Biochemical and structural characterisation of cutinase mutants in the presence of the anionic surfactant AOT

V. Brissos a,⁎, E.P. Melo a,b, J.M.G. Martinho c, J.M.S. Cabral a

⁎ IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

a Institute for Biotechnology and Bioengineering, Centre for Molecular and Structural Biomedicine, Campus de Gambelas, 8005-139 Faro, Portugal

b Centro de Química-Física Molecular, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

A R T I C L E   I N F O

Article history:
Received 12 February 2008
Received in revised form 8 April 2008
Accepted 22 April 2008
Available online 4 May 2008

Keywords:
Cutinase
AOT
Kinetics
Stability
Protein folding

A B S T R A C T

The reactivity, stability and unfolding of wild-type (WT) Fusarium solani pisi cutinase and L153Q, S54D and T179C variants were studied in the absence and presence of the dioctyl sodium sulfate (AOT) surfactant. In the absence of surfactant the S54D variant catalytic activity is similar to that of the WT cutinase, whereas L153Q and T179C variants show a lower activity. AOT addition induces an activity reduction for WT cutinase and its variants, although for low AOT concentrations a small increase of activity was observed for S54D and T179C. The enzyme deactivation in the presence of 0.5 mM AOT is relatively slow for the S54D and T179C variants when compared to wild-type cutinase and L153Q variant. These results were correlated with secondary and tertiary structure changes assessed by the CD spectrum and fluorescence of the single tryptophan and the six tyrosine residues. The WT cutinase and S54D variant have similar secondary and tertiary structures that differ from those of T179C and L153Q variants. L153Q, S54D and T179C mutations prevent the formation of hydrophobic crevices responsible for the unfolding by anionic surfactants, with the consequent decrease of the AOT–cutinase interactions.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Cutinases are enzymes whose name derives from their ability to degrade cutin polymers, but they additionally hydrolyse a wide variety of both water-soluble esters and emulsified triacylglycerols [1,2]. Cutinase has the enzymatic capabilities of true esterases and true lipases, being an efficient catalyst both in solution and at water–lipid interfaces. In addition, cutinase is stable under alkaline conditions and its structural integrity does not depend on calcium [3]. These properties make cutinases good candidates to improve the removal of fat-based stains in detergent formulations. However, anionic surfactants present in detergent formulations rapidly inactivate wild-type (WT) Fusarium solani pisi cutinase, which limits their use [1,4,5]. Electrostatic interactions were already identified as being important for the stabilization of cutinase in the presence of the anionic surfactants. For instance, in the presence of lithium dodecyl sulphate, a cutinase variant with a more positively charged surface in position 172, (N172K), was found to be less stable than the WT cutinase, while the introduction of negative charged residues in position 17 and 196 (R17E and R196E) increased the stability of the mutants [6].

This work studies three mutants of the WT F. solani pisi cutinase found by directed evolution to improve the stability of cutinase in the presence of AOT (S54D, L153Q and T179C), schematically represented in Fig. 1 [7,8].

S54D mutant was chosen because in the high-throughput screening it showed the highest stability against dioctyl sodium sulfate (AOT) without a significant decrease in activity. This residue is located in a α-helix (51–63) far from the active site (Fig. 1). The substitution of the serine residue by an aspartic acid is thought to prevent the creation of a large hydrophobic crevice that is observed in the course of unfolding of cutinase due to the separation of the helices encompassing residues 51–63 and 191–211 [4]. L153Q mutant was selected to study the importance of changing the hydrophobic residue (leucine) by a less hydrophobic amino acid (glutamine) in the loop 151–166 (Fig. 1), that was previously identified to be a weak spot regarding the stability of cutinase [4]. However, several mutants were found in this region in the high-throughput screening with a slight stability improvement in the presence of AOT [7,8]. Finally, T179C mutant, located in one of the binding loops (171–191) (Fig. 1), was chosen to understand the effect of introducing a cysteine (more space filling residue), near the active centre and next to another cysteine involved in a disulphide bridge (Cys171–Cys178), on the protein structure and catalytic activity.

The enzymatic activity and the stability of the WT cutinase and the three mutants were measured using the hydrolysis reaction of p-nitrophenylbutyrate (p-NPB) in the absence and presence of several amounts of the anionic surfactant, AOT. The hydrolysis reaction of the WT cutinase and its variants follows Michaelis–Menten kinetics, in the
absence and presence of AOT. The catalytic activity of the enzymes and the inhibition effect of AOT were correlated with structural changes of the protein, assessed by both the intrinsic fluorescence of the single tryptophan (Trp69) and the six tyrosine residues (Tyr38, 77, 119, 149, 162 and 191) distributed along the backbone and by far-UV circular dichroism (CD) spectra. The creation of hydrophobic patches induced by the addition of AOT was probed by the dichroism (CD) spectra. The concentration of GdnHCl is identical for the WT cutinase and its variants and does not vary with the amount of AOT. The amount of p-nitrophenol produced during the reaction was calculated by UV absorption at 400 nm (ε=15,400 M−1 cm−1), using a Hitachi U-2000 spectrophotometer.

Using the Michaelis–Menten kinetics scheme with competitive inhibition,

\[ E + S \xrightarrow{k_1} ES \xrightarrow{k_{cat}} E + P \]

where the reversible complex (ES) is formed between the substrate (S) and enzyme (E) with equilibrium constant, \( K_m \). The product (P) results from the dissociation of the complex with rate constant \( k_2 \). The presence of the surfactant (I) a competitive binding was considered by the formation of an inactive complex (EI) with equilibrium constant, \( K = [E]/[E]I \).

The kinetic scheme gives for the initial rate the Lineweaver–Burk equation:

\[ \frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \]

\[ \text{Relative activity} = \frac{1}{1 + \frac{[I]}{K_i}} \frac{k_1}{k_2} \frac{\beta_1}{\beta_2} \frac{E+I}{E} \]

where \([E]\) and \([S]\) are the total concentrations of enzyme and substrate, respectively. From the linear plot, \( K_m \) and \( V_{max} \) were calculated from the intercept and slope, respectively. The inhibition constant \( K_i \) was finally obtained from the slope ratios of the Lineweaver–Burk plots in the presence and absence of surfactant.

2.4. Stability measurements in the presence of AOT

The inactivation of WT cutinase and its variants by AOT was determined using 300 nM cutinase in assay buffer at 40 °C containing 0.5 mM of AOT. At several times, 15 μl aliquots were removed and diluted 100 fold in assay buffer containing 0.5 mM of surfactant. The residual activity of the samples was determined by adding 0.7 mM p-NPB and measuring the absorbance of p-nitrophenol at 400 nm over 1 min periods at 30 °C. The assays were made in triplicate. The stability plots were fitted with the Henley and Sadana model [11,12] that incorporates two consecutive first-order deactivation steps,

\[ E \rightarrow E_1 \rightarrow E_2 \]

where \( k_1 \) and \( k_2 \) are the deactivation rate coefficients and \( \beta_1 \) and \( \beta_2 \) the relative ratios of the specific activities of enzyme conformational states, \( E_1 \) and \( E_2 \), respectively. The integration of the rate equations, considering that each species is weighted by its own activity, gives the following expression for the relative activity.

\[ \text{Relative activity} = \frac{1}{1 + \frac{[I]}{K_i}} \frac{k_1}{k_2} \frac{\beta_1}{\beta_2} \frac{E+I}{E} \]

2.5. Circular dichroism

The CD spectra were acquired with a Jasco 720-spectropolarimeter at room temperature. Cutinase solutions (5×10^{-6} M) at pH 8.0 containing several amounts of AOT were incubated overnight. The far-UV CD spectra (190–300 nm) were recorded under nitrogen flow in a 1×1 mm square quartz cuvette, setting the experimental conditions to: 0.5 mm bandwidth; 100 mdeg sensitivity; 0.2 nm resolution; 4 s response; 20 nm/min scanning speed. The mean residue ellipticity [θ] (deg cm² dmol⁻¹) was calculated from the CD spectra considering 114.8 g mol⁻¹ as the mean residue weight for cutinase. The ellipticity at 222 nm is inversely proportional to the α-helix content and was used to follow the loss of secondary structure.

2.6. Fluorescence measurements

The fluorescence spectra were recorded in a SLM-AMINCO spectrophotometer (450 W ozone-free Xenon lamp), using a right angle geometry. The excitation light was selected by a double monochromator (4 nm bandwidth), being the fluorescence selected by a double monochromator (4 nm bandwidth) and detected by a cooled photomultiplier (Hamamatsu, R928). Protein solutions (5×10^{-6} M) were excited at 280 nm (excitation of both tryptophan and tyrosine residues) and at 296 nm (for selective excitation of tryptophan residue), and emission was collected between 290–450 nm and 305–450 nm, respectively. The emission of the six tyrosine residues of cutinase was obtained from the fluorescence spectrum by excitation at 280 nm, after subtracting the emission due to the single tryptophan. The fluorescence spectra were recorded after overnight incubation of the samples. The spectra were corrected for the background using the spectrum of the solvent recorded in identical conditions.

2.3. Enzymatic hydrolysis kinetics

The hydrolysis of p-NPB (0.1–0.8 mM) catalyzed by WT cutinase and its variants was studied in the absence and presence of several amounts of AOT. The amount of p-nitrophenol produced during the reaction was calculated by UV absorption at 400 nm (ε=15,400 M−1 cm−1), using a Hitachi U-2000 spectrophotometer.

Using the Michaelis–Menten kinetics scheme with competitive inhibition,
2.7. 1.8-ANS fluorescence

Solutions of WT cutinase and its variants (5 × 10⁻⁶ M) containing several amounts of AOT were incubated with 1.25 × 10⁻⁴ M of 1.8-ANS. Fluorescence spectrum of ANS was recorded between 420–650 nm, by excitation at 410 nm.

2.8. Unfolding equilibrium studies

Guaniidinium hydrochloride unfolding curves were obtained from both the ellipticity at 222 nm and the tryptophan fluorescence intensity at the maximum by excitation at 296 nm. The stock solution containing the surfactant was let to incubate with the required concentration of AOT for 1.5 h. By dilution, several solutions with the required concentration of AOT for 1.5 h. By dilution, several solutions with the required concentration of AOT for 1.5 h. By dilution, several solutions with a constant concentration of cutinase (5 × 10⁻⁶ M) and several concentrations (0–3 M) of GdnHCl and AOT (0–10 MR) were prepared. Samples were allowed to equilibrate overnight prior to the measurements.

The unfolding curves were fitted with a two-state model. The apparent equilibrium constant of unfolding (Keq) and the free-energy change (ΔG°), were calculated by:

\[ K_{eq} = \frac{(y_f - y_u) \cdot (K_{eq})}{(y_i - y_u)} \]  

(3)

\[ \Delta G^0 = -R \ln(K_{eq}) \]  

(4)

where y is the spectroscopic property (ellipticity or fluorescence intensity) at the given denaturant concentration and yf and yi are the corresponding values for the folded and unfolded protein states, respectively. These values were extrapolated from the pre- and post-transition baselines obtained by a linear fit. The ΔG° varies linearly with the concentration of denaturant,

\[ \Delta G^0 = \Delta G^0(H_2O) + \frac{\Delta G^m(H_2O) \cdot (\text{denaturant})}{m} \]  

(5)

where ΔG°(H₂O) is the free-energy change in the absence of denaturant and m is a measure of the ΔG° dependence with the denaturant concentration. The midpoint of denaturation concentration (Cm) corresponds to the denaturant concentration at which ΔG°=0.

The unfolding curves were fitted with Eq. (6) [13], using a nonlinear least-squares procedure,

\[ F = \frac{a_0 + b_0 \cdot c + (a_1 + b_1) \cdot m \cdot \exp(c \cdot m)}{1 + 10^{d \cdot m \cdot \exp(c \cdot m)}} \]  

(6)

where F is the spectroscopic property, c the denaturant concentration, m0–m8 the sensitivity of the equilibrium constant to unfolding, c0–c3 the denaturant concentration corresponding to half unfolded protein, a0, a1, b0, b1 the intercepts and c0, c1 the slopes of the fluorescence baselines at low (N) and high (D) denaturant concentrations before and after the transition region, respectively.

3. Results

3.1. Catalysed hydrolysis of p-NPB

After purification, enzymes were analysed by SDS-PAGE and the molar mass found for the WT cutinase and its mutants was identical (~22 KDa).

![Fig. 2](image2.png)

**Fig. 2.** Variation of the initial rate (v₀) of the p-NPB hydrolysis with the concentration of AOT: WT (●), L153Q (●), S54D (▲), and T179C (○). The reaction was performed in 20 mM Tris–HCl (pH 8) containing 3 mM cutinase, 0.7 mM p-NPB and several concentrations of AOT.

![Fig. 3](image3.png)

**Fig. 3.** Deactivation profile of cutinase WT (●), L153Q (●), S54D (▲), T179C (○) in the presence of 0.5 mM AOT. The experimental results were fitted (full line) with the Sadana model (Eq. 2).

The initial rate of p-NPB hydrolysis catalysed by WT cutinase and its mutants was studied as a function of AOT concentration. The activity in the absence of AOT is identical for the WT cutinase and S54D variant, but much lower for the L153Q and T179C mutants (Fig. 2). The shape of the curves is also different, decreasing with the AOT concentration for the WT cutinase and T179C variant, while the other two mutants (S54D and L153Q) display a maximum resulting from an initial increase at low concentrations followed by a continuous decrease. The initial rates follow Michaelis– Menten kinetics, being the parameters, in the absence and presence of AOT (below the CMC), summarized in Table 1. In the absence of AOT, the kinetic parameters of the S54D mutant are practically equal to those of the WT cutinase, while the catalytic efficiency of the L153Q and T179C mutants are lower. The addition of AOT leads to Km values higher than Km (1+|I|/Km). The L153Q and T179C mutants (Km=0.44±0.04 mM and Km=0.46±0.05 mM, respectively) show higher and the S54D mutant (Km=1.38±0.14 mM) lower inhibition by AOT than the WT cutinase (Km=0.72±0.06 mM). The decrease of the initial rate with the increase of AOT concentration observed for the WT cutinase and T179C variant is essentially due to the strong inhibition of the surfactant because kcat is practically invariant with the AOT concentrations. The shapes of the S54D and L153Q curves are due to two competing effects: the increase of kcat and the inhibition with increasing AOT concentrations.

| Table 1 Effect of AOT on kinetic parameter values for cutinase and its mutants L153Q, S54D and T179C in aqueous solutions |
| --- | --- | --- | --- |
| Protein | [AOT] (mM) | kcat (10⁴ s⁻¹) | K_m (mM) | K_i (mM) |
| WT | 0 | 1.56±0.29 | 0.35±0.02 | 0.72±0.06 |
| 0.5 | 1.55±0.10 | 0.48±0.05 | 1.02±0.01 |
| 1 | 1.74±0.13 | 0.85±0.01 | 1.12±0.12 |
| 1.5 | 1.52±0.14 | 1.00±0.14 | 1.08±0.16 |
| 2 | 1.38±0.17 | 1.00±0.14 | 1.08±0.16 |
| S54D | 0 | 1.47±0.13 | 0.33±0.03 | 1.38±0.14 |
| 0.5 | 2.47±0.15 | 0.74±0.07 | 1.38±0.14 |
| 1 | 2.42±0.27 | 0.72±0.01 | 1.38±0.14 |
| 1.5 | 2.52±0.15 | 1.14±0.11 | 1.38±0.14 |
| 2 | 2.32±0.29 | 1.74±0.75 | 1.38±0.14 |
| L153Q | 0 | 1.10±0.77 | 0.31±0.03 | 0.44±0.04 |
| 0.5 | 1.60±0.84 | 0.69±0.07 | 0.44±0.04 |
| 1 | 1.79±0.22 | 1.31±0.13 | 0.44±0.04 |
| 1.5 | 2.58±0.18 | 3.57±0.21 | 0.75±0.11 |
| 2 | 2.85±0.25 | 4.33±0.41 | 0.75±0.11 |
| T179C | 0 | 0.79±0.09 | 0.20±0.02 | 0.46±0.05 |
| 0.5 | 0.96±0.05 | 0.49±0.05 | 0.46±0.05 |
| 1 | 1.11±0.07 | 0.66±0.06 | 0.46±0.05 |
| 1.5 | 1.10±0.12 | 1.09±0.11 | 0.46±0.05 |
| 2 | 1.15±0.12 | 1.56±0.16 | 0.46±0.05 |
3.2. Stability measurements in the presence of AOT

The loss of initial activity of the WT cutinase and its variants upon incubation with 0.5 mM AOT was monitored over the course of 1 h at 40 °C (Fig. 3). The stability curves of the WT cutinase and their mutants in the absence of AOT are identical, and were used as a control. In the presence of AOT, the three mutants show different stability profiles.

The L153Q mutant stability curve can be fitted with a single exponential (pseudo-first-order kinetics) with an inactivation rate constant of $k_1 = 0.025 \text{ min}^{-1}$ (Table 2). The deactivation curves of the other variants are more complex and were fitted with the Henley and Sadana model\[11,12\]. A good fit was obtained, as judged by the plot of the residuals and the correlation coefficients. The S54D variant decays to an intermediate state $E_1$ with rate constant $k_1 = 0.083 \text{ min}^{-1}$. This intermediate has lower activity than the initial enzyme ($\beta_1 = 0.81$) and deactivates with rate constant $k_2 = 0.004 \text{ min}^{-1}$ to a final state $E_2$ with residual specificity ($\beta_2 = 0.006$). The T179C mutant decays with rate constant $k_1 = 6.85 \text{ min}^{-1}$ to $E_1$ with specific activity higher than the initial enzyme ($\beta_1 = 1.16$), followed by the deactivation with rate constant $k_2 = 0.014 \text{ min}^{-1}$ to the final state $E_2$ with very low activity ($\beta_2 = 0.011$).

The WT cutinase and L153Q mutant display the same average activity of 37 mM s$^{-1}$, while the S54D mutant has a significantly higher average activity of 86 mM s$^{-1}$ (Table 2). The T179C variant has an average activity (46 mM s$^{-1}$) higher than the WT cutinase, despite the low initial activity due to the high activity of intermediate $E_1$ and slow deactivation paths that compensate for the low initial activity of the enzyme.

3.3. Fluorescence and CD measurements

The fluorescence spectra of the WT cutinase and its variants were recorded in buffer solutions at pH 8.0 (Fig. 4). The fluorescence of cutinase by excitation at 280 nm is due to both the single tryptophan and the six tyrosine residues. In proteins having both tyrosine and tryptophan aromatic residues the fluorescence of tryptophan generally dominates\[14\]. This does not occur in the WT cutinase because the single tryptophan (Trp69) fluorescence is strongly quenched ($\phi = 0.01$, [15]) by the adjacent disulfide bond between Cys31 and Cys109, located approximately 4 Å away from tryptophan\[16–19\]. Thus, the emission of native cutinase is dominated by six tyrosines (Tyr38, 77, 119, 149, 162 and 191) as was observed for the WT cutinase and S54D, L153Q variants in buffer solution. The fluorescence of the native T179C mutant has a major contribution from tryptophan, which suggests that for this mutation the tryptophan is not in the vicinity of the disulfide bridge.

The emission of the unfolded proteins (WT and variants) is dominated by the tryptophan fluorescence because upon denaturation by GdnHCl, the tryptophan residue is released from the vicinity of

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_1 (\text{min}^{-1})$</th>
<th>$k_2 (\text{min}^{-1})$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>Average activity (mM s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.83</td>
<td>0.026</td>
<td>0.82</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>S54D</td>
<td>0.083</td>
<td>0.004</td>
<td>0.81</td>
<td>0.006</td>
<td>86</td>
</tr>
<tr>
<td>L153Q</td>
<td>0.025</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>T179C</td>
<td>6.85</td>
<td>0.014</td>
<td>1.16</td>
<td>0.011</td>
<td>46</td>
</tr>
</tbody>
</table>

Fig. 4. Fluorescence spectra ($\lambda_{\text{em}} = 280$ nm) of 5 μM cutinase WT (A), S54D (B) L153Q (C), and T179C (D) dissolved in 20 mM Tris–HCl, pH 8.0. Native (full line); 3.0 M GdnHCl (dotted line); MR= 100 (dashed line).
the disulfide bridge and in turn the strong tryptophan quenching disappears. By addition of AOT the fluorescence spectra approach those of the unfolded proteins. At MR ([AOT]/[protein])=100 the spectra of the WT cutinase and L153Q variant are identical (intensity and shape), suggesting that unfolding is occurring. The shape of the spectrum of the T179C variant does not change significantly with AOT addition, although a large increase of the fluorescence intensity at 340 nm was observed. The S54D variant fluorescence spectrum is composed of both the tryptophan and tyrosine emissions. This indicates that at MR=100 the enzymes still exist in the folded state (fluorescence dominated by tyrosines) however, a fraction of proteins show already structural changes (fluorescence dominated by the tryptophan).

In order to further study the influence of AOT, the fluorescence intensity at the maximum of tryptophan (λ<sub>exc</sub>=296 nm) and tyrosine residues (obtained from the spectrum by excitation at λ<sub>exc</sub>=280 nm after subtracting the fluorescence of tryptophan) as function of MR was studied (Fig. 5A and B respectively). The tryptophan fluorescence intensity of the WT cutinase and S54D, L153Q variants are identical until MR~40 increasing substantially for higher MR values (Fig. 5A). This indicates that the enzymes begin to denature at this point. After this value the tertiary structure of all the enzymes changes, with the S54D variant being the most resistant to unfolding by AOT. The T179C variant has much higher tryptophan fluorescence intensity and varies in a non-monotonic way with the addition of AOT, showing a maximum at MR~10 and a minimum at MR~40, increasing afterwards until reaching a plateau.

The tyrosine fluorescence of the WT cutinase and its variants as a function of MR is shown in Fig. 5B. In the absence of surfactant the WT cutinase and S54D mutant have the same intensity, while the L153Q and T179C mutants show much smaller values. The tyrosine fluorescence intensity of the WT cutinase slightly increases at low MR and then decrease. The S54D variant has a similar profile to the WT cutinase at low MR but for higher MR values remains almost constant with increasing amounts of surfactant. The tyrosine...
fluorescence of the L153Q and T179C variants does not change with MR, being the intensity of the T179C mutant much lower than that of L153Q.

The ellipticity of the WT cutinase and its variants is shown in Fig. 6. The far-UV absorption spectra of proteins (typically 240 nm to 190 nm) are mainly due to the peptide bond. There is a weak but broad $\pi \rightarrow \pi^*$ transition centred around 210 nm and an intense $\pi \rightarrow \pi^*$ transition around 190 nm. The CD spectra in this spectral region can be used to quantify the overall secondary structure content of proteins namely by following the 222 nm signal, which is assigned to and considered inversely proportional to the $\alpha$-helix content $[20–22]$. The unfolding by GdnHCl leads to the loss of the secondary structure, while by addition of surfactant the secondary structure is almost conserved. This is consistent with earlier studies showing that proteins denatured by detergents contained a large degree of order structure $[23–25]$. The WT cutinase and S54D variant have much higher $\alpha$-helix content than the L153Q and T179C variants.

In order to further study the influence of AOT in the secondary structure, the ellipticity at 222 nm of all the proteins was plotted as a function of MR (Fig. 7). The ellipticity of the WT cutinase and S54D variant is identical in the absence of AOT. Upon AOT addition up to MR~40 there is a decrease of the S54D mutant ellipticity (corresponding to an $\alpha$-helix content increase), to increase afterwards ($\alpha$-helix content decrease) slightly for the S54D variant and more pronouncedly for the WT cutinase. The $\alpha$-helix content of the L153Q and T179C variants is much lower than that of WT cutinase and S54D variant (reduction of 38% and 45%, respectively) and practically does not vary with MR.

3.4. 1,8-ANS binding studies

The fluorescence of 1,8-ANS is very sensitive to its own environment. In water the ANS fluorescence is very weak, while in hydrophobic media it is relatively strong $[26–29]$. This property was explored to monitor the development of hydrophobic patches on cutinase surface induced by the binding/interactions of AOT molecules. AOT is expected to promote less rigid packing of hydrophobic clusters upon binding thus allowing greater interactions between the ANS and the protein to occur.

The ANS fluorescence intensity at 500 nm is practically constant or slightly increases for all the enzymes until MR=30 (Fig. 8). By increasing the AOT concentration, the fluorescence intensity increases rapidly for the WT cutinase and moderately for its variants. The S54D mutant shows the smaller increase, followed by the T179C and L153Q variants.

Table 3

<table>
<thead>
<tr>
<th>Protein</th>
<th>MR</th>
<th>$\Delta G^0$ (H2O) (kcal mol$^{-1}$)</th>
<th>$m$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$C_m$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Steady state fluorescence</td>
<td>0</td>
<td>6.7±1.0 4.05±0.07 1.40±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.7±1.4 4.89±0.23 1.61±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.9±1.5 4.92±0.03 1.61±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>0</td>
<td>10.3±0.4 6.08±0.22 1.69±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.0±0.2 3.60±0.10 1.68±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.9±0.1 4.64±0.08 1.71±0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S54D Steady state fluorescence</td>
<td>0</td>
<td>8.0±0.2 4.72±0.10 1.69±0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.0±1.3 4.17±0.70 1.67±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.8±0.0 4.76±0.03 1.64±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>0</td>
<td>9.3±0.5 5.51±0.33 1.68±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.1±0.8 5.56±0.48 1.63±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.8±0.5 5.95±0.32 1.65±0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L153Q Steady state fluorescence</td>
<td>0</td>
<td>6.9±1.1 4.25±0.08 1.62±0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.0±1.2 4.35±0.14 1.61±0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.1±1.0 4.05±0.09 1.51±0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>0</td>
<td>6.9±1.6 4.27±0.94 1.62±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.4±1.2 5.40±1.16 1.57±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.8±1.3 3.56±0.84 1.63±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T179C Steady state fluorescence</td>
<td>0</td>
<td>7.0±1.5 4.00±0.96 1.75±0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.8±1.0 3.81±0.03 1.77±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.9±1.3 3.50±0.07 1.68±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>0</td>
<td>8.3±0.1 4.82±0.05 1.77±0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.2±1.2 5.30±0.71 1.80±0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.8±0.4 5.54±0.20 1.77±0.13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5. Unfolding equilibrium studies in GdnHCl

The WT cutinase unfolding induced by GdnHCl, obtained from the ellipticity at 222 nm and the tryptophan fluorescence intensity in the absence of surfactant and for MR=5 and MR=10, is shown in Fig. 9. The curves calculated from the CD and fluorescence signals are identical and the influence of AOT is minor. Similar curves were obtained for the three variants (results not shown). The shape of the curves suggests that unfolding proceeds without intermediates, involving only the folded and unfolded states. However, ΔG°, (H2O) and m values in the absence and in the presence of AOT are consistently higher for CD than for the fluorescence intensity (Table 3). This can indicate the presence of an intermediate state, in accordance with a previous work where an unfolding intermediate (molten globule) was identified in the unfolding of cutinase by GdnHCl [30].

4. Discussion

In the absence of surfactant, the catalytic performance of the S54D variant, whose mutation is located far from the active site, is identical to that of the WT cutinase. The other variants, L153Q and T179C, whose mutations are near the active centre, have much lower activity (Table 1). The kcat values of the L153Q and T179C variants are much lower (higher inhibition effect) than those of the WT cutinase and S54D mutant indicating that these mutants have a higher affinity to AOT. In the presence of AOT, the S54D variant shows the highest kcat and a substantial increase of kcat when compared to WT cutinase. The effect of AOT on the catalytic activity is complex and a simple interpretation what was observed experimentally as both activation and inhibition seem to occur. The maximum changes in the activity occur before the CMC, suggesting that these changes result from the binding of monomeric AOT to regions other than the substrate binding site. It was also observed that the anionic surfactant sodium dodecyl sulphate (SDS) increases the reactivity of active site residues presumably as a result of localised unfolding [131]. Some authors [5] suggested that cutinase interactions with SDS result in a hyperbolic mixed inhibition behaviour, for surfactant concentrations above CMC. This anionic surfactant has also been shown to have an activation effect on Aspergillus niger catalase [32], on the tetrameric enzyme β-galactosidase from E. coli [33], on BSA [34] and on Sulfolobus solfataricus β-glycosidase [35]. The catalytic performance of L153Q and T179C decreases in the presence of AOT as was observed for the WT cutinase. The variation of the initial rates with the amount of AOT for the L153Q variant is similar to that of the WT cutinase, except for low AOT concentrations where some activation seems to occur, while for the T179C variant the partial unfolding occurs at higher AOT concentrations than those observed for the WT cutinase. The inactivation evolution with time of the enzymes in the presence of 0.5 mM of AOT is faster for the WT cutinase and L153Q variant than for the S54D and T179C mutants. This suggests that the interaction of AOT is stronger with the WT cutinase and L153Q than with the S54D and T179C variants. The initial activity of the WT cutinase and S54D mutant is much higher than that of L153Q and T179C variants and this is attributed to their different secondary structures. Indeed, the α-helix content of the S54D is similar to that of the WT but much higher than that observed for the L153Q and T179C variants (Fig. 6). The presence of AOT did not have a significant effect on the secondary structure of L153Q and T179C variants (Fig. 7). For the S54D mutant low AOT concentrations seem to induce a gradual increase in α-helical content up to MR=40, which is constant for WT cutinase. For MR=40 the α-helical content decreases for the WT cutinase and remains practically constant for the S54D variant. It was observed that SDS also promoted an increase in α-helical content of ovalbumin, carbonic anhydrase [36] and of β-glycosidase from the thermophilic archaeon S. solfataricus [37].

The tryptophan fluorescence intensity (Fig. 5A) of the WT cutinase and S54D, L153Q mutants does not change until MR=40. This indicates that the tertiary structure around the single tryptophan located in the opposite site of the active centre does not change. For MR=40 the intensity increases substantially, suggesting the beginning of denaturation. The increase is less prominent for the S54D mutant indicating a stronger resistance of this mutant against AOT. Tryptophan fluorescence intensity of the T179C variant is much higher and tyrosine fluorescence lower than that of the WT cutinase and the other mutants, i.e. the tryptophan suffers less quenching from the disulphide bridge, without a significant variation with AOT concentration change. As the tryptophan is located in the opposite side of the mutation (Fig. 1), this suggests that large structural changes occurred. A possibility is that Cys179, introduced by this mutation, forms an intramolecular disulphide bridge with Cys171 or Cys178, that are involved in a disulphide bridge in WT cutinase. This could imply a distortion on the structure and lead to the displacement of the tryptophan from the disulphide bridge (Cys31–Cys109), and/or the approach of a tyrosine with the consequent increase in tyrosine–tryptophan energy transfer efficiency. The structural conformation changes that probably occur near the active site correlate well with the decrease of activity of this mutant.

The β-strand (residues 67–73) in which Trp69 is located lies between two α-helices (residues 51–63 and 91–108) that have been identified, by molecular dynamics simulations, as the lowest mobile secondary structure elements [4]. It is believed that once the binding sites are saturated with AOT, the interaction proceeds to other regions with higher mobility, allowing the access of surfactant tails to hydrophobic regions. From 1.8-ANS fluorescence (Fig. 8) it is clear that all the three mutations prevent the development of hydrophobic solvent accessible patches. The serine 54 is located in the α-helix 51–63 and an aspartic acid in this position probably promotes a hydrogen bond between carboxylate D54 and the main carbonyl chain of A195 [78]. This would prevent the separation of helices encompassing residues 51–63 and 191–211, thus avoiding the formation of the larger hydrophobic crevice that is thought to be the most important weak region for unfolding by anionics [4]. This mutation is the one that leads to the lowest fluorescence of the probe ANS for the whole range of AOT concentrations indicating the development of fewer hydrophobic solvent patches when compared to the WT cutinase. Since no significant changes in the tertiary structure near the tryptophan region or near the active centre were observed, it is reasonable to assume that these hydrophobic crevices that are less exposed in the structure of this mutant are involved in hydrophobic interactions with the surfactant, inducing the unfolding of cutinase. The replacement of a hydrophobic amino acid (L153Q) also reduced the development of hydrophobic solvent accessible patches (the relative fluorescence intensity of ANS was two orders of magnitude lower than that of the WT cutinase). However, the effect of AOT in the vicinity of the tryptophan is similar to the observed for the WT cutinase and probably this is also true near the active centre. This may suggest the existence of stronger electrostatic interactions between AOT and this mutant, responsible for a high destabilisation of the tertiary structure. The T179C mutation in one of the binding loops leads to a decrease in the fluorescence intensity of the probe ANS by 2.5 orders of magnitude when compared to the WT cutinase. This suggests that this mutation also avoids the exposition of hydrophobic crevices since both regions near the tryptophan and the active centre exhibit a weaker destabilisation in the presence of the surfactant. Generally, the ANS fluorescence increases before changes in the tryptophan and tyrosine fluorescence occur, indicating that the access of ANS to the protein hydrophobic regions occurs before any significant changes in the tertiary structure can be detected by the tryptophan and tyrosine fluorescence. The unfolding curves of the mutants suggest the presence of an intermediate, that
clearly shares some of the characteristics found in other proteins, that is molten globe in character, e.g., native-like secondary structure, with disordered tertiary structure and higher ANS fluorescence [38–41].

It is worth to note that the susceptibility to unfolding of cutinases cannot be explained solely by electrostatic interactions. The degree of stabilisation of the native state, which in turn might reveal the vulnerability of surfactant association and the stabilisation of transition states and intermediates in the unfolding pathway should also be considered. To clarify this point the equilibrium unfolding of the WT cutinase and mutants induced by GdnHCl was studied. However, the unfolding does not allow a better understanding since WT cutinase and variants show the same unfolding profiles, not being influenced by the addition of AOT until MR = 10.

5. Concluding remarks

The results show that the S54D mutant of cutinase is significantly more resistant to AOT denaturation than the WT. This mutant shows a secondary and tertiary structure similar to that of the WT and the increased resistance to AOT may result from the establishment of a hydrogen bond between the carboxyl group of the aromatic acid and the carboxyl chain of the residue A195. This prevents the separation of helices encompassing residues 51–63 and 191–211, thus avoiding the formation of large hydrophobic crevices, responsible for the unfolding by anionic surfactants.

The L153Q mutation also reduced the development of hydrophobic solvent accessible patches. However, stronger electrostatic interactions may occur between the anionic surfactant and this mutation, as the effect of AOT in the tertiary structure is the same as that observed for the WT cutinase.

The T179C mutation located close to the active centre and to disulﬁde bond Cys171–Cys178 introduced changes in the cutinase structure that were observed even in the cutinase region around the tryptophan residue. Nevertheless, this mutation also reduced the development of hydrophobic solvent accessible patches.

After the saturation of the binding sites by AOT, this surfactant can interact with cutinase regions of higher mobility, allowing access of the surfactant to the hydrophobic regions. As cutinase has certainly more than one independent binding site, the interaction of the surfactant with all sites at the protein surface cannot be avoided. However, if the L153Q, S54D and T179C mutations prevent the development of hydrophobic crevices formed upon initial unfolding or even by the initial binding of surfactant to specific sites, the interaction between surfactant and protein may be reduced. These regions can be detergent sensitive zones at the cutinase surface and furnish the key for the design of biocatalysts with sufficient stability for practical applications in detergent industry.

Acknowledgements

V. Brissos gratefully acknowledges PhD grant SFRH/BD/9019/2002 from Fundação para a Ciência e Tecnologia. J. M. G. Martinho acknowledges the financial support from the IN – Instituto de Nanociências e Nanotecnologias. The authors are indebted to Prof. João Pessoa for Circular Dichroism facilities.

References

1334

V. Brissos et al. / Biochimica et Biophysica Acta 1784 (2008) 1326–1334


