NaTDC than the wild-type TLL. However, all these studies involved bulk experiments and there was no means of monitoring the "interfacial quality."

In the present work, we used an approach inspired by the original article by Bougis et al.,[11] who measured the surface fluorescence emission of mixed monomolecular films of phospholipids and cardiotoxins spread together at the air–water interface. The method used by these authors ensured that the fluorescence signal originated from the interface. Both the didodecanoylphosphatidylglycerol and the cardiotoxin molecules were soluble in a $CHCl_3$/MeOH (85/15, v/v) mixture and spread spontaneously at the air–water interface without any significant desorption into the aqueous phase. By contrast, TLL is a larger protein that is not soluble in organic solvents and that partitions between the bulk phase and the air–water interface when injected into the aqueous subphase. One key question therefore arose as to whether, under these experimental conditions, the measured FRET originated from the interaction between TLL and *cis*-PnA present at the interface or from the same molecules solubilized into the bulk phase.

The new interfacial fluorescence spectroscopic method that we developed can be used to observe the FRET occurring between TLL and mixed films of *cis*-PnA/NaTDC present at the air–water interface, using a linearly polarized UV pulsed laser beam. In order to check the validity of this technique, we measured the fluorescence emission intensity simultaneously at various wavelengths ranging from 300 to 500 nm (excitation at 280 nm), as well as the variations in the surface pressure with time. Independent surface excess measurements of TLL and TLL(S146A) are in agreement with the fact that higher surface FRET values were observed with TLL(S146A) than with TLL.

MATERIALS AND METHODS

Materials

The NaTDC was purchased from Sigma and used without further purification, and *cis*-PnA was procured from Molecular Probes and used without further purification. A stock solution of 3.2 m*M* of *cis*-PnA in ethanol containing 0.001% (w/v) butylhydroxytoluene as an antioxidant was stored in the dark at −20°C under an argon atmosphere. These precautions ensure that any polyene decomposition that may occur is negligible.[12]

TLL and its mutant TLL(S146A), in which the active site S146 was mutated to Ala,[13,14] as well as monoclonal and polyclonal antibodies directed against TLL, were kindly provided Dr. S. A. Patkar (Novo Nordisk, Denmark). The buffer used was 10 m*M* acetate (pH 5.0), 100 m*M* NaCl, and 20 m*M* $CaCl_2$.

A rectangular 46-mL Plexiglas trough (12 cm in length and 6 cm in width) was used for the surface fluorescence experiments [see Fig. 1(B)].

Methods

In Situ Surface Fluorescence: Experimental Setup and Measurements

The experimental setup [see Fig. 1(A)] was composed of an excimer laser (pulsed, 25-ns full width at half-maximum at 308 nm) pumping a dye laser (Rhodamine 560 chloride), followed by a KDP crystal in order to reach the tryptophan excitation wavelength of 280 nm. The laser beam falls upon

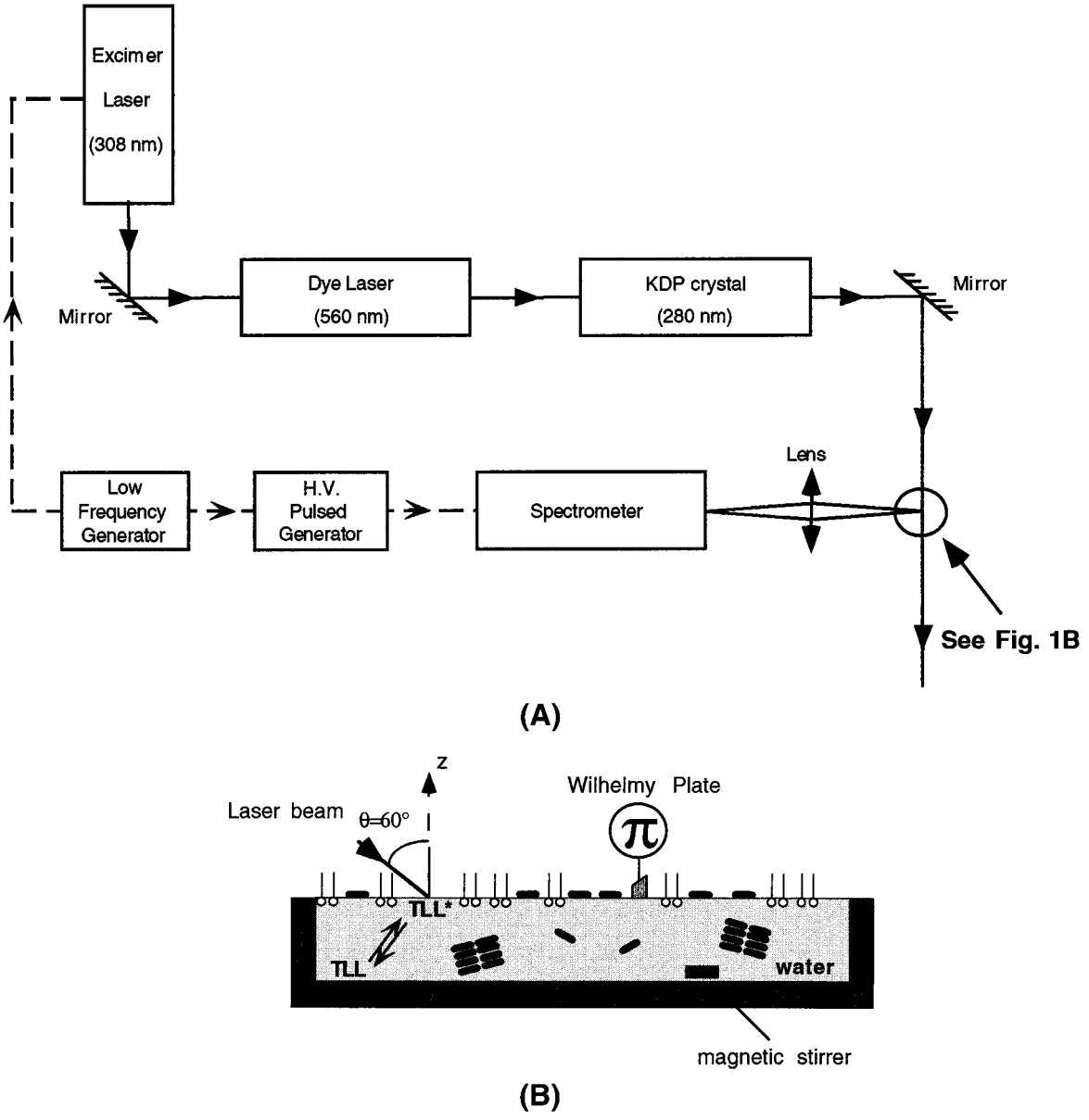


Figure 1. (A) The scheme of the *in situ* surface fluorescence experimental setup. (B) A diagram of the trough used for *in situ* surface fluorescence experiments. TLL, the lipase molecules present in the bulk phase; TLL*, the protein adsorbed at the air–water interface. (▬) The NaTDC molecules; (⏚) the *cis*-PnA molecule spread at the air–water interface. The incident angle of the laser beam was set to 60° according to the sample normal. The dark arrow indicates the incident laser beam and the dotted arrow (normal to the sample) indicates the direction of the fluorescence measurements. The surface pressure (π) was continuously monitored simultaneously with the surface fluorescence data collection.

the surface of an aqueous buffered solution placed in a Plexiglas trough [see Fig. 1(B)]. The incident angle θ was set to 60°, according to the sample normal. A set of lenses images the irradiated surface in the slit of the spectrometer. The spectrometer consists of a monochromator (500-mm focal length) associated with an ICCD working in the pulse mode (from 5 ns to the microsecond range). Because of the short lifetime of tryptophan fluorescence (in the nanosecond range), good synchro-

nization is required between the excitation light and the fluorescence signal detection. The use of a low frequency generator allows the synchronization of a laser pulse and a spectrum acquisition. The gate of the ICCD was fixed to 50 ns to prevent any interference from any parasitic light arising from the sample, and a long-pass edge filter (WG 320, Schott) was also placed in front of the spectrometer slit. The spectra presented here were obtained as the result of 100 accumulations. The 290–460 nm region was explored. The spectra were not corrected on the basis of the long-pass edge filter response.

Fluorescence measurements using the experimental setup described above were carried out at room temperature in the dark. The trough was first filled with 46 mL of buffer containing 10 m*M* acetate (pH 5.0), 20 m*M* $CaCl_2$, 100 m*M* NaCl, and 1 m*M* NaTDC. A spectrum was recorded and used as the reference spectrum. A TLL sample (final concentration of 300 n*M*) was then injected into the aqueous subphase, which was stirred for 30 s. Another spectrum was recorded. Four aliquots of *cis*-PnA (1, 1, 2, and 2 μL) were finally spread consecutively from a chloroform solution of *cis*-PnA (3.2 m*M*). After spreading each aliquot, a spectrum was recorded. All the fluorescence emission spectra were corrected by subtracting them from the reference spectrum.

Formation of Mixed Films

An ethanolic solution of *cis*-PnA (3.2 m*M*) was spread over an aqueous subphase, which contained NaTDC (1 m*M* final concentration) and TLL (0–300 n*M* final concentration) in a Plexiglas trough.

Simultaneously with the surface fluorescence measurements, the surface pressure was measured via the Wilhelmy method, using a thin platinum plate (3.94-cm perimeter) attached to an electromicrobalance (made in our laboratory), which was connected in turn to a microprocessor [see Fig. 1(B)].

Surface Film Recovery

At the end of the surface fluorescence measurements, the film was aspirated as previously described.[2] The same volume as that corresponding to the aspirated film was collected from the subphase. Two kinds of measurements were then performed: classical fluorescence spectroscopy and determination of the surface excess of TLL and TLL(S146A) adsorbed to the monomolecular films.

Classical Fluorescence Spectroscopy. After the last surface spectrum was recorded, the recovered surface film, as well as an equal volume of the subphase, were placed in a 3.5-mL quartz cuvette (1-cm optical path length) and analyzed using a SFM 25 spectrofluorimeter from Kontron Instruments. Fluorescence measurements were carried out at 29°C under constant stirring. The optical density of the samples was less than 0.1 in the 280–500 nm spectral range to ensure that no inner filter effects would occur. The fluorescence emission spectra were recorded at an excitation wavelength of 280 nm by scanning over an emission wavelength ranging from 350 to 500 nm.

Determination of Surface Excess of TLL and TLL(S146A) Adsorbed to Monomolecular Films. The surface excess of TLL and TLL(S146A) was measured using the monomolecular film technique combined with an ELISA. At each bulk concentration of TLL or TLL (S146A), the amounts of adsorbed lipase were determined after aspirating the surface film and an equal volume of the bulk phase. The amounts of TLL recovered were subsequently measured using an ELISA, as described in Aoubala et al.[3]

RESULTS

Surface Pressure Measurements

After NaTDC (1 m*M* final concentration) was injected into the aqueous buffer subphase, the surface pressure increased rapidly and reached a steady state at 32 mN m^{-1} [see Fig. 2(A)]. The subsequent spreading of *cis*-PnA aliquots from an ethanolic solution increased the surface pressure to a final value of 35 mN m^{-1}. Similar results were obtained when TLL (300 n*M*) was injected into the subphase before *cis*-PnA was spread [see Fig. 2(B)].

Comparisons of Fluorescence Emission Spectra Corresponding to Recovered Film and Aqueous Bulk Sample

The fluorescence spectra corresponding to the recovered film and the bulk sample are presented in Figure 3(A,B). The fluorescence emission spectrum of the bulk sample containing TLL [Fig.

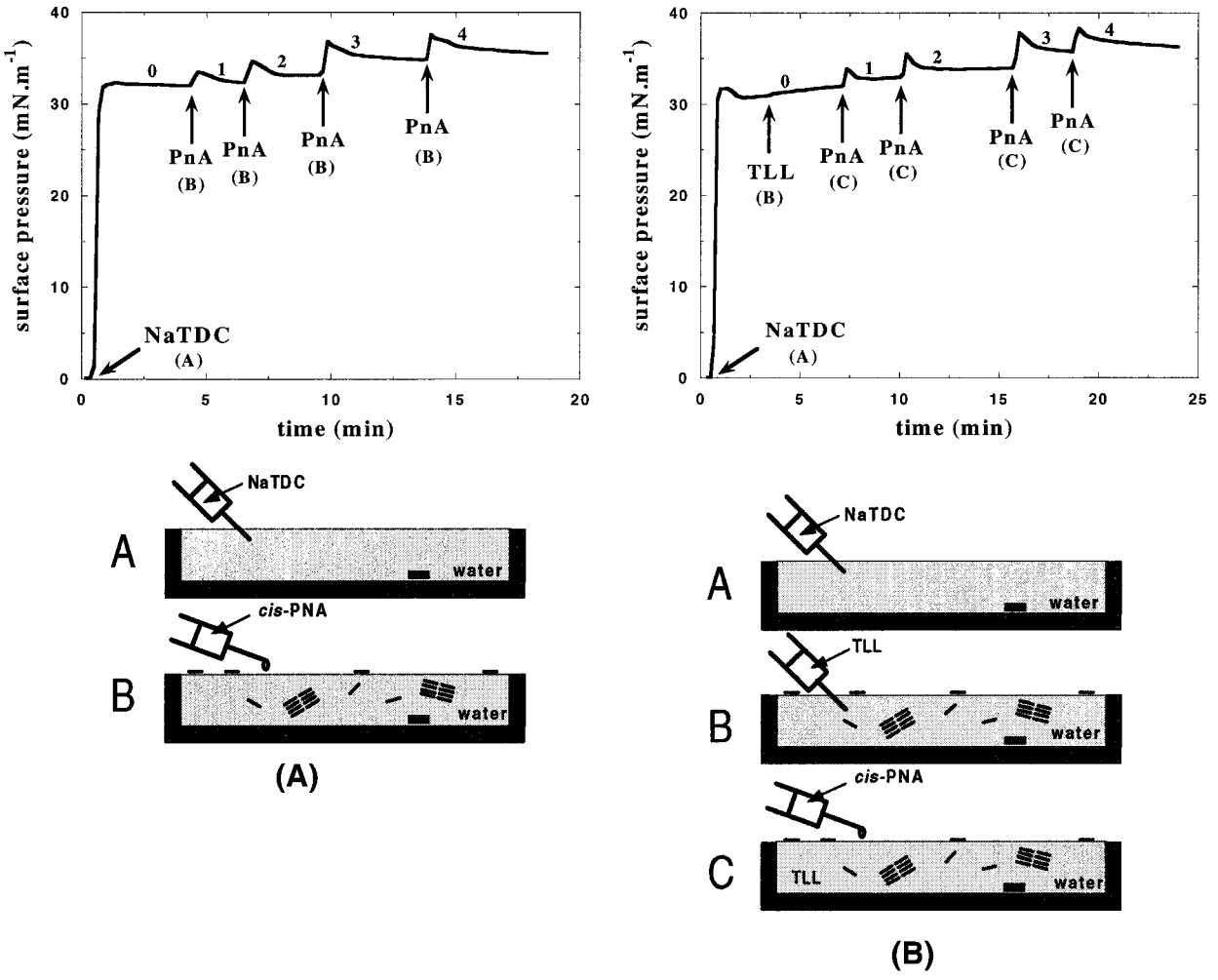


Figure 2. The changes with time in the surface pressure upon spreading *cis*-PnA in the (A) absence or (B) presence of TLL (300 n*M* final bulk concentration). (A,B) Experiments were performed according to the scheme presented at the bottom. (A) Step A consisted of injecting NaTDC into the water subphase (1 m*M* final concentration). Step B consisted of spreading *cis*-PnA at the air–water interface. Four aliquots of *cis*-PnA (1, 1, 2, and 2 μL) were spread consecutively from a ethanolic stock solution at 3.2 m*M*. (B) Step A consisted of injecting NaTDC into the water subphase (1 m*M* final concentration). Step B consisted of injecting TLL into the water subphase (300 n*M* final concentration). Step C consisted of spreading *cis*-PnA at the air–water interface. Four aliquots of *cis*-PnA (1, 1, 2, and 2 μL) were spread consecutively from a ethanolic stock solution at 3.2 m*M*. The time marks 0, 1, 2, 3, and 4 correspond to the recording of the *in situ* surface fluorescence spectra. The buffer used contained 10 m*M* acetate (pH 5.0), 100 m*M* NaCl, and 20 m*M* $CaCl_2$.

3(B)] was comparable to those previously obtained by Yapoudjian et al.[10] when TLL was simply dissolved in the aqueous buffer along with 1 m*M* NaTDC at pH 5.0. Any desorbed *cis*-PnA present had negligible effects on the TLL fluorescence spectrum. It is worth noting that, in the presence of TLL in the aqueous subphase, the fluorescence emission spectrum of the recovered film underwent a blueshift in the 300–370 nm wavelength region, as compared to the spectrum of the bulk sample [see Fig. 3(B)]. Furthermore, a new fluorescence signal appeared in the 400–450 nm spectral region in the recovered film. In the absence of TLL, as control experiments, a fluorescence signal was observed only with the recovered film [Fig. 3(A)], giving the fluorescent properties

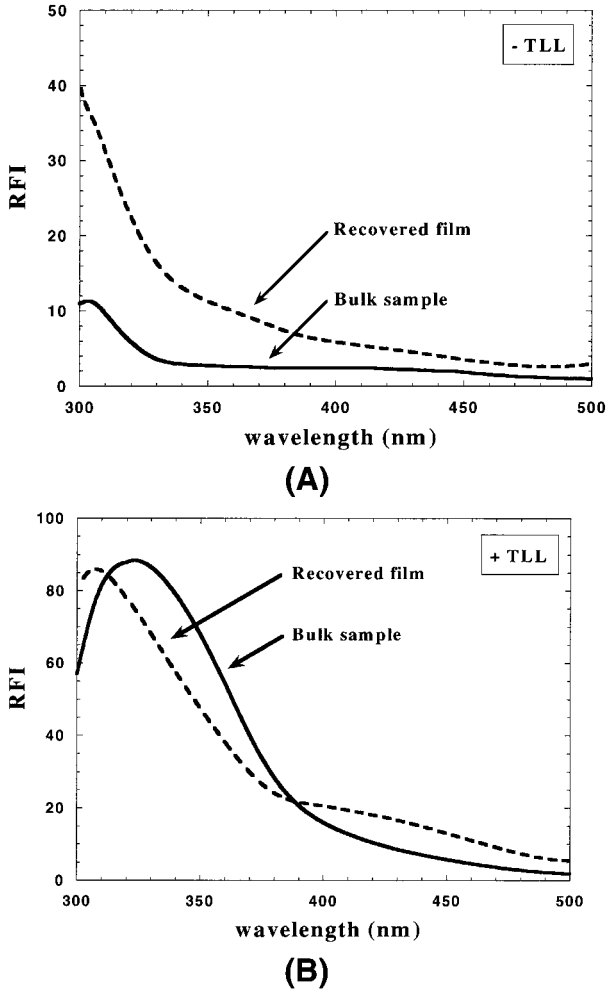


Figure 3. The fluorescence emission spectra corresponding to the (- - -) recovered film and (—) the bulk sample in the (A) absence or (B) presence of TLL in the subphase. The experimental conditions are the same as Figure 2. The recovered film and the bulk sample were collected after the last *in situ* surface fluorescence recording.

of the *cis*-PnA collected during the aspiration procedure.

In Situ Surface FRET

The surface fluorescence emission spectra of TLL [Fig. 4(A)] and TLL(S146A) [Fig. 4(B)] were recorded after spreading increasing amounts of *cis*-PnA, using the experimental setup described in the Methods section. With both lipases, a clear-cut decrease in the relative fluorescence intensity (RFI) occurred within the 300–370 nm spectral region when the surface concentrations of *cis*-PnA were increased. Concomitantly, we observed an increase in the RFI in the 400–450 nm spectral region. The lower panels in Figure 4(A) (TLL) and 4(B) [TLL(S146A)] were added for the sake of comparison: these give data previously obtained by Yapoudjian et al.[10] and presented under the results of standard RFI measurements performed in a classical spectrofluorimeter cuvette.

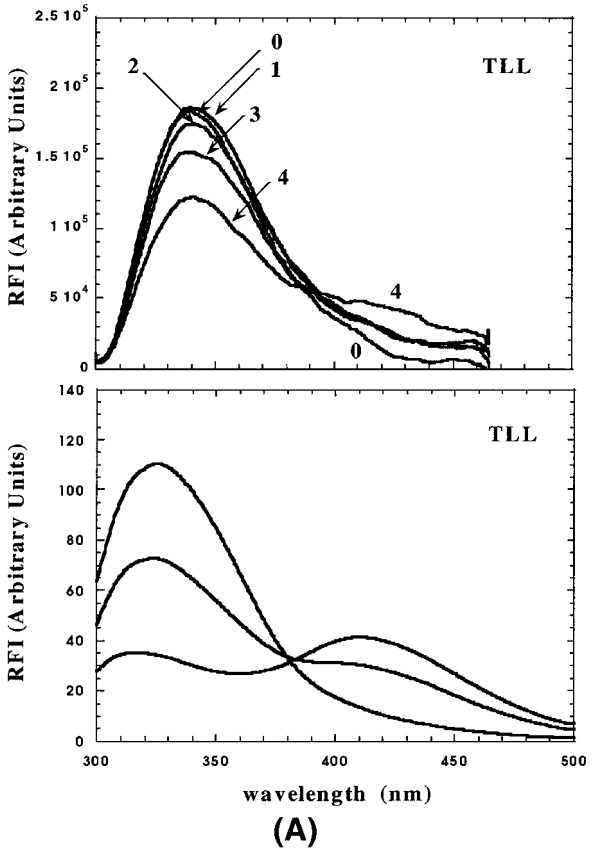
Surface Excess of TLL and TLL(S146A) Adsorbed to NaTDC/*cis*-PnA Mixed Monomolecular Films

The TLL and TLL(S146A) surface excesses measured using the ELISA (see Methods) are presented in Figure 5. It can be noted that at a 300-n*M* final concentration, which is the lipase bulk concentration used for surface FRET experiments, surface saturation is almost reached for both TLL and TLL(S146A). Moreover, the surface excess of TLL(S146A) was found to be around twice that of TLL. It is important that we also performed another type of experiment using a different protocol (the second protocol in Fig. 5) in which *cis*-PnA was first spread at the air–water interface (at a final surface pressure of 25 mN m^{-1}), followed by injections into the bulk phase of NaTDC (1 m*M* final concentration) and TLL (156 n*M* final concentration). In this case (second protocol, Fig. 5), no lipase surface excess was detectable. With this experimental protocol at a TLL or TLL(S146A) bulk concentration of 300 nm, no surface FRET was observed.

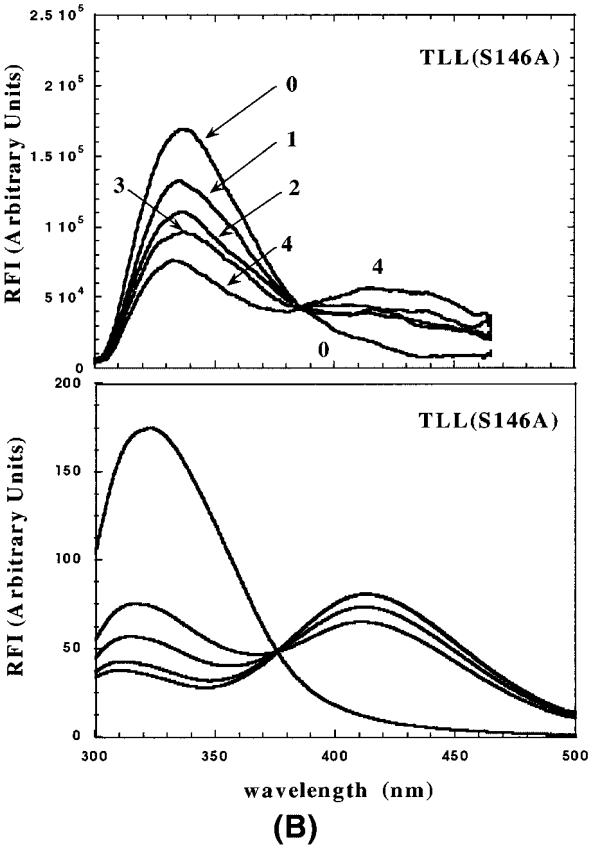
DISCUSSION

Surface FRET Origination from Interface?

Yapoudjian et al.[10] present fluorescence spectroscopic data showing that NaTDC (1 m*M*) solubilized *cis*-PnA in the form of mixed aggregates of *cis*-PnA/NaTDC. It was therefore necessary to determine whether any *cis*-PnA remains at the interface when spread over a subphase containing NaTDC. First, the fact that *cis*-PnA was still present at the air–water interface was indicated by the increase in the surface pressure that occurred whenever *cis*-PnA aliquots were spread in the presence of NaTDC in the bulk phase [Fig. 2(A,B)]. Second, the *cis*-PnA fluorescence emission spectrum was only observed with the recovered film [Fig. 3(A)]. It can thus be concluded that our experimental procedure led to the formation of mixed *cis*-PnA/NaTDC films, as illustrated in Figure 1(B).



(A)



(B)

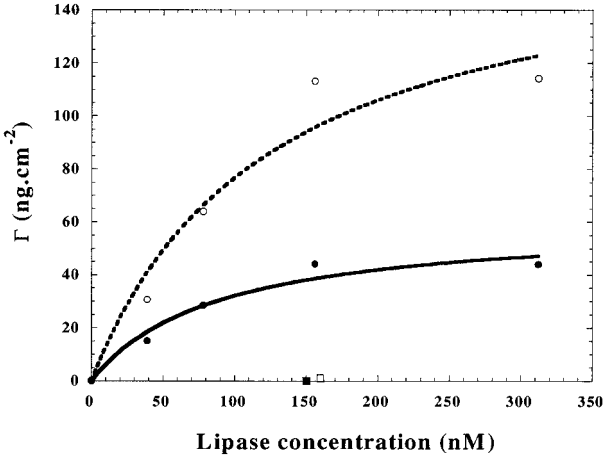


Figure 5. The surface excesses as measured by ELISA of (●, ■) TLL and (○, □) TLL(S146A) at various final bulk concentrations of lipase. Experiments were performed according to two different experimental protocols. The first protocol corresponds to the method illustrated in Figure 2(B). (●, ○) The final bulk concentration of lipase ranged from 0 to 312 n*M*. (■, □) In the second protocol, we used the following order of addition: spreading of *cis*-PnA at a final surface pressure of 25 mN m^{-1} (step C) followed by injections of NaTDC (step A) and lipase at a final bulk concentration of 156 n*M* (step B).

Upon comparing the similarities between the various FRET experiments performed under classical conditions [Fig. 4(A), lower panel] and with the newly developed *in situ* surface fluorescence device [Fig. 4(A), upper panel], the question arose as to whether the fluorescence signal actually originated from the interface. In order to address this key point, we recorded classical fluorescence spectra that we used as control spectra after recovering the films and comparing them with those obtained from bulk samples. From the data presented in Figure 3(B), the spectrum given by the surface recovered film can be clearly seen to have undergone a blueshift in comparison with the spectrum of the bulk sample. This indicates that the tryptophan residues present in TLL were exposed to a hydrophobic environment when in con-

Figure 4. *In situ* surface FRET recorded with (A) TLL or (B) TLL(S146A). The experimental conditions are the same as Figure 2. Spectra were recorded at the time marks 0, 1, 2, 3, and 4 as indicated in Figure 2. For the sake of comparison, the bottom panels are data from Yapoudjian et al.[10] and correspond to previous bulk experiments performed with classical FRET.

tact with a *cis*-PnA/NaTDC mixture. This conclusion is further supported by the increase observed (in the spectral region of 390–500 nm) in the level of *cis*-PnA fluorescence emission, which was probably due to FRET and was observed only with the recovered film.

When *cis*-PnA was spread before the injection of NaTDC and TLL, no lipase surface excess was measured by ELISA (second protocol, Fig. 5). Under these conditions, no surface FRET was observed, which argues in favor of the surface origin of the observed FRET.

In Situ Surface FRET

The RFI decrease occurring in the 300–370 nm spectral region and the concomitant RFI increase in the 400–450 nm spectral region can be taken to be the signature of the FRET occurring between TLL tryptophans and *cis*-PnA [Fig. 4(A,B)]. In view of the points discussed above, the FRET between adsorbed TLL and spread *cis*-PnA was then successfully measured at the air–water interface. The maximum quenching rates of the RFI (%) measured at the λ_{max} with TLL and TLL(S146A) were 36 and 56%, respectively. Assuming that TLL and TLL(S146A) form similar complexes with the *cis*-PnA molecule (resulting in identical relative orientations and distances between *cis*-PnA and the four tryptophan residues), the quenching amplitude can be attributed to the surface excess of the complex. It is thus possible to calculate theoretically that the surface excess of TLL(S146A) is 1.6 times greater than in the TLL.

In order to directly check the validity of this conclusion, we performed independent surface excess measurements using an ELISA (Fig. 5). One can note that at all concentrations of lipase tested, TLL(S146A) is around 2 times more adsorbed to mixed monomolecular films of NaTDC/*cis*-PnA than the wild-type TLL. This increased adsorption of TLL(S146A) may be explained by the fact that the mutation of the S146 to Ala destabilizes the close conformation of the lid and subsequently favors the open conformation of the enzyme, thus facilitating the interaction with the interface.

Contrary to what was initially thought, the point mutation of an active site serine into a lipase can drastically affect its surface behavior and such a mutant cannot be used as a model of the wild-type lipase form. As a conclusion, one can attribute the structural role to the catalytic serine.

The new *in situ* surface fluorescence technique developed during this study could be used in the future to detect surface FRET occurring between an adsorbed protein and a lipid film (containing an appropriate fluorescent fatty acid) spread at the air–water interface.

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