J|A|C|S
COMMUNICATIONS

Published on Web 03/02/2006

CEESY: Characterizing the Conformation of Unobservable Protein States

Hugo van Ingen, Geerten W. Vuister, Sybren Wijmenga, and Marco Tessari*

Department of Physical Chemistry, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands

Received October 7, 2005; E-mail: tessari@nmr.ru.nl

Many biological processes such as ligand binding, enzyme catalysis, allosterics, and protein folding involve conformational rearrangements on a micro- to millisecond time scale to transient protein states with a low population, also termed excited states.[1] These unobservable minor states can only be studied indirectly by exploiting their continuous interconversion into the major or ground state. Kinetic and thermodynamic parameters of this exchange process can be determined along with the frequency difference between excited and ground state for a number of backbone and side-chain nuclei using well-established relaxation dispersion techniques.[2] These frequency differences are of great interest because they allow the structural characterization of the excited-state, provided their sign is known.[3] However, only the absolute value is accessible using relaxation dispersion methods. Experiments to measure the sign have so far only been designed for backbone ^{15}N nuclei.[4,5] Here, we present a new method, named CEESY (for Chemical Exchange to Excited States spectroscopY), to establish the sign of the frequency difference between ground- and excited-state signals using a single 2D spectrum. Importantly, this method is not limited to ^{15}N but can also be applied to most relevant spins in bioorganic materials and will, therefore, allow for a more complete spectroscopic characterization of the excited state.

The essential pulse sequence element of the CEESY (Pronounce as /'keisi:/ (International Phonetic Alphabet notation)) experiment involves two heteronuclear spins *I* and *S* (Figure 1a). It consists of a modified spin-echo sequence comprised of two periods of duration τ, separated by a refocusing 180° pulse on spin *S*. Single quantum *S* spin coherence evolves in the first τ period and *IS* multiple quantum coherence in the second τ period. In the absence of exchange, the chemical shift evolution of spin *S* is completely refocused and a perfect echo is obtained at point *a*. Conversely, chemical shift refocusing is only partially achieved in the presence of exchange, as a result of a difference in the exchange induced shift for SQ and MQ coherences[4] and a small net rotation of *S* coherence at point *a* results (Figure 1b,c). Thus, a large, *main* component (projection on echo axis) and a small, *orthogonal* component (projection on orthogonal axis) are present at the end of this modified spin-echo. The sign of this rotation is determined by the sign of the Larmor frequency difference between excited and ground-state resonances in most cases of practical interest, i.e., fast-to-intermediate two-site exchange.[4] Observation of identical signs for the two components can therefore be directly translated into an increased Larmor frequency in the excited state ($\omega_{S,e} > \omega_{S,g}$), while opposite signs denote a decreased Larmor frequency in the excited state with respect to the ground state ($\omega_{S,e} < \omega_{S,g}$). The signs of the two components can readily be obtained by a proper choice of the phase ϕ of the 90° pulse on spin *S* at time point *a* (Figure 1a). The CEESY building block is robust with respect to interferences from *J*-couplings and cross-correlated relaxation. The requirement for two-site exchange is not so restrictive. In the case of multisite exchange, meaningful average spectral information on the conformations in the ensemble of excited states can be obtained.[2a]

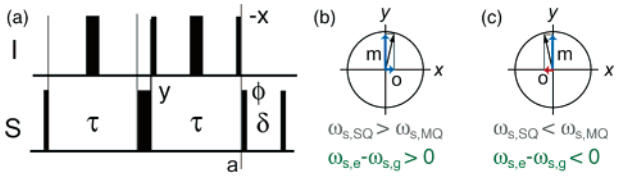

Figure 1. (a) Diagram of the CEESY pulse sequence element. Narrow (wide) bars represent 90° (180°) pulses along the *x*-axis unless indicated otherwise. (b,c) In the presence of exchange to an excited state, the echo formed at time point *a* is rotated. The relative sign of the orthogonal (o) and main (m) component matches the sign of the frequency separation between excited and ground state. Passive heteronuclear *J*-couplings involving either spin *I* or *S* are refocused. Passive homonuclear couplings introduce a scaling factor $\cos(\pi J_{S-S}2\tau)$ that identically affect the orthogonal and main component. Double antiphase terms $4I_zS_xS_z$ and $4I_zS_xS_y$, generated by homonuclear coupling J_{S-S}, are removed by the *z*-filter. Cross-correlation effects are suppressed by the 180° pulses on spin *I*.

We have applied the approach outlined above to determine the sign of the frequency difference for the amide proton ^{1}H$_N$ ($\Delta\omega_{HN} = \omega_{HN,e} - \omega_{HN,g}$) and the backbone ^{15}N nuclei ($\Delta\omega_N = \omega_{N,e} - \omega_{N,g}$). CEESY experiments for ^{1}H$_N$ and ^{15}N were tested on the 12 kDa PAH2 protein domain, for which fast-to-intermediate conformational exchange has been observed between a structured form and a low-populated, partially unfolded state.[6] The CEESY building block was inserted in a regular sensitivity enhanced HSQC (pulse sequences and experimental parameters shown in Supporting Information) and for both the main and the orthogonal component a 2D ^{15}N,^{1}H correlation spectrum was recorded.

Positive and negative peaks were found in the 2D spectrum of the orthogonal component for both ^{1}H$_N$ and ^{15}N nuclear spins. Representative results are shown in Figure 2. Starting with the ^{1}H$_N$ CEESY, panel (a) shows the main component of the echo for residue L22 in three spectra recorded with increasing duration of the spin-echo delay τ. These 1D traces demonstrate the decay of signal intensity due to relaxation and exchange. In panel (b) the traces for orthogonal components of residues L22, D23 and Q98 are plotted. For L22 a negative signal is observed, whose magnitude increases with increasing spin-echo delay. Likewise, an increasing positive signal is seen for D23. For Q98, no signal is observed, implying negligible chemical exchange for the ^{1}H$_N$ nucleus of this residue. Panel (c) displays two typical results of the ^{15}N CEESY. A clear, negative signal is observed for L22, and a positive peak for D23.

As discussed above, opposite signs for the two components imply a smaller Larmor frequency in the excited state with respect to the ground state, while identical signs indicate a larger Larmor frequency in the excited state. To translate this into a chemical shift difference, one has to consider that the direction of the frequency axis runs in opposite directions for ^{1}H and ^{15}N as it depends on the sign of the gyromagnetic ratio.[7] Thus, the ^{1}H$_N$ and

10.1021/ja0568749 CCC: $33.50

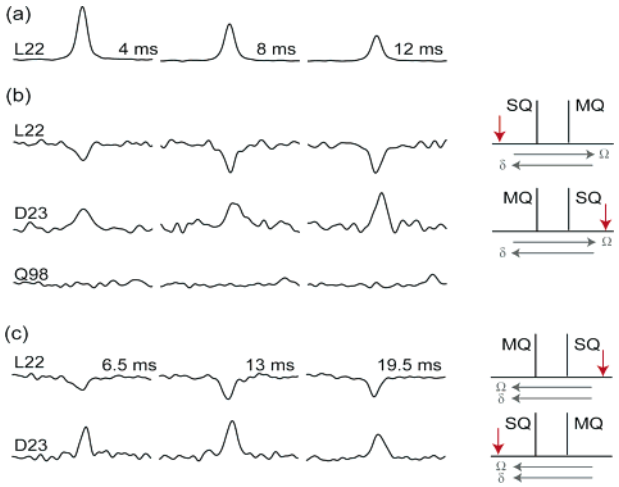

Figure 2. Representative results of the $^{1}H_N$ (a,b) and ^{15}N CEESY (c), showing slices through the peak maximum along the acquisition axis of the 2D spectrum. (a) main component of L22 at three echo delay values (indicated above the traces). (b) Orthogonal component of L22 (decreased $\omega_{HN,e}$), D23 (increased $\omega_{HN,e}$) and Q98 (no exchange). (c) Orthogonal components of L22 (decreased $\omega_{N,e}$) and D23 (increased $\omega_{N,e}$). Stick spectra on the right indicate the corresponding relative positions of ground state SQ- and MQ-peaks and the excited-state resonance (red arrow). Directions of the frequency and chemical shift axis are shown with gray arrows. Data acquired on 0.9 mM uniformly $^{13}C/^{15}N$ labeled PAH2 sample at 11.7 T, 298 K and a room-temperature probe head.

^{15}N resonances of L22 (δ_{HN} 7.3 ppm; δ_N 126.0 ppm) are shifted to higher and, respectively, lower ppm values in the excited state. The opposite behavior was found for D23 (δ_{HN} 8.7 ppm; δ_N 113.1 ppm), with $^{1}H_N$ and ^{15}N resonances shifted to respectively lower and higher ppm values in the excited state. For both residues these results indicate a resonance shift toward the random coil values, consistent with a less structured excited state.[6]

The sign of $\Delta\omega_{HN}$ and $\Delta\omega_N$ was classified using the 2D spectrum of the orthogonal component recorded with the longest spin-echo delay (i.e., τ is 12 ms for $^{1}H_N$ and 19.5 ms for ^{15}N). Taking a conservative approach, only those signals were considered for which the spin-echo was rotated by more than 1° and for which the intensity, $|I_{ortho}|$, exceeded 3 times the noise level. The maximum rotation observed, defined as $\arctan(I_{ortho}/I_{main})$, was $-5 \pm 1°$ for the $^{1}H_N$ CEESY and $+19 \pm 2°$ for the ^{15}N CEESY. Out of 72 nonoverlapping resonances, 15 $\Delta\omega_{HN}$ and 34 $\Delta\omega_N$ signs could be classified. The latter accounts for nearly all residues with significant dispersion of their ^{15}N transverse relaxation rates (Figure 3). Notably, even for the fast-relaxing amide proton a significant number of $\Delta\omega$ signs were determined. Interestingly, $\Delta\omega_N$ and $\Delta\omega_{HN}$ have opposite signs for 10 out of 13 possible comparisons.

To validate the CEESY method, the sign of $\Delta\omega_N$ was also determined using the H(S/M)QC approach, as proposed by Skrynnikov et al.[4] A triplicate set of interleaved HSQC and HMQC spectra was recorded and using a threshold of 0.3 Hz,[4] 16 signs could be classified, which were found in perfect agreement with the outcome of the ^{15}N CEESY experiment (Figure 3). Only 9 out of these 16 residues showed differences in ^{15}N peak position beyond 1 Hz, with a maximum of 3.2 Hz and an average of 1.1 Hz (taking absolute values). These small differences in peak position are related to the low population (ca. 1%) of the excited state.[6] Since the sensitivity of the CEESY experiment is primarily limited by signal-to-noise and spectral analysis is less hampered by signal overlap, a more complete classification of the sign of the shift difference $\Delta\omega$ was possible, even in this case of extremely skewed populations.

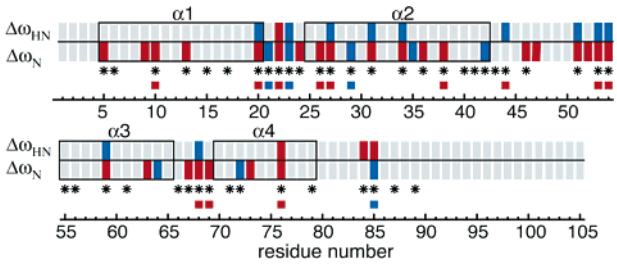

Figure 3. Resulting signs (red: negative; blue: positive) of $\Delta\omega_{HN}$ (top row) and $\Delta\omega_N$ (bottom row) between ground and excited state. Boxes indicate helices. Stars indicate residues with significant dispersion of $^{15}N-R_2$ rates.[6] Signs of $\Delta\omega_N$ from the H(S/M)QC method are shown with squares.

In conclusion, we propose a new method to determine the position of unobservable excited-state resonances relative to the observed resonances using a single 2D spectrum. The obtained information is crucial for the structural characterization of excited states. Our CEESY experiment can be applied to proteins, nucleic acids, or any system in fast-to-intermediate two-site exchange. Here, we have reported the experimental determination of the sign of $\Delta\omega$ of the backbone nitrogen and, for the first time, of the amide proton. Importantly, this approach can be extended to different nuclear species. We anticipate that application to other nuclei, such as the C_α and C_β of proteins, will provide spectroscopic information to characterize the structure of unobservable protein conformations in more detail. Ultimately, combination of relaxation−dispersion and CEESY experiments applied to multiple backbone nuclei will allow main-chain structure determination of these excited states.

Acknowledgment. We thank Prof. Dr. Cees W. Hilbers for his interest, and NWO for financial support (Grant JC 99-03).

Supporting Information Available: Pulse sequences (Varian pulse sequence available upon request) and detailed results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Mulder, F. A.; Mittermaier, A.; Hon, B.; Dahlquist, F. W.; Kay, L. E. *Nat. Struct. Biol.* **2001**, *8*, 932−935. (b) Volkman, B. F.; Lipson, D.; Wemmer, D. E.; Kern, D. *Science* **2001**, *291*, 2429−2433. (c) Eisenmesser, E. Z.; Bosco, D. A.; Akke, M.; Kern, D. *Science* **2002**, *295*, 1520−1523. (d) Korzhnev, D. M.; Salvatella, X.; Vendruscolo, M.; Di Nardo, A. A.; Davidson, A. R.; Dobson, C. M.; Kay, L. E. *Nature*, **2004**, *430*, 586−590. (e) Dyson, H. J.; Wright, P. E. *Methods Enzymol.* **2005**, *394*, 299−321.

(2) (a) Palmer, A. G.; Kroenke, C. D.; Loria, J. P. *Methods Enzymol.* **2001**, *339*, 204−238. (b) Akke, M. *Curr. Opin. Struct. Biol.* **2002**, *12*, 642−647. (c) Palmer, A. G.; Grey, M. J.; Wang, C. *Methods Enzymol.* **2005**, *394*, 430−465. (d) Ishima, R.; Torchia, D. A. *J. Biomol. NMR* **2003**, *25*, 243−248. (e) Ishima, R.; Baber, J.; Louis, J. M.; Torchia, D. A. *J. Biomol. NMR* **2004**, *29*, 187−198. (f) Lundström, P.; Akke, M. *ChemBioChem*, **2005**, *6*, 1685−1692.

(3) (a) McElheny, D.; Schnell, J. R.; Lansing, J. C.; Dyson, H. J.; Wright, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5032−5037. (b) Beach, H.; Cole, R.; Gill, M. L.; Loria, J. P. *J. Am. Chem. Soc.* **2005**, *127*, 9167−9176. (c) Grey, M. J.; Wang, C.; Palmer, A. G. *J. Am. Chem. Soc.* **2003**, *125*, 14324−14335. (d) Eissenmesser, E. Z.; Millet, O.; Labeikovsky, W.; Korzhnev, D. M.; Wolf-Watz, M.; Bosco, D. A.; Skalicky, J. J.; Kay, L. E.; Kern, D. *Nature* **2005**, *438*, 117−121.

(4) Skrynnikov, N. R.; Dahlquist, F. W.; Kay, L. E. *J. Am. Chem. Soc.* **2002**, *124*, 12352−12360.

(5) (a) Trott, O.; Palmer, A. G. **2002**, *J. Magn. Res. 154*, 157−160. (b) Korzhnev, D. M.; Orekhov, V. Y.; Dahlquist, F. W.; Kay, L. E. *J. Biomol. NMR* **2003**, *26*, 39−48. (c) Korzhnev, D. M.; Orekhov, V. Y.; Kay, L. E. *J. Am. Chem. Soc.* **2005**, *127*, 713−721.

(6) van Ingen, H.; Baltussen, M. A. H.; Aelen, J. M. A.; Vuister, G. W. *J. Mol. Biol.* **2006**, in press.

(7) Levitt, M. H. *J. Magn. Reson.* **1997**, *126*, 164−182.

JA0568749