THE JOURNAL OF PHYSICAL CHEMISTRY B LETTERS

2008, *112,* 3581−3585
Published on Web 03/01/2008

Orientation of Cutinase Adsorbed onto PMMA Nanoparticles Probed by Tryptophan Fluorescence

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Received: December 19, 2007; In Final Form: February 13, 2008

The fluorescence of the single tryptophan (Trp69) of cutinase from *Fusarium solani pisi*, free in aqueous solution and adsorbed onto the surface of poly(methyl methacrylate) (PMMA) latex particles, was studied at pHs of 4.5 and 8.0. The monodisperse PMMA particles ($d = 106.0 \pm 0.1$ nm) were coated with a quite compact monolayer of cutinase at both pH values. The Trp decay curve of the folded free cutinase in solution can only be fitted with a sum of four exponentials with lifetimes of 0.05, 0.3−0.4, 2−3, and 6−7 ns, irrespective of pH. The 50 ps lifetime is attributed to the population of Trp residues hydrogen bonded with the Ala32 and strongly quenched by a close disulfide bridge, while the other lifetimes are due to the non-hydrogen-bonded Trp rotamers. The 50 ps Trp lifetime component disappears by temperature melting and upon protein adsorption, owing to the disruption of the Trp−Ala hydrogen bond and the release of the Trp residue from the vicinity of the disulfide bridge. This shows that cutinase adsorption occurs by the region of the protein where the Trp is located, which agrees with the retention of cutinase enzymatic activity by adsorption at basic pH.

1. Introduction

Protein immobilization is an important phenomenon in bioseparations, biocatalysis, artificial tissues and organs, biosensors, solid-phase immunoassays, and drug delivery systems.[1−7] Proteins physically adsorb on different surfaces in several amounts and orientations, with changes in the secondary structure whose extent depends on the kind and intensity of the interactions established with the substrate.[8−12] Protein−surface interactions include van der Waals, electrostatic, hydrogen-bonding, and hydrophobic components. The adsorption depends on the protein properties (surface charge, hydrophobic domains) and medium but is also strongly influenced by the surface energy, surface roughness, surface functional groups, and hydrophobicity of the substrate surface.[8] Proteins can also be immobilized by a small number of strong bonds between the protein and the surface.[13] Among the available methodologies, the photonic activation of disulfide bridges, proposed by Neves-Petersen et al.,[14] looks very promising.

Generally, proteins adsorb onto surfaces forming a non-compact monolayer even at the highest surface coverage due to the impossibility to fulfill all of the surface area available. The maximum amount of protein adsorbed is still dependent on the orientation of the adsorbed protein, which is strongly influenced by the type and strength of the interaction that different protein domains establish with the support surface. The structural and functional properties of the immobilized protein are dependent on its orientation upon adsorption, which can be a key issue in many technological applications.[15]

This work describes the fluorescence of the single Trp of cutinase from *Fusarium solani pisi*, adsorbed onto the surface of monodisperse PMMA nanospheres with a diameter equal to 106.0 ± 0.1 nm, bearing carboxylic groups at the surface. Cutinase is a lypolitic enzyme constituted by 197 amino acid residues with an ellipsoidal shape ($45 \times 30 \times 30$ Å^3), whose crystal structure has been determined to 1.0 Å resolution.[16] It is an α/β hydrolase with a core formed by a β-sheet of five parallel strands surrounded by four α-helices (Figure 1). The active center is formed by the triad Ser120, Asp175, and His188 as well as by an oxianionic cavity.[17] This enzyme is able to degrade cutin, the insoluble lipid polyester matrix that covers plant surfaces, being also active in the hydrolysis of a wide variety of synthetic esters and triglycerides.[18]

The intrinsic fluorescence of cutinase is essentially due to six tyrosines and a single tryptophan (Trp69) located in the region opposite to the active site (Figure 1). At room temperature, the majority of the native cutinase molecules adopt a conformation in which the Trp forms an unusual hydrogen bond between its π aromatic electronic system and the NH of alanine (Ala32).[19] Nevertheless, other protein conformations exist in which the Trp residue is free or is involved in a ground-state charge-transfer complex with a close disulfide bridge. The Trp fluorescence decays can only be fitted with a sum of four exponentials, with lifetimes of 0.05, 0.3−0.4, 2.0−3.0, and 6−7 ns. The 50 ps lifetime component is due to the hydrogen-bonded

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10.1021/jp711903e CCC: $40.75

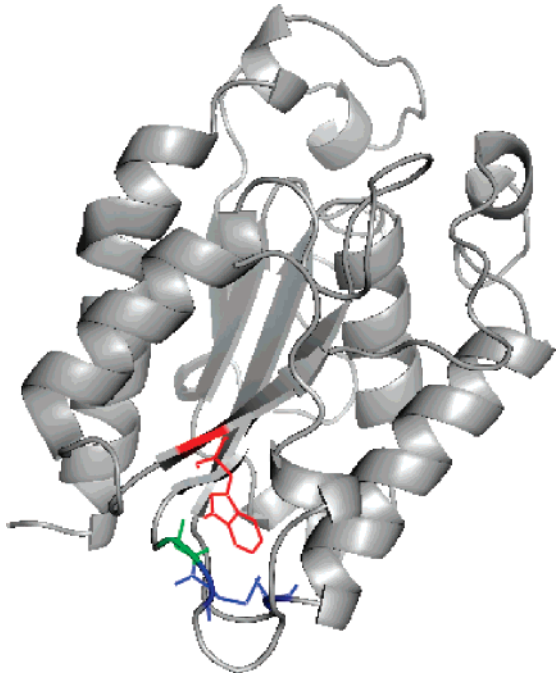

Figure 1. The structure of folded cutinase showing the Trp69 residue (red), the disulfide bridge (Cys31−Cys109) (blue), and the Ala32 (green).

Trp residues[20,21] that are forced to be in the vicinity of a strong quencher, a cystine formed by a disulfide bridge between two adjacent cysteine (Cys31 and Cys109) residues. The other lifetimes are similar to those found for other proteins and are associated with the rotamers of the non-hydrogen-bonded tryptophan residues. The low quantum yield of the single Trp in cutinase (~0.01) is a consequence of the negligible contribution of the hydrogen-bonded Trp to the quantum yield and the noncontribution of those forming charge-transfer complexes with the disulfide bridge. Upon protein melting by temperature increase, the shortest decay component disappears, and the fluorescence intensity increases more than 3 times, owing to the disruption of the Trp−Ala hydrogen bond with the subsequent suppression of the quenching by the cystine residue.[20]

Cutinase has a high affinity for the PMMA surface, with a "binding constant" of $\sim 5 \times 10^{-3}$ m^2 mg^{-1}, irrespective of pH. The maximum amount of protein adsorbed varies between $\Gamma =$ 1.1 (pH 4.5) and 1.7 mg m^{-2} (pH 7.0−9.0), which are closer to the values predicted by the formation of a hydrated protein monolayer.[22] The main driving force for cutinase adsorption arises from the hydrophobic effect, although specific hydrogen bonds with the carboxylic groups of PMMA and electrostatic interactions cannot be discarded.[22] Upon adsorption, the secondary structure and activity of the enzyme is maintained in neutral and basic media. At pH 4.5, the activity is reduced to ~ 44.5% of its value in solution, with substantial loss of the secondary structure, which reflects the lower stability of the enzyme at low pH.[22] The retention of activity at neutral and basic pH suggests that cutinase adsorbs onto the PMMA particles by the surface regions opposite to the active center. This was further confirmed by the disappearance of the 50 ps Trp fluorescence decay component upon cutinase adsorption onto the PMMA particles surface. Upon adsorption, the disulfide bridge remains intact, but the Trp-Ala hydrogen bond breaks, releasing the Trp residues from the vicinity of the disulfide bridge that effectively quenches the Trp fluorescence. Upon a temperature increase, the free and adsorbed cutinase melt at temperatures around 50 °C. The melting temperature is higher at a pH of 8.0, owing to the higher stability of the enzyme in a basic medium.[20]

2. Experimental Section

Materials. The *Fusarium solani pisi* cutinase (MW = 22.5 kDa, pI 7.6) was produced and purified following the methods described elsewhere.[23] Buffer aqueous solutions of cutinase (10^{-5}−10^{-6} M) were prepared at pHs of 4.5 (acetate buffer) and 8 (tris-HCl buffer).

The PMMA latex particles with a diameter of 106.0 ± 0.1 nm, determined by dynamic light scattering, were synthesized by emulsion polymerization using methyl methacrylate (5.0 g), sodium dodecyl sulfate (0.034 g/100 g), and 4,4′-azobis(4-cyanopentanoic) acid (0.051 g). A reactor of 50 mL volume equipped with a glass anchor-type stirrer running at 300 rpm, a condenser, and a nitrogen inlet and outlet was used. The polymerization was performed in an aqueous buffer solution of $NaHCO_3$ (10^{-2} M, pH ~ 8) at 70 °C for 4 h.[24] The PMMA latex particles, bearing carboxylic groups on the surface resultant from the initiator dissociation, were washed by several centrifugation−redispersion cycles, with the substitution of the supernatant by milli-Q water in each step.

Cutinase Adsorption. The cutinase adsorption was performed on PMMA particles (0.5% w/v dispersion) at 25 °C. The adsorption process was carried out in 0.01 M buffer solutions at pH 4.5 and 8.0, in a volumetric flask of 10 mL. The maximum amount of cutinase adsorbed is Γ=1.1 mg m^{-2} at pH 4.5 and Γ=1.7 mg m^{-2} at pH 8.0.

Fluorescence Spectra. The fluorescence spectra were recorded in a Spex Fluorolog 112 spectrofluorimeter. The fluorescence was recorded from 310 to 500 nm (9 nm bandwidth) by excitation at 300 nm (9 nm bandwidth). A cutoff filter (λ_{cut} < 300 nm) was used before the entrance slit of the emission monochromator to eliminate the Rayleigh scatter light component. The spectra were corrected for the response of the fluorescence-detecting system using a previously determined correction curve. The temperature was controlled within ±0.5 °C with a home-built thermostat, and the solutions were stirred during the measurements. For each spectrum, a freshly prepared solution was used to minimize photobleaching and the disruption of the disulfide bridge that occurs upon Trp irradiation.[25]

Fluorescence Decay Curves. The fluorescence decay curves were obtained by the single-photon timing technique using picosecond laser excitation. The 300 nm excitation pulse (5−6 ps) was achieved by doubling the laser output light of a Coherent 701-2 dye (Rhodamine 6G) laser synchronously pumped by a mode-locked Coherent Innova 400-1 argon ion laser. The excitation light beam was expanded before entering the sample solution, and neutral density filters were used in order to minimize the photodegradation of cutinase. The fluorescence was selected by a Jovin-Yvon HR320 monochromator with a grating of 100 lines mm^{-1} (20 nm bandwidth) and detected by a Hamamatsu 2809U-01 microchannel plate photomultiplier. The fluorescence decay curves were acquired using vertically polarized light for excitation and selecting the fluorescence at the magic angle (54.7°). The decays were recorded with both short (3 ps/channel) and long (22 ps/channel) time spans in a multichannel analyzer working with 1024 channels. A global analysis of the decays with the lifetimes linked in both decays was performed by a nonlinear least-squares method based on the Marquard algorithm.[26]

3. Results and Discussion

Fluorescence Spectra. Figure 2A shows the fluorescence spectra of the single Trp of cutinase in a 10^{-5} M aqueous

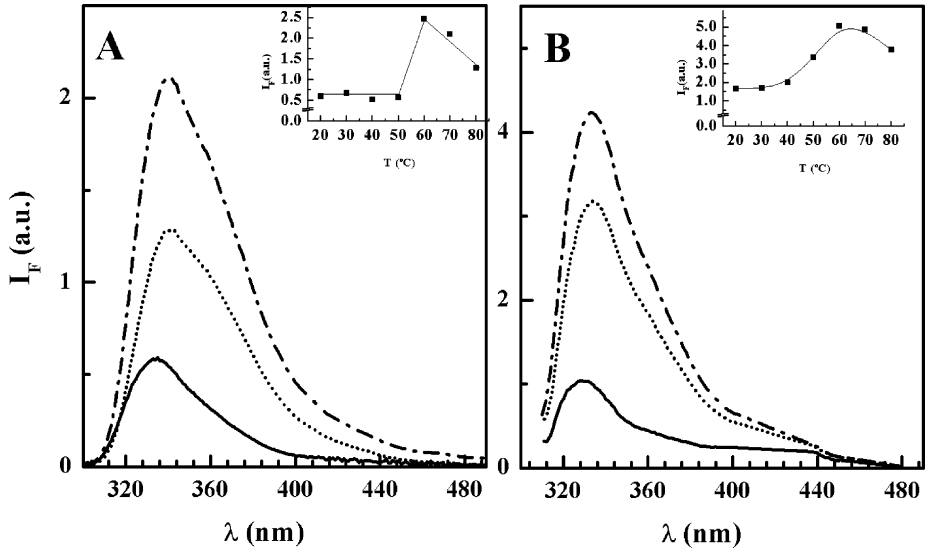

Figure 2. Fluorescence spectra of a fresh cutinase solution (10^{-5} M; tris-HCl 0.01 M; pH 8.0) free in solution (A) and adsorbed onto the surface of PMMA particles (B) by excitation at $\lambda_{exc} = 300$ nm: 20 °C (—); 70 °C (— · — · —); 80 °C (· · ·). Inset: fluorescence intensity at the maximum versus temperature.

solution (pH 8.0) at several temperatures. The shape and intensities of the fluorescence spectra are practically invariant until 50 °C. At this temperature, an abrupt increase of the fluorescence signal and a red shift of the fluorescence maximum wavelength occur. The variation of both the fluorescence intensity and wavelength shift are due to the denaturation of cutinase. Figure 2B show the fluorescence spectra of cutinase adsorbed onto PMMA latex particles (0.5% w/v dispersion) at a pH of 8.0 and $\Gamma = 1.7$ mg m^{-2}.

The fluorescence intensities in the inset of Figure 2A and B show that cutinase has a sharper denaturation when free in solution than upon adsorption onto the PMMA particle surface. This results from the fact that protein denaturation is a highly cooperative process, and when adsorbed, the multiple interactions between the protein groups and the support limit the necessary structural flexibility required for a sharp transition. After protein melting, the Trp fluorescence intensity decreases with temperature for both the free and adsorbed cutinase, owing to the increase of the nonradiative processes and fluorescence quenching.

Figure 3 shows the wavelength of the maximum fluorescence intensity of Trp for the free and adsorbed cutinase as a function of temperature.

The Trp fluorescence maximum deviates to longer wavelengths as temperature increases from 20 to 70 °C for the free and the adsorbed protein. Near the melting temperature, the wavelength shift is more pronounced since the Trp residue becomes more and more exposed to the polar aqueous environment. The solvatochromic shifts show, in agreement with the fluorescence intensity measurements, that the adsorbed cutinase has a lower melting temperature and a broader transition.

Tryptophan has two nearly isoenergetic excited singlet sates, the ^1L_a and ^1L_b. The Trp emission occurs from the ^1L_a state, whose dipole moment of ~6 D is substantially higher than its ~2 D ground-sate dipole moment.[27,28] This dipole moment increase (~4 D) explains the red shift of the Trp fluorescence by increasing the polarity of the solvent. The maximum wavelength curve for the adsorbed cutinase is shifted to smaller wavelengths compared to that of the free cutinase in solution. This indicates that the Trp region of the protein interacts with the PMMA surface and feels a less polar environment, certainly caused by the dehydration of the protein upon adsorption. The

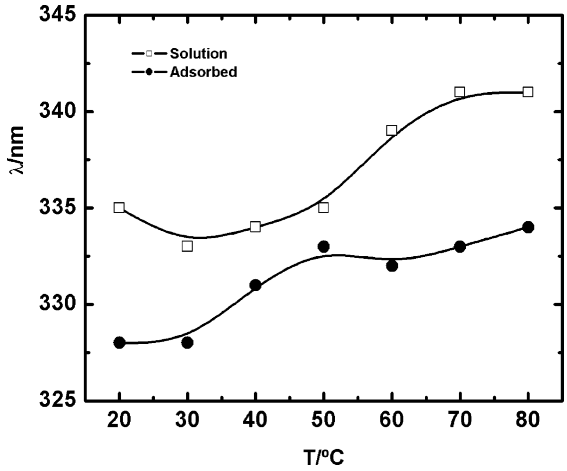

Figure 3. Variation of the wavelength of the maximum of the Trp fluorescence intensity versus temperature at a pH of 8.0; (□) free cutinase in solution; (●) adsorbed cutinase on the surface of PMMA particles.

Trp fluorescence spectra are identical at pHs of 4.5 and 8.0,[20] indicating that the fluorescence of Trp is not sensitive to the structural changes that occur upon cutinase adsorption at a pH of 4.5.

Fluorescence Decay Curves. Figure 4A shows the Trp decay curve for free cutinase in solution (10^{-6} M; pH 8.0) at 23 °C. The excitation and emission wavelengths were set at 300 and 354 nm, respectively. The decay can only be well-fitted with a sum of four exponentials with amplitude a_i and lifetime τ_i (0.05, 0.4, 2.5, 7.0 ns), plus a scattering component, a_0 (see the weighted residues in Figure 4A)

$$I(t) = \sum_{i=1}^{4} a_i e^{-t/\tau_i} + a_0 \qquad (1)$$

The scattering component considers not only the scattered light but also other very fast processes (such as the solvent dielectric relaxation) that cannot be resolved by our single-photon timing equipment.[29]

The short component of ~50 ps is due to a subpopulation of cutinase molecules in which the Trp establishes a hydrogen bond

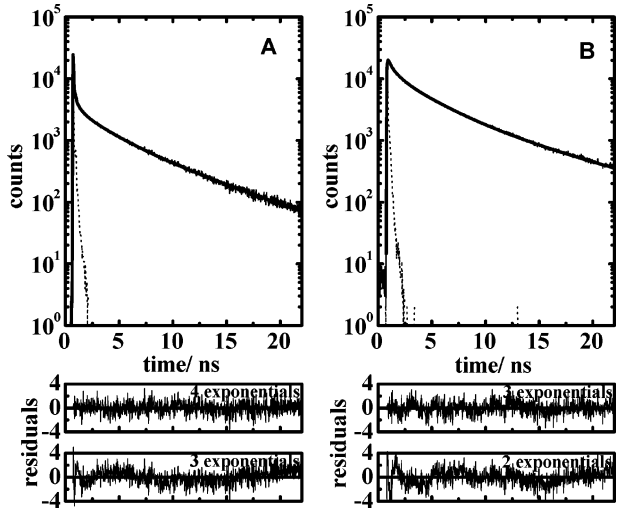

Figure 4. Fluorescence decay curves of the Trp of cutinase (10^{-6} M; pH 8.0) at 23 °C, $\lambda_{exc} = 300$ nm, and $\lambda_{em} = 354$ nm (A) free in solution and (B) adsorbed on PMMA particles (0.05% w/v dispersion). The decays (—) were fitted (**—**) with a sum of exponentials plus a scattering component after convolution with the instrumental response function (· · ·). For each sample, two decay curves were recorded with 22 and 3 ps/channel (not shown) in a MCA working with 1024 channels. The decays were globally fitted, and the quality of the fit was judged by the chi-square (χ^2), the plot of the residuals, and the autocorrelation of the residuals (not shown).

with Ala32. This was identified by ^{15}N NMR and results from the interaction between the hydrogen of Ala and the π electrons of the Trp aromatic ring. The hydrogen-bonded tryptophans are forced to be in the vicinity of the cystine residue, which, in turn, quenches Trp fluorescence very efficiently.[30] The other components with lifetimes of 0.4, 2.5, and 7.0 ns are attributed to the ground-state conformers of the tryptophan side-chain (rotamers). Each conformation has a different environment leading to distinct quenching rates because of the different distances and relative orientations of Trp to the quencher groups.[31,32] Apart from the quenching by the carbonyl group of the peptide bond, the stronger quencher of the cutinase Trp fluorescence is the cystine (Cys31−Cys109) residue.[33]

Figure 4B shows the fluorescence decay curve of Trp adsorbed on the surface of PMMA particles (0.05% w/v dispersion) at $\Gamma = 1.7$ mg m^{-2}, a pH of 8.0, and 23 °C. The decays can only be well-fitted with a sum of three exponentials with 0.8, 3.3, and 9.4 ns lifetimes, plus a scattering component (see the weighted residuals in Figure 4B). From the decay parameters, two major differences can be inferred from the free and the adsorbed cutinase; (i) the short component of 50 ps disappears upon cutinase adsorption, which indicates the disruption of the Trp−Ala hydrogen bond, and (ii) the Trp lifetimes increase upon cutinase adsorption onto the PMMA surface, suggesting a slower dynamic quenching motivated by a reduction of the mobility of the protein region where the Trp is located.

Table 1 summarizes the pre-exponential factors, a_i, and lifetimes, τ_i, for the Trp decay curves at 23 and 70 °C and pHs of 4.5 and 8.0.

The decay parameters of the Trp fluorescence of the free cutinase are similar at both pH values, suggesting that protein conformations vary slightly with pH. Upon a temperature increase from 23 (below the melting temperature of cutinase) to 70 °C (above the melting temperature of cutinase), the non-hydrogen-bonded Trp lifetimes decrease on both the free and the adsorbed cutinase, while the short lifetime component (50 ps), only present in the free protein at low temperatures, disappears. The reduction of lifetimes is less significant for the adsorbed than that for the free protein due to the reduction of the protein mobility around the Trp region, which implies a slower dynamic quenching.

TABLE 1: Tryptophan Fluorescence Decay Times (τ_i), Pre-exponential Factors for the Long Time Scale (22 ps Per Channel) Decay (a_i), and Average Lifetime ($\bar{\tau}$) of 10^{-6} M Cutinase Solutions Free (bold) and Adsorbed onto PMMA Particles (0.05% w/v Dispersions) at pHs of 4.5 and 8.0 and at 23 and 70 °C

T (°C)	a_1	τ_1 (ns)	a_2	τ_2 (ns)	a_3	τ_3 (ns)	a_4	τ_4 (ns)	$\bar{\tau}$ (ns)	χ^2
					pH 4.5					
23	**0.08**	**6.3**	**0.06**	**0.30**	**0.04**	**2.1**	**0.82**	**0.05**	**0.6**	**1.1**
	0.25	7.3	0.26	0.64	0.49	3.1			3.5	1.1
70	**0.10**	**3.0**	**0.34**	**0.40**	**0.56**	**1.2**			**1.1**	**1.1**
	0.18	6.8	0.48	0.65	0.34	2.4			2.4	1.1
					pH 8.0					
23	**0.05**	**7.0**	**0.07**	**0.40**	**0.07**	**2.5**	**0.82**	**0.05**	**0.6**	**1.0**
	0.18	9.4	0.34	0.80	0.48	3.3			3.6	1.2
70	**0.08**	**4.0**	**0.34**	**0.40**	**0.59**	**1.2**			**1.2**	**1.1**
	0.10	6.5	0.39	0.60	0.51	2.3			2.1	1.1

The amplitude-weighted average Trp lifetime, $\bar{\tau} = \sum a_i\tau_i / \sum a_i$, increases (around 2 fold) for the free protein and slightly decreases for the adsorbed cutinase, upon a temperature increase from 23 to 70 °C. The contradictory behavior for the free and the adsorbed protein is a consequence of the short lifetime component (50 ps), which provides the major contribution to the average lifetime of the free protein and is absent when the protein adsorbs. For the free protein melting, the average lifetime increase is mainly due to the disappearance of the short lifetime component, while for the adsorbed cutinase, the slight lifetime reduction is caused by the increase of dynamic quenching.

The increase of the fluorescence intensity (>3 times) upon temperature melting is slightly higher than the increase in the average lifetime (~2 times) for the free protein, while for the adsorbed protein, the fluorescence intensity similarly increases by about 3 times, whereas the average lifetime decreases. Differences in the variation of the fluorescence intensities and the corresponding amplitude-weighted average lifetimes are due to static quenching from charge-transfer ground-state complexes between Trp and the disulfide bridge.[20] The small influence of static quenching upon melting of the free protein is a consequence of the small percentage of Trp residues accessible to form charge-transfer complexes because the majority of them are hydrogen bonded to alanine. The hydrogen bond is highly directional, which prevents the hydrogen-bonded Trp residues from being involved in charge-transfer complexes with the disulfide bridge. On the contrary, the strong static quenching observed for the adsorbed cutinase is allowed by the disruption of the Trp−Ala hydrogen bonding upon adsorption, turning the Trp residues accessible to make charge-transfer complexes with the disulfide bridge.

The fluorescence results (fluorescence spectra, solvatochromic shifts, fluorescence decay curves) clearly show that cutinase adsorbs onto the PMMA surface by the region where the tryptophan is located, irrespective of pH.

4. Conclusions

The fluorescence of the single Trp of cutinase shows that cutinase adsorbs onto the surface of PMMA particles in a one-side orientation by the Trp region. This correlates well with the retention of the enzyme activity upon adsorption at basic pH. The decrease of the enzymatic activity upon adsorption at

a pH of 4.5 is not due to a different protein orientation but simply to the loss of secondary structure induced by the interaction with the support. In conclusion, irrespective of pH, cutinase adsorbs onto the surface of PMMA particles by the high hydrophobic Trp region of cutinase, in accordance with the relevance of the hydrophobic interactions for protein adsorption.

Acknowledgment. Centro de Química-Física Molecular (CQFM) and IN-Instituto de Nanociências e Nanotecnologias acknowledge the finantial support from Fundação para a Ciência e a Tecnologia (FCT) Project POCTI/2000/QUI/33866. Andrea M. Santos thanks FCT for the Ph.D. Grant SFRH/BD/1227/2000.

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