J|A|C|S COMMUNICATIONS

Published on Web 06/28/2010

A Heteronuclear Zero Quantum Coherence N_z-Exchange Experiment That Resolves Resonance Overlap and Its Application To Measure the Rates of Heme Binding to the IsdC Protein

Scott A. Robson, Robert Peterson, Louis-S Bouchard, Valerie A. Villareal, and Robert T. Clubb*

University of California, Los Angeles, Department of Chemistry and Biochemistry, UCLA-DOE Institute for Genomics and Proteomics and the California NanoSystems Institute, UCLA, 611 Charles E. Young Drive, Los Angeles, California 90095

Received March 2, 2010; E-mail: rclubb@mbi.ucla.edu

Abstract: Chemical exchange phenomena in NMR spectra can be quantitatively interpreted to measure the rates of ligand binding, as well as conformational and chemical rearrangements. In macromolecules, processes that occur slowly on the chemical shift time scale are frequently studied using 2D heteronuclear ZZ or N_z-exchange spectroscopy. However, to successfully apply this method, peaks arising from each exchanging species must have unique chemical shifts in both dimensions, a condition that is often not satisfied in protein−ligand binding equilibria for ^{15}N nuclei. To overcome the problem of ^{15}N chemical shift degeneracy we developed a heteronuclear zero-quantum (and double-quantum) coherence N_z-exchange experiment that resolves ^{15}N chemical shift degeneracy in the indirect dimension. We demonstrate the utility of this new experiment by measuring the heme binding kinetics of the IsdC protein from *Staphylococcus aureus*. Because of peak overlap, we could not reliably analyze binding kinetics using conventional methods. However, our new experiment resulted in six well-resolved systems that yielded interpretable data. We measured a relatively slow k_{off} rate of heme from IsdC (<10 s^{-1}), which we interpret as necessary so heme loaded IsdC has time to encounter downstream binding partners to which it passes the heme. The utility of using this new exchange experiment can be easily expanded to ^{13}C nuclei. We expect our heteronuclear zero-quantum coherence N_z-exchange experiment will expand the usefulness of exchange spectroscopy to slow chemical exchange events that involve ligand binding.

NMR spectroscopy is a powerful method to investigate macromolecular motions that mediate important processes such as ligand binding, enzyme catalysis, and folding. These motions typically occur on the microsecond to millisecond time scales and are manifested in the NMR spectra as chemical exchange phenomena.[1,2] For a two-site exchange process that is in slow exchange, the effective rate constant of interconversion is significantly smaller than the chemical shift difference between the two sites. In this situation, distinct chemical shifts are observed for each species if they are sufficiently populated and experience magnetically inequivalent environments. Slow exchange kinetics in proteins were initially measured using 1D and 2D homonuclear methods that suffered from resonance overlap.[3] This problem was partially alleviated by Montelione and Wagner[4] who developed a 2D I_zS_z exchange heteronuclear correlation experiment that resolves the exchange data using the chemical shift of a heteronucleus. Subsequently, Kay and colleagues developed a 2D N_z-exchange experiment with improved sensitivity and the ability to simultaneously measure chemical exchange and ^{15}N longitudinal relaxation rates.[5] To quantify the kinetics of exchange using this experiment *both* the ^1H_N and ^{15}N nuclei of each interchanging species must have distinct chemical shifts to generate exchange and auto peaks (Figure 1A). However, this condition is often not satisfied as the ^{15}N chemical shifts of the backbone amide atoms of each exchanging species may be degenerate because the relatively small gyromagnetic ratio of the ^{15}N nucleus makes its chemical shift less sensitive to changes in its magnetic environment (Figure 1B). This problem is particularly acute for ligand binding equilibria, as ligand binding often does not significantly alter the structure of the protein and thus the chemical shift of the ^{15}N nucleus is unperturbed.

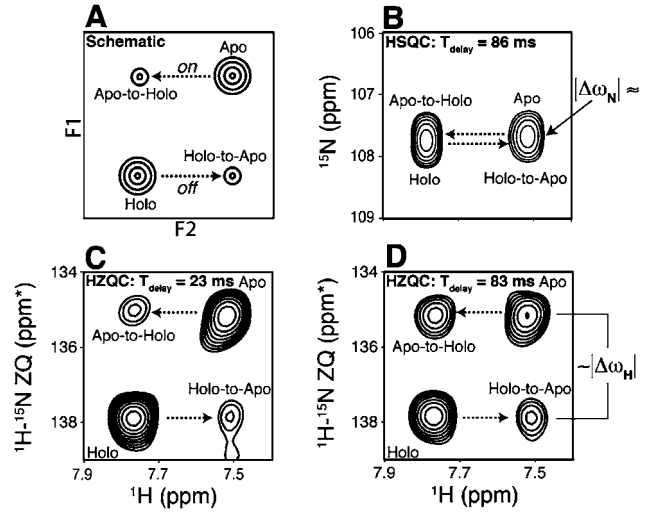

Figure 1. (A) Schematic showing the idealized N_z-exchange spectrum.[5] Resolved auto and exchange peaks are generated when the apo and holo forms of a protein are in slow exchange and they have nondegenerate chemical shifts in F_1 and F_2. (B) Conventional 2D N_z-exchange spectrum showing data from the backbone amide of G80 in the [^{15}N]IsdC-heme complex ($T_{delay} = 86$ ms). Exchange cross peaks are not resolved because the apo and holo forms have similar ^{15}N chemical shifts. Panels (C) and (D) show the ^{1}H−^{15}N HZQC N_z-exchange spectrum of G80 recorded using T_{delay} values of 23 and 83 ms, respectively. Exchange cross peaks are resolved by $\sim|\Delta\omega_H|$ in the F_1 dimension yielding interpretable data.

We encountered this problem in our studies of the IsdC protein from the bacterial pathogen *Staphylococcus aureus*. IsdC is a key component of the iron regulated surface determinant (Isd) system that captures heme from hemoglobin during infections.[6] In this relay system IsdC captures heme from upstream surface exposed receptors and then transfers it to the IsdDEF complex that imports heme into the cytoplasm.[7−10] In an effort to understand how IsdC captures and releases heme during the cell wall transfer process, we initially used the 2D N_z-exchange heteronuclear experiment to study [^{15}N]IsdC, 50% saturated with heme. Although the heme binding reaction is in slow exchange on the chemical shift time scale, only the backbone amide of K131 exhibited sufficiently distinct ^{15}N chemical shifts in the apo

10.1021/ja1017865

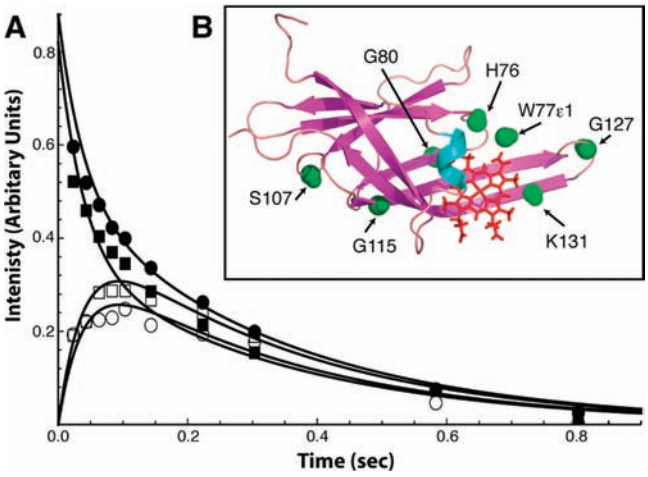

Figure 2. (A) G80 peak intensities for apo (■), holo (●) auto peaks, apo-to-holo (□) and holo-to-apo (○) exchange peaks. Solid lines are fits of the data used to extract the rate constants.[5] (B) Structure of IsdC (ribbon diagram) with the heme molecule indicated in red. Amides used to quantify the exchange kinetics using the HZQC N_z-exchange experiment are shown as green spheres and labeled. Only K131 could be measured using the conventional N_z-exchange experiment.

and holo forms of protein. However the K131 cross peaks were weak in intensity and thus not reliably quantified (Figure S1A). Thus, overlap in the 2D N_z-exchange spectrum presented a serious obstacle in our effort to assess the kinetics of heme binding.

To circumvent the problem of ^{15}N chemical shift degeneracy we developed a heteronuclear zero quantum coherence (HZQC) N_z-exchange experiment (Figure S2). This experiment is similar to the conventional 2D N_z-exchange experiment; however it evolves zero quantum ^1H and ^{15}N coherence in the indirect (F_1) dimension instead of single quantum ^{15}N. Peaks in the indirect dimension of the HZQC spectrum resonate at $\omega_H-\omega_N$, where ω_H and ω_N are the frequency offsets of the ^1H and ^{15}N atoms relative to the corresponding carrier frequency, respectively.

This is advantageous when the ^{15}N chemical shifts of the amide atom in the two exchanging species are irresolvable. This is because the auto peaks in the HZQC N_z-spectrum are displaced from one another by $|\Delta(\omega_H - \omega_N)|$ in F_1, enabling observation of the exchange peaks, even when $\Delta\omega_N \approx 0$ (Figure 1C−D). Application of the experiment to the [^{15}N]IsdC-heme complex enables exchange data from five residues to be quantified that were previously recalcitrant to analysis using the conventional 2D N_z-exchange experiment (Figure S1B). Notably, simple modification of the pulse scheme also enables the recording of a heteronuclear double quantum coherence (HDQC) N_z-exchange spectrum in which the chemical shifts of the cross peaks in the indirect dimension are positioned at $\omega_H + \omega_N$ (Figures S2,S3). The auto peaks in each exchanging pair in the HDQC spectrum are displaced from one another by $|\Delta(\omega_H + \omega_N)|$. Since the HZQC displacement between exchanging species does not equal the HDQC displacement ($|\Delta(\omega_H - \omega_N)|$ versus $|\Delta(\omega_H + \omega_N)|$), both HZQC and HDQC versions should be performed when attempting to resolve degenerate frequencies in F_1 because overlapped signals in a HZQC spectrum may not be overlapped in the HDQC spectrum and *vice versa*. For our system, the HZQC N_z-exchange spectrum exhibited the least amount of overlap and was used to quantify the exchange kinetics of heme binding to IsdC.

Figure 2A shows data for G80 in the [^{15}N]IsdC-heme complex and illustrates that the HZQC N_z-exchange experiment can be used to study slow exchange. In a conventional 2D N_z-exchange heteronuclear spectrum of the 50% saturated IsdC protein, the ^{15}N frequencies of G80 in the apo (upfield in ^1H) and holo (downfield in ^1H) forms of the protein are degenerate, precluding the observation of exchange cross peaks at all mixing times (Figure 1B). However, in the HZQC N_z-exchange spectrum the auto peaks have nondegenerate shifts, enabling the observation of exchange crosspeaks when a variety of mixing times are employed (Figure 1C−D). A plot of crosspeak intensities for G80 versus mixing time yields the expected dependence (Figure 2A). Simultaneous fitting of the data yields effective on ($k_{on\text{-}NMR}$) and off (k_{off}) rates of heme binding to IsdC of 7.8 s^{-1} and 7.0 s^{-1}, respectively. Importantly, employment of the HZQC N_z-exchange experiment enabled the exchange behavior of several other amides that surround the heme binding site to be quantified, further verifying the binding kinetics (Figure 2B, Figure S4, and Table S1). The average values obtained from these data are 6.2 ± 1.8 s^{-1} and 5.1 ± 1.5 s^{-1} for the $k_{on\text{-}NMR}$ and k_{off} values, respectively. Based on our k_{off} value of 5.1 s^{-1} and our previously determined K_D for heme:IsdC binding of ~0.34 μM,[10] we calculate an intrinsic k_{on} rate of $\sim 1.5 \times 10^7$ s^{-1} M^{-1}. Our determined $k_{on\text{-}NMR}$ value of 6.2 s^{-1} is a pseudo-first-order rate constant and is equal to [heme]*k_{on}, where [heme] is the concentration of free heme in solution. Thus we calculate a free heme concentration of ~0.4 μM, which is in agreement with previously published data on the solubility of heme under our NMR conditions.[11] The slow release kinetics presumably affords IsdC sufficient time to encounter the downstream IsdDEF transporter which it loads with heme via a specific protein−protein complex.[12,13] Furthermore, the k_{on} rate suggests that heme binding to IsdC is rapid and close to diffusion limiting. Similar binding kinetics are probably exhibited by other gram-positive pathogens that capture heme using IsdC homologues.

In summary, we have shown that zero and double quantum coherence exchange spectra effectively resolve ^{15}N degeneracy enabling exchange kinetics to be quantified. This method is widely applicable and can also be used for resolving degenerate ^{13}C signals.

Acknowledgment. We thank Arthur Pardi for providing example Mathematica files for curve fitting of the exchange equations. This work was supported by NIH Grant AI52217 to R.T.C. and NIH Training Grant F31GM075564 to V.A.V.

Supporting Information Available: Further NMR spectra, details of the pulse sequences and other methods used are contained within the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Mittermaier, A.; Kay, L. E. *Science* **2006**, *312*, 224–8.
(2) Wang, C.; Palmer, A. G., III. *Magn. Reson. Chem.* **2003**, *41*, 866–876.
(3) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546–4553.
(4) Montelione, G.; Wagner, G. *J. Am. Chem. Soc.* **1989**, *111*, 3096–3098.
(5) Farrow, N.; Zhang, O.; Forman-Kay, J.; Kay, L. *J. Biomol. NMR* **1994**, *4*, 727–734.
(6) Grigg, J. C.; Ukpabi, G.; Gaudin, C. F. M.; Murphy, M. E. P. *J. Inorg. Biochem.* **2010**, *104*, 341–348.
(7) Pilpa, R. M.; Robson, S. A.; Villareal, V. A.; Wong, M. L.; Phillips, M.; Clubb, R. T. *J. Biol. Chem.* **2009**, *284*, 1166–76.
(8) Muryoi, N.; Tiedemann, M. T.; Pluym, M.; Cheung, J.; Heinrichs, D. E.; Stillman, M. J. *J. Biol. Chem.* **2008**, *283*, 28125–36.
(9) Liu, M.; Tanaka, W. N.; Zhu, H.; Xie, G.; Dooley, D. M.; Lei, B. *J. Biol. Chem.* **2008**, *283*, 6668–76.
(10) Villareal, V. A.; Pilpa, R. M.; Robson, S. A.; Fadeev, E. A.; Clubb, R. T. *J. Biol. Chem.* **2008**, *283*, 31591–600.
(11) Lebrun, F.; Bazus, A.; Dhulster, P.; Guillochon, D. *J. Agric. Food Chem.* **1998**, *46*, 5017–5025.
(12) Maresso, A.; Schneewind, O. *BioMetals* **2006**, *19*, 193–203.
(13) Grigg, J. C.; Vermeiren, C. L.; Heinrichs, D. E.; Murphy, M. E. P. *J. Biol. Chem.* **2007**, *282*, 28815–22.

JA1017865