

## One-Sample Approach to Determining the Relative Orientations of Proteins in Ternary and Binary Complexes from Residual Dipolar Coupling Measurements

Marco Tonelli,<sup>†</sup> Larry R. Masterson,<sup>‡</sup> Gabriel Cornilescu,<sup>†</sup> John L. Markley,<sup>†</sup> and Gianluigi Veglia<sup>\*,†,§</sup>

*National Magnetic Resonance Facility at Madison, Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706-1544, and Departments of Biochemistry, Molecular Biology, and Biophysics, and Chemistry, University of Minnesota, Minneapolis, Minnesota 55455-0431*

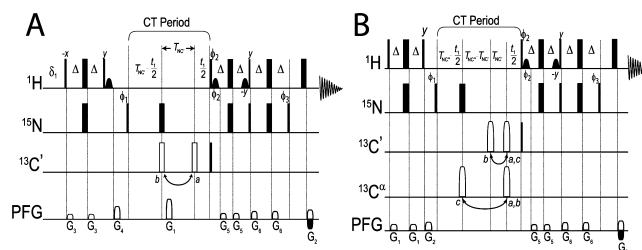
Received June 17, 2009; E-mail: vegli001@umn.edu

NMR spectroscopy is a powerful method for analyzing changes at the atomic level in the structure and dynamics of macromolecular complexes.<sup>1–4</sup> NMR distance restraints used to define the conformational space of protein–protein assemblies are commonly derived from intermolecular nuclear Overhauser effects,<sup>5–7</sup> paramagnetic relaxation enhancements,<sup>8,9</sup> cross-saturation,<sup>10</sup> and chemical shift perturbations.<sup>11</sup> However, because full analysis requires spectral information from each partner in the complex, multiple samples are used for such measurements. Thus the full atomic details of protein assemblies, which only NMR can offer *in solution*, can be time and cost restrictive. Other strategies have exploited amino acid selective<sup>12</sup> or asymmetric labeling patterns<sup>13,14</sup> to facilitate measurements of intermolecular distances. Unfortunately, only the interface is characterized and the full backbone conformation and relative orientation of protein–protein complexes is not defined. In some cases, the approach requires sequences which are insensitive for large assemblies with inherently short transverse relaxation times ( $T_2$ ).

The conformational space of macromolecular complexes also can be determined from orientational restraints derived from residual dipolar couplings (RDCs) under weak alignment.<sup>15,16</sup> Again, a common strategy for avoiding spectral overlap and for associating NMR signals with a particular subunit is to prepare multiple samples with differential labeling patterns. Particular care is necessary in the case of RDC measurements because slight changes in the experimental conditions can alter the alignment tensor, such that information from different samples cannot be correlated directly.

The implementation of a spin–echo difference during a constant time period of an HSQC was originally introduced by Bax and co-workers<sup>17</sup> for the measurement of side chain dihedral angles. We recently applied a similar constant time spin–echo filter element to replace a two-dimensional version of the triple resonance HNCOC pulse sequence to identify sequential pairs of amino acids in large proteins and enzymes<sup>18</sup> and extended this idea to enable chemical shift perturbation mapping of samples containing three isotopically labeled species in solution.<sup>19</sup>

Here we report an approach that, combined with an asymmetric isotopic labeling scheme, enables simultaneous measurement of RDCs from subunits of binary and ternary complexes with high sensitivity. Unlike recently reported schemes,<sup>20,21</sup> the pulse sequence used in this approach is shorter and provides higher sensitivity, particularly for large systems. Also, the approach supports the unambiguous identification of spectral information from each subunit of a binary or ternary complex.



**Figure 1.** Schematic for the gradient-selected TROSY-based pulse sequences for binary (A) or ternary (B) protein mixtures. A reference spectrum is obtained by applying the  $180^\circ$   $^{13}\text{C}$  pulses (open pulses) at position *a*, while  $^{13}\text{C}'$  or  $^{13}\text{C}^\alpha$  suppression is obtained with pulses position *b* or *c*, respectively. Delay durations:  $\Delta = 2.4$  ms;  $\delta = 0.11$  ms;  $T_{\text{NC}'} = 16.5$  ms,  $T_{\text{NC}\alpha} = 23.5$  ms. Further details, including spectral editing to obtain each subunit, are provided in the Supporting Information.

The complete details for the detection and deconvolution of a spectrum containing three isotopically labeled species were provided in a previous publication,<sup>19</sup> and only a brief description is provided here. The subunits of the ternary complex are labeled differentially as follows: uniformly  $^{15}\text{N}$  ( $\text{U-}^{15}\text{N}$ ) labeled (species A), uniformly  $^{15}\text{N}$  and  $^{13}\text{C}$  ( $\text{U-}^{15}\text{N},^{13}\text{C}$ ) labeled (species B), and  $\text{U-}^{15}\text{N}$  and selectively  $^{13}\text{C}'$  or  $^{13}\text{C}^\alpha$  labeled (species C). Selective  $^{13}\text{C}'$  or  $^{13}\text{C}^\alpha$  labeling can be achieved for recombinant proteins expressed in *Escherichia coli* by utilizing 1- $^{13}\text{C}$  or 2- $^{13}\text{C}$  glucose, respectively, as the sole carbon source.<sup>22</sup> Signals from either or both  $^{13}\text{C}$  labeled species (species B or C) can be suppressed in a *selective manner* by using a [ $^1\text{H}/^{15}\text{N}$ ]-HSQC sequence containing a modified constant-time period which leads to  $J_{\text{NC}'}$  or  $J_{\text{NC}\alpha}$  modulation (Figure 1). Thus resonances from either species A or species A and C can be produced in subspectra. Linear combinations of these subspectra lead to the observation of the three individual subunits.<sup>19</sup>

To measure RDCs, we adapted these sequences to utilize sensitivity-enhanced TROSY or anti-TROSY spin-state selection and allowed  $J_{\text{HN}}$  coupling to become active during chemical shift evolution so that  $^1\text{H-}^{15}\text{N}$  splittings could be measured accurately (Figure 1). Alternatively, RDCs can be extracted by measuring the  $^1\text{H-}^{15}\text{N}$  half-splitting between a pair of TROSY and decoupled HSQC spectra.<sup>23</sup> Because the introduction of  $J_{\text{NC}'}$  or  $J_{\text{NC}\alpha}$  modulation is used only for the purpose of suppressing the detection of resonances, this modulation has no effect on the accuracy of the  $^1\text{H-}^{15}\text{N}$  splittings measured. As shown in Figure 1, the sequence is simplified to increase sensitivity in cases of a binary complex (Figure 1A), or the full pulse sequence (Figure 1B) is used in the case of a ternary complex. The total constant time period is  $\sim 33$  ms ( $1/2 J_{\text{NC}'}$ ) or  $\sim 49$  ms ( $1/2 J_{\text{NC}\alpha}$ ), respectively.<sup>19</sup>

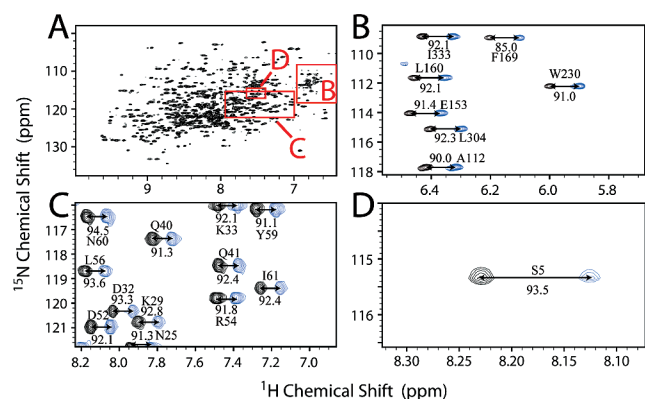
As a proof of concept, we applied this approach to a ternary mixture of noninteracting proteins: maltose binding protein (MBP,

<sup>†</sup> University of Wisconsin—Madison.

<sup>‡</sup> Departments of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota.

<sup>§</sup> Departments of Chemistry, University of Minnesota.

## COMMUNICATIONS



**Figure 2.** Spectral analysis of the convoluted spectrum containing  $[U-^2H, U-^{15}N]$ -MBP,  $[U-^{15}N, ^{13}C]$ -ubiquitin, and  $[^{15}N$ -Ser<sup>5</sup>,  $^{13}C$ -Ala<sup>4</sup>]-Kemptide. TROSY-detection was used to obtain all  $^{15}N$  resonances in the sample (A). Isotropic  $J$ -coupling values were obtained from spectral editing of TROSY and anti-TROSY data sets as shown for MBP (B), ubiquitin (C), and Kemptide (D).

85 44 kDa), ubiquitin (8 kDa), and Kemptide (0.7 kDa). Proteins were  
86 either expressed in *Escherichia coli* BL21(DE3) to obtain uniform  
87 labeling ( $[U-^2H, U-^{15}N]$ -MBP and  $[U-^{15}N, ^{13}C]$ -ubiquitin) or  
88 synthesized using solid phase synthesis with standard Fmoc  
89 chemistry ( $[^{15}N$ -Ser<sup>5</sup>,  $^{13}C$ -Ala<sup>4</sup>]-Kemptide). The NMR sample con-  
90 tained 1.5 mM MBP, 1 mM ubiquitin, and 0.5 mM Kemptide.

91 The pulse sequence from Figure 1B was used to obtain the  
92 isotropic couplings for the ternary mixture shown in Figure 2. All  
93  $^1H-^{15}N$  correlations were observed in the reference spectrum  
94 (Figure 2A), and the subspectra for each component were obtained  
95 by using spectral editing of the suppressed spectra (Figure 2B–D).  
96 Weak alignment was then introduced using pf1 phage (12 mg/mL),  
97 and the couplings were remeasured to obtain backbone RDC values  
98 for the well-folded proteins ubiquitin and MBP. Back-calculated  
99 RDC values based on the crystal structures 1UBQ (ubiquitin)<sup>24</sup> and  
100 1OMP (MBP)<sup>25</sup> matched well with experimentally measured values  
101 obtained in this study (Figure S1, Supporting Information). Outliers  
102 from the MBP structure may arise from a small difference in pH  
103 ( $\sim 7.3$  used here vs 7.0 used previously) and possibly from the  
104 slightly different construct used in this study which has an  
105 N-terminal His<sub>6</sub> tag and an artifact of a TEV-cleavage site that left  
106 four extra residues at the C-terminus.<sup>26</sup> Thus, coupling values from  
107 this single sample were sufficient to define the backbone confor-  
108 mational space of these structured proteins, along with their relative  
109 alignment tensors.

110 This new approach to measuring backbone RDC values in a  
111 ternary complex eliminates the need for multiple samples, removes  
112 errors from sample inconsistencies, and ultimately reduces costs  
113 related to the preparation of multiple samples. Although shown only  
114 for a tertiary mixture of proteins, this approach will work for soluble  
115 and membrane proteins, so long as the reference HSQC experiment  
116 can be obtained. In the case of membrane proteins, lanthanides or  
117 polyacrylamide gels may be used to introduce weak alignment so  
118 that the detergent or bicelle are not perturbed.<sup>16,27</sup>

119 When a binary mixture of proteins is studied, the signal-to-noise  
120 ( $S/N$ ) resulting from the pulse sequence (Figure 1A) used to detect  
121 the  $^{15}N$ -labeled species is  $\sqrt{2}$  higher than that of approaches that  
122 rely on a HNCQ-based sequence,<sup>18</sup> as has been proposed recently.<sup>20,21</sup>  
123 In addition, the time period in which magnetization is transverse  
124 in HNCQ-based experiments is  $\sim 68$  ms, whereas the sequences

presented here only require half this time period ( $\sim 33$  ms) for a  
125 binary mixture or  $\sim 49$  ms for a ternary mixture. Our laboratories  
126 have shown that, for large proteins, the constant-time spin-echo  
127 filter used in this approach has superior  $S/N$  and works well when  
128 HNCQ-based sequences provide insufficient  $S/N$ , particularly in  
129 cases in which  $T_2$  values are less than  $\sim 50$  ms.<sup>18</sup>

130 In summary, we present a new approach for the acquisition of  
131 backbone amide RDCs for binary or ternary complexes using a  
132 single sample. Together with the applicability of these sequences  
133 to monitor chemical shift perturbations in titration experiments,<sup>19</sup>  
134 this method provides useful orientational restraints for high-  
135 resolution studies of protein complexes.<sup>11</sup> Since numerous biological  
136 processes rely on protein assemblies or transient interactions, this  
137 approach should be well suited for a wide range of applications.  
138

**Acknowledgment.** Financial support provided by NIH Grants  
139 GM64742, GM072701, and HL080081 (G.V.), RR02301 and  
140 GM66326 (J.L.M.), and AHA support from 0615546Z (L.R.M.).  
141 NMR data were collected at NMRFAM (NIH: P41RR02301,  
142 P41GM66326, RR02781, and RR08438; NSF: (DMB-8415048,  
143 OIA-9977486, BIR-9214394) and USDA. U. of Minnesota NMR  
144 Facility is supported by NSF (BIR-961477).  
145

**Supporting Information Available:** pulse sequence and spectral  
146 editing details, and experimental RDC fitting to available structures.  
147 This material is available free of charge via the Internet at [http://](http://pubs.acs.org)  
148 [pubs.acs.org](http://pubs.acs.org).  
149

## References

- 125 (1) Teague, S. J. *Nat. Rev. Drug Discovery* **2003**, *2*, 527–541. 152
- 126 (2) Clarkson, J.; Campbell, I. D. *Biochem. Soc. Trans.* **2003**, *31*, 1006–1009. 153
- 127 (3) Kay, L. E. *J. Magn. Reson.* **2005**, *173*, 193–207. 154
- 128 (4) Betz, M.; Saxena, K.; Schwalbe, H. *Curr. Opin. Chem. Biol.* **2006**, *10*, 155
- 129 (5) Clore, G. M.; Gronenborn, A. M. *Nat. Struct. Biol.* **1997**, *4*, 849–853. 156
- 130 (6) Zwahlen, C.; Legault, P.; Vincent, S. J. F.; Greenblatt, J.; Konrat, R.; Kay, 157
- 131 L. E. *J. Am. Chem. Soc.* **1997**, *119*, 6711–6721. 158
- 132 (7) Post, C. B. *Curr. Opin. Struct. Biol.* **2003**, *13*, 581–588. 159
- 133 (8) Clore, G. M.; Tang, C.; Iwahara, J. *Curr. Opin. Struct. Biol.* **2007**, *17*, 160
- 134 (9) Arora, A.; Tamm, L. K. *Curr. Opin. Struct. Biol.* **2001**, *11*, 540–547. 161
- 135 (10) Shimada, I. *Methods Enzymol.* **2005**, *394*, 483–506. 162
- 136 (11) Zuiderweg, E. R. *Biochemistry* **2002**, *41*, 1–7. 163
- 137 (12) Igarashi, S.; Osawa, M.; Takeuchi, K.; Ozawa, S.; Shimada, I. *J. Am. Chem.* 164
- 138 (13) Walters, K. J.; Ferentz, A. E.; Hare, B. J.; Hidalgo, P.; Jasanoff, A.; Matsuo, 165
- 139 (14) Traaseth, N. J.; Ha, K. N.; Verardi, R.; Shi, L.; Buffy, J. J.; Masterson, 166
- 140 (15) Prestegard, J. H.; Bougault, C. M.; Kishore, A. I. *Chem. Rev.* **2004**, *104*, 167
- 141 (16) Bax, A.; Grishaev, A. *Curr. Opin. Struct. Biol.* **2005**, *15*, 563–570. 168
- 142 (17) Vuister, G. W.; Wang, A. C.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 5334– 169
- 143 (18) Tonelli, M.; Masterson, L. R.; Hallenga, K.; Veglia, G.; Markley, J. L. 170
- 144 (19) Shimada, I. *Methods Enzymol.* **2005**, *394*, 483–506. 171
- 145 (20) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 172
- 146 (21) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 173
- 147 (22) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 174
- 148 (23) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 175
- 149 (24) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 176
- 150 (25) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 177
- 151 (26) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 178
- (27) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 179
- (28) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 180
- (29) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 181
- (30) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 182
- (31) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 183
- (32) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 184
- (33) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 185
- (34) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 186
- (35) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 187
- (36) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 188
- (37) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 189
- (38) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 190
- (39) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 191
- (40) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 192
- (41) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 193
- (42) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 194
- (43) Masterson, L. R.; Tonelli, M.; Markley, J. L.; Veglia, G. *J. Am. Chem.* 195

JA904766G