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The Assignment of Downfield Proton Resonances in an Enzyme Inhibitor Complex Using Time-Dependent Saturation Transferred NOEs

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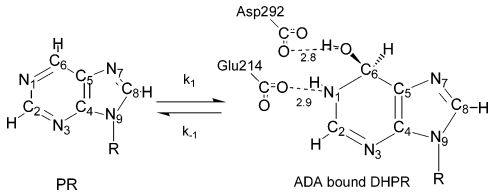
When an inhibitor binds to an enzyme, new downfield resonances (>11 ppm) often appear in the proton NMR spectrum. These resonances usually belong to the protons of NH and OH groups that form strong hydrogen bonds upon binding. Typically, the assignment of these resonances is important for understanding binding and/or mechanism. The assignment of NH proton resonances can be achieved via isotopic labeling of either the enzyme or the inhibitor with ^{15}N and use of a $^{1}H-^{15}N$ correlation NMR experiment such as HMQC.[1−3] Assignment is more difficult for OH proton resonances, and one often needs to rely on the sequential assignments of most protein resonances[4] or elimination of all other possibilities.[2]

Here, we show that when these well-resolved resonances cannot be assigned by transient NOE methods due to their fast relaxation rates, it may be possible to achieve the assignments, under favorable conditions, using a time-dependent saturation transferred NOE (STNOE) experiment. STNOE is based on the principles of truncated driven NOE and the saturation/exchange transferred NOE to unbound ligand.[5] It is expected to work with complexes in which the dissociation constant is in the submillimolar to submicromolar range, typical for ligand binding to enzymes. The implementation of the STNOE measurement is similar to recently published STD studies,[6] except that just one proton resonance is selected for saturation, and the saturation period is limited to the initial NOE build-up phase. By selectively saturating one of the downfield proton resonances observed in the protein−ligand complex, an NOE may be observed on one or more of the free ligand protons through relaxation between the bound ligand and protein protons within the complex followed by exchange between bound and free ligands. This type of NOE should occur when the proton of the bound ligand is spatially close to the proton being saturated, or via spin diffusion when the saturation time is long enough. The assumption is made that the closer the bound ligand proton is relative to the proton being saturated, the faster the STNOE buildup will be on this proton in the free ligand. Thus, by monitoring the NOE buildup of the proton resonance of the free ligand versus the saturation time, one can determine the proximity of this proton to that being saturated. Our relaxation and exchange matrix analyses (to be published) indicate that when a short internal reference is available, the downfield proton resonance may be unambiguously assigned.

We employ this approach on adenosine deaminase (ADA) to investigate how the enzyme brings about its catalytic rate enhancement. ADA catalyzes the hydrolysis of adenosine or deoxyadenosine to their respective inosine product and ammonia. Purine ribonucleoside (PR) is a competitive inhibitor of ADA, with an apparent inhibition constant of 2.8 μM.[7] Upon binding to ADA, PR is hydrated at N1 and C6 to yield 6-hydroxyl-1,6-dihydropurine

Scheme 1



(DHPR), which is believed to resemble the enzyme reaction intermediate (Scheme 1).[8] Cleary, strong hydrogen bonds to N1H and O6H would stabilize this structure and promote catalysis. Crystallographic studies show that the active site Zn^{2+} coordinates with O6 and hydrogen bonds on N1H and O6H of the bound DHPR are formed with the active site carboxyl groups.[9] Although the crystallographic distance between N1 and oxygen of Glu214 is not very short, 2.9 Å,[9] indicating only a modest hydrogen bond, Raman studies suggest that N1H of DHPR must be strongly hydrogen bonded in the complex.[10] Thus, assignments of the N1H and O6H resonances in the complex will be helpful to characterize these hydrogen bonds. Of the five resolved resonances in the downfield region >12 ppm, at 14.9, 14.2, 13.7, 13.3, and 12.9 ppm (inset, Figure 1), we show below that the resonances at 14.9 and 13.3 ppm belong, respectively, to the O6H and N1H protons of DHPR complexed with ADA, thus confirming that the enzyme does indeed supply important and strong hydrogen bonds to these key protons.

STNOE measurements were conducted on the downfield resonances at 14.9, 14.2, 13.7, 13.3, 12.9, and 11.9 ppm. The STNOE buildup of CH resonances of free PR, located at 8.8, 8.6, and 8.4 ppm from C6H, C2H, and C8H protons, respectively, in the ADA plus excess PR mixture upon saturation of the resonance at 14.9 ppm (indicated by the arrow in the inset) is shown in Figure 1A. The STNOE on the C6H resonance appears at a saturation time of ∼150 ms and increases as the saturation time increases. The STNOE on the C2H appears at ∼500 ms, suggesting that it results from spin diffusion, possibly through the C6H/N1H protons. No STNOE is observed on the C8H resonance with saturation times up to ∼1 s. These results establish that the 14.9 ppm resonance belongs to a proton that is closer to the C6H of bound DHPR in the complex than to the C2H proton and still further away from the C8H proton.

The STNOE buildup of the free PR CH resonances upon saturation of the resonance at 13.3 ppm is shown in Figure 1B. The STNOEs on both the C6H and the C2H resonances appear at about the same time, ∼350 ms, and increase at the same rate as the saturation time on the 13.3 ppm resonance increases. On the other hand, the STNOE on the C8H appears at ∼700 ms. Hence, the 13.3 ppm resonance likely belongs to a proton that is about equidistance from the C6H and C2H protons of bound DHPR. Furthermore, the results shown in Figure 1A and B also suggest that the proton with resonance at 14.9 ppm is closer to the C6H of

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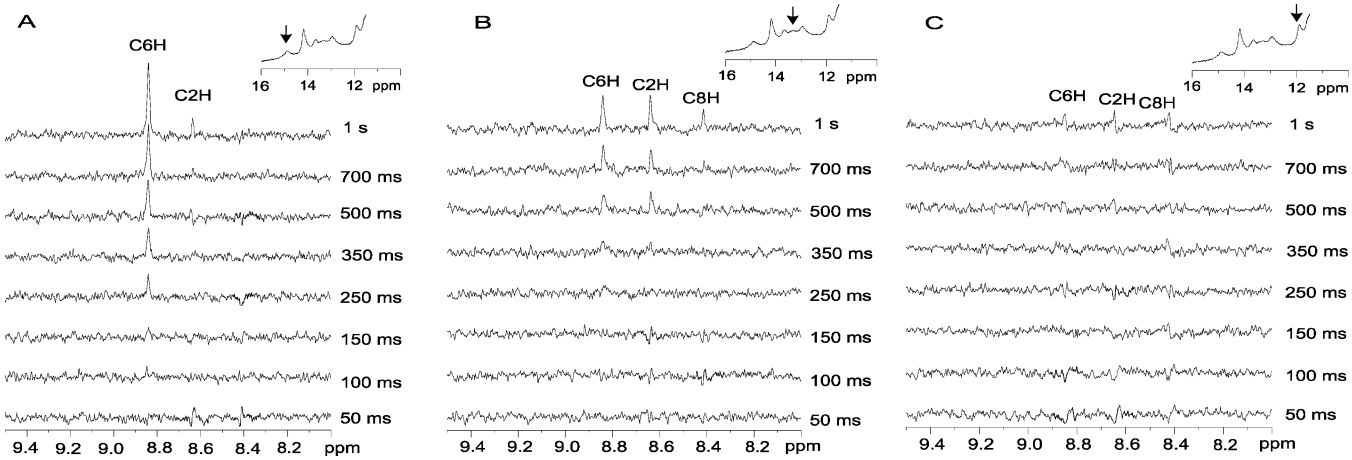

Figure 1. STNOE build-up experiments with the calf intestine ADA−PR mixture (0.3 mM:3 mM) in 5 mM tris-d_{11}, 0.1 M NaCl, pH 7.0 (5% D_2O) at 10 °C. (A) In the pulse sequence, a selective Gaussian shaped inversion pulse (25 ms) is applied repeatedly on or off the resonance at 14.9 ppm (see arrow in inset, in which the jump-return spectrum of the complex is shown) in alternative runs for a specified saturation time, and then a spin lock pulse of 10 ms duration and 4 kHz field strength is applied, followed by the observation pulse. The difference is achieved by a 180° shift of the receiver phase in alternative runs. A DPFGSE pulse sequence is used for water suppression.[11] The acquisition time was set to 0.92 s, and the recycle delay was 2.5 s. The data were sampled with 16k points with 512 scans for each spectrum. (B) The selective pulse was set on resonance at 13.3 ppm (see arrow in inset). (C) The selective pulse was set on resonance at 11.9 ppm (see arrow in inset). All experiments are conducted on a Bruker Avance DRX300 spectrometer.

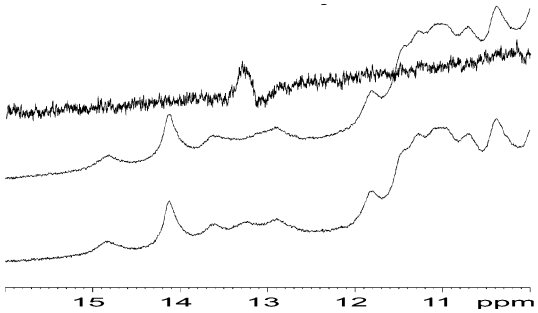

Figure 2. Jump-return proton spectra of the ADA−[^{15}N1]PR mixture (0.5 mM:2 mM) with (bottom) or without (middle) ^{15}N decoupling during acquisition. The top spectrum is the difference spectrum obtained by positioning the carrier of the ^{15}N decoupling pulse in and out of the ^{15}N spectral region and with a 180° shift of the receiver phase in alternative runs. Other sample conditions were the same as those in Figure 1, except the temperature was 5 °C.

DHPR in the complex than the proton with resonance at 13.3 ppm because the STNOE on C6H appears about 200 ms earlier when the resonance at 14.9 ppm is saturated than when the resonance at 13.3 ppm is saturated.

Upon saturation of the resonance at 11.9 ppm, no STNOE is observed on any resonance of the free PR until the saturation time is about 1 s (Figure 1C). Such observations indicate that the resonance at 11.9 ppm belongs to a proton that is far away from the bound PR base in the complex. No significant STNOE is observed on C6H or C2H upon saturation of the resonances at 14.2, 13.7, and 12.9 ppm, suggesting that these resonances belong to protons not close to C2H or C6H of bound DHPR in the complex (data not shown).

The above experiments demonstrate that the time-dependent STNOE can be used to establish the relative proximity of the protons that show resolved resonances in the downfield region to the carbon protons of the bound inhibitor and potentially useful for the assignments of these resonances once an internal distance reference can be obtained. One way to obtain this distance reference is by using an ^{15}N labeled compound.

Figure 2 shows the downfield region of the proton spectrum of the ADA−[^{15}N1]PR mixture. The bottom and the middle spectrum were obtained with and without ^{15}N decoupling during acquisition, respectively. The top spectrum is a difference spectrum obtained with and without ^{15}N decoupling in alternative runs. The results clearly show a ^{1}H−^{15}N coupling between ^{15}N1 and the 13.3 ppm proton resonance. Hence, the 13.3 ppm resonance can be assigned to the N1H of the bound DHPR in the complex. The fact that the 13.3 ppm resonance belongs to N1H indicates that the relative late appearance of the STNOE on the C2H and C6H resonances of the free PR upon saturation of the 13.3 ppm resonance is mostly due to the relatively slow exchange between free PR and bound DHPR. In addition, it also suggests that when the selective saturation is limited to just one isolated resonance, the effect of the spin diffusion is negligible until the saturation time is longer than about 0.5 s under our experimental conditions. Because the time-dependent STNOE results have shown that the resonance at 14.9 ppm belongs to a proton closer to C6H than N1H in the ADA−DHPR complex, it can be assigned to the O6H proton of bound DHPR.

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References

(1) Halkides, C. J.; Wu, Y. Q.; Murray, C. J. *Biochemistry* **1996**, *35*, 5, 15941−15948.
(2) Harris, T. K.; Abeygunawardana, C.; Mildvan, A. S. *Biochemistry* **1997**, *36*, 14661−14675.
(3) Li, C. M.; Tyler, P. C.; Furneaux, R. H.; Kicska, G.; Xu, Y.; Grubmeyer, C.; Girvin, M. E.; Schramm, V. L. *Nat. Struct. Biol.* **1999**, *6*, 582−587.
(4) La Mar, G. N.; Asokan, A.; Espiritu, B.; Yeh, D. C.; Auclair, K.; Ortiz De Montellano, P. R. *J. Biol. Chem.* **2001**, *276*, 15676−15687.
(5) Balaram, P.; Bothner-B, A. A.; Breslow, E. *J. Am. Chem. Soc.* **1972**, *94*, 4017−4018. Wagner, G.; Wüthrich, K. *J. Magn. Reson.* **1979**, *33*, 675−680. Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402−417.
(6) Mayer, M.; Meyer, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 1784−1788. Haselhorst, T.; Espinosa, J. F.; Jimenez-Barbero, J.; Sokolowski, T.; Kosma, P.; Brade, H.; Brade, L.; Peters, T. *Biochemistry* **1999**, *38*, 6449−6459.
(7) Frieden, C.; Kurz, L. C.; Gilbert, H. R. *Biochemistry* **1980**, *19*, 5303−5309.
(8) Kurz, L. C.; Frieden, C. *Biochemistry* **1987**, *26*, 8450−8457.
(9) Kinoshita, T.; Nishio, N.; Nakanishi, I.; Sato, A.; Fujii, T. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2003**, *59*, 299−303.
(10) Deng, H.; Kurz, L. C.; Rudolph, F. B.; Callender, R. *Biochemistry* **1998**, *37*, 4968−4976.
(11) Hwang, T. L.; Shaka, A. J. *J. Magn. Reson., Ser. A* **1995**, *112*, 275−279.

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