Thermodynamic characterizations of an intramolecularly hydrogen bonded C_5-structure across proteinogenic residue

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Abstract Thermodynamic investigations of a smallest possible intramolecularly hydrogen bonded C_5-structure, across a Thr residue, in model peptides Boc-Xxx-Thr-NH_2 (Xxx = Ile, 1 or Leu, 2), indicated unusual thermal stability of the structure in non-polar medium. An analysis of van't Hoff plots, constructed from variable temperature ^1H NMR data, yielded the thermodynamic parameters of a hydrogen bonded five-membered ring. The non-significance of the spatial organizations of the preceding $C^{\delta}H_3$ bearing hydrophobic proteinogenic residue on the thermal stability of the C_5-structure has been observed. The results revealed that the contribution of this element of secondary structure is quantifiable and the stability appeared to be roughly comparable to other intramolecularly hydrogen bonded reverse turn structures frequently observed in polypeptides and proteins.

Key words: C_5-structure; NMR, ^1H-spectroscopy; van't Hoff analysis; β- and γ-turn secondary structure

1. Introduction

Polypeptide and protein de novo design principles require conformational parameters of relevance and quantitative understanding of thermodynamic measurements of enthalpies, entropies and free energetics, responsible for stabilization of three-dimensional structures [1–4]. Short peptides having a strong tendency to adopt a unique compact structure, mimicking various secondary structural features, stabilized by an intramolecular hydrogen bond, are of outstanding thermodynamic interest for understanding folding-unfolding processes in polypeptides and proteins, since they contribute to the net stability of the folded native state [5–8]. Amongst the various intramolecularly hydrogen bonded structures, conformationally an intraresidue hydrogen bonded five-membered ring, also known as C_5-structure, constitutes the smallest possible hydrogen bonded secondary structural element across an α-amino acid [9,10]. To the best of our knowledge, the thermodynamic characterization of this element of secondary structure, particularly across a proteinogenic residue, has never been investigated experimentally, and as a result its contribution towards the net stability in polypeptides and proteins remains unknown.

In a recent study we have unambiguously established the presence of this hydrogen bonded C_5-structure, across a chiral $C^{\alpha}H$ bearing proteinogenic Thr residue, and attempts have been made to lay out its ^1H NMR spectroscopic signatures [10]. In this report, we wish to probe the thermodynamic characterization of the unfolding process of the C_5-structure and obtain the energetic parameters from variable temperature ^1H NMR in two model peptides, Boc-Ile-Thr-NH_2, **1** and Boc-Leu-Thr-NH_2, **2**, in a non-interacting, poor hydrogen bond accepting solvent. The investigation offers an opportunity to quantitate the strength of this hydrogen bonded five-membered ring which in turn allows us to evaluate the possible role that this element of secondary structure can play during early events in protein folding and in determining the net stability of the three-dimensional native structure(s).

2. Materials and methods

2.1. Synthesis and characterization

The peptides were synthesized using a solution phase methodology. Each peptide was prepared by DCC mediated condensation of Boc-Ile-OH or Boc-Leu-OH and HCl·Thr-OMe in DMF in presence of TEA followed by treatment with gaseous ammonia in anhydrous methanol. The fully protected peptide amides were purified by silica gel (60–120 mesh) column chromatography using chloroform and chloroform-methanol mixtures as eluents. Both peptides were found to be homogeneous by TLC (Merck, type G-60 aluminum based silica). Physical properties: **1**: white solid, m.p. 192°C, $[\alpha]_D^{25} = +4.8°$ (c = 0.25 in MeOH), $R_f = 0.77$; **2**: white solid, m.p. 182°C, $[\alpha]_D^{25} = -22.8°$ (c = 0.25 in MeOH), $R_f = 0.77$ (R_f values are in 8% MeOH:$CHCl_3$). The peptides were fully characterized by 1D and 2D ^1H NMR and found to be consistent with the structure. Detailed synthetic procedures and characterizations of peptide **1** and **2** will be presented elsewhere.

2.2. ^1H NMR studies

Variable temperature ^1H NMR experiments were carried out on a Bruker WH-270 MHz FT-NMR spectrometer, equipped with variable temperature accessory as described earlier [11,12]. Each time, the temperature was stabilized for an additional 10 min before acquiring the FID and the ppm values were obtained with respect to internal reference TMS. In order to make direct comparisons between the thermodynamic parameters, **1** and **2** were treated similarly. The temperature coefficients ($\Delta\delta/\Delta T$) for the Thr backbone amide NH were obtained from the variable temperature experiments in $CDCl_3$. The van't Hoff plots were generated from the equilibrium constant obtained at each temperature studied by employing the limiting chemical shift values reported by Gellman and coworkers [13,14]. The regression coefficient was set at 1.0 in order to get the best fit of van't Hoff plots. The thermodynamic parameters ΔH and ΔS were derived from the slope and the intercept, respectively.

3. Results and discussion

Extremely well resolved ^1H NMR spectra for peptides **1** and **2** were obtained in $CDCl_3$. Resonance assignments were made using ^1H-^1H 2D correlated spectroscopy (COSY). Our preliminary conformational analysis of **1** and **2** has established that the Thr backbone amide NH is a strongly intramolecu-

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Abbreviations: DCC, *N,N'*-dicyclohexylcarbodiimde; TEA, triethylamine; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; $\Delta\delta/\Delta T$, temperature coefficient; ΔH, ΔS, ΔG and ΔC_P, changes in enthalpy, entropy, Gibbs free energy and heat capacity

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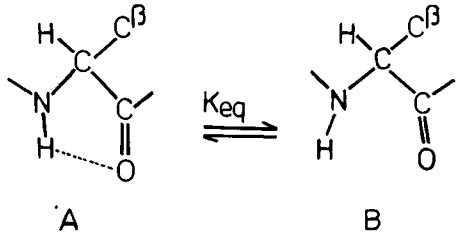

Fig. 1. Proposed equilibrium between an intramolecularly hydrogen bonded state **A** and a non-hydrogen bonded state **B**. Hydrogen bond is indicated by a dotted line.

larly hydrogen bonded secondary structural element. These conclusions were substantiated by the facts that the Thr amide NH was significantly insensitive towards $CDCl_3$-$(CD_3)_2SO$ solvent mixtures of varying compositions and temperature in an aprotic polar medium, $(CD_3)_2SO$. Based on the χ_1 value determined from $^3J_{\alpha\beta}$ coupling constant, we excluded any significant contribution from the Thr O^γ atom in an intramolecular hydrogen bond [10].

In $CDCl_3$, the Thr amide NH exhibited very little temperature dependence of chemical shifts in both peptides. The observed significantly low temperature coefficient ($\Delta\delta/\Delta T$) values $\leq 3.1\times10^{-3}$ ppm/K (Table 1) are typical of either strongly intramolecularly hydrogen bonded (solvent shielded) or fully solvated (solvent exposed) amide protons. These values are indeed in agreement with those reported earlier in short linear peptides in a non-interacting apolar medium [15–17]. Generally, large $\Delta\delta/\Delta T$ ($>4.0\times10^{-3}$ ppm/K) values in $CDCl_3$ are diagnostic of intermolecular hydrogen bonds between peptide molecules which are disrupted on raising the temperature. From the combined analysis of ^1H NMR data one can safely conclude that these $\Delta\delta/\Delta T$ values can be attributed entirely to the presence of intramolecular hydrogen bonds rather than free amide NH, indicating that a significant population of hydrogen bonded structures remains largely unaffected by temperature over the range studied. It may be worth underlining that in $CDCl_3$, the observed $\Delta\delta/\Delta T$ value of $\sim3.0\times10^{-3}$ ppm/K in general would represent a more realistic value for a strong intramolecularly hydrogen bonded structure stable at higher temperatures [12,15–17]. The linearity of the plots, i.e. chemical shifts vs. temperatures, ruled out the presence of any conformational transition.

In recent years, variable temperature ^1H NMR data of amide protons have extensively been employed to obtain thermodynamic parameters and information on their relative stability pertaining to specific type of intra- or intermolecularly hydrogen bonded ring structures [13,14,18–20]. Considering the case of intramolecularly hydrogen bonded five-membered rings in peptides **1** and **2**, the assumption is made that at room temperature there exists an equilibrium between two states: intramolecularly hydrogen bonded state **A** and a non-hydrogen bonded state **B** (Fig. 1). Also, the population of state **B** increases on raising the temperature.

As expected, the chemical shifts of the Thr NH in **1** varies between 6.87 and 7.35 ppm over the temperature range 318–235 K, indicating a significant increase in the population of state **A** on lowering the temperature. The equilibrium constant, K_{eq}, for the two-state process was determined at several temperatures according to the equation $K_{eq}=(\delta_{obs}-\delta_n)/(\delta_b-\delta_{obs})$, where δ_n and δ_b represent the limiting chemical shift values for non-hydrogen bonded and fully hydrogen bonded states, respectively, in an apolar medium of the amide NH proton in model system suggested by Gellman's group [14]. Since the relative orientations of hydrogen bond donors and acceptors are uniquely different in various elements of secondary structures, an estimation of structure specific limiting chemical shift value for a hydrogen bonded amide NH does not seems to be straightforward. In fact, in order to make direct comparisons of the thermodynamic parameters between various hydrogen bonded geometries it is quite reasonable to argue for employing common upper as well as lower limiting chemical shift values.

The temperature dependence of K_{eq} was then used to construct a van't Hoff plot for the hydrogen bonded Thr amide NH by using the van't Hoff equation $\ln K_{eq}=(-\Delta H/R)\cdot1/T+(\Delta S/R)$ from which thermodynamic parameters of the C_5-structure were derived (Fig. 2). The thermodynamic parameters ΔH, ΔS and ΔG obtained for the unfolding process of the hydrogen bonded five-membered ring for **1** and **2** are listed in Table 1. The results of van't Hoff analysis clearly indicate that in an apolar, hydrophobic environment state **A** is 0.78 and 0.66 kcal/mol more favored enthalpically and disfavored entropically by 2.66 and 2.07 e.u. than state **B** in **1** and **2** respectively, under identical conditions. It is important to note that these results are valid only if $\Delta C_p\approx0$ (almost negligible), as strongly evidenced from the linearity of the van't Hoff plots over the temperature range studied [21,22]. Interestingly, the

Table 1
Relevant thermodynamic parameters of the C_5-structure as deduced from van't Hoff plots, in $CDCl_3$

Parameter	1	2
$\Delta\delta/\Delta T\times10^{-3}$ (ppm/K)[a]	3.10	2.50
ΔH (kcal/mol)	−0.78	−0.66
ΔS (e.u.)	−2.66	−2.07
ΔG (kcal/mol)[b]	0.01	−0.05

[a]$\Delta\delta/\Delta T$ determined over the temperature range 293–318 K.
[b]ΔG calculated at 298 K.

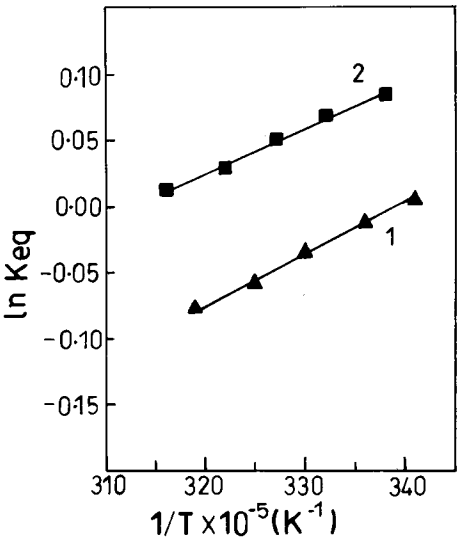

Fig. 2. van't Hoff plots of ln K_{eq} vs. 1/T (K^{-1}) constructed for an internally hydrogen bonded Thr backbone amide NH over a range of temperatures in **1** and **2** (regression coefficient ≈1). A peptide concentration of ~3 mg/0.5 ml in $CDCl_3$ was employed to obtain variable temperature ^1H NMR data.

data are likely to suggest that the five-membered ring system is thermodynamically quite stable at ambient temperature and juxtaposition of hydrogen bond donor and acceptor of the Thr residue favors optimal C_5 geometry enthalpically. The most striking feature of this study indicates that the net contribution of enthalpic as well as entropic parameters in overall ΔG at ambient temperature is roughly comparable with those reported for other hydrogen bonded secondary structural elements mimicking various reverse turns, e.g. β- and γ-turn conformations [14,18–20,23,24]. It is pertinent to note that the enthalpy-entropy internal compensation effects are the consequences of the unique complex phenomenon of flexible and/or conformationally restricted geometries of the structures and the solvent properties interacting with them. Further, it is worth mentioning that though the reverse turn structures, frequently observed in small biologically active peptides and proteins, have been investigated conformationally for more than two decades, only limited thermodynamic information is available from the studies performed on model peptides in apolar hydrophobic media. A comparison of ΔG at 298 K indicates that in both **1** and **2** the hydrogen bonded state **A** is substantially stable. The data also reflect that in non-interacting apolar media the presence of C^{β} or C^{γ} branching does not significantly stabilize/destabilize this element of backbone conformation.

While thermodynamic information of proteins has become available during the past two decades, corresponding thermodynamic parameters of peptides and related molecules, having well defined conformations stabilized by an intramolecular hydrogen bond(s), have only become available in recent years [4,24–26]. The present investigation describes the first thermodynamic characterization and the relevant energetic parameters of a locally extended intramolecularly hydrogen bonded C_5-structure across a chiral proteinogenic residue. The absence of any long-range tertiary interactions in these model peptides may suggest the potential of the primary structure for dictating the formation of a thermodynamically stable intramolecularly hydrogen bonded C_5-structure in physiologically relevant peptides. The results of analysis promise to provide insight into a quantitative understanding of the stability of polypeptides and proteins containing this element of secondary structure. The data also suggest that the presence of $C^{\delta}H_3$ bearing hydrophobic side chains with different geometrical orientations in the proximity are unlikely to play a critical role in quantitative stabilization of C_5 conformation, particularly in an apolar, hydrophobic environment, unless otherwise influenced by other factors and/or interactions. The available results indicate that the intrinsic potential of the Thr residue in these model peptides to form the C_5-structure is sufficient to be influenced by $C^{\delta}H_3$ bearing residues.

Surprisingly, the available thermodynamic parameters associated with 10- and 7-membered hydrogen bonded ring structures indicate that the thermal stability of the C_5-structure is comparable in hydrophobic media despite dramatic deviation in the N-H–O angle in the latter. The average N-H–O angle for C_5-structures, incorporated into peptides containing $C^{\beta,\beta'}$ symmetrically disubstituted non-proteinogenic, achiral glycine residues, determined from X-ray diffraction studies is $\sim 110°$, which is significantly reduced from linearity [27,28]. Usually, such non-planarity about N-H–O=C torsion angles is expected to result in a weak intraresidue hydrogen bond. The observed unusual thermal stability clearly emphasizes that the C_5-structure in proteins and polypeptides merits further consideration and requires immediate attention.

One of the mechanistic models, the 'framework model' proposed for protein folding, receives strong support for the transient existence of partly folded or folded hydrogen bonded structures in short linear peptides representing protein fragments [1,6,7]. The thermodynamically stable C_5-structure, more convincingly a 'metastable intermediate', in a non-polar environment is biologically more relevant for elucidating the protein folding behavior in non-aqueous environments and may reveal its energetics in the interior hydrophobic core of the protein and proteins and polypeptides in membrane-like environments. Further, the 'unusual thermal stability' of a hydrogen bonded five-membered ring is likely to provide strong support for the notion that this structure may be a 'seeding precursor' of a subsequent folding pathway and would contribute significantly to the stability of the native structure. At present, though our study does not provide direct experimental evidence for the existence of this folded structure during protein folding under physiological conditions, its substantial stability is expected to contribute fundamentally in initiation of protein folding pathways presumably by 'restricting the available conformational space' in the Ramachandran map and triggering subsequent events for cooperative growth towards the native structure.

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