Protein Structures

DOI: 10.1002/anie.200702842

Structure of the Protein BPTI Derived with NOESY in Supercooled Water: Validation and Refinement of Solution Structures**

*Yang Shen and Thomas Szyperski**

Very high quality protein structures are important to gain insight into conformational preferences of proteins, for molecular-mechanics force-field calibration, and for validation of structure-refinement procedures.[1] Highly resolved X-ray structures (< 1.0 Å resolution) are routinely obtained at cryogenic temperatures ($T \approx -150$ to -170 °C) to reduce radiation damage. Flash-cooling[1] of crystals within 0.1–1 s prevents protein cold denaturation.[2] However, cooling is slow compared to most internal motional modes, so that the ambient-temperature equilibrium ensemble is not accurately trapped[1b] and changes in the protein structure, primarily its surface, are induced.[1c,d] Thus, some accuracy is traded off against greatly increased precision, and the development of new methodology for validating and refining protein solution structures at the highest possible resolution is desirable.

NMR-based structural biology in supercooled water[3] promises[4] to yield highest-quality structures by the use of NOESY at about −15 °C. Aromatic ring flipping[5] is slowed and labile hydrogen exchange[6] is negligible.[4a,b] Additional NOEs can then be detected for refinement. Potential structural/dynamic changes are assessed by monitoring chemical shifts against temperature. The chemical shift measurements can readily confirm the absence of cold denaturation and reveal if the most populated ambient- and low-temperature states are virtually identical.[4b] NOE constraints determined in supercooled water and at ambient temperature can then be combined, and consistency of constraints allows both validation and refinement of an ambient-temperature NMR solution structure.

Slowing of ring flipping requires the slowing of larger-amplitude motional modes that allow the bulky rings to flip.[4b,5] Thus, the duration of the flip is orders of magnitude shorter than the inverse flip rate.[5] Although ring flipping affects NMR spectra,[7] this dynamic phenomenon can be neglected when describing the structure of a protein (for example, ring flipping hardly increases Phe and Tyr $C^{\delta/\varepsilon}$ B-factors in X-ray structures[5] and can be ignored for interpreting diffraction patterns). Furthermore, initial and final conformations of a Phe/Tyr ring flip are identical, so that lowering the temperature cannot impact on equilibration of states connected by flipping rings. Freezing-in of aromatic rings thus offers a unique potential for validating and refining NMR solution structures.

We present the refinement of a high-quality structure of 6-kDa protein BPTI (bovine pancreatic trypsin inhibitor) obtained with the constraint input (protein data bank[8] entry 1PIT) previously used[9] for an ambient-temperature (36 °C) structure determination by using constraints derived from 2D [^{1}H,^{1}H] NOESY with spectra acquired at low temperature (−15 °C). Virtually identical aliphatic proton chemical shifts at 36 and −15 °C[4b] show that the conformation of BPTI is hardly affected by supercooling, if at all,[4b] and allowed straightforward identification of 138 additional NOEs (113 aromatic and 25 hydroxy protons).

The ambient-temperature data[9] (642 distance and 96 dihedral-angle constraints) were combined with the 138 low-temperature distance constraints. CYANA[10] structure calculations (Supporting Information, Table S1) showed that including low-temperature constraints does not lead to any significant constraint violations, showing consistency of the two independently derived constraint sets and validating the ambient-temperature structure.[9a] For backbone and best-defined side chains, average root-mean-square deviation (RMSD) values of atomic coordinates relative to mean coordinates are reduced by about 40–60 % because of low-temperature constraints (Supporting Information, Table S1), reflecting an apparent increase in precision (Figure 1, Supporting Information, Figure S3). Importantly, flexibly disordered surface side chains are not frozen-in at −15 °C (Supporting Information, Figure S4 and Table S1).

We compared the NMR solution structures (Figure 1) with a 1.0-Å room-temperature X-ray crystal structure.[11] The BPTI structure can be refined simultaneously against ambient-temperature NMR constraints and 1.0-Å X-ray diffraction data,[12] that is, the crystal structure must be almost entirely located within the conformational space allowed by the NMR constraints. Furthermore, conformations of the polypeptide backbone and molecular core are hardly affected by crystallization.[13] Those conformations were therefore chosen as structural references to assess the accuracy of NMR solution structures. A reduction of RMSD values between mean NMR and X-ray coordinates of about 30 % occurred upon inclusion of low-temperature constraints (Supporting Information, Table S2), demonstrating an apparent increase

[*] Dr. Y. Shen,[+] Prof. T. Szyperski
Department of Chemistry
State University of New York at Buffalo
Buffalo, New York 14260 (USA)
Fax: (+1) 716-645-2250
E-mail: szypersk@chem.buffalo.edu
Homepage: http://www.chem.buffalo.edu/szyperski.php

[+] Current address:
Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases Bethesda, MD 20892 (USA)

[**] This work was supported by a Research Innovation Award of the Research Corporation and the National Science Foundation (MCB 0416899).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

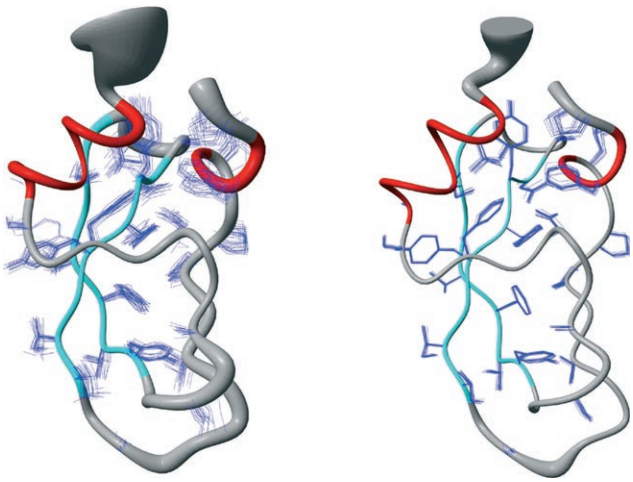

Figure 1. BPTI NMR solution structures derived with ambient-temperature (36 °C) constraints[9] (left, CYANA[10] target function 0.28 Å^2) and refined with low-temperature (−15 °C) constraints (right, 0.48 Å^2). The thickness of the rod representing the backbone is proportional to the mean of the C$^\alpha$ global displacements in 20 conformers, calculated after superposition of backbone atoms N, C$^\alpha$, and C′ of secondary structure elements. Helices red, β strands cyan, other polypeptide segments gray, best-defined side chains blue. Mean RMSD values calculated for the backbone of residues 2 to 56 relative to the mean coordinates are 0.38 ± 0.09 Å (left) and 0.22 ± 0.05 Å (right). A superposition of heavy atoms of best-defined side chains is also shown (RMSD values 0.65 ± 0.10 Å (left) and 0.37 ± 0.03 Å (right); residues 2,4,6,8,9,11,16,18,19,21–25,27,30,32–35,38,40,43,45,47,48,51,54 with global displacements smaller than 0.65 Å).

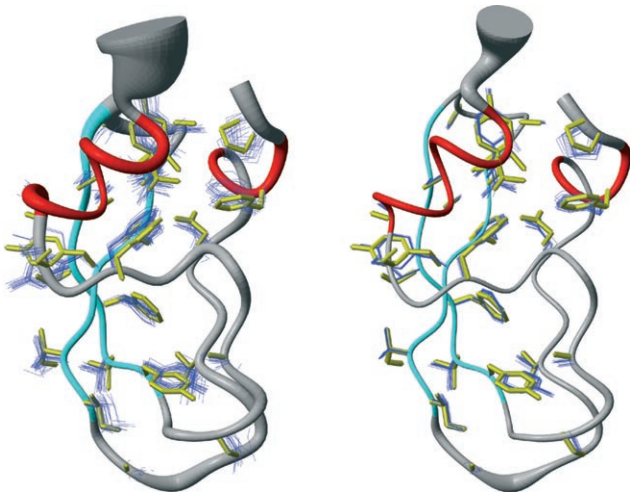

Figure 2. Comparison of 1.0-Å X-ray crystal structure (5PTI)[11] with ambient-temperature NMR (left) and low-temperature refined NMR solution structure (right) from Figure 1. The thickness of the rod representing the backbone is proportional to the global displacements calculated for C$^\alpha$ atoms between mean NMR and X-ray structure coordinates after superposition of the backbone atoms N, C$^\alpha$, and C′ of secondary structure elements and side-chain heavy atoms of the molecular core. Helices red, β strands cyan, other polypeptide segments gray, best-defined side chains of the molecular core blue; side chains from X-ray analysis yellow. RMSD values for the backbone heavy atoms N, C$^\alpha$, and C′ between X-ray and mean NMR coordinates are 0.91 Å (left) and 0.64 Å (right); a mean value of 0.38 Å was obtained between three different crystal structures.[13] A superposition of molecular core side-chains (residues 2,4,11,16,18,19,21–25,27,30,32–35,38,40,43,45,47,48,51,55) is shown for the 20 conformers determined by NMR spectroscopy. RMSD values calculated for the backbone heavy atoms N, C$^\alpha$, and C′ and core heavy atoms between X-ray and mean NMR coordinates are 1.02 Å (left) and 0.78 Å (right); a value of 0.39 Å was obtained between the three different crystal structures.[13]

in accuracy arising from these constraints (Figure 2, Supporting Information, Figure S5).[14]

Taken together, low-temperature NOE constraints can serve to validate and refine NMR solution structures of those proteins that do not exhibit significant shifts of their ground state upon supercooling. Owing to the high viscosity of supercooled water,[2] the approach described here is primarily suited for smaller proteins. In contrast to cryogenic X-ray crystallography, however, the protein surface is hardly affected by the supercooling. Moreover, further refinement may be achieved by use of residual dipolar coupling constraints.[15] In the future, refined very high quality NMR solution structures of smaller proteins may well be of importance for validating and complementing insights obtained from high resolution X-ray structures.[1a] Structural genomics research networks[16] may efficiently identify the best suited targets to pursue this goal.

Experimental Section

Two 2D [^{1}H,^{1}H] NOESY spectra were recorded with mixing times of 40 and 10 ms, respectively, at −15 °C on a VARIAN INOVA 750 spectrometer using H_2O– or D_2O–BPTI solutions (6 mM, pH 3.5) in 1.0-mm outside-diameter glass capillary tubes. For further details, see reference [4b], and for plots and comparison with ambient-temperature spectra, see Figures 4 and 8 thereof. CH···HC NOEs were identified in the D_2O spectrum (total 113; 14 intraresidue, 3 sequential, 22 medium-range, 74 long-range), whereas NOEs involving hydroxy protons were identified in the H_2O spectrum (25; 14, 4, 2, 5).

Received: June 26, 2007
Revised: September 27, 2007
Published online: November 12, 2007

Keywords: aromatic ring flipping · NMR spectroscopy · protein structures · structure elucidation · supercooled water

[1] a) E. Garman, *Curr. Opin. Struct. Biol.* **2003**, *13*, 545–551; b) B. Halle, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4793–4798; c) D. H. Juers, B. W. Matthews, *J. Mol. Biol.* **2001**, *311*, 851–862; d) K. V. Dunlop, R. T. Irvin, B. Hazes, *Acta Crystallogr. Sect. D* **2005**, *61*, 80–87.
[2] F. Franks, *Adv. Protein Chem.* **1995**, *46*, 105–139.
[3] C. A. Angell in *Water: a Comprehensive Treatise* (Ed.: F. Frank), Plenum, New York, **1982**.
[4] a) J. J. Skalicky, D. K. Sukumaran, J. L. Mills, T. Szyperski, *J. Am. Chem. Soc.* **2000**, *122*, 3230–3231; b) J. J. Skalicky, J. L. Mills, S. Sharma, T. Szyperski, *J. Am. Chem. Soc.* **2001**, *123*, 388–397; c) J. L. Mills, T. Szyperski, *J. Biomol. NMR* **2002**, *23*, 63–67; d) C. R. Babu, V. J. Hisler, J. Wand, *Nat. Struct. Mol. Biol.* **2004**,

11, 352 – 357; e) T. Szyperski, J. L. Mills, D. Perl, J. Balbach, *Eur. Biophys. J.* **2006**, *35*, 363 – 366.
[5] a) G. Wagner, *Q. Rev. Biophys.* **1983**, *16*, 1 – 57; b) The majority of Tyr ($> \approx 95\,\%$) and Phe rings ($> \approx 97\,\%$) flip rapidly at ambient temperature.[4b]
[6] J. M. Scholtz, A. D. Robertson, *Methods Mol. Biol.* **1995**, *40*, 291 – 311.
[7] Rapid rotation about χ^2 (ring flipping) averages shifts of stereochemically equivalent protons (e.g., $^1H^{\delta 1}$ and $^1H^{\delta 2}$ of Tyr). Then, NOE constraints are referred to pseudoatoms or treated as being ambiguous. Stereochemically equivalent protons of stalled rings usually exhibit nondegenerate shifts because of anisotropic shielding. This fact allows accurate distance constraints to individual ring protons to be derived.[4b]
[8] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, I. N. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235 – 242.
[9] a) K. D. Berndt, P. Güntert, L. P. M. Orbons, K. Wüthirch, *J. Mol. Biol.* **1992**, *227*, 757 – 775; b) The constraint input of Ref. [9a] was used using the same calculation protocol with and without low-temperature constraints.
[10] T. Herrmann, P. Güntert, K. Wüthrich, *J. Mol. Biol.* **2002**, *319*, 209 – 227.
[11] A. Wlodawer, J. Walter, R. Huber, L. Sjolin, *J. Mol. Biol.* **1984**, *180*, 301 – 329.
[12] C. A. Schiffer, R. Huber, K. Wüthrich, W. F. van Gunsteren, *J. Mol. Biol.* **1994**, *241*, 588 – 599.
[13] The average RMSD value calculated for backbone atoms N, C^α, and C' of residues 2 to 56 in three X-ray crystal structures (protein data bank entries 4PTI, 5PTI, and 6PTI) relative to their mean coordinates is 0.22 Å. The corresponding RMSD value calculated for backbone heavy atoms and molecular core side-chain heavy atoms is 0.23 Å.
[14] See also information-theoretical analysis of distance constraints in the Supporting Information.
[15] a) J. R. Tolman, J. M. Flanagan, M. A. Kennedy, J. H. Prestegard, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 9279 – 9283; b) N. Tjandra, A. Bax, *Science* **1997**, *278*, 1111 – 1114.
[16] G. T. Montelione, D. Zheng, Y. Huang, C. Gunsalus, T. Szyperski, *Nat. Struct. Biol.* **2000**, *7*, 982 – 984.

