





Structure discrimination for the C-terminal domain of Escherichia coli trigger factor in solution

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Abstract NMR measurements can give important information on solution structure, without the necessity for full-scale solution structure determination. The C-terminal protein binding domain of the ribosome-associated chaperone protein trigger factor is composed of non-contiguous parts of the polypeptide chain, with an interpolated prolyl isomerase domain. A construct of the C-terminal domain of Escherichia coli trigger factor containing residues 113–149 and 247–432, joined by a Gly-Ser-Gly-Ser linker, is well folded and gives excellent NMR spectra in solution. We have used NMR measurements on this construct, and a longer construct that includes the prolyl isomerase domain, to distinguish between two possible structures of the C-terminal domain of trigger factor, and to assess the behavior of the trigger factor C-terminal domain in solution. Crystal structure comparison of two X-ray crystal structures, of intact trigger factor from E. coli (Ferbitz et al., Nature 431:590–596, 2004), and of a truncated trigger factor from Vibrio cholerae (Ludlam et al., Proc Natl Acad Sci USA 101:13436–13442, 2004) showed significant differences in the structure of the C-terminal domain, such that the two structures could not be superimposed. We show using NMR chemical shifts and long range nuclear Overhauser effects that the secondary and tertiary structure of the E. coli C-terminal domain in solution is consistent with the crystal structure of the E. coli trigger factor and not with the V. cholerae protein. Given the similarity of the amino acid sequences of the E. coli and V. cholerae proteins, it appears likely that the structure of the V. cholerae protein has been distorted as a result of truncation.

Keywords Chaperone Trigger factor · Peptide binding domain · C-terminal domain · Crystal structure comparison

Two X-ray crystal structures, of intact trigger factor from E. coli (Ferbitz et al., Nature 431:590–596, 2004), and of a truncated trigger factor from Vibrio cholerae (Ludlam et al., Proc Natl Acad Sci USA 101:13436–13442, 2004)

Introduction
The first chaperones that a newly synthesized protein chain encounters are thought to be bound to the ribosome. Trigger factor, a chaperone protein from E. coli, has been shown to bind to the large subunit of ribosomes with a 1:1 stoichiometry, and has been proposed as one of the first chaperones encountered by a nascent peptide. The N-terminus of a newly synthesized protein binds to trigger factor while it is still attached to the ribosome. The trigger factor–nascent protein complex subsequently dissociates from the ribosome, following the protein to be handed off to other cytosolic chaperones, such as the DnaK system (Hesterkamp et al. 1996; Deuerling et al. 1999; Bukau et al. 2000).

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Trigger factor has a modular structure consisting of three domains, an N-terminal domain, which is the ribosome binding site (Hesterkamp et al. 1997), a central proline isomerase (PPIase) domain (Hesterkamp and

Bukau 1996) identified initially by sequence homology with FK506-binding protein (Callebaut and Moras 1995), and a C-terminal domain, the putative binding site for nascent peptides (Hesterkamp et al. 1997). Much work has been done to characterize the PPIase domain of trigger factor from a number of organisms. Interest has been particularly high in this domain because, for some primitive organisms, it represents the only proline isomerase activity present in the genome (Bang et al. 2000). Thus, trigger factor is thought to be one of the earliest chaperones to have evolved.

A considerable amount of structural information has been published on trigger factor domains, as well as the complete protein (Vogtherr et al. 2002; Kristensen and Gajhede 2003; Ferbitz et al. 2004; Ludlam et al. 2004). These structures are illustrated in Fig. 1. Interestingly, there are significant differences between these structures, particularly in the C-terminal peptide-binding domain (blue/cyan). The “cradle” for accommodation of the

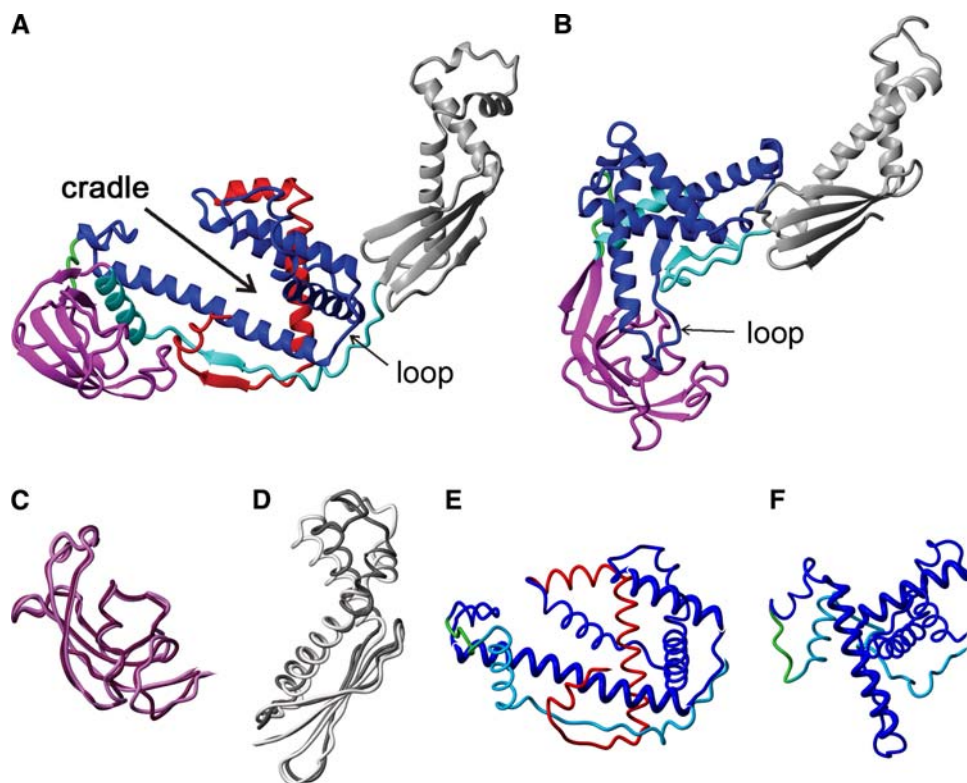


Fig. 1 (a) The structure of *E. coli* trigger factor (Ferbitz et al. 2004). The ribosomal binding domain (residues 1–111) is in gray, the PPIase domain (residues 151–242) is in magenta. The linker sequences between the C-terminal and PPIase domains are in green. The sequence between residues 112–148, which connects the ribosomal binding domain and the PPIase domain, and which is folded as part of the C-terminal domain, is shown in cyan. The part of the C-terminal domain common to both *E. coli* and *V. cholerae* trigger factors (residues 250–379) is shown in blue. The part of the C-terminal domain that is missing from the *V. cholerae* structure is shown in red. The loop between helices 2 and 3 of the C-terminal domain is labeled, as is the peptide-binding “cradle” identified by Ferbitz et al. (2004). (c) Superposition of the PPIase domains from *E. coli* and *V. cholerae* trigger factors. In parts (d) and (e), the lighter colors represent the *E. coli* and the darker colors the *V. cholerae* trigger factors. (f) C-terminal domain, is shown in cyan. The part of the C-terminal domain common to both *E. coli* and *V. cholerae* trigger factors (colors the same as both crystal structures (residues 250–379) is shown in blue. The part of the C-terminal domain that is missing from the *V. cholerae* structure is shown in red. Helices 2 and 3 of the two structures are shown with thicker lines

the secondary structure consistent with the *E. coli* or *V. cholerae* structure in solution? Is the C-terminus of the protein (red in Fig. 1) necessary for the folded structure of the protein? Are the tertiary structure and interdomain contacts in solution consistent with one or other of the structures? Are the orientation of the helices consistent with the crossing angles in Fig. 4e and f? We have addressed these questions using NMR measurements of the trigger factor C-terminal domain in solution, and concluded that the trigger factor C-terminal domain shows greater consistency in secondary structure and tertiary contacts with the *E. coli* X-ray structure (Ferbitz et al. 2004) than the *V. cholerae* X-ray structure (Ludlam et al. 2004), but that the structure in solution is most probably dynamic rather than fixed in a single conformation.

Materials and methods

Protein expression and purification

The genes for TIG(113–432) and TIG(113–432/150–246) were cloned into a pET21a vector using standard methods. Triple $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ labeled proteins were prepared by growing transformed *E. coli* BL21(DE3) in minimal medium in 99.99% D_2O with $^{15}\text{NH}_4\text{Cl}/(^{15}\text{NH}_4)_2\text{SO}_4$ and $^{13}\text{C}_6$ -glucose as the sole nitrogen and carbon sources. Cells were grown at 37 °C, induced with 1 mM IPTG at an OD_{600} of ~ 0.7 , and induced at 15 °C. Cells were harvested at an OD_{600} of ~ 1.8 , suspended in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl and 20 mM imidazole), and lysed by sonication. The lysed cells were centrifuged and the supernatant was loaded onto a 15 ml Ni-NTA column. Protein was eluted from the column with elution buffer (20 mM Tris pH 7.5, 150 mM NaCl and 200 mM imidazole). Elutions were combined, diluted down to 35 mM NaCl and further purified through a 10 ml Q-Hi trap column with a linear gradient of buffer (20 mM Tris pH 7.5, 2 mM DTT, 1 mM EDTA and 0.6 M NaCl). The yield of purified soluble TIG(113–432) and TIG(113–432/150–246) in the *E. coli* expression system are about 20 mg/l culture.

NMR spectroscopy

Fractions containing TIG(113–432/150–246) from the Q column were combined, concentrated and dialyzed against the NMR buffer (10 mM Tris pH 6.8, 50 mM NaCl, 2 mM DTT, 1 mM EDTA). NMR samples were at a concentration of approximately 1 mM in 0.5 ml of NMR buffer containing 95% $\text{H}_2\text{O}/5\%$ D_2O . 2D ^{15}N - ^1H TROSY (Pervushin et al. 1997), 3D TROSY-based deuterium decoupled HNCA (Salzmann et al. 1998), HN(CA)CB and 4D ^{15}N HSQC-NOESY-HSQC (Tugarinov et al. 2002) were recorded to obtain sequence specific backbone assignments. All spectra were

acquired at 20 °C on Bruker DRX600 with cryoprobe and AVANCE 900 spectrometers. Spectra were processed with NMRPipe (Delaglio et al. 1995), and analyzed with NMRView (Johnson and Blevins 1994). Proton chemical shifts were referenced to external DSS at 0 ppm ^{13}C and ^{15}N chemical shifts were referenced indirectly using absolute frequency ratios. Residual dipolar coupling measurements were acquired under the same conditions (buffer, temperature, pH) as above on the Bruker AVANCE 750 spectrometer. Isotropic samples were at a concentration of approximately 0.8 mM in 0.5 ml of NMR buffer. Anisotropic samples were obtained using 8% stretched acrylamide gels (Sass et al. 2000). These gels were prepared, washed for 5 days, dehydrated at 3 °C and rehydrated overnight with 0.6 ml isotropic sample. ^{15}N 2D IPAP (Wang et al. 1998) experiments were used to obtain residual dipolar couplings but, due to the size of the protein, the standard IPAP experiment gave ambiguous results, with missing anti-phase peaks and low signal/noise. Better results were obtained using standard ^{15}N HSQC (Norwood et al. 1990) and TROSY (Yang and Kay 1999) experiments, both of which gave excellent spectra. Data were processed in NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994). RDCs were calculated for each cross peak from the difference of the TROSY peaks for isotropic and anisotropic media, and the RCSA, calculated from the difference of the HSQC peaks for isotropic and anisotropic media: $\text{RDC} = (\Delta\delta_{\text{TROSY}} - \text{RCSA}) \times 2$ (Tate et al. 2004). Theoretical RDC values of both structures were calculated using the program PALES (Zweckstetter and Billek 2000). T_1 and T_2 relaxation measurements were obtained at 750 MHz using published pulse programs (Farrow et al. 1994). For T_1 experiments delays of 10*, 40, 80, 160, 240, 400*, 800, 1,200* and 2,000 ms were used. For T_2 experiments delays of 6*, 10, 14*, 18, 22, 34*, 42, 50, 62, 74*, 98, 122* and 162 ms were used. For the heteronuclear ^1H - ^{15}N NOE measurements (3 complete independent determinations), saturated and unsaturated spectra were recorded in an interleaved manner. Delay times with an asterisk indicate that experiments with these delay times were repeated. Data were processed using NMRPipe and analyzed using NMRView and the program Curvet (Mandel et al. 1995).

Design of C-terminal domain constructs

Both crystal structures show that the C-terminal domain of the trigger factor protein contains parts of the amino acid

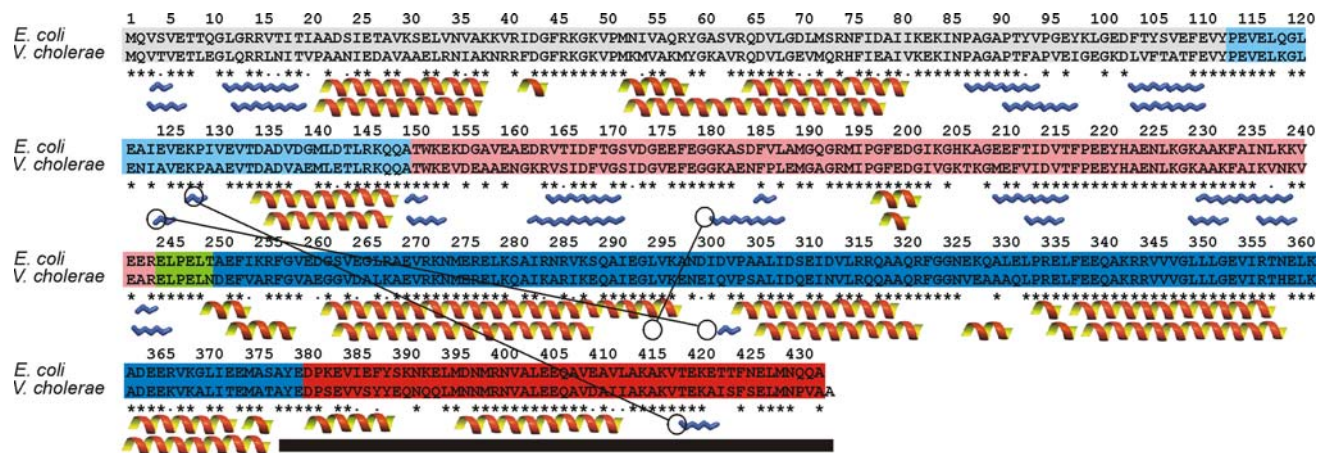
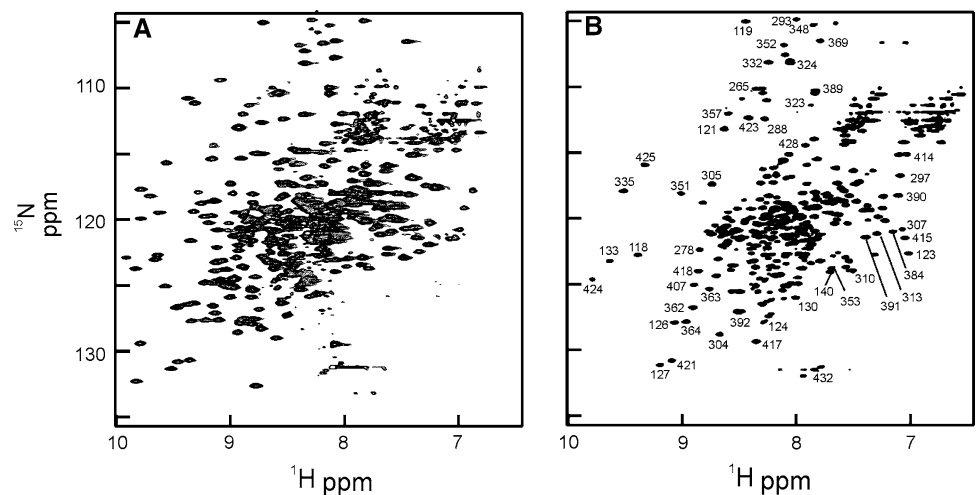


Fig. 2 Alignment of the amino acid sequences of *E. coli* and *V. cholerae* for the free *V. cholerae* structure (Ludlam et al. 2004). The black box indicates *V. cholerae* trigger factor. Colored boxes on the sequences correspond to residues missing from the *V. cholerae* structure. Small circles and lines indicate examples of close contacts inferred from the *V. cholerae* structure (no box) were not present in the construct coordinates of the two structures. Asterisks and dots below the sequences indicate residue identity and homology, respectively, between the two sequences. Symbols under the sequences indicate helices (red/yellow) or beta-strands (blue) for the ribosome-bound *E. coli* trigger factor (Ferbitz et al. 2004) and

sequence that are not contiguous (Fig. 1). This is illustrated in Fig. 2, in which the sequence is colored according to the protein TIG(1–432). The full length protein gave an NMR spectrum that indicated it was aggregated (data not shown). In order to study the solution structure of the C-terminal domain, constructs of TIG(113–432) and the full-length protein were prepared corresponding to the proteins used in the two crystal structures (Ferbitz et al. 2004; Ludlam et al. 2004).

Constructs TIG(113–389) (C-terminal domain) and TIG(1–389) (full-length protein, minus C-terminal residues not visible in the *V. cholerae* structure) were well expressed, soluble and well-behaved in solution, but gave poor NMR spectra, indicative of incomplete folding. We reasoned that the C-terminal domain, TIG(113–432) (50–246), where the C-terminal 43 residues of the PPLase domain was excised and replaced with a short linker, might be required for structural stability. Two alternative constructs were made, TIG(113–432) and the full-length protein. The full length protein gave an NMR spectrum that indicated it was aggregated (data not shown). In order to study the solution structure of the C-terminal domain, constructs of TIG(113–432) and the full-length protein were prepared corresponding to the proteins used in the two crystal structures (Ferbitz et al. 2004; Ludlam et al. 2004).

Fig. 3 (a) 600 MHz clean-TROSY spectrum of TIG (113–432). (b) 800 MHz HSQC spectrum of TIG (113–432/150–246), showing selected assignments



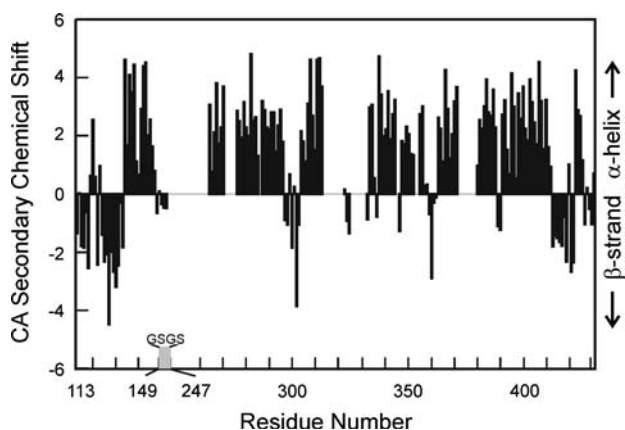


Fig. 4 Plot of secondary chemical shifts (observed chemical shift minus sequence-corrected random coil chemical shift) versus residue number for the single-domain C-terminal construct TIG(113–432Δ150–246)

β -strands are observed, strand 1 between residues 125 and 132 and strand 2 between 416 and 421. A total of 8 helices is observed in the set of assigned residues, mapped onto the amino acid sequence in Fig. 5, which also shows for comparison the 8 helices observed for the structure of *E. coli* trigger factor (Ferbitz et al. 2004) and the 6 helices of the structure of the *V. cholerae* trigger factor (Ludlam et al. 2004). In most cases, the helices inferred from the solution NMR chemical shifts are located in the same parts of the sequence as in both crystal structures. Significantly, the NMR studies show unequivocal evidence for well-formed helical and β -structure in the C-terminal section of the protein, which was present in the *E. coli* structure but not in the *V. cholerae* structure. An additional helix was observed by NMR at the very C-terminus, residues 426–432, the site of an irregular helix in the *E. coli* structure (see Fig. 1a).

extremely well in soluble form and we were able to obtain

excellent well-resolved spectra (Fig. 3). The cross peaks in

Fig. 3b can be almost exactly matched with a subset of those

in the spectrum of the 2-domain construct in Fig. 3a. This

observation is consistent with the idea that the domains of

Figure 2 shows the contacts between secondary structure

trigger factor are independently folded, and that there is little

or no contact between the PPIase domain and the C-terminal

domain, as seen in the *E. coli* structure, rather than the

interdomain contact between the PPIase domain and the loop

between helices 2 and 3 seen in the *V. cholerae* structure.

Resonance assignments for TIG(113–432Δ150–246)

About 80% of the backbone resonance assignments for

TIG(113–432Δ150–246) were readily made using standard

triple resonance techniques. The assigned residues include

the complete non-contiguous region 113–149, the GSGS

linker, and residues 264–269, 276–313, 322–326, 334–370

and 382–432. Consistent with both crystal structures shown

in Fig. 1, the majority of the domain is helical, according to

a plot of C^α secondary chemical shifts (Fig. 4). Two short



Fig. 5 Location of secondary structure observed for the TIG(113–432Δ150–246) construct. The amino acid sequence is shown outlined in the analogous regions of the two X-ray crystal structures is shown, in boxes colored according to Figs. 1 and 2. The GSGS linker is underlined. Secondary structures obtained from NMR chemical shifts are shown in the bottom line (red: helix; blue: β -strand; black: structure)

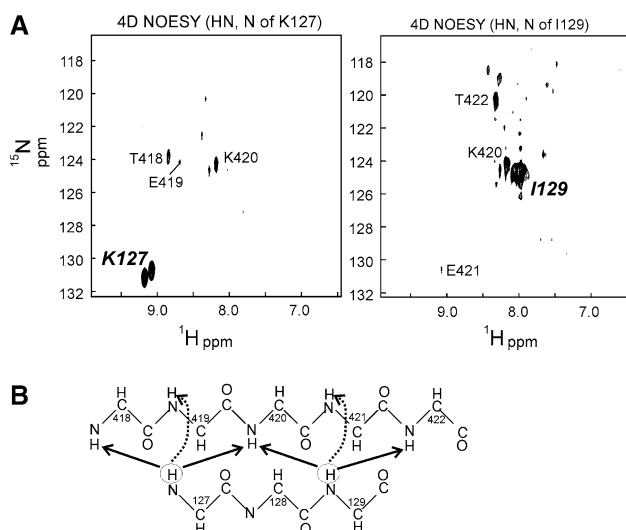


Fig. 6 (a) Planes from a 600 MHz 4-dimensional NOESY spectrum, showing long-range NH–NH NOEs that delineate sheet connectivities between residues 127–129 and 418–422. Schematic diagram showing the connectivities mapped onto the two amino acid sequences

Residual dipolar coupling measurements

Residual dipolar couplings (RDCs) for the nitrogen-amide nature of the construct used, since this is the area where proton (NH) and nitrogen-carbonyl (NC) vectors were measured to obtain alignment tensors and deduce the relative orientation of the helices within the C-terminal domain. There is no correlation of the experimentally determined RDCs with theoretical couplings obtained (Zweckstetter and Bax 2000; Zweckstetter et al. 2004) for the C-terminal domain of either X-ray structure (Ludlam et al. 2004; Ferbitz et al. 2004) (data not shown). A good correlation between the observed RDCs and those derived from an independently calculated structure is an indication that the structure of the molecule giving rise to the RDCs corresponds well with the model, but a poor correlation does not necessarily indicate that the structures do not share common features (Simon et al. 2005). In addition, dynamics and

flexibility can cause local averaging of RDCs to the point that correlation with structure is obscured. Taking into account the trigger factor's biological role as a chaperone for many different protein molecules, we hypothesized that the poor correlation of the data for the protein-binding C-terminal domain with either crystal structure may be due to the presence of multiple different conformations in solution. Our results suggest that the structure of the C-terminal domain in solution is not consistent with the truncated *V. cholerae* crystal structure. Our evidence for this includes the observed lack of a stable folded structure in solution for constructs lacking the C-terminus, whereas the constructs containing the C-terminus, both the two-domain construct

Relaxation parameters indicate flexible regions of TF C-terminal domain

In order to determine whether the lack of correlation between the measured RDC data and the two crystal structures of *V. cholerae* (113–432 and 150–246) are not consistent with either X-ray structure.

structures was caused by the inherent dynamics of the molecule, we measured T_1 and T_2 relaxation parameters and heteronuclear NOEs for TIG(113–432 and 150–246). The average heteronuclear NOE is 0.68, significantly lower than expected for a well-structured protein (usually 0.8), and there are several regions that show groups of particularly low values (Fig. 7a). These include residues 130–140, 141–150 and 365–385, as well as an area of sparse data around residue 320. A loop region between residues 415–423 also shows evidence of flexibility on a ps–ns timescale, with heteronuclear NOE values of 0.6. The values are color coded and plotted onto the *E. coli* X-ray structure, with spheres of varying radius showing the extent of motion. The T_2 parameters (Fig. 7b) indicate that motion on the μ s–ms timescale is limited to a few areas that may represent points of hinge-bending in large-scale motion of subdomains of this molecule. Apart from the termini, there are a few residues (149, 299, 323, 361 and 403) with above-average T_2 values. Residue 299 shows outlying values for T_1 and heteronuclear NOE as well as for T_2 . Figure 7c shows that this residue forms part of a cluster of residues associated with the N-terminal non-contiguous sequence of the protein. The high amplitude and heterogeneous time scales of the motions of this region may be due to the presence of a heterogeneous conformational ensemble in solution. Another indication of interdomain motion is the location of two of the residues with increased T_2 values at the intersection of two of the helices that cross in the *E. coli* X-ray structure. This is illustrated in the inset to Fig. 7b.

Our results suggest that the structure of the C-terminal domain in solution is not consistent with the truncated *V. cholerae* crystal structure. Our evidence for this includes the observed lack of a stable folded structure in solution for constructs lacking the C-terminus, whereas the constructs containing the C-terminus, both the two-domain construct TIG(113–432) and the C-terminal domain alone TIG(113–432 and 150–246) are well-folded and give NMR spectra consistent with each other. Our results indicate that the construct with the PPlase domain excised, but including the entire C-terminal sequence, shows secondary and tertiary structure that is consistent with the *E. coli* structure, but not with the *V. cholerae* structure. The RDC measurements for TIG(113–432 and 150–246) are not consistent with either X-ray structure.

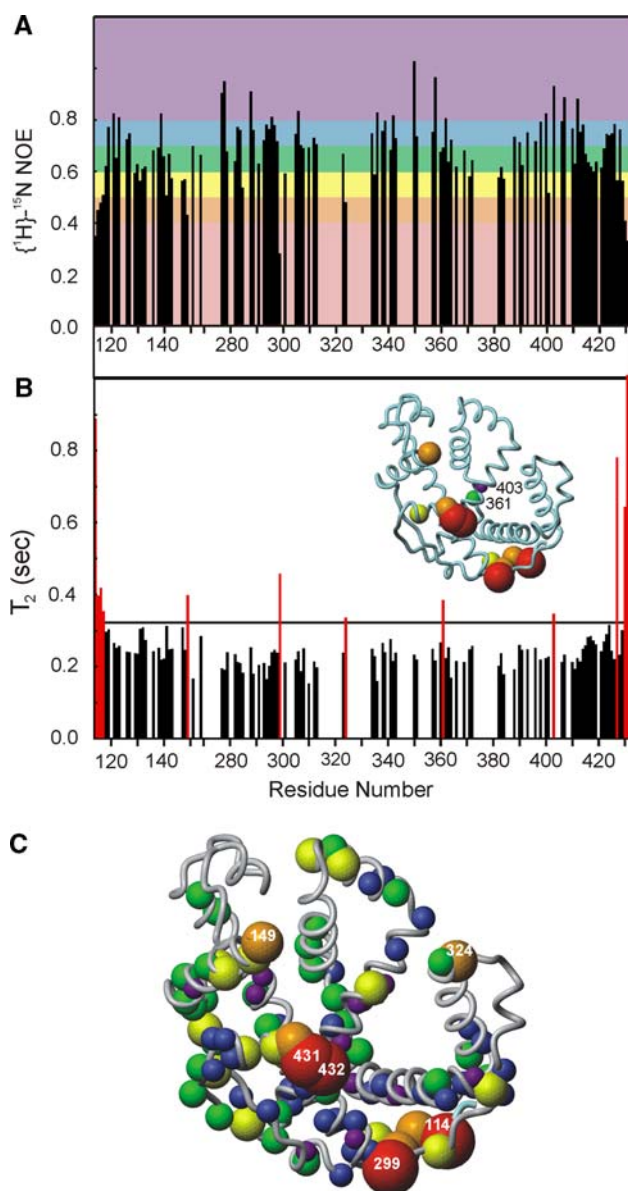


Fig. 7 (a) Heteronuclear ^1H - ^{15}N NOE values as a function of residue number. Background is shaded red ($\text{NOE} < 0.4$), orange ($0.4 < \text{NOE} < 0.5$), yellow ($0.5 < \text{NOE} < 0.6$), green ($0.6 < \text{NOE} < 0.7$), blue ($0.7 < \text{NOE} < 0.8$) and violet ($\text{NOE} > 0.8$). (b) Measured relaxation time T_2 as a function of residue number. Residues with values above the horizontal line at 0.33 s are designated by red bars. (Inset: Structure of the *E. coli* C-terminal domain of trigger factor {Ferbit2004:27321/id} showing residues that have elevated values as a subset of the backbone nitrogens shown in part (c)). (c) Structure of the *E. coli* C-terminal domain of trigger factor {Ferbit2004:27321/id} showing heteronuclear NOE values from part (a) mapped onto the corresponding backbone nitrogen atoms. Colors correspond to the NOE values described in part (a) and the radius of the spheres increases in inverse proportion to the NOE value to indicate higher-amplitude motions at these sites

This is an indication that the overall topology of this domain in solution differs from that shown in the *E. coli* protein X-ray structure, or that there is conformational heterogeneity in this domain, resulting in averaged values of the RDCs.

Why are the two crystal structures of the C-terminal domain of trigger factor so different? The protein sequences are from different organisms, *E. coli* in the NMR measurements and in one of the X-ray structures, and *V. cholerae* in the second X-ray structure, but this factor should not greatly influence the three-dimensional structures, since these proteins have highly homologous amino acid sequences (Fig. 2) (71% identical, 77% homologous over the entire 432-residue sequence, 74% identical, 75% homologous in the C-terminal domain alone, and even 64% identical and 74% homologous in the C-terminal sequence that was truncated in the *V. cholerae* protein). The most likely explanation for the observed differences between the two X-ray structures is the absence of the C-terminal 44 residues that were truncated from the *V. cholerae* protein used for structure determination (Ludlam et al. 2004). We find that these residues are critical for folding of the *E. coli* trigger factor into its native conformation: NMR spectra of truncated constructs indicated that they are unfolded, and a trigger factor mutant lacking the C-terminal 53 residues is functionally inactive: the truncation of these residues has been shown to result in complete loss of trigger factor chaperone activity in vitro and in vivo (Merz et al. 2006). Our results imply that this loss of function is due to improper folding and inability to adopt the correct native conformation. These observations provide an example of a case where crystallization conditions and crystal packing forces appear to have stabilized a structure that is not representative of the solution conformational ensemble of the protein.

Significantly, our residual dipolar coupling measurements do not show a good correlation even with the *E. coli* crystal structure. We initially set out to discriminate orientations of the helices in both structures using RDCs. However, our RDC data for two independent vectors is inconsistent with the *E. coli* crystal structure. From the relaxation data, we see that the C-terminal domain is quite flexible on a number of time scales, perhaps including large-scale hinge-bending motion of subdomains within the molecule, which could be responsible for the presence of a range of different orientations of the helices, even though the overall topology and structural contacts remain the same as in the *E. coli* structure.

We show in this paper that a series of simple NMR measurements can discriminate between two structures that differ in essential points. Two- and three-dimensional NMR spectra rapidly gave information on the secondary and tertiary structure that enabled us to discriminate between the two possible structures. In the case of the trigger factor C-terminal domain, which appears to be more dynamic in solution than would be suggested by either of the two crystal structures, the overall topology was not well-described by RDC measurements. RDCs would be

more appropriate in distinguishing the structures of more rigid domains.

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