Effects of proline *cis-trans* isomerization on TB domain secondary structure

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Abstract

The transforming growth factor β (TGF- β) binding protein-like (TB) domain is found principally in proteins localized to extracellular matrix fibrils, including human fibrillin-1, the defective protein in the Marfan syndrome. Analysis of the nuclear magnetic resonance (NMR) data for the sixth TB module from human fibrillin-1 has revealed the existence of two stable conformers that differ in the isomerization states of two proline residues. Unusually, the two isoforms do not readily interconvert and are stable on the time scale of milliseconds. We have computed independent structures of the major and minor conformers of TB6 to assess how the domain fold adjusts to incorporate alternatively *cis*- or *trans*-prolines. Based on previous observations, it has been suggested that multiple conformers can only be accommodated in flexible regions of protein structure. In contrast, P22, which exists in *trans* in the major form and *cis* in the minor form of TB6, is in a rigid region of the domain, which is confirmed by backbone dynamics measurements. Overall, the structures of the major and minor conformers are similar. However, the secondary structure topologies of the two forms differ as a direct consequence of the changes in proline conformation.

Keywords: dynamics; NMR; proline *cis-trans* isomers; TB domain; topology

The transforming growth factor β binding protein-like (TB, see Bork et al., 1996) domain is one of the most common types of module found in the fibrillins and latent transforming growth factor β binding proteins (LTBPs), both of which exist in matrix fibrils and are involved in extracellular matrix architecture and storage of latent transforming growth factor β (TGF- β) (Sakai et al., 1991; Dallas et al., 1995; Taipale et al., 1996). TB domains have been reported to mediate cell adhesion of fibrillin-1 (Pfaff et al., 1996; Taipale et al., 1996) and the association of LTBP-1 with latent TGF- β 1 (Gleizes et al., 1996; Saharinen et al., 1996). In addition, the identification of Marfan syndrome causing mutations that change TB domain residues (Collod-Bérout et al., 1998) underscores the functional significance of this module.

Recently the solution structure of the major form of the sixth TB domain from human fibrillin-1 was reported (Yuan et al., 1997). Here we present NMR data for TB6, which demonstrate the co-existence in solution of proline *cis-trans* isomers. Unusually, these data suggest that the TB6 isomers are independently stable on both the millisecond and hour time scales. Using a highly concentrated ¹⁵N-labeled NMR sample and a novel treatment of the NOESY data, we have computed independent structures of two isoforms. Backbone relaxation data confirm that one of the *cis-trans* prolines is found in a rigid region of the TB domain structure. Detailed analysis of these structures indicates that while they are globally similar, the proline isoforms are associated with different secondary structure topologies. The significance of these results with respect to protein fold classification is discussed.

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Abbreviations: CPMG, Carr-Purcell-Meiboom-Gill; DSSP, dictionary of secondary structure of proteins; cbEGF, calcium binding epidermal growth factor; HSQC, heteronuclear single quantum correlation; LTBP, latent transforming growth factor β binding protein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; RMSD, root-mean-square deviation; T_1 , nuclear spin-lattice relaxation time; T_2 , nuclear spin-spin relaxation time; TB, transforming growth factor β binding protein-like; TGF, transforming growth factor.

Results and discussion

Identification of proline *cis-trans* isomers

In the ¹H-¹⁵N HSQC spectrum of TB6, in addition to the expected 78 backbone and side-chain NH peaks, 20 backbone NH resonances and four side-chain NH resonances of weaker intensity were observed (Fig. 1). As in the case of the stronger intensity resonances, they could be sequentially assigned on the basis of

been estimated to be 77 kJ mol⁻¹ for calbindin D_{9k}, and >78 kJ mol⁻¹ for anthopleurin-A; rates of 0.26 s⁻¹ (52°C) and 0.2 s⁻¹ (25°C) are associated with proline *cis-trans* isomerization in calcitonin and calbindin D_{9k}, respectively.

Structure calculation of the conformers

Since both Pro22 and Pro52 can exist in either *cis* or *trans* conformation, in principle there are four possible conformations for TB6. However, the two prolines are far apart in both the sequence and the three-dimensional structure, and the 27 residues with non-degenerate signals can be divided into two disjoint groups each

Proline isomerization has been observed by NMR in several folded proteins including staphylococcal nuclease (Fox et al., 1986; Evans et al., 1987), calbindin D_{9k} (Chazin et al., 1989; Kördel et al., 1990), calcitonin (Amodeo et al., 1994), anthopleurin-A (Scanlon & Norton, 1994), the Lqh-8/6 toxin-like peptide (Adjadj et al., 1997), and an active IL-3 variant (Feng et al., 1997). As first demonstrated by Fox et al., proline *cis-trans* isomers of a globular protein can interconvert directly as well as via the unfolded state. The barrier to interconversion between the two forms ($\Delta G^\ddagger$) has

Fig. 2. Representative NMR data for the major and minor conformers. **A:** A region from a two-dimensional D_2O NOESY spectrum showing the two sets of intraresidue crosspeaks for Pro22, labeled in upper case and lower case for the major and minor conformations, respectively. **B:** Strips taken from a three-dimensional ^{15}N -NOESY-HSQC spectrum for sequential residues Thr50-Glu51 and Phe56-Cys60 in their major (left) and minor (right) isoforms. The Glu51 HN-Pro52 $H\delta^*$ NOEs indicate that Pro52 is *trans* in the minor isoform. In the major form, a strong crosspeak is observed between Glu51 $H\alpha$ and Pro52 $H\alpha$ indicative of a *cis* conformation of Pro52 (data not shown).

affected either directly or indirectly by one of the two prolines. Hence two representative structures were calculated according to the populations of the nondegenerate resonances and sequential NOE assignments. In the major conformer, Pro22 is in *trans* and Pro52 is in *cis*, and in the minor conformer the conformations of both Pro22 and Pro52 are inverted. The separability of the effects of the two Pro *cis-trans* isomerizations was confirmed by calculation of an additional family of structures with both prolines in the *cis* conformation (data not shown).

Cis-trans isomerization of a single proline can cause peak doubling of a number of residues on either side of the proline. Con-

sequently, the presence of multiple isoforms in solution complicates the conversion of NOE crosspeaks to distance constraints used in structure calculations. Strategies used previously to circumvent this problem include site-directed mutagenesis (Kördel et al., 1990) and calculation of both conformers followed by free-energy minimization (Amodeo et al., 1994). However, both of these methods have drawbacks. Since proline is the only naturally occurring amino acid for which both the *cis* and *trans* conformations are energetically favorable, a mutation changes the conformationally accessible space and may therefore remove alternative structures. When all of the NOE constraints are equally weighted in the calculation

of both conformers neither the *cis* nor the *trans* structure can fulfill the entire set of experimentally derived distances. We have utilized an alternative solution to the problem of NOE intensity calibration. Crosspeaks between protons with degenerate chemical shifts are taken to represent unique distances compatible with a single population of structures. Hence, crosspeak intensities of nondegenerate signals are scaled to compensate for the differences in populations of the two forms. The populations were calculated from the intensity ratio of the major and minor form (2:1) measured for each of the nondegenerate backbone NH resonance pairs in the ^1H - ^{15}N HSQC spectrum (see Materials and methods). This method relies on the assumptions that (1) when crosspeaks for the major and minor forms are degenerate, the structure for the two forms is the same in this region, and (2) differences in the dynamics of the two forms for this purpose can be neglected. Given that the overall correlation times for the two forms are virtually identical, the assumptions represent a good first approximation, particularly for residues in the interior of the protein.

The structures of the major and minor forms of TB6 are illustrated schematically in Figure 3. The two isoforms adopt a similar globular structure with the same 1-3, 2-6, 4-7, 5-8 disulfide bonding pattern, each comprising six β -strands and two α -helices. The

backbone (C^α , C, N; residues 7–64) atomic RMS difference for the two average structures is 1.11 Å. The two structures both fit the experimental data well with no violations greater than 0.4 Å or 3° for experimentally derived distance and angle constraints, respectively. Table 1 provides NMR structural statistics for both the major and the minor conformations.

Source of peak doubling in experimental data

Detailed comparison of the two structures allows us to speculate on the cause of peak-doubling in the TB6 spectra. Residues with nondegenerate proton resonances are mainly surrounding Pro52. A detailed analysis of the structural differences between the major and minor forms in this region shows that the residues containing protons with nondegenerate chemical shifts cluster around the aromatic ring of Phe56, which is in close proximity to Pro52. Since aromatic ring currents strongly affect chemical shift, this suggests that the doubling is caused mainly by changes in position and orientation of specific protons with respect to the Phe56 ring. The overlay of the backbones of the major and minor form shows that the ring itself undergoes only a minor reorientation, whereas the protons of the surrounding residues are displaced up to 3.5 Å.

Fig. 3. MOLSCRIPT (Kraulis, 1991) stereoview of the average minimized structures calculated from the NMR data for the (A) major and (B) minor conformations of TB6. β -strands are shown as arrows and α -helices are depicted as coils. The two prolines (Pro22 and Pro52) with *cis/trans* conformations are displayed in ball-and-sticks.

Table 1. Structural statistics

Major conformation	$\langle SA \rangle^a$	(SA)	Minor conformation	$\langle SA \rangle$	(SA)
RMSD from experimental distance restraints (Å)			RMSD from experimental distance restraints (Å)		
All (1636)	0.020 ± 0.003	0.013	All (1501)	0.057 ± 0.007	0.059
Interproton distances			Interproton distances		
Intraresidue (491)	0.022 ± 0.004	0.016	Intraresidue (485)	0.033 ± 0.006	0.019
Sequential (366)	0.013 ± 0.004	0.007	Sequential (345)	0.055 ± 0.014	0.059
Interresidue short-range ($ i-j \leq 4$) (254)	0.021 ± 0.004	0.013	Interresidue short-range ($ i-j \leq 4$) (206)	0.060 ± 0.007	0.045
Interresidue long-range ($ i-j > 4$) (402)	0.018 ± 0.004	0.013	Interresidue long-range ($ i-j > 4$) (348)	0.085 ± 0.026	0.097
Ambiguous (99)	0.016 ± 0.005	0.018	Ambiguous (93)	0.028 ± 0.009	0.021
H-bonds (24)	0.042 ± 0.028	0.013	H-bonds (24)	0.010 ± 0.008	0.006
RMSD from experimental ϕ restraints (deg) (34)	0.681 ± 0.098	0.613	RMSD from experimental ϕ restraints (deg) (31)	0.352 ± 0.114	0.310
Deviations from idealized covalent geometry			Deviations from idealized covalent geometry		
Bonds (Å)	0.002 ± 0.0003	0.002	Bonds (Å)	0.003 ± 0.0001	0.002
Angles (deg)	0.454 ± 0.029	0.399	Angles (deg)	0.487 ± 0.014	0.397
Impropers (deg)	0.370 ± 0.033	0.308	Impropers (deg)	0.328 ± 0.020	0.221
F_{NOE} (kJ mol ⁻¹)	126.9 ± 37.1	59.2	F_{NOE} (kJ mol ⁻¹)	162.2 ± 17.6	80.1
F_{CDIH} (kJ mol ⁻¹)	4.1 ± 1.2	3.2	F_{CDIH} (kJ mol ⁻¹)	1.1 ± 0.6	0.8
F_{repe1} (kJ mol ⁻¹)	45.4 ± 18.5	19.7	F_{repe1} (kJ mol ⁻¹)	71.5 ± 10.5	30.7
RMSD for (C $^\alpha$, C, N) residues 7–64 to (SA) (Å)	0.44 ± 0.17		RMSD for (C $^\alpha$, C, N) residues 7–64 to (SA) (Å)	0.72 ± 0.17	

^aThe notation is as follows: $\langle SA \rangle$ denotes the 20 structures calculated from the NMR data; (SA) refers to the average minimized structure.

indicating that TB6 is more malleable in this region than in the vicinity of Pro22. This is in agreement with the observation of μ s-ms dynamics for a number of residues near Pro52.

Backbone dynamics of the two forms

A complete summary of the dynamics data for TB6 is shown in Figure 4. Here we present a qualitative interpretation of these data due to suspected sample aggregation (see Materials and methods). Overall, the T_1 and T_2 relaxation time constants of the major as well as the minor conformer show very little variation except for the data of two residues in the N-terminus and four residues in the C-terminus and a few residues in the vicinity of Pro52 (Fig. 4A,B), indicating that the effective correlation times of the majority of backbone amide vectors are very similar. The average order parameter ($\langle S^2 \rangle$) of the residues in the central region of TB6 (Met7 to Ser64) is 0.85 ± 0.04 and the order parameters (S^2) decrease smoothly to 0.2 toward the N- and C-termini (Fig. 4D). This implies that TB6 adopts a well-ordered conformation in the central region with a short flexible N-terminus (six residues), and a longer flexible C-terminal tail (10 residues). In particular, residues surrounding P22 are well ordered, in contradiction of the hypothesis that rigid regions of structure can only accommodate a single proline conformer (Feng et al., 1997).

In contrast, the majority of residues with significant exchange contributions are located toward the C-terminus of TB6, and particularly in the vicinity of P52. Hence in addition to the coexistence of two populations in this region, both forms exhibit significant dynamics on the ms- μ s time scale. The specific behavior of the two forms differs: detailed analysis of the data shown in Figure 4 suggests that the unraveling of the beginning of α -helix 2 in the minor conformation is correlated with a significant change in the slow backbone dynamics of this region. However, the absence of exchange peaks on the millisecond time scale (see above) indicates that these motions are not correlated with *cis-trans* isomerization

of P52. Generally speaking, it is unclear whether or not the mobility of the region near α -helix 2 is of biological relevance since a specific function for the TB domain in human fibrillin-1 has yet to be derived. It will be interesting to see whether or not this helix is more rigid in the presence of the cEGF-like domain 32, which is linked to the C-terminus of TB6 in the intact protein.

Residues exhibiting fast internal motions are mainly located in the flexible termini. In the central region of TB6, only residues Gly17 and Lys18 in the short loop between β -strands B and C and residue Thr50 in the loop between β -strand F and α -helix 2 show fast internal motions. Since most of the loops in the TB module are as rigid as secondary structure regions, it is interesting to note that the BC loop, which manifests increased flexibility, contains the site of an RGD insertion in TB domain four from human fibrillin-1 (Yuan et al., 1997). The RGD-containing loop in a fibronectin type III module has also been observed as flexible, which is consistent with its biological function (Main et al., 1992).

Topologies of the two conformers

Analysis of the secondary structures of the TB6 isomers (see Materials and methods) reveals that the minor structural changes associated with *cis-trans* isomerization of P22 and P52 result in measurably different secondary structure topologies (Fig. 5). Specifically, in structures calculated with Pro22 in the *trans* (major) conformation, strands C-B-E-F form a four-stranded antiparallel β -sheet, while strands A-D form a short two-stranded antiparallel β -sheet. Alternatively, in the minor form strands B-C comprise a double stranded antiparallel β -sheet, while strands D-A-E-F are in a four-stranded antiparallel β -sheet. This topological change results from the presence of an interstrand NOE between the C $^\alpha$ H protons of residue 11 and residue 22 in the major form but not in the minor form, which changes the break points between strands A,B and strands C,D. Less significantly, small differences in the lengths of strands shown in Figure 5 are primarily a reflection of

Fig. 4. Relaxation parameters for TB6 (major conformation in black and minor in red). Shown are (A) ^{15}N T_1 , (B) ^{15}N T_2 relaxation time constants, (C) heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE ratios, (D) generalized order parameters S^2 , and (E) internal correlation times (τ_c , shaded lines) and chemical exchange contributions (R_{ex} , solid lines) determined from ^{15}N spin relaxation measurements as a function of protein sequence. The secondary structure elements of TB6 in the major conformation are also displayed in A and D.

the statistical distribution of topologies in the family of NMR structures (see Materials and methods). The family of test structures with both Pro22 and Pro52 in the *cis* conformation showed the same β -strand topology as the minor form (data not shown). Conformational change of Pro52 shortens α -helix 2 by two residues. All of the residues in α -helix 2 are doubled in the NMR spectra, and the shortening of this helix is the direct consequence of different NOE connectivities and intensities for the two forms.

Chothia and Finkelstein (1990) reported that proteins with as little as 40% sequence identity usually have the same fold. This redundancy of sequence information is exploited in fold classification schemes (Murzin et al., 1995; Orengo et al., 1997). Changes

in β -sheet bifurcation are commonly observed amongst members of the same fold class (A. Murzin, pers. comm.). However, here we show such a change directly effected by a proline *cis-trans* isomerization. To the best of our knowledge, this is the first time that a change in proline isomerization state has been linked to such a topological adjustment.

Conclusions

The coexistence of proline *cis* and *trans* isomers for Pro22 and Pro52 in the TB6 domain results in the doubling of proton resonances for a total of 27 residues (i.e., about a third of the total

Fig. 5. Secondary structure elements of TB6 (A) in the major conformation and (B) in the minor conformation. β -strands are shown as arrows and α -helices are depicted as cylinders. The positions of the two prolines with *cis* and *trans* conformations are each highlighted with a star. The conformation is indicated by a circle for *trans* and a square for *cis*. The starting and ending residue numbers of the secondary structure elements are displayed based on consensus secondary structures derived using PROCHECK-NMR (see Materials and methods). Strands that are outlined by dashed lines were identified in approximately 50% of the calculated structures, while the other strands were identified in at least 75% of the structures.

number of residues). The NMR data suggest that the major and minor forms of TB6 are stable and do not interconvert on the NMR time scale. We have described a new method to calibrate the NOE crosspeak intensities, which we used in the calculations of the major and minor conformations of this domain. The ^{15}N relaxation measurements agree with a well-defined globular fold for TB6. While the region surrounding P22 is relatively rigid, residues with ms- μs time scale motions in the major and minor form are located in the region around Pro52, suggesting slow conformational exchange in addition to the coexistence of two conformers. Fast internal motion is mainly restricted to the flexible N- and C-termini and the BC loop that contains an RGD sequence in the fourth TB domain from human fibrillin-1.

Comparison of the structures of the two isoforms reveals that the pairing of two β -strands is interchanged (B-E in the major form and A-E in the minor form), resulting in different topologies. Also, the isomerization state of Pro52 leads to a shortening of α -helix 2. While these changes are significant, consideration of the backbone RMSD for the two average structures (~ 1 Å) suggests that the difference between the two structures may be less profound than

looking at topology only. Investigation of the structure and dynamics of the TB6-cbEGF32 domain pair are currently in progress. It will be interesting to see whether or not two conformations for Pro22 and Pro52 are observed in this domain pair, the structure of which will be of significant relevance to the domain folding of intact fibrillin.

Materials and methods

NMR data acquisition

The preparation of NMR samples and NMR data acquisition for the major conformer of the TB domain have been described previously (Yuan et al., 1997). Throughout this manuscript TB domain residues 3–74 correspond to residues 2054–2125 of the intact fibrillin protein numbered according to Pereira et al. (1993). Additional NMR experiments described here were recorded on the uniformly ^{15}N -labeled sample at 25 °C. A three-dimensional gradient enhanced ^1H - ^{15}N edited ROESY experiment with a mixing time of 30 ms was acquired at 600 MHz (Bax & Davis, 1985; Kay et al., 1992a). The acquisition times were 114 ms in the direct ^1H (F_3) dimension, 11 ms in the ^{15}N (F_2) dimension, and 57 ms in the indirect ^1H (F_1) dimension.

The ^{15}N T_1 , ^{15}N T_2 , and ^1H - ^{15}N heteronuclear NOE data were measured at 500 MHz and 25 °C using previously described methods (Farrow et al., 1994; Kay et al., 1989). In T_1 and T_2 experiments the acquisition times were 114 ms in the ^1H (F_2) and 12.9 ms in the ^{15}N (F_1) dimensions, respectively. Eight scans were recorded for each acquisition with a recycle delay of 2 s to ensure sufficient recovery of ^1H magnetization. Relaxation delays used in collection of T_1 data were 40 (measured twice), 100, 160, 240, 340.6, 460.8, 601, 861.5, and 1,202 ms. Transverse relaxation time constants (T_2) were measured with a spin-echo sequence and a CPMG delay of 325 μs . The relaxation periods were 17.9 (measured twice), 35.8, 59.7, 89.5, 125, 167, 209, 298.4, 358, and 447.6 ms. Dipolar and chemical shift anisotropy cross-correlations were removed by application of a proton 180° pulse every 5 ms (T_1) and in the middle of the basic CPMG block (Boyd et al., 1990; Kay et al., 1992b). A pair of ^1H detected ^1H - ^{15}N heteronuclear NOE experiments with and without NOE were recorded with acquisition times of 114 ms in the ^1H dimension and 19.4 ms in the ^{15}N dimension. In the experiment with NOE, ^1H saturation was effected by means of a train of 225° flip-angle pulses at 10-ms intervals for 3 s. In both experiments 128 scans were recorded for each t_1 transient.

Calibration of NOE data for structure calculation of major and minor conformers

The NMR data were characterized by doubled signals for a minority of residues adjacent to the prolines with differing isomerization states. Residues with nondegenerate proton chemical shifts fell into two groups according to the proton chemical shift difference between the two isoforms. The first group includes 13 residues that exhibit exactly the same NOE connectivities and very similar proton chemical shifts (maximum ^1H chemical shift difference < 0.1 ppm in all spectra) between the two conformations indicating only minor changes in structure. The remaining 14 residues form the second group, which show large chemical shift differences (^1H chemical shift difference > 0.1 ppm for at least one

resonance). These include the 2 prolines (Pro22 and Pro52), nine residues close to Pro52 (Pro49, Thr50, Glu51, Asp53, Ala55, Phe56, Arg57, Ile59, and Cys60), and three residues in the flexible C-terminal tail (Gly69, Asp71, and Asp72). Among these, three residues (Pro22, Glu51, and Pro52) also showed different NOE patterns that are indicative of the proline conformational differences in the structure of each isoform.

The calibration of NOE intensities was based on the crosspeaks between protons with degenerate autocorrelation peaks according to known secondary structure distances (Wüthrich, 1986). Previous studies have confirmed that the effects of proline *cis-trans* isomerization are highly localized (Kördel et al., 1990). Also, chemical shift is very sensitive to structural perturbation. Hence it was assumed that degenerate peaks correspond to the same distance interaction in both forms. Doubled peaks corresponding to the same residue were designated as "major" or "minor" based on the independent assignment of the two conformers. Based on comparison of the volumes of major and minor ^1H - ^{15}N peaks for all well-resolved pairs in the HSQC spectrum (apart from I59 which manifests exchange broadening), the ratio of conformers is 2:1. To correctly scale nondegenerate NOEs, the intensity of each doubled peak was multiplied by the inverse of its corresponding intensity proportion. That is, major peak intensities were multiplied by 3/2 and minor peaks by three before they were classified into distance restraint categories.

Two representative families of structures for TB6 were calculated, one using the NOE crosspeaks with major intensities (the major conformation), and one using those with minor intensities (the minor conformation). In the major isoform, Pro22 is *trans*, while Pro52 is *cis*; whereas in the minor conformation, the conformations of these prolines are inverted. The residues manifesting nondegenerate signals in the NMR spectra can be divided into two disjoint groups affected either directly or indirectly by one of the two prolines. Hence the separability of the effects of each proline isomerization was tested by calculation of a set of structures with both Pro22 and Pro52 in the *cis* conformation, using distance constraints derived from minor crosspeaks for residues affected by Pro22. For the major conformation, 1,670 constraints were derived from NMR spectra including 491 intraresidue ($|i - j| = 0$), 366 sequential ($|i - j| = 1$), 254 short-range ($|i - j| \leq 4$), 402 long range ($|i - j| > 4$), and 99 ambiguous interproton distance restraints; 24 distance restraints for 12 backbone hydrogen bonds; and 34 backbone torsion angle ϕ restraints (Yuan et al., 1997). The structures in the minor conformation were calculated using 1,532 constraints, which included 485 intraresidue ($|i - j| = 0$), 345 sequential ($|i - j| = 1$), 206 short-range ($|i - j| \leq 4$), 348 long range ($|i - j| > 4$), and 93 ambiguous interproton distance restraints; 24 distance restraints for 12 hydrogen bonds; and 31 backbone torsion angle ϕ restraints. Fewer NOE derived constraints were tabulated for the minor form because nondegenerate crosspeaks corresponding to this conformer were of lesser intensity. One thousand three hundred sixty-three constraints were the same for both structure calculations.

Structures were calculated from the experimental restraints using *ab initio* simulated annealing from an extended template structure with ideal covalent geometry (Nilges et al., 1988, 1991), using the program X-PLOR v3.851 with v4.02 parameter and v4.01 topology files (kindly provided by M. Nilges). Since more recent versions of X-PLOR parameter and topology files were used in calculation of the minor conformer, the major conformer was recalculated with respect to the structure reported previously (Yuan

et al., 1997) so that the major and minor structures would be comparable. The use of the new parameter set did not have appreciable effects on the structure of the major form, and the two families of structures representing the major conformer are structurally and energetically equivalent. In this study final structures (20 major, 20 minor) were selected based on agreement with the experimental data, with no distance violations greater than 0.4 Å, no torsion angle violations greater than 3°, F_{NOE} less than 183 kJ mol⁻¹ for the major conformation, and F_{NOE} less than 186 kJ mol⁻¹ for the minor conformation. For each conformer an average coordinate structure was calculated and energy minimized (Brünger, 1992). A consensus secondary structure for each family of conformers was derived using PROCHECK-NMR (Laskowski et al., 1996), which uses the DSSP algorithm (Kabsch & Sander, 1983). Due to the statistical nature of this method for secondary structure definition, small variations in start and end positions of β -strands are sometimes observed from one family of NMR structures to the next.

Analysis of ^{15}N relaxation data

All NMR spectra were processed using Felix 2.3 (Biosym, Inc., San Diego, California). The ^{15}N T_1 and ^{15}N T_2 spectra were processed with mild resolution enhancement in F_2 and linear prediction in F_1 to optimize resolution while maintaining a good signal to noise ratio. The T_1 and T_2 relaxation time constants were derived from two-parameter exponential fits to the resonance intensities. Errors were determined from the standard deviations of differences in the peak intensities in the spectra, which were recorded twice (Palmer et al., 1991). The T_2 and the majority of the T_1 data for 64 well-resolved resonances could be fit satisfactorily to a two-parameter model. For the T_1 data of the remaining residues, the use of more complicated models was statistically not justified and the two-parameter model was retained. The heteronuclear NOE effect was calculated as the ratio of resonance intensities in the spectra recorded with and without the NOE. Errors in the heteronuclear NOE were estimated from the signal to noise ratio of each spectrum.

The ^{15}N relaxation data of 56 residues with either degenerate or stronger intensity NH resonances could be measured. In addition, for 8 of the 20 less intense backbone NH resonances relaxation data that correspond to the minor form could be analyzed. Hydrodynamic modeling confirmed that the diffusion tensors of the major and minor forms of TB6 are similar and spherical within a good approximation (Delatorre & Bloomfield, 1981; Delatorre et al., 1994). Hence, an isotropic correlation time of about 7.0 ns was estimated from the mean value of the T_1/T_2 ratio of all except six residues located at the N- and C-termini. The large value of the overall correlation time is most likely a result of the high sample concentration. Uniformly high values of the $\{^1\text{H}\}$ - ^{15}N NOEs of these residues with an average of 0.68 ± 0.07 indicates the absence of significant contributions from sub-ns motions and is consistent with a well-ordered molecule (Fig. 4C). Since both forms of TB6 are spherical, the relaxation data were analyzed using the model-free approach (Lipari & Szabo, 1982a, 1982b). The analysis was performed using the program Modelfree (Palmer et al., 1991). However, the inclusion of internal correlation times and exchange contributions were not based on the statistical analysis of the data, because in the case of a population of two conformers the assumption of a single overall correlation time already represents a simplification. Instead, internal correlation times and exchange terms were introduced for residues with a T_1/T_2 ratio that was smaller or

greater than the mean ratio by more than one standard deviation. A generalized order parameter S^2 was derived for each of the 56 residues in the major form, 37 of which have T_1/T_2 ratios within one standard deviation of the average (Fig. 4D). For 11 residues with T_1/T_2 less than the average by more than one standard deviation, an internal correlation time (τ_e) was incorporated to account for fast motions; while for eight residues with T_1/T_2 ratio greater than the average by one standard deviation, an exchange term (R_{ex}) was calculated (Fig. 4E).

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