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ARTICLE

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# Secondary structure of prothymosin α evidenced for conformational transitions induced by changes in temperature and concentration of *n*-dodecyltrimethylammonium bromide

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**Abstract** Conformational changes of prothymosin α (ProTα) induced by changes in temperature and concentration of the denaturant *n*-dodecyltrimethylammonium bromide ($C_{12}$TAB) were studied by difference spectroscopy. The conformational transition of ProTα by $C_{12}$TAB was followed as a function of denaturant concentration by absorbance measurements at 230 nm and the data were analyzed to obtain the Gibbs energy of the transition in water $\left(\Delta G^0_w\right)$ and in a hydrophobic environment $\left(\Delta G^0_{hc}\right)$ for saturated protein-surfactant complexes. The value of $\Delta G^0_w$ was 6.38 kJ mol$^{-1}$ and that for $\Delta G^0_{hc}$, which is not affected by temperature, was –18.62 kJ mol$^{-1}$. Changes of absorbance at 230 nm of ProTα with temperature can be assumed to resemble a transition in the secondary structure. The parameters characterizing the thermodynamics of unfolding, melting temperature ($T_m$), enthalpy ($\Delta H_m$), entropy ($\Delta S_m$) and heat capacity ($\Delta C_p$) were determined. The values obtained for $T_m$, $\Delta H_m$ and $\Delta S_m$ are smaller that those found for other globular proteins; $\Delta C_p$ was found to be much smaller. These results suggest that ProTα exhibits some type of secondary structure under these conditions (10 mM glycine buffer, pH 2.4).

**Keywords** Protein stability · Prothymosin α · Difference spectroscopy · Thermal denaturation · Secondary structure

**Abbreviations** *CMC*: critical micelle concentration · *$C_{12}$TAB*: *n*-dodecyltrimethylammonium bromide · *ProTα*: prothymosin alpha

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## Introduction

Prothymosin α (ProTα) is a 109 amino acid protein first isolated from rat thymus and initially classified as one of the so-called thymic hormones (Haritos et al. 1984). It is one of the most abundantly expressed human genes together with myosin light chain, 90 kDa heat shock protein and ribosomal proteins. Two physiological roles have been claimed for ProTα (Piñeiro et al. 2000): (1) it has been shown to be an essential protein for cell growth and (2) its immunogenic functions suggest that it is a biological response modifier (BRM). ProTα is considered one of the most acidic peptides in the eukaryotic world with an isoelectric point of 3.5, exhibiting 18 aspartic and 39 glutamic acid residues. The primary structure is highly unusual, since aromatic and sulfur amino acids are totally absent; consequently, it does not absorb at 280 nm. Figure 1 shows a simple scheme of its structure.

The conformation of ProTα under physiological conditions is also a controversial issue. Although preliminary research suggested the absence of secondary structure (Watts et al. 1990; Schmidt and Werner 1991) an exhaustive study by Gast et al. (1995) using circular dichroism, mass spectrometry, dynamic light scattering and small-angle X-ray scattering revealed that, in solution, ProTα adopts a random coil-like conformation with persistent direction and curvature. This absence of

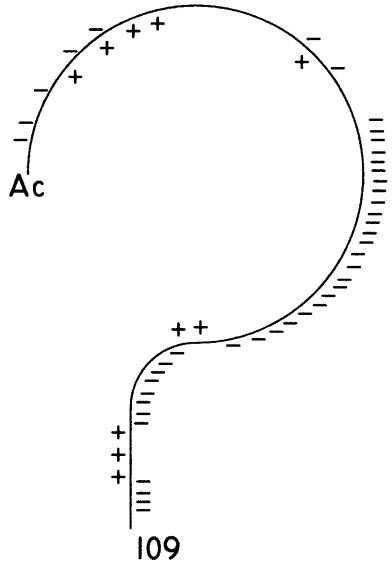


**Fig. 1** An illustration of the structure of prothymosin α (ProTα) showing the acid (–) and basic (+) residues (From Piñeiro et al. 2000)

secondary structure could possibly introduce even more questions concerning this atypical protein. The lack of tertiary structure does not present in itself a problem because, although it is true that the three-dimensional structure of a protein defines its function, it is also true that some proteins take on a tertiary structure when they interact with other molecules or with a receptor (Stanfield and Wilson 1995; Piñeiro et al. 2000). A truly random coil represents a fully denatured structure that cannot be unfolded or denatured further, but it is of course possible that some secondary-type structure is present in a largely random coil conformation and this could be broken down with temperature or by surfactants, which is the subject of our study in this work.

One can judge the stability of any structure by studying its disruption; thus the stability of a protein can be determined by studying its denaturation. Since a protein molecule is a macroscopic system, the disruption of this structure should be regarded as a change of the macroscopic state of the system. However, the main problem is to define the macroscopic states that are realized under a given range of external conditions. The native protein structure can be disrupted by changing different intensive variables specifying the external conditions, such as temperature, pressure, pH and the concentration of denaturants. However, information on the stability of a protein structure can be obtained only by using the temperature as the variable, because the temperature and energy of a system are the conjugate variables determining the state of a macroscopic system (Privalov 1989).

In previous work we have contributed to the study of conformational transitions of different proteins induced by amphiphilic molecules with denaturant capacity: insulin by *n*-alkyl sulfates (Sarmiento et al. 1992; Housaindokht et al. 1993), by *n*-alkyltrimethylammonium bromides (Pombo et al. 1996) and by imipramine (Lopez-Fontan et al. 1999); lysozyme by *n*-alkyltrimethylammonium bromides (Jones et al. 1995; Mosquera et al. 1996) and by *n*-alkyl sulfates (Sarmiento et al. 1998); and albumin by the penicillins cloxacillin, dicloxacillin, flucoxacillin and nafcillin (Taboada et al. 2000a, 2000b). We have used different physical techniques such as microcalorimetry, difference spectroscopy, ζ-potential and equilibrium dialysis. These studies have permitted us to analyze in detail the process of disruption of native protein structures, obtaining all the thermodynamic parameters corresponding to the structural changes. Now we investigate the conformation of ProTα in vivo to determine whether it adopts a specific structure under cellular conditions, such as interactions with membranes or receptor molecules, or whether its unfolded conformation might be advantageous for its interaction with other biological molecules.

As a starting point, in this work we studied the conformation of ProTα in solution at low pH in terms of thermodynamic parameters. We demonstrate by means of spectroscopic techniques that ProTα exhibits some type of secondary structure in these conditions.

## Materials and methods

### Reagents

ProTα was a generous gift from Thymoorgan Pharmazie (Vienenburg, Germany). It was extracted from calf thymus according to the methods of Haritos et al. (1984). The surfactant *n*-dodecyltrimethylammonium bromide was obtained from Lancaster MTM. The buffer solution for the experiments was 10 mM glycine-HCl (pH 2.4). All other materials were of analytical grade and solutions were made up in doubly distilled water.

### Critical micelle concentrations

Critical micelle concentrations (CMCs) were measured conductimetrically (del Rio et al. 1994) using a conductivity meter (Kyoto Electronics, type C-117). The conductivity cell (Kyoto, type K-121) was calibrated with KCl solutions in the appropriate concentration range. The CMC values obtained for *n*-dodecyltrimethylammonium bromide ($C_{12}$TAB) in a low pH medium were: 13.10 mM at 15 °C, 13.02 mM at 20 °C, 13.02 mM at 25 °C, 13.15 mM at 30 °C and 13.41 mM at 35 °C.

### Difference spectroscopy

Difference spectra were measured using a Beckman spectrophotometer (model DU 640) with six microcuvettes, which operates in the UV-visible region (from 190 to 1100 nm) of the electromagnetic spectrum. For absorbance measurements with changing temperature, a Beckman (DU series) temperature controller was used, following the Peltier methods of controlling temperature, in the range 15–100 °C.

All measurements were made using ProTα solutions with a concentration of 0.5 g $L^{-1}$ in carefully matched quartz cuvettes (50 μL capacity) in the wavelength range 200–345 nm. For absorbance difference spectra, the cells were filled with protein and ligand solutions, using the protein solutions as reference. Stock solutions of defined protein concentration were prepared by

dissolving a certain amount of freeze-dried protein in an appropriate buffer volume. All cuvettes were filled and placed in the same orientation for all tests. Measurements were made after ProTα and surfactant had been incubated for over 30 min, after which the difference spectra did not change.

All measurements reported refer to surfactant concentrations below the CMC of $C_{12}$TAB.

## Results and discussion

### Effect of the denaturant

To study the effect of $C_{12}$TAB and temperature on the conformation of ProTα, we first established the absorption spectra for the thermal denaturation of active ProTα in 10 mM glycine (pH 2.4) at different temperatures in the wavelength range 200–345 nm.

Figure 2 shows the spectra of the native protein and, as we can see, similar plots were obtained over the temperature range. The results also show a dramatic increase in absorbance, reaching a maximum at 230 nm followed by a progressive decline in the magnitude of the absorbance between 240 and 260 nm. Most noticeably, as the temperature reaches a certain value ( > 30 °C) the absorbance of the 230 nm band increases dramatically.

To evaluate the effect of $C_{12}$TAB on the denaturation of ProTα, we carried out difference spectra for the 230 nm band for native versus $C_{12}$TAB-treated protein. The results (Fig. 3) show two different sets of plots, depending on the temperature used to measure the difference spectra at 230 nm, 35 °C being the cut-off temperature. Thus, experiments carried out at temperatures below 35 °C show a similar pattern for the different surfactant concentrations studied. According to the thermal denaturation results, the difference spectra at 230 nm corresponding to the native versus $C_{12}$TAB-treated ProTα, increase as the temperature (40, 50, 60 and 70 °C) and the surfactant concentrations (2–10 mM) increase.

The plots corresponding to temperatures above 35 °C show practically the same difference spectra values at 230 nm for the different surfactant concentrations. Taking into account the data of Fig. 2, it can be said that under our conditions the interaction of the surfactant with ProTα undergoes a significant increase in the difference spectra, which is proportional to the concentration of surfactant. Even more, the sigmoidal-like shape of these plots supports a significant change in the ProTα conformation. Thus the data show a transition region (4–6 mM $C_{12}$TAB) that is similar for all temperatures below 35 °C. This transition occurs at a concentration of surfactant below the CMC.

Thermodynamically, as a first approximation the denaturation process can be considered as a transition between the two macroscopic states: the native state (N) and a denaturated state with $\bar{v}$ bound surfactant ligands ($DS_{\bar{v}}$) ::

$$N + \bar{v}S \rightleftarrows DS_{\bar{v}} \tag{1}$$

where $\bar{v}$ is the average number of surfactants molecules bound to the denaturated complex $DS_{\bar{v}}$. The two-state character of the transition is a consequence of the cooperative nature of the protein unfolding. The analysis of the equilibrium unfolding transition requires extrapolation of the baseline for the native and unfolded protein into the transition region to determine the fraction of denaturated molecules, $F_D$, as a function of the unfolding parameters (Pace 1990):

$$F_D = \frac{A_N - A_{OBS}}{A_N - A_D} \tag{2}$$

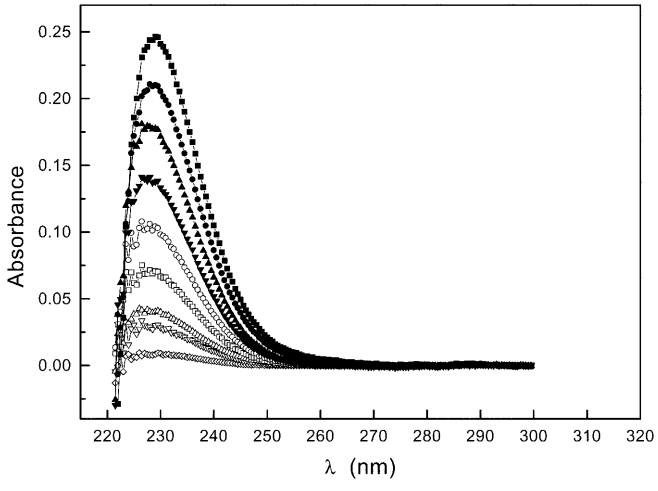


**Fig. 2** Equilibrium studies of thermal denaturation of bovine ProTα. Absorbance spectra of ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$) in 10 mM glycine buffer (pH 2.4) at different temperatures: (◇) 20 °C, (▽) 25 °C, (Δ) 30 °C, (□) 40 °C, (○) 50 °C, (▼) 60 °C, (▲) 70 °C, (●) 80 °C, (■) 90 °C

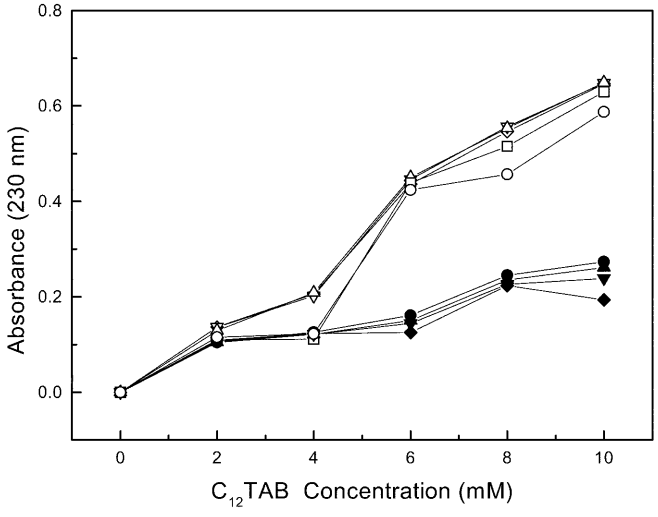


**Fig. 3** Effect of $C_{12}$TAB on the ProTα conformation. Difference spectral band (230 nm) of ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4) in the presence/absence of *n*-dodecyltrimethylammonium bromide ($C_{12}$TAB) at different temperatures: (◇) 20 °C, (▽) 25 °C, (Δ) 30 °C, (□) 35 °C, (○) 40 °C, (◆) 45 °C, (▼) 50 °C, (▲) 60 °C, (●) 70 °C

where $A_{OBS}$ is the absorbance observed and $A_N$ and $A_D$ are the absorbance for the native and denaturated conformations.

The difference in the standard Gibbs energy between the folded and unfolded conformations can then be calculated as:

$$\Delta G^0 = -RT \ln\left[\frac{F_D}{1-F_D}\right] = -RT \ln\left[\frac{A_N - A_{OBS}}{A_{OBS} - A_D}\right] \quad (3)$$

where $R$ is the gas constant and $T$ is the absolute temperature.

The equilibrium constant ($K$) for Eq. (1) is:

$$K = \frac{[DS_{\bar{v}}]}{[N][S]^{\bar{v}}} = \frac{K_S}{[S]^{\bar{v}}} = \frac{F_D/(1-F_D)}{[S]^{\bar{v}}} \quad (4)$$

where $K_S$ is the ratio of denatured to native molecules. Figures 4 and 5 show that $\Delta G°$ and $\ln K_S$ are a linear function of [S] at 25 °C, consistent with the relationships:

$$\Delta G° = \Delta G°_w - m[S] \quad (5)$$

$$\ln K_S = lnK_w + \frac{m}{RT}[S] \quad (6)$$

where $\Delta G°_w$ is the Gibbs energy at zero concentration of denaturant. Thus, to obtain the values $\Delta G°_w$, a reliable procedure for the extrapolation of $\Delta G°([S])$ to zero denaturant concentration, i.e.:

$$\Delta G°_w = \Delta G°([S])_{\lim[S]\to 0} \quad (7)$$

is required. Numerous observations on a large number of different proteins showed that, in the transition range, the Gibbs energy of unfolding is, in a relatively narrow range, a linear function of denaturant concentration (Myers et al. 1995). These observations led to the so-called linear extrapolation method (LEM) for determination of the Gibbs energy of unfolding in the absence of denaturant (Eq. 5), where the so-called denaturant $m$ value is a measure of the dependence of $\Delta G°$ on surfactant concentration, [S], and the $m$[S] term thus represents the difference in transfer Gibbs energy between the unfolded and native states. An equation of the form of Eq. (5) is found for protein denaturation by urea (Pace et al. 1989; Pace 1990).

The most attractive feature of this analysis is that it allows one to estimate the stability of a protein over a range of conditions of interest. On this point, following Plaza del Pino and Sanchez-Ruiz (1995), the difference should be noted between the *thermodynamic stability*, which is the work required to disrupt the native protein structure under particular solution conditions, and temperature and the *thermal stability*, which is understood simply as the temperature at which a protein denatures with a particular solvent and pH.

Thermodynamic stability (stabilization of Gibbs energy) is directly related to the equilibrium between native and denatured states of the protein. This indicates that the higher the thermodynamic stability, the less the probability for the native protein molecule to unfold due to thermal fluctuations and the shorter the time interval during which each protein molecule is unfolded. This, in turn, determines the sensitivity of the protein to hydrolysis or inactivation due to chemical modifications or aggregation of unfolded polypeptide chains. Thus, thermodynamic stability (stabilization of Gibbs energy) of a protein is a parameter which is directly related to the real lifetime of proteins in solutions under native conditions (Kovrigin and Potekhin 2000).

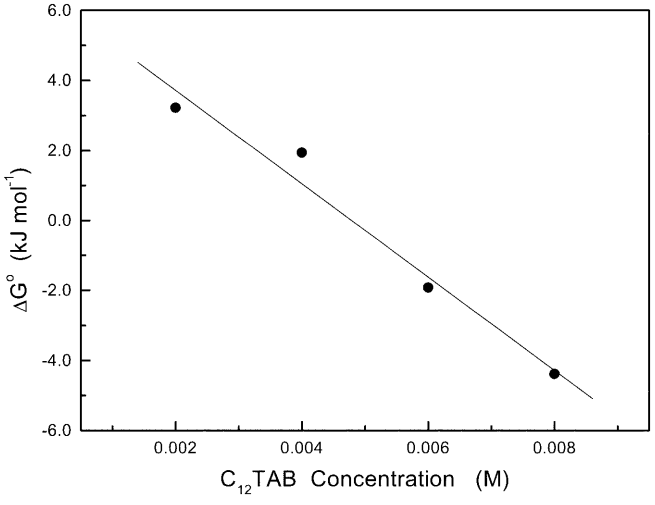


**Fig. 4** Standard Gibbs energy difference between the folded and the unfolded conformations ($\Delta G°$) of ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4) as a function of $C_{12}$TAB concentration. The $\Delta G°$ values were calculated using Eq. (3). The *solid line* represents Eq. (5) with the average parameters given in Table 1

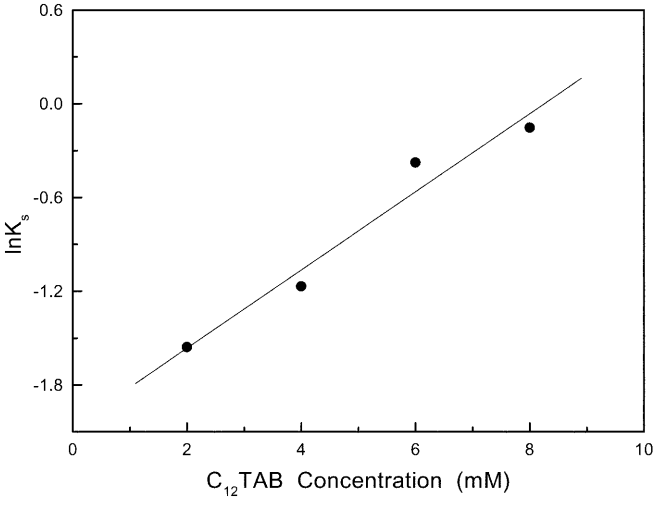


**Fig. 5** Relationship between logarithm of the ratio of denatured to native molecules of ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4), 25 °C, $\ln K_S$, and $C_{12}$TAB concentration

The values of $\Delta G^{\circ}_{w}$ and $m$ have been calculated using Eqs. (5) and (6). These parameters have been derived both from the assumption that $A_N$–$A_D$ is constant, i.e. $A_N$ and $A_D$ were taken from the start and finish of the transition. Table 1 shows the values of $\Delta G^{\circ}_{w}$ and $m$.

It follows from Eq. (4) that:

$$\ln K = \ln K_S - \bar{v} \ln[S] \quad (8)$$

At a surfactant concentration of 1 mol dm$^{-3}$, $\ln K = \ln K_S$ and the equilibrium constant $K$ corresponds to the unfolding transition in a surfactant saturated complex, approximating that in a very hydrophobic environment with a corresponding Gibbs energy change $\Delta G^{\circ}_{hc}$. Using least-squares analysis of the plots of $\ln K_S$ versus ln[$C_{12}$TAB], the values of $\Delta G^{\circ}_{hc}$ were obtained. The values of $\Delta G^{\circ}_{hc} - \Delta G^{\circ}_{w}$, defined as $\Delta(\Delta_{tr}G^{\circ})$, correspond to the difference in the Gibbs energies of transfer of the unfolded and native state from water to a hydrophobic environment, as previously described (Housaindokht et al. 1993; Pombo et al. 1996). All values of the denaturant unfolding parameters are shown in Table 1. $\Delta G^{\circ}_{hc}$ is not affected by the temperature over the transition range observed.

The results of the ProTα denaturation and the values of the parameters obtained from the chemical denaturation suggest that the studied protein has some type of secondary structure which is affected by the denaturant. Thus the values of the thermodynamics parameters of denaturation are in agreement with the values for the unfolding of other proteins, although slightly lower. Moreover, these results confirm the idea developed in several works (Schmidt and Werner 1991; Gast et al. 1995) about a kind of secondary structure for ProTα in an acid medium.

## Effect of the temperature

Another commonly used method for estimating the conformational stability of proteins is an analysis of thermal unfolding curves, like those shown in Fig. 2. Similar plots exhibiting a maximum at 230 nm were obtained for all temperature ranges studied. The absorbance at 230 nm as a function of temperature, in buffered medium (pH 2.4), is shown in Fig. 6. The data show that there is a transition region over which the absorbance changes with temperature. Thermal denaturation studies on ProTα in acid media (pH 2.4) were carried out using the same spectrophotometer described previously. Similar to unfolding with $C_{12}$TAB, a two-state mechanism was assumed in order to evaluate the thermodynamic parameters obtained from spectroscopic techniques, based on the equilibrium constant $K_{eq}$ for $N \rightleftarrows D$ conversion for the transition between these two states, where N represents the native state and D the denaturated state. The equilibrium constant was deduced from the equation

$$K_{eq} = \frac{[\text{Unfolded}]}{[\text{Native}]} = \frac{F_D(T)}{1 - F_D(T)} \quad (9)$$

and the Gibbs energy at a given temperature, $\Delta G(T)$, is related to the equilibrium constant and can be obtained experimentally only in the temperature range where the transition is observed experimentally:

$$\Delta G = -RT \ln K_{eq}(T) \quad (10)$$

Plots of $\Delta G$ versus temperature (Fig. 7) show that the variation of $\Delta G$ with temperature is not linear, which is consistent with thermal denaturations analyzed by other authors with other proteins (Thomson et al. 1989; Farruggia and Pico 1999; Makhatadze 1999) in the transition region. A least-squares analysis of plots of $\Delta G$ versus temperature yields the midpoint of the thermal unfolding curve ($T_m = T$ at $\Delta G = 0$).

The thermal denaturation curves are analyzed in exactly the same way as in the chemical denaturant unfolding curves, but yield $\Delta G$ as a function of temperature rather than denaturant concentration. These results can be used to determine the melting temperature ($T_m$), the entropy change at $T_m$ ($\Delta S_m$), the enthalpy change at $T_m$ ($\Delta H_m$) and the difference in heat capacity ($\Delta C_p$) between the folded and unfolded conformations.

The temperature dependence of $\Delta G$ can be expressed by a modified form of the Gibbs-Helmholtz equation

**Table 1** Parameters characterizing the $C_{12}$TAB unfolding of ProTα at 25 °C, pH 2.4, in 10 mM glycine buffer

| $\Delta G^{\circ}_{w}$ (kJ mol$^{-1}$) | $m$[a] (kJ mol$^{-1}$ M$^{-1}$) | $\ln K$[b] | $\Delta G^{\circ}_{hc}$ (kJ mol$^{-1}$) | $\Delta(\Delta_{tr}G^{\circ})$ (kJ mol$^{-1}$) |
|---|---|---|---|---|
| 6.38 | 1333.3 | 4.94 | –12.24 | –18.62 |

[a] The correlation coefficient of the plots in Fig. 4 is 0.98
[b] The correlation coefficient of linear plot $\ln K_S$ versus ln[$C_{12}$TAB] is 0.97

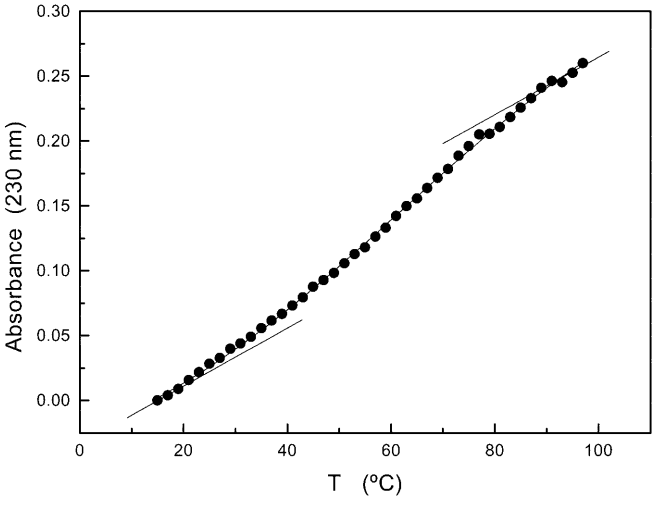


**Fig. 6** Thermal unfolding curve for ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4) using the difference absorbance at 230 nm. The lines shown in the pre- and post-transition region were used to determine the $A_N$ and $A_D$ values used in Eqs. (9) and (10)

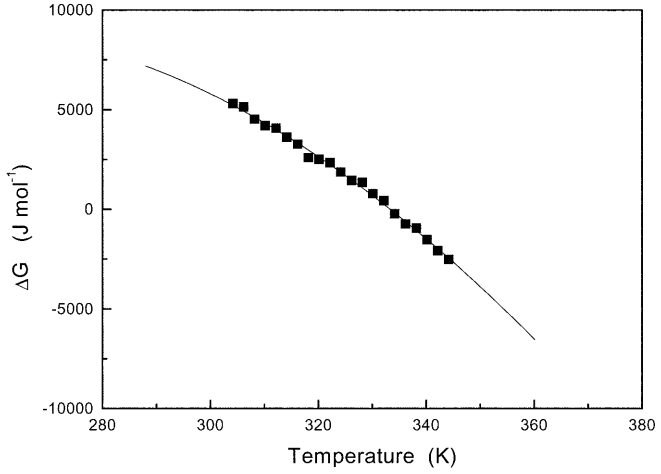


**Fig. 7** Gibbs energy of unfolding ($\Delta G$) of ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4) as a function of temperature. The $\Delta G$ values were calculated using Eq. (10). The *solid line* represents a best fit of Eq. (10) used to determinate the thermodynamic parameters of melting temperature ($T_m$), enthalpy change at the melting temperature ($\Delta H_m$) and heat capacity at the melting temperature ($\Delta C_p$)

**Fig. 8** Thermal unfolding curve for ProTα ($0.5\times10^{-3}$ kg dm$^{-3}$, 10 mM glycine buffer, pH 2.4), using the difference absorbance at 230 nm, in the presence of different concentrations of $C_{12}$TAB: **A** 2 mM, **B** 4 mM, **C** 6 mM, **D** 8 mM, **E** 10 mM

(Pace and Laurents 1989; Pace 1990; Kaushik and Bhat 1998):

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + \ln(T/T_m)] \quad (11)$$

where $\Delta G(T)$ is $\Delta G$ at a temperature $T$. Equation (11) was used to fit the thermal denaturation data by using a non-linear least-squares fit and successive iterations using the Marquardt-Levenberg routine. A minimum of 30 iterations or more was performed until the fractional change in the $\chi^2$ value was within the tolerance limit, which was set to 0.005. The values of the thermal unfolding parameters are listed in Table 2. $T_m$, $\Delta S_m$ and $\Delta H_m$ are of the same order as those found for different globular proteins. However, the $\Delta C_p$ value is smaller than those for other globular proteins.

Figure 8 shows the absorbance changes of ProTα at 230 nm versus temperature at several denaturant concentrations of $C_{12}$TAB. All the measurements were made in a pH 2.4 medium. Furthermore, this figure shows a denaturation transition which is greater when the $C_{12}$TAB concentration is above 4 mM. At a temperature range between 30 and 40 °C a sudden change in the absorbance is observed, which increases as the $C_{12}$TAB concentration rises. These results show two effects: first, that of thermal unfolding; and second, that of surfactant-induced unfolding.

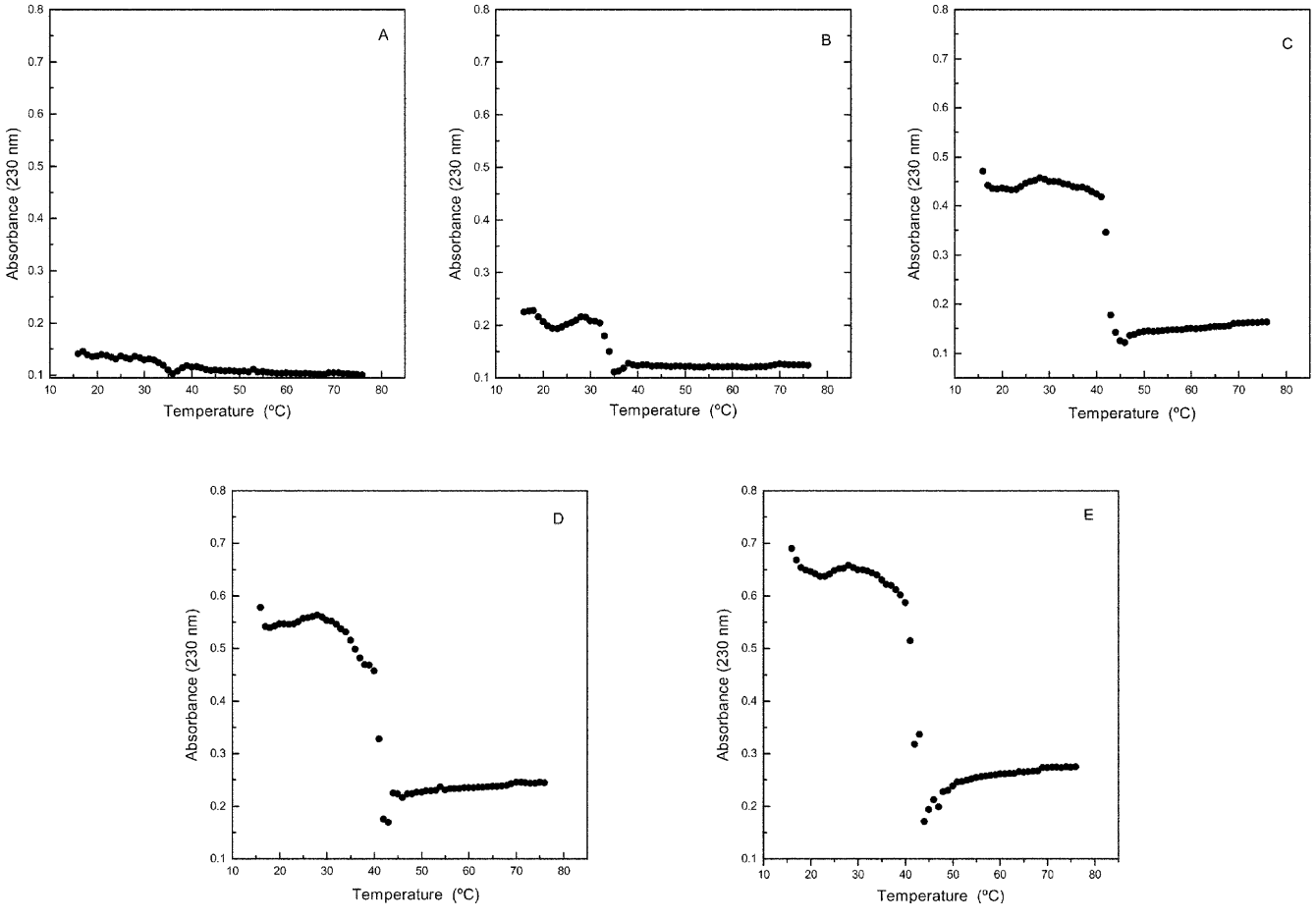

**Table 2** Parameters characterizing the thermal unfolding of some proteins

| Protein | $T_m$ (°C) | $\Delta H_m$ (kJ mol$^{-1}$) | $\Delta C_p$ (kJ mol$^{-1}$ K$^{-1}$) | $\Delta S_m$ (kJ mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|
| ProTα (this work) | 60.1 | 71.3 | 0.77 | 0.21 |
| Rnase A[a] (pH 2.8) | 42.6 | 376.7 | 8.23 | 1.19 |
| Human serum albumin[b] (pH 5.4) | 65.4 | 417.2 | 21.4 | 1.23 |
| Lysozyme[c] (pH 2.0) | 66.8 | 205.0 | – | 0.60 |
| Human lysozyme[d](pH 3.4) | 59.9 | 440.0 | 6.02 | 1.32 |

[a]From Kaushik and Bhat (1998)
[b]From Freire and Biltonen (1978a, 1978b, 1978c)
[c]From Velicelebi and Sturtevant (1978)
[d]From Kuroki et al. (1992)

## Conclusion

Using spectroscopic techniques and analyzing the results of denaturation experiments by two different methods, thermal and denaturant concentration, we can suggest that ProTα undergoes a change in its conformation. Then we can assume that this protein has some kind of secondary structure as other authors (Gast et al. 1995) observed and it is characterized as a random coil conformation in similar external conditions. The conformational transition induced in ProTα by $C_{12}$TAB has characteristics analogous to those induced by $C_{12}$TAB in globular proteins. However, the conformational transition due to temperature presents some differences; the thermodynamic quantities of enthalpy and entropy changes at melting temperature are smaller than the denaturation induced in globular proteins and the change in heat capacity is much smaller.

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