Fluorescence of the Single Tryptophan of Cutinase: Temperature and pH Effect on Protein Conformation and Dynamics

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ABSTRACT

The cutinase from Fusarium solani pisi is an enzyme with a single \( \lambda \)-tryptophan (Trp) involved in a hydrogen bond with an alanine (Ala) residue and located close to a cysteine formed by a disulfide bridge between two cysteine (Cys) residues. The Cys strongly quenches the fluorescence of Trp by both static and dynamic quenching mechanisms. The Trp fluorescence intensity increases by about fourfold on protein melting because of the disruption of the Ala–Trp hydrogen bond that releases the Trp from the vicinity of the cysteine residue. The Trp forms charge-transfer complexes with the disulfide bridge, which is disrupted by UV light irradiation of the protein. This results in a 10-fold increase of the Trp fluorescence quantum yield because of the suppression of the static quenching by the cysteine residue. The Trp fluorescence anisotropy decays are similar to those in other proteins and were interpreted in terms of the wobbling-in-cone model. The long relaxation time is attributed to the Brownian rotational correlation time of the protein as a whole below the protein-melting temperature and to protein-backbone dynamics above it. The short relaxation time is related to the local motion of the Trp, whose mobility increases on protein denaturation.

INTRODUCTION

The intrinsic fluorescence of proteins may provide important information on its structure, conformation and dynamics. These properties are relevant for the full understanding of protein activity. Fusarium solani pisi cutinase belongs to a group of homolog enzymes capable of degrading cutin, the insoluble lipid–polyester matrix covering the surface of plants (1). The relevant applications of this enzyme emerge from its lypoLytic activity because it degrades both monomeric and aggregated triglycerides.

The crystal structure of F. solani pisi has been determined to 1.0 Å resolution (2,3). It is an \( \alpha/\beta \) hydrolase with a core constituted by a central \( \beta \)-sheet of five parallel strands covered by four \( \alpha \)-helices. The catalytic center is composed of the triad serine 120, histidine 188 and aspartate 175 and an oxygen hole (4). In the opposite side of the active center there is a single \( \lambda \)-tryptophan (Trp) residue, located in the \( \beta \)-sheet, close to one of the two disulfide bridges of this esterase. The \( ^{14} \)N relaxation nuclear magnetic resonance (NMR) data show that the \( \beta \)-sheet has very low mobility in both the picosecond and the nanosecond timescales (5). The Trp located in the \( \beta \)-strand of residues 68–79 forms a hydrogen bond with alanine 32 (\( ^{13} \)Ala) located in the loop of residues 27–33, assuring the stability of the N-terminal side of the \( \beta \)-sheet by holding these two strands.

On UV light irradiation in the absorption band of Trp, a 10-fold increase in the fluorescence quantum yield of Trp was observed, which was attributed to the breakdown of the disulfide bridge between the cysteine (Cys) residues, \( ^{13} \)Cys and \( ^{13} \)Cys (6,7). Cutinase is a well-known example where the photoreduction of a disulfide bridge occurs by electronic excitation of Trp. Despite the importance of this phenomenon on the inactivation of enzymes and hormones, very few cases were studied until now (8,9).

This article describes both the fluorescence spectra, fluorescence decay curves and fluorescence anisotropy decays of the single Trp of cutinase at different pH values and several temperatures, below and above the protein-melting temperature. The Trp fluorescence spectrum is redshifted and sharply increases in intensity (four-fold) on protein melting. The Trp fluorescence decay curves at room temperature can be fitted only with a sum of four exponentials, with lifetimes around 0.05–0.06, 0.3–0.5, 2–3 ns and 6–7 ns, slightly dependent on pH. After protein melting (T = 50°C), the shortest component of the decay (50–60 ps) disappears because of the suppression of a strong quenching by the cysteine caused by the proximity of the hydrogen-bonded Trp to this residue. On protein melting, the Trp is released from the vicinity of the cystine residue because of the break of the hydrogen bond.

The Trp fluorescence quantum yield increases by an order of magnitude by UV light irradiation of the protein because of the suppression of the static quenching of Trp by the disulfide bridge. The disulfide bridge forms ground-state charge-transfer complexes with Trp, and it is disrupted by a complex mechanism involving the transfer of an electron from the excited Trp to the cystine residue.

The anisotropy decays of Trp below and above protein-melting temperature are well described by a sum of two exponentials and were interpreted in terms of the wobbling-in-cone model (10,11). At room temperature the long relaxation time of the anisotropy decay is associated with a Brownian rotational correlation time of the protein as a whole, whereas the short lifetime (0.5 ns) is...
due to the local motion of the Trp. After protein denaturation the short lifetime decreases (0.3-0.4 ns) because of the rise of Trp mobility, whereas the long lifetime (~7 ns) is assigned to the dynamics of the protein backbone.

MATERIALS AND METHODS

Materials. The F. solani pisi cutinase (M = 22.5 kDa) was produced and purified following methods described elsewhere (12). Buffer aqueous solutions of cutinase (0.2 × 10^{-3} - 1.0 × 10^{-3} M) were prepared covering the pH range between 4 and 8 using 0.01 M acetate (pH 4) or phosphate (pH 6 and 8) buffers.

Fluorescence. Fluorescence spectra were recorded in a Spex Fluorolog 112 spectrofluorimeter using right-angle geometry. The excitation was at 300 nm (9 nm bandwidth) and the fluorescence recorded from 310 to 500 nm (9 nm bandwidth). A cutoff filter (λcut > 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 homebuilt thermostat, and the solution was stirred during the measurements. For each spectrum, a freshly prepared solution was used.

Decay curves. The fluorescence decay curves were obtained by the single-photon timing technique using a 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 (13). The excitation light beam was expanded before it entered the sample solution, and neutral density filters were used to minimize the photobleaching 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 vertical polarized light for excitation and selecting the fluorescence at the magic angle (54.7°). A global analysis of the decay curves obtained with two time spans was performed by a convolution procedure using a nonlinear least squares method based on the Marquardt algorithm (14). The decay curves were fit with a sum of exponentials and a scattering component that considers both the scattering light and the very short components that cannot be resolved by our single-photon timing equipment. The anisotropy decay curves were constructed from the I_{VR}(t), I_{VP}(T) fluorescence decays obtained with vertical polarized excitation light and selecting the vertical (I_{VV}) or horizontal (I_{HH}) components of the fluorescence

\[ r(t) = \frac{I_{VR}(t) - G I_{HH}(t)}{I_{VV}(t) + 2GI_{HH}(t)}. \]

where G = I_{VR}/I_{HH} is an experimental correction factor that considers the artifacts introduced by the detecting system on the polarized fluorescence light components. For our experimental setup G = 1.0 because the fluorescence after being selected with an appropriate polarizer was depolarized before the entrance slit of the monochromator.

RESULTS

Fluorescence spectra

Figure 1 shows the Trp fluorescence spectra normalized to 1.0 at the wavelength of the maximum intensity of a cutinase solution (1.0 × 10^{-3} M, pH 4) by selective excitation at 300 nm at different temperatures.

The spectra were measured with a fresh solution at each temperature to minimize the variation of fluorescence intensity with irradiation time. The Trp fluorescence wavelength maximum is around 332 nm below 40°C, increasing to 338 nm for temperatures higher than 50°C. According to Burstein (15) this indicates that Trp is buried within the protein but still exposed to water below 40°C, increasing the exposure to the solvent for higher temperatures. The shape of the Trp fluorescence spectra changes slightly, and the wavelengths of maximum intensity shifts to the red end by excitation from 295 to 302 nm, which was attributed to ground-state heterogeneity (see next section). The intensities in the maximum of the fluorescence spectra plotted as an insert are practically constant between 20°C and 40°C, increasing sharply between 40°C and 50°C, to decrease afterward. Both the solvatochromic shifts and the fluorescence intensities display a break between 40°C and 50°C, which was identified as the protein-melting temperature (16). At pH 8, the fluorescence spectra (Fig. 2) are slightly shifted to the red at all temperatures, and the melting occurs at higher temperatures (~55°C), indicating that cutinase is more stable at higher pH values (12).

The Trp fluorescence quantum yield of cutinase is around 0.01 (17). The low quantum yield is mainly a result of Trp fluorescence quenching by the disulfide bridge between the Cys. 31Cys and 69Cys. The increase of the fluorescence quantum yield on protein melting is because of the disappearance of both static and dynamic quenching processes, as was confirmed by decay curve analysis (see next section). After protein melting, the Trp fluorescence intensity decreases with temperature as a result of the increase of both the intrinsic nonradiative decay processes and the dynamic quenching.

Fluorescence decay curves

The Trp quantum yield of fresh solutions of cutinase at room temperature is very low, and the decay curves are complex. To obtain reliable results, for each sample two decays with different time-span ranges were obtained. Figure 3 shows the decay curve of a cutinase solution (2.0 × 10^{-6} M, pH 8) at 80°C, recorded with a timescale of 11 ps per channel (longer time range) in a multi-channel analyzer working with 1024 channels.
This decay was analyzed simultaneously with another one (timescale of 3 ps per channel), with a sum of three exponentials plus a scattering component. The lifetimes were linked in both decays. The global fit is good ($\chi^2 = 1.1$), with common lifetimes $\tau_1 = 0.37$ ns, $\tau_2 = 1.1$ ns and $\tau_3 = 5.0$ ns and preexponential factors, $a_1 = 0.43$, $a_2 = 0.54$ and $a_3 = 0.032$ for the shorter time range and $a_1 = 0.44$, $a_2 = 0.53$ and $a_3 = 0.031$ for the longer time range. The scattering component has contributions from light scattering and a fast microscopic solvent dielectric relaxation that is not resolved by our single-photon timing equipment. Fluorescence heterogeneity of Trp in proteins may have several origins, such as Trp rotational isomers (rotamers), multiple protein conformations, protein dynamics, quenching by nearby groups and solvent relaxation as pointed out by Lakowicz in a recent review (18). The rotational isomers of Trp are generally invoked to explain Trp fluorescence in proteins (19,20), but this explanation has been questioned because the model compound of Trp in proteins, N-acetyl-l-tryptophanamide, has a single exponential decay in water (18). However, ground-state heterogeneity can still persist because of multiple protein conformations for which the Trp fluorescence can be quenched diversely by the peptide bond (21), other amino acids and disulfide bridges in its surroundings (22). The complexity of Trp fluorescence was also attributed to a nonfluorescent state (dark state) (23,24) associated with a charge-transfer complex between the excited Trp and a neighboring quenching side chain residue. This is rather speculative because the “dark” state was never detected.

The spectral relaxation is also a reasonable cause for the complexity of the decays because on Trp electronic excitation in the $^1L_u$ state, the dipole moment increases substantially (25,26). The dynamic spectral shifts involved in solvent relaxation add a fast-decay component to the fluorescence decay curves measured in the blue side of the fluorescence spectrum and a rise time to decays obtained at the red edge of the spectrum. For Trp in water, the solvent relaxation is very fast, occurring within 2 ps after Trp electronic excitation (26). The increase of solvent viscosity slows down the process: e.g., the indole relaxation in glycerol at room temperature occurs in the nanosecond time range (27). For many proteins in water, solvent relaxation is a fast process occurring in the picosecond time range (26,28,29). Therefore, solvation dynamics does not explain the Trp lifetime components in the nanosecond range, which for single Trp proteins are probably produced by multiple protein conformations and fluorescence quenching.

Figure 4 shows the decay recovered with a long timescale (22 ps per channel) of a dilute cutinase solution at pH 8 and 22°C by excitation with light of 300 nm, the fluorescence being recovered at 354 nm.

The decay was fitted simultaneously with another one recorded with a smaller timescale (3 ps per channel). The lifetimes were linked in both decays. The fit begins with a sum of three exponentials plus a scattering component. The fitting results were not acceptable (see the residuals in Fig. 4), being an adequate fit achieved only by adding an extra exponential with a very short lifetime (–0.05 ps). This very short component was already observed by other authors (6,9). Table 1 summarizes the lifetimes and the normalized preexponential factors (long timescale decay) for all temperatures at pH 4 and 8.
The lifetimes remain invariant for temperatures below ~50°C, which supports the fact that Trp fluorescence heterogeneity results from ground-state microstates. Indeed, if Trp fluorescence heterogeneity resulted from the spectral relaxation, the Trp lifetimes should decrease with temperature increase. This was corroborated by the broadening of the time-resolved fluorescence spectra when this was acquired at longer times after Trp excitation. For temperatures higher than the protein-melting temperature (T > 50°C), the short lifetime component disappears, whereas the others decrease with temperature increase. It was observed using 15N NMR that 32Ala at room temperature (when the protein is folded) forms an hydrogen bond with the π electrons of the aromatic ring of Trp. On increasing the temperature, this hydrogen bond breaks, suggesting that the short lifetime is associated with the Ala–Trp complex. It is known that in immunophilins (30), homeodomain (31) and human interleukin-2 (32) proteins, the NH of Trp participates in a hydrogen bond with the π electrons of an aromatic residue in proximity. The hydrogen bond can explain the quenching of Trp fluorescence by a mechanism that involves the dissipation of electronic energy as vibrational energy through the hydrogen bond, in a way similar to the quenching of pyrrole-containing heteroaromatics by pyridine and derivatives (33) and the quenching of aminoanthraquinones by alcohols and hydroperoxides (34). In our case, such a mechanism is not operative because the hydrogen bond does not involve any atom of Trp. Therefore, the most plausible explanation lies in the fact that the hydrogen-bonded Trp are forced to be closer to the cystine residue, which in turn quenches the Trp fluorescence very efficiently. This is consistent with the decrease in the weight of the short component to the decay with temperature increase (see a4 in Table 1).

The population of fluorescent Trp residues hydrogen bonded to Ala can be estimated from the ratio of the pre-exponential factor of this component to the sum of all preexponential factors (excluding the scattering component) as long as the Trp radiative rate constant is maintained for all the Trp. The Trp residues forming charge-transfer complexes are not included in the above calculation because their fluorescence is instantaneously quenched. The fraction of fluorescent hydrogen-bonded Trp residues is very high (0.8 at 22°C) before protein melting and reduces to zero after melting, at which time the very short lifetime (~0.05 ns) disappears. This lifetime is linked to the Trp quenching by the disulfide bridge, but its disappearance does not explain the fourfold increase in the fluorescence quantum yield on protein melting (see Figs. 1 and 2). Indeed, the pre-exponential factors weighted average lifetime, $\tau = \sum a_i \tau_i / \sum a_i$, increases only by about three-fold.

<table>
<thead>
<tr>
<th>pH 8</th>
<th>a1</th>
<th>τ1 (ns)</th>
<th>a2</th>
<th>τ2 (ns)</th>
<th>a3</th>
<th>τ3 (ns)</th>
<th>a4</th>
<th>τ4 (ns)</th>
<th>1</th>
<th>2</th>
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<td>0.07</td>
<td>0.4 (2)</td>
<td>0.07</td>
<td>2.5</td>
<td>0.05</td>
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<td>0.9</td>
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<td>0.07</td>
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<td>0.05</td>
<td>0.09</td>
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<td>2.7</td>
<td>0.05</td>
<td>7.8</td>
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<td>0.16</td>
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<td>0.04</td>
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<td>0.7</td>
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<td>0.30</td>
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<td>0.59</td>
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<td>80</td>
<td>—</td>
<td>—</td>
<td>0.44</td>
<td>0.3 (7)</td>
<td>0.53</td>
<td>1.1</td>
<td>0.03</td>
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<th>a2</th>
<th>τ2 (ns)</th>
<th>a3</th>
<th>τ3 (ns)</th>
<th>a4</th>
<th>τ4 (ns)</th>
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<th>2</th>
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<td>0.82</td>
<td>0.05</td>
<td>0.05 (8)</td>
<td>0.3 (3)</td>
<td>0.04</td>
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<td>0.056</td>
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<tr>
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<td>0.12</td>
<td>0.07</td>
<td>0.32</td>
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<td>0.38</td>
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<td>0.18</td>
<td>5.0</td>
<td>1.9</td>
<td>1.1</td>
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<tr>
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<td>—</td>
<td>—</td>
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<td>1.4</td>
<td>0.19</td>
<td>3.5</td>
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<td>—</td>
<td>0.34</td>
<td>0.3 (9)</td>
<td>0.56</td>
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<tr>
<td>80</td>
<td>—</td>
<td>—</td>
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<td>0.3 (1)</td>
<td>0.37</td>
<td>0.89</td>
<td>0.07</td>
<td>2.5</td>
<td>0.7</td>
<td>1.1</td>
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Figure 5. Trp lifetimes obtained from the fit of the decays curves at several temperatures for a solution of cutinase \(2.0 \times 10^{-6} \, M\), pH 4. The quenching rate constant, \(k_q\), after protein denaturation was calculated using Eq. 2.

Figure 6. Variation of the Trp fluorescence intensity (\(\lambda_{ex} = 345\) nm) of a cutinase solution \(1.0 \times 10^{-5} \, M\), pH 4 under irradiation with UV light of 300 nm.

(see Table 1) on melting, which indicates that a static quenching mechanism attributable to the Trp-disulfide bridge complexes should also be involved. The hydrogen bond with Ala is highly directional, which probably impairs the approach of the hydrogen-bonded Trp to the \(^3\)Cys residue to form charge-transfer complexes. This is certainly true because \(^3\)Cys is connected with \(^3\)Ala, and the bonding of Trp to one residue excludes the bonding to the other. According to this reasoning, Trp in cutinase are divided into three sets: Trp hydrogen bonded to Ala, Trp forming charge-transfer complexes with cystine and the remaining free Trp. The Trp fluorescence quantum yield is due to the free and the hydrogen-bonded Trp. The very low quantum yield of Trp in cutinase (~0.01), compared with the quantum yield of Trp in water (0.14) (22), is consistent with the negligible contribution of the hydrogen-bonded Trp to the quantum yield and the substantial fraction of Trp forming charge-transfer complexes with the disulfide bridge. The stability of the N-terminal side of the \(\beta\)-sheet is assured by both the hydrogen-bonded Trp and the ones forming charge-transfer complexes with the cystine residue. Because the disulfide bridge remains intact after protein melting, the Ala–Trp hydrogen bond break is probably the main reason for the denaturation of cutinase by temperature increase. To explain the time-resolved and steady-state fluorescence results, it is necessary to assume that on cutinase melting the Ala–Trp hydrogen bond breaks, which releases the Trp from the vicinity of the cystine residue and induces the disruption of some Trp–disulfide bridge complexes.

Figure 5 shows the Trp lifetimes vs temperature for a cutinase solution at pH 4.

The lifetimes before protein denaturation (22–50°C) are practically constant, reflecting not only the invariance of the Trp deactivation processes in this temperature interval but also the low mobility of the \(\beta\)-sheet, which lead to similar dynamic quenching processes. After denaturation, the short lifetime remains invariant within experimental error, whereas the other two lifetimes decrease with temperature. This is because of the dynamic quenching of Trp by a quencher, whose rate constant can be calculated by the Stern–Volmer equation (35)

\[
\frac{\tau_i}{\tau_i^0} = 1 + k_q \tau_i,
\]

where \(\tau_i\) is the lifetime of Trp in the microstate \(i\) in the plateau (before protein melting) and \(\tau_i^0\) their lifetime after protein denaturation. The values calculated using Eq. 2 are shown in Fig. 5 for a cutinase solution at pH 4. The quenching constants cannot be calculated for the short lifetime component because their expected variation is within experimental error. The quenching mechanism can be decomposed in two steps: the approach of Trp to the quencher and the quenching reaction on the encounter. For strong quenchers, the global process is controlled by the dynamic step, and the rate constant depends on both the separation between the Trp and the quencher and on the dynamics of the protein backbone in the solvent. The side chain amino acid residues located within 10 Å from Trp that quench its fluorescence are aspartate, glutamate, lysine, asparagine, glutamine and the disulfide bridge. The disulfide bridge is the most effective quencher as shown by Chen and Burkle (22), using model compounds that mimic the quenchers of Trp in proteins. The Trp quenching rate constants are high \((k_q = 0.15 \, \text{ns}^{-1} \, \text{and} \, 0.26 \, \text{ns}^{-1})\), indicating that the Trp is in the vicinity of the disulfide bridge or that the backbone dynamics is fast. The differences in the quenching rate constants suggest that the Trp–disulfide bridge separation changes with protein conformation.

**Protein irradiation by UV light**

Figure 6 shows the variation of the fluorescence intensity (at 345 nm) with irradiation time for a cutinase solution \(1.0 \times 10^{-5} \, M\), pH 4) stirred and irradiated by 300 nm (9 nm bandwidth) light from a 150 W xenon lamp.
Table 2. Tryptophan fluorescence decay times ($\tau_i$), pre-exponential factors (long timescale decay) ($a_i$), average lifetime ($\tau$) and $\chi^2$ at pH 8 and 4 of an irradiated cutinase solution, $2.0 \times 10^{-6} \text{ M}$ at 40°C. The excitation wavelength is 300 nm, and the fluorescence was observed at 354 nm.

<table>
<thead>
<tr>
<th>pH</th>
<th>$a_1$</th>
<th>$\tau_1$ (ns)</th>
<th>$a_2$</th>
<th>$\tau_2$ (ns)</th>
<th>$a_3$</th>
<th>$\tau_3$ (ns)</th>
<th>$a_4$</th>
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<td>0.11</td>
<td>0.5 (6)</td>
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<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.39</td>
<td>0.05</td>
<td>0.19</td>
<td>0.5 (5)</td>
<td>0.31</td>
<td>2.5</td>
<td>0.12</td>
<td>6.0</td>
<td>1.6</td>
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</table>

The fluorescence intensity increases by more than 10-fold with irradiation, after which it reaches a plateau and then decreases afterward. The fluorescence maximum is reached at shorter times by increasing the power of the excitation light. The fluorescence decay curves measured after irradiation (in the plateau region of Fig. 6) are identical for several pH values between 4 and 8 at a given temperature. Table 2 summarizes the lifetimes and preexponential factors for irradiated samples at 40°C at both pH 4 and 8.

From these decays we can conclude that irradiation does not change the lifetimes significantly but influences the pre-exponential factors. The pre-exponential factor $a_1$ is the shortest lifetime component decreases with the disruption of the disulfide bridge, reducing the number of hydrogen-bonded Trp in the vicinity of the quencher. On the other hand, because the fluorescence intensity increases more that 10-fold and the average lifetime increases by about three-fold (see Tables 1 and 2), we must conclude that the quenching is mainly static. This results from the Trp residues involved in charge-transfer complexes with the disulfide bridge. On Trp electronic excitation, we expect a mechanism similar to the one proposed by Grossweiner and Usui (36,37) for the photolysis of indole or Trp in the presence of cystine. A radical anion of cystine is formed by the capture of the electron generated by the electronic excitation of the Trp, followed by the dissociation of the cystine radical anion, to give a thiolate ion and the thyl radical. In a new step, the thiolate ion abstracts a hydrogen from the cation radical of Trp to give the free thiol in solution, whereas the thyl radical can initiate the sulfinylation of Trp (8). This was supported by the work of Prompers et al. (7), in which thiol groups in the irradiated solution were detected and which in addition shows that the Ala–Trp hydrogen bond remains intact. The Trp released by disruption of the disulfide bridge have fluorescence decays identical to the free Trp before irradiation because the thiol group has dynamic fluorescence-quenching properties similar to those of the disulfide bridge. In fact, both quenchers suppress the indole fluorescence by a diffusion-controlled mechanism (22).

### Fluorescence polarization

Figure 7 shows the Trp fluorescence anisotropy decay of a cutinase solution ($2.0 \times 10^{-6} \text{ M}$, pH 6) at 22°C by excitation at 300 nm. The anisotropy decays are practically invariant in the pH range between 4 and 8.

The anisotropy at time zero is around 0.25, which is the typical value for Trp in solution and in proteins at this excitation wavelength (38,39). By excitation of the Trp at the red edge of the absorption spectrum ($\lambda > 300 \text{ nm}$), it is mainly the $^1L_6$ state that is populated. As the excitation and emission states coincide, a theoretical value of $r(0) = 0.4$ (collinear transition dipole moments for the absorption and emission processes) is expected. The lower value found can be in part attributed to mixed polarization because even by excitation at 300 nm, a small fraction of light can populate the $^1L_6$ state and the transition dipole moments to the $^1L_3$ and $^1L_4$ states are perpendicular (40). Another possibility results from the large variation of the permanent dipole moment (25,41) on electronic excitation to the $^1L_4$ state that induces fluctuations in the local fields. This perturbs the excited state, leading to different orientations of the transition dipole moments for absorption and emission.

The fluorescence anisotropy decays were analyzed by a unidimensional model, which correlates all the lifetimes with all the rotational correlation times. The anisotropy decays were fitted with a sum of two exponentials,

$$r(t) = \beta_1 \exp(-t/\theta_1) + \beta_2 \exp(-t/\theta_2), \quad (3)$$

where $\theta_{1,2}$ are the two correlation times and the preexponential factors, $\beta_{1,2}$, take into account the contributions of the two components to the decay. Table 3 summarizes the values obtained at pH 6 and several temperatures.

The values of $r(0)$ after protein denaturation are slightly lower than before, which can indicate that a very fast relaxation process occurs within the time resolution of our experimental setup. Different dynamic models have been used to interpret the intrinsic Trp fluorescence anisotropy behavior in proteins (10,42–44). Probably, the most appropriate model is the wobbling-in-cone one in which the indole ring is subjected to a fast rotational motion that decays exponentially with relaxation time $\theta_\text{r}$ and reaches a plateau. In this case, the anisotropy is given by (10)

$$r(t) = r(0) \times [(1-P_x) \exp(-t/\theta_\text{r}) + P_x \exp(-t/\theta_\text{a})], \quad (4)$$

where $\theta_\text{a}$ is the Brownian correlation time of the protein and $r(0)$ is the anisotropy at the initial time. Considering that the rotational correlation time of the proteins is much larger than the local
Table 3. Tyroshpophylfluorescence anisotropy correlation times (θᵢ), pre-exponential factors (βᵢ), anisotropy at zero time (r₀) and cone semiangle (wᵦₘₜ) at several temperatures of fresh prepared cutinase solutions (2.5 × 10⁻⁷ M, pH 6). The excitation wavelength is 300 nm, and the fluorescence was observed at 354 nm.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>β₁</th>
<th>θ₁ (ns)</th>
<th>β₂</th>
<th>θ₂ (ns)</th>
<th>rₐ</th>
<th>wₘₜ (°)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>0.21</td>
<td>14.1</td>
<td>0.05</td>
<td>0.54</td>
<td>0.26</td>
<td>21</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>0.18</td>
<td>12.2</td>
<td>0.06</td>
<td>0.53</td>
<td>0.24</td>
<td>25</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>0.21</td>
<td>12.0</td>
<td>0.04</td>
<td>0.35</td>
<td>0.25</td>
<td>20</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>0.15</td>
<td>6.8</td>
<td>0.06</td>
<td>0.35</td>
<td>0.21</td>
<td>26</td>
<td>1.1</td>
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<tr>
<td>70</td>
<td>0.12</td>
<td>8.6</td>
<td>0.07</td>
<td>0.41</td>
<td>0.19</td>
<td>30</td>
<td>1.1</td>
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<tr>
<td>80</td>
<td>0.16</td>
<td>6.8</td>
<td>0.07</td>
<td>0.31</td>
<td>0.23</td>
<td>27</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The long correlation time is given by

\[ θₘₜ = \eta V RT, \]

where η is the solvent viscosity, V the molar volume of the protein, T the absolute temperature and R the ideal gas constant. From the correlation times before protein denaturation, a value of the hydrodynamic radius of 27 ± 2 Å was obtained. A similar hydrodynamic radius (Rₕ = 30 ± 4 Å) was measured at room temperature by dynamic light scattering. This confirms that the long correlation time is associated with the rotation of the protein as a whole. After protein denaturation, the long correlation time should be associated with the dynamics of the protein backbone and not with the rotational motion of the protein. The correlation times for the denatured cutinase corrected to room temperature using the Stokes–Einstein equation are ∼15 ns. These values are identical to the corresponding ones calculated for other proteins (45), although lower than the values found for Trp in synthetic polypeptide chains (46). The short correlation time is associated with the restricted diffusion motion of the indole ring. When the Trp fluorescence results from ground-state heterogeneity, this correlation time should be considered an average value over all the cutinase microstates. After denaturation, the short correlation lifetime decreases and the wobbling-in-cone angle increases, reflecting the mobility increase of Trp at higher temperatures.

CONCLUSIONS

The fluorescence of the single Trp of cutinase was studied at different temperatures below and above protein melting at pH 4 and 8. The melting temperature obtained from the fluorescence spectral shifts and intensities is higher at pH 8, indicating that cutinase is more stable at higher pH. The fluorescence decays below the melting temperature can be fitted only with a sum of four exponentials with a very fast decay component (0.05 ns) attributed to the Trp fluorescence quenching by a disulfide bridge in its vicinity. Above the melting temperature, the shortest lifetime component disappears, possibly because of the break of the Ala–Trp hydrogen bond that keeps the Trp in the vicinity of the disulfide bridge. The fourfold increase in fluorescence intensity observed after protein melting is not completely explained by the increase in the average lifetime, which means that a static quenching component exists, probably from the Trp–cystine complexes.

By irradiation with UV light at room temperature, the disulfide bridge breaks, whereas the short lifetime component of the Trp decay remains, suggesting that irradiation preserves the Ala–Trp hydrogen bond. The 10-fold increase in Trp fluorescence quantum yield is mainly due to the suppression of the static quenching from the Trp–cystine complexes.

The Trp fluorescence anisotropy decays can be fitted with a sum of two exponentials above and below protein denaturation. The short component results from the local motion of the Trp. The longer one is due to protein rotation below protein melting and to the backbone protein dynamics above it.

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