Journal of the Korean Magnetic Resonance Society **2001**, *5*, 130-137

^{15}N NMR Relaxation Studies of Backbone Motion of the catalytic Residues in Free and Steroid-bound Δ ^{5}-3-Ketosteroid Isomerase

Sunggoo Yun and Hee Cheon Lee*

Department of Chemistry/Division of Molecular and Life Sciences
Pohang Universtity of Science and Technology, Pohang, 790-784, Korea
Received November 25, 2001

Abstract: Backbone dynamics of the catalytic residues in free and steroid-bound Δ ^{5}-3- ketosteroid isomerase from *Pseudomonas testosteroni* has been examined by ^{15}N relaxation measurements. The relaxation data were analyzed using the model-free formalism to extract the model-free parameters (S^2, τ_e, and R_{ex}). Tyr-14 and Asp-99 exhibit enhanced high-frequency (pico- to nanosecond) internal motions in the free enzyme, which are restricted upon ligand binding, while Asp-38 experiences severe restriction of the internal motions in the free enzyme, suggesting that Tyr-14 and Asp-99 are more actively involved in the ligand binding than Asp-38. The results also indicate that the H-bond network in the catalytic cavity might be slightly strengthened upon ligand binding, which may have some implications on the enzyme mechanism.

INTRODUCTION

Protein dynamics is a subject of great interest, because it can markedly influence the important aspects of protein functions, such as folding,[1,2] molecular recognition, stability, and enzyme action.[3] Especially, the specific intermolecular interactions between a ligand and the protein can alter the internal motions in a wide range of time scales. Heteronuclear NMR has been widely used to study such internal motions of proteins, because ^{13}C and ^{15}N NMR relaxation measurements provide unique experimental data for the side chain and backbone dynamics in a wide range of time scales from pico- to milliseconds.[4-13] For instance, the model-free analysis[14,15] of the relaxation data provides dynamical information of the internal motions on the pico- and nanosecond time-scale parameterized as the spatial restriction of the N-H (or C-H) vector orientation (S^2: generalized order parameter), the

*To whom : hcl@postech.ac.kr

effective correlation time of the internal motion (τ_e: pico- to nanosecond time scale) and rotational tumbling of the whole molecule (τ_m : nanosecond time scale), and the micro-environment change on the micro- to millisecond time scale (R_{ex}).

Ketosteroid isomerase (KSI) is a homodimeric enzyme with 125 amino acid residues per subunit which catalyzes the conversion of Δ^5-to Δ^4-3-ketosteroid via a dienolic intermediate with diffusion-controlled rate. This enzyme has a molecular mass of 26.8 kDa and consists of three α-helices and a six-strand mixed β-pleated sheet that contains three β-bulges. Three-dimensional structures in solution as well as those in crystal state have been identified by NMR and X-ray crystallography.[16,17] Fig. 1 shows that the structure of KSI is a conical closed barrel formed by a highly curved β-sheet and three α-helices. The α-helices are adjacent to each other and arranged in an antiparallel orientation. The narrow end of the structure is blocked, while the wide end is open to the bulk solvent forming an active site. A narrow and long patch of the β-sheet of each monomer forms a dimer interface, where the interactions are mostly between side chains and hydrophobic in nature.

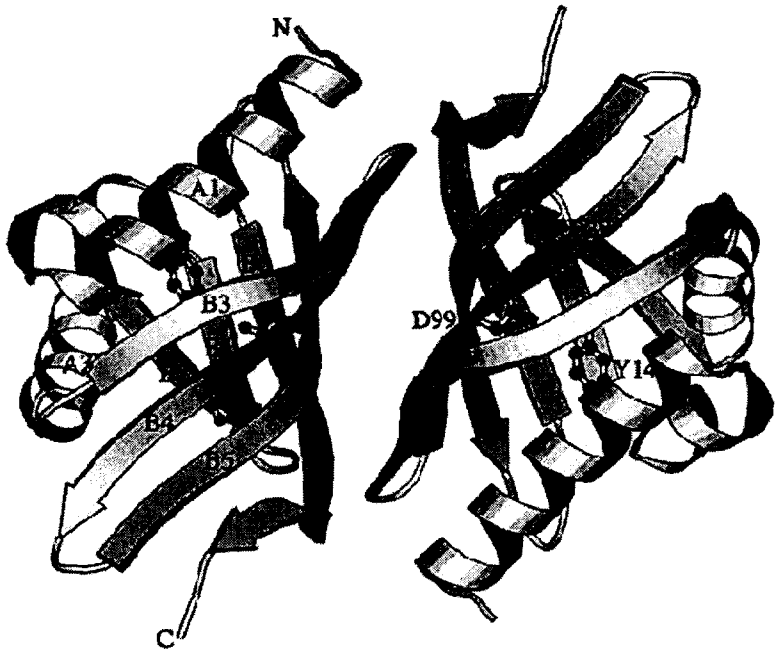

Fig. 1. Dimeric structure of KSI viewed roughly along the molecular 2-fold axis. A and B correspond to α-helix and β-strand, respectively. The three major catalytic residues are shown in ball-and-stick model.

Zhao *et al.*[4] examined the side chain and backbone dynamics of the catalytic residue, Tyr-14, in a mutant KSI, Y55F/Y88F, by ^{13}C NMR relaxation measurement in the presence and absence of a steroid ligand, 19-nortestosterone hemisuccinate (19-NTHS). The results showed that the high-frequency (pico- to nanosecond) motion of the phenolic side chain C_ε became more restricted than that of the backbone C_α upon binding of 19-NTHS, while the ligand binding decreased the low-frequency (micro- to millisecond) motion of C_α, but produced no change in that of C_ε. To further elucidate the motional changes in the active site of KSI upon ligand binding, the backbone dynamics of the key catalytic residues in free KSI and its complex with 19-NTHS was examined by ^1H-detected ^{15}N NMR relaxation measurements. Motional parameters (S^2, τ_e, and R_{ex}) were extracted from the relaxation data using the model-free formalism.[14,15]

EXPERIMENTAL PROCEDURES

Materials. 19-nortestosterone hemisuccinate obtained from Steraloids (Wilton, NH) showed a single spot on thin layer chromatography and were used without further purification. Buffer salts and DMSO-d_6 were from Sigma (St, Louis, MO). ^{15}N-labeled NH_4Cl was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Preparation of NMR Sample. NMR samples were prepared to contain 1.0 mM of uniformly ^{15}N labeled-protein in 10 mM potassium phosphate, 9 % (v/v) DMSO-d_6 and 91 % H_2O. ^{15}N labeled KSI were prepared as described previously.[13] The pH of the sample was adjusted to 7.0. In order to obtain the protein complexed with a steroid, 19-NTHS was added to the protein solution with a slightly excess amount of inhibitor relative to that of the protein.

NMR Measurements and Processing. All NMR data were collected at 27 ℃ on a Bruker DRX500 spectrometer (500.13 MHz for ^1H and 50.7 MHz for ^{15}N). The pulse sequences used to record ^{15}N T_1 and T_2 were those described by Barbato *et al.*[18] with a slight modification to include water flip-back[19] and WATERGATE [20] techniques for eliminating the water resonance. Decoupling of ^{15}N spins during acquisition was performed using WALTZ-16 composite pulse sequence with a field strength of 2 kHz. The T_1 and T_2 measurements were performed using total 60 transients per t_1 experiment. 128× 2048 complex points were acquired in the t_1× t_2 dimensions. The cross peak intensities were measured as peak volumes in order to increase sensitivity,[21] and T_1 and T_2 were obtained by non-linear fitting of single exponential decays to the experimental data. The error levels in T_1 and T_2 were estimated by a 500 Monte Carlo simulation.[22] T_2 measurements utilized a 100 μs delay between sequential ^{15}N pulses in the CPMG pulse train for attenuating the ^{15}N signal loss during a T_2 relaxation period. The field strength of the

refocusing pulses in the CPMG pulse sequence was 3.3 kHz. In order to suppress effects of cross-correlation between ^{1}H-^{15}N dipolar and ^{15}N CSA relaxation mechanism in the T_1 and T_2 experiments, WALTZ-16 and ^{1}H 180° pulses were applied during the recovering delays as described.[23-25] A 4 s relaxation delay was used between scans. The t_1 dimension was zero-filled to 256 real data points, and 90° phase-shifted sine bell window function was applied prior to Fourier transformation and baseline correction in both dimensions.

RESULTS AND DISCUSSION

The assignments of backbone amide ^{1}H and ^{15}N chemical shifts of free KSI and its complex with 19-NTHS were published elsewhere.[13] To obtain the correct model-free parameters S^2, τ_e, and R_{ex}, it is necessary to estimate an exact value for the overall rotational correlation time, τ_m, from the ^{15}N T_1/T_2 ratio because small amounts of rotational anisotropy may contribute to R_{ex}.[26] Ignoring rotational anisotropy can also lead to a distortion of the internal correlation time when the extended model-free formalism is required for an adequate fit.[27] Therefore, an anisotropic model for rotational diffusion is essential in the case of highly asymmetric or multidomain proteins.[27-29] Free KSI is highly asymmetric having a relative ratio of 1.00: 0.95: 0.55 for the principal components of the inertia tensor based on the solution structure.[16] It has also been shown[13] that the relaxation data for KSI are best described by an axially symmetric rotational diffusion tensor. Hence, the τ_m and $D_{\parallel}/D_{\perp}$ values[13] from the axially symmetric model were used to analyze the internal motions of free and steroid-bound KSI.

^{15}N relaxation parameters were analyzed with the model-free method[14,15] by using the program Modelfree v. 4.0.[22,30] The model-free parameters were selected by extensive Monte Carlo simulations and F-statistical testing, as described in the literature.[30] The models and the optimized parameters were (1) S^2, (2) S^2 and τ_e, (3) S^2 and R_{ex}, (4) S^2,τ_e and R_{ex} and (5) S_s^2, S_f^2, and τ_s, where S^2 is the square of the generalized order parameter characterizing the amplitude of the internal motions, τ_e is the effective correlation time for the internal motions, R_{ex} is the exchange contribution to T_2, and the subscripts f and s indicate fast and slow time scales, respectively. The difference between parallel and perpendicular components of the ^{15}N chemical shift tensor ($\sigma_{\parallel}-\sigma_{\perp}$) was taken to be -170 ppm,[31,32] and the value of 1.02 Å for the N-H bond length was used for the calculations. The spectral density functions used for calculations of the relaxation data in an axially symmetric diffusion case are given by [33-37]

$$J(\omega)=\frac{2}{5}\left[S^2\sum_{j=1}^{3}\frac{A_j\tau_j}{1+(\omega\tau_j)^2}\right] \quad (1)$$

$$J(\omega)=\frac{2}{5}\left[S^2\sum_{j=1}^{3}\frac{A_j\tau_j}{1+(\omega\tau_j')^2}+\frac{(1-S^2)\tau}{1+(\omega\tau)^2}\right] \quad (2)$$

$$J(\omega)=\frac{2}{5}\left[S^2\sum_{j=1}^{3}\frac{A_j\tau_j}{1+(\omega\tau_j)^2}+\frac{(S_f^2-S^2)\tau}{1+(\omega\tau)^2}\right] \quad (3)$$

where $\tau^{-1}=6D+\tau_e^{-1}$, $\tau_1^{-1}=6D_\perp$, $\tau_2^{-1}=5D_\perp+D_\parallel$, $\tau_3^{-1}=2D_\perp+4D_\parallel$, $A_1=(3\cos^2\theta-1)^2/4$, $A_2=3\sin^2\theta\cos^2\theta$, and $A_3=(3/4)\sin^4\theta$, and θ is the angle between the N-H bond vector and the principal axis of the diffusion tensor. D is the isotropic diffusion constant, $D_\parallel$ and $D_\perp$ are the components of the diffusion tensor parallel and perpendicular to the principal axis of the axial symmetry, respectively. The isotropic correaltion time, τ_m, can be newly defined and is related to the isotropic diffusion constant D by $\tau_m=(6D)^{-1}$. The parameter A_j, which reflects the orientation of the amide N-H vector with respect to the principal axis of the diffusion tensor, was estimated from the solution structure of free wild type KSI or Y55F/Y88F complexed with 19-NTHS as input structures. The equations (1), (2) and (3) were used for models 1, 2, and 5, respectively, and additional R_{ex} term was introduced to the equations (1) and (2) for models 3 and 4. In model 5 (eq. 3), $S^2=S_s^2S_f^2$.

Table 1 summarizes the model-free parameters of the three active site residues, Tyr-14, Asp-38 and Asp-99 for free and complexed KSI. The order parameters of Tyr-14 and Asp-99 increased upon ligand binding, indicating severe restriction of the high-frequency internal motions of the residues in the complexed KSI, while the order parameter of Asp-38 decreased significantly upon ligand binding. This result may suggest that Tyr-14 and Asp-99 are involved more actively in the ligand binding with relatively enhanced high-frequency (pico- to nanosecond) motions in the free enzyme and make strong interactions with the ligands in the complexed enzyme. On the other hand, all three residues exhibit no sizable R_{ex} contribution to the linewidth. This result is in sharp contrast with the results of ^{13}C NMR relaxation measurements for the active site residue, Tyr-14, of a mutant KSI, Y55F/Y88F by Zhao *et al.*[4], in which the 19-NTHS binding decreased the sizable R_{ex} term

Table 1. Model-free Parameters of the Active Site Residues in Free and Complexed KSI[a]

	Tyr-14		Asp-38		Asp-99	
	free	complexed	free	complexed	free	complexed
S^2	0.96±0.03	0.98±0.02	0.99±0.02	0.95±0.03	0.97±0.03	0.98±0.02
$R_{ex}(s^{-1})$	0	0	0	0	0	0

[a] The uncertainties in the parameters are the standard deviations from the simulations using the program Modelfree v. 4.0.

of the C_α of Tyr-14. In general, large R_{ex} terms indicate the existence of low-frequency motions associated with conformational and/or chemical exchange processes on time scales ranging from micro- to milliseconds.[38]

One of the most noticeable features of KSI active-site geometry is that the polar functional groups are maintained in the highly apolar site by forming a hydrogen-bond network. The carboxyl group of the catalytic residue Asp-99 is connected to the hydroxyl group of Tyr-14 via a water molecule, and in turn Tyr-14 is linked to Tyr-55, forming a H-bond network, Asp99-Wat504- Tyr14-Tyr55. Among the three catalytic residues, only Asp-38 is not involved in this H-bond network. Recent study on KSI revealed that this H-bond network in the active site is important for both stability and function of the enzyme.[39] As can be seen in Table 1, the order parameter of D38 decreased significantly, while those of Tyr-14 and Asp-99 increased. This result suggests that the H-bond network in the catalytic cavity might be slightly strengthened upon ligand binding, which might have some implications on the enzyme mechanism.

Acknowledgements

This work was supported by a grant from POSCO R & D (2001) and by KOSEF.

REFERENCES

1. B. Brutscher, B. Bruschweiler, and R. R. Ernst, *Biochemistry,* **36**, 13043 (1997).
2. N. A. Farrow, O. Zhang, J. D. Forman-Kay, and L. E. Kay, *Biochemistry,* **36**, 2390 (1997).
3. S. Mine, S. Tate, T. Ueda, M. Kainosho, and T. Imoto, *J. Mol. Biol.,* **286**, 1547 (1999).
4. L. K. Nicholson, L. E. Kay, D. M. Baldisseri, J. Arango, P. E. Young, A. Bax, and D. A. Torchia, *Biochemistry,* **31**, 5253 (1992).
5. Q. Zhao, C. Abeygunawardana., and A. S. Mildvan, *Biochemistry,* **35**, 1525 (1996).

6. J. T. Stivers, C. Abeygunawardana, A. S. Mildvan, and C. P. Whitman, *Biochemistry,* **35**, 16036 (1996).
7. M. E. Hodson, and D. P. Cistola, *Biochemistry,* **33**, 2278 (1997).
8. E. T. Olejniczak, M. M. Zhou, and S. W. Fesik, *Biochemistry,* **36**, 4118 (1997).
9. P. Yuan, V.P. Marshall, G. L. Petzold, R. A. Poorman, and B. J. Stockman, *J. Biomol. NMR,* **15**, 55 (1999).
10. N. A. Farrow, R. Muhandiram, A. U. Singer, S. M. Pascal, C. M. Kay, G. Gish, S. E. Shoelson, T. Pawson, J. D. Forman-Kay, and L. E. Kay, *Biochemistry,* **33**, 5984 (1994).
11. L. E. Kay, D. R. Muhandiram, N. A. Farrow, Y. Aubin, and J. D. Forman-Kay, *Biochemistry,* **35**, 361 (1996).
12. L. Yu, C. X. Zhu, Y. C. Tse-Dinh, and S. W. Fesik, *Biochemistry,* **35**, 9661 (1996).
13. S. Yun, D. S. Jang, D. H. Kim, K. Y. Choi, and H. C. Lee, *Biochemistry,* **40**, 3967 (2001).
14. G. Lipari, and A. Szabo, *J. Am. Chem. Soc.,* **104**, 4546 (1982).
15. G. Lipari, and A. Szabo, *J. Am. Chem. Soc.,* **104**, 4559 (1982).
16. Z. R. Wu, S. Ebrahimian, M. E. Zawrotny, L. D. Thornburg, G. C. Perez- Alvarado, P. Brothers, R. M. Pollack, and M. F. Summers, *Science,* **276**, 415 (1997).
17. H. S. Cho, G. Choi, K. Y. Choi, and B. –H. Oh, *Biochemistry,* **37**, 8325 (1998).
18. G. Barbato, M. Ikura, L. E. Kay, R. W. Pastor, A. Bax, *Biochemistry,* **31**, 5269 (1992).
19. S. Grzesiek, and A. Bax, *J. Am. Chem. Soc.,* **115**, 12594 (1993).
20. M. Piotto, M. Saudek, and V. Sklenar, *J. Biomol. NMR,* **2**, 661 (1992).
21. C. Rischel, J. C. Madsen, K. V. Anderson, and F. M. Poulsen, *Biochemistry,* **33**, 13997 (1994).
22. A. G., III, Palmer, M. Rance, and P. E. Wright, *J. Am. Chem. Soc.,* **113**, 4371 (1991).
23. J. Boyd, U. Hommel, and I. D. Campbell, *Chem. Phys. Lett.,* **175**, 477 (1992).
24. L. E. Kay, P. Keifer, and T. Saarinen, *J. Am. Chem. Soc.,* **114**, 10663 (1992).
25. A. G., III, Palmer, N. J. Skelton, W. J. Chazin, P. E. Wright, and M. Rance, *Mol. Phys.,* **75**, 699 (1992).
26. N. Tjandra, S. E. Feller, R. W. Paster, and A. Bax, *J. Am. Chem. Soc.,* **117**, 12562 (1995).
27. A. P. Hansen, A. M. Petros, R. P. Meadows, and S. W. Fesik, *Biochemistry,* **33**, 15418 (1994).
28. R. Bruschweiler, X. Liao, and P. E. Wright, *Science,* **268**, 886 (1995).
29. J. P. Mackay, G. L. Shaw, and G. F. King, *Biochemistry,* **35**, 4867 (1996).
30. A. M. Mandel, M. Akke, and A. G., III, Palmer, *J. Mol. Biol.,* **246**, 144 (1995).
31. D. Fushaman, and D. Cowburn, *J. Am. Chem. Soc.,* **120**, 7109 (1998).
32. N. Tjandra, A. Szabo, and A. Bax, *J. Am. Chem. Soc.,* **118**, 6986 (1996).
33. B. Halle, and H. Wennerström, *J. Phys. Chem.,* **75**, 1928 (1981).
34. L. Banci, I. Bertini, F. Cramaro, R. D. Conte, A. Rosato, and M. S. Viezzoli, *Biochemistry,* **39**, 9108 (2000).

35. J. Zajicek, Y. Chang, and F. J. Castellino, *J. Mol. Biol.*, **301**, 333 (2000).
36. S. C. Sahu, A. K. Bhuyan, A. Majumdar, and J. B. Udgaonkar, *Proteins,* **41**, 460 (2000).
37. D. E. Woessner, *J. Chem. Phys.*, **3**, 647 (1962).
38. G. M. Clore, P. C. Driscoll, P. T. Wingfield, and A. M. Gronenborn, *Biochemistry,* **29**, 7387 (1990).
39. D. H. Kim, D. S. Jang, G. H. Nam, G. Choi, J. S. Kim, N. C. Ha, M. S. Kim, B. –H. Oh, K. Y. Choi, *Biochemistry*, **39**, 4581 (2000).