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A spectroscopic analysis of thermal stability of the *Chromobacterium viscosum* lipase

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Abstract

The thermal stability of the lipase from *Chromobacterium viscosum* was assessed by deactivation (loss of activity), fluorescence, circular dichroism (CD) and static light scattering (SLS) measurements. Lipase fluorescence emission is dominated by the tryptophyl contribution. An increase in the tyrosyl contribution from 2 to 16% was only observed upon prolonged incubation at 60°C. The effect of temperature on the tryptophyl quantum yield was studied and two activation energies were calculated. Tryptophan residues in the native structure have an activation energy of 1.9 kcal mol^{-1} for temperature-dependent non-radiative deactivation of the excited state. A structural change occurs at approximately 66.7°C and the activation energy increases to 10.2 kcal mol^{-1}. This structural change is not characterized by tryptophan exposure on the surface of the protein. The deactivation and the evolution of structural changes with time after lipase incubation at 60°C were assessed by fluorescence, CD and SLS measurements. CD spectra show that both secondary and tertiary structures remain native-like after incubation at 60°C in spite of the fluorescence changes observed (red-shift from 330 to 336 nm on the trytophyl emission). SLS measurements together with the CD data show that deactivation may be due to protein association between native molecules. Deactivation and the decrease on the fraction of non-associated native lipase evaluated by changes in fluorescence intensity with time, show apparent first order kinetics. According to the rate constants, fluorescence changes precede deactivation pointing to an underestimation of the deactivation. Reactivation upon dilution during the activity assay and substrate-induced reactivation due to lipase interfacial adsorption are possible causes for this underestimation.

Keywords: Chromobacterium viscosum lipase; Thermal stability; Fluorescence; Circular dichroism; Static light scattering

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1. Introduction

Lipases are capable of catalysing a variety of reactions, many of which have great potential importance in industry. The best known example is the modification of their natural substrates, triacylglycerols, mainly carried out in non-aqueous media [1]. Lipase-catalysed hydrolysis in aqueous media have also industrial relevance in the bioconversion of polyunsaturated fatty acids to other bioactive compounds with desirable nutritional, biomedical or surfactant attributes [2], in the resolution of racemic alcohols by hydrolysis of racemic esters [3] and in the detergent industry. Enhanced stability, particularly at high temperatures, make them more attractive for industry.

Fluorescence methods are being increasingly used in biochemical characterization because of the inherent sensitivity of this technique. Proteins have tryptophan and tyrosine as intrinsic fluorophores, the former being highly sensitive to microenvironment polarity. The protein fluorescence is sensitive to the substrate binding, association reactions and to denaturation. In addition to the fluorescence, other spectroscopic techniques such as circular dichroism and light scattering are very useful techniques to assess structure–function relationships in proteins. In these types of studies some authors have examined the comparison of the conformation and activity changes during the time course of enzyme denaturation. Tsou and co-workers [4,5] show considerable evidence that inactivation precedes conformational changes because the active site is located in a more flexible region than the protein molecule as a whole. Other studies using different systems have shown a reasonable agreement between the deactivation rate and the rate constant for the structural transition [6–8].

Chromobacterium viscosum lipase with 33 kDa mol. wt. was purified from a commercially available preparation [9,10]. Its biochemical, kinetic and stability characterization was carried out and homology to the *Pseudomonas glumae* lipase was demonstrated [11]. This includes immunological cross-reaction with monoclonal antibodies specific to the *C. viscosum* lipase and 100% homology between the N-terminal sequences. More recently, the X-ray structure of *C. viscosum* was obtained and some structural differences were observed between *C. viscosum* and *P. glumae* lipase, despite the identity of amino acid sequences (with the exception of the prospective proteolytic loss of three amino acids) [12]. A reason for these structural changes may be the different crystallization conditions. The lipase from *C. viscosum* is of industrial interest, owing to its potential in the detergent industry where stability in aqueous solution at high temperatures is very important [12]. The thermal stability of *C. viscosum* lipase in aqueous solution was further studied in this paper. The elucidation of the deactivation pathway is important to devise a way of increasing stability. Steady-state fluorescence emission from native and inactivated lipase is characterized. Circular dichroism (CD) and static light scattering (SLS) measurements were used to give insight on the deactivation pathway of the lipase.

2. Experimental

2.1. Chemicals

Chromobacterium viscosum lipase was purified from a commercial preparation supplied by Toyo Jozo Enzyme (Japan) as previously described [9]. The pure lipase was lyophilized in distilled water and stored at −20°C. A pH 9.0 solution in 50 or 20 mM Tris–HCl buffer was prepared for the fluorescence and SLS and CD, respectively. Sigma Lipase Substrate, 50% (v/v) triolein, was used as the stock solution.

2.2. Activity measurements

Lipolytic activity was measured in a closed 25-ml reaction vessel thermostated at 60°C, using triolein as the substrate. A stable emulsion 10% (v/v) in triolein was prepared by dilution of the commercial preparation from Sigma in 50 mM Tris–HCl buffer (pH 9.0). Lipase concentration was 0.1 mg ml^{-1} in the reaction vessel. The lipolysis reaction was followed by magnetic stir-

ring for 5 min, and samples (200 μl) were taken from the vessel every 1 min. Colorimetric determination of free oleic acid in the samples was carried out using the Lowry–Tinsley method [13].

2.3. Fluorescence measurements

Fluorescence measurements were obtained by excitation at 280 or 300 nm with excitation and emission slits set at 8 nm on a Perkin-Elmer MPF-3 fluorescence spectrophotometer with 90° geometry. The sample absorbance was kept below 0.1 at the excitation wavelength to avoid inner filter effects. Current lipase concentration was 0.08 mg ml^{-1} for excitation at 280 nm and 0.86 mg ml^{-1} for excitation at 300 nm. The instrumental response at each wavelength was corrected by means of a curve obtained using appropriate fluorescence standards [14].

Emission spectra were decomposed on tyrosyl and tryptophyl contributions as described elsewhere [15,16]. The emission spectrum of tryptophan residues (obtained with 300 nm excitation light) after appropriate normalization includes the energy transferred from tyrosine residues. Quantum yields of tryptophyl fluorescence ($\varnothing_F$) were determined as previously described [16] using free tryptophan in aqueous solution at pH 7.0 as standard [$\varnothing_F = 0.1$ (S. Andrade and S.M.B. Costa, unpublished)] and were not corrected for tyrosinate absorption.

2.4. CD measurements

Spectra were obtained with a Jasco spectropolarimeter, model J-720. A thermostated rectangular quartz cell with 1-cm path length and a cylindrical quartz cell with 0.1-cm path length were used to obtain spectra at 60°C and room temperature, respectively. The current lipase concentration was 0.8–1.5 mg ml^{-1} for near-UV and 0.3–1.0 mg ml^{-1} for far-UV. Results were expressed in terms of mean residue ellipticity ($\theta_{mrw,\lambda}$) in units of deg cm^2 $dmol^{-1}$ and were determined according to the following equation:

$$\theta_{mrw,\lambda} = MRW\theta_\lambda / 10lc \quad (1)$$

where MRW is the mean residue weight ($MRW_{C.\ viscosum} = 104.1$), θ_λ is the observed ellipticity in deg, l is the path length in cm and c is the lipase concentration in g ml^{-1}. All the spectra were obtained after base line subtraction.

2.5. SLS measurements

SLS measurements were carried out at 60°C in a standard luminescence spectrometer, model LS-50B from Perkin-Elmer, using a fluorescence quartz cell with 1-cm path length. Incident light of 500 nm was used and the scattered light was also collected at 500 nm at the scattering angle of 90°. 'Excitation' and 'emission' slits were kept at 2.5 nm. Lipase concentration was 0.6 mg ml^{-1}.

2.6. Data analysis

The quantum yield of fluorescence is defined by considering the rate constants which compete with the radiative fluorescence rate constant [15]:

$$\varnothing_F = k_1/(k_1 + k_0 + k_2) \quad (2)$$

where k_1 is the radiative rate constant which accounts for fluorescence emission of photons from the excited singlet (temperature independent), k_0 is the sum of intersystem crossing and temperature-independent processes including a part of internal conversion [17] and k_2 is the sum of non-radiative rate constants comprising the temperature-dependent internal conversion. Therefore, k_2 follows Arrhenius' law and considering that at temperatures above 0°C, k_0/k_1 is small compared with $\ln(\varnothing_F^{-1} - 1)$:

$$\ln\left(\varnothing_F^{-1} - 1\right) = \ln(A/k_1) - E_A/RT \quad (3)$$

The fluorescence intensity (FI) at 330 nm ($\lambda_{excit} = 300$ nm) was used to calculate the fraction of native lipase:

$$f_{N(t)} = (FI_{(t)} - FI_A)/(FI_N - FI_A) \quad (4)$$

where f_N is the fraction of native lipase calculated for different incubation times, $FI_{(t)}$ is the fluorescence intensity at time t and FI_N (FI at

time zero) and FI_A (FI after incubation at 60°C for 110 min) are the fluorescence intensity of native alone and native lipase which associates to form assemblies, respectively.

The decrease in the fraction of lipase native, f_N, and lipase active, f_a, (assessed by the ratio activity$_{t=t}$/activity$_{t=0}$) upon incubation at 60°C was described by a first order process by using the Levenberg–Marquardt non-linear least-squares method and the following equation:

$$f_{N(t)} \text{ or } f_{a(t)} = \text{offset} + A\exp^{(-tk)} \quad (5)$$

where the offset is the f_N or the f_a at the plateau where the FI or the activity do not change, A is the amplitude of the decrease and k is the rate constant. Activity measurements were carried out at 60°C to avoid reversibility upon cooling.

3. Results

Emission spectra of *Chromobacterium viscosum* lipase at pH 9.0 were recorded and the temperature effects on both the quantum yield and on the emission maxima were determined (Fig. 1). At low temperatures the lipase is native but there is a shift on the emission maximum to longer wavelengths with an increase in temperature. Tryptophyl emission is sensitive to the microenvironment and a red-shift is observed with an increase in the polarity [18]. The red-shift observed with the temperature increase means that excited state non-radiative deactivation of tryptophan residues emitting at lower wavelengths is more temperature-dependent. The *C. viscosum* lipase has three tryptophan and 10 tyrosine residues. The calculated activation energy within the temperature range where lipase remains native was 1.9 kcal mol^{-1}. Above 60°C a greater quantum yield temperature dependence was clearly defined, reflecting a protein structural change. The activation energy increases then to 10.2 kcal mol^{-1}. Emission maxima at 329 nm do not reflect an increased polarity for the tryptophyl microenvironment. The interception point of the two straight lines in Fig. 1 occurs at 66.7°C. This temperature

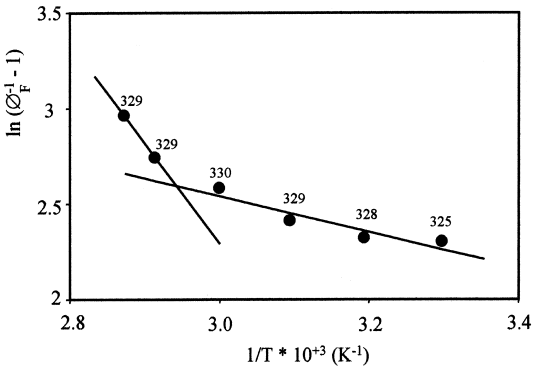

Fig. 1. Thermal dependence of the tryptophyl fluorescence (excitation light of 300 nm) of *Chromobacterium viscosum* lipase. Numbers above each data point are the emission maxima.

value, which is indicative of the transition temperature from the native to the structurally different state, occurs approximately 5° after the plateau of the activity–temperature profile in a range where activity measurements display a large standard deviation [19].

The fluorescence emission from tryptophan and tyrosine residues of *C. viscosum* lipase incubated at 60°C is shown in Fig. 2. The structural change evolution of enzymes with time is an approximation to operational stability despite the absence of substrate. Incubation at 60°C leads to a shift of the tryptophyl emission maximum from 330 to 336 nm after 40 min which remains constant during 110 min. This red-shift reflects either an

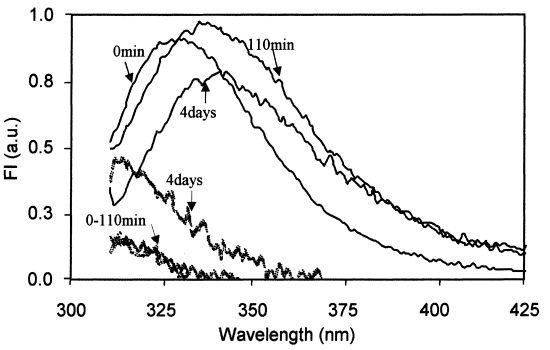

Fig. 2. Lipase tryptophyl (thin solid line) and tyrosyl (thick dotted line) fluorescence emission after 0, 110 min and 4 days at 60°C.

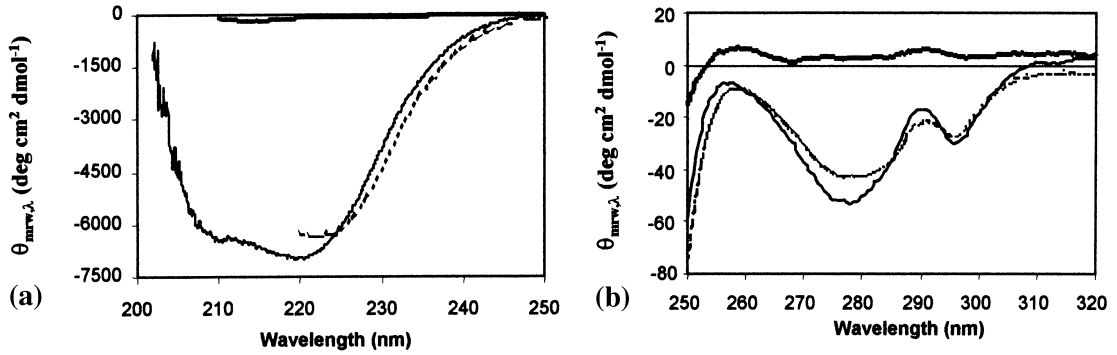

Fig. 3. Far (a) and near-UV (b) circular dichroism spectra of *C. viscosum* lipase. The spectra depicted by the thin solid line and thick solid line were obtained at room temperature in the absence and presence of 6 M guanidine hydrochloride, respectively. The spectra depicted by the dotted line was obtained at 60°C, 0 min incubation time.

increased polarity on the microenvironment of the emitting tryptophans or a larger non-radiative process for the tryptophans emitting at lower wavelengths. Tyrosyl emission is not affected until 110 min and is approximately 2% of the total emission. After 4 days at 60°C, a tryptophyl maximum occurs at 341 nm and the tyrosyl contribution increases to 16% indicating most probably a less structured state. Emission maximum obtained with an excitation wavelength of 280 nm are blue-shifted (maxima occurring at 323 and 335 nm after 0 and 40 min, respectively) because tyrosyl emission occurs near 305 nm. After 4 days, excitation at 280 nm shows a more significant blue-shift (from 341 to 331 nm) because the tyrosyl contribution increases (Fig. 2).

The circular dichroism spectra of the *C. viscosum* lipase at 25°C show the native-like secondary and tertiary structures completely disrupted in the presence of 6 M guanidine hydrochloride (Fig. 3a,b). A negative ellipticity is observed between 250 and 202 nm since α-helix displays negative ellipticities at this wavelength range. β-Sheet also displays negative ellipticities down to approximately 205 nm [20]. *C. viscosum* has 35.1% α-helix and 13.5% β-sheet content. The typical α-helical conformation transitions at 208nm ($\pi-\pi^*$) and 222 nm ($n-\pi^*$) show a 222/208 nm amplitude ratio of 1.12, similar to that of most water-soluble helical proteins (from approx. 0.85 to approx. 1.15) [21]. The intensities of aromatic CD bands depend on the protein rigidity with the more mobile side chains having lower intensities, on the interaction between aromatic amino acids and on the number of aromatic amino acids [20]. At 60°C, a low resolution smoothed spectra were recorded due to the high scan rate and large integration time used. These conditions were imposed to assure reproducible measurements and that the time needed to record the spectra was negligible when compared with the half-life deactivation time. Buffer absorption on the thermostated 1-cm path length cell prevents measurements to be taken below 218 nm but allows repro-

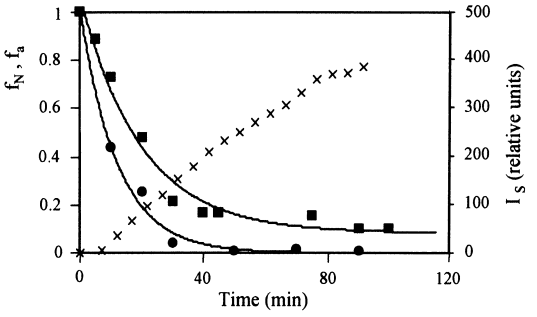

Fig. 4. Evolution of the fraction of native lipase (●) and of the fraction of active lipase (■) with time were fitted to a first order process (solid lines) as described in material and methods. Rate constants were 0.083 and 0.05 1 min^{-1} respectively. Also shown is the evolution of the total intensity of scattered light (I_S) at 500 nm (×) with time.

ducible measurements at 222 nm which reflect mainly α-helical content [22].

Fig. 4 shows the lipase deactivation (loss of activity) upon incubation at 60°C and the comparison of the evolution of protein structural changes with time assessed by fluorescence and SLS measurements. The fraction of native (assessed by fluorescence changes) and active lipase (assessed by activity measurements) were well fitted to a first order process and the rate constants were 0.083 and 0.05 1 min^{-1} to fluorescence changes and deactivation, respectively. In opposition to fluorescence changes, CD spectra show no significant changes after incubation at 60°C both on the near and far-UV range (data not shown). The molar ellipticity at 296 nm in the region of the tryptophan residues spectrum remains constant during incubation at 60°C. The same occurs at 280 nm where tyrosine residues display a peak of optical activity [20] and at 222 nm. The intensity of the scattered light increases during lipase incubation at 60°C indicating protein association/aggregation [23].

4. Discussion

The tryptophyl quantum yield of *Chromobacterium viscosum* lipase with increasing temperature indicates a structural change occurring at approximately 67°C (Fig. 1). Excited-state electron transfer to the peptide bond has a low activation energy (3.2 kcal mol^{-1}) for non-radiative deactivation processes [17]. In the low temperature range covered in the present work a low activation energy (1.9 kcal mol^{-1}) was also obtained. For native proteins E_A values range from 2 to 3 kcal mol^{-1} [15], except for some proteinases where E_A varies between 7.9 and 12.9 kcal mol^{-1} [24]. However, in the high temperature range the activation energy increases to 10.2 kcal mol^{-1}. This value points to water quenching of tryptophyl emission which has an activation energy of 11–13 kcal mol^{-1} [25,26]. Water quenching should become important to tryptophan residues exposed on the surface of the protein upon denaturation. However, emission maxima do not indicate a more exposed localization on the protein surface since no red-shift was observed.

Incubation of *C. viscosum* at 60°C leads to a shift of the tryptophyl emission from 330 to 336 nm after 40 min (Fig. 2). Tyrosyl contribution is very low (2%) and increases to 16% only upon prolonged incubation at 60°C (4 days). Tryptophan residues are usually the main contributors to the emission of globular proteins due to Tyr-to-Trp energy transfer and quenching by nearby groups on the peptide chain [18], with a few exceptions [16]. The increase on the tyrosyl contribution probably reflects a less efficient Tyr-to-Trp energy transfer [27]. Tyrosyl contribution increases for other proteins upon denaturation [28]. The emission maximum at 336 nm does not reflect a large exposure on the protein surface since the free amino acid emits at 351–361 nm, depending on the pH value [16]. These data point to no lipase denaturation occurring initially upon incubation at 60°C. Additionally these data also point to the absence of proteolytic activity as the cause for deactivation. Proteolytic activity will cause lipase unfolding. Denaturation seems to be detectable only after prolonged incubation causing an increased tyrosyl contribution and a red-shift to 341 nm on the tryptophyl maximum. The three-dimensional structure of *C. viscosum* lipase (Protein data bank entry 1CVL) specifically on the tryptophan localization and microenvironment was analysed to understand the observed steady-state fluorescence characteristics. The accessibility of the three tryptophan residues to the water on the surface of the protein was calculated by using the WHAT IF program [29]. Side-chain accessibility to the solvent was 2, 1 and 0% for Trp30, 209 and 283, respectively. A very small percentage of the van der Waals' radii of the tryptophan atoms is accessible to the solvent explaining the tryptophyl emission at 325–330 nm. A sphere of specified radius was drawn around tryptophan residues to analyse their microenvironment (Fig. 5). Tryptophan residues are mainly surrounded by hydrophobic side-chains as expected from buried residues. Tyrosine 29 and 31 are within 6.0 Å around Trp30 (Fig. 5a). Tyrosine 281 and 207 are within the 6.0 Å around Trp209 (Fig. 5b). Tyrosine 281 is also within the 6.0 Å

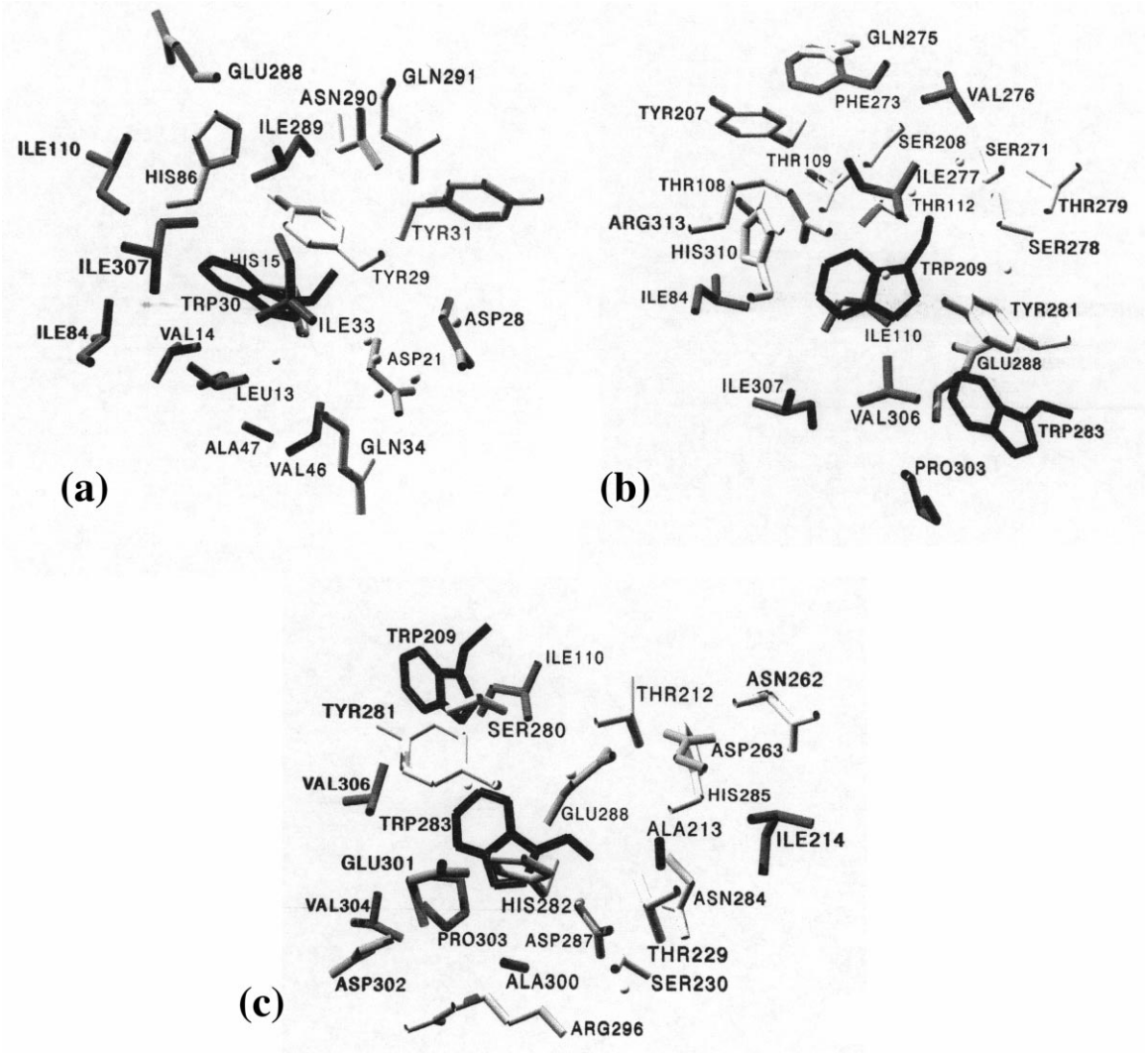

Fig. 5. Tryptophan residue microenvironment on the *C. viscosum* lipase (Protein data bank 1CVL). A 6.0 Å radium was defined around Trp30 (a) Trp209 (b) and Trp283 (c). Within this sphere the peptide backbone was removed. The non-polar side chains were coloured dark grey and the acidic, basic and polar side chains were light grey to white. Tryptophan side chains were coloured black and water molecules associated to the protein crystal were depicted by white balls. Representation drawn with a Swiss-Pdb Viewer [34].

around Trp283 (Fig. 5c). This proximity between tryptophan and four of 10 tyrosine residues should contribute to the lower tyrosyl emission. The efficiency of non-radiative energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor [30] and the distance at which the efficiency is 50% is 10–18 Å for a Tyr–Trp pair [18].

The CD data indicate that secondary and tertiary structures remain native during deactivation observed after incubation at 60°C. Therefore, deactivation is not due to protein denaturation as could be suggested by the small red-shift observed for the fluorescence spectra shown in Fig. 2. The excited state of tryptophan is more sensitive to the microenvironment than the ground state and

subtle conformational rearrangements can be detected by fluorescence measurements. SLS measurements show protein association (Fig. 4). Interactions occur between native protein molecules leading to protein association, distinct from protein aggregation which is usually assigned to denaturated proteins. Lipase deactivation and fluorescence changes seem to result from protein association. This point of view is supported by the high specific activity value (approx. 800–1000 μmol min^{-1} mg^{-1}) obtained at the plateau of deactivation. Deactivation may also result from minor changes in the protein conformation, not detectable by CD measurements, that may lead to protein association but this hypothesis is difficult to distinguish. In this case protein association just correlates to deactivation. The *C. viscosum* lipase contains a calcium binding site [12]. Loss of the calcium ion can disrupt the hydrogen bonding network, which extends to the catalytic His 285 of the active site, resulting in enzyme deactivation.

Data in Fig. 4 show that deactivation and fluorescence intensity changes display first-order kinetics. Loss of the calcium ion can lead to first-order kinetics or, alternatively, second or higher order processes such as association of native proteins can display apparent first-order kinetics when assessed by activity and fluorescence measurements. According to the rate constants determined in Fig. 4, protein fluorescence changes precede deactivation. Tsou [4] pointed out two explanations for the underestimation of deactivation: (i) reactivation upon protein dilution during the activity assay; and (ii) substrate-induced reactivation. The dilution effect is effective if the association/aggregation is a pathway to deactivation as seems to occur with this lipase. The equilibrium between assembled and non-assembled native states can be shifted to the non-associated form upon dilution during the activity assay. The reversibility of the assembling step can be achieved also upon cooling. If the activity assay was carried out at 37°C instead of 60°C the deactivation is well fitted to a first-order process with a rate constant of 0.015 min^{-1} [19]. Substrate-induced reactivation is likely to occur also especially during a lipase activity assay in aqueous solution. Triglycerides are emulsified in aqueous solution forming microdroplets to which lipases should adsorb to display full activity (the so-called interfacial activation) [31]. This phenomenon might be due to the presence of an amphiphilic lid covering the active site of the enzyme. The lid opens upon contact with a lipid–water interface making the active site accessible. A motion of the helix-α5 of *C. viscosum* lipase seems to be necessary for the interfacial activation of this lipase [12]. The interfacial adsorption would shift the equilibrium to the non-assembled native state leading to reactivation. The importance of the substrate on the rate of deactivation was shown for pancreatic lipases which deactivate differently when trioleoylglycerol or dioleoylglycerol were used as substrates [32].

Brown and Yada [8] have found similar activation energies for the isothermal denaturation (followed by difference ultraviolet spectroscopy) and irreversible deactivation (incubation at high temperature and assay for activity at room temperature) of two fungi proteinases. Their study showed that irreversible deactivation was coincident with a gross conformational change providing support for a two-state model where only native and irreversible denaturated states were present in significant concentrations. In the case of proteinases, autolysis is an important factor for the irreversibility of protein denaturation and for the agreement between conformational and deactivation experiments. For the tetrameric β-galactosidase the decrease of activity and the decrease of tetramer population as a function of temperature are coincident [33]. The diversity of results described in the literature for different proteins shows that comparison between structural changes and deactivation should take into account different activity assays and the specificity of the deactivation pathway. An approach based on several spectroscopic techniques was important to have insight on the thermal deactivation pathway of the *C. viscosum* lipase.

5. Conclusions

The temperature effects on the tryptophyl fluorescence quantum yield and on the emission max-

ima of the *Chromobacterium viscosum* lipase show two ranges. The activation energies of temperature-dependent non-radiative deactivation of the excited state were 1.9 kcal mol^{-1} for the native state and 10.2 kcal mol^{-1} after a structural change occurring above 60°C. This structural change is not characterized by a tryptophan exposure to the protein surface as expected from a denaturation process since no red-shift and no increase on the tyrosyl contribution were detected. Further insight on this structural change was obtained by comparing the deactivation to the evolution of the fluorescence intensity, CD and SLS measurements with time after incubating the lipase at 60°C. The deactivation shows an apparent first-order kinetics with a rate constant of 0.05 1 min^{-1} which it is likely due to the association of native molecules as shown by CD and SLS measurements. Minor changes in the protein conformation, not detectable by CD measurements, may also lead to deactivation and to lipase association. Far and near-UV range CD spectra show native-like secondary and tertiary structure. In spite of the native-like tertiary structure tryptophyl emission changes upon assembly of the native states showing the greater sensitivity of the excited state of tryptophan to the microenvironment when compared to the ground state. These changes were characterized by a red-shift from 330 to 336 nm occurring during the first 40 min of incubation and show apparent first-order kinetics with a rate constant of 0.083 min^{-1}. Reactivation upon protein dilution during the activity assay and substrate-induced reactivation are the two possible mechanisms to explain the large rate constant observed for fluorescence changes when compared with the deactivation process.

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References

[1] J. Bosley, Turning lipases into industrial biocatalysts, in: A.R. Macrae (Ed.), Microbial Lipases: Structures, Function and Industrial Applications, Biochemistry Society Transactions, 25, 1997, pp. 174–178.

[2] I. Gill, R. Valivety, Polyunsaturated fatty acids, part 2: biotransformation and biotechnological applications, Trends Biotechnol. 15 (1997) 470–478.

[3] K. Hult, M. Holmquist, Kinetics, molecular modelling, and synthetic applications with microbial lipases, Methods Enzymol. 286 (1997) 386–405.

[4] C.-L. Tsou, Inactivation precedes overall molecular conformation changes during enzyme denaturation, Biochim. Biophys. Acta 1253 (1995) 151–162.

[5] Y.-L. Zhang, J.M. Zhou, C.L. Tsou, Inactivation precedes conformation change during thermal denaturation of adenylate kinase, Biochim. Biophys. Acta 1164 (1993) 61–67.

[6] E.P. Melo, C.M.L. Carvalho, M.R. Aires-Barros, S.M.B. Costa, J.M.S. Cabral, Deactivation and conformational changes of cutinase in reverse micelles, Biotechnol. Bioeng. 58 (1998) 380–386.

[7] D.G. Wallace, J.W. Donovan, P.M. Schneider, A.M. Meunier, J.L. Lundblad, Biological activity and conformational stability of the domains of plasma fibronectin, Arch. Biochem. Biophys. 212 (1981) 515–524.

[8] E.D. Brown, R.Y. Yada, A kinetic and equilibrium study of the denaturation of aspartic proteinases from the fungi *Endothia parasitica* and *Mucor miehei*, Biochim. Biophys. Acta 1076 (1991) 406–415.

[9] M.A. Taipa, M.R. Aires-Barros, J.M.S. Cabral, Purification of lipases from *Chromobacterium viscosum*: is there a high molecular weight lipase? Biotechnol. Technol. 6 (1992) 533–538.

[10] M.A. Taipa, P. Moura-Pinto, J.M.S. Cabral, Identification and characterization of *Chromobacterium viscosum* lipase, Biotechnol. Technol. 8 (1994) 27–32.

[11] M.A. Taipa, K. Liebeton, J.V. Costa, J.M.S. Cabral, K.-E. Jäeger, Lipase from *Chromobacterium viscosum*: biochemical characterization indicating homology to the lipase from *Pseudomonas glumae*, Biochim. Biophys. Acta 1256 (1995) 396–402.

[12] D. Lang, B. Hofmann, L. Haalck et al., Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 Å resolution, J. Mol. Biol. 259 (1996) 704–717.

[13] R.R. Lowry, J.J. Tinsley, Rapid colorimetric determination of free fatty acids, J. Am. Chem. Soc. 53 (1976) 470–472.

[14] G.M.M. Medeiros, M.F. Leitão, S.M.B. Costa, Fluorescence of acridine and acridine 9-carboxylic acid in anionic micelles, J. Photochem. Photobiol. A: Chem. 72 (1993) 225–233.

[15] J.W. Longworth, Luminescence of polypeptides and proteins, in: R.F. Steiner, I. Weinryb (Eds.), Excitated States

of Proteins and Nucleic Acids, Plenum Press, New York, 1971, pp. 319–484.
[16] E.P. Melo, S.M.B. Costa, J.M.S. Cabral, Denaturation of a recombinant cutinase from *Fusarium solani* in AOT-*iso*-octane reverse micelles: a steady-state fluorescence study, Photochem. Photobiol. 63 (1996) 169–175.
[17] Y. Chen, B. Liu, H.T. Yu, M.D. Barkley, The peptide bond quenches indole fluorescence, J. Am. Chem. Soc. 118 (1996) 9271–9278.
[18] J.R. Lakowicz, Protein fluorescence, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1983, pp. 341–381.
[19] M.R. Castellar, M.A. Taipa, J.M.S. Cabral, Kinetic and stability characterization of *Chromobacterium viscosum* lipase and its comparison with *Pseudomonas glumae* lipase, Appl. Biochem. Biotechnol. 61 (1996) 299–314.
[20] S.M. Kelly, N.C. Price, The application of circular dichroism to studies of protein folding and unfolding, Biochim. Biophys. Acta 1338 (1997) 161–185.
[21] G.E. Arnold, L.A. Day, A.K. Dunker, Tryptophan contributions to the unusual circular dichroism of fd bacteriophage, Biochemistry 31 (1992) 7948–7956.
[22] J.D. Morriset, J.S.K. David, H.J. Pownall, A.M. Gotto, Jr., Interaction of an apolipoprotein (ApoLP-alanine) with phosphatidylcholine, Biochemistry 12 (1973) 1290–1299.
[23] B.A. Chrunyk, R. Wetzel, Breakdown in the relationship between thermal and thermodynamic stability in an interleukin-1β point mutant modified in a surface loop, Protein Eng. 6 (1993) 733–738.
[24] P. Dolashka, I. Dimov, N. Genov, I. Svendsen, K.S. Wilson, C. Betzel, Fluorescence properties of native and photooxidised proteinase K: the X-ray model in the region of the two tryptophans, Biochim. Biophys. Acta 1253 (1992) 151–162.
[25] Y. Chen, M.D. Barkley, Toward understanding tryptophan fluorescence in proteins, Biochemistry 37 (1998) 9976–9982.
[26] H.T. Yu, M.A. Vela, F.R. Fronczek, M.L. McLaughlin, M.D. Barkley, Microenvironmental effects on the solvent quenching rate in constrained tryptophan derivatives, J. Am. Chem. Soc. 117 (1995) 348–357.
[27] Y. Saito, H. Tachibane, H. Hayashi, A. Wada, Excitation-energy transfer between tyrosine and tryptophan in proteins evaluated by the simultaneous measurement of fluorescence and absorbance, Photochem. Photobiol. 33 (1981) 289–295.
[28] J.G. Brand, R.H. Cagan, Fluorescence characteristics of native and denaturated monellin, Biochim. Biophys. Acta 493 (1977) 178–187.
[29] G. Vriend, What if: a molecular modelling and drug design program, J. Mol. Graph. 8 (1990) 52–56.
[30] J.R. Lakowicz, Energy transfer, Principles of Fluorescence Spectroscopy, New York: Plenum Press, (1983) 305–339.
[31] H. van Tilbeurgh, M.P. Egloff, C. Martinez, N. Rugani, R. Verger, C. Cambillau, Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography, Nature 362 (1993) 814–820.
[32] A. Lykidis, V. Mougios, P. Arzoglou, Kinetics of the two-step hydrolysis of triacylglycerol by pancreatic lipases, Eur. J. Biochem. 230 (1995) 892–898.
[33] R.A. Edwards, A.L. Jacobson, R.E. Huber, Thermal denaturation of β-galactosidase and of two site-specific mutants, Biochemistry 29 (1990) 11001–11008.
[34] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modelling, Electrophoresis 18 (1997) 2714–2723.