Journal of Magnetic Resonance **146,** 381–384 (2000)
doi:10.1006/jmre.2000.2172, available online at http://www.idealibrary.com on IDEAL®

Lanthanide Ions Bind Specifically to an Added "EF-Hand" and Orient a Membrane Protein in Micelles for Solution NMR Spectroscopy

Che Ma and Stanley J. Opella[1]

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received June 8, 2000

Twelve amino acid residues corresponding to an "EF-hand" calcium-binding site were added to the N-terminus of a protein, providing a specific lanthanide ion binding that weakly orients the protein in solution. A comparison of spectra of the protein with and without the EF-hand residues demonstrates that the structure of the native protein is not perturbed by this modification, since there are minimal chemical shift changes. With a lanthanide but not calcium bound to the EF-hand, the protein is weakly oriented by the magnetic field, since residual dipolar couplings can be measured. Since the signs and magnitudes of the couplings varied with the type of lanthanide, this demonstrated the ability to obtain multiple orientations of the protein in solution. The sample is a membrane protein in lipid micelles that disrupted the commonly employed bicelle and filamentous phage solutions; therefore, the addition of a specific metal binding site in the form of an EF-hand may provide a general approach to orienting proteins where the addition of external agents is problematic. An additional benefit is that the lanthanide ions perturb the protein resonances in ways that provide unique orientational and distance constraints.

Key Words: **residual dipolar couplings; membrane protein; micelles; lanthanide; Vpu.**

INTRODUCTION

Membrane proteins are challenging samples for NMR spectroscopy. In solution, the resonances are broad because of the relatively slow reorientation of the proteins in lipid micelles, and the chemical shift dispersion is generally quite limited because of the predominantly helical secondary structure. Although the application of solid-state NMR methods to lipid bilayer samples is an attractive and effective alternative, there are a number of reasons for determining the structures of membrane proteins in micelles. It is feasible to obtain reasonably well resolved two-dimensional heteronuclear correlation spectra of uniformly ^{15}N-labeled membrane proteins in selected types of lipid micelles, at high fields, and at relatively high temperatures; uniform labeling with ^{2}H and ^{13}C is essential for making backbone assignments. However, even in the most favorable examples it has proven difficult to resolve and assign a sufficient number of "long-range" NOEs to determine the tertiary fold. Thus, membrane proteins in micelles would seem to be ideal candidates for the use of residual dipolar couplings for characterizing their overall topology, which often consists of helical segments separated by turns or mobile loops, and refining their three-dimensional structures. However, hydrophobic membrane proteins and their associated lipids disrupt the most commonly employed orienting agents, including bicelles (*1*) and filamentous phages (*2, 3*). There are examples of other types of proteins and complexes that are ill suited to NMR studies in solutions containing foreign orienting agents.

Initially, residual dipolar couplings were measured on cyanometmyoglobin, which was weakly oriented in a high magnetic field by the anisotropic paramagnetic susceptibility of the iron in the heme group (*4*). Lanthanide ions (Ln^{3+}) also induce weak alignment of proteins in solution, as demonstrated with calcium-binding proteins (*5*) and proteins with chelating tags (*6*). Cobalt bound to a zinc finger tag has also been shown to orient a protein in solution (*7*).

In this Communication, we demonstrate that weak alignment of a membrane protein in lipid micelles in solution can be accomplished in a controlled way by adding an "EF-hand" calcium-binding site to the N-terminus of the protein. This allows the measurement of residual dipolar couplings and other spectroscopic parameters resulting from the presence of a single lanthanide ion in a unique, well-defined location (*8*). Further, the use of more than one type of lanthanide makes it possible to obtain multiple orientations of the protein in the magnetic field, which is valuable for eliminating ambiguities in the orientational data (*9*).

RESULTS AND DISCUSSION

Experiments were carried out on Vpu, an 81-residue membrane-associated accessory protein encoded in the HIV-1 genome. The protein has two biological activities that contribute to the virulence of HIV-1 infections in humans; it facilitates the budding of newly formed virus particles from infected cells and enhances the degradation of gp160–CD4 complexes

[1] To whom correspondence should be addressed. Current address: Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, 0307, La Jolla, CA 92093-0307. Fax: (858) 822-4821. E-mail: sopella@ucsd.edu.

1090-7807/00 $35.00

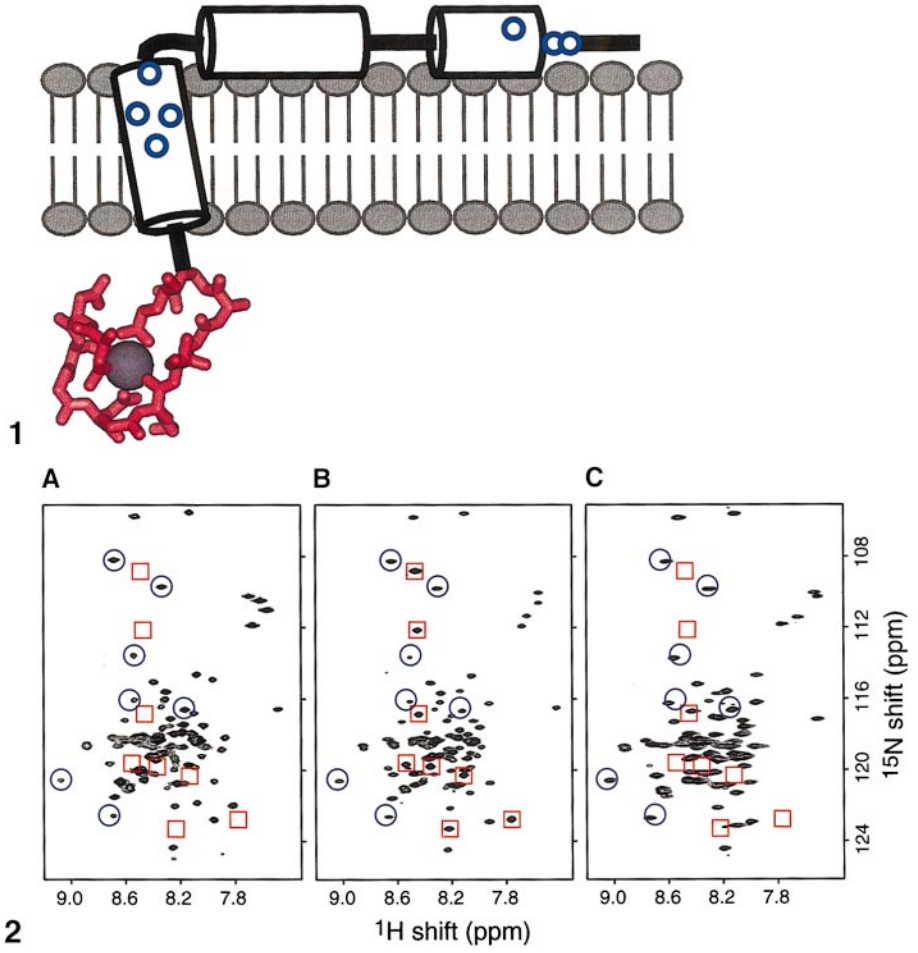

FIG. 1. Schematic model of EF-Vpu. The 92-residue polypeptide was prepared using standard molecular biology techniques to add the 12 residues that constitute one of the "EF-hand" motifs to the N-terminus of Vpu. The amino acid sequence of EF-Vpu is DNDGDGKIGADE QPIQIAIVAL VVAIIIAIVV WSIVIIEYRK ILRQRKIDRL IDRLIERAED SGNESEGEIS ALVELGVELG HHAPWDVDDL; the residues of the EF-hand are underlined. This polypeptide was expressed in bacteria, isolated, and purified using procedures similar to those used for full-length Vpu (*12*). The sequence of the underlined residues and the three-dimensional structure of the EF-hand correspond to that of residues 90–101 of oncomodulin (PDB code: 1RRO) (*15*).

FIG. 2. Two-dimensional ^{15}N–^{1}H HSQC spectra of Vpu and EF-Vpu in dihexanoyl phosphatidylcholine (DHPC) micelles in aqueous solution. (A) Vpu. (B) Ca^{2+}-bound form of EF-Vpu. (C) Yb^{3+}-bound form of EF-Vpu. The sample conditions are 1 mg of protein, 200 mM DHPC, pH 4.0, and 323 K. The spectra were obtained on a Bruker DMX 750 NMR spectrometer. The IPAP ^{15}N–^{1}H HSQC experiment (*16*) was used to measure the one-bond ^{15}N–^{1}H residual dipolar couplings from the amide resonances within the blue circles. The values are listed in Table 1.

(*10, 11*). Although the polypeptide expressed and purified from bacteria is missing its N-terminal residue and has the two other methionines replaced by leucines, it retains full biological activities (unpublished results). Using a combination of solid-state NMR and solution NMR methods, we have shown that its secondary structure is nearly all α-helical and is arranged with a hydrophobic *trans*-membrane helix near the N-terminus and two amphipathic in-plane helices in the cytoplasmic C-terminal domain (*12*). Despite extensive experimental efforts to determine its tertiary fold in lipid micelles by solution NMR methods, the spectral overlap in crucial regions makes the reliable identification and assignment of long-range NOEs extremely difficult. Attempts to overcome the limitations of the chemical shift and NOE data by supplementing them with residual dipolar couplings have been hampered by interactions with and subsequent degradation of bicelle- and phage-containing solutions. Although we have found that many membrane proteins have adventitious lanthanide binding that enables their orientation in solution (unpublished results), this was not the case for Vpu.

Figure 1 shows a schematic structure of the 92-residue EF-Vpu polypeptide, which has a 12-residue sequence corresponding to the EF-hand of a well-characterized calcium-binding protein added to its N-terminus. Since lanthanide and calcium ions have similar atomic radii, they both bind tightly to the same calcium-binding sites in proteins (*13*). The N-terminal attachment of the EF-hand sequence was designed to minimize the potential for undesirable structural perturbations,

TABLE 1

Residual Dipolar Couplings (rdc) Measured for Representative Backbone ^{15}N–^{1}H Sites on Vpu with Bound Lanthanide in DHPC Micelles

Residue	Yb^{3+} rdc (Hz)	Dy^{3+} rdc (Hz)
Ile19	6.23	4.18
Ser23	0.63	−5.02
Val25	7.80	4.78
Tyr29	5.65	0.84
Gly67	1.58	1.56
Gly71	−1.08	4.19
His72	0.49	−5.79

Note. The molar ratio of lanthanide to protein was 10:1 for Yb^{3+} and 2:1 for Dy^{3+}.

which might result from interactions between the Ln^{3+} ions and polar residues in the C-terminal cytoplasmic domain. The choice of an N- or C-terminal location for the EF-hand will depend on the properties of the individual protein, and it may be advantageous to make both constructs in some cases to check on structural perturbations and provide an alternative mechanism for obtaining multiple alignment directions of the protein.

Figure 2 compares the two-dimensional heteronuclear single-quantum correlation (HSQC) spectrum of the 80-residue native Vpu (Fig. 2A) to those of the 92-residue EF-Vpu in the presence of Ca^{2+} (Fig. 2B) and Yb^{3+} (Fig. 2C). Red boxes are drawn around the backbone amide resonances from residues in the added EF-hand portion of the polypeptide that are not present in native Vpu (*12*). Notably, the red boxes are empty in the spectra in both Fig. 2A, where the corresponding residues are not present in the polypeptide, and Fig. 2C, where all of the resonances associated with residues in the EF-hand are shifted or broaden beyond experimental detection when the paramagnetic Yb^{3+} is bound instead of Ca^{2+}. Nearly all of the amide resonances from residues 14–81 of the native Vpu portion of the polypeptides have similar ^{1}H and ^{15}N chemical shifts in the presence and absence of the EF-hand at the N-terminus; only the resonances from 13 residues closest to the N-terminus show detectable changes of chemical shifts. Thus, the added residues do not perturb the structure or environment of most of the native protein, and it may be that those residues close to the N-terminus only have altered environments. Moreover, the resonances of the native Vpu portion of the polypeptide are similar whether Ca^{2+} (Fig. 2B) or Yb^{3+} (Fig. 2C) are bound. This indicates that both calcium and lanthanide ions bind specifically to the EF-hand at the N-terminus of the protein.

The blue circles in all three spectra enclose representative resonances from residues in the native Vpu portion of the polypeptide; the locations of these residues in the polypeptide are indicated by the circles superimposed on the structure in Fig. 1. The residual dipolar couplings associated with these resonances are listed in Table 1 for Yb^{3+} and Dy^{3+} bound to EF-Vpu. Significantly, different residual dipolar couplings are measured for each of these resonances upon binding Yb^{3+} and Dy^{3+}. This demonstrates that the two lanthanides result in different directions of orientation of the protein. This is a significant advantage for structure determination, since it can be used to eliminate many ambiguities in relative molecular orientations derived from the residual dipolar couplings.

The EF-hand is a well-characterized metal binding motif found in a large number of calcium-binding proteins (*13*), and lanthanide ions replace calcium ions in these binding sites with minimal structural perturbations. The addition of a Ln^{3+} binding site to the N-terminus of a membrane protein makes it possible to measure residual orientations in micelle samples using solution NMR methods. The residual dipolar couplings available from these experiments help to overcome the limitations inherent in having only a few assigned long-range NOEs and enable the determination of the three-dimensional structures of membrane proteins in micelles. In addition, many other proteins and their complexes have been found to be unstable in bicelle or phage solutions, and this approach allows these agents to be avoided. Moreover, the recent demonstrations of structure determination based on spectral parameters from paramagnetic species (*8, 14*) offer further potential for the use of lanthanide ions bound to specifically located, nonperturbing EF-hand to the N- and C-termini of proteins that do not contain naturally occurring metal-binding sites.

ACKNOWLEDGMENTS

We thank G. Veglia for helpful discussions. This research was supported by Grant PO1 GM56538 from the National Institute of General Medical Sciences.

REFERENCES

1. N. Tjandra and A. Bax, Direct measurement of distances and angles in biomolecules by NMR in a dilute liquid crystalline medium. *Science* **278,** 1111–1114 (1997).
2. G. M. Clore, M. R. Starich, and A. M. Gronenborn, Measurements of residual dipolar couplings of macromolecules aligned in the nematic phase of a colloidal suspension of rod-shaped viruses. *J. Am. Chem. Soc.* **120,** 10571–10572 (1998).
3. M. R. Hansen, L. Mueller, and A. Pardi, Tunable alignment of macromolecules by filamentous phage yields dipolar coupling interactions. *Nat. Struct. Biol.* **5,** 1065–1074 (1998).
4. J. R. Tolman, J. M. Flanagan, M. A. Kennedy, and J. H. Prestegard, Nuclear magnetic dipole interactions in field-oriented proteins—Information for structure determination in solution. *Proc. Natl. Acad. Sci. USA* **92,** 9279–9283 (1995).
5. M. A. Contreras, J. Ubach, O. Millet, J. Rizo, and M. Pons, Measurement of one bond dipolar couplings through lanthanide-induced orientation of a calcium-binding protein. *J. Am. Chem. Soc.* **121,** 8947–8948 (1999).
6. A. Tuechelmann, H. Schwalbe, and C. Griesinger, Magnetically anisotropic tags for biomolecules. Abstract, 3rd European Conference, Oxford (1998).

7. V. V. Gaponenko, C. J. Walsey, B. M. Hoffman, and P. R. Rosevear, Determination of residual dipolar couplings using a cobalt binding tag. *Biophys. J.* **78,** 1705Pos (2000).
8. M. Allegrozzi, I. Bertini, M. B. L. Janik, Y. M. Lee, G. H. Lin, and C. Luchinat, Lanthanide-induced pseudocontact shifts for solution structure refinements of macromolecules in shells up to 40 angstrom from the metal ion. *J. Am. Chem. Soc.* **122,** 4154–4161 (2000).
9. H. M. Al-Hashimi, H. Valafar, M. Terrell, E. R. Zartler, M. K. Eidsness, and J. H. Prestegard, Variation of molecular alignment as a means of resolving orientational ambiguities in protein structures from dipolar couplings. *J. Magn. Reson.* **143,** 402–406 (2000).
10. K. Strebel, T. Klimkait, F. Maldarelli, and M. A. Martin, Molecular and biochemical analyses of human immunodeficiency virus type 1 vpu protein. *J. Virol.* **63,** 3784–3791 (1989).
11. R. L. Willey, F. Maldarelli, M. A. Martin, and K. Strebel, Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J. Virol.* **66,** 7193–7200 (1992).
12. F. M. Marassi, C. Ma, H. Gratkowski, S. K. Straus, K. Strebel, M. Oblatt-Montal, M. Montal, and S. J. Opella, Correlation of the structural and functional domains in the membrane protein Vpu from HIV-1. *Proc. Natl. Acad. Sci. USA* **96,** 14336–14341 (1999).
13. T. C. Williams, D. C. Corson, and B. D. Sykes, Calcium-binding proteins: Calcium(II)–lanthanide(III) exchange in carp parvalbumin. *J. Am. Chem. Soc.* **106,** 5698–5702 (1984).
14. J.-C. Hus, D. Marion, and M. Blackledge, *De novo* determination of protein structure by NMR using orientational and long-range order restraints. *J. Mol. Biol.* **298,** 927–936 (2000).
15. F. R. Ahmed, D. R. Rose, S. V. Evans, M. E. Pippy, and R. To, Refinement of recombinant oncomodulin at 1.30-angstrom resolution. *J. Mol. Biol.* **230,** 1216–1224 (1993).
16. M. Ottiger, F. Delaglio, and A. Bax, Measurement of *J* and dipolar couplings from simplified two-dimensional NMR spectra. *J. Magn. Reson.* **131,** 373–378 (1998).