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ARTICLE

Klaus Gast · Dietrich Zirwer · Gregor Damaschun

Are there temperature-dependent structural transitions in the "intrinsically unstructured" protein prothymosin α?

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Abstract Prothymosin α, a typical member of the class of the so-called "intrinsically unstructured" proteins, adopts a random-chain conformation under physiological environmental conditions. An apparent formation of ordered secondary structure and a moderate compaction are observed upon the change from neutral to acid pH at room temperature. We have addressed the question of whether there are temperature-dependent changes of the conformational state of prothymosin α at low pH using circular dichroism spectroscopy and static and dynamic light scattering. In contrast to previous investigations, we did not observe a heat-induced conformational transition. For comparison, we have also carried out the same experimental procedures with acid-unfolded phosphoglycerate kinase from yeast. In this case we observed a weak compaction and a slight apparent increase in ordered secondary structure with increasing temperature, probably caused by the higher average hydrophobicity as compared to prothymosin α. In the absence of a clear structural transition, we deduce the observed effects result mainly from a progressive redistribution in the population of ϕ-ψ angles of the polypeptide backbone when the temperature is increased. Furthermore, the paper should demonstrate the difficulties in distinguishing between such a progressive change amongst a continuum of states within the ensemble of unfolded conformations from the formation of authentic stable secondary structures in highly unfolded proteins. This problem is not solved presently and convincing evidence can only be supplied by the combination of various experimental techniques.

Keywords Protein folding · Secondary structure · Stokes radius · Light scattering · Circular dichroism

K. Gast (✉) · D. Zirwer · G. Damaschun
Max-Delbrück-Centrum für Molekulare Medizin Berlin-Buch, Robert-Rössle Strasse 10, 13125 Berlin, Germany
E-mail: gast@mdc-berlin.de
Fax: +49-30-94062548

Abbreviations *CD*: circular dichroism · *DLS*: dynamic light scattering · *PGK*: phosphoglycerate kinase · *ProTα*: prothymosin α · *SLS*: static light scattering

Introduction

Unlike the defined three-dimensional structure in the folded native state, proteins in unfolded states represent a large ensemble of different conformations. The question of whether proteins in highly unfolded states, e.g. at high concentrations of denaturant or at extreme pH, can be considered as true random coils, or whether the astronomically large number of possible chain configurations is reduced due to steric constraints as well as chain regions or clusters of persistent ordered structure, has attracted much attention during the past few years (Pappu et al. 2000; Plaxco and Gross 2001; Shortle and Ackerman 2001; van Gunsteren et al. 2001; Baldwin 2002; Klein-Seetharaman et al. 2002). Persistent or transiently formed ordered secondary structure within the unfolded state could play an important role in the initiation of folding towards the native state. In addition to proteins unfolded by extreme environmental conditions, so-called "natively unfolded" or "intrinsically unstructured" proteins have been discovered (Schweers et al. 1994; Gast et al. 1995; Weinreb et al. 1996; Wright and Dyson 1999). The number of known sequences of this kind is rapidly increasing (Uversky et al. 2000; Dunker et al. 2001; Uversky 2002). Among them, particularly very interesting are those polypeptide chains which are very flexible and highly expanded and have only little or no ordered secondary structure under physiological conditions. These proteins apparently escape the structure-function paradigm (Wright and Dyson 1999) and adopt an ordered three-dimensional structure (entirely or in parts) only under specific environmental conditions, e.g. during interaction with other macromolecules or membranes. One member of this category of proteins is prothymosin α (ProTα), an acidic protein (pI = 3.5) consisting of 109 amino acids. Despite

the many intracellular and extracellular effects described for ProTα, the exact biological function remains to be unraveled (Piñeiro et al. 2000).

The existence of intrinsically unstructured proteins has considerably stimulated the attempts towards a better understanding of the conformational properties of unfolded proteins in general. Noteworthy, many of the intrinsically unstructured proteins play an important role in protein conformational diseases (Weinreb et al. 1996; Rochet and Lansbury 2000). The detailed structural characterization of the unfolded state is difficult because standard high-resolution methods cannot be applied. Therefore, a variety of physical methods has to be used in order to characterize the conformational properties in terms of chain dimensions or compactness, persistent secondary structure, backbone conformational preferences and constraints, and long-range ordering. Particularly concerning the last two aspects, recent progress in NMR spectroscopy appears to yield new insights (Barbar 1999; Shortle and Ackerman 2001; Klein-Seetharaman et al. 2002). Small-angle X-ray scattering (SAXS) (Glatter and Kratky 1982), measuring the geometric radius of gyration R_G, as well as dynamic light scattering (DLS) (Schmitz 1990) and pulsed-field-gradient NMR (Wilkins et al. 1999), measuring the hydrodynamic Stokes radius R_S, are suitable techniques to measure the average chain dimensions. Circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopy are equally important and somewhat alternative methods to monitor changes in protein secondary structure. CD is one of the most sensitive techniques for studying variations in the average protein conformation. In the case of unfolded proteins, however, there exist problems in unequivocally relating the observed spectral changes to changes in the average backbone conformation and/or to the formation of stable elements of regular secondary structure. These problems are even more severe for simpler, but less specific, methods like UV difference spectroscopy. In summary, only the combination of at least some alternative physical methods can yield convincing insights into structural changes occurring in unfolded states.

In this work, we are dealing with one particular aspect concerning structural changes in the unfolded state, namely the question of how increasing temperature influences the average conformational properties of an essentially unfolded polypeptide chain, using the intrinsically unstructured protein ProTα. We applied DLS to monitor changes in the average chain dimensions and far-UV CD spectroscopy to detect changes in secondary structure. The combination of static light scattering (SLS) and DLS was used to probe the state of association at different temperatures. Previous studies have shown that ProTα has a random chain conformation at neutral pH (Gast et al. 1995). ProTα apparently adopts some ordered secondary structure and becomes slightly more compact when the pH is lowered down to 2.4 (Gast et al. 1995; Uversky et al. 1999), but it remains in an essentially unfolded state. Extensive formation of ordered secondary structure was observed only in the presence of trifluoroethanol (Gast et al. 1995).

How changes in temperature alter the conformational preferences of an unfolded polypeptide chain is an essentially open question. Pombo et al. (2001) have inferred a heat unfolding transition for ProTα at pH 2.4 according to their UV difference spectroscopic measurements. This result has to be substantiated because of the far-reaching consequences and the fact that only this rather indirect method has been used in these studies. ProTα has a rather unusual amino acid composition. It contains 49% aspartic and glutamic acid residues, aromatic amino acid residues are totally absent and it has a low average hydrophobicity. Accordingly, the question arises of whether or not the response to heating is typical for unfolded protein chains in general. Therefore, we have carried out the same experiments with acid-unfolded phosphoglycerate kinase (PGK) from yeast, which has a typical average hydrophobicity and is in a compact, natively folded conformation at neutral pH.

We find that ProTα retains its average conformational properties and does not undergo a structural transition when the temperature is varied between 10 °C and 80 °C. The observed minor changes of the far-UV CD spectrum and their temperature dependence would rather hint at the formation of regular secondary structure with increasing temperature. This finding is different from that of Pombo et al. (2001), who have suggested that an increase in temperature causes unfolding. Apparent formation of ordered secondary structure at elevated temperatures is even far more pronounced for acid-denatured PGK and is accompanied by a weak contraction of the polypeptide chain.

Materials and methods

Materials

ProTα was a gift from Thymoorgan Pharmazie (Germany). It was extracted from calf thymus according to the method of Haritos et al. (1984). Yeast PGK was purchased from Roche Diagnostics (Germany). The acid-denatured protein was prepared as described elsewhere (Damaschun et al. 1999) and was finally dialyzed against 10 mM glycine/HCl buffer, pH 2.4. The heptapeptide GCLGNSK was a gift from Dr. Margitta Dathe (Forschungsinstitut für Molekulare Pharmakologie, Germany). All other chemicals were of analytical grade.

Protein concentrations

Stock solutions of defined concentrations of ProTα were produced by dissolving a certain amount of the freeze-dried protein in 10 mM glycine/HCl buffer, pH 2.4. Glycine/HCl is a very useful buffer for experiments over a wide temperature range since its temperature coefficient is practically zero (Fasman 1989). Protein concentrations after subsequent dilution and filtration were determined spectrophotometrically using the absorbances in the far-UV region A(215 nm, 0.1%, 1 cm) = 7.32 and A(225 nm, 0.1%, 1 cm) = 2.75 since ProTα is lacking aromatic amino acid residues. The concentration of PGK was determined using A(278 nm, 0.1%, 1 cm) = 0.495. The heptapeptide solution of 0.25 mg/mL was prepared by dissolving the corresponding amount of the freeze-dried peptide in buffer.

Methods

Circular dichroism

CD measurements were done on a JASCO J-720 spectrometer (Japan) using 1 mm rectangular cells (Hellma, Germany). Protein concentrations amounted to 0.25 mg/mL. Mean residue ellipticities were calculated using mean residue weights of 109.6 and 107.4 for ProTα and PGK, respectively. The spectrometer was calibrated with (+)-10 camphorsulfonic acid at 290.5 and 192.5 nm (Johnson 1990).

Light scattering

DLS and SLS were measured simultaneously with one and the same instrument at a scattering angle of 90°. The laboratory-built apparatus consisting mainly of a diode-pumped Nd:YVO_4 cw laser, Millenia IIs (Spectra-Physics, USA), operating at 532 nm wavelength and 0.5 W output power, a thermostated cell holder and a photon detection system, presently equipped with a high quantum yield avalanche photodiode, has been described in detail (Gast et al. 1992). The solvents and protein solutions were filtered through 100 nm pore-size Anotop filters (Whatman, UK) directly into 100 μL flow-through cells (Hellma, Germany). Relative scattering intensities and Rayleigh ratios were obtained using toluene as a reference sample. For the estimation of apparent molar masses we have used a refractive index increment $(\partial n/\partial c) = 0.19$ mL/g typical for proteins in aqueous solutions. The homodyne time-autocorrelation functions of the scattered light intensity $g(\tau)$ were calculated by a 90-channel multibit multiple-τ correlator. Distribution functions of the translational diffusion coefficient D were calculated from $g(\tau)$ using the program CONTIN (Provencher 1982) and consisted mainly of one component representing the diffusion coefficient of the monomeric protein. The diffusion coefficients were converted into Stokes radii via the Stokes-Einstein equation $R_S = k_B T/(6\pi\eta_0 D)$, where k_B is Boltzmann's constant, T is the temperature in K, and η_0 is the solvent viscosity. Solvent viscosities and densities were measured using an Ubbelohde-type viscometer, Viscoboy 2 (Lauda, Germany), and a digital density meter, DMA 58 (Anton Paar, Austria), respectively. Refractive indices of the solvents were measured with an Abbe-type refractometer.

UV difference spectroscopy

Difference spectra were measured using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Germany). Because both solvents and protein solutions show temperature-dependent changes in absorbance A in the far-UV region, the temperature-induced difference spectrum of the protein $\Delta A_P(\lambda, T–T_0)$ was calculated from solution and solvent absorption spectra $A(\lambda)$ by:

$$\Delta A_P(\lambda, T - T_0) = A_{solution}(\lambda, T) - A_{solution}(\lambda, T_0) - (A_{solvent}(\lambda, T) - A_{solvent}(\lambda, T_0)) \quad (1)$$

where T_0 is the reference temperature (20 °C in the present case).

Results

Temperature-induced changes of the far-UV CD spectra

CD spectra within the wavelength range from 195 nm up to 260 nm were measured at temperatures between 10 °C and 80 °C in steps of 10 °C. CD spectra of ProTα at some chosen temperatures are shown in Fig. 1a. The most striking spectral change is a decrease in the amplitude of the negative CD band at about 200 nm. The spectrum after heating and cooling down to 10 °C slightly deviates from that before heating. This is due to degradation of the protein during the measurements at temperatures above 60 °C (see below). The relative changes of the CD signal at two characteristic wavelengths are shown in Fig. 1b. The CD at 222 nm is particularly sensitive to changes in helical secondary structure, while the negative band at about 200 nm is characteristic of random secondary structure. The specific ellipticities were normalized to the value at 20 °C.

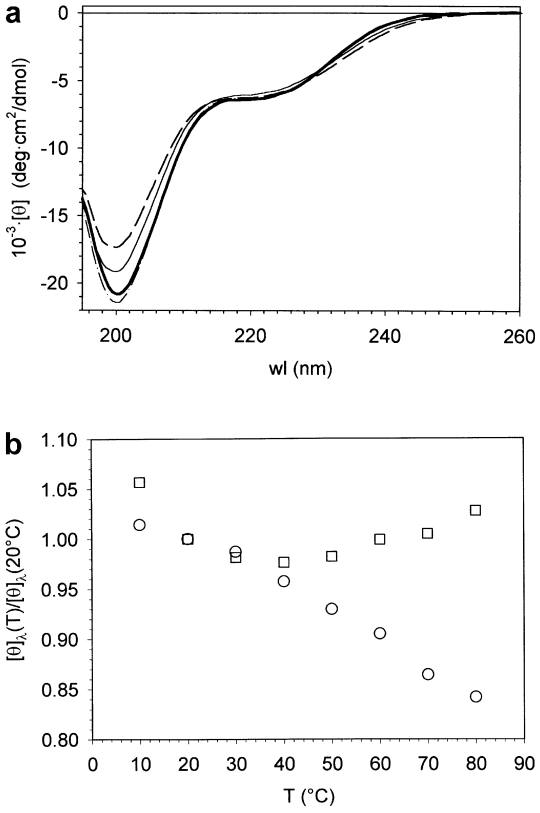

Fig. 1 **a** CD spectra of prothymosin α, $c = 0.25$ mg/mL, in 10 mM glycine/HCl buffer, pH 2.4, $d = 1$ mm at different temperatures: 10 °C (*thick line*), 50 °C (*thin line*), 80 °C (*dashed line*), and 10 °C after heating (*dot-dashed line*). **b** Relative changes of the specific ellipticities at 200 nm (*circles*) and 222 nm (*squares*)

The corresponding CD spectra for acid-denatured PGK are shown in Fig. 2a. The spectra at 10 °C before and after heating are practically identical. The higher reversibility of the CD spectrum was achieved essentially by keeping the measurement times at elevated temperatures as short as possible. Figure 2b shows the temperature-induced relative changes of the CD signal for PGK at 200 and 222 nm.

Static and dynamic light scattering experiments

From static and dynamic light scattering measurements at finite protein concentrations, only apparent molar masses M_{app} and apparent translational diffusion coefficients D_{app} can be obtained. Extrapolation to zero protein concentration yields the true values of M and D,

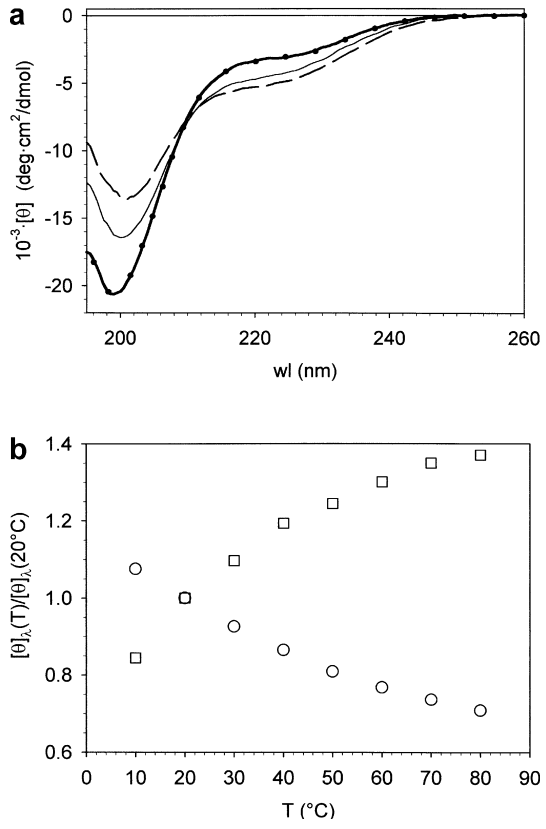

Fig. 2 **a** CD spectra of PGK, $c = 0.25$ mg/mL, in 10 mM glycine/HCl, pH 2.4, $d = 1$ mm at different temperatures: 10 °C (*thick line*), 40 °C (*thin line*), 80 °C (*dashed line*), and 10 °C after heating (*large dots*). **b** Relative changes of the specific ellipticities at 200 nm (*circles*) and 222 nm (*squares*)

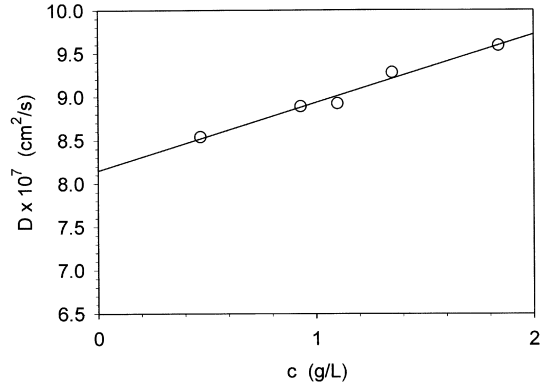

Fig. 3 Concentration dependence of the translational diffusion coefficient D for prothymosin α in 10 mM glycine/HCl buffer, pH 2.4, at 20 °C

respectively. From the slopes of the fits the second virial coefficient A_2 or the diffusive virial coefficient k_D can be calculated. As an example, we have drawn in Fig. 3 the apparent diffusion coefficients measured by DLS versus protein concentration for ProTα at pH 2.4. The data obtained from linear fits according to the equation $D(c) = D(0)(1 + k_D c)$ for ProTα and PGK under different conditions are summarized in 0 > Table 1. Similar data treatments for $1/M_{app}$ obtained from SLS yielded molar masses of 11,700 and 10,400 g/mol for ProTα at pH 7.4 and 2.4, respectively. This confirms that ProTα is in a monomeric state under these environmental conditions. Up to 60 °C the change in scattering intensity was less than 5%, proving the absence of heat-induced aggregation. Above 60 °C, a slow decrease of the scattering intensity indicated the onset of protein degradation. Protein degradation at elevated temperatures and low pH was also detectable in the case of PGK. The changes in the molecular dimensions in dependence on temperature were monitored by measuring the apparent Stokes radius $R_{S,app}$. The relative changes of $R_{S,app}$ for ProTα and PGK at acid pH are shown in Fig. 4. $R_{S,app}$ for ProTα is essentially constant up to 60 °C. The decrease in $R_{S,app}$ above 60 °C is not due to compaction, but results from partial degradation. This becomes evident from the reduced value of $R_{S,app}$ after cooling down to 20 °C. Partial degradation of the protein was confirmed by subsequent SDS gel electrophoresis (data not shown). $R_{S,app}$ of PGK steadily decreases between 10 °C and 50 °C. In order to be able to check the reversibility of the observed size change, we did not heat the sample for DLS measurements above 60 °C. $R_{S,app}$ for PGK after heating and subsequent cooling down to 20 °C coincides with the initial value within the experimental error of ±1%.

Temperature dependence of the UV absorption difference spectra

These experiments were carried out for two reasons. Firstly, we wanted to check the usefulness of temperature-dependent UV difference spectroscopic measurements for monitoring changes in protein conformation. Secondly, these experiments were necessary in order to relate our findings to previous results concerning temperature-induced changes in secondary structure of ProTα (Pombo et al. 2001). Figure 5a shows UV absorption difference spectra of ProTα at temperatures between 25 and 80 °C with 20 °C as reference temperature. The experiments have been carried out under identical experimental conditions ($c = 0.5$ mg/mL,

Table 1 Diffusion coefficients $D_{20,W}$, Stokes radii R_S and diffusive virial coefficients k_D for ProTα and PGK under different solvent conditions at 20 °C

Protein; condition	$D_{20,W}\times10^7$(cm^2/s)	R_S (nm)	k_D (mL/mg)
ProTα; PBS, pH 7.4	6.99 ± 0.07	3.07 ± 0.03	0.030 ± 0.004
ProTα; 10 mM glycine/HCl, pH 2.4	8.15 ± 0.08	2.63 ± 0.03	0.096 ± 0.010
PGK; 10 mM glycine/HCl, pH 2.4	2.85 ± 0.03	7.51 ± 0.08	0.56 ± 0.03
PGK; 10 mM HCl, pH 2.4	2.86 ± 0.03	7.49 ± 0.08	0.55 ± 0.04

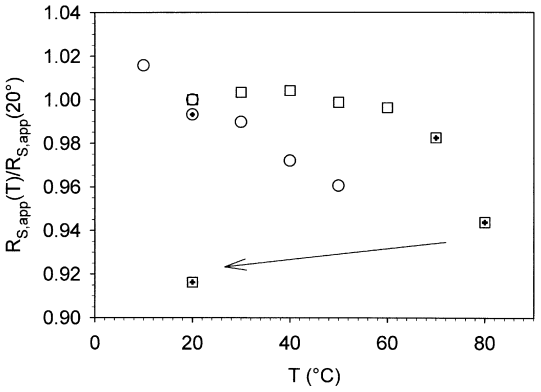

Fig. 4 Relative changes of the apparent Stokes radius for ProTα, $c = 1.1$ mg/mL (*squares*) and PGK, $c = 0.5$ mg/mL (*circles*) in 10 mM glycine/HCl buffer, pH 2.4, in dependence on temperature. The *crossed symbols* mark the values measured after heating or those values, which have to be omitted because of distortions due to protein degradation

pathlength $d = 1$ cm) and the results are consistent with those obtained by Pombo et al. (2001). However, it is noteworthy that the total absorbance of the protein solution (dashed line in Fig. 5a) exceeds 2 at wavelengths below 230 nm. At absorbances above 2, which means that less than 1% of the light is transmitted by the sample, spectral distortions due to stray light must be taken into consideration. Indeed, spectral distortions under conditions described above became evident by measurements at about five times lower protein concentration (Fig. 5b). Unfortunately, the reduction in protein concentration does not considerably improve the experimental conditions. The critical absorbance of about 2 is only shifted from 230 nm to 215 nm. This is mostly due to the strong absorption of the glycine buffer in the far-UV region. Our results confirm the necessity of using a solvent of low absorption in the far-UV region (Roseneck and Doty 1961). Despite the spectral distortions constraining the usefulness of UV difference spectroscopy in this case, the data indicate an increase in protein absorbance at 230 nm with increasing temperature. For comparison we have also measured difference spectra for PGK at 0.12 mg/mL (Fig. 5c) and for a short polypeptide GCLGNSK at 0.25 mg/mL (Fig. 5d). The changes of the specific mean-residue difference absorbance $\Delta\epsilon_r = \Delta A_P/(c_{MR}d)$ in dependence on temperature are shown for both ProTα and PGK in Fig. 6. c_{MR} is the molar mean amino acid residue concentration and d is the optical pathlength.

Discussion

The monomeric state of ProTα is preserved at low pH and elevated temperatures

Many proteins undergo aggregation when the temperature is increased. This process is favored by the exposure of hydrophobic patches during thermal unfolding and by the increase of hydrophobic interactions when the temperature is raised. Temperature-dependent protein aggregation prevents any reliable studies of the molecular dimensions and may severely influence optical-spectroscopic data. The monomeric nature of ProTα at pH 2.4 and in a wide temperature range was demonstrated by our SLS data. The measured molar mass of 10,400 g/mol is close to the molar mass $M = 11{,}983$ g/mol calculated from the amino acid composition and does not change with temperature according to the essentially constant light scattering intensity. These findings are important preconditions for our studies, showing that all observed temperature-induced variations in experimental parameters result from the monomeric protein. The absence of protein aggregation further means that the measured absorption spectra are not influenced by light scattering. The same holds for acid-denatured PGK. However, another factor influencing the molecular integrity of both unfolded proteins had to be taken into consideration. Protein degradation became evident at temperatures above 60 °C. This fact is not surprising for highly unfolded proteins. We believe that this is mostly due to acid hydrolysis rather than digestion by proteases, because the effect emerged at rather high temperatures. Since these findings were taken into consideration during further experiments or distorted data points were excluded from the analysis (see Fig. 4), the outcome of our studies is not influenced by this fact.

The average conformation of ProTα is not essentially changed on heating according to the results of CD and DLS investigations

The CD spectrum of ProTα at pH 2.4 and near room temperature differs somewhat from that at neutral pH, indicating an apparent increase in helical structure from ~0% up to 13% (Gast et al. 1995) or 15% (Uversky et al. 1999). Such a quantitative estimation can be obtained by taking the specific ellipticity at 222 nm as a measure of helical content (Chen et al. 1972). This conclusion is also consistent with the decrease of the amplitude of the negative CD band at about 200 nm, which is characteristic of unordered structure. Uversky et al. (1999) have monitored the specific ellipticity at 222 nm between pH 2 and pH 9. Practically all changes proceed within the pH range between 3.5 and 5.5. It is, however, an open question whether the observed changes in the CD spectrum result from the formation of stable, hydrogen-bonded secondary structure or reflect only a redistribution of the occupancy of allowed ϕ-ψ angles in the Ramachandran map within the random chain configuration in favor of ϕ-ψ angles corresponding to helical structure. The latter interpretation is supported by the fact that no apparent long-range NOE cross-peaks were observed in the NOESY spectrum of ProTα at pH 2.8 (Uversky et al. 1999). It is conceivable that the average chain configuration is

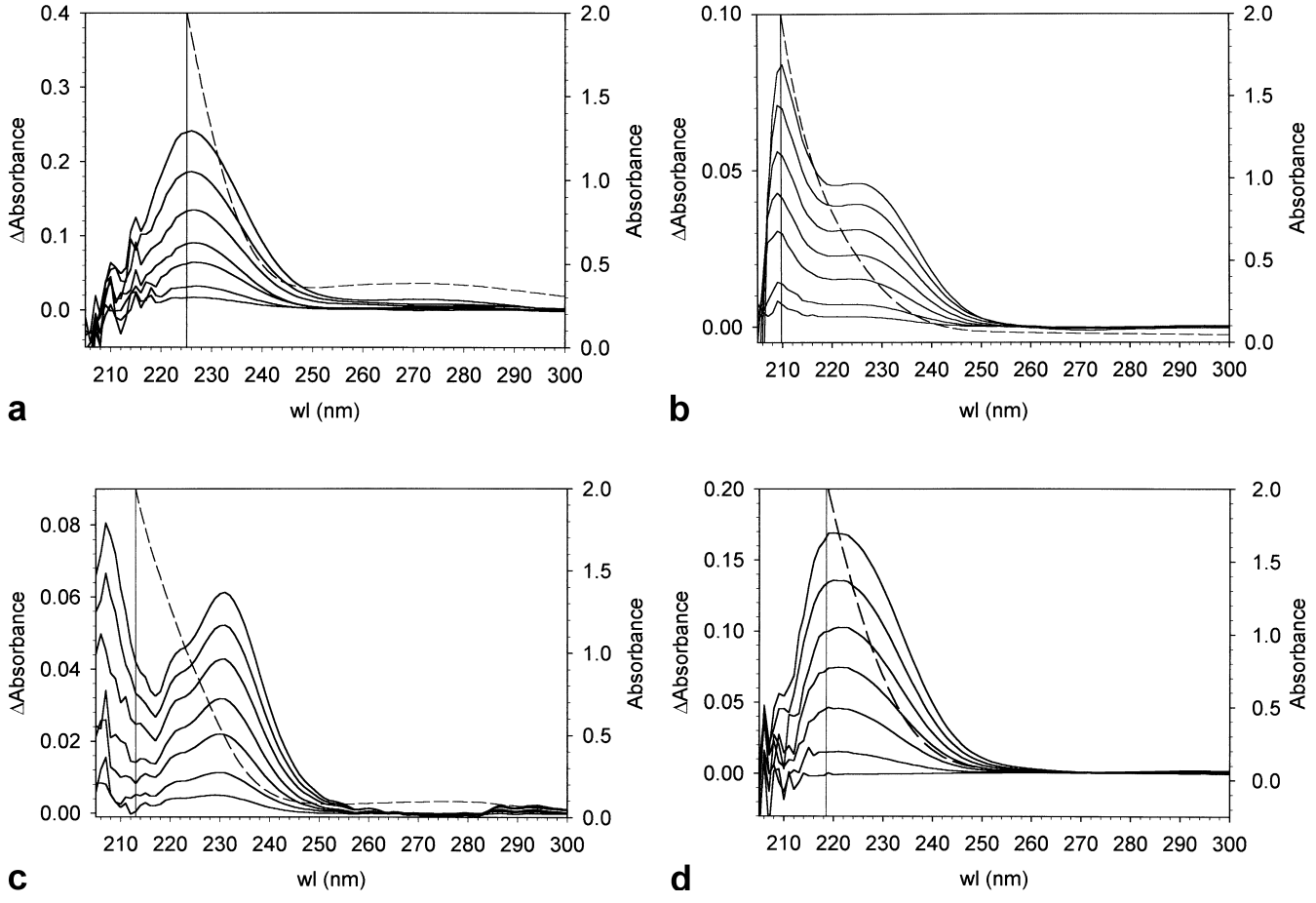

Fig. 5 **a** Absorption difference spectra of ProTα, $c = 0.5$ mg/mL in 10 mM glycine/HCl buffer, pH 2.4, $d = 1$ cm, at 25, 30, 40, 50, 60, 70, and 80 °C (*from bottom to top*). The *dashed line* shows the solution spectrum at 80 °C. The reference temperature is 20 °C. The *thin vertical line* marks the wavelength below which the difference spectrum is distorted due to a high total absorption of the sample ($A > 2$). **b** The corresponding spectra for ProTα at $c = 0.11$ mg/mL. **c** Absorption difference spectra of PGK, $c = 0.12$ mg/mL in 10 mM glycine/HCl buffer, pH 2.4, $d = 1$ cm, at 25, 30, 40, 50, 60, 70, and 80 °C (*from bottom to top*). The *dashed line* shows the solution spectrum at 80 °C. **d** Absorption difference spectra of the heptapeptide GCLGNSK, $c = 0.25$ mg/mL in 10 mM glycine/HCl buffer, pH 2.4, $d = 1$ cm, at 25, 30, 40, 50, 60, 70, and 80 °C (*from bottom to top*). The *dashed line* shows the solution spectrum at 80 °C

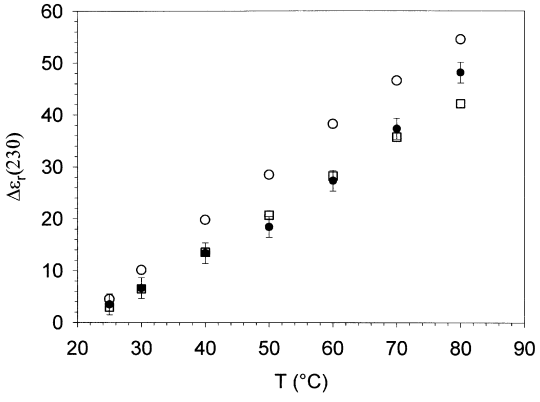

Fig. 6 Changes of the mean-residue difference absorbances at 230 nm in dependence on temperature for ProTα, $c = 0.5$ mg/mL (*filled circles*) and 0.11 mg/mL (*open squares*) in 10 mM glycine/HCl buffer, pH 2.4, and PGK, 0.12 mg/mL (*open circles*), in 10 mM HCl, pH 2. The experimental error, which is shown for ProTα at $c = 0.5$ mg/mL, is practically the same for all data points

notably changed when the charge distribution along the polypeptide chain is altered by lowering the pH from a value above to a value below the isoelectric point (p$I = 3.5$) of the protein. The change in pH from 7.5 to 2.4 also leads to a decrease in the average chain dimensions according to the drop in Stokes radius R_S from 3.07 nm to 2.63 nm (Table 1). The Stokes radii measured by Uversky et al. (1999) using size exclusion chromatography differ slightly from that determined by us using DLS and reflect a somewhat stronger compaction.

The specific mean-residue ellipticity [θ] at 222 nm remains essentially constant when the temperature is raised from 10 to 80 °C (Fig. 1b), pointing to the absence of perceptible changes in secondary structure. The origin of the shallow minimum of the relative ellipticity at 222 nm at temperatures near 40 °C is unclear. Furthermore, there are no remarkable changes in the average chain dimensions within the temperature range up to 60 °C, where the DLS results are unaffected by protein degradation. On the other hand, the decrease

of the amplitude of the negative CD signal at 200 nm could be taken as an indication of a decrease in unordered secondary structure. The origin of these apparently inconsistent changes of the CD spectum at 200 and 222 nm is presently not clear. However, in summary, the results are consistent with the absence of pronounced temperature-dependent changes in secondary structure with only some indications of an apparent formation of ordered secondary structure. Therefore, the results of our CD investigations contradict the conclusions drawn by Pombo et al. (2001) from difference spectroscopic measurements.

The situation is somewhat different for acid-unfolded PGK. In this case, the increase in the CD amplitude at 222 nm and the decrease in the amplitude at about 200 nm consistently point to an apparent formation of ordered secondary structure when the temperature is raised. Additionally, the changes in the CD are accompanied by a slight decrease in the average chain dimensions, reflected by the temperature dependence of the apparent Stokes radius (Fig. 4). The reduction of chain dimensions and the changes in secondary structure are obviously driven by strengthening of the hydrophobic forces with increasing temperature. The observed changes proceed gradually and cannot be attributed to a cooperative transition. Similar behavior concerning temperature-induced changes of the CD spectrum of unfolded proteins was also found for acid-denatured apocytochrome *c* and acid-denatured staphylococcal nuclease (Privalov et al. 1989), as well as for α-synuclein (Uversky et al. 2001). Concomitant reduction of the chain dimensions with increasing temperature was demonstrated for apocytochrome *c* and staphylococcal nuclease by measuring intrinsic viscosities (Privalov et al. 1989). Assuming a temperature-dependent increase in hydrophobic interactions as the driving force for the observed effects, it is not surprising that remarkable changes in secondary structure and chain compaction are not observed for ProTα because of the low average hydrophobicity of this protein.

Do the changes in far-UV absorption correlate with temperature-induced alterations of the protein conformation?

Our results (Fig. 5a, b) have demonstrated that far-UV difference spectroscopic investigations must be performed with care in order to prevent distortions of the spectra due to stray light when the basic absorption of the solution exceeds a value of about 2. This value is probably typical of most of the commercial instruments. We found an essentially linear increase of the specific difference absorption per residue at 230 nm for both ProTα and PGK (Fig. 6) on heating. The slightly steeper rise in the case of PGK is probably due to the contribution of aromatic side chain absorption in this spectral region. A heat-induced increase in absorbance is also detectable in the near-UV region (Fig. 5c). The differences between ProTα and PGK in the thermal responses with respect to the CD and the Stokes radius are not reflected in the temperature-induced changes of $\Delta\epsilon_r(230)$. The strictly linear dependence of $\Delta\epsilon_r(230)$ on temperature raises the question of whether these changes indeed reflect conformational variations or are mostly or entirely caused by temperature-induced alterations of the environment of the chromophores. According to the large hydrodynamic dimensions measured by DLS, the peptide chromophores are expected to be exposed to solvent. It must be taken into consideration that temperature-induced changes of the solvent properties may have a direct influence on the optical transitions. The results of additional measurements with a short polypeptide support this view. The far-UV difference spectra of the heptapeptide GCLGNSK show similar unspecific changes in dependence on temperature as was observed in the case of ProTα and PGK. A particularly unspecific characteristic of the thermal-induced difference spectra is the absence of detectable wavelength shifts. Therefore, it is questionable whether the capability to monitor changes in secondary structure by far-UV difference absorption measurements can be improved in general as compared to the higher potentials of CD and infrared spectroscopic investigations. This is probably the reason why, except for some earlier far-UV difference spectroscopic investigations in the 1960s (Glazer and Smith 1960, 1961; Rosenheck and Doty 1961), the method did not find application as a tool for characterizing conformational transitions of proteins. Rosenheck and Doty (1961) have mostly analyzed the strong absorption bands between 190 and 200 nm, which can only be measured in solvents of low absorbance. These authors also found characteristic absorbance differences between 220 and 240 nm for polypeptides in the random coil and helical conformations. Interestingly, the absorbance within this spectral region in the helical conformation is higher than in the random coil conformation. The opposite is observed at 190 nm within a spectral region, which can be clearly attributed to the π-π^* transition. According to our knowledge, the origin of the observed arbsorbance changes between 220 and 240 nm for proteins and polypeptides (Glazer and Smith 1961; Rosenheck and Doty 1961) has not been fully explained. The spectral changes at about 230 nm probably originate mostly from the n-π^* transition, while at $\lambda < 215$ nm contributions from the π-π^* transition are expected to dominate. However, because of the coupling between the amide chromophores of the peptide chain a clear separation of the transitions is difficult, particularly outside the absorption maxima. Additionally, the interference with the strong temperature-dependent absorption of the buffer has to be taken into consideration below 210 nm. In contrast to these observations, absorption difference spectroscopy in the near-UV region has been widely used to study changes in protein conformation (Demchenko 1986).

Conclusions

The basic question of this work concerning temperature-induced structural transitions of the intrinsically unstructured protein ProTα can be clearly answered. There is no thermal unfolding of the apparent ordered secondary structure that is formed when the pH is lowered from 7.5 to 2.4. This conclusion is essentially derived from CD measurements, which are sensitive indicators of any changes of secondary structure. If there are small alterations in the average conformation, the CD results hint rather at the formation instead of the disruption of regular secondary structure. The apparent formation of ordered secondary structure with increasing temperature is much more pronounced for acid-denatured PGK. Similar behavior was observed for some other unfolded proteins. The structure of unfolded proteins is represented by an ensemble of different molecular conformations of the polypeptide chain, which are characterized by a particular set of ϕ-ψ angles. The measured physical quantities are averages over such an ensemble. Our interpretation of the apparent changes in secondary structure supposes a redistribution of the occupied ϕ-ψ angles of the polypeptide backbone, whereby the polypeptide chain remains essentially in the random chain conformation. In the case of PGK, where the apparent changes in secondary structure are more pronounced, a concomitant reduction of the average chain dimensions is observed. A similar interpretation of the apparent formation of defined structures was already proposed by Sreerama et al. (2000) concerning the differences between the low- and high-temperature CD spectra of unfolded apomyoglobin and staphylococcal nuclease. It is conceivable that the changes in secondary structure and compactness of ProTα upon lowering the pH from 7.5 to 2.4 can be interpreted in the same manner. Both temperature and pH may influence the free energy surface, which determines the distribution of occupied ϕ-ψ angles of the unfolded polypeptide chain. These changes in the energy surface must not lead to a cooperative transition in the thermodynamic sense as is observed in folding transitions of natively folded proteins. A detailed understanding of these conformational transformations requires the application of a variety of biophysical methods, particularly those with the potential for distinguishing authentic secondary structural states from the rapidly interchanging constituents of a dynamic population of unstructured conformations.

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