An alternate conformation of the hyperthermostable HU protein from *Thermotoga maritima* has unexpectedly high flexibility

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Abstract The homodimeric HU protein from the hyperthermophile *Thermotoga maritima* (HU*Tmar*) is a model system which can yield insights into the molecular determinants of thermostability in proteins. Unusually for a thermostable protein, HU*Tmar* exists in a structurally heterogeneous state as evidenced by the assignment of two distinct and approximately equally populated forms in solution. Relaxation measurements combined with chemical shift, hydrogen exchange, and nuclear Overhauser enhancement data confirm the main structural features of both forms. In addition, these data support a two-state model for HU*Tmar* in which the major form closely resembles the X-ray structure while the very flexible minor form is less structured. HU*Tmar* may therefore be a new example of the small class of hyperthermostable proteins with unexpected flexibility.

Key words: Conformational flexibility; Protein dynamics; Thermostability; Nuclear magnetic resonance spin relaxation

1. Introduction

Proteins from thermophilic organisms display properties that reflect the environment in which they have evolved. The general consensus reached from biophysical studies of many different systems is that thermal stability results from conformational rigidity due to the increased packing efficiency in the structures of thermophilic proteins [1]. Recent data from studies of selected thermostable systems with unexpected flexibility challenge this paradigm however [2].

The HU-IHF family of dimeric histone-like proteins have various roles in DNA remodelling, bending, compaction, and negative supercoiling of the bacterial nucleoid [3–5]. Members of this family include HU, integration host factor (IHF), H-NS, TF1, and Fis, and can bind DNA specifically or non-specifically. The intrinsic dynamics of HU proteins is highlighted by X-ray structures of HU proteins of *Bacillus stearothermophilus* (HU*Bst*) and *Thermotoga maritima* (HU*Tmar*) (refined to resolutions of 2.1 Å and 1.53 Å respectively) in which no electron density was observed for the DNA binding β-arms [6,7]. The nuclear magnetic resonance (NMR) structure of HU*Bst* provided the first details on the structure and dynamics of the highly mobile yet internally structured β-arms in the free protein [8,9]. The β-arms stabilise and wrap around the DNA upon binding as clearly shown by the crystal structures of the DNA complexes of both IHF and HU [10–12]. These studies present a dramatic bending of the DNA mediated by proline intercalation by residues in the β-arms which in turn become much less mobile. The dynamics of the β-arms therefore play an essential role in mediating DNA binding.

The HU*Tmar* protein ($T_m = 80.5$°C) is a model thermostable system with unusually high-affinity and temperature-independent DNA binding properties [13]. The extreme stability and versatility of HU*Tmar* ensures protection of tightly bound DNA substrates which has allowed the development of biotechnological applications, for instance, as a highly effective gene delivery agent [14].

The results from an NMR analysis of the behaviour of HU*Tmar* in solution are complementary to those from a recent site-directed mutagenesis study characterising destabilising mutations [15]. Our results characterise an anomalous dynamic property of HU proteins. We noted that wild-type and mutants of HU*Tmar* are unexpectedly heterogeneous in solution. The data also support a two-state model in which the major form resembles the X-ray structure while the flexible minor form seems to result from a structural rearrangement of the dimer architecture. The use of the major/minor terminology therefore refers to structural integrity rather than populations of the two conformations. The observed dynamics of HU*Tmar* in solution are unforeseen when compared with other thermostable proteins.

2. Materials and methods

2.1. Sample preparation

Cloning of the HU*Tmar* gene, overexpression, and purification of wild-type and mutant proteins were performed as described [16]. Fully labelled protein was produced using ^{15}N-NH_4Cl and ^{13}C-glucose in minimal medium. Protein samples (1 mM) were prepared in a 50 mM phosphate buffer with 200 mM KCl at pH 5.8. Addition of the pro-

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Abbreviations: HU*Tmar*, *Thermotoga maritima* HU protein; HU*Bst*, *Bacillus stearothermophilus* HU protein; HU*Bsu*, *Bacillus subtilis* HU protein; IHF, integration host factor; NOE, nuclear Overhauser enhancement; R_1, R_2, longitudinal and transverse relaxation rates; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser enhancement spectroscopy

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tein to the buffer solution changes the pH to approximately 6.2 since the protein is positively charged. 7.5% D_2O was added for NMR measurements.

2.2. NMR spectroscopy

NMR spectra were recorded on Bruker Avance spectrometers operating at proton frequencies of 500, 600, 700, and 750 MHz at a temperature of 311 K. The backbone and side chain resonances were assigned using standard triple resonance techniques [17]. The ^{15}N heteronuclear nuclear Overhauser enhancement (NOE), longitudinal and transverse relaxation rate (R_1 and R_2) data sets were recorded using modified versions of the standard Bruker pulse sequences. The NOE experiment was repeated three times, R_1 data sets were recorded with relaxation delays of: 0.1 (×2), 0.2, 0.3, 0.4 (×2), 0.5, 0.6, 0.8, 1.0 s; and R_2 data sets with delays of 0 (×2), 2, 16, 32, 48, 64 (×2), 80, 96, 128, and 160 ms. All NMR data were processed using NMRPipe [18] and analysed using NMRView [19]. Relaxation data were fitted as described [20]. Hydrogen exchange protection factors were calculated as described [21].

3. Results

A sequence alignment of HU proteins and the secondary structure elements of the crystal structure of HU*Tmar* are presented in Fig. 1. Surprisingly, 140 peaks (excluding side chains) were counted in the heteronuclear single quantum coherence (HSQC) spectrum where 84 were expected (Fig. 2). The extra peaks could be assigned to residues throughout the protein and two slowly exchanging and approximately equally populated forms of the protein exist under the experimental conditions. This equilibrium seems to persist indefinitely and no time-dependent changes in the intensities of peaks from either form were observed. All residues in the primary sequence were assigned in the major form while 75 residues were assigned in the minor form. The remaining residues in the minor form are degenerate with those in the major form. The temperature, pH, salt concentration and protein concentration were all varied with no significant change in the spectra and no degradation was observed on sodium dodecyl sulphate gels. Urea–Triton gels, which are very sensitive and can identify point mutations, show a single homogeneous band which indicates that HU*Tmar* is chemically homogeneous (data not shown). One fraction of the protein sample was thermally denatured and refolded into the same conformational equilibrium. Major chemical shift

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          <β1><----α1----><---------α2---------> <β2> <β3->
Tmar -----MTKKELIDRVAKKAGAKKKDVKLILDTILETITEALAKGEKVQIVGFGSF 50
Bst  -----MNKTELINAVAETSGLSKKDATKAVDAVFDSITEALRKGDKVQLIGFGNF 50
Bsu  -----MNKTELINAVAEASELSKKDATKAVDSVFDTILDALKNGDKIQLIGFGNF 50
Tth  AAKKTVTKADLVDQVAQATGLKLLDVKAMVDALLAKVEEALANGSKVQLTGFGTF 55
Tvo  ----MVGISELSKDVAKKANTTQKVARTVIKSFLDEIVAQANGGQKINLAGFGIF 51
          : : :*:  **   :  :::     :     ::::: :* *::  ***

     <β3><--flexible β-arms--> <β4><---α3--->
Tmar EVRKAAARKGVNPQTRKPITIPERKVPKFKPGKALKEKVK-- 90
Bst  EVRERAARKGRNPQTGEEMEIPASKVPAFKPGKALKDAVK-- 90
Bsu  EVRERSARKGRNPQTGEEIEIPASKVPAFKPGKALKDAVAGK 92
Tth  EVRKRKARTGVKPGTKEKIKIPATQYPAFKPGKALKDKVK-- 95
Tvo  ERRTQGPRKARNPQTKKVIEVPSKKKFVFRASSKIKYQQ--- 90
     **:*  ::*:  :*:*   :*  ::: *:::::::*  ::
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Fig. 1. ClustalW [32] alignment of HU sequences from *Thermotoga maritima* (Tmar), *Bacillus stearothermophilus* (Bst), *Bacillus subtilis* (Bsu), *Thermus thermophilus* (Tth) and *Thermoplasma volcanium* (Tvo). Asterisks indicate residues conserved in all five sequences and colons indicate residues additionally conserved between HU*Tmar* and HU*Bst*. The secondary structure elements in the X-ray structure of HU*Tmar* are indicated.

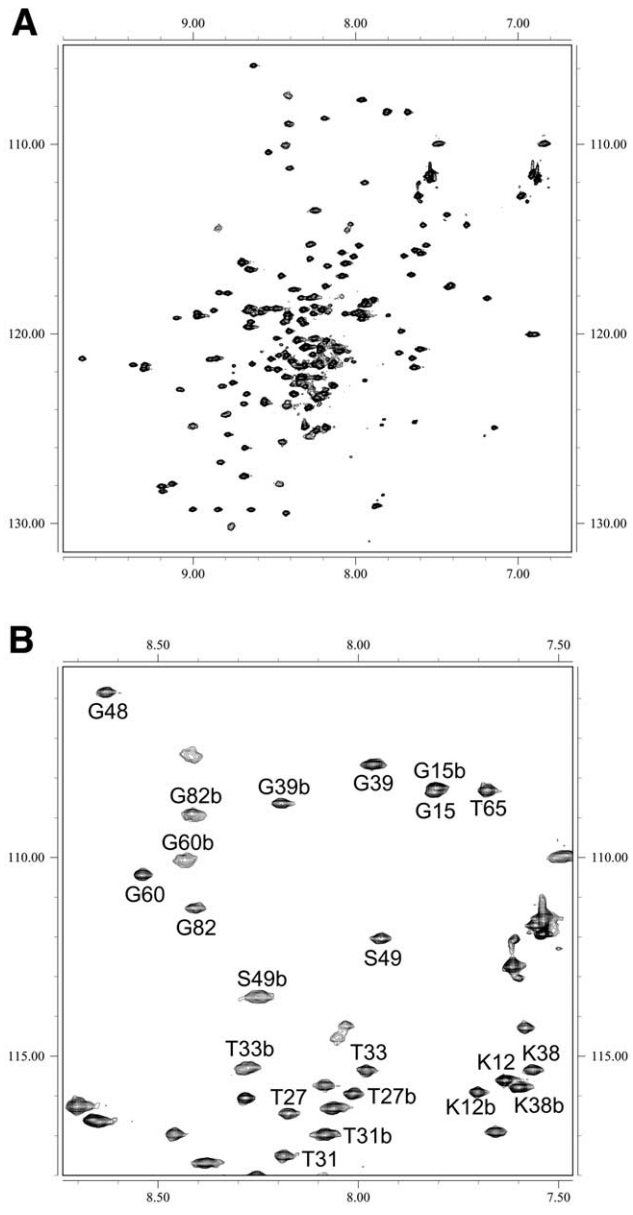

Fig. 2. 2D ^{15}N HSQC spectrum (A) and excerpt (B) of uniformly ^{15}N-labelled HU*Tmar* recorded at 750 MHz proton frequency and 311 K. Selected resonances are marked with their assignments where b indicates the minor form.

differences indicating conformational differences between the two forms are found throughout the sequence and notably in the short C-terminal α-helix (Fig. 3). The HSQC spectra of two destabilising point mutants (E34D and V42I) are almost identical to that of the wild-type protein, showing the same doubled set of NMR signals. The heterogeneity of HU*Tmar* in solution is therefore an intrinsic property of the structure. Analysis of the ^{13}C chemical shift data [22] rule out *cis-trans* proline isomerisation as an explanation for the two forms.

Considering first the major form, the secondary structure determined by the $^{13}C^{\alpha}$ secondary chemical shifts is consistent with that observed in the NMR and X-ray structures of HU*Bst* and HU*Tmar* (Fig. 4). The general pattern observed in the relaxation data (Fig. 5) is that the residues of the β-arms are more flexible than the core of the protein. The more

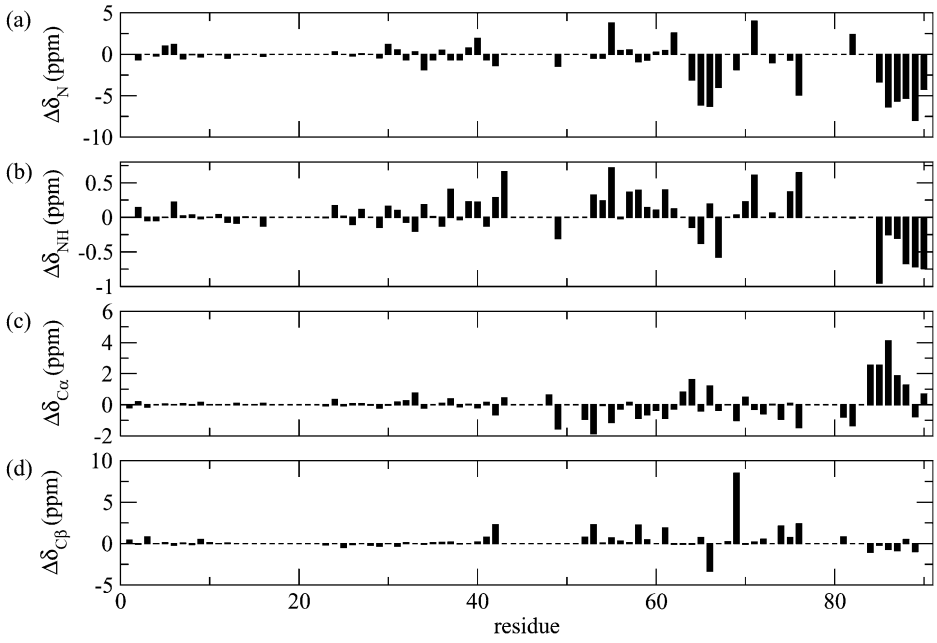

Fig. 3. Chemical shift mapping, $\Delta\delta$ = major−minor (ppm), to identify conformational differences between the major and minor forms of HU*Tmar*.

negative values for the ^{15}N heteronuclear NOE and the lower values for transverse relaxation rates compared to the core of the protein indicate enhanced mobility. In addition the values of the NOE and R_2 for residues N62–I69 indicate a maximum degree of flexibility for the turns at the tips of the β-arms. The dynamics of the major form are therefore highly similar to those observed for HU*Bst* [9].

In contrast, the minor form shows a number of critical differences compared to the major form. The $^{13}C^{\alpha}$ shifts (Fig. 4) indicate a change in conformation for the C-terminal residues G82–K90. In addition, the minor form clearly has different relaxation rates for residues for which data are available (Fig. 6). In particular, the substantially more negative values of the heteronuclear NOE and the lower values of the R_2 rates for the β-arms and the C-terminus indicate even higher mobility compared to the major form. In support of this the nuclear Overhauser enhancement spectroscopy (NOESY) spectra show no crosspeaks for the majority of residues of the β-arms and the C-terminus in the minor form. This is in contrast with the major form in which the characteristic NOEs defining the structure of the β-arms and the C-terminal α-helix were assigned in the same way as for

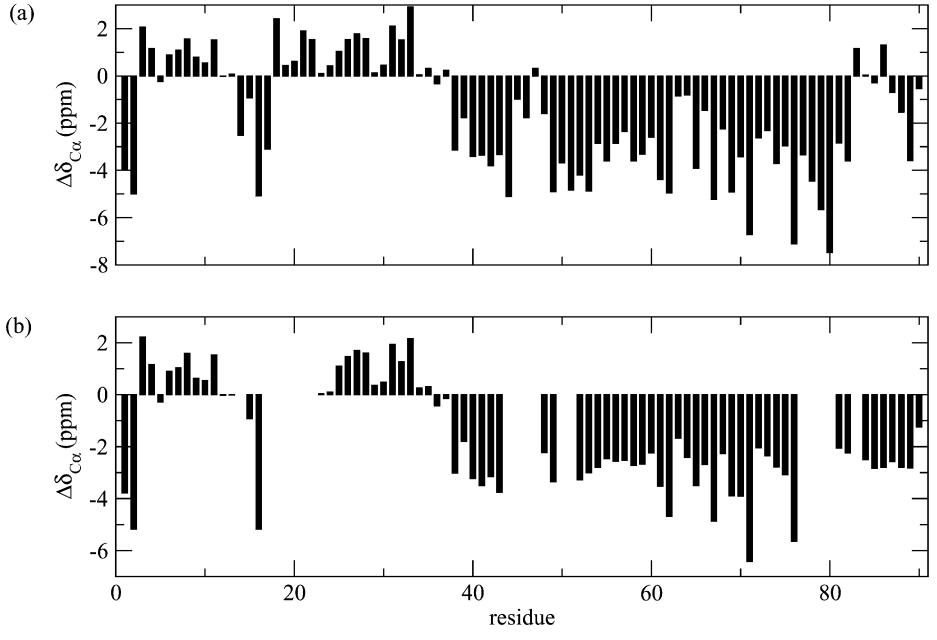

Fig. 4. Backbone $^{13}C^{\alpha}$ secondary chemical shifts for the (a) major and (b) minor forms of HU*Tmar*. Positive and negative shifts are indicative of α-helical and β-strand conformations respectively [33].

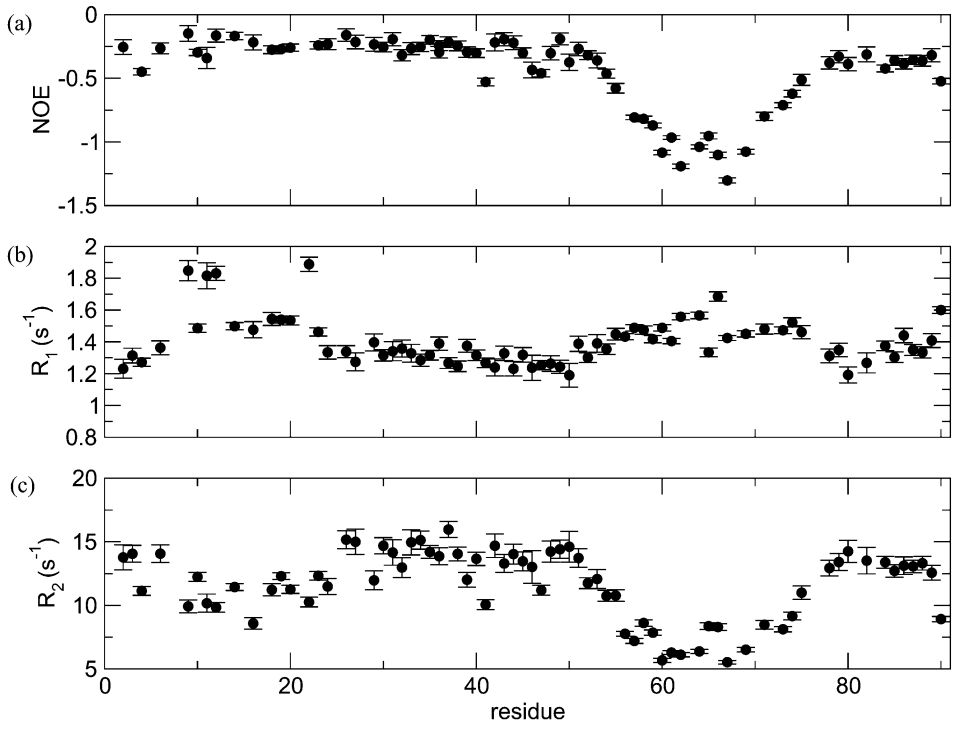

Fig. 5. Backbone ^{15}N (a) NOE, (b) R_1 and (c) R_2 data for the major form of HU*Tmar*. Lower values of NOE and R_2 indicate increased flexibility on a ps–ns timescale.

HU*Bst* [23]. Protection factors calculated from amide proton exchange also indicate a less stable α2 in the minor form (Fig. 7).

The R_2/R_1 ratio reflects the orientation of secondary structure elements (or complete molecules) with respect to the overall rotational symmetry axis. The data clearly indicate differences between the two forms (Fig. 6). The major form of HU*Tmar* and HU*Bst* have similar values of the R_2/R_1 ratios throughout the sequence. This indicates that the major form has a diffusional anisotropy which is comparable to that of HU*Bst*. Furthermore, the R_2/R_1 values for α1 are lower on average than those for α2. In the minor form the opposite effect is seen since the R_2/R_1 values for α1 are higher than those for α2. While increased R_2 rates (and consequently R_2/R_1 ratios) can be due to conformational exchange, decreased R_2 rates can only be due to increased mobility. Since the average magnitudes of the deviations are similar for both the increased and the decreased rates and uniform in the α1

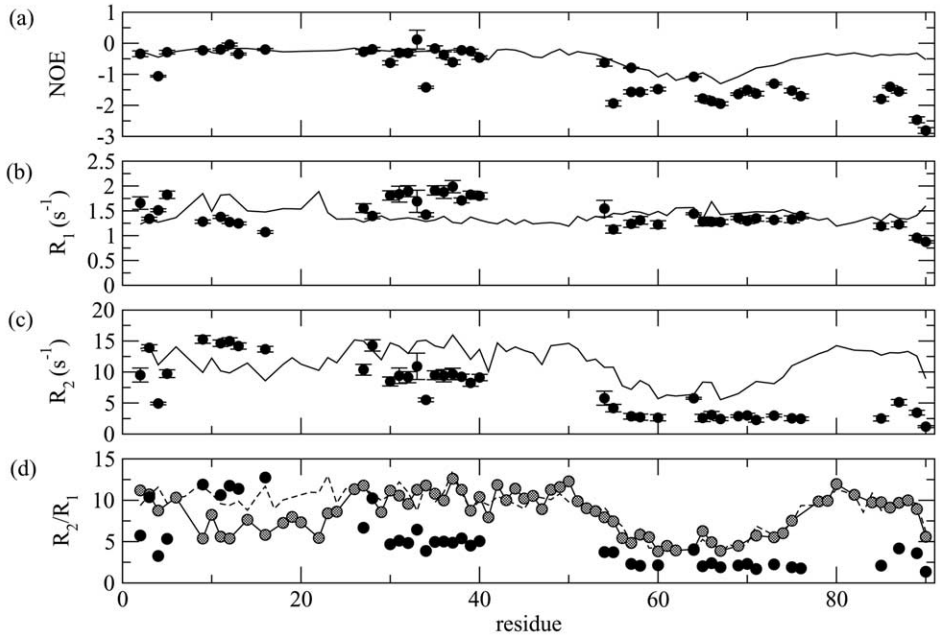

Fig. 6. Comparison of (a) NOE, (b) R_1 and (c) R_2 values for the major (line) and minor (circles) forms of HU*Tmar*; (d) comparison of R_2/R_1 ratios for the major (shaded circles) and minor (filled circles) forms of HU*Tmar* with the values for HU*Bst* (dashed line) for reference.

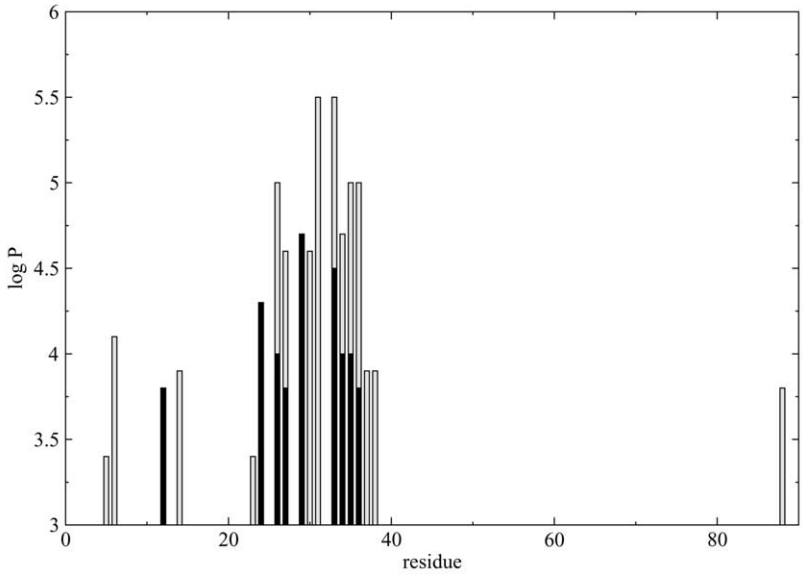

Fig. 7. Protection factors (P) calculated for the major (shaded bars) and minor (black bars) forms of HU*Tmar*. Higher values indicate backbone amide protons which are less solvent-accessible due to either structural location or conformational fluctuations.

and α2 helices, conformational exchange is not likely. The R_2/R_1 values show rather that the relative positions of the two helices with respect to the diffusional symmetry axis are different for the major and minor forms.

4. Discussion

There are several sources of data which illustrate the different conformational properties of the two forms of HU*Tmar* in solution. The two most likely explanations for this conformational heterogeneity are: (1) monomer–dimer equilibrium and (2) different dimeric structures due to dynamic effects possibly leading to a structural rearrangement. We propose that the latter is the case for HU*Tmar* based on our analysis of the available data. Furthermore, based on the chemical shift, NOESY, and relaxation data we conclude that the major form bears close resemblance to both the X-ray and NMR structures of HU*Tmar* and HU*Bst* respectively.

The monomeric state of HU*Tmar* is likely to exist only very transiently in solution due to the high stability of the dimer [24] and therefore cannot account for the observed differences in the relaxation data. This is supported by the observation that changes in the protein and salt concentrations do not significantly change the spectra. In addition the average R_2/R_1 values for the core of the protein are very similar for the major and minor forms which would not be expected for a monomeric minor form. A comparison of the R_2/R_1 values for the α1 and α2 helices however indicates a difference in the relative positions of the two helices with respect to the molecular diffusion axis for the two forms of HU*Tmar*. Whether this is due to a molecular rearrangement of the helices or due to a reorientation of the major diffusion axis cannot be concluded at this stage. This would require a high-resolution structural analysis of the minor form.

The high stability of the HU*Tmar* dimer results from intermonomer contacts in the compact core of the protein. The flexible β-arms extend from the core and do not participate in formation of the dimer but are instead available to wrap around a DNA substrate [8]. A recent Raman spectroscopy deuterium exchange study of the solution conformation of HU*Bst* found unexpectedly high exchange rates for helical elements which the authors attributed to flexibility at the dimeric interface [25]. The backbone relaxation rates measured for both forms of HU*Tmar* also indicate unusual flexibility at both the homotypic and heterotypic dimeric interfaces formed by the two pairs of α1 and α2 helices. Flexibility at the dimeric interface could account for the critical role of residue G15 in the turn between the α1 and α2 helices since mutation of this residue is known to dramatically reduce the thermostability of HU proteins [15,26].

The biophysical properties of HU proteins from various sources have been documented in detail [6]. In marked contrast to HU*Tmar*, protein concentration and ionic strength have been shown to affect both the structure and stability of HU*Bsu* in solution as measured by circular dichroism [27]. These observations were extended with electrospray ionisation mass spectrometry measurements of the influence of the ionic strength on the monomer–dimer equilibrium in HU*Bsu* [28]. The NMR data presented here expand on the earlier biophysical results for HU*Tmar* [24] since information is now available for individual amino acids and the existence of dual conformations is surprising.

Recent research has raised interesting questions about correlating thermostability with rigidity or flexibility in thermostable proteins [1,30,31]. For instance, the most thermostable protein known, rubredoxin from *Pyrococcus furiosus*, with a melting temperature close to 200°C has been shown to have similar flexibility to the mesophilic rubredoxin from *Clostridium pasteurianum* [29].

5. Conclusion

We have demonstrated that HU*Tmar* is unexpectedly heterogeneous and dynamic in solution on a broad timescale range. The major form in solution has features consistent with those determined in the X-ray structure as evidenced by chemical shift, NOE, and ^{15}N relaxation data. The presence of the two forms in both wild-type and several mutants

of HU*Tmar* suggests that the equilibrium is an intrinsic property of the native structure. The dynamical behaviour of HU*Tmar* suggests that this protein may be a new example of the small class of extremely thermostable proteins which display unexpected structural heterogeneity in solution. To the best of our knowledge HU*Tmar* is the first example of a homodimeric thermostable protein displaying these properties.

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