Thermal unfolding of proteins at high pH range studied by UV absorbance

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Abstract

This work describes a methodology to monitor protein unfolding by using the well known changes in tyrosine absorbance with the ionization of the side chain phenol group. It can be applied to proteins that are functionally active at pH values higher than 9.0 where the current UV differential spectroscopy technique can not be used. The simplicity and facility of the proposed methodology (only two absorbance measurements have to be acquired) can make it very useful namely for technological applications. Thermal unfolding of cutinase and \( \alpha \)-chymotrypsin were followed using this methodology and the thermodynamic stability data was obtained assuming a two-state mechanism. The transition from the folded to the unfolded state was further confirmed by fluorescence maxima for both proteins proving the validity of the methodology based on UV measurements. © 1997 Elsevier Science B.V.

Keywords: Protein structure and analysis; Protein unfolding; Cutinase; \( \alpha \)-Chymotrypsin

Abbreviations: NATrpA, \( N \)-acetyl-l-tryptophanamide; NATyrA, \( N \)-acetyl-l-tyrosinamide; NAPhe, \( N \)-acetyl-l-phenylalanine; cut, cutinase; prot, protein; A, absorbance.

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1. Introduction

The three-dimensional structure of a protein is essential to its biological activity. Structural alterations at this level, namely the unfolding or denaturation of proteins, are frequently responsible for the loss of activity. Adverse environmental conditions such as heat, oxidation, detergents and other denaturing agents, weaken or even break the intramolecular forces responsible for maintaining the active form of a protein, thereby causing the protein molecule to unfold.

Several techniques can be used to follow the unfolding of a protein, such as absorbance, fluorescence, circular dichroism, optical rotatory dispersion, nuclear magnetic resonance, viscosity and other hydrodynamic methods [1]. Most of these techniques need expensive equipment and/or extensive technical knowledge. On the other hand, UV absorbance is a current laboratory technique that does not have special requirements.

The UV absorbance changes following tyrosyl ionization can be used to monitor protein unfolding. Buried tyrosine residues are not affected by the pH. The unfolding of the protein molecule leads to the exposure of these residues at the protein surface and therefore tyrosyl ionization is detected. In this paper we measured the degree of ionization of tyrosine residues and used it to follow protein unfolding. The methodology proposed is very simple and easy to perform making it very useful to evaluate protein denaturation. Provided the number and nature of aromatic amino acids of the protein is known, two absorbance measurements, one at 250 and the other at 278 nm, are sufficient to obtain the degree of tyrosine ionization in the protein. The requisite of knowing the number and nature of the aromatic amino acids is not highly restrictive since currently molecular biology techniques supply this information. Since the unfolding of the protein is detected from the ionization of the side chain phenol group of tyrosine residues, the method can only be applied in the alkaline pH range (pH \textless 9.0) where the phenolic hydrogen ionization is measurable. At these high pH values the current UV differential spectroscopy is hardly used to follow protein unfolding. The UV differential technique is based on the exposition of some aromatic residues buried within the hydrophobic core of the protein to the aqueous solvent during unfolding, giving rise to a decrease in absorbance in the 285–295 nm range. Tyrosinate formation at high pH during unfolding leads to an increase in absorbance in the same wavelength range (280–310 nm). This absorbance increase overcomes the red-shift resulting from solvent exposure, thus preventing the correct protein unfolding analysis by UV differential spectroscopy. Additionally, the methodology proposed in this paper requires smaller amounts of protein than UV differential spectroscopy.

As a model system to validate the method, a recombinant cutinase from \textit{Fusarium solani} was used. Cutinase is a term originally proposed to describe an enzyme capable of degrading the insoluble lipid polyester matrix, i.e., cutin, which covers plant surfaces. This enzyme also displays lipolytic activity, being able to hydrolyse ester bonds of fatty acid esters (E.C.3.1.1.) [2]. Cutinase can be studied by this methodology since it displays activity between pH 7.5 and 11 [3]. The method was further applied to \(\alpha\)-chymotrypsin (E.C. 3.4.21.1) from bovine pancreas to show its more general applicability. \(\alpha\)-Chymotrypsin also displays activity in the alkaline pH range [4]. The thermal unfolding
transition detected by this methodology was further confirmed by fluorescence maxima for both proteins.

2. Materials and methods

2.1. Chemicals

Recombinant cutinase (MW 22KD, pI 7.0) was a gift from CORVAS International NV (Gent, Belgium). This preparation was obtained as a lyophilized powder of over 95% purity (w/w). α-Chymotrypsin type II from bovine pancreas (MW 25KD) was obtained from Sigma.

N-acetyl-l-tryptophanamide (NATrpA) (crystalline), N-acetyl-l-tyrosinamide (NATyrA) (crystalline), N-acetyl-l-phenylalanine (NAPhe) (Sigma grade) were purchased from Sigma. All salts were of analytical reagent grade and were purchased from Merck.

2.2. pH measurements

The effect of temperature on the pH of carbonate–bicarbonate buffer and on the pK of NATyrA was assessed by using a Crison micropH 2001 pH meter with an automatic temperature probe.

2.3. Spectroscopy

Absorbance measurements were carried out on a JASCO V-560 UV–VIS spectrophotometer.

Fluorescence measurements were obtained by excitation at 280 nm on a Perkin-Elmer MPF-3 fluorescence spectrophotometer with 90° geometry. The instrumental response at each wavelength was corrected by the use of a curve obtained using appropriate fluorescence standards [5].

The absorbance spectra of NATyrA, NATrpA and NAPhe were obtained using concentrations of $3.6 \times 10^{-5}$, $6 \times 10^{-6}$ and $3.6 \times 10^{-5}$ M, respectively, in a 5 mM Tris-HCl buffer at pH 7.6. Sodium hydroxide was used to titrate the ionizing groups. These concentrations were maintained when the mixture of the three components was studied and hence the ratio of concentrations in the mixture was 6:1:6 for NATyrA, NATrpA and NAPhe, respectively. This ratio of concentrations was chosen as this is also the ratio of tyrosine, tryptophan and phenylalanine residues in the primary structure of cutinase [6].

2.4. Thermal unfolding curves

Thermal unfolding of cutinase and α-chymotrypsin was carried out with the cell temperature calibrated against the bath (refrigerated circulator Haake DC3-K15) temperature. Cutinase fluorescence and absorbance spectra were obtained at the reported
cell temperature with a protein concentration of $6 \times 10^{-6} \text{ M}$ in a 50 mM Tris-HCl buffer at pH 8.0 and in a 50 mM carbonate–bicarbonate buffer above pH 9.5. α-Chymotrypsin fluorescence and absorbance spectra were obtained at the reported cell temperature with a protein concentration of $1.5 \times 10^{-6} \text{ M}$ in a 50 mM carbonate–bicarbonate buffer. Analysis of the UV data was based on a two-state model where the equilibrium constant for the unfolded and the folded state of the protein is given by:

$$K_{u/f} = \frac{Y_f - Y}{Y - Y_u}$$

where $Y_f$ and $Y_u$ are the values of $\alpha_{prot}/\alpha$ (degree of exposure of tyrosine residues at the protein surface—see results Section 3.2) characteristic of the folded and unfolded state, respectively, under the temperature where $Y$ is being measured. $Y_f$ and $Y_u$ are linear functions dependent on temperature, both in pre- and post-transition regions. The temperature at the midpoint of the thermal unfolding curve ($T_m$) where $\Delta G = 0$ was calculated from a plot of $\Delta G$ versus temperature using $\Delta G$ values within the range $\pm 2$ Kcal/mol.

3. Results

3.1. Principles

The ionic behavior of the N-acetyl-amide derivatives of aromatic l-amino acids were studied in the alkaline pH range. The utilization of these derivatives (except for phenylalanine where the N-acetyl derivative was used) simulate the ionic behavior of amino acids in the primary structure of proteins. Both the $\alpha$-carboxyl and the $\alpha$-amino group are derivatized as they would be in the peptide bond and thus the titration of side chains is decoupled from charge effects of $\alpha$-substituents.

The near-UV absorption spectra of NATyrA changes dramatically when the pH is raised from 7.6 to 12.9 (Fig. 1). There is an increase in the molar absorption coefficient in the 250 nm region and in the peak of maximum absorbance which shifts from 275 to 293 nm. These changes result from the ionization of the phenolic hydrogen [7] from the lateral chain of NATyrA. From the absorbance at 250 nm (Fig. 1) a value of 10.1 was determined for the pK of this phenolic hydrogen at 25°C. Two isosbestic points, at 269 and 278 nm (phenol and phenolate ion have the same molar absorption coefficient at these wavelengths), were also determined from the intersection point of each absorption spectrum of Fig. 1.

In contrast, the near-UV spectra of NATrpA and NAPhe do not change within the same range of pH (Figs. 2 and 3), reflecting the absence of ionizable groups in the side chain of these amino acid derivatives.

Fig. 4 shows the pH effect on the absorbance at 250 and 278 nm for a mixture of NATyrA, NATrpA and NAPhe with a ratio of concentrations of 6:1:6, respectively (see Section 2.3) for cutinase in aqueous solution and for thermal denaturated cutinase at 25°C. For the mixture of free amino acid derivatives, the absorbance at 250 nm increases dramatically with the increase of pH due to ionization of phenolic hydrogen of NATyrA.
Fig. 1. Titration of N-acetyl-L-tyrosinamide (NATyrA) (3.6 × 10^{-5} M) between pH 7.6 and 12.9 by near-UV absorbance.

Fig. 2. Titration of N-acetyl-L-tryptophanamide (NATrpA) (6 × 10^{-4} M) between pH 7.6 and 12.9 by near-UV absorbance.
Fig. 3. Titration of N-acetyl-l-phenylalanine (NAPhe) (3.6 × 10^{-3} M) between pH 7.6 and 12.6 by near-UV absorbance.

The absorbance at 278 nm does not change with the pH since at this wavelength phenol and phenolate ion have the same molar absorption coefficient ($\varepsilon = 1623$ M^{-1} cm^{-1} (Table 1)).

When cutinase was studied in the same range of pH at room temperature, no significant changes were noted. The absorbance at 250 nm is constant when the pH is raised from 8.0 to 10.5 (Fig. 4). However, if unfolding of the cutinase molecule is induced by thermal denaturation, the near-UV spectra begin to change with the same pattern as NATyrA, which is mainly characterized by an increase in the absorbance at 250 nm (Figs. 4 and 5). Since the main contributors to the near-UV spectra of proteins are the aromatic amino acids [7], the alterations observed for denaturated cutinase relative to native cutinase are due to changes in the absorption spectra of tyrosine residues. These are the sole aromatic amino acids whose near-UV spectrum is affected by pH, as it was shown by the N-acetyl-amide derivatives. This means that for native cutinase, the phenol groups of tyrosine residues are not affected by pH due to a buried location or to a higher pK than the one obtained for NATyrA (pK = 10.1). Solvent contact surface [8] for tyrosine residues in cutinase was calculated from cutinase structure [9]. The values were very low (from 7 for Tyr 119 to 0% for the others tyrosine residues) showing clearly a buried location. When the protein molecule unfolds tyrosine residues are exposed at the surface or the interactions responsible for the increase in the pK value are broken and the ionization of the phenolic hydrogen occurs.

It is also shown in Fig. 4 that at 278 nm the absorbance of cutinase in the folded state
Fig. 4. pH effect on the absorbance of native cutinase (♦ $A^{258}$, □ $A^{278}$), thermal denaturated cutinase (▲ $A^{258}$, △ $A^{278}$) and of a mixture of NATyrA, NATrpA and NAPhe with a ratio of concentration of 6:1:6, respectively (■ $A^{258}$, □ $A^{278}$) at 25°C. The 6:1:6 ratio is that of tyrosine, tryptophan and phenylalanine residues in cutinase, respectively [6]. Full symbols are the absorbance values at 250 nm and empty symbols are the absorbance values at 278 nm.

(native cutinase) and in the unfolded state (thermal denaturated cutinase), is close to the absorbance of the mixture of free amino acid derivatives. Thus, at 278 nm the following approximation can be made:

$$A_{prot}^{278} = (\varepsilon_{NATyrA}n_{Tyr}^{\text{prot}}[prot]) + (\varepsilon_{NATrpA}n_{Trp}^{\text{prot}}[prot]) + (\varepsilon_{NAPhe}n_{Phe}^{\text{prot}}[prot])$$

$$= (\varepsilon_{NATyrA}n_{Tyr}^{\text{prot}}[prot]) + (\varepsilon_{NATrpA}n_{Trp}^{\text{prot}}[prot])$$

(1)

since NAPhe dode not absorb at 278 nm (Fig. 3).

$\varepsilon_{\text{NATyrA, NATrpA, NAPhe}}$ are the molar absorption coefficient of NATyrA, NATrpA and

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>$\varepsilon_{\text{NATyr(OH)A}}$</th>
<th>$\varepsilon_{\text{NATyr(OH)+A}}$</th>
<th>$\varepsilon_{\text{NATyrA}}$</th>
<th>$\varepsilon_{\text{NAPhe}}$</th>
</tr>
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<tbody>
<tr>
<td>250</td>
<td>390</td>
<td>9460</td>
<td>2260</td>
<td>140</td>
</tr>
<tr>
<td>278</td>
<td>1623*</td>
<td>1623*</td>
<td>5640</td>
<td>0</td>
</tr>
</tbody>
</table>

*Average of the determination made at pH 7.7 and 12.4 (1623±216 M$^{-1}$ cm$^{-1}$).
Fig. 5. Absorbance spectra of cutinase at pH 9.6 (25°C) with increasing temperature from 23 to 65°C.

NaPhe, respectively, $n^\text{Tyr,Trp,Phe}$ are the number of tyrosine, tryptophan and phenylalanine residues per molecule of protein, $[\text{prot}]$ is the protein concentration and $l$ is the optical path length.

The absorbance of cutinase at 250 nm depends on the ionization state of tyrosines. At pH 8.0, where the percentage of phenol ionization obtained from the Henderson–Hasselbalch equation is 0.8% (% of ionization in the mixture of free amino acid derivatives), folded cutinase and the mixture have the same value of absorbance (Fig. 4). When the pH is raised, the increase in absorbance only occurs for unfolded cutinase, where the phenol group of tyrosine residues can be ionized. The lower absorbance value at 250 nm obtained for thermal denaturated cutinase relative to the mixture of free amino acid derivatives is due to the fact that tyrosine residues are probably not completely exposed to solvent or not totally free of intramolecular interactions in the unfolded state [10]. Thus the approximation made at 278 nm can also be made at 250 nm but at this wavelength phenylalanine residues contribute to the absorbance which is greatly affected by the degree of phenol ionization:

\[
A_{250}^{\text{prot}} = (\varepsilon_{\text{NATyr(OH)A}}n^\text{Tyr}A)[\text{prot}]l + (\varepsilon_{\text{NATyr(OH)^{-}A}}n^\text{Tyr}A)[\text{prot}]l
\]
\[
+ (\varepsilon_{\text{NATrpA}}n^\text{Trp}A)[\text{prot}]l + (\varepsilon_{\text{NaPhe}}n^\text{Phe}A)[\text{prot}]l
\]  

$\varepsilon_{\text{NATyr(OH)A}}, \varepsilon_{\text{NATyr(OH)^{-}A}}, \varepsilon_{\text{NATrpA}}, \varepsilon_{\text{NaPhe}}$ are the molar absorption coefficients of NATyrA at pH 7.7 (percentage of phenol ionization obtained from the Henderson–Hasselbalch is 0.4%), NATyrA at pH 12.4 (percentage of phenol ionization obtained from the
Henderson–Hasselbalch is 99.5%), NATrpA and NAPhe, respectively (Table 1) and $\alpha_{prot}$ is the degree of phenol ionization of tyrosine residues in the protein.

### 3.2. Methodology

The degree of phenol ionization of tyrosine residues in a protein can be calculated from Eq. (2), but it is highly affected by errors in the value of the protein concentration. If the ratio of absorbances $A_{250}/A_{278}$ is used, the degree of phenol ionization becomes independent of the protein concentration and thus a more accurate value of $\alpha_{prot}$ is obtained. Therefore, once the absorbances at 250 and 278 nm of a protein sample are determined and the values of molar absorption coefficient of the different aromatic amino acid derivatives (Table 1) are provided, $\alpha_{prot}$ can be obtained from the equation:

$$
\alpha_{prot} = \frac{a + b + c - d - e}{a - f}
$$

where

- $a = A_{prot}^{278} e_\text{NATyr(OH)}^{250} n_\text{Tyr}^o$
- $b = A_{prot}^{278} e_\text{NATrpA}^{250} n_\text{Trp}^o$
- $c = A_{prot}^{278} e_\text{NAPhe}^{250} n_\text{Phe}^o$
- $d = A_{prot}^{250} e_\text{NATyr}^{278} n_\text{Tyr}^o$
- $e = A_{prot}^{250} e_\text{NATrpA}^{278} n_\text{Trp}^o$
- $f = A_{prot}^{278} e_\text{NATyr(O)}^{250} n_\text{Tyr}^o$

Applying the absorbance values of Fig. 4 to Eq. (3) the degree of phenol ionization for folded cutinase ($\alpha_{\text{native cut}}$) and for unfolded cutinase ($\alpha_{\text{denatured cut}}$) at pH 9.5 and 10.5 can be calculated. $\alpha_{\text{native cut}}$ is 0.00 and 0.03 for pH 9.5 and 10.5, respectively, as would be expected, since there is no significant increase in the absorbance value at 250 nm when the pH was raised from 8.0 to 10.5. When unfolding of cutinase was induced, $\alpha$ increases to 0.15 and 0.61 for pH 9.5 and 10.5, respectively, and thus an increase in the absorbance at 250 nm was observed (Fig. 4).

If the protein structure acquires an ideal unfolded state with residues isolated from one another by the solvent it will be expected that tyrosine residues behave as NATyr and the degree of phenol ionization will be given by the Henderson–Hasselbalch equation:

$$
\alpha = \frac{10^{pH - pK}}{1 + 10^{pH - pK}}
$$

Then the ratio between the degree of phenol ionization in a protein ($\alpha_{prot}$) and the degree predicted by the Henderson–Hasselbalch ($\alpha$),
describes the degree of tyrosine residues exposure at the protein surface. This degree can be used to follow the transition from the folded to the unfolded state (see Section 3.3 below) since tyrosine residues are more solvent exposed in the unfolded state. Obviously this methodology can only be applied if the increase in the tyrosine solvent exposure is detectable, i.e., when the pH causes some degree of phenol ionization ($\text{pH} \geq 9.0 \Rightarrow \alpha \
lineq 0.07$).

### 3.3. Application

Fig. 5 shows the temperature effect on the absorption spectra of cutinase at pH 9.6. Absorbance changes occurring with the increase in temperature reflect only tyrosine ionization since there is no temperature effect on the molar absorption coefficient of the $N$-acetyl-amide derivatives of aromatic amino acids (data not shown). It is interesting to note that the isosbestic points of tyrosine and tyrosinate as protein chromophores are different from the ones obtained for NATyrA (269 and 278 nm) and that they depend on the pH. They occur at 274 and 290 nm for cutinase at pH 9.6 (Fig. 5) and at 273 and 283 nm for cutinase at pH 10.7 (data not shown). $\alpha$-Chymotrypsin shows also two isosbestic points occurring respectively at 273 and 297 nm (data not shown). The small difference between the isosbestic point of NATyrA used in Eq. (3) and the ones obtained for cutinase and for $\alpha$-chymotrypsin justify the utilization of Eq. (3) to calculate $\alpha_{\text{prot}}$.

The temperature range where cutinase unfolds is clearly identified in Fig. 5. Tyrosine solvent exposure increases mostly when temperature raises from 46.4 to 55°C and can be easily detected by the increase in the absorbance at 250 nm. Absorbance data obtained from the thermal unfolding of cutinase at pH 9.6 (Fig. 5) and at pH 10.7 (data not shown) was used to calculate $\alpha_{\text{prot}}$ by using Eq. (3). $\alpha$ was then calculated by the Henderson–Hasselbalch Eq. (4) taking into account the temperature effect on the buffer pH and on the tyrosine pK. We calculate for carbonate–bicarbonate buffer $dpH/dT = -0.009 \text{ unit/degree}$ and for NATyrA $dpK/dT = -0.016 \text{ unit/degree}$. pH buffer and tyrosine pK at temperature $T$ are thus $[\text{pH} + (T-25°C) \times -0.009]$ and $[\text{pK} + (T-25°C) \times -0.016]$, respectively. Borate buffer (pH 9.3–10.7) can also be used since $dpH/dT = -0.008 \text{ unit/degree}$ is small [11].

The ratio $\alpha_{\text{prot}}/\alpha$ was then used to follow the thermal unfolding transition of cutinase at pH 9.6 and 10.7 (Fig. 6). This transition is irreversible at these conditions (high temperature and pH) probably due to chemical reactivity of thiol groups or disulfide bonds [12] but no protein aggregates leading to light scattering are observed. The same approach was used to follow the thermal unfolding of $\alpha$-chymotrypsin at pH 10.0 (Fig. 7) which has eight tryptophan, four tyrosine and six phenylalanine residues [13]. Accurate curves were obtained with a single-step unfolding process observed for both proteins. The thermal unfolding transition was further confirmed by fluorescence maxima for both proteins (Figs. 6 and 7). Tryptophan residues emitting at longer wavelengths indicate a more solvent exposed localization which characterize the
Fig. 6. Thermal unfolding curves (continuous lines) for cutinase at pH 9.6 (●) and 10.7 (○) followed by the degree of tyrosine exposure at the protein surface ($\alpha_{\text{max}}/\alpha$). Thermal unfolding transition detected by fluorescence maxima ($\bullet$ pH 9.6; □ pH 10.7) occurs within the same range of temperature. The dashed lines were drawn to facilitate visual inspection of the unfolding transition detected by fluorescence maxima.

Thermal induced transition detected by UV data and by fluorescence maxima occur within the same range of temperature.

Thermal unfolding curves followed by the degree of tyrosine exposure at the protein surface were analysed assuming a two-state mechanism and the data is shown in

Fig. 7. Thermal unfolding curve (continuous lines) for α-chymotrypsin at pH 10.0 (●) followed by the degree of tyrosine exposure at the protein surface ($\alpha_{\text{max}}/\alpha$). Thermal unfolding transition detected by fluorescence maxima ($\bullet$ pH 10.0) occurs within the same range of temperature. The dashed line was drawn to facilitate visual inspection of the unfolding transition detected by fluorescence maxima.
Table 2. The temperature at the midpoint of the thermal unfolding curve, $T_m$, is lower for cutinase at pH 10.7 than for cutinase at pH 9.6. Higher pH values destabilize the native conformation of cutinase.

4. Discussion

Current UV differential spectroscopy cannot be used to follow protein unfolding at high pH values. The absorbance increase resulting from tyrosyl ionization cancels out the red-shift caused by solvent exposure, thus preventing the correct analysis of the unfolding process. However, some proteins have their active conformation in the alkaline pH range where they display activity. For these proteins, we propose a different methodology which makes use of the UV absorbance changes resulting from tyrosyl ionization at high pH range to follow the thermal unfolding of proteins.

The method was applied to two proteins displaying activity in the alkaline pH range, a cutinase from *Fusarium solani* and $\alpha$-chymotrypsin from bovine pancreas. Higher ratios of tyrosine to tryptophan in the protein structure increases the magnitude of the absorbance change used to follow the unfolding of the protein. Cutinase with a tyrosine to tryptophan ratio of 6:1, respectively, is a favourable case. But the method is also adequate when the ratio is much more unfavourable. This is the case for $\alpha$-chymotrypsin with a tyrosine to tryptophan ratio of 4:8, respectively.

The methodology proposed accounts for the protein absorbance as the sum of its aromatic residues. In spite of its previous application [7], this assumption should be used with caution since the well-known red-shift in the peak position of aromatic amino acids, when they are buried inside the protein molecule, as well as the changes in absorbance produced by the transfer of a residue from the protein core to water [15] can cause some deviation. These changes are well documented by Li et al. [16]. They found that tyrosine residues of ketosteroid isomerase located in a hydrophobic environment can present a molar absorption coefficient up to 32% higher than a tyrosine residue near the surface of the protein. However, these deviations between the protein absorbance and that of aromatic amino acids are negligible for the proposed method since the protein absorbance at 250 nm can increase up to 2426% (9460/390) with tyrosyl ionization.

It is also assumed that the absorbance of protein at 278 nm does not change with the
degree of phenol ionization. This is not exactly true since the phenol–phenolate isosbestic point in proteins can be different from 278 nm as shown for cutinase and α-chymotrypsin. However, changes in the absorbance at 278 nm are certainly very little when compared to the changes that occur at 250 nm, as demonstrated by the two proteins, and thus do not greatly affect the accuracy of the method. The existence of well-defined isosbestic points for both proteins prove that the absorbance changes result exclusively from two species, tyrosine and tyrosinate.

Another important question that can be raised is whether the pK of tyrosine residues may be different from 10.1. For example, Li et al. [16] found an unusually high value of 11.6 for the pK of a tyrosine residue located in a hydrophobic environment. This method only assumes that the pK of tyrosine residues is 10.1 at 25°C when the protein acquires an ideal unfolded state. This assumption should be valid when residues were isolated from one to another by the solvent. This ideal state is hardly attained. The unfolded state of cutinase shows a value for $\alpha_{\text{prot}}/\alpha$ slightly higher at pH 10.7 than at pH 9.6 but less than one in both cases (Fig. 6). The value for $\alpha_{\text{prot}}/\alpha < 1$ means that the net pK of tyrosine residues in the unfolded state is higher than 10.1. This can be due to the fact that tyrosine residues are not completely solvent exposed or not totally free of intramolecular interactions in the unfolded state. On the contrary for α-chymotrypsin the $\alpha_{\text{prot}}/\alpha > 1$ value results in a net pK lower than 10.1. Lower pK values for ionizable residues in proteins can result, for example, from neighboring positive charges. The possibility that the unfolded state in the absence of chemical denaturants retains a significant amount of residual structure and electrostatic interactions, especially at extremes of pH, has been refereed [17]. The two and the five disulphide bonds that exist in cutinase and in α-chymotrypsin, respectively, can contribute to structural constraints in the unfolded state.

Both folded and unfolded state of the two proteins were independently confirmed by fluorescence spectroscopy. The thermal induced transition detected by the two techniques occur within the same range of temperature proving the validity of the methodology based on UV measurements. The method was shown to be appropriate for the two proteins studied but its principles should be valid for a broader range of proteins and applications. For example, this methodology can be very useful for technological applications where detecting protein denaturation easily can be a very important aid to developing improved enzyme stability. The alternative use of destabilizing agents to study denaturation such as urea or guanidinium chloride is not straightforward since they change the polarity of the solvent [15]. Their effects upon the pK of the N-acetyl-L-tyrosinamide need to be evaluated beforehand.

5. Simplified description of the method and its (future) applications

Thermal unfolding of proteins at pH values higher than 9.0 was studied by an UV absorbance methodology.

A protein solution in carbonate–bicarbonate or borate buffer at pH > 9.0 was incubated at room temperature. Lower protein concentrations ($A_{\lambda^0} < 0.05$) should be used to prevent any protein precipitation due to thermal denaturation. Stepwise
temperature increases were carried out and absorbance at 250 and 278 nm was measured at each temperature after the sample had reached equilibrium. These absorbance values applied to Eq. (3) were used to calculate the degree of protein phenol ionization ($\alpha_{\text{prot}}$). The Henderson–Hasselbalch equation (Eq. (4)), taking into account the temperature effect on the buffer pH and on the tyrosine $pK_a$, was then used to calculate the phenol ionization for an ideal protein unfolded state ($\alpha$). The transition from the folded to the unfolded state was followed by the ratio $\alpha_{\text{prot}}/\alpha$ since protein phenol ionization increases for the unfolded state.

The methodology is useful for studying unfolding of proteins in the alkaline pH range where current UV differential spectroscopy can not be applied. The simplicity and facility of this methodology can make it very useful namely for technological applications. Long-term stability under denaturing conditions (e.g., organic solvents) can also be studied by this method provided their effect upon the $pK_a$ of the $N$-acetyl-tyrosinamide is evaluated.

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