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Analysis of the conformation and stability of rat TTF-1 homeodomain by circular dichroism

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Abstract The conformational stability of TTF-1HD has been determined by CD-monitored thermal denaturation and isothermal urea unfolding studies. The Gibbs free energy of stabilization found are 1.44 and 1.26 kcal·mol^{-1}, respectively. TTF-1HD exhibits a T_m of 42°C and a ΔC_p of 80 cal·mol^{-1}·K^{-1} indicating that TTF-1HD, when free in solution, is a mobile flexible segment folded into loose helices. Such a flexibility would be relevant for the DNA-binding function of this homeodomain. In fact, a small reduction of the α-helical content of TTF-1HD significally modifies its DNA-binding activity.

Key words: Homeodomain; Circular dichroism; Transcription factor; DNA binding; Protein structure; α-Helix

1. Introduction

The homeodomain is a 61-amino acid structure conserved along evolution from yeast to higher mammals [1]. Essential molecular function of the homeodomain is the binding to specific DNA sequences [2]. Even if divergent on the basis of the amino acid sequence, homeodomains have a very similar tridimensional structure, with three α-helices, two of which are involved in a helix–turn–helix motif [3]. Homeodomains bind DNA as monomers and base-pair contacts are made mainly by the third (recognition) helix and by the extended N-terminal arm [4–7]. Several studies have been performed to understand the molecular basis of the DNA-binding specificity [8–10]. The side chain of amino acid at position fifty appears to have a major role in controlling the binding specificity [8,9]. However the nature of the amino acid at position fifty is not the only determinant of this phenomenon. In fact, for example, TTF-1 and Antennapedia have glutamine at position fifty, but while Antennapedia binds to a sequence having the TAAT as core-motif and not to others, TTF-1 binds very efficiently to sequences having the CAAG core-motif and, by contrast, binds very poorly to TAAT sequences [10–13]. By a Nuclear Magnetic Resonance study, we have demonstrated that the structure of TTF-1HD is similar to that reported for others homeodomains, though a relatively large degree of flexibility at the end of the recognition helix was found [14]. This finding suggests that the peculiar DNA-binding specificity of TTF-1HD could be due to the interplay of several subtle structural characteristics of this homeodomain and not only to the nature of some side chain of the DNA-contacting amino acids. A study as a function of temperature and urea concentrations, is presented here, in order to evaluate the stability and folding properties of TTF-1HD. The data indicate that the structure of TTF-1HD is more flexible than those reported for others HDs [15,16].

2. Materials and methods

2.1. Expression and purification of TTF-1HD

Construction of plasmid pT7.7 TTF-1 was as described in [10]. The expression and purification of the TTF-1HD were done essentially as described in [2]. The purified protein appeared homogeneous as judged by SDS-PAGE and reverse-phase HPLC (data not shown). The homeodomain concentration was determined using a molar absorption coefficient of 9570 M^{-1}·cm^{-1} at 280 nm, calculated according to Wetlaufer [17].

2.2. Circular dichroism and fluorescence studies

CD spectra were performed on a Jasco J-600 CD/ORD spectropolarimeter calibrated with a 0.1% (w/v) D-canphorsulfonic acid solution. Far-ultraviolet circular dichroism spectra are presented in terms of mean residue molecular ellipticity [θ] (in deg·cm^2·dmol^{-1}), based on a mean residue weight of 124. Thermal denaturation studies were performed on a solution of TTF-1HD (10 μM) in 10 mM phosphate buffer, 100 mM NaF, pH 7.5. The solution was cooled from room temperature to 4°C, allowed to equilibrate for 30 min, then the temperature was raised in 5°C increments up to 85°C, for each time the solution was allowed to equilibrate for 30 min, and its CD spectrum was recorded three times. The reported results are the smoothed average over the three measurements. The melting curve was analyzed according to [18]. Spectra were analyzed using the program of Menendez-Arias et al. [19]. Isothermal unfolding of TTF-1HD was achieved by adding increasing volumes of 8 M urea up to a final volume of 500 μl. Samples were equilibrated at room temperature for 30 min prior to recording the CD spectra. Additionally, homeodomain unfolding was monitored by changes in the tryptophan fluorescence using a Perkin Elmer LC 50 fluorometer. Excitation of the single tryptophan residue (Trp-48) at 290 nm (slit 5) allowed monitoring of the emission at 340 nm (slit 5) using a cell-path length of 1 cm. The free energy of unfolding of TTF-1HD was calculated from the changes in molar ellipticity at 222 nm which was measured at increasing concentrations of urea. TTF-1HD conformational stability was estimated from denaturation curve using the 'Denaturant Binding Model' as reported by Pace [20].

2.3. Gel-retardation assay

DNA-binding activity of native and heat-treated TTF-1HD was measured by gel retardation assay. The oligodeoxynucleotides used as probes were the C site of Thyroglobulin promoter (to monitor the specific DNA-binding) and a mutant of C, here named Cm (mutation 14 in [21]), to monitor the non-specific DNA binding. Either native or

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Abbreviations: TTF-1HD, thyroid transcription factor 1 homeodomain; CD, circular dichroism.

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heat-treated TTF-1HD were incubated with labelled oligodeoxynucleotides in a buffer containing 20 mM Tris, 0.1 mM EDTA, 5 mM DTT, 50 μg/ml BSA, 10% glycerol at pH 7.4, for 10 min at 24°C in a final volume of 20 μl. Reaction mixtures were loaded on a 8% polyacrilamide non denaturing gel and run for 1.5 h at 4°C.

3. Results and discussion

3.1. Thermal denaturation

Fig. 1 shows some of the CD spectra recorded at different temperatures (pH 7.5); the changes seen upon thermal denaturation of TTF-1HD reflect the transition from a native folded state to a more disordered one. The curves in Fig. 1 show an isodichroic point (204 nm), indicating the presence of only two independent spectral forms. The thermal unfolding curve corresponding to a smoothed plot of the ellipticity observed at 222 nm vs. absolute temperature is reported in Fig. 2. A T_m (Fig. 2) value of 41°C is found for the helix–coil transition. This T_m value, at pH 7.5 showed no concentration dependence over a 10-fold range (10–100 μM, data not shown). According to a two-state mechanism of unfolding [18], we have calculated the equilibrium constants of unfolding at the indicated temperatures. The van 't Hoff plot, obtained when the experimental data are analyzed on the unfolded-folded two-state model, is reported in Fig. 3. The van 't Hoff $\Delta H°$ and $\Delta S°$, calculated from the intercept, are 25.41 kcal/mol and 80.4 cal/K·mol, respectively. A folding free energy of 1.44 kcal·mol^{-1} at 25°C is estimated. A T_m value of 42.8°C is obtained using the van 't Hoff $\Delta H°$ and $\Delta S°$. This value is in agreement with the one determined as the first derivative of the melting curve but is significantly lower than the value reported for Antennapedia homeodomain (48°C) [15]. The inset of Fig. 3 shows that the van 't Hoff enthalpy, associated with the thermal unfolding of TTF-1HD (i.e. calculated as the first derivative of the k values given in Fig. 3), is linearly related to the temperature with a positive slope. Such a behavior is expected for a two-state transition [22]. The observed slope (inset Fig. 3) of about 80 cal·mol^{-1}·K^{-1} represents the difference in thermal capacity at constant pressure (ΔC_p) between the folded and the unfolded states of the molecule. This difference reflects the change in the degree of exposure of hydrophobic residues caused by unfolding the protein [22]. TTF-1HD is highly soluble at pH 7.5

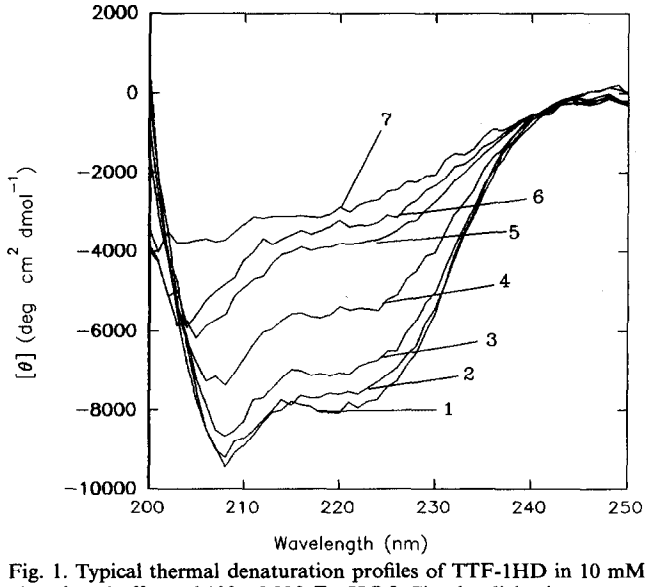


Fig. 1. Typical thermal denaturation profiles of TTF-1HD in 10 mM phosphate buffer and 100 mM NaF, pH 7.5. Circular dichroism spectra recorded at (1) 4, (2) 24, (3) 34, (4) 44, (5) 54, (6) 68 and (7) 85°C. The protein concentration was 10 μM.

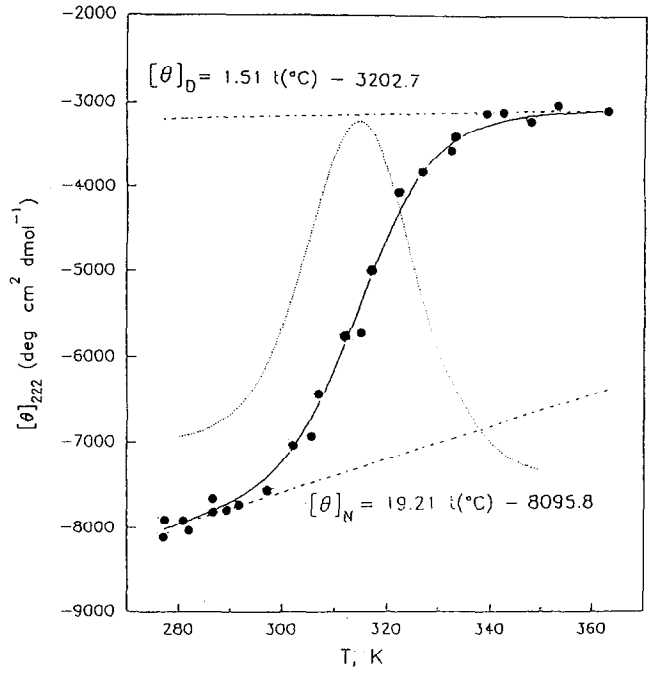


Fig. 2. Transition curve for the thermal denaturation of TTF-1HD obtained from the ellipticity at 222 nm ($[\theta]_{222}$) vs. absolute temperature (T). The dashed lines and equations are based on a least-squares analysis of the pre- and post-transition base line. The solid line represents the fitted function as described in [19], dots represent the ellipticity values calculated from four denaturation profiles as in Fig. 1. The equations for $[\theta]_N$ and $[\theta]_D$ give the value of $[\theta]_{222}$ for the native and denaturated states, respectively, where t is the temperature in °C. The dotted line is the first derivative of the transition curve.

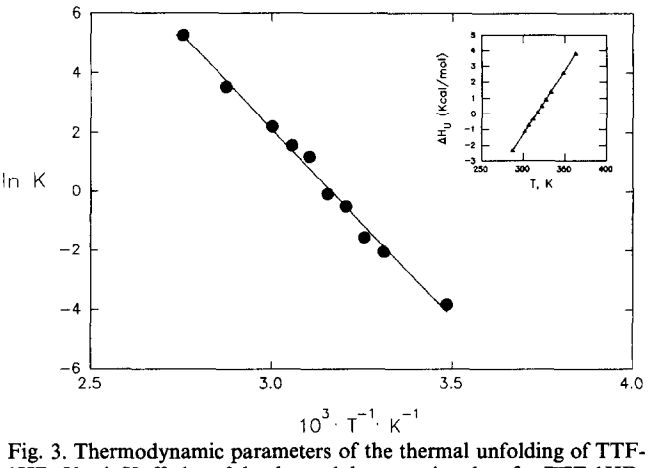


Fig. 3. Thermodynamic parameters of the thermal unfolding of TTF-1HD. Van 't Hoff plot of the thermal denaturation data for TTF-1HD. The equilibrium constants were calculated as described in [18] using the ellipticity data reported in Fig. 2. The solid line represents the fitted function of the data points. Inset: dependence of the enthalpy of unfolding, ΔH_u, of TTF-1HD on absolute (T) temperature. ΔH_u values were obtained from the first derivative of the van 't Hoff plot. The slope of the least-squares line indicates the change in heat capacity associated with denaturation (ΔC_p = 80 cal·mol^{-1}·K^{-1}).

implying that hydrophobic side chains (residues) are shielded from the solvent [24]. The relatively low ΔC_p value of TTF-1HD suggests that the unfolding reaction starts from a non-compact structure in which the hydrophobic moieties are rather poorly sequestered from contact with water.

3.2. Isothermal denaturation

Fig. 4 shows the sharp unfolding curve for native TTF-1HD obtained when changes in CD signals or fluorescence intensity are monitored at varying urea concentrations. The trend indicates that no stable intermediate species are present in appreciable concentration at any stage of the transition. The changes observed were found to be completely reversible. TTF-1HD in the presence of 8.0 μM urea was refolded by dialyzing the sample overnight with 10 mM NH_4HCO_3 buffer, pH 7.5. The ellipticity of TTF-1HD at high temperature is considerably more intense than the ellipticity at high urea concentration (Figs. 2 and 4). Similar findings have already been reported and ascribed to the distinct molecular processes which proteins undergo upon thermal or urea-driven denaturation. The phenomenon has been interpreted as indicative of the presence of residual structure in thermally unfolded proteins [23]. The data in Fig. 4 have been replotted (data not shown) and analyzed according to Pace [20]. The moles of urea bound during denaturation (Δn) and the free energy of unfolding ΔG_u, determined by regression analysis, are 11.42 and 1.26 kcal·mol^{-1} at 25°C, respectively. The calculated free energies of unfolding either with urea or with temperature are similar but lower than those determined by similar methods for the unfolding of small globular proteins, such as ribonuclease A (7.5 kcal·mol^{-1}) [24], isolated homeodomains such as the Oct-2 Pou domain (>10 kcal·mol^{-1}) [16] and the DNA-binding domain transcription factor LFB1 (11.5 kcal·mol^{-1}) [25]. The extremely low ΔG_u (1.26 and 1.64 kcal·mol^{-1} at 25°C obtained urea and temperature, respectively) found for TTF-1HD indicates an intrinsic high degree of flexibility. This result is consistent with our previous result obtained in a NMR study [14].

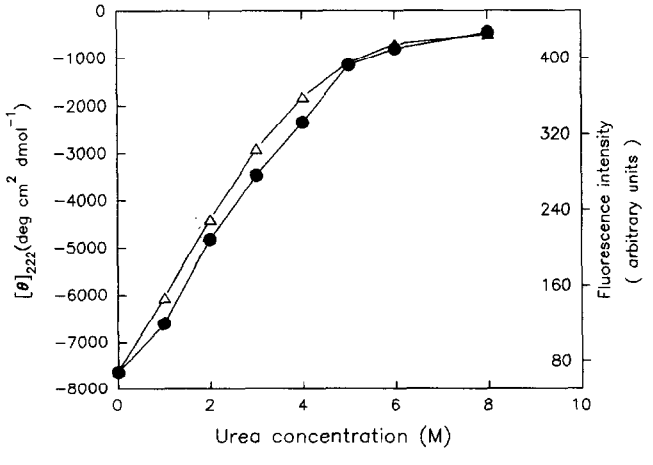
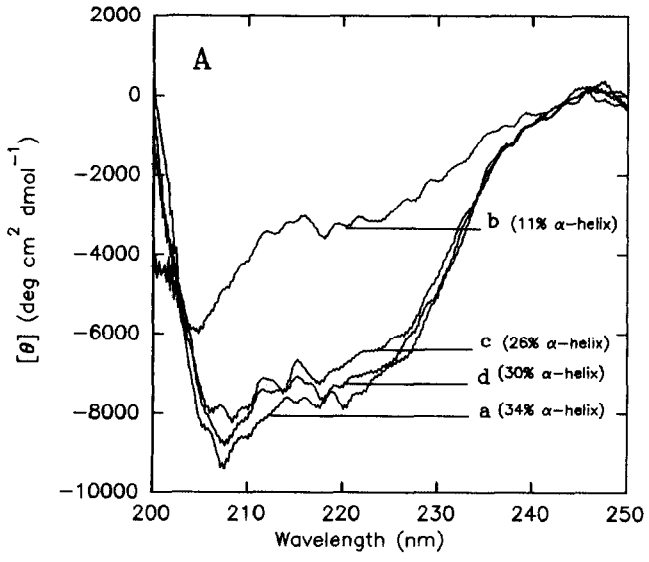


Fig. 4. Unfolding transition of TTF-1HD as a function of urea concentration. (▽) and (●) refer to ellipticity at 222 nm and fluorescence intensity at 340 nm, respectively. The homeodomain concentration are 10 and 11 μM for CD and fluorescence experiments respectively. The solid line represents the fitted function of the data points.



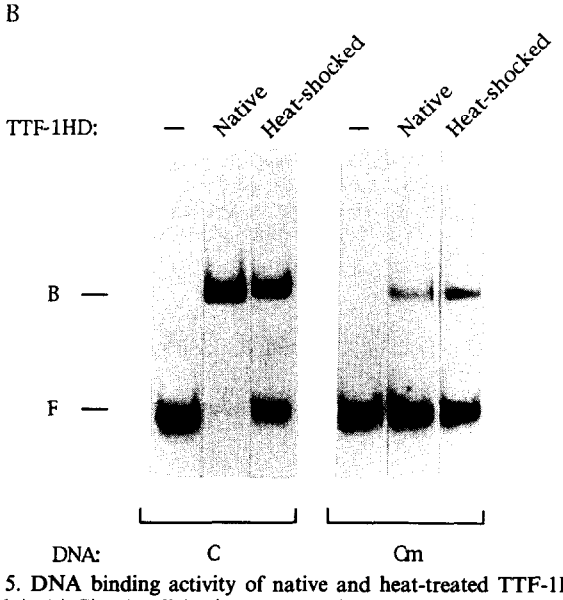


Fig. 5. DNA binding activity of native and heat-treated TTF-1HD. Panel A: (a) Circular dichroism spectra of TTF-1HD recorded a 24°C in 10 mM phosphate buffer and 100 mM NaF, pH 7.5; (b) CD spectra at 60°C; (c) CD spectra at 24°C after 10 min; (d) CD spectra at 24°C after 60 min. The estimated α-helical contents are indicated in parentheses. Panel B: DNA-binding activity. F and B indicate free and protein-bound DNA, respectively.

3.3. Thermal perturbation modifies TTF-1HD DNA recognition

As reported in Fig. 1 the helical content of TTF-1HD is very sensitive to temperature variations. Such temperature-induced perturbations are reversible as reported in Fig. 5A. After heating the solution at 60°C, the refolding of TTF-1HD at 24°C, expressed as percentage of helical content from the experimental value of $[\theta]_{222}$, proceeds towards the limiting value expected at 24°C (Fig. 5A) (very close to the maximum observed at 4°C,

Fig. 1). The helical percentage recovery, with respect to the native homeodomain, was found 76% after 10 min and 90% after 60 min of cooling at 24°C, respectively. Thus, from 10 min after the end of the heat shock up to 60 min, the structure of TTF-1HD is slightly perturbed. Such a perturbation reduces the specific DNA-binding activity of TTF-1HD. In fact, the heat-treated TTF-1HD binds less efficiently to the C sequence, compared to the native protein. Vice versa, the heat-treated and native protein bind similarly to the Cm sequence (Fig. 5B). The quantitation of this phenomenon is not possible, since the heat-treated form of the peptide is not in a conformational equilibrium state. Attempts are in progress in order to set up a quantitative approach. Nevertheless, these results suggest that small changes, induced on the conformation of the TTF-1HD, are sufficient to alter the capability of the homeodomain to discriminate specific and non-specific DNA sequences.

Present results indicate that the polypeptide chain of the homeodomain of TTF-1, is 'loosely' folded [14]. NMR experiments show that typical α-helical interproton connectivities are present for residues 52–58 of TTF- 1HD. However, a fast H–D exchange is also present in the same region. Such data suggest that the structure flexibility of TTF-1HD could be mostly due to the C- terminal of the recognition (III) helix [14]. Similar results have been previously reported for Antennapedia [15] and Oct 2 [26] homeodomains. Since the side chains in this region are able to make specific contacts with DNA, one can argue that a flexible α-helix might better fit into the major grove of cognate DNA.

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