Chinese Journal of Chemistry, **2004**, *22*, 1395—1398 **Communication**

Clean SEA-TROSY Experiments to Map Solvent Exposed Amides in Large Proteins

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It is well known that the SEA-TROSY experiment could alleviate some of the problems of resonance overlap in $^{15}N/^{2}H$ labeled proteins as it was designed to selectively map solvent exposed amide protons. However, SEA-TROSY spectra may be contaminated with exchange-relayed NOE contributions from fast exchanged hydroxyl or amine protons and contributions from longitudinal relaxation. Also, perdeuteration of the protein sample is a prerequisite for this experiment. In this communication, a modified version, clean SEA-TROSY, was proposed to eliminate these artifacts and to allow the experiment to be applied to protonated or partially deuterated proteins and protein complexes.

Keywords proteins, solvent accessibility, hydrogen exchange, TROSY, NMR

Spectral overlap is usually one of the major obstacles for structural determination of very large proteins. Recently, a new experiment, SEA (Solvent Exposed Amides)-TROSY,[1] was proposed to partly resolve this problem. Since the solvent exposed amide protons can exchange rapidly with water, they are expected to appear in the SEA-TROSY spectra as stronger peaks, while those buried in the interior of the protein will disappear or appear as weaker peaks. As solvent exposed residues can be involved in protein-protein or protein-ligand contacts, this experiment is particularly useful to study these interactions.

However, perdeuteration of the protein sample is a prerequisite for SEA-TROSY experiments to work properly. For partially deuterated or protonated proteins and protein complexes, SEA-TROSY spectra may contain NOE contributions from aliphatic protons. Moreover, SEA-TROSY spectra may be contaminated with exchange-relayed NOE contributions from fast exchanged hydroxyl or amine protons[2] and artifacts due to longitudinal relaxation processes during τ_m.[3]

Regarding to the potential problems described above, we developed the clean SEA element, a modified version of the SEA element, which not only effectively eliminates these NOE contributions but also suppresses the longitudinal relaxation contributions by using an appropriate phase cycling scheme. NOE contributions are eliminated by applying a spin-echo filter,[4,5] or a double $^{13}C/^{15}N$ filter for $^{15}N/^{13}C$ labeled samples[3,6-8] along with the CLEANEX-PM spin locking sequence during τ_m.[9] Therefore, perdeuteration of the sample is not required. In our previous work, clean SEA-HSQC was proposed to detect the signals from solvent exposed residues in small proteins.[10] However, for large proteins, TROSY-type experiments have to be employed to record spectra with optimized transverse relaxation rates and the highest spectral resolution.[11] In this communication, clean SEA-TROSY is proposed to detect the signals from solvent exposed residues in large proteins.

The clean SEA-TROSY pulse scheme shown in Figure 1a was designed for $^{15}N/^{13}C$ labeled proteins. This scheme starts with a double $^{15}N/^{13}C$ filter,[3] which serves to eliminate all the magnetization generated from protons attached to nitrogen and carbon atoms. A pair of gradient pulses, $g1$ (0.5 ms×1.1 G/cm) bracket the double ^{15}N filter to prevent water magnetization loss caused by radiation damping. After the filter, a gradient pulse, $g2$ (0.5 ms×25.8 G/cm) is used to remove non-zero-order coherences. Water magnetization is allowed to pass the filter and exchange with amide protons during the subsequent mixing period. The CLEANEX-PM mixing scheme[9] is used to eliminate both exchange-relayed NOE contributions from the fast exchanged hydroxyl or amine protons and intramolecular NOE contributions from aliphatic protons. Radiation damping during τ_m is removed by a weak bipolar gradient, gm (0.05 G/cm), while that during the t_1 evolution period was suppressed by another bipolar gradient, *gd* (0.05 G/cm). The delays and strengths of the other gradient pulses were $g3$=(0.5 ms×10.7 G/cm), $g4$=(0.5 ms×4.3 G/cm), $g5$=(0.5 ms×0.4 G/cm), $g6$=(0.5 ms×17.2 G/cm). The phase cycle is as follows: φ_1=16(x),

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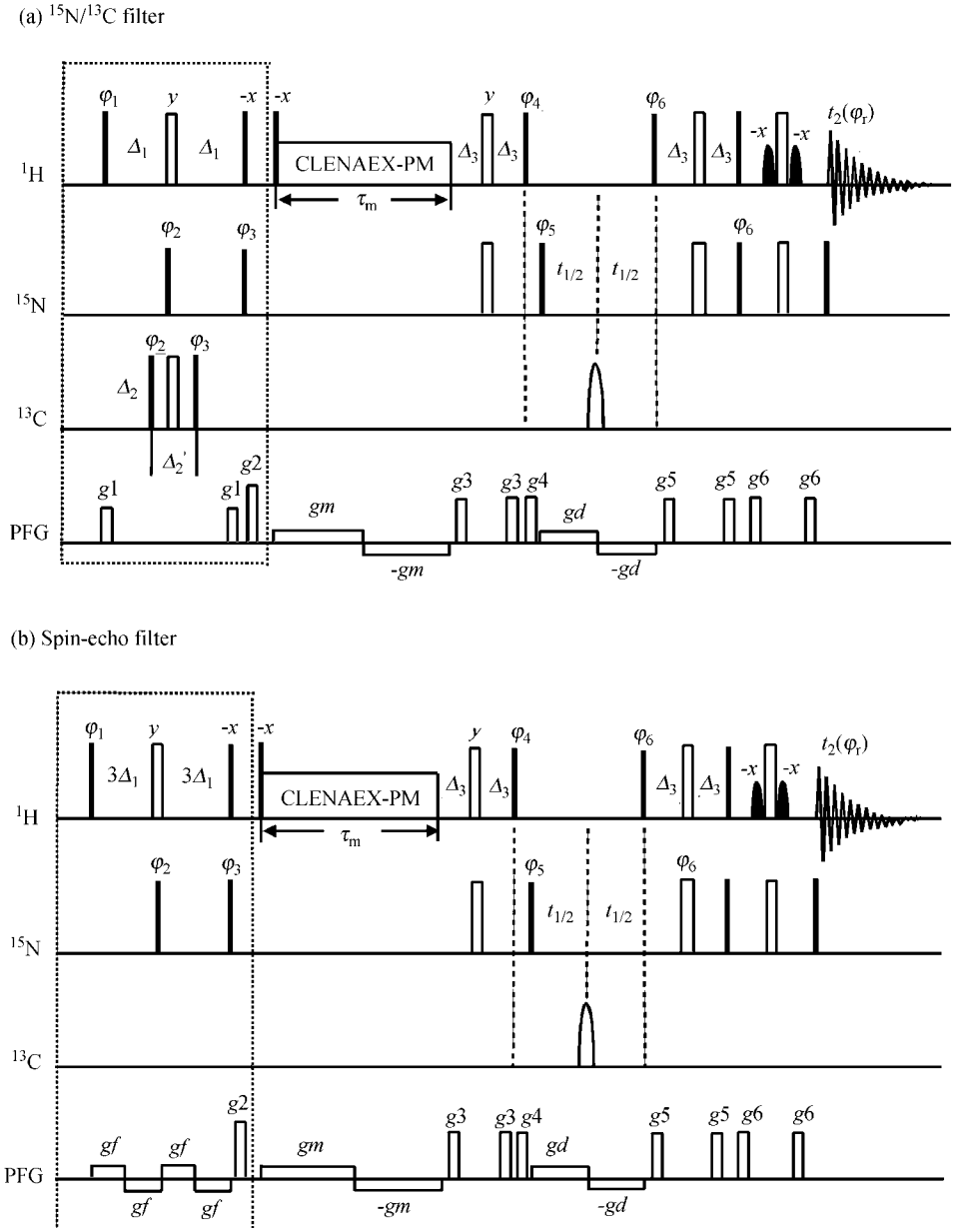

Figure 1 Clean SEA-TROSY pulse sequences for the selective observation of solvent exposed amide protons. Narrow and wide squares denote 90° and 180° hard pulses, respectively. Unless indicated, pulse phases were applied along *x*. (a) A double ^{15}N/^{13}C filter served to eliminate NOE contributions from aliphatic protons in ^{15}N/^{13}C labeled proteins. (b) The spin-echo filter combined with the double ^{15}N filter served to eliminate NOE contributions from aliphatic protons in ^{15}N labeled proteins.

16($-x$); φ_2=4(x), 4($-x$); φ_3=8(x), 8($-x$); φ_4=16(y), 16($-y$); φ_5=y, x, $-y$, $-x$; φ_6=y; φ_r=x, $-y$, $-x$, y. The delays used in the pulse sequence were Δ_1=5.5 ms, Δ_2=3.7 ms, Δ'_2=3.6 ms, Δ_3=2.3 ms. The length of the double ^{15}N filter was set to $2\Delta_1=1/{}^1J_{NH}$=11.0 ms. The proton carrier was switched during the CLEANEX-PM pulse train from the water resonance to the middle of the NH range. The carbon carrier was placed on the aliphatic carbons (35 ppm) at the beginning of the pulse sequence, then shifted to the center between $^{13}C_\alpha$ and ^{13}CO (116 ppm) after the double ^{15}N/^{13}C filter. A selective ^{13}C 180° pulse on $^{13}C_\alpha$ and ^{13}CO was applied to refocus ^{15}N-^{13}C couplings during t_1. A water flip-back version of the TROSY scheme[12] is used to detect the signals.

The peak intensities in a clean SEA-TROSY spectrum depend on a number of factors including the local environment of the residue in the protein, the amino acid type and its solvent accessibility.[13,14] In general, residues located in loop regions on the protein surface are in very fast exchange with water and appear as stronger peaks in the spectrum, whereas those located in hydrogen-bonded secondary structures or buried in the interior of proteins are in relatively slow exchange with water and appear as weaker peaks or do not appear in the clean SEA-TROSY spectrum.

Figure 1b shows another version of the clean SEA-TROSY pulse scheme, which integrates a double ^{15}N filter with a spin-echo filter.[4,5] This scheme can be applied to ^{15}N labeled proteins. The length of the double

^{15}N filter is extended to $6\Delta_1 = 6/^1J_{HN} = 33$ ms, during which most of the protein magnetization decays away completely because of *J*-coupling evolution and the much shorter longitudinal relaxation time when compared with that of water protons. A bipolar gradient, *gf* (0.05 G/cm) is applied to suppress radiation damping in the spin-echo filter.

The longitudinal relaxation processes during the mixing period can be also contributed to exchange spectra,[3] and will normally obscure the effect of the exchange with water and distort the peak intensities, or give rise to peaks in the spectrum from non-labile amide protons. This artifact can be effectively suppressed by applying appropriate phase cycling to the first 90° proton pulse and the receiver (Figure 1). The water magnetization prior to τ_m is alternatively aligned along the axes $+y$ and $-y$ in subsequent scans.

The clean SEA-TROSY experiment has been applied to a 1.5 mmol•L^{-1} uniformly ^{15}N labeled sample of dihydrofolate reductase (DHFR, 162 amino acid residues) complexed with unlabeled methotrexate (MTX) in an NMR buffer (200 mmol•L^{-1}, KCl, 50 mmol•L^{-1} KH_2PO_4, pH 6.5, in 90% H_2O/10% D_2O). All the 2D NMR experiments were performed on a Varian Unity INOVA 500 spectrometer at 35 ℃. The mixing time was 5, 30, 60, 100, 200 ms, the pre-delay was 2.3 s, and the spectral widths were 8000 and 1587 Hz for ^{1}H and ^{15}N, respectively. 128(t_1)×1024(t_2) complex points were recorded and processed with nmrPipe.[16] 32 and 128 scans were acquired for the TROSY and clean SEA-TROSY experiments, respectively. As a reference, the spectrum shown in Figure 2a is a normal TROSY spectrum. Figure 2b was recorded with the clean SEA-TROSY pulse scheme described in Figure 1b. The spectrum is simplified significantly because, in principle, only water-accessible amide protons are likely to appear in the spectrum. This shows that the degree of spectral overlap in clean SEA-TROSY spectra can be greatly reduced when compared with that in normal TROSY spectra.

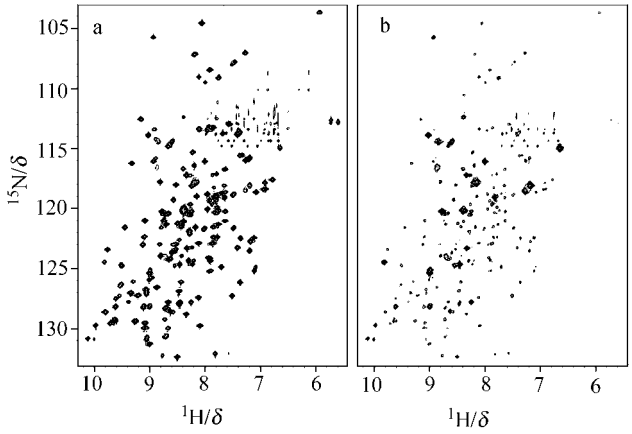

Figure 2 (a) TROSY spectrum. (b) Clean SEA-TROSY spectrum recorded with the pulse scheme of Figure 1b. Spectrum (b) was drawn with contour levels being 50% of those in spectrum (a). The mixing time and predelay were 100 ms and 2.3 s, respectively.

The 3D solution structure of DHFR complexed with MTX has been solved by NMR spectroscopy[17] (PDB ID 1A08). The relative solvent accessibilities of several typical residues Q71, D67, E56, Q65, Y68, T63, T62 were calculated to be 60.8%, 51.2%, 38.5%, 28.9%, 21.1%, 13.7%, 8.0%, respectively, by the program MOLMOL.[15]

Figure 3 displays the peak intensities in the clean SEA-TROSY spectrum for these residues as a function of mixing time. The evaluation of the buildup rates from these curves demonstrates that the exchange rates of these residues are in the order of Q71>D67>E56>Q65>Y68>T63>T62, as expected from their respective relative solvent accessibilities.

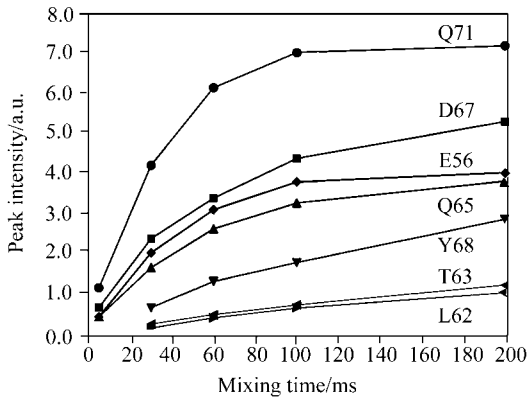

Figure 3 Buildup curves of amide crosspeak intensities in clean SEA-TROSY spectra of DHFR complexed with MTX with mixing time of 5, 30, 60, 100, and 200 ms. The predelay used was 2.3 s.

In conclusion, the clean SEA-TROSY experiment described above allows the mapping of solvent exposed amides in protonated or partially deuterated large proteins. This method not only effectively eliminates both intramolecular or intermolecular NOE contributions from aliphatic protons and exchange-relayed NOE contributions from fast exchanged hydroxyl or amine protons, but also suppresses contributions from longitudinal relaxation. Clean SEA-TROSY would prove useful for resonance assignment, protein-ligand binding studies and the precise measurement of exchange rates.

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