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Rapid Mapping of Active Site of KSI by Paramagnetic NMR

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Active site mapping has been done for Δ^5-3-ketosteroid isomerase (KSI) by analyses of paramagnetic effect on ^{1}H-^{15}N HSQC spectra using 4-hydroxyl-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO) and an intermediate analog (equilenin). Our result revealed that residues in hydrophobic cavity of KSI, particularly active site region, mainly experienced a high line-broadening effect of NMR signal with HyTEMPO, while they experienced full recovery of a lineshape upon the addition of equilenin. The mapped region was very similar to the active site of KSI as described by the crystal structure. These observations indicate that a combined use of paramagnetic reagent and substrate (or analog) could rapidly identify the residues in potential active site of KSI, and can be applied to the analysis of both active site and function in unknown protein.

Key Words : NMR, Ketosteroid isomerase, Active site, Paramagnetic agent, Equilenin

Introduction

It has been known that the protein active site plays a significant role in various physiological processes. The catalytic process is a binding reaction of protein similar to a lock and key model where the substrate behaves like a key fitting in the lock by interaction between substrate and residues of active site in the protein. The active site of the protein fits to the substrate conformation in size, shape, charge, and hydrophobic or hydrophilic character, or a little conformational change is induced in order to ensure precise binding to the substrate.[1,2] Thus, the knowledge of active site on protein is very important to understand the functions in proteins. Computational methods were mainly utilized for the preliminary studies of active site prediction in proteins, where the accuracies were low or not demonstrated experimentally.[3-5]

Studies of enzyme mechanism carried out by several methods like X-ray crystallography, NMR, florescence, and IR spectroscopy form an important part of structural biology.[6-8] Among them, X-ray crystallography and NMR are powerful methods to determine three-dimensional structure of various molecules, including inorganic compounds, DNA and proteins.[9] These two methods, however, generally need much time and efforts to obtain structural information. Thus, it is difficult to quickly ascertain the functions of unknown proteins, and the development of rapid mapping method for enzyme active site determination becomes vital.

4-Hydroxyl-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO) is a nitroxide spin label shown in Figure 1(a). It leads to line-broadening of NMR resonances of proximal atoms without affecting the chemical shift, since magnetic dipole of unpaired electron increases their transverse relaxation rate. These paramagnetic perturbations have been used to distinguish the solvent exposed surface area of protein using multi-dimensional NMR experiments.[10,11] Up till now, however, they have not been intensively applied to the identification of residues located in the active site of enzyme.

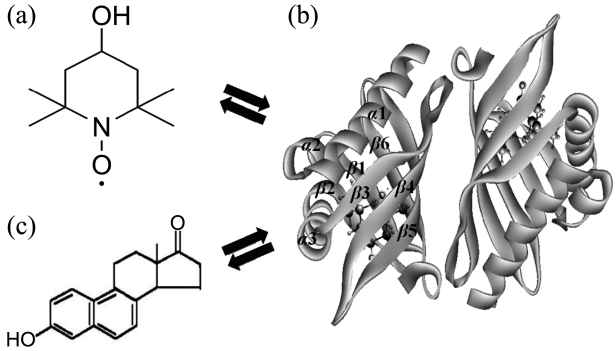

Figure 1. Structures of (a) HyTEMPO, (b) KSI, and (c) equilenin. α and β in KSI correspond to α-helix and β-strand, respectively.

Δ^5-3-ketosteroid isomerase (KSI; EC 5.3.3.1) from *Pseudomonas putida* biotype B is a homodimeric enzyme with 131 amino acid residues per monomer which catalyzes the proton position conversion of C4β- to C6β.[12] It has been studied extensively to understand the catalytic mechanism of this allylic rearrangement.[13,14] The structure of KSI using X-ray crystallography and NMR has revealed that this enzyme folds into three α-helices and six β-strands in each monomer as shown in Figure 1(b) and contains many hydrophobic residues at the active site.[15,16] The active site residues such as Tyr16, Asp40, Asp103, Trp120 are located deep in the hydrophobic cavity, and involved in the hydrogen bond network. 3-Hydroxy-1,3,5(10),6,8-estrapentaen-17-one (equilenin) geometrically resembles the dienolate reaction intermediate as shown in Figure 1(c), and binds tightly at the KSI active site with a dissociation constant (K_d) of 1.9 μM.[17,18] In presence of equilenin, the active site of KSI is blocked from outside by the steroid substrate and apolar residues, making it completely inaccessible to any type of solvent effects such as HyTEMPO.

In the present study, a rapid NMR method to identify the residues in active site of enzyme has been described. Active site mapping has been done for KSI by analyses of paramagnetic effect on ^{1}H-^{15}N HSQC spectra using HyTEMPO and equilenin as an indirect probe. The mapped region by NMR experiments was very similar to the active site of KSI as described by the crystal structure. These observations suggest that a combined use of paramagnetic reagent and substrate (or analog) could rapidly map the potential active site of KSI, and can be applied to the analysis of both active site and function in unknown protein.

Experimental

HyTEMPO, deuterium oxide (D_2O) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Company (St. Louis, MO, 99.9% purity). ^{15}N-labeled NH_4Cl was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). These chemicals were used without further purification. The intermediate analog (equilenin) was purchased from Steraloids (Newport, RI, USA). It was dissolved in DMSO for the experiments and stored at room temperature.

^{15}N-labeled KSI was overproduced in *Escherichia coli* strain BL21 (DE3) grown in 2 L of minimal medium (M9) supplemented with 0.1% (w/v) ^{15}N-labeled NH_4Cl (99% ^{15}N). The labeled protein was purified by deoxycholate affinity chromatography and Superose 12 gel filtration chromatography (Pharmacia) as previously described.[19] The homogeneity of protein was confirmed by the presence of a single band on SDS-polyacrylamide gels stained with coomassie blue.

NMR samples (~1.5 mM) used for ^{1}H-^{15}N TROSY-HSQC NMR spectra were prepared in a buffer (4 ºC) containing 20 mM potassium phosphate (pH 7.0, 10% v/v D_2O), 1 mM DTT and a small amount of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for internal chemical shift reference of ^{1}H and ^{15}N. Stock solution of HyTEMPO was added to the ^{15}N-labeled KSI sample making its concentration as 4.5 mM. A slightly excess amount of equilenin was added to the ^{15}N-labeled KSI solution with HyTEMPO. All NMR samples were equilibrated at room temperature for 48 hours.

NMR experiments for backbone assignment of KSI-equilenin complex were performed on a Bruker 800 MHz spectrometer operating at 800.25 MHz for proton frequency (Korea Basic Science Institute at Ochang) equipped with a triple resonance probe and pulsed field x-, y-, z- gradient capabilities. HNCA and HNCOCA spectra were recorded with 70 and 48 complex data points along t_1 and t_2 domain, and 1024 data points along t_3 domain, using States-TPPI method in t_1 dimension, and Echo-Antiecho method in t_2 dimension with a relaxation delay of 1 s. All ^{1}H-^{15}N TROSY-HSQC NMR experiments were collected at 298 K on a Bruker DRX-500 spectrometer operating at 500.13 MHz for proton frequency equipped with triple resonance probe. ^{1}H-^{15}N HSQC NMR spectra of NMR sample were recorded using a 90° pulse of 8.8 μs, 128 data points along t_1 domain and 2048 data points along t_2 domain using State-TPPI method in t_1 dimension with a relaxation delay of 2 s. The ^{1}H chemical shifts were calibrated to DSS, and ^{15}N chemical shifts were determined by indirect referencing.[20]

^{1}H-^{15}N HSQC NMR spectra of free KSI, KSI with HyTEMPO and KSI with both HyTEMPO and equilenin were recorded at the same experimental conditions except number of scans. The NMR data were processed with an SGI octane 2 workstation using the NMRPipe software package.[21] All NMR data were analyzed using the Sparky software.[22]

Results and Discussion

Multi-dimensional NMR experiments were performed for KSI to observe the effect of HyTEMPO as a paramagnetic agent. Amide ^{1}H and ^{15}N resonances are sensitive probes for the environment of a particular residue in proteins.[23] The backbone ^{1}H and ^{15}N resonance assignment of free KSI was reported earlier, and a total of 120 out of 131 residues were assigned.[24] The backbone assignments for KSI-equilenin complex were carried out by comparing cross-peaks from ^{1}H-^{15}N HSQC spectra with the published assignment of free KSI. HNCA and HNCOCA experiments were additionally performed to clarify the unambiguous assignments, where the characteristic chemical shifts of the ^{13}Cα of thirteen glycine residues were used as a starting point. Figure 2 shows the fingerprint ^{1}H-^{15}N TROSY-HSQC NMR spectrum of KSI with equilenin, which is very similar to that of free KSI. A total of 103 residues except those with severe overlaps were assigned for the KSI sample with equilenin.

^{1}H-^{15}N HSQC experiment of free KSI was performed in the presence of 4.5 mM HyTEMPO. The spectrum provides sufficient resolution for a lineshape analysis, and the peak height value of all of cross-peaks changed with HyTEMPO. Specifically, for some residues it was particularly decreased, while some other residues disappeared, which indicates that HyTEMPO causes line-broadening for NMR signals of KSI residues. A detailed evaluation of the paramagnetic attenuation was done by calculating decreases (A_i) of peak height according to the formula.

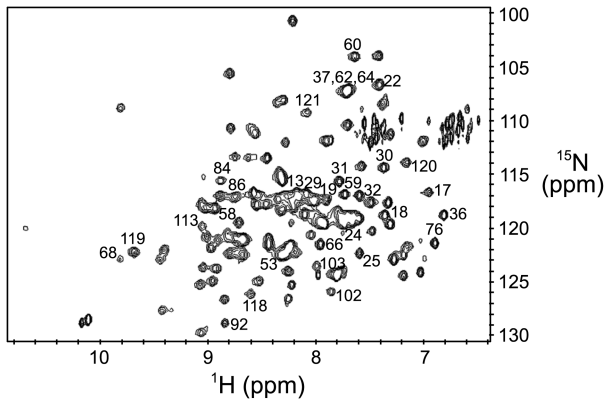

Figure 2. Fingerprint ^{1}H-^{15}N HSQC spectrum of KSI with equilenin. Only selected residues are labeled by numbers on the spectrum.

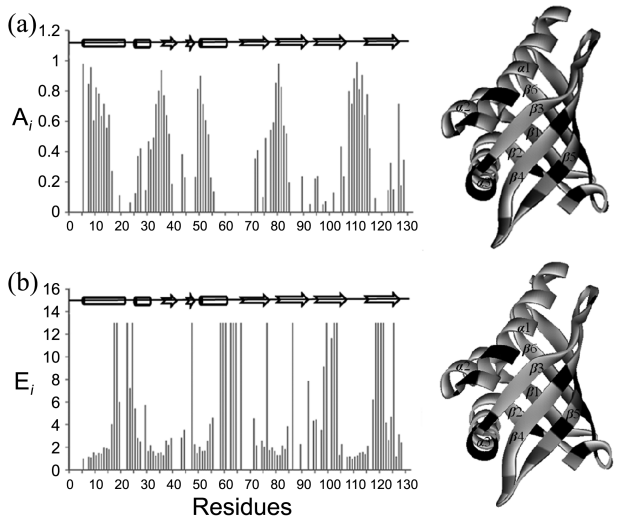

Figure 3. Analysis of A_i and E_i values calculated from peak heights for each residue of various KSI conditions. (a) A_i: native KSI *versus* KSI-HyTEMPO. (b) E_i: KSI-HyTEMPO *versus* KSI-HyTEMPO-equilenin. Some of labels are not shown or are shown to be maximum values because of either severe overlaps or extreme line-broadening. Each α- and β- secondary structures are illustrated as rectangles and arrow, relatively. Remarkable changes are marked by black (disappearance or appearance) and navy grey (highly attenuation or increase) color on the ribbon diagram of each KSI monomer.

$$A_i = \frac{V_p^i}{V_d^i} \quad (1)$$

Here, V_p and V_d represent the peak height value of the cross-peak in ^{1}H-^{15}N HSQC spectrum of KSI with and without HyTEMPO, respectively. Figure 3(a) shows A_i values as a function of amino acid residue number of KSI. As shown in Figure 3(a), a number of residues, especially the residues in hydrophobic cavity of KSI, experienced noticeable line-broadening. Among them, some resonances such as Ile17, Glu18, Val22, Asp24, Ile47, Arg58, Gln59, Gly60, Gly62, Gly63, Gly64, Val66, Ala76, Phe86, Leu99, Ile102, Asp103, Ala118, Tyr119, Trp120, Ser121, and Leu125 disappeared in ^{1}H-^{15}N HSQC spectrum of KSI with HyTEMPO, and some other residues such as Trp92 and Cys97 experienced severe attenuation of the peak height under a specific cut-off (average A_i minus standard deviation). These residues are marked by black (disappearance) and navy grey (highly attenuation) on the three-dimensional structure of native KSI, and mainly located around active site residues in the hydrophobic cavity of KSI.

HyTEMPO has been known to cause line-broadening of NMR signals through specific or non-specific (random collision) interactions.[25-27] Line-broadening of NMR resonances may relate to solvent exposure of residues in the protein. KSI has a hydrophobic cavity with approximate dimensions of 8.5 × 9.5 Å surface and 16 Å deep.[15] In contrast, HyTEMPO is approximately 6.5 × 8.7 Å.[28] Thus, HyTEMPO can easily enter in hydrophobic cavity due to the slightly large size of hydrophobic cavity. These characteristics of HyTEMPO can enable rapid mapping of binding site between the substrate (or analog) and KSI. However, the use of HyTEMPO only may not be sufficient to accomplish the purpose of this work, since some proteins can have multiple hydrophobic cavities without any active site.[29] Thus, the use of substrate (or analog) could be helpful to distinguish normal hydrophobic cavities from the active site.

In order to evaluate the effect of substrate (or analog) on KSI-HyTEMPO solution, we monitored the peak height value of each resonance in ^{1}H-^{15}N HSQC spectrum on KSI solution with both HyTEMPO and equilenin. Upon the addition of equilenin, most of residues experienced the recovery of peak height in ^{1}H-^{15}N HSQC spectrum. Specifically, some residues mainly reappeared in ^{1}H-^{15}N HSQC spectrum, and some other residues particularly experienced a sharp increase of peak height. Enhancements in the peak height of NMR signals for each residue of KSI due to the binding of equilenin on its active site were also calculated. The formula on the NMR signal enhancement (E_i) is almost similar with that of attenuation, but denominator and numerator are reversed. Figure 3(b) shows E_i values as a function of amino acid residue number of KSI. As shown in Figure 3(b), lots of residues experienced significant enhancements of NMR signals due to the binding of equilenin on the active site of KSI. Specifically, residues which had been disappeared by HyTEMPO were perfectly reappeared in ^{1}H-^{15}N HSQC spectrum of KSI-HyTEMPO solution with equilenin, and some other residues such as Trp92, Cys97, and Val101 experienced the significant enhancements of peak height over a specific cut-off (a sum of the average and standard deviation of E_i). These residues are marked by black (reappearance) and navy grey color (enhancement) on the ribbon diagram of native KSI, and also mainly located in the active site of KSI.

Once equilenin was accordingly added to the solution of native KSI with HyTEMPO, HyTEMPO comes out of hydrophobic cavity due to the strong binding between KSI and equilenin.[30] The crystal structure of KSI with equilenin was introduced and analyzed as described before.[16] Residues such as Tyr16, Tyr32, Asp40, Tyr57, Trp92, Asp103, Trp120 in KSI play an important role about binding between KSI and equilenin.[31] Furthermore, active site residues are surrounded closely by non-polar groups (Met13, Ile17, Leu19, Val20, Ile25, Ile28, Val29, Met31, Ala36, Val38, Ile47, Ile53, Phe56, Leu61, Ala68, Met84, Phe86, Val101, Met105, Ile113, Met116, and Ala118).[16] Upon the addition of equilenin, therefore, active site residues were surrounded completely by both hydrophobic inhibitor and non-polar residues with solvent accessibilities of zero. In other words, the access of bulk solvent during enzyme catalysis is blocked by the layer of non-polar residues and steroid substrate.[16] Hence, HyTEMPO does not affect residues in hydrophobic cavity anymore, and NMR signals of lots of peaks were dramatically reappeared or experienced the restoration of a lineshape by strong binding between KSI and equilenin.

Our experiments using both HyTEMPO and substrate (or analog) propose a rapid NMR method to find the residues in potential active site of enzyme. Although this method could

not describe the accurate active site between an enzyme and a substrate, but still it can provide rapid mapping for potential active site region of enzyme which can be used to study the functions of proteins irrespective to the use of three-dimensional crystal structure for the same.

Conclusion

We have investigated the active site region of KSI using paramagnetic reagent (HyTEMPO), intermediate analog (equilenin) and multi-dimensional NMR technique. Lots of NMR resonances experienced noteworthy line-broadening in ^{1}H-^{15}N HSQC spectrum after injecting HyTEMPO on the native KSI solution. The peak height analysis revealed that HyTEMPO affects the residues in hydrophobic cavity, particularly the active site region. Meanwhile, many residues experienced the significant signal enhancements upon the addition of equilenin on the KSI-HyTEMPO solution. Our result shows that the residues in potential active site region of protein can be easily detected by a combined use of paramagnetic reagent and substrate (or analog) using multi-dimensional NMR spectroscopy. This mapping method of active site based on paramagnetic NMR experiments may enable the functional study of enzyme without requirement of its three-dimensional structure.

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