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Direct Identification of Enzyme Active Site Residues by Solid-State REDOR NMR: Application to KDO8P Synthase

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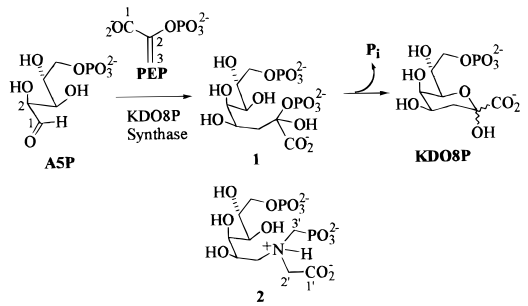
In this communication we report the first direct identification of active site residues of the enzyme 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase (KDO8PS). This is accomplished by the application of rotational-echo double-resonance[1] (REDOR) solid-state NMR experiments to lyophilized *binary* complexes of uniformly ^{15}N-labeled KDO8PS with each of its natural substrates, phosphoenolpyruvate (PEP) and D-arabinose-5-phosphate (A5P). Its complex with the mechanism-based inhibitor, **2** (Scheme 1), the most potent inhibitor known to date[2] ($K_i = 0.4$ μM), is studied as well and its binding is compared to that of the natural substrates.

The REDOR technique[1] is based on selective reintroduction of (magnetic) dipolar interactions between pairs of specific types of nuclei, and in favorable cases enables their accurate measurement from which distance constraints can be obtained.[3] Its application for the study of protein−ligand interactions is achieved by incorporation of stable isotopes at known positions in the protein that are proximate to (labeled) ligand moieties. In previous studies, REDOR measured distances were shown to be in excellent agreement with X-ray data.[3a,b] Moreover, ^{31}P{^{13}C} REDOR was employed to investigate the conformation of the bound inhibitor glyphosate in the enzyme−substrate−inhibitor ternary complex of 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS),[3c] for which no X-ray structure is available. REDOR, e.g., ^{31}P{^{13}C} and ^{15}N {^{31}P}, was also used for the determination of structural constraints in the above ternary complex,[3d] and to directly monitor transient enzyme−intermediate complexes of EPSPS[3e] and of UDP-GlcNac enolpyruvoyl transferase (*Mur Z*).[3f]

The enzyme KDO8PS catalyzes the condensation reaction between A5P and PEP to form KDO8P and inorganic phosphate (P_i, Scheme 1).[4] This important enzymatic reaction plays a crucial role in the assembly process of lipopolysaccharides of most Gram-negative bacteria,[5] and is therefore an attractive target for the design of novel antibacterial drugs. Interestingly, unlike most PEP-utilizing enzymes that cleave the high-energy P−O bond of PEP, KDO8PS and its family members (*Mur Z*, EPSPS, and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase[6] (DAHPS)) cleave

Scheme 1



the C−O bond of PEP.[7] While the mechanisms of EPSPS[8a] (targeted by the herbicide glyphosate) and *Mur Z*[8b] (targeted by the antibiotic fosfomycin) were unambiguously characterized, the mechanisms of KDO8PS and DAHPS continue to be uncertain. Recent studies[9a,2] on KDO8PS, including the synthesis and evaluation of the first acyclic bisubstrate inhibitor (**2**),[2] supported a mechanism involving the formation of an acyclic bisphosphate intermediate **1** (Scheme 1).[9b] Mutagenesis studies[10] identified in *E. coli* KDO8PS two vital cysteines, Cys38 and Cys166, yet their specific role still remains unclear. In addition, since to date neither the crystal structure nor the identity of the active site residues of KDO8PS are known, rational design of potent inhibitors for this enzyme is impaired. As part of our study on the structure−function relationship of KDO8PS, [*u*-^{15}N]KDO8PS was prepared and interactions between ^{15}N enzyme labels to ^{31}P nuclei of the ligands were examined by REDOR NMR.[11]

The ^{15}N NMR CPMAS echo spectrum of [*u*-^{15}N]KDO8PS binary complex with PEP (Figure 1a) shows resolved peaks for the nitrogen atoms of 10 side-chain arginine (guanidino: internal ϵ, 60 ppm; terminal η, 49 ppm), 22 lysine (ϵ 9.0 ppm) residues, and an intense amide peak (95 ppm). Peaks of six side-chain histidines (δ_1 224; ϵ_2 143 ppm) are not shown. In addition, detailed inspection of this spectrum reveals fine structure for the arginine (56.0 ppm), and a high field shoulder for lysine (3.0 ppm), attributed to a single arginine and to a single lysine residue residing in a distinct chemical environment. Interestingly, these two peaks are also present in the ^{15}N CPMAS reference spectra of the other two complexes, KDO8PS−A5P and KDO8PS−**2** (spectra not shown), implying that they arise from chemically distinct environments inherent to the enzyme itself, and are therefore not a result of interaction with a specific ligand. As will be shown below, these residues will be associated with specific recognition and binding of PEP by the enzyme.

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(11) [*u*-^{15}N]KDO8PS was isolated from *E. coli* DH5α (pJU1) grown on minimal media (M9 salts), supplemented with $(^{15}NH_4)_2SO_4$ and purified as previously reported.[4] [1-^{13}C]A5P was prepared from [1-^{13}C]D-arabinose (CIL) and hexokinase (Sigma). [1-^{13}C]PEP was purchased from CIL. [1′-^{13}C]**2** and [3′-^{13}C, ^{15}N]**2** were prepared by using a recently reported one-step procedure.[2] The PEP-free enzyme was prepared as previously published,[9a] followed by dialysis against a buffer containing 120 mM MOPS, 0.1 mM DTT, pH 7.3. This solution was adjusted to contain 150 μM enzyme, 0.4% PEG, 8 mM Sucrose, and 3.8 mM MOPS. Binary complexes were prepared by adding stoichiometric amounts of each ligand, followed by fast freezing and overnight lyophilization.[3c] We assumed that this procedure along with the binding constants of each of these ligands (see text) should optimize the specific active site enzyme−ligand bound forms in the lyophilized solids.

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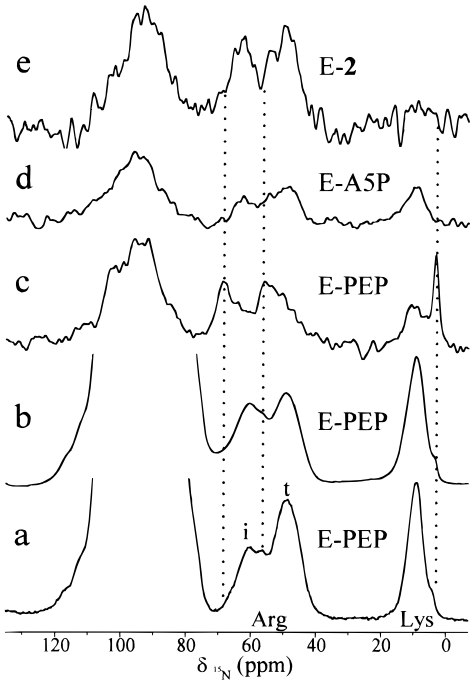

Figure 1. ^{15}N NMR CPMAS echo spectrum and ^{15}N{^{31}P} REDOR spectra of lyophilized binary complexes of [*u*-^{15}N]KDO8PS with (a) [1-^{13}C]-PEP; CPMAS echo, (b) [1-^{13}C]PEP; REDOR reference spectrum (S_0), (c) [1-^{13}C]PEP; REDOR difference spectrum (ΔS), (d) [1-^{13}C]A5P; ΔS, (e) [1′-^{13}C]**2**, ΔS. Dotted lines correspond to the distinct arginine and lysine peaks exhibited in spectrum c. Spectra c−e are vertically magnified by 2.5 relative to their reference spectra. All spectra were recorded on a 300 MHz CMX-*infinity* three-channel Chemagnetics/Varian spectrometer, using a triple-resonance Chemagnetics probe. Samples were spun in 5 mm thin wall Zirconia rotors at 5000 ± 2 Hz and maintained at 1 ± 0.2 °C. REDOR experiments (spectra b−e) utilized 64 rotor cycles followed by an additional pair for Hahn-echo formation. Spectrum a employed only the Hahn-echo part (differences in relative peak intensity between spectra a and b arise from T_2 differences). A contact time of 0.7 ms with matched RF levels at 50 kHz, followed by 100 kHz proton decoupling, and 2 s repetition time were employed. REDOR π-pulses employed xy-8 phase cycling scheme [Gullion, T.; Baker, D. B.; Conradi, M. S. *J. Magn. Reson.* **1990**, *89*, 479]. Up to 80000 scans were collected for 50−80 mg samples. ^{15}N chemical shifts are relative to solid $(^{15}NH_4)_2SO_4$.

The ^{15}N{^{31}P} REDOR difference spectra of the binary complexes are depicted in Figure 1c−e, along with the REDOR reference spectrum of the complex with PEP in Figure 1b. Spectra c and d show peaks exclusively for residues in the enzyme active site whose ^{15}N nuclei are within 6 Å of ^{31}P nuclei of the phosphate groups of PEP and A5P, respectively. Additional to the broad difference peak of the amide nitrogens (95 ppm), Figure 1c exposes two lysine peaks at 9.5 and 3.0 ppm, and two (complex) arginine peaks at 56.0 and 68.7 ppm. Thus, apart from the 56.0 ppm arginine and the 3.0 ppm lysine peaks associated with chemically distinct enzyme environments, this spectrum reveals yet an additional distinct environment manifested by the substantially shifted 68.7 ppm arginine peak (dotted lines). Figure 1c therefore identifies at least two different lysine and at least one arginine residues that interact with PEP phosphate moiety, where the role of the chemically distinct residues is clearly demonstrated.

The spectrum of KDO8PS−A5P binary complex (Figure 1d), however, exposes a different set of peaks than those observed in Figure 1c, with two arginine peaks at 49.0 and 62.0 ppm, and only one lysine peak at 9.0 ppm. Since peaks of the distinct residues that bind PEP (dotted lines) are present in the CPMAS spectrum of the binary complex with A5P, but absent from its REDOR difference spectrum, it is concluded that these residues are not involved in A5P phosphate binding. Hence, although the dissociation constants for these two binary complexes are not known ($K_m^{PEP} = 6\ \mu$M, $K_m^{A5P} = 25\ \mu$M),[12] the above observations suggest that different sets of enzyme residues bind the two substrates, and correspond to two (adjacent) subsites capable of independent and sufficiently strong binding.

The REDOR difference spectrum of the KDO8PS−**2** complex in Figure 1e reveals that unlike PEP and A5P binding, no lysine residues are involved in inhibitor binding. Additional to the backbone nitrogen peak (95.0 ppm), it depicts only arginine peaks (49.5 and 63.0 ppm) similar to those observed for the A5P complex in Figure 1d. This suggests that binding of **2** by the enzyme partially mimics A5P binding, and has little, if any, interaction at the PEP binding site.[13] Note that although data on the above binary complexes cannot depict the interactions existing in the KDO8PS−PEP−A5P ternary complex, or in the KDO8PS−**1** binary complex, combined kinetic[2] and REDOR NMR data suggest that **2** is neither a good bisubstrate mimic nor a good mimic of intermediate **1**, and it can be best characterized as an A5P-based substrate-analogue inhibitor of KDO8PS.

It should be emphasized that the exceptional narrowness of the 3.0 ppm lysine shoulder (Figure 1a,b), as manifested by its 30 Hz difference peak (Figure 1c), implies that this lysine side chain is preserved by the enzyme in a unique chemical environment. Moreover, its attribution to a single lysine residue (out of 22) enabled ^{15}N{^{31}P} REDOR distance determination of 5.0 Å as the maximum internuclear separation from PEP phosphate.[14]

Solid-state REDOR NMR employed in this study provided a clear distinction between the binding of each substrate and inhibitor by directly exposing enzyme active-site residues. The data obtained also reveal for the first time that PEP, the first substrate in a sequentially ordered mechanism,[12] is bound by KDO8PS via a set of structurally and chemically distinct lysine and arginine residues. In light of their specific role for PEP binding, and the fact that their unique chemical environments are preserved intact in each of the three complexes, we suggest that these residues function also as "sensors" for PEP recognition.[15] These observations therefore pose an intriguing evolutionary question regarding the commonality of this motif in other PEP-utilizing enzymes, especially in DAHPS, *Mur Z*, and EPSPS, where the same C−O bond cleavage of PEP and 2-*si* face addition of cosubstrates at C-3 of PEP were exclusively demonstrated.[7]

Finally, based on the important role of arginines alluded to by this study, five highly conserved arginines of KDO8PS were replaced with alanine, and the resulting mutants were kinetically characterized.[16] Of those, while R226A retained 34% of wild-type activity, R70A, R120A, and R168A displayed only 1.2%, 0.7%, and 0.1% of wild-type activity, and R63A was completely inactive. These results further substantiate the NMR results, manifesting that these arginines are vital for substrate binding and/or catalysis. Further kinetic and solid-state NMR studies of these mutants and wild-type enzyme are underway.

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(13) ^{31}P{^{15}N} REDOR on [*u*-^{15}N]KDO8PS-[1′-^{13}C]**2** and ^{31}P{^{13}C} REDOR on KDO8PS-[3′-^{13}C,^{15}N]**2** identify unambiguously that only the ^{31}P phosphate of **2** interacts with ^{15}N labels of the enzyme (data not shown).

(14) ^{15}N{^{31}P} REDOR experiments were performed for n = 32, 48, 64 rotor cycles; S_0 and ΔS peak intensities for each n were estimated by deconvolution. $\Delta S/S_0(n)$ were best fit by a 40 Hz dipolar coupling corresponding to 5.0 ± 0.2 Å. Relying on full active site occupancy by PEP, 5.0 Å is therefore an upper limit for the internuclear distance.

(15) Consistent with our observations that KDO8PS is isolated with 1 equiv of tightly associated PEP and is less stable when the PEP is removed.[9a]

(16) The mutant enzymes were overexpressed in *E. coli* XL1-Blue (pKS$^+$II) and purified similar to the wild-type enzyme.