Long-Range Distance Measurements of Protein Binding Sites by Rotational-Echo Double-Resonance NMR

Daniel R. Studelska,[†] Christopher A. Klug,[†,‡] Denise D. Beusen,[§,||] Lynda M. McDowell,[†] and Jacob Schaefer*[,†]

Department of Chemistry, Washington University
Center for Molecular Design, Washington University
School of Medicine, St. Louis, Missouri 63130

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Both hetero-[1−3] and homonuclear[4−7] solid-state NMR experiments are being used to characterize the binding sites of proteins and protein complexes that are unsuited for diffraction studies and too big for solution-state NMR. The basic approach is to introduce stable-isotope labels, either as clusters of specific labels[8,9] or by uniform labeling,[10] and to use one or more of the recently developed pulse sequences to reintroduce dipolar coupling between the labels in the presence of dipolar decoupling of the protons and high-speed magic-angle spinning. Accurate distance determinations for $^{13}C-^{15}N$, $^{13}C-^{13}C$, and $^{13}C-^{31}P$ of 5, 6, and 8 Å, respectively, are practical. Because the range increases with the gyromagnetic ratios of the labels, ^{19}F NMR offers the prospect of the longest distance determinations. In this communication we describe the use of ^{19}F stable-isotope labeling of two domains surrounding a protein binding site and report the measurement of a 16-Å $^{31}P-^{19}F$ internuclear distance from one of those domains to ligands in the binding site.

The 46-kD enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase was ^{19}F labeled. This enzyme catalyzes the condensation of phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) to produce 5-enolpyruvylshikimate-3-phosphate and inorganic phosphate.[11−13] The reaction is inhibited by the herbicide *N*-(phosphonomethyl)glycine, also known as glyphosate[14,15] (Glp). X-ray diffraction studies of EPSP synthase have been successful,[16] but there is no structure for the stable ternary complex that EPSP synthase forms with S3P and Glp. We used site-directed mutagenesis to remove and introduce tryptophans into an *Escherichia coli* isoform of EPSP synthase. Clones were screened by restriction analysis,

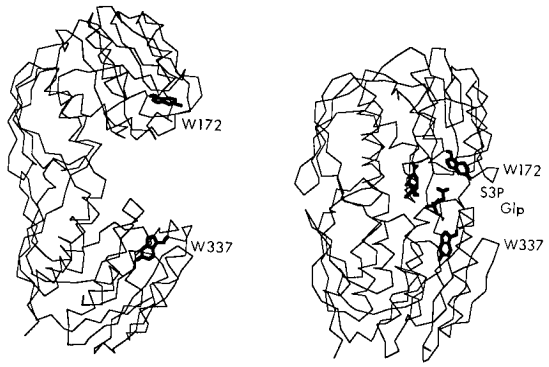

Figure 1. (Left) Cα trace of 5-enolpyruvylshikimate-3-phospate synthase, based on the X-ray diffraction results of ref 16, showing the approximate locations of W172 (upper domain) and W337 (lower domain) of the double mutant W289Q;F172W. The side chain of F172 in the wild-type structure can be modified to tryptophan with retention of the wild-type rotameric state and only slight adjustment to remove van der Waals contacts with nearby residues. The F−F distance in this structure is 38 Å. (Right) Cα trace of a model of the EPSPS ternary complex consistent with $^{19}F-^{31}P$ REDOR distance measurements (*cf.* Figure 3). Ligands were docked into the X-ray structure on the basis of the results of REDOR studies involving complexes of specifically ^{13}C- and ^{15}N-labeled protein and ligands (refs 17−21). The hinge was identified by searching for residues in the connecting strands that were not part of a secondary structural element in either the upper or lower domain. Torsions in the two hinge strands in EPSPS were manually adjusted to bring the two protein domains into proximity. Ligand position and hinge torsions were adjusted iteratively to accommodate the P−F distances measured in this study and C−N, C−P, and N-P distances measured previously.[17−21] The F−F distance in this model is 21 Å. The F−P distances for W337-S3P, W337-Glp, W172-S3P, and W172-Glp in the model are 18, 13, 7, and 11 Å, respectively.

and the absence of errors in the EPSP synthase coding region of each final plasmid construct was confirmed by commercial DNA sequencing (National Biosciences, Inc., Plymouth, MN).

Fluorine label was biosynthetically introduced into the enzyme via DL-[6-^{19}F]tryptophan using bacteria auxotrophic for tryptophan. The positioning of the ^{19}F labels is illustrated using the structure[16] shown in Figure 1 (left). Wild-type EPSP synthase has only two tryptophan residues (W289 and W337), and both are in the lower domain.[16] We created a single mutant (W289Q) by removing the lower-domain tryptophan which was the more distant of the two from the cleft region. We then created a double mutant (W289Q;F172W) by introducing tryptophan into the upper domain near the binding site. When the tryptophans are specifically labeled by ^{19}F, measurements of long-range $^{19}F-^{31}P$ (and, in principle, $^{19}F-^{19}F$) distances monitor directly the extent of closure of the cleft on binding of ^{31}P-containing ligands. Details of the binding-site reorganization are revealed by specific ^{13}C labeling of the negatively charged ligands[17−20] and ^{15}N labeling of lysine, histidine, and arginine residues,[18,21] followed by solid-state NMR analysis.

The ligands S3P and Glp were complexed[20] to [6-^{19}F]Trp−W289Q−EPSPS in a solution that was 2.7 mg/mL protein, 1% poly(ethylene glycol)-8000, 20 mM sucrose, 2 mM 4-morpholinepropanesulfonic acid, and 1 mM dithiothreitol. The solution containing the ternary complex was frozen at −20 °C and then

[†] Department of Chemistry.
[‡] Present address: Department of Chemical Engineering, Stanford University, Stanford, CA 94305.
[§] Center for Molecular Design.
[||] Present address: Tripos Inc., St. Louis, MO 63144.

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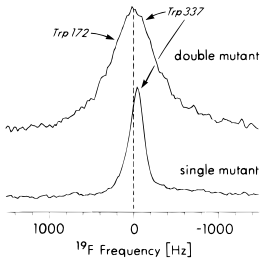

Figure 2. 188-MHz CPMAS Hahn-echo ^{19}F NMR spectra of 3 μmol of [6-^{19}F]Trp−W289Q−EPSPS−S3P−Glp (bottom) and 1 μmol of [6-^{19}F]Trp−W289Q;F172W−EPSPS−S3P−Glp (top) with 95-kHz proton decoupling and 5-kHz magic-angle spinning. Only the center bands are shown. The assignment for the double mutant assumes that the resonance from W172 is shifted 0.3 ppm to low field relative to that from W337.

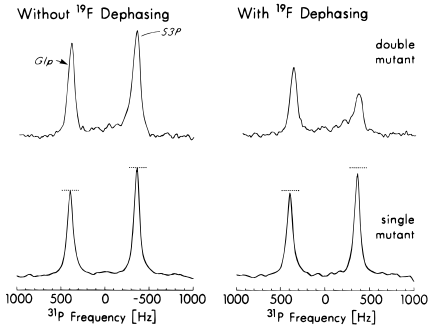

Figure 3. 80-MHz REDOR ^{31}P NMR spectra of 3 μmol of [6-^{19}F]-Trp−W289Q−EPSPS−S3P−Glp after 96 rotor cycles of 5-kHz magic-angle spinning (bottom) and of 1 μmol of [6-^{19}F]Trp−W289Q;F172W−EPSPS−S3P−Glp after 64 rotor cycles of 5-kHz magic-angle spinning (top), with no ^{19}F dephasing (left) and with ^{19}F dephasing (right). Dephasing for the single mutant averages about 4% (compare dotted lines), corresponding to ^{19}F−^{31}P distances of about 16 Å between W337 and the ^{31}P of both S3P and Glp. Dephasing for the double mutant is about 50% and is dominated by W172 which is less than 10 Å from the binding site.

cooled with liquid nitrogen before lyophilization. The ^{19}F NMR spectrum of the lyophilized powder shows a single line with a width of about 200 Hz (Figure 2, bottom). A spin count indicates ^{19}F labeling of W337 of more than 95%. Rotational-echo double-resonance[1] (REDOR) ^{31}P NMR with ^{19}F dephasing[22] (Figure 3, bottom) is consistent[2] with a 16-Å separation (±2 Å) of the ^{31}P of S3P and Glp from the ^{19}F of W337. Similar labeling and sample preparation of [6-^{19}F]Trp−W289Q;F172W−EPSPS−S3P−Glp ternary complex results in a broader ^{19}F NMR spectrum (Figure 2, top) with double the integrated intensity. Assuming that the substantial ^{31}P REDOR dephasing (Figure 3, top) arises only from ^{31}P coupling to the ^{19}F of W172, the ^{19}F−^{31}P separation is 8.5 ± 0.5 Å for S3P and 10.1 ± 0.5 Å for Glp. Error estimates are based on signal-to-noise ratios of the spectra; ^{31}P−^{19}F REDOR quantitation was calibrated by determination of the 3.6-Å distance in fluoroapatite[23] and by observation of a null ^{31}P REDOR difference signal for phosphoserine. Weak ^{13}C−^{19}F and ^{13}C−^{31}P dipolar couplings have been measured[9,20] for known distances using the same probe and spectrometer. The P−F distances for both ligands determined by the ^{31}P REDOR NMR experiments (with ^{19}F dephasing) of Figure 3 prove that the cleft region has closed on binding of S3P and Glp (Figure 1, right).

About half of the ^{19}F line widths of Figure 2 arise from residual dipolar coupling to the protons, as established by the 3-ms lifetimes of rotor-synchronized Hahn echo trains (data not shown). These short lifetimes preclude ^{19}F−^{19}F measurements on the present sample and, in general, will need to be increased to 15 ms or more if long-range, ^{19}F-observed homonuclear and heteronuclear distance measurements are to be realized. We anticipate increasing proton-decoupling fields to 150 kHz for such experiments; improved phase-modulated decoupling methods may also help.[24] Specific deuteration of tryptophan will not help because the nearest-neighbor proton for ^{19}F is probably not on the tryptophan itself. Because much of the ^{19}F line width is homogeneous, NMR experiments performed at higher static fields will result in higher resolution, and this will be important for systems likely to produce complicated spectra. The homogeneous line shape for a tryptophan side chain means that the buffered,[21] cryo- and lyoprotected[25,26] protein has maintained a native conformation at least 20 Å from the binding site.

Both mutants resemble wild-type EPSP synthase in their binding of S3P and Glp.[19,20] The single-mutant EPSP synthase has the full catalytic activity of the wild-type enzyme, while the double mutant has greatly reduced catalytic activity. Kinetic parameters for S3P were determined for the forward reaction of the wild-type, single, and double mutants of EPSP synthase by determining phosphate release using S3P concentrations between 2 and 192 μM while holding PEP concentration constant at 96 μM. The wild-type and single-mutant reactions were run for 1 min with 9.85 nM enzyme. The double-mutant reactions were run for 5 min with 98.5 nM enzyme. The reactants, in 200 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid/tetramethylammonium hydroxide buffer (pH 7.0), were in a final volume of 100 μL, and the reaction temperature was 30 °C. The reactions were stopped by the addition of reagents for phosphate determination.[27] The results of the kinetic measurements (mean ± standard deviation) are $K_{m,apparent}$-[μM S3P] = 4.5 ± 0.4, 4.1 ± 0.7, 18.7 ± 0.8, and $V_{max,apparent}$-[μM Pi min^{-1}] = 9.1 ± 0.9, 7.8 ± 0.9, 2.7 ± 0.4 for the wild-type, single-, and double-mutant EPSP synthases, respectively.

Considering enzyme dilution, catalysis by the wild-type enzyme is approximately 30 times faster than catalysis by the double-mutant enzyme. We believe that the decreased rate for the double-mutant EPSP synthase is due to slower catalysis and not due to a population of unfolded, enzymatically incompetent enzyme molecules. The best evidence for this conclusion is the sharp lines in the ^{31}P NMR spectrum of the double-mutant ternary complex (Figure 3, top). Sharp ^{31}P lines are not observed for structurally inhomogeneous populations of complexes.[20] In addition, the decreased rate of catalysis for the double-mutant enzyme was accompanied by a marked increase in $K_{m,apparent}$ for S3P. This result suggests that the polar fluorotryptophan residue in the upper domain (which is within 10 Å of the binding site) is perturbing the rate of catalysis by reducing the affinity of S3P binding.

The reversible conversion of natural substrates to intermediates to products *in the lyophilized state* is slowed sufficiently by low temperature to permit REDOR measurements of distances between ^{31}P and ^{13}C labels in the ligands and ^{15}N and ^{19}F labels in the enzyme.[28] REDOR analysis of the complete reaction pathway for both wild-type and double-mutant EPSP synthases is in progress.

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