J|A|C|S
COMMUNICATIONS

Published on Web 12/01/2006

Magnetic Susceptibility-Induced Alignment of Proteins in Reverse Micelles

Kathleen G. Valentine, Maxim S. Pometun, Joseph M. Kielec, Robert E. Baigelman, Jayme K. Staub, Kristy L. Owens, and A. Joshua Wand*

Johnson Research Foundation and Department of Biochemistry & Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059

Received March 1, 2006; E-mail: wand@mail.med.upenn.edu

With the completion of several grand-scale genome sequencing efforts, it has become possible to undertake a comprehensive analysis of the structural basis for the function of the proteins encoded by the human and other genomes.[1] The sheer number of proteins involved is daunting.[2] The task is made even more difficult by the observation that a significant fraction of the proteomes of various species is somewhat ill-suited for analysis by the two main structural methods: X-ray crystallography and solution NMR spectroscopy.[1−4] In particular, proteins of marginal stability in vitro are problematic for both approaches. In addition, solution NMR spectroscopy is somewhat limited by the relaxation properties of slowly tumbling macromolecules. One approach is to employ extensive deuteration and the TROSY effect.[5] Another approach actively seeks to increase the effective rate of molecular reorientation by encapsulating the protein of interest within the protective shell of a reverse micelle and dissolving the resulting particle in a low-viscosity fluid.[6] This method also allows the study of marginally stable proteins, where the confined space of the reverse micelle is used to stabilize the compact native state.[7]

Human ubiquitin is the only example of a structure of an encapsulated protein determined to high resolution.[8] A current significant deficiency for structure determinations of encapsulated proteins has been the absence of longer range restraints derived from residual dipolar couplings (RDCs) arising from partial alignment of the protein in the magnetic field.[9,10] RDCs are an extremely powerful structural restraint.[11,12] Here we report that encapsulated proteins partially align within a magnetic field.

We have examined the magnetically induced alignment of three encapsulated proteins (Table 1). The apparent splittings (J + D) in the IPAP ^{15}N−^{1}H HSQC spectra were measured[13] at 17.6 and 11.7 T. Encapsulated ubiquitin gave RDCs that were small (−0.9 to +0.5 Hz) but still ∼5-fold greater than those measured in aqueous solution.[14] Encapsulated cytochrome *c* (cyt *c*), both the paramagnetic (oxidized) and diamagnetic (reduced) states, and oxidized (diamagnetic) encapsulated flavodoxin all showed significant RDCs (Figure 1). The RDCs for encapsulated oxidized cyt *c* are roughly 5 times larger than those seen in free solution.[15] Interestingly, the

Table 1. Magnetic Susceptibility Tensors and Refinement Statistics of Encapsulated Proteins[a]

				ref structure[d]	tensor alignment[e]	
	W_0[b]	$\Delta\chi$[c]	R[c]	rmsd (Å)	θ	ϕ
ox cyt *c*	11.4	−2.45	0.50	0.90	150(3)	76(2)
red cyt *c*	8.6	−3.19	0.55	0.90	104(1)	65(1)
ubiquitin	14	−0.41	0.57	1.13	56(1)	91(6)
flavodoxin	17	−4.12	0.54	0.52	78(1)	168(7)
flavodoxin	27	+3.02	0.56	0.53	18(2)	172(3)

[a] Encapsulated proteins were prepared in pentane as described in the Supporting Information. [b] W_0, water loading, defined as the molar ratio of water to surfactant molecules. Determined directly by NMR signal integration. [c] Tensor parameters were estimated from histograms of the RDC distributions.[16] $\Delta\chi = 1/3[\chi_{33} - (1/2)(\chi_{11} + \chi_{22})]$ in units of 10^{-27} J T^{-2}, and $R = 1/3(\chi_{22} - \chi_{11})/\Delta\chi$, where $|\chi_{33} - \chi\text{iso}| \geq |\chi_{11} - \chi_{\text{iso}}| \geq |\chi_{22} - \chi_{\text{iso}}|$.[9,10] [d] Proteins were refined with preservation of ideal covalent geometry using simulated annealing in CNS.[17] Variance of the lowest energy refined encapsulated protein structure from the starting reference structure: cytochrome *c* (PDB code 1HRC[18]), ubiquitin (PDB code 1G6J[8]), and flavodoxin (PDB code 1FLV[19]). [e] The alignment axis system was translated to the amide N atom of the Ala 28 plane in ubiquitin, the Fe atom of the heme plane in cyt *c*, and the N10 atom of the flavin plane in flavodoxin. θ is the angle the perpendicular to the plane makes with respect to the χ_{33} axis of the alignment tensor; ϕ is the angle the χ_{11} axis makes with the bond contained in the plane (C′−N of Ala 28 in ubiquitin, N−D−Fe of the heme plane in cyt *c*, and C9A−N10 of the flavin ring in flavodoxin). The standard deviations of the tensor alignment angles across each family of refined structures are shown in parentheses.

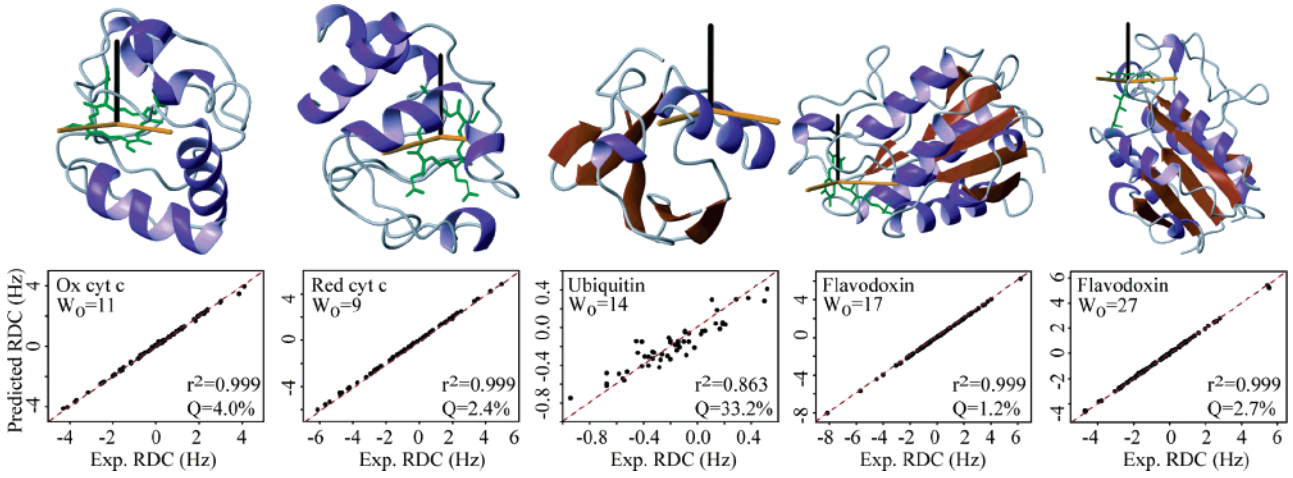

Figure 1. Correlation plots of predicted ^{15}N−^{1}H RDCs based on the determined alignment tensor and refined structure versus the experimentally observed ^{15}N−^{1}H RDCs for each of the encapsulated proteins examined (lower panels). The axis system for the determined alignment tensor is positioned in a MolMol[21] ribbon representation of the refined structures (upper panels).

10.1021/ja061438n CCC: $33.50

character of the alignment tensor for flavodoxin changes significantly with an increase in water loading.

A simple strategy was used to evaluate whether the measured RDCs were structurally meaningful. Previously determined structures were used as a starting point in the evaluation (Table 1). Distance restraints were generated for all heavy atoms within ~5 Å and given ±0.20 Å variance. A random selection of 15% of these distance restraints was combined with the experimental RDCs and the estimated magnitudes of the axial and rhombic components of the susceptibility anisotropy tensor (Table 1) as the total constraints list.

The structures were refined using a simulated annealing protocol implemented in CNS[17] and employing torsion angle dynamics for the high-temperature stages and Cartesian coordinate dynamics for the final cooling and energy minimization stages. In addition to the usual restraints on covalent geometry, the amide N−H geometry was explicitly constrained to prevent deviations of the ω angle from planarity. The unusually extensive set of relatively tight heavy-atom distance restraints, combined with strictly enforced covalent geometry, particularly at the amide N−H, was designed to largely ameliorate concern about local structural distortion resulting from the satisfaction of a limited set of RDC-based structural restraints.[20] Essentially ideal geometry was maintained in the final structures (see Supporting Information). Refined structures were selected using an acceptance criterion of a maximum of two RDCs >0.2 Hz deviation and no significant violation of the imposed distance restraints. The alignment of the magnetic susceptibility tensor within the set of structures was found to be common to all members of the set (Table 1). Analysis of the refined structures with REDCAT[22] gave essentially identical results. REDCAT solutions were not found for the unrefined starting structures. The final minimum energy structures showed good agreement with the starting structure with respect to overall rmsd (Table 1) and to the ψ,ϕ Ramachandran angles. The fit of the predicted to the experimental RDCs is shown in Figure 1. The precision is excellent. This indicates that the RDCs are structurally meaningful and that the structures of the encapsulated proteins are closely similar to that of the corresponding reference structure.

Figure 1 indicates the orientation of the magnetic susceptibility tensor in each of the proteins. The χ_{33} element of the tensor in oxidized cyt *c* makes an angle of ~30° with the normal to the heme plane. This deviates significantly from the free aqueous solution of oxidized cyt *c*, where the angle was ~7°.[15] This suggests that an alignment mechanism that overrides the protein's contributions to the magnetic susceptibility is operative. This is not unexpected. The magnetic susceptibility tensor of the reverse micelle assembly includes contributions not only from the protein and its bound prosthetic group but also from the surrounding surfactants, water, and ions comprising the reverse micelle. Indeed, the simple addition of water to the flavodoxin sample significantly changed the alignment orientation. The individual susceptibilities combine with the three-dimensional geometric constraints imposed by the organization of the assembly to result in a nonspherical distribution. Motion of charged species may also contribute. Though the details are quite involved and alignment is therefore not easily predicted, the magnetic basis for the observed alignment is clear and would appear to be general. In addition, using the normalized scalar product as a measure,[23] there appears to be no correlation of the observed alignment tensors with those predicted[24] by steric alignment. Importantly, the proteins need not be paramagnetic in order to show significant alignment. The ability to vary the orientation of the alignment tensor by changing the water content of the reverse micelle should also provide additional independent sets of RDCs for refinement by numerical analysis.

In summary, we have shown several examples of encapsulated proteins that partially align in a magnetic field. The alignment results in residual dipolar couplings of a magnitude measurable by standard methods. The RDCs are structurally meaningful and, when comprehensively utilized,[20] should provide a powerful set of restraints for the determination of high-resolution structures of encapsulated proteins.

Acknowledgment. Supported by NIH grant GM35940, NSF grant DMR05-20020, and the Mathers Foundation. J.M.K. holds an NSF predoctoral fellowship.

Supporting Information Available: Details of reverse micelle sample preparation; RDC values and histograms for the determination of the principle elements of the magnetic susceptibility tensors; structural refinement statistics; comparison of the determined magnetic alignment tensors to that predicted for steric alignment; and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Yakunin, A. F.; Yee, A. A.; Savchenko, A.; Edwards, A. M.; Arrowsmith, C. H. *Curr. Opin. Chem. Biol.* **2004**, *8*, 42−48.
(2) Liu, J. F.; Hegyi, H.; Acton, T. B.; Montelione, G. T.; Rost, B. *Proteins* **2004**, *56*, 188−200.
(3) Peti, W.; Etezady-Esfarjani, T.; Herrmann, T.; Klock, H. E.; Lesley, S. A.; Wüthrich, K. *J. Struct. Funct. Genomics* **2004**, *5*, 205−215.
(4) Arrowsmith, C. H.; et al. *Proc. Nat. Acad. Sci. U.S.A.* **2002**, *99*, 1825−1830.
(5) Wider, G.; Wüthrich, K. *Curr. Opin. Chem. Biol.* **1999**, *9*, 594−601.
(6) Wand, A. J.; Ehrhardt, M. R.; Flynn, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15299−15302.
(7) Peterson, R. W.; Anbalagan, K.; Tommos, C.; Wand, A. J. *J. Am. Chem. Soc.* **2004**, *126*, 9498−9499.
(8) Babu, C. R.; Flynn, P. F.; Wand, A. J. *J. Am. Chem. Soc.* **2001**, *123*, 2691−2692.
(9) Tolman, J. R.; Flanagan, J. M.; Kennedy, M. A.; Prestegard, J. H. *Proc. Nat. Acad. Sci. U.S.A.* **1995**, *92*, 9279−9283.
(10) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114.
(11) Bax, A. *Protein Sci.* **2003**, *12*, 1−16.
(12) Prestegard, J. H.; Bougault, C. M.; Kishore, A. I. *Chem. Rev.* **2004**, *104*, 3519−3540.
(13) Ottiger, M.; Delaglio, F.; Bax, A. *J. Magn. Reson.* **1998**, *131*, 373−378.
(14) Tjandra, N.; Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1996**, 6264−6272.
(15) Bertini, I.; Janik, M. B.; Liu, G.; Luchinat, C.; Rosato, A. *J. Magn. Reson.* **2001**, *148*, 23−30.
(16) Clore, G. M.; Gronenborn, A. M.; Bax, A. *J. Magn. Reson.* **1998**, *133*, 216−221.
(17) Brunger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr. D: Biol. Crystallogr.* **1998**, *54*, 905−921.
(18) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595.
(19) Rao, S. T.; Shaffie, F.; Yu, C.; Satyshur, K. A.; Stockman, B. J.; Markley, J. L.; Sundarlingam, M. *Protein Sci.* **1992**, *1*, 1413−1427.
(20) Bax, A.; Grishaev, A. *Curr. Opin. Struct. Biol.* **2005**, *15*, 563−570.
(21) Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graphics* **1996**, *14*, 51−55, 29−32.
(22) Valafar, H.; Prestegard, J. H. *J. Magn. Reson.* **2004**, *167*, 228−241.
(23) Sass, J.; Cordier, F.; Hoffmann, A.; Cousin, A.; Omichinski, J. G.; Lowen, H.; Grzesiek, S. *J. Am. Chem. Soc.* **1999**, *121*, 2047−2055.
(24) Zweckstetter, M.; Bax, A. *J. Am. Chem. Soc.* **2000**, *122*, 3791−3792.

JA061438N