SEA-TROSY (Solvent Exposed Amides with TROSY): A Method to Resolve the Problem of Spectral Overlap in Very Large Proteins

Maurizio Pellecchia,*,† David Meininger,† Anna L. Shen,‡ Rick Jack,† Charles B. Kasper,‡ and Daniel S. Sem†

Triad Therapeutics Inc., 5820 Nancy Ridge Dr. San Diego, California 92121
McArdle Laboratory for Cancer Research, Medical School University of Wisconsin, Madison, Wisconsin 53706

Received December 5, 2000
Revised Manuscript Received April 3, 2001

The recent advances of ^{2}H labeling in combination with the revolutionary spectral improvement provided by the development of TROSY-type experiments[1] largely reduce the problems of nuclear spin relaxation particularly in ^{15}N,^{1}H experiments and allowed the study of complexes of molecular mass >50 kDa where only one of the components (with ∼200 amino acid residues) was ^{15}N/^{2}H labeled.[2] However, for even larger proteins with a large number of amino acid residues (>300) the problem of resonance overlap still represents a major obstacle for resonance assignment and chemical shift perturbation studies. Recently, several research groups proposed the use of segmental labeling to conduct studies on selectively labeled domains in multidomain proteins.[3] Although these methods are very promising for studying interdomain interactions or performing structural characterization in multidomain proteins, low expression yields still represent a major hurdle. Recently, Sattler and Fesik[4] proposed the use of lanthanide-induced shifts to increase the chemical shift dispersion. However, the increase in dispersion is rather limited (∼1 ppm at most) and a small detrimental relaxation effect has also been observed.

In this communication we present a modification of ^{15}N,^{1}H correlation experiments that is designed to reduce the problem of resonance overlap in very large proteins (>70 kDa) with a large number of amino acid residues (>300). Our idea is based on the concept that for binding studies only amides that are exposed to the solvent are of interest whereas those buried in the interior of the protein are not likely to be involved in intermolecular interactions. The selection of solvent exposed amide protons in a perdeuterated ^{15}N-labeled sample dissolved in H_2O is obtained with the pulse scheme of Figure 1a. The sequence starts with a ^{15}N double filter[5] that serves to eliminate all the magnetization generated from amide protons. Water magnetization is not affected by the ^{15}N filter and subsequently is returned along the *z*-axis by the last 90° ^{1}H pulse prior to τ_m (Figure 1a). At this time point, water *z* magnetization is allowed to exchange with amide protons during a variable mixing time, τ_m (Figure 1a). Backbone amides that are exposed to the solvent will acquire magnetization from the solvent that can be subsequently detected with a TROSY-type experiment.[1,6] A water flip-back version of

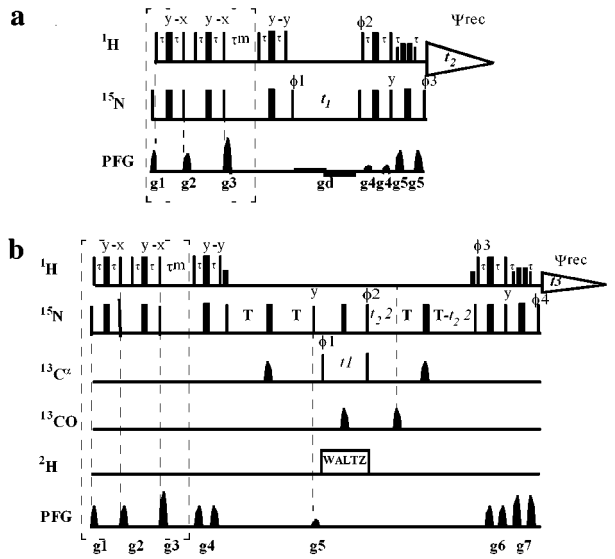

Figure 1. (a) Pulse sequence to selectively observe solvent exposed amide protons with TROSY (SEA-TROSY). Narrow and thin bars represent 90° and 180° radio frequency pulses, respectively. Unless specified otherwise, pulse phases are along the *x*-axis. The pulsed field gradients are 500 μs duration with strengths of $g_1 = 20$ G/cm, $g_2 = 30$ G/cm, $g_3 = 40$ G/cm, $g_4 = 15$ G/cm, $g_5 = 55$ G/cm. The bipolar gradient g_d is 0.5 G/cm and it is used to avoid radiation damping effects during $t1$.[15] The delay τ was set to 2.7 ms. The phase cycle was as follows: $\phi_1 = y, -y, -x, x$; $\phi_2 = y$; $\phi_3 = x$; $\Psi_{rec} = x, -x, -y, y$. A phase sensitive spectrum in the ^{15}N dimension is obtained by recording a second FID for each $t2$ value, with $\phi_1 = -y, y, -x, x$, $\phi_2 = -y$, and $\phi_3 = -x$, and the data were processed as described by Pervushin et al.[6] The SEA element is outlined by the dashed rectangle. (b) Pulse scheme for the 3D SEA-HNCA-TROSY. ^{13}C$^\alpha$ 180° pulses are RE-BURP pulses[9] of 250 μs duration centered at 53 ppm and are designed to selectively excite the aliphatic region (excitation of ∼8000 Hz) without exciting the ^{13}CO region (∼177 ppm). This avoids losses of magnetization due to ^{15}N−^{13}CO ^1J coupling constants (∼15 Hz). ^{13}CO decoupling pulses are off-resonance Gaussian shaped pulses of 120 μs duration shifted to 177 ppm. A phase sensitive spectrum in the ^{15}N dimension is obtained by recording a second FID for each $t2$ value, with $\phi_1 = 2(x), 2(-x)$ and $\phi_2 = x, -x$, and the data were processed as described by Pervushin et al.[6] States-TPPI[10] quadrature detection in the ^{13}C$^\alpha$ dimension was achieved by incrementing ϕ_1. The pulsed field gradients are 500 μs duration and strengths of $g_1 = 20$ G/cm, $g_2 = 30$ G/cm, $g_3 = 40$ G/cm, $g_4 = 25$ G/cm, $g_5 = 20$ G/cm, $g_6 = 15$ G/cm, $g_7 = 55$ G/cm. ^{2}H decoupling during ^{13}C$^\alpha$ evolution is achieved with a WALTZ-16 composite pulse[11] at a field strength of 2.5 kHz. For both schemes, suppression of residual water is achieved with a WATERGATE sequence using a 3−9−19 composite pulse.[12]

TROSY [1,6] has been adopted as it is a requisite for the successful implementation of the SEA selection. The appearance of the resulting spectrum is the same as a ^{15}N,^{1}H TROSY spectrum, but containing only backbone amides that are solvent exposed and therefore with much fewer resonances. The signal intensity (I) of a given amide proton in the resulting ^{15}N,^{1}H TROSY spectrum at a given mixing time, τ_m, is related to the exchange rate, k_{ex}, according to:[7]

$$I = I_\infty(1 - e^{-k_{ex}\tau_m}) \quad (1)$$

where I_∞ is the intensity at infinite mixing time (complete

* Address correspondence to this author. E-mail: mpellecchia@triadt.com.
† TRIAD Therapeutics, Inc. San Diego.
‡ University of Wisconsin.

(1) (a) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366−12371. (b) Wider, G.; Wüthrich, K. *Curr. Op. Struct. Biol.* **1999**, *9*, 594−601. (c) Saltzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13585−13590.
(2) Pellecchia, M.; Sebbel, P.; Hermanns, U.; Wüthrich, K.; Glockshuber, R. *Nat. Struct. Biol.* **1999**, *6*, 336−339.
(3) (a) Yamazaki, T.; Otomo, T.; Oda, N.; Kyogoku, Y.; Uegaki, K.; Ito, N.; Ishino, Y.; Nakamura, H. *J. Am. Chem. Soc.* **1998**, *120*, 5591−5592. (b) Xu, R.; Ayers, B.; Cowburn, D.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 388−393.
(4) Sattler, M.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 7885−7886.
(5) (a) Otting, G.; Wüthrich, K. *Q. Rev. Biophys.* **1990**, *23*, 39−96. (b) Breeze, A. L. *Prog. NMR Spectrosc.* **2000**, *36*, 323−372.
(6) (a) Pervushin, K.; Wider, G.; Wüthrich, K. *J. Biomol. NMR* **1998**, *12*, 345−348. (b) Zhu, G.; Kong, X. M.; Sze, K. H. *J. Biomol. NMR* **1999**, *13*, 77−81.
(7) Gemmeker, G.; Jahnke, W.; Kessler, H. *J. Am. Chem. Soc.* **1993**, *115*, 11620−11621.

10.1021/ja005850t CCC: $20.00
Published on Web 04/20/2001

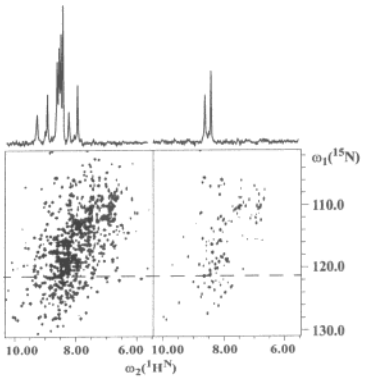

Figure 2. TROSY (left) and SEA-TROSY (right) ^{15}N,^1H correlation spectra of 0.5 mM ^2H,^{15}N labeled P450 reductase from rat liver (95% H_2O, 5% D_2O; $T = 303$ K, pH 7.5). U-^2H/^{15}N- and U-^2H/^{15}N/^{13}C-labeled P450 reductase from rat liver, lacking the N-terminal 56 residues and the C-terminal WS sequence (Shen and Kasper, manuscript in preparation), was expressed on Celltone (Martek Biosciences Corp.) supplemented M9 minimal media and purified as previously described.[13] The TROSY spectrum was obtained with the same pulse scheme but without the SEA element (dashed box in Figure 1a). The SEA-TROSY spectrum was obtained with the pulse scheme reported in Figure 1a with a mixing time τ_m of 100 ms. The spectra were recorded on a Bruker DRX700 spectrometer operating at 700 MHz ^1H frequency. 128 ($t1$) × 1024 ($t2$) complex points were recorded. Traces along $\omega 2$ are taken at the position indicated by the dashed line. Spectra were acquired and processed with XWINNMR (Bruker AG) and analyzed using XEASY.[14]

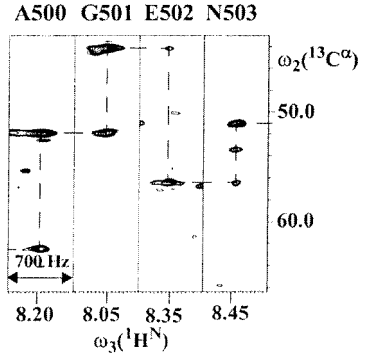

Figure 3. Representative $\omega_2(^{13}C^\alpha)-\omega_3(^1H^N)$ strips from a 3D SEA-HNCA-TROSY spectrum recorded with the pulse scheme of Figure 1b with a 0.5 mM sample of ^{15}N/^2H/^{13}C-labeled sample of P450 reductase. The assignment of the loop of residues Ala 500 to Asn 503 has been obtained via the sequential $^{13}C^\alpha(i)/^{13}C^\alpha(i-1)$ connectivities indicated by the dashed line.

exchange). The individual exchange rates, k_{ex}, depend on the amino acid type, the sequence, the pH of the solution, and the degree of protection.[8] Generally, backbone amides located in loop regions are very fast to exchange whereas residues located in secondary structure elements are relatively slow to exchange with high protection factors. Other amide proton resonances that can in principle also appear in the spectrum are those in direct contact with bound water molecules or those close to Thr, Ser, and Tyr hydroxyl groups. As we are mainly interested in obtaining backbone resonance assignment for solvent exposed loop regions, the SEA element can be combined with triple resonance TROSY-type experiments,[1c] performed on triply ^{13}C/^2H/^{15}N-labeled samples, as in the SEA-HNCA-TROSY (Figure 1b), SEA-HNCACB-TROSY, etc. As an application we recorded the SEA-TROSY experiment with 200 μL of a 0.5 mM sample of the uniformly ^{15}N/^2H-labeled catalytic portion (residues 57−678, 71 kDa molecular mass) of rat NADPH cytochrome P450.[13] As a reference we recorded the ^{15}N,^1H TROSY spectrum of P450 (Figure 2, left). Here deuteration and TROSY dramatically improved the quality of the spectrum that contained virtually no signals when recorded with a nondeuterated sample and without TROSY (data not shown). However, only roughly ∼300 out of ∼600 expected cross-peaks can be resolved whereas most of the spectrum is characterized by severe resonance overlap that makes resonance assignments an improbable task and chemical shift mapping studies practically impossible. The spectrum resulting from the SEA-TROSY experiment at 100 ms mixing time is shown in Figure 2 (right). The spectral simplification is dramatic (Figure 2) as is also evident from the 1D traces taken from a crowded region of the spectra. The SEA-TROSY spectrum reported in Figure 2 exhibits a loss in signal-to-noise ratio of a factor of 1.4 on average. This small loss, however, is largely compensated by the spectral simplification achieved. The cross-peak intensity is modulated by eq 1 so that a best compromise between spectral simplification and signal-to-noise ratio has to be empirically derived by comparing SEA-TROSY spectra recorded at different mixing times. To demonstrate that the cross-peaks in the SEA-TROSY belong mainly to loop regions of the molecule, we also recorded a 3D SEA-HNCA-TROSY (Figure 1b). Loop regions can be easily assigned in the SEA-HNCA-TROSY sub-spectrum as the number of possible $^{13}C^\alpha$ connectivities is largely reduced (Figure 3). The process of mapping the sequentially connected stretches of amino acid onto the sequence of the protein can also be facilitated by the knowledge of the three-dimensional structure of the enzyme, which allows identification of the sequences of solvent exposed loop regions. However, in most cases additional triple resonance experiments such as 3D SEA-HNCACB-TROSY may also be needed. In conclusion, we show that the selection of solvent exposed amide protons with SEA-TROSY-type experiments represents an attractive solution to the problem of resonance overlap encountered with very large proteins with a very large number of amino acid residues, and that the method can be used to obtain partial backbone assignments without requiring selective labeling. As the SEA element can be combined with available experiments to obtain information on protein structure, backbone dynamics, ligand binding, enzyme inhibition, etc., we believe it constitutes a very simple and general tool to enlarge the field of application of NMR spectroscopy to macromolecules that are otherwise not amenable to NMR analysis.

Acknowledgment. We thank Prof. Dr. Kurt Wüthrich for his valuable advise and suggestions.

JA005850T

(8) Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Protein Struct. Func. Gen.* **1993**, *17*, 75−86.

(9) Geen, H.; Freeman, R. *J. Magn. Reson.* **1991**, *93*, 93−96.

(10) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.

(11) Shaka, A. J.; Keeler, J.; Frenkiel, T.; Freeman, R. *J. Magn. Reson.* **1983**, *52*, 335−338.

(12) Sklenar, V.; Piotto, M.; Leppik, R.; Saudek, V. *J. Magn. Reson. A* **1993**, *102*, 241−244.

(13) Shen, A. L.; Porter, L. H.; Wilson, T. E.; Kasper, C. B. *J. Biol. Chem.* **1989**, *264*, 7584−7589.

(14) Bartels, C.; Xia, T.; Billeter, M.; Guntert, P.; Wüthrich, K. *J. Biomol. NMR* **1995**, *5*, 1−10.

(15) Sklenar, V. *J. Magn. Reson. A* **1995**, *114*, 132−135.