

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

Michael A. Durney<sup>a,b</sup>, Rainer W. Wechselberger<sup>a</sup>, Charalampos G. Kalodimos<sup>a,1</sup>,  
Robert Kaptein<sup>a</sup>, Constantinos E. Vorgias<sup>c</sup>, Rolf Boelens<sup>a,\*</sup>

<sup>a</sup>*Bijvoet Center for Biomolecular Research, Universiteit Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

<sup>b</sup>*Department of Chemistry, University of Southampton, Southampton SO17 1BJ, UK*

<sup>c</sup>*Department of Biochemistry and Molecular Biology, National and Kapodistrian University of Athens, 15784 Athens, Greece*

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**Abstract** The homodimeric HU protein from the hyperthermophile *Thermotoga maritima* (HUTmar) is a model system which can yield insights into the molecular determinants of thermostability in proteins. Unusually for a thermostable protein, HUTmar 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 HUTmar in which the major form closely resembles the X-ray structure while the very flexible minor form is less structured. HUTmar may therefore be a new example of the small class of hyperthermostable proteins with unexpected flexibility.

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**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), HNS, 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* (HUBst) and *Thermotoga maritima* (HUTmar) (refined to resolutions of 2.1 Å and 1.53 Å respectively) in which no electron density was observed for the DNA binding  $\beta$ -arms [6,7]. The nuclear magnetic resonance (NMR) structure of HUBst provided the first details on the structure and dynamics of the highly mobile yet internally structured  $\beta$ -arms in the free protein [8,9]. The  $\beta$ -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  $\beta$ -arms which in turn become much less mobile. The dynamics of the  $\beta$ -arms therefore play an essential role in mediating DNA binding.

The HUTmar protein ( $T_m = 80.5^\circ\text{C}$ ) is a model thermostable system with unusually high-affinity and temperature-independent DNA binding properties [13]. The extreme stability and versatility of HUTmar 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 HUTmar 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 HUTmar 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 HUTmar in solution are unforeseen when compared with other thermostable proteins.

## 2. Materials and methods

### 2.1. Sample preparation

Cloning of the HUTmar gene, overexpression, and purification of wild-type and mutant proteins were performed as described [16]. Fully labelled protein was produced using  $^{15}\text{N}$ - $\text{NH}_4\text{Cl}$  and  $^{13}\text{C}$ -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-

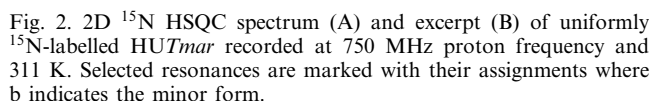
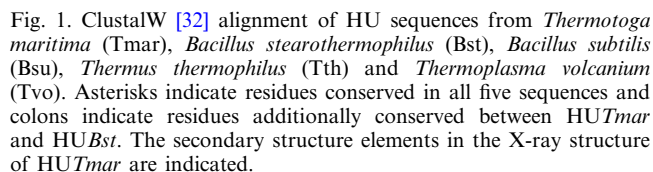
\*Corresponding author. Fax: (31)-30-2537623.

E-mail address: r.boelens@chem.uu.nl (R. Boelens).

<sup>1</sup> Present address: Department of Chemistry, Rutgers University, Newark, NJ 07102-1811, USA.

**Abbreviations:** HUTmar, *Thermotoga maritima* HU protein; HUBst, *Bacillus stearothermophilus* HU protein; HUBsu, *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

A sequence alignment of HU proteins and the secondary structure elements of the crystal structure of HUT<sub>mar</sub> 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 HUT<sub>mar</sub> 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



differences indicating conformational differences between the two forms are found throughout the sequence and notably in the short C-terminal  $\alpha$ -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 HUTmar in solution is therefore an intrinsic property of the structure. Analysis of the  $^{13}\text{C}$  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  $^{13}\text{C}\alpha$  secondary chemical shifts is consistent with that observed in the NMR and X-ray structures of HUBst and HUTmar (Fig. 4). The general pattern observed in the relaxation data (Fig. 5) is that the residues of the  $\beta$ -arms are more flexible than the core of the protein. The more

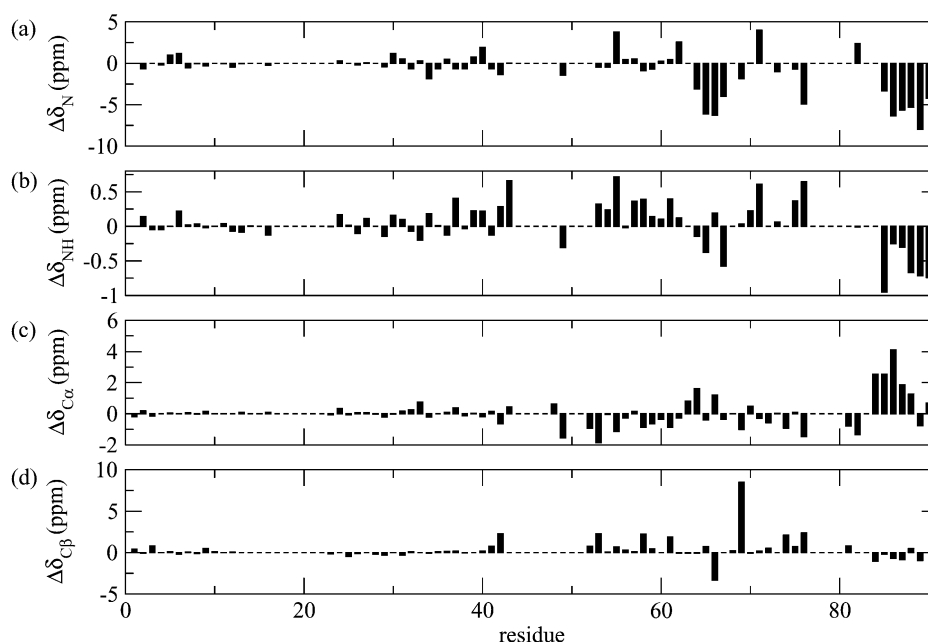


Fig. 3. Chemical shift mapping,  $\Delta\delta$ =major–minor (ppm), to identify conformational differences between the major and minor forms of HUTmar.

negative values for the  $^{15}\text{N}$  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  $\beta$ -arms. The dynamics of the major form are therefore highly similar to those observed for HUBst [9].

In contrast, the minor form shows a number of critical differences compared to the major form. The  $^{13}\text{C}\alpha$  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  $\beta$ -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  $\beta$ -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  $\beta$ -arms and the C-terminal  $\alpha$ -helix were assigned in the same way as for

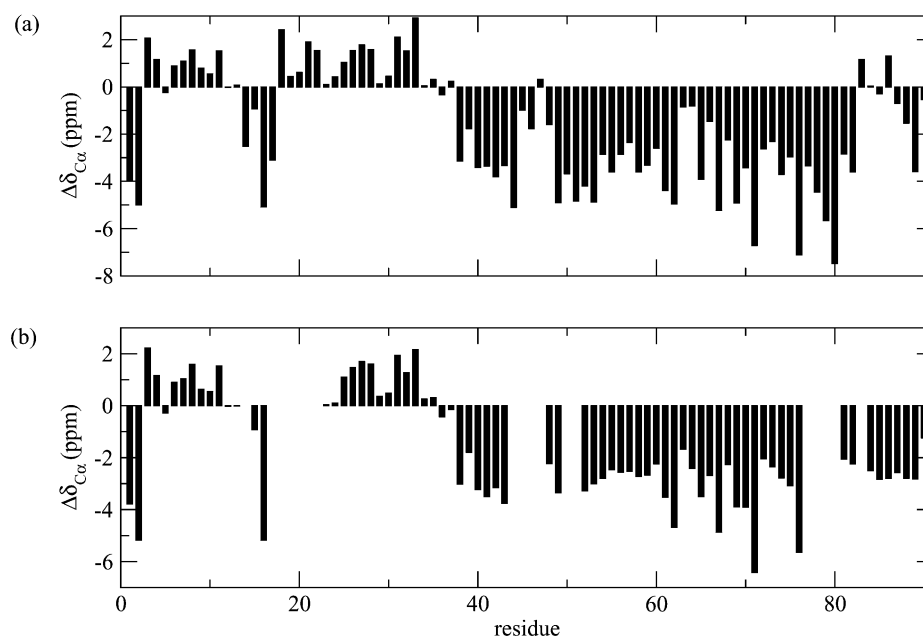


Fig. 4. Backbone  $^{13}\text{C}\alpha$  secondary chemical shifts for the (a) major and (b) minor forms of HUTmar. Positive and negative shifts are indicative of  $\alpha$ -helical and  $\beta$ -strand conformations respectively [33].

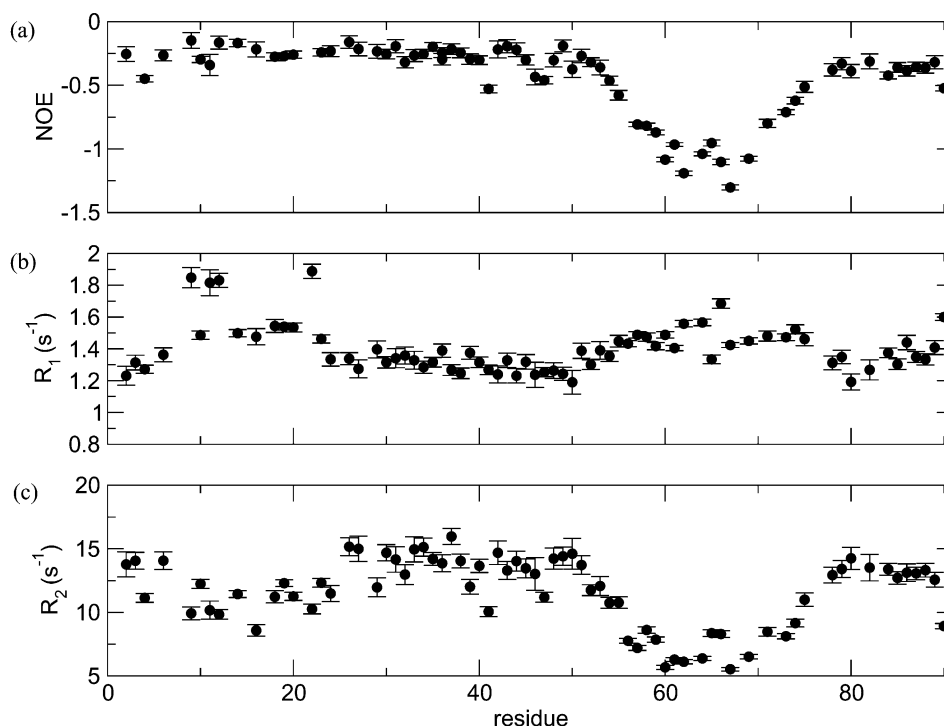


Fig. 5. Backbone  $^{15}\text{N}$  (a) NOE, (b)  $R_1$  and (c)  $R_2$  data for the major form of HUTmar. Lower values of NOE and  $R_2$  indicate increased flexibility on a ps–ns timescale.

HUBst [23]. Protection factors calculated from amide proton exchange also indicate a less stable  $\alpha 2$  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 HUTmar and HUBst 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 HUBst. Furthermore, the  $R_2/R_1$  values for  $\alpha 1$  are lower on average than those for  $\alpha 2$ . In the minor form the opposite effect is seen since the  $R_2/R_1$  values for  $\alpha 1$  are higher than those for  $\alpha 2$ . 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  $\alpha 1$

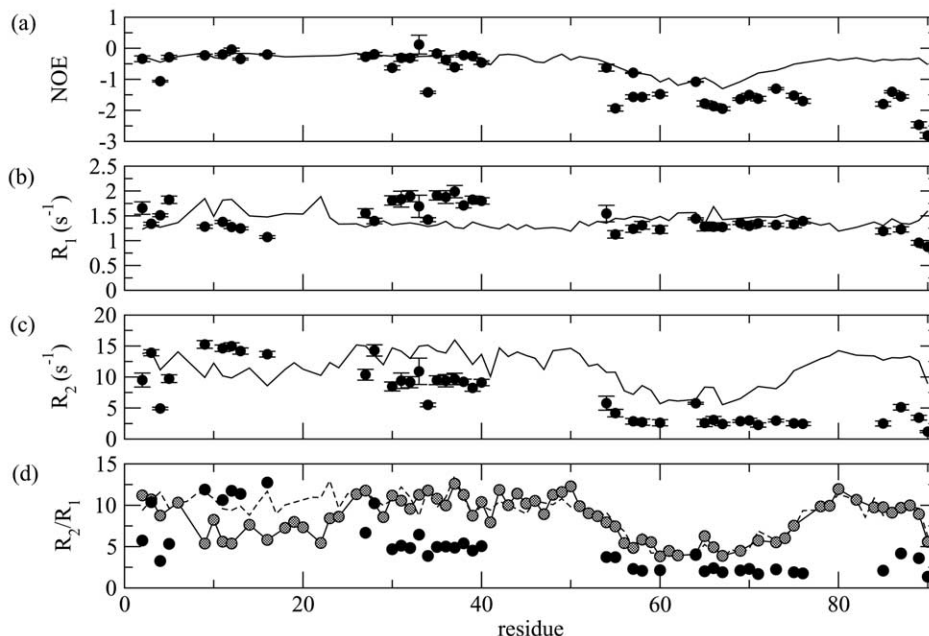


Fig. 6. Comparison of (a) NOE, (b)  $R_1$  and (c)  $R_2$  values for the major (line) and minor (circles) forms of HUTmar; (d) comparison of  $R_2/R_1$  ratios for the major (shaded circles) and minor (filled circles) forms of HUTmar with the values for HUBst (dashed line) for reference.

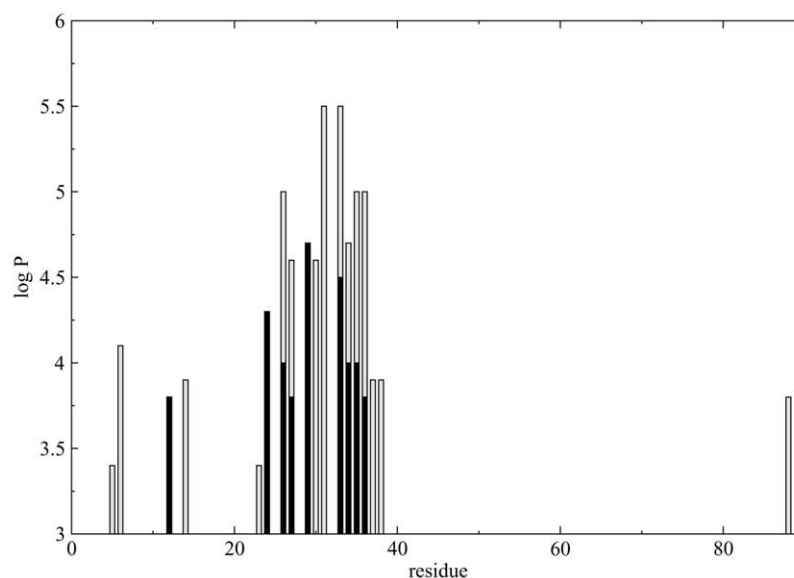


Fig. 7. Protection factors ( $P$ ) calculated for the major (shaded bars) and minor (black bars) forms of HUTmar. Higher values indicate backbone amide protons which are less solvent-accessible due to either structural location or conformational fluctuations.

and  $\alpha 2$  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 HUTmar 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 HUTmar 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 HUTmar and HUBst respectively.

The monomeric state of HUTmar 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  $\alpha 1$  and  $\alpha 2$  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 HUTmar. 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 HUTmar dimer results from inter-monomer contacts in the compact core of the protein. The flexible  $\beta$ -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 HUBst 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 HUTmar also indicate unusual flexibility at both the homotypic and heterotypic dimeric interfaces formed by the two pairs of  $\alpha 1$  and  $\alpha 2$  helices. Flexibility at the dimeric interface could account for the critical role of residue G15 in the turn between the  $\alpha 1$  and  $\alpha 2$  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 HUTmar, protein concentration and ionic strength have been shown to affect both the structure and stability of HUBsu 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 HUBsu [28]. The NMR data presented here expand on the earlier biophysical results for HUTmar [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 HUTmar 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  $^{15}\text{N}$  relaxation data. The presence of the two forms in both wild-type and several mutants

of HUTmar suggests that the equilibrium is an intrinsic property of the native structure. The dynamical behaviour of HUTmar 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 HUTmar is the first example of a homodimeric thermostable protein displaying these properties.

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