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Photophysics of the single tryptophan residue in *Fusarium solani* Cutinase: Evidence for the occurrence of conformational substates with unusual fluorescence behaviour

Petra C.M. Weisenborn [a,*], Hans Meder [a], Maarten R. Egmond [a], Ton J.W.G. Visser [b], Arie van Hoek [b]

[a] *Unilever Research Laboratorium, Olivier van Noortlaan 120, 3133 AT Vlaardingen, Netherlands*
[b] *Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, Netherlands*

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Abstract

The single tryptophan residue, at position 69 in the amino acid sequence, was used as an intrinsic probe to obtain structural and dynamical information on the lipolytic enzyme *Fusarium solani* cutinase. In the enzyme's native state the tryptophan fluorescence is highly quenched. Time-resolved experiments reveal that the majority of the excited state species is characterized by an unusually fast decay time of approximately 40 ps, indicating the occurrence of a very efficient nonradiative relaxation process, possibly via the adjacent disulphide bond or via the peptide bonds of a nearby loop. A minority of the excited state species relaxes on a nanosecond time scale. Irradiation of the enzyme in the tryptophan absorption band causes an increase by an order of magnitude of the fluorescence quantum yield. This increase is ascribed to a photo-induced, subtle structural change of a minor subset of species whose fluorescence is not highly quenched. The structural change is accompanied by a tightening of the local environment of the tryptophan moiety, as indicated by results from time-resolved fluorescence anisotropy which reveal a complete disappearance of the segmental flexibility of the tryptophan moiety.

Keywords: Cutinase; Lipase; Fluorescence; Conformation

1. Introduction

Lipolytic enzymes catalyze the hydrolysis of fatty esters such as triacylglycerols. Their activity on triacylglycerols is often greatly enhanced at lipid–water interfaces. One of the industrial applications of lipolytic enzymes involves their addition to detergent products for fabric washing in order to improve the product's effectiveness in the removal of fat stains. It is realized, however, that the conditions in a typical detergent solution (such as pH, ionic strength, surfactant micelles) generally do not provide an optimal environment for an enzyme to be fully active or even to retain its native conformation. Much research is therefore devoted to fully understand the effects of

* Corresponding author.

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detergents on the structural and functional properties of lipolytic enzymes.

One of the lipolytic enzymes presently studied is the recombinant cutinase from *Fusarium solani pisi*, expressed in *Escherichia coli*. The native enzyme consists of 199 amino acids. The cutinase reported about here, contains on its N terminus an additional peptide chain consisting of 15 amino acids. From X-ray diffraction of cutinase it was concluded that this peptide does not adopt a specific structure [1]. On the C terminus two additional residues with respect to the native enzyme are present. Therefore, the recombinant cutinase consists of 217 amino acids, whose numbering is based on the recombinant sequence. It is a compact one-domain molecule with dimensions of approximately 45 Å × 30 Å × 30 Å [1].

For studying the effects of surface active molecules on the structural properties of *Fusarium solani* cutinase we employed fluorescence spectroscopy, a sensitive tool to study internal protein structure and dynamics. We discovered that the intrinsic single tryptophan residue (Trp-69) exhibits unusual behaviour. For a buffered aqueous solution of the enzyme it was found that the tryptophan fluorescence is extremely weak. Furthermore, the intensity of the tryptophan fluorescence increases significantly upon prolonged irradiation of the solution with 295 nm light, extracted from the mild 250 W Xenon source of the fluorescence spectrometer. We have now studied the fluorescence properties of cutinase in buffer solution in more detail, in order to obtain more insight into the origin and consequences of the observed photo-induced process and to more unambiguously interpret the effects of surface active agents on the enzyme's fluorescence properties. The present paper deals with the photophysics of *Fusarium solani* cutinase in a neat buffer solution.

2. Materials and methods

2.1. Chemicals and enzymes

Fusarium solani cutinase was expressed in *Escherichia coli*. All purification steps were carried out at 4°C. Crude enzyme was liberated from the cells using osmotic shock treatment. This material was bound to SP Sephadex C50, added batchwise at pH 4.8 and eluted with 30 mM Tris buffer pH 8.2 over a glass filter. Subsequently, the enzyme was passed over DEAE cellulose (DE52, Whatmann) in order to remove contaminating material, which is tightly bound whereas the enzyme is eluted under the conditions used. The enzyme was finally purified by HPLC using an HP-Q column (Pharmacia) in 5 mM Tris, 0.1 M EDTA pH 8.2) eluted with a salt gradient (0–0.4 M NaCl). Fractions containing enzyme activity were pooled and dialysed against 1 mM ammonium bicarbonate pH 7.5 and finally lyophilized. The purified enzyme gives a single band in iso-electric focussing yielding an iso-electric point (p*I*) of 6.88. This p*I* was also calculated from the amino acid composition using standard pK_a values for titrating amino acid residues and found to be 6.82. The molecular mass of the enzyme was determined by electrospray mass spectrometry yielding a value of 22212 Da, which agrees with the calculated mass of the enzyme based on the amino acid composition. The specific activity of the pure enzyme is 695 LU/mg and has not been reported before. The observed OD at 280 nm for a 1% solution of the pure enzyme ($OD^{1\%}_{280\ nm} = 6.61$) is in agreement with the value calculated from the content of aromatic residues.

Tris [tris(hydroxymethyl)aminomethane] was of analytical grade from Merck. Buffer solutions were prepared with doubly distilled water.

All experiments were performed in aqueous solutions containing 10 mM Tris and 10 mM NaCl. The pH was adjusted to 9.0.

2.2. Experimental set-up

Steady-state fluorescence measurements were carried out using a Spex Fluoromax spectrofluorimeter equipped with a thermostated cuvette holder which was routinely set at 20°C. Quartz cuvettes (1-cm paths lengths) were used. During measurements the sample solution was continuously stirred with a magnetic stirrer to maintain homogeneity in the solution. The excitation and emission slits were both set at 4 nm.

Time-resolved fluorescence measurements were performed at 20°C using the time-correlated single photon counting method [2,3]. Excitation came from

the frequency-doubled output of a cavity-dumped cw dye laser, synchronously pumped by the frequency-doubled output of a mode-locked cw Nd:YLF laser. The frequency of excitation pulses was 951 kHz, the wavelength 302 nm and the pulse energy in the pJ region. The detected emission of fluorescence was filtered using a WG 335 nm cut-off filter and a Schott 348.8-nm interference filter with a 5.4-nm band pass; the selection of the direction of polarization of detected fluorescence light was by computer-controlled rotation (90°) of a Polaroid HNP'B filter. The fluorescence and anisotropy decays were obtained via a repeated cycle in which the parallel and perpendicularly polarized fluorescence intensities, with respect to the direction of polarization of the excitation light, are measured during 10 s. Background was taken into account by measuring the fluorescence of the solvent in the same way. For deconvolution purposes the fluorescence of a solution of *p*-terphenyl in ethanol (fluorescence lifetime 1.06 ns) was measured before and after measurement of each sample.

2.3. Data analysis

Experimental data were transferred to a Silicon Graphics workstation for data analysis. Analysis of the decay of the fluorescence intensity and of the fluorescence anisotropy was performed using the commercially available maximum entropy method software (Maximum Entropy Data Consultants Ltd., Cambridge, England). The principle and applications of MEM have been described in the literature [3–6].

3. Results and discussion

3.1. Steady-state fluorescence spectroscopy

3.1.1. Fresh solutions of cutinase

Fig. 1 shows the fluorescence spectra of freshly prepared 2.5 μM solutions of cutinase in 10 mM Tris/NaCl buffer pH 9, excited with 280 and 295 nm light respectively. Excitation with 280 nm light results in a spectrum with a maximum at 305 nm which is typical for a tyrosine fluorescence spectrum [7]. Cutinase contains six tyrosine residues. Excitation with 295 nm light, aimed at selective excitation

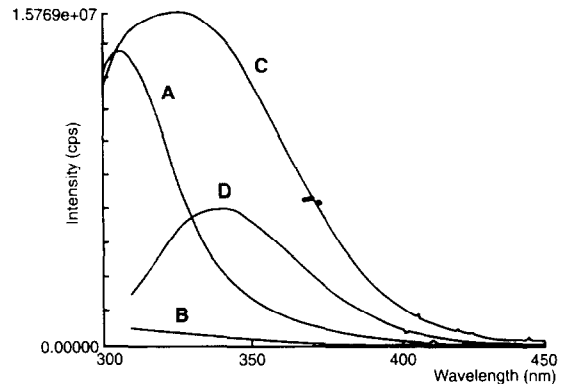

Fig. 1. Fluorescence spectra of 2.5 μM solutions of cutinase in 10 mM Tris/NaCl buffer pH 9. Freshly prepared solutions excited with (A) 280 and (B) 295 nm light, irradiated solutions excited with (C) 280 and (D) 295 nm light.

of the tryptophan residue, results in a spectrum which does not exhibit any characteristic tryptophan features.

The spectra in Fig. 1 are different from the fluorescence spectra obtained under similar conditions for most (native) proteins which contain tyrosine and tryptophan residues; in most cases the tryptophan contribution dominates their fluorescence spectra due to the intrinsically larger fluorescence quantum yield of tryptophan and due to energy transfer from the excited tyrosine residues to the tryptophan moiety. This is considered a general rule [8]. Exceptions to this rule are known but very rare [9].

The extremely weak tryptophan fluorescence spectrum indicates the occurrence of a very efficient nonradiative relaxation process by which the excited singlet state loses its energy. Such efficient nonradiative decay should find its origin in the nature of the microenvironment of the tryptophan residue. The protein structure surrounding Trp-69 and possible mechanisms for the fast radiationless decay will be discussed further on.

The low intensity spectrum shown in Fig. 1A is associated with the native state of the enzyme. When dissolved in a solution containing e.g. the denaturing component dithiothreitol, the degree of quenching is considerably reduced.

3.1.2. Photosensitivity of the tryptophan fluorescence quantum yield

Repeated recordings of the fluorescence spectrum of the same solution of cutinase were found not to

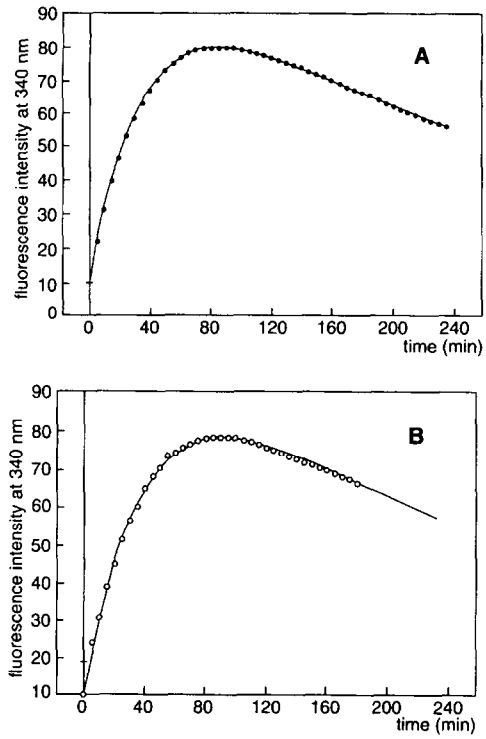

Fig. 2. Intensity of the fluorescence at 340 nm, emitted by a 2.5 μM solution of cutinase solution as a function of time under conditions of constant irradiation in the spectrofluorimeter at (A) 20°C and (B) 40°C. The lines represent the best fit of the biexponential function in Eq. (1) to the experimental points. Fit parameters are: $k_r = 0.026$ min^{-1}, $k_d = 0.0038$ min^{-1} (A) and $k_r = 0.023$ min^{-1} and $k_d = 0.0039$ min^{-1} (B).

coincide. Surprisingly, the fluorescence quantum yield increased with each successive recording of the spectrum. The variation of the fluorescence quantum yield upon continuous irradiation is illustrated in Fig. 2A, showing the time dependence of the fluorescence intensity at 340 nm for a 2.5 μM solution of cutinase. The solution was continuously stirred. The excitation wavelength was set at 295 nm.

From the curve in Fig. 2A it can be concluded that in fact two consecutive photo-induced processes take place. In a first step, non-fluorescing molecules are converted into fluorescing molecules. The fluorescence spectra corresponding to the time when the intensity of the 340 nm emission is at its maximum are given in Fig. 1C and D for 280 and 295 nm excitation, respectively. The broad emission band in Fig. 1D with its maximum at 340 nm is characteristic for a tryptophan fluorescence spectrum. The spectrum in Fig. 1C, resulting from 280 nm excitation, contains both tyrosine and tryptophan emission bands. It may be concluded that the increased fluorescence quantum yield originates from a change in the fluorescence properties of the tryptophan residue resulting from photo-excitation. This observation is in agreement with findings reported by Kolattukudy [10] who suggested that irradiation releases the tryptophan residue from its quenched state by bringing about conformational changes in the protein.

In a second, slower step the fluorescing molecules created in the first step are photo-bleached leading to a decrease in the fluorescence intensity. Photobleaching is a more generally encountered process, observed to a greater or less extent for most tryptophan residues in proteins. The prevailing explanations for a decreasing tryptophan fluorescence yield involve a range of photochemical reactions starting from one of the electronically excited states of tryptophan. These reactions may include e.g. radical reactions following electron ejection or following a reaction with oxygen [11]. The result of these photoreactions is an irreversible conversion of the indole chromophore into a nonfluorescing group.

The curve in Fig. 2A can be described satisfactorily by a biexponential function as given in Eq. (1),

$$IF(t) = A + B\exp(-k_r t) + C\exp(-k_d t) \qquad (1)$$

where $IF(t)$ is the intensity of the fluorescence measured at time t, k_r and k_d the rate constants for the rise and decay of the fluorescence intensity, respectively. A, B and C are constants. The value for k_r yielding the best fit is $26 \cdot 10^{-3}$ min^{-1}. The applicability of Eq. (1) to Fig. 2A indicates that photo-excitation of tryptophan induces the occurrence of two consecutive first order reactions, i.e. taking place within one enzyme molecule. This conclusion is corroborated by the observed independence of the rate of the reactions on the concentration of the enzyme in the solution. It seems likely that the first reaction involves a structural reorganization of the tryptophan residue and its protein environment, leaving the indole chromophore intact, but providing it with a more favourable surrounding for light emission. According to the Arrhenius' theory for reaction rates, the rate constant of a unimolecular

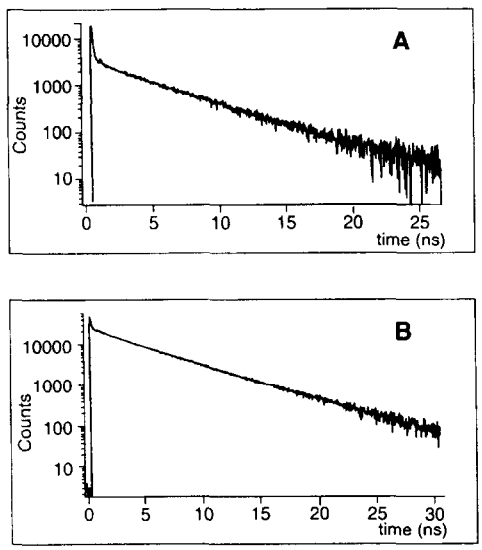

Fig. 3. Decay of the fluorescence intensity after pulse excitation of (A) fresh and (B) irradiated 10 μM solution of cutinase.

reaction depends on the activation energy and on the friction exerted by the surrounding (protein) medium. The latter factor will probably be more important when larger conformational changes take place. Both factors, however, are expected to cause the rate of the reaction to be temperature-dependent. Studying the photo-reaction at a different temperature may thus provide insight into its mechanism. In Fig. 2B a plot of the fluorescence intensity versus time, obtained at 40°C is given. The value of k_r yielding the best fit of Eq. (1) to the curve in Fig. 2B amounts to $23 \cdot 10^{-3}$ min^{-1}. This value differs by only 10% from the value obtained for the curve recorded at 20°C. This difference is negligible in view of the uncertainty in the calculation. It may thus be concluded that the reaction is characterized by an activation energy which is virtually temperature independent. This implies that the activation energy of the reaction, which is the amount of energy necessary to be available for bond breaking and rearrangement, is very small. It may be speculated that in its ground state the tryptophan residue is hydrogen bonded by its indole nitrogen atom with the hydrogen atom of either a water molecule or a part of the protein matrix. The redistribution of electron density over the indole ring in the photo-excited state may cause weakening of this bond causing it to break. This in turn alleviates the restraints posed by the hydrogen bond and allows the tryptophan residue and its environment to relax into an energetically more favourable structure.

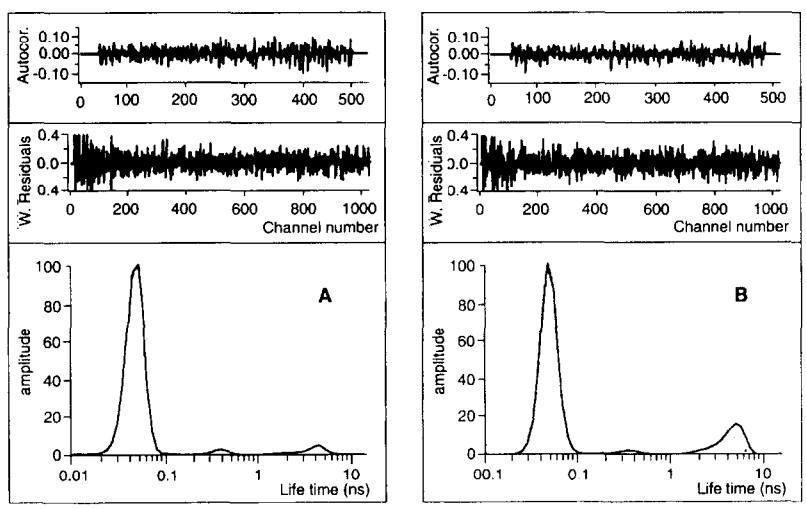

Fig. 4. Lifetime distribution recovered by MEM analysis of the fluorescence decay of (A) fresh and (B) irradiated solution of cutinase. Values obtained for barycenters (relative areas in %) of the peaks are for the fresh solution: 0.048 ns (93%), 0.425 ns (2%), 4.3 ns (5%), and for the irradiated solution: 0.047 ns (81%), 0.345 ns (2%), 4.4 ns (17%). Chi square amounts to 1.63 (A) and 2.27 (B). The top panels show the weighted residuals and autocorrelation of the residuals.

3.2. Time-resolved fluorescence and anisotropy

3.2.1. Fresh solution of cutinase

Fig. 3A shows the time-dependence of the tryptophan fluorescence, detected at 348.8 nm, resulting after 302 nm pulsed excitation of a 10 μM fresh solution of cutinase. Data accumulation was stopped after two cycles instead of the usual ten cycles in order to get a picture of the Trp fluorescence characteristics before prolonged irradiation has contaminated the sample with photo-product. The amplitude versus decay time profile, recovered from this curve by the Maximum Entropy Method (MEM) of analysis is shown in Fig. 4A. Several classes of lifetimes can be discerned. The dominating contribution to the fluorescence is made by the first class of lifetimes, characterized by a decay time of 40 ± 10 ps. This excited state lifetime is extremely short for a tryptophan residue and indicates that the specific surroundings of this residue cause efficient nonradiative relaxation leading to the very small fluorescence quantum yield actually observed.

The presence of two or more lifetime classes in the fluorescence decay of a single tryptophan containing protein may be attributed to the existence of multiple conformational substates [12–16]. The explanation of different lifetimes invokes various degrees of fluorescence quenching in the different conformations. The sensitivity of indole fluorescence to changing environmental conditions is well recognized. Within a protein matrix, intramolecular groups likely to interact with the tryptophan residue can quench the fluorescence. Groups that can quench tryptophan fluorescence include ionic species (e.g. carboxyl groups), disulphide bonds and carbonyl groups of peptide bonds [17]. Examination of the X-ray structure reveals that Trp-69 is shielded from the solvent by a flexible peptide loop (high B factors) extending from residue 25 to 31. Residue 31 is a cysteine residue which forms a disulphide bond with residue 109. The distance in the crystal structure between the indole ring and the disulphide bond is about 5 Å. The high B factors found for the loop covering Trp-69 indicate, however, that this distance may well be different in the solution structure. The proximity of a disulphide bond may explain the highly quenched nature of the majority of the fluorescing species.

The fractional contributions of the peaks in the lifetime distribution in Fig. 4A can be considered proportional to the relative populations of different substates only when exchange between protein conformations is negligible during depopulation of the excited state [17]. The contribution of the short (40

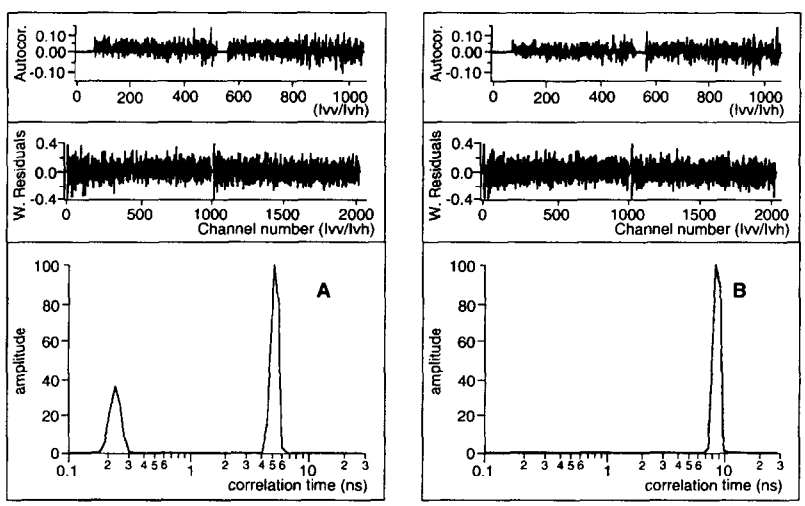

Fig. 5. Amplitude profile of the rotational correlation times of (A) fresh and (B) irradiated solution of cutinase, recovered by MEM analysis of the decay of the fluorescence anisotropy. Values obtained for barycenters (relative areas in %) of the peaks are for the fresh solution: 0.24 ns (33%), 5.5 ns (67%), and for the irradiated solution: 8.9 ns (100%). Chi square amounts to 1.38 (A) and 2.11 (B). The top panels show the weighted residuals and autocorrelation of the residuals.

ps) component to the emission of a fresh solution of cutinase at 20°C is found to amount to 93%; the overall contribution of the longer lived components is limited to only a few %.

The distribution of anisotropy decay times recovered by MEM analysis is given in Fig. 5A. The plot shows two peaks centered at 240 ps and at 5.5 ± 0.5 ns. This indicates that the fluorescence anisotropy decays due to a rapid rotational motion and a slower one. The rapid motion reflects the internal motion or segmental flexibility of the tryptophan residue within the protein matrix. The slower motion may be explained by the global motion of a large part of the enzyme. However, a time constant of 5.5 ns is not fully in agreement with the value expected for the rotational correlation time of a molecule of the same mass as cutinase (21.7 kDa). A calculation of the expected value of the rotational correlation time ϕ uses a modified Stokes–Einstein relation [18,19] as given in Eq. (2),

$$\phi = \frac{V\eta}{kT} = \frac{M(\upsilon + h)\eta}{RT} \qquad (2)$$

in which V is the volume of the spherical protein molecule, η is the viscosity of the solvent, k is the Boltzmann constant, T is the absolute temperature, M is the molecular mass, υ is the partial specific volume, h is the degree of hydration, and R is the gas constant. For a spherical molecule having the same molecular mass as cutinase, at 20°C, a value for ϕ of 8.3 ns is calculated, assuming $\eta = 10^{-3}$ Pa s, $\upsilon = 0.73$ cm^3/g, and $h = 0.2$ cm^3/g. The choice for the values of the parameters υ and h follows the generally adopted practice [18]. Taking into account that the shape of cutinase resembles an ellipsoid rather than a sphere would result in a further increase of the expected rotational correlation time [20]. It thus seems unlikely that the observed slow relaxation time of 5.5 ns is related to the motion of the entire enzyme. It is more likely, as will also turn out from results described in the next sections, that the occurrence of a larger scale segmental motion of a substantial part of the enzyme (floppiness) which contains the Trp residue accounts for the correlation time of 5.5 ns.

The discussion in the previous paragraph about the time-resolved anisotropy data assumes that all excited species have the same rotational properties. It is, however, obvious that during the lifetime of the species characterized by a decay time of 40 ps, the anisotropy decrease which takes place on a nanosecond time scale is negligible. These short lived species therefore cannot account for the observed anisotropy decay times. Only the species with nanosecond fluorescence decay times may be associated with (sub)nanosecond anisotropy decay times.

3.2.2. Photosensitivity of time-resolved fluorescence behaviour

The cutinase solution used to obtain the data on fresh solutions was kept in the sample compartment and irradiated with 302 nm pulses while monitoring the fluorescence intensity. Irradiation was stopped when the increase in signal intensity levelled off, indicating that the first photo-reaction was more or less completed. Then accumulation of 10 cycles of data was started. During data acquisition the signal intensity remained more or less constant. In Fig. 3B the time-dependence of the fluorescence decay is shown. Comparison of the decay curves in Fig. 3A and B reveals that the relative contribution of the fast component to the fluorescence is considerably smaller in the irradiated sample. A quantification of the difference in relative contributions of shorter and longer lived species before and after irradiation of the sample may be derived from calculation of the peak areas in the amplitude versus lifetime distributions, see Fig. 4A and B. Thus it turns out that the contribution of the 40 ps species has decreased from 93 to 81%, that of the 4.4 ns species has increased from 5 to 17%, whereas that of the 300 ps species has remained constant and small. Such a component is always associated with single Trp proteins [21]. A straightforward explanation for this observation involves photo-induced conversion from species in the conformational substate characterized by a fluorescence lifetime of 40 ps to species in the conformational substate characterized by a lifetime of 4.4 ns. This explanation agrees with the observed increase in the fluorescence quantum yield. The persistence of the 4.4 ns lifetime component of the Trp fluorescence after irradiation does not necessarily imply that the Trp is in a unique environment (note that the distribution is broad). In this way the fluorescence anisotropy decay can be explained.

In Fig. 5B the distribution of rotational correlation times of the irradiated cutinase solution is shown. The distribution exhibits only one peak, with a barycentre at 8.9 ns. This indicates that irradiation has resulted in the conversion of flexible tryptophan residues ($\phi = 0.24$ and 5.5 ns) into rather immobilized tryptophans ($\phi = 8.9$ ns). The latter value is in good agreement with the value calculated according to Eq.(2) for the rotational correlation time of a spherical hydrated particle of the same weight as cutinase (8.3 ns). The difference between calculated and observed value may well be explained by the more ellipsoid shape of cutinase [20].

4. Conclusion

Irradiation of cutinase only induces a photo-reaction in the conformational substates characterized by a fluorescence decay time of ca. 4.4 ns. These species upon photo-excitation instantaneously relax into a more favourable and stable conformation, in which they are tightly enclosed by the surrounding protein matrix. The majority of species, however, remains unchanged by photo-excitation because of ultrafast radiationless decay to the ground state, probably due to the vicinal disulphide bond. The activity of the enzyme, which was tested before and after irradiation of a cutinase sample on a monomolecular substrate, turned out to have remained unchanged. This was expected, since the Trp-69 residue is localized far away from the active site of the enzyme.

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