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^{15}N NMR Relaxation Study of the Catalytic Residues in Y14F Mutant Ketosteroid Isomerase

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Abstract: ^{1}H-detected ^{15}N NMR was employed to investigated the effect of mutation (Y14F) on the dynamic properties of catalytic residues in Δ^5-3- ketosteroid isomerase (KSI) from *Conamonas testosteroni*. In particular, the backbone dynamics of the catalytic residues have been studied in free enzyme and its complex with a steroid ligand, 19-nortestosterone hemisuccinate, 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}). The results show that the mutation causes a significant decrease in the order parameter (S^2) for the catalytic residues of free Y14F KSI, presumably due to breakdown of the hydrogen bond network by mutation. In addition, the order parameters of Phe-14 and Asp-99 increased slightly upon ligand binding, indicating a slight restriction of the high-frequency (pico- to nanosecond) internal motions of the residues in the complexed Y14F KSI, while the order parameter of Tyr-55 decreased significantly upon ligand binding.

Keywords : KSI, NMR, relaxation, dynamics, order parameter, model-free.

INTRODUCTION

The dynamical properties of proteins are as important as their average structural properties, because the protein dynamics can markedly influence the important aspects of protein functions, such as enzyme action,[1] stability, and protein folding.[2,3] In addition, the specific intermolecular interactions between a ligand and a protein can alter the internal

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motions in a wide range of time scale. Heteronuclear relaxation is now widely used to describe the protein dynamics, since ^{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.[4-12] For folded globular proteins, the model-free analysis[13,14] 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 effective correlation time of the internal motion (τ_e : pico- to nanosecond time scale), 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 using four major catalytic residues of Tyr 14 as a general acid, Asp 38 as a general base, Tyr 55, and Asp 99. This enzyme has a molecular weight of 26.8 kDa as a dimer and consists of three-helices and a six-strand mixed-pleated sheet that contains three-bulges per subunit. Three-dimensional structures in solution as well as those in crystal state have been identified by NMR and X-ray crystallography.[15,16]

A number of NMR relaxation studies on the backbone or side chain dynamics of KSI have been carried out for understanding the enzymatic nature of KSI.[5, 17-19] Zhao *et al.*[5] 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. On the other hand, the relaxation studies on the backbone dynamics of wild-type KSI by Yun *et al.*[17, 18] showed that 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.

It is expected that the backbone dynamics of mutant as well as wild-type protein could show some important physical aspects about the protein functions, and the backbone and side chain dynamics in several mutant proteins have been investigated using ^{13}C and ^{15}N NMR relaxation experiments.[20-26] In the present work, we have prepared the Y14F (Phe replacing Tyr-14) mutant KSI by site-directed mutagenesis to further elucidate the motional changes in the active site of KSI upon ligand binding. Backbone dynamics of the key catalytic residues in free mutant KSI and its complex with 19-NTHS has been studied by ^{1}H-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.

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). Expression and purification of ^{15}N labeled mutant (Y14F) KSI was performed as previously described.[17]

Preparation of NMR Sample. NMR samples were prepared to contain ca. 0.7 mM of uniformly ^{15}N labeled mutant (Y14F) KSI in 20 mM potassium phosphate, 9 % (v/v) DMSO-d_6 and 91 % H_2O. The pH of the sample was adjusted to 7.0. In order to obtain the steroid-bound protein, 19-NTHS was added to the protein solution with a slightly excess amount of inhibitor relative to that of the protein, and the unbound steroid was separated by ultracentrifugation.

NMR Measurements and Processing. All NMR data were collected at 300 K on a Bruker DRX500 spectrometer (500.13 MHz for ^{1}H 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.*[27] with a slight modification to include water flip-back[28] and WATERGATE[29] techniques for eliminating the water resonance. Decoupling of ^{15}N spins during acquisition was performed

using a GARP composite pulse sequence with a field strength of 1.3 kHz. The T_1 and T_2 measurements were performed using total 96 transients per t_1 experiment. 128×2048 complex points were acquired in the $t_1 \times t_2$ dimensions. A total of 9 and 10 data sets were collected to measure T_1 and T_2, respectively. The cross peak intensities were measured as peak volumes in order to increase sensitivity,[30] 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.[31] T_2 measurements utilized a 100μs delay between sequential ^{15}N pulses in the CPMG pulse train for attenuating the ^{15}N signal loss during a T_2 relaxation period. 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, ^{1}H 180°pulses were applied during the recovering delays as described.[32-34] A 4 s relaxation delay was used between scans. Heteronuclear steady state ^{15}N{^{1}H} NOE were determined from spectra recorded with (NOE) and without (control) saturation of protons, where saturation was achieved by a train of 120°pulses separated by 5 ms for 4 s. The NOE measurements were performed using total 96 transients per t_1 experiment. 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.

Analysis of Relaxation Parameters. ^{15}N relaxation parameters were analyzed with the model-free method[14,15] by using the program Modelfree v. 4.1.[31,35] Anisotropic model for rotational diffusion is essential in the case of highly asymmetric or multidomain proteins.[37-39] It has also been shown[17] that the relaxation data for wild-type KSI are best described by an axially symmetric rotational diffusion tensor. Thus, the τ_m and $D_{\|} / D_{\perp}$ values from the axially symmetric model were used to analyze the internal motions of free and steroid-bound KSI.

The model-free parameters were selected by extensive Monte Carlo simulations and F-statistical testing, as described in the literature.[35] 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 $(\sigma_{\|} - \sigma_{\perp})$ was taken to be -170 ppm, and the value of 1.02 Å for the N-H bond length was used for the calculations. Detailed procedures for the analysis of relaxation data can be found in our previous work. [17]

RESULTS AND DISCUSSION

The assignments of backbone amide ^1H and ^{15}N chemical shifts of mutant Y14F KSI and its complex with 19-NTHS were carried out by comparing cross-peaks from HSQC spectra with those of previously reported wild-type KSI.[17] Comparison of the chemical shifts between free and complexed Y14F KSI shows that there are large chemical shift changes in the active site residues upon ligand binding. Especially the chemical shift changes of Phe14 and Tyr55 are much larger than those of wild-type KSI upon ligand binding, which might be related to the breakdown of hydrogen bond between Tyr55 and Phe14 by mutation. According to the X-ray structure[16] and to the proposed reaction mechanism of KSI, Tyr55 forms a hydrogen bond network and assists the catalytic reaction in free wild-type KSI.

For an initial estimate of the overall rotational correlation times (τ_m) and diffusion tensor (D), residues were selected on the basis of criteria suggested by Tjandra.[36] The statistical result indicates that the ^{15}N relaxation rates are best described by an axially symmetric rotational diffusion tensor. Therefore, the diffusion anisotropy value ($D_{\|}/D_{\perp}$) from the axially symmetric model as well as τ_m were used to analyze the internal motions of free Y14F KSI. After selection of appropriate internal dynamics models and model-free calculation, the optimized effective τ_m was 20.08 ± 0.03 ns with a diffusion anisotropy $D_{\|}/D_{\perp}$ = 1.19 ± 0.02. The same calculation was carried out for the complexed Y14F KSI. The best acceptable model was also the axially symmetric one from F-test and Monte Carlo simulation. The calculated value of τ_m from final optimization was 18.09 ± 0.03 ns with

$D_{\parallel}/D_{\perp} = 1.33 \pm 0.01$. The τ_m value of complexed Y14 F KSI is slightly smaller than that of free form, indicating that Y14F KSI becomes more compact upon ligand binding.

Table 1 summarizes the model-free parameters of the active site residues, Phe-14, Tyr-55 and Asp-99 for free and complexed Y14F KSI. The catalytic residues of free Y14F KSI have significantly reduced order parameters (S^2) when compared with those of free wild type KSI (0.96 for Tyr-14 and 0.97 for Asp-99), indicating enhanced high-frequency (pico- to nanosecond) internal motions in Y14F mutant KSI, presumably due to the breakdown of the hydrogen bond network by mutation. In addition, the order parameters of Phe-14 and Asp-99 slightly increased (0.852 vs. 0.872) or remained nearly constant (0.842 vs. 0.847) upon ligand binding, indicating a slight restriction of the high-frequency motions in the complexed Y14F KSI, while the order parameter of Tyr-55 decreased significantly upon ligand binding. 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.*[5], in which the 19-NTHS binding decreased the sizable R_{ex} term of the $C_\alpha$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.

Table 1: Model-free Parameters of the Active Site Residues in Y14F Mutant KSI

	Phe-14		Tyr-55		Asp-99	
	free	complexed	free	complexed	free	complexed
S^2	0.852	0.872	0.842	0.847	0.913	0.785
R_{ex} (s^{-1})	0	0	0	0	0	0

One of the most noticeable features of KSI active-site geometry is that the polar functional groups are maintained in the highly polar 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. Recent study on KSI revealed that this H-

bond network in the active site is important for both stability and function of the enzyme.[40] The large decrease in the order parameter of Tyr-55 upon ligand binding might be a direct consequence of the breakdown of this hydrogen bond network by Y14F mutation.

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