Time-Resolved Solid-State REDOR NMR Measurements on 5-Enolpyruvylshikimate 3-Phosphate Synthase

Yan Li, Richard J. Appleyard, Wendy A. Shuttleworth, and Jeremy N. S. Evans*

Department of Biochemistry and Biophysics
Washington State University
Pullman, Washington 99164-4660

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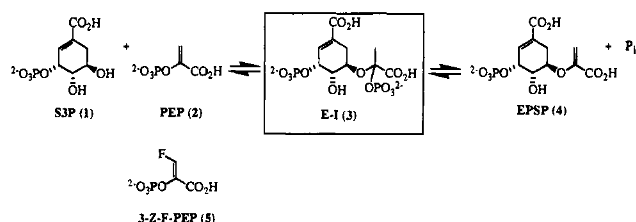
Recently this laboratory introduced the novel technique of time-resolved solid-state NMR spectroscopy for the direct detection of transient enzyme intermediates of 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase[1] and uridine diphosphate *N*-acetylglucosamine enolpyruvyl transferase.[2] The time-resolved method[3] involves rapid freeze quench of enzyme–substrate mixtures at discrete time intervals, enabling the pre-steady-state kinetic trapping of transient species as a function of time, followed by low-temperature solid-state NMR analysis of the samples at each time point. Enzymatic reactions which have either single or multiple intermediates can in theory be stopped along the reaction coordinate, and the most populated ground-state species examined. If used in conjunction with solid-state NMR methods for measuring internuclear distances, the method also has the potential to be complementary to Laue X-ray diffraction methods,[4] since the latter approach suffers from the problem of kinetic asynchronicity, in which after a short time, substrate, intermediate, and product are present simultaneously. In this communication, we report the first application of a time-resolved rotational echo double resonance (REDOR) solid-state NMR experiment to the direct detection of the intermolecular dipolar coupling in the transient enzyme–intermediate complex of 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase.

The REDOR technique was introduced by Schaefer and co-workers[5] and relies on the fact that the effect of the dipolar interaction between two spins on the rotational echo can be manipulated by π pulses, enabling its measurement. Since the dipolar coupling is related to internuclear distance,[5] the REDOR method can measure quite long distances (e.g., <6 Å for ^{31}P–^{15}N up to <12 Å for ^{13}C–^{19}F) to an accuracy of around ±0.1 Å. EPSP synthase (EC 2.5.1.19) is a well-characterized enzyme,[6,7] which catalyzes the penultimate step in the aromatic amino acid biosynthetic pathway in higher plants and bacteria. EPSP (**4**) is formed from shikimate 3-phosphate (S3P, **1**) and phosphoenolpyruvate (PEP, **2**) (see Scheme 1). The enzyme is a monomer with molecular weight M_r = 46 000, and the cloned *Escherichia coli* gene has been used to generate a hyperexpressing strain,[8] so that the bacterial enzyme is available in gram quantities. Furthermore, EPSP synthase is the primary site of action of the herbicide glyphosate,[9] or *N*-phosphonomethylglycine.

Scheme 1



Schaefer and co-workers[10] have used ^{31}P–^{13}C solid-state REDOR NMR experiments to investigate the conformation of the bound inhibitor glyphosate in the enzyme•S3P•glyphosate ternary complex for which no X-ray structure is available. An intermolecular ^{31}P–^{13}C distance of 7.2 Å was measured between the phosphate of S3P and [1-^{13}C]glyphosate implying that S3P and glyphosate are relatively close in the enzyme binding site. Another pertinent study on EPSP synthase found[11] that the 3-(*Z*)-fluoro-PEP (**5**) is converted to the fluoro derivative of the intermediate, which remains tightly bound to the enzyme active site and not converted to product.

Figure 1 shows ^{15}N CP-MAS REDOR difference solid-state NMR spectra of [U-^{15}N]EPSP synthase•S3P mixed with glyphosate, 3-(*Z*)-fluoro-PEP, or [2-^{13}C]PEP under steady-state and pre-steady-state conditions, followed by lyophilization at low temperature. The substrates and/or inhibitor were present only in a slight excess over the enzyme, in order to minimize nonspecific binding, which would lead to erroneous interpretations of the data. At this stage, lyophilized solids have been employed because of the extreme technical difficulties of performing REDOR measurements on frozen solutions. In the first case, the complex with the inhibitor glyphosate is stable. However, in the latter two cases, where either the substrate analogue 3-(*Z*)-fluoro-PEP or the true substrate was used and trapped under time-resolved conditions (the time-resolution being 30 min with 3-(*Z*)-fluoro-PEP and 50 ms with [2-^{13}C]-PEP), special handling was required, particularly with the true substrate, which was kept at low temperature throughout the lyophilization. The presence of intermediate in the samples in Figures 1B and 1C was confirmed by CP-MAS TOSS solid-state ^{31}P and ^{13}C NMR, respectively (hence the use of [2-^{13}C]-PEP in the sample of Figure 1C), obtained after the lyophilization and 3-day REDOR data accumulations (data not shown). This provides evidence that even the unstable transient intermediate of EPSP synthase, formed from the true substrate under pre-steady-state conditions, can survive in the lyophilized solid. We have shown that, in frozen solution, under conditions in which both substrates are in excess, the relative ^{13}C intensity of the intermediate derived from [2-^{13}C]PEP correlates with the progress of the reaction.[1] Work is underway to determine the extent of reaction in a lyophilized solid under conditions of equimolar substrates. These results therefore represent the first

* To whom correspondence should be addressed.

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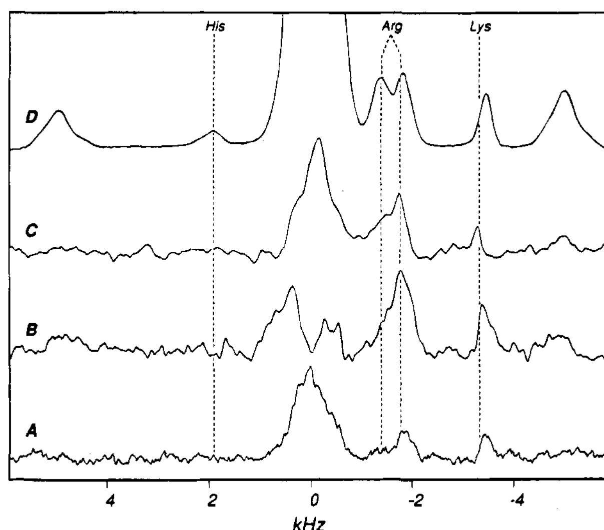

Figure 1. 9.4 T ^{15}N CP-MAS solid-state REDOR difference NMR spectra of [U-^{15}N]EPSP synthase•S3P plus (A) glyphosate (GLP), (B) 3-(*Z*)-fluoro-PEP, and (C) [2-^{13}C]PEP under time-resolved conditions. (D) The full echo spectrum of the sample in (A). The sample in (A) was prepared by mixing 0.05 mM [U-^{15}N]EPSP synthase with 0.10 mM S3P and 0.11 mM GLP in 2 mM MOPS (pH 7.5), 5 mM DTT buffer at 295 K for 1 h and lyophilization at <273 K for the first 4 h and then at 295 K for 44 h, and the spectra (using ca. 30 mg of protein) were acquired at ~295 K. The sample in (B) was prepared by mixing 0.8 mM [U-^{15}N]EPSP synthase with 1.25 mM S3P and 1.25 mM 3-(*Z*)-fluoro-PEP in 2 mM MOPS (pH 7.5), 5 mM DTT buffer at 295 K for 30 min, dilution in buffer to 0.05 mM protein, and lyophilization at <273 K for the first 4 h and then at 273 K for 20 h, and spectra (using ca. 40 mg of protein) were acquired at 273 K. The sample in (C) was prepared by a rapid mix and freeze quench (after 50 ms) of a solution of [U-^{15}N]EPSP synthase and S3P with a solution of [2-^{13}C]-PEP in 50 mM Tris•HCl (pH 7.8) buffer to final concentrations of 4, 6, and 6 mM, respectively. The rapid frozen sample was then lyophilized at <273 K for 24 h, and the spectra (using ca. 60 mg of protein) were acquired at 223 K. The freeze quench apparatus is described in detail elsewhere.[1] [U-^{15}N]EPSP synthase was isolated from *E. coli* BL21-(λDE3)(pLysS)(pWS230) grown on minimal media (M9 salts) supplemented with $^{15}NH_4Cl$ and purified by literature methods.[8] NMR spectroscopy was carried out on a Chemagnetics CMX-400 solid-state NMR spectrometer and a 5 mm triple resonance probe, with 4 ± 0.01 kHz spinning speed and ±0.1 K temperature regulation. Data were acquired using a REDOR pulse sequence[5] with the *xy*-8 phase cycling, interleaving the full echo and dephased spectra, 3 μs ^{1}H 90° pulse width, 1 ms contact time, 2 s recycle time, and 83 kHz ^{1}H decoupling field and processed off-line on a Silicon Graphics 4D25TG computer using FELIX (Biosym).

example of time-resolved solid-state REDOR NMR spectra of enzyme–intermediate complexes.

The spectra also show which residues in the enzyme active site are within approximately 6 Å of the phosphates in the bound substrates/inhibitor, intermediate, or product. In addition to the amide nitrogen resonance at 0 kHz, Figure 1A shows two additional resonances which arise from arginine and lysine residues. Note that only one of the two types of nitrogen in the guanidino group of arginine appears in the spectrum (for comparison, see the full echo spectrum shown in Figure 1D), consistent with only the terminal nitrogens being involved in phosphate binding. This is also consistent with the chemical modification and mutagenesis data on EPSP synthase suggesting that there are at least three lysine[12] (out of a total of 17) and two arginine[13] (out of a total of 22) residues which are both highly conserved and appear to be located in the enzyme active site. Inspection of the X-ray structure of the uncomplexed EPSP synthase[14] also shows that active site lysine and arginine residues implicated by chemical modification and mutagenesis are located close to other residues known to be present in the active site. Figure 1B shows that both 3-(*Z*)-fluoro-PEP and the fluorinated intermediate that is formed[11] interact with the enzyme active site in a different manner compared to the enzyme•S3P•glyphosate complex. In particular, there appear to be additional interactions with arginine and different interactions with backbone amide residues. Figure 1C shows a spectrum similar to that in Figure 1A, with the exception of displaying additional interactions to arginine residues, including the internal arginine side chain nitrogen. None of the spectra display interactions to histidine, suggesting that His-385, which has been shown to be located in the active site,[15] is >6 Å from the phosphates. The minor variations in chemical shift for the lysine resonances in the different samples arise presumably because of minor perturbations in the local structure, which could be due to the fact that the three samples were obtained at different temperatures or because the three different complexes differ slightly in their enzyme active site structures. However, comparison of the full echo spectra for the three samples (data not shown) shows identical chemical shifts for the bulk lysine resonance.

Although in this report, we have not distinguished between substrate, intermediate, and product, and therefore also have a kinetically asynchronous population, the advent of solid-state NMR methods which can measure the dipolar interaction selectively[16,17] will make this distinction possible in cases in which their chemical shifts are different. Such a potential ability to select these species is one of the unique advantages this method may have over the Laue approach. Although the X-ray structure of the enzyme active site is crucial for interpreting the data, since there is no structure available for the EPSP synthase•substrate/inhibitor complex, our solid-state NMR approach is the only technique available for interrogating these interactions. We believe that this new technique will ultimately enable the delineation of the molecular events of enzymatic catalysis.

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