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Time-resolved solid-state REDOR NMR studies of UDP *N*-acetylglucosamine enolpyruvyl transferase

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Abstract The new method of time-resolved solid-state rotational echo double resonance (REDOR) NMR spectroscopy introduced recently by this laboratory has been applied to the enzyme uridine *N*-acetylglucosamine (UDP-NAG) enolpyruvyl transferase (EPT), with the goal of probing the interactions between reactive species and their enzyme active site. The approach has been used in a qualitative fashion with the enzyme-inhibitor and enzyme-intermediate complexes of uniformly ^{15}N-labeled UDP-NAG EPT, trapped under steady-state and pre-steady-state conditions. A different set of intermolecular interactions between the substrates UDP-NAG, UDP-NAG plus 3-*Z*-fluorophosphoenolpyruvate, covalent *O*-phosphothioketal, and UDP-NAG plus phosphoenolpyruvate trapped under time-resolved conditions (after 50 ms reaction time), and the EPT enzyme active site were observed, and this is contrasted to a similar study of the interactions in a related enzyme, 5-enolpyruvyl-shikimate-3-phosphate synthase.

Key words: Enolpyruvyl transferase; Enzyme active site; Enzyme mechanism; Rotational echo double resonance; Time-resolved solid-state NMR; Uridine diphosphate *N*-acetylglucosamine enolpyruvyl transferase

1. Introduction

This laboratory has been developing 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 [1–4], and uridine diphosphate *N*-acetyl-glucosamine enolpyruvyl transferase [5]. The time-resolved method [6] 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. The method also has the potential to be complementary to Laue X-ray diffraction methods [7–11], since the latter approach suffers from the problem that diffusion of the substrate results in a kinetically heterogeneous population of enzyme-bound states (i.e. substrate, intermediate and product present simultaneously) and transient lattice disorder during substrate entry results in diffuse electron densities just at the time when the reaction takes place. Even when caged substrate molecules are employed [12,13], maintaining kinetic synchronicity can be a major problem with the Laue approach. In contrast, NMR has the potential to distinguish between a distribution of populated states of kinetic species on the basis of chemical shifts, provided the species have well-dispersed resonances. Thus, in principle the application of time-resolved solid-state rotational echo double resonance (REDOR) solid-state NMR can provide enzyme active site distance information as a function of the reaction coordinate. This laboratory first applied the technique to the direct detection of the intermolecular dipolar coupling in the transient enzyme-intermediate complex of the enzyme EPSP synthase [14]. In this paper, we report the second example of this technique, to probe the intermolecular interactions in UDP-NAG EPT. In both cases the results are qualitative, although efforts to generate quantitative data are in progress.

Uridine diphosphate-*N*-acetylglucosamine enolpyruvyltransferase (UDP-NAG EPT), the first enzyme in bacterial peptidoglycan biosynthesis, catalyzes the condensation of PEP and UDP-*N*-acetylglucosamine (UDP-NAG, **1**) to produce enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UDP-NAG, **3**), as shown in Scheme 1. The enzyme is a monomer (with no metal or cofactor requirements) with molecular weight M_r = 45,000. It is inhibited [15] by the antibiotic phosphonomycin (also known as fosfomycin), which is bacteriocidal for both gram-positive and gram-negative bacteria. Early work on small amounts of partially purified enzyme from two different sources has suggested that either a covalent enolpyruvyl [16] with UDP-NAG EPT from *Enterobacter cloacae*, or an *O*-phosphothioketal [17] with UDP-NAG EPT from *Micrococcus leisodeikticus* covalent enzyme intermediate is involved in the enzymatic mechanism. The gene for the enzyme from *Ent. cloacae* has been cloned in *E. coli* by Wanke et al. [18], and also the gene from *E. coli* has been cloned in *E. coli* by Marquardt et al. [19]. Marquardt et al. [20] conclude that the mechanisms of EPSP synthase and UDP-NAG enolpyruvyl transferase are similar, and report the detection of a non-covalent tetrahedral intermediate.

Recent kinetic and biochemical analysis of the enzyme-intermediate [21] has shown that, contrary to the implications of Marquardt et al. [20], a stable and isolable covalent intermediate forms, in which PEP is attached to the enzyme through cysteine-115 in a *O*-phosphothioketal. This has also since been detected by NMR in this laboratory [5] and independently by Anderson and co-workers [22]. They also carried out prelimi-

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Abbreviations: DTT, dithiothreitol; EPSP, 5-enolpyruvylshikimate-3-phosphate; EPT, enolpyruvyl transferase; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PEP: phosphoenolpyruvate; REDOR, rotational echo double resonance; UDP-NAG, uridine diphosphate *N*-acetylglucosamine.

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nary pre-steady-state kinetics, which appear to suggest that the covalent species is turned over at rates consistent with being on the reaction pathway, although the kinetic analysis is somewhat suspect. Walsh and co-workers [23] also provide some ^{19}F-NMR evidence in support of a covalent intermediate formed from 3-*Z*-fluoro-PEP and UDP-NAG enolpyruvyl transferase from *E. coli*. Use of 3-*Z*-fluoro-PEP is an approach which was used for EPSP synthase [24] and in that study resulted in accumulation of fluorinated intermediates, which are unable to proceed to product. Although a broad resonance was observed [23] consistent with a covalent species, it was obtained in samples quenched in 0.2 M KOH, in which it is unclear to what extent the presumably denatured protein is soluble and therefore whether the observed species is relevant. Apparently the same broad resonance is also observed when the enzyme is incubated with the fluorinated derivative of the postulated non-covalent intermediate, and again quenched in base. Since kinetic competence was not illustrated for this study, and this is particularly important for chemical quench studies in which chemical artefacts can be generated, this does not provide particularly compelling evidence for the covalent intermediate. Turnover of the enzyme-bound *O*-phosphothioketal to product was demonstrated by Amrhein's group, on a timescale consistent with being a kinetically competent intermediate. The role of Cys-115 was also established unequivocally through site-directed mutagenesis of C115S, which resulted in inactive enzyme [21]. This was confirmed by Walsh and co-workers [25], who also found that fosfomycin inactivates UDP-NAG enolpyruvyl transferase by specifically modifying Cys 115. Also, more recently Walsh and co-workers [26] have reported a kinetic analysis of inactivation of UDP-NAG EPT by 3-*Z*-fluoro-PEP. They interpret their data as supporting the notion that the covalent intermediate is on a branched pathway in the enzymatic mechanism, rather than on the primary pathway. Further work from the same laboratory [27] with the (*E*)- and (*Z*)-isomers of the substrate analogue phosphoenolbutyrate imply that irrespective of the substrate regiochemistry, the product is formed from a common intermediate with an sp^3 locus at C3 by *syn-anti* or *anti-syn* addition-elimination. This was interpreted as being inconsistent with a covalent intermediate being on the primary pathway, and supporting the branched pathway hypothesis.

2. Materials and methods

2.1. Chemicals and enzymes

All chemicals were purchased from Sigma (St. Louis, MO) except [2,3-$^{13}C_2$]PEP (>98% ^{13}C) which was obtained from MSD Isotopes (Canada), and $^{15}NH_4Cl$ (>98% ^{15}N), which was obtained from Cambridge Isotopes (Andover, MA).

2.2. Purification of UDP-NAG enolpyruvyl transferase

The enzyme was purified from *E. coli* according to the method previously reported [21]. All manipulations were carried out at 4°C. [U-^{15}N]UDP-NAG EPT was isolated from *E. coli* grown up on minimal media (M9 salts) with $^{15}NH_4Cl$ as the sole nitrogen source, and purified as usual.

2.3. Enzyme activity assay and protein determination

UDP-NAG enolpyruvyl transferase activity was determined by measuring the rate of release of inorganic phosphate [28]. Protein was determined as described [29].

2.4. Modification of UDP-NAG enolpyruvyl transferase.

The covalent enzyme intermediate was prepared by incubating 0.01 mM [U-^{15}N]UDP-NAG enolpyruvyl transferase with 0.01 mM UDP-NAG and 0.1 mM [2,3-$^{13}C_2$]PEP in 50 mM Tris-HCl, pH 7.8, 1 mM DTT at 37°C for 15 min. The solution was concentrated by ultrafiltration using an Amicon YM-10 filter to about 25 ml, and then loaded onto a Mono Q 16/10 (Pharmacia Biotechnology, Piscataway, NJ) FPLC column. Bound material was eluted with a gradient of 0.0 – 0.2 M KCl in 50 mM Tris buffer, pH 7.4. Fractions corresponding to the enzyme intermediate was pooled, washed extensively with 50 mM Tris buffer, pH 7.8, by repeated dilution and ultrafilration and concentrated to about 200 μl by ultrafiltration (approx. 51 mg protein).

2.5. Preparation of complex of UDP-NAG enolpyruvyl transferase with 3-Z-fluoro-PEP

The complex was prepared by incubating 1.07 mM [U-^{15}N]UDP-NAG enolpyruvyl transferase with 1.28 mM UDP-NAG and 1.28 mM 3-*Z*-fluoro-PEP at 25°C for 15 min in 50 mM Tris-HCl, pH 7.6, 1 mM DTT. After this time, 15 ml of 2 mM MOPS, 2.5 mM DTT, 10 μM EDTA, pH 7.6 was added to the solution, followed by quick freezing in liquid nitrogen and lyophilization at low temperature (< 243 K).

2.6. Rapid-freeze quench experiments

The fast-frozen samples were prepared at a mixing time of 50 ms according to the method previously described [3–5]. The enzyme-to-substrate ratio, UDP-NAG enolpyruvyl transferase (3.65 mM): UDP-NAG:[2,3-$^{13}C_2$]PEP, was 1:1.2:1.2 in 50 mM Tris-HCl buffer, pH 7.6.

2.7. Time-resolved solid-state REDOR NMR spectroscopy

The REDOR technique was introduced by Schaefer and co-workers [30–32], 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. The dephasing of magnetization of one spin involved in dipolar coupling to another heteronucleus in the presence and absence of these π-pulses, and subsequent refocusing as a function of the magic angle spinning frequency, leads to a variation of resonance intensities. This intensity variation is related to the dipolar coupling constant. The first spectrum is obtained using a standard cross polarization pulse sequence with π-pulses on the observed nucleus (e.g. ^{15}N) in the middle of the evolution period. During this period, the observable magnetization evolves under the influence of the chemical shifts and the heteronuclear dipolar interaction. The π pulses refocus both interactions, leading to a signal S during the acquisition period. The second spectrum is obtained with an additional train of π-pulses on the dipolar-coupled spin (e.g. ^{31}P). These pulses affect the observed signal by preventing rotational refocusing of the dipolar interaction. The magnetization is therefore not completely refocused, and the signal intensity drops by an amount ΔS. For weak dipolar coupling, the change in signal intensity is related to the distance between the coupled spins by [33],

$$\Delta S/S = KD^2N_c^2\nu_r^{-2} \quad (1)$$

where N_c is the number of rotor cycles during the evolution period, ν_r is the spinning speed (in Hz), D is the dipolar coupling (in Hz), and K is a dimensionless constant (= 1.066). Measurement of D can be used to calculate internuclear distance (r) according to the following relationship:

$$D = \left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_I\gamma_S\hbar}{2\pi r^3} \quad (2)$$

where γ_I and γ_S are the gyromagnetic ratios for the two heteronuclear spins involved, and $\hbar$ is Planck's constant divided by 2π. If performed at relatively slow spinning speeds or with samples of high chemical shielding anisotropy, alternative pulse sequences need to be adopted [34,35]. This experiment therefore yields the internuclear distance, which for an isolated spin pair (e.g. ^{13}C-^{15}N or ^{31}P-^{13}C) can be determined to an accuracy around ± 0.1 Å. Furthermore, the distances which can be measured can be quite long, for example: < 5 Å for ^{13}C-^{15}N, < 6 Å for ^{31}P-^{15}N, < 8 Å for ^{13}C-^{31}P, and < 18 Å for ^{13}C-^{19}F. This of course assumes that in the case of an enzyme-substrate complex, there is essentially 100% labelling of the sites involved in the spin pairing, and that the substrate is completely bound, without any free species present. Such conditions can be met, if proper care is taken (as is the case in the experiments reported here).

All the [U-^{15}N]EPT samples were lyophilized in 2 mM MOPS (pH 7.5) buffer with 5 mM DTT, and the final concentration of the protein in buffer was 0.05 mM. In the case of unstable complexes, the temper-

ature during the lyophilization was kept below 243 K, by using a mixture of liquid nitrogen and ice.

NMR spectroscopy was carried out on a Chemagnetics CMX-400 solid-state NMR spectrometer equipped with 3 RF channels, and a Pencil® triple resonance probe using a 5 mm zirconia rotor, with 6 ± 0.01 kHz spinning speed and temperature ± 0.1 K regulation. Data were acquired using a REDOR pulse sequence [30, 33] with supercycled phase cycling [36–38] with 96 π-pulses, and interleaving of the full echo and dephased spectra. Spectra were acquired with a 3 μs ^{1}H 90° pulse width, 1 ms contact time, 2 s recycle time, 83 kHz ^{1}H decoupling field, and the data was processed off-line on a Silicon Graphics 4D25TG computer using FELIX (Biosym). ^{15}N chemical shifts are reported referenced to $^{15}NH_4Cl$ at 0 ppm.

3. Results and discussion

Fig. 1 shows ^{15}N CP-MAS REDOR difference (ΔS) solid-state NMR spectra of [U-^{15}N]UDP-NAG EPT covalently modified with PEP in the presence of UDP-NAG, and mixed with UDP-NAG alone and with 3-*Z*-fluoro-PEP or [2,3-$^{13}C_2$]PEP

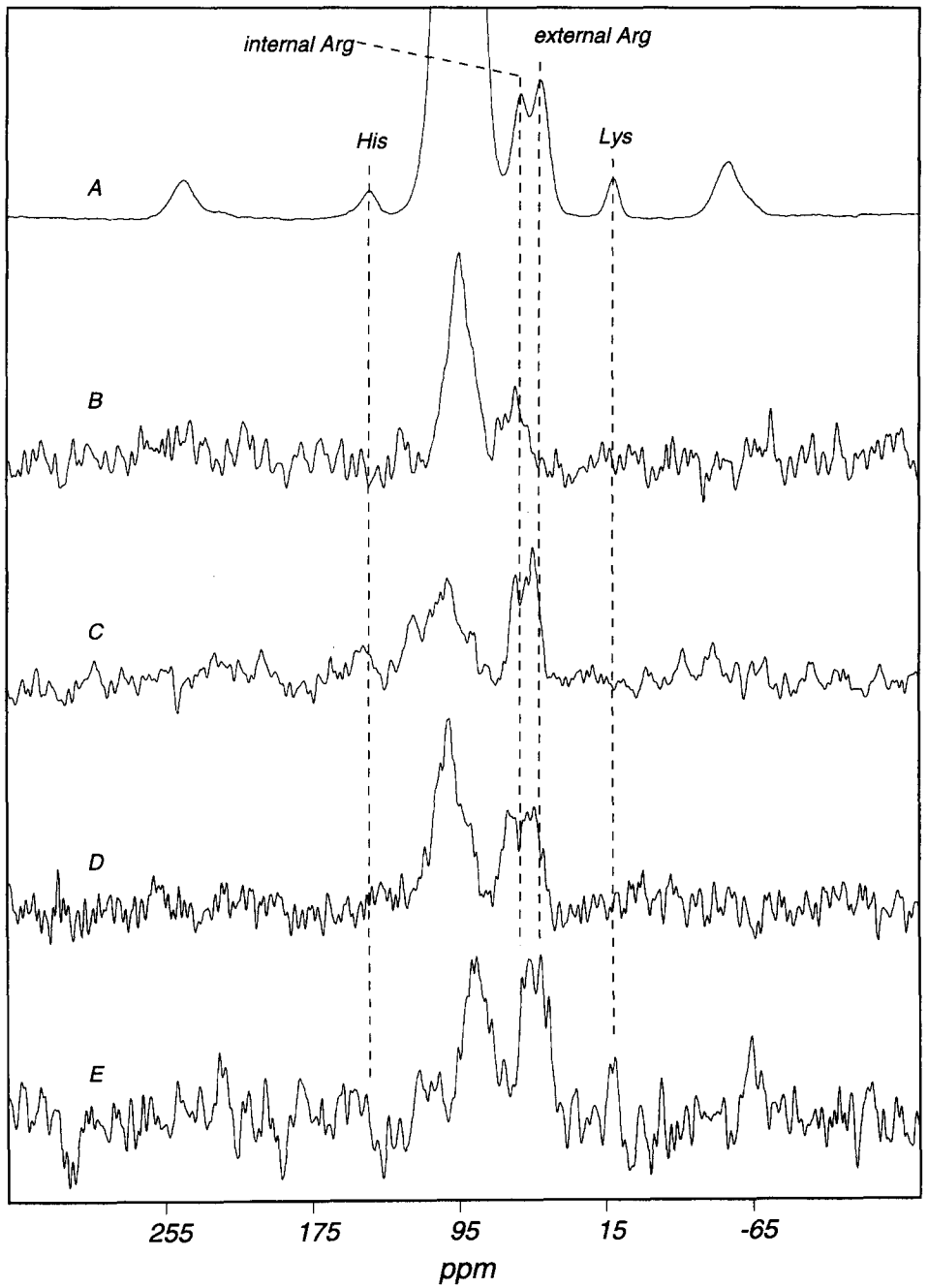
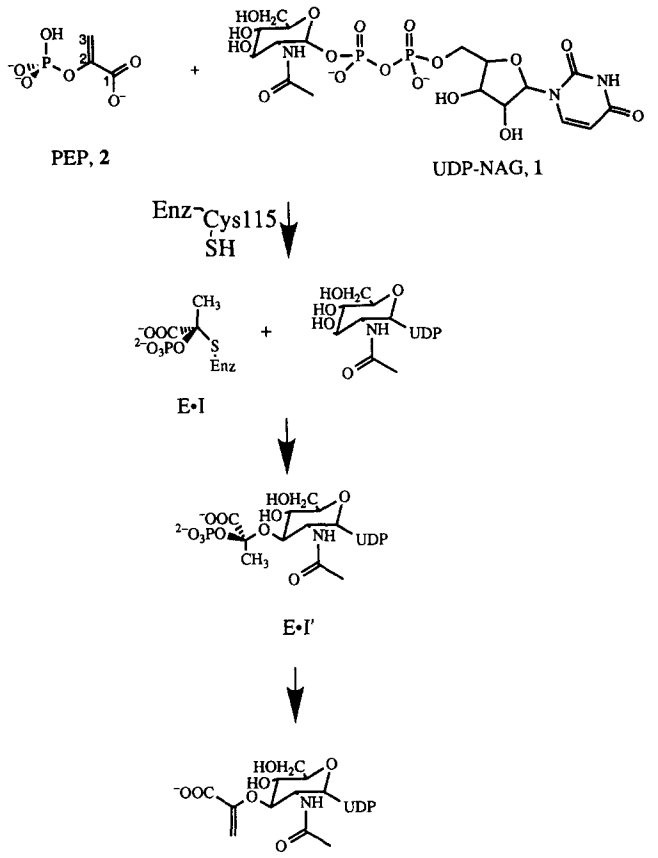


Fig. 1. The 9.4 T ^{15}N CP-MAS solid-state REDOR difference (ΔS) NMR spectra of [U-^{15}N]UDP-NAG EPT: (A) the full echo spectrum; (B) plus UDP-NAG (EPT:UDP-NAG = 1:1); (C) plus UDP-NAG and 3-*Z*-fluoro-PEP (EPT:UDP-NAG:3-*Z*-fluoro-PEP = 1:1.2:1.2); (D) covalently modified with [2,3-$^{13}C_2$]PEP (purified from mixture of EPT:UDP-NAG:[2,3-$^{13}C_2$]PEP = 1:1:10); and (E) plus UDP-NAG and [2,3-$^{13}C_2$]PEP (EPT:UDP-NAG:[2,3-$^{13}C_2$]PEP = 1:1.2:1.2) under time-resolved conditions (50 ms into the reaction). The samples were lyophilized at <243 K and the spectra obtained at 223 K. The NMR data were obtained with approximately the same number of scans for each spectrum, and they are displayed with normalized intensities (i.e. the noise level is the same in B–E), referenced to $^{15}NH_4Cl$ at 0 ppm.

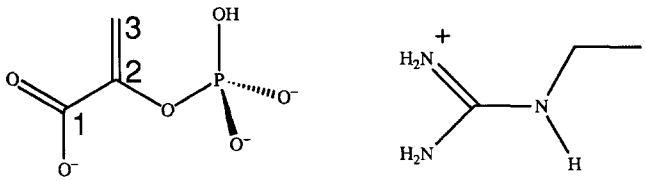


Enolpyruvyl UDP-NAG, 3

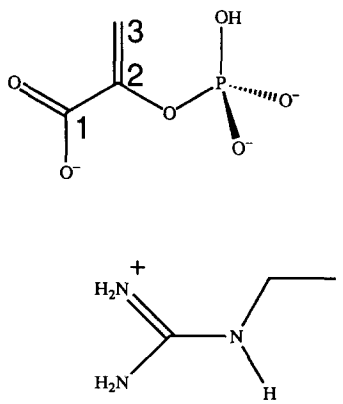
Scheme 1.

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 non-specific binding, which would lead to erroneous interpretations of the data. At this stage lyophilized solids have been employed; methods for performing REDOR measurements on frozen solutions are under development in this laboratory. In the case of the complex of the enzyme with UDP-NAG alone, this is stable, and therefore did not require special handling. However, in the cases where either the covalent intermediate, the substrate analogue 3-*Z*-fluoro-PEP or the true substrate were used and trapped under time-resolved conditions (the time-resolution being 50 ms with [2,3-$^{13}C_2$]PEP), the samples were lyophilized and maintained at low temperature. The presence of bound substrate or intermediate in the samples in Fig. 1B–E was confirmed by CP-MAS TOSS solid-state ^{31}P-NMR obtained after the lyophilization and 3-day REDOR data accumulations (data not shown), where in most cases there is sufficient ^{31}P chemical shift dispersion to resolve the species present.

The REDOR difference spectra also show which residues in the enzyme active site are within approximately 6 Å of the phosphorus nuclei in the bound substrates/inhibitor and either intermediate or product (without distinguishing between phosphorus nuclei if there are more than one). The intensities observed in the REDOR difference spectra are proportional to distance as indicated in Eqs. 1 and 2 given above, and since substrates are present in essentially equimolar concentrations with enzyme, and exceed the K_M values for the substrates (the K_M for UDP-NAG and PEP are in the μM regime) or K_i (the K_i for 3-*Z*-fluoro-PEP is also in the μM regime) for inhibitors, represent specific intramolecular complexes without complications from adjacent molecules, which would be too far away. In addition to the large central resonance at 95 ppm, which arises from the amide nitrogens, the spectra in Fig. 1B–E show additional resonances which arise from arginine residues –there are a total of 24 arginine residues in EPT. For some reason, the intensity of the lysine resonance in the full echo spectrum (Fig. 1A) is not as large as predicted by the number of lysine resiues, which is 20 in EPT, and this is being investigated further. Some interaction to lysine is apparent in Fig. 1E, but not for the other complexes. Interestingly somewhat different interactions appear with the different substrates to those seen with EPSP synthase [14], principally to arginine and the amides. Of particular note is the fact that it appears that the phosphates interact with both the terminal and internal nitrogens of the guanidino group of arginine in the complexes with 3-*Z*-fluoro-PEP or PEP, implying that they are in a side-by-side arrangement (see Scheme 2). To date there is no chemical modification or mutagenesis data to confirm or refute these observed interactions, although considering the relatively high sequence similarity between EPSP synthase and UDP-NAG EPT (for example, using the *Z*-scores of the BestFit program from the GCG package, EPSPS from *E. coli* versus *Ent. cloacae* UDP-NAG EPT gives $Z = 13.3$, where generally $Z > 9$ indicates significant sequence similarity, and $Z < 3$ indicates no significant sequence similar-



End-on Arrangement for EPSP Synthase



Side-by-side Arrangement for UDP-NAG EPT

Scheme 2.

ity), sequence alignments reveal a number of conserved lysine and arginine residues. There is a considerable amount of chemical modification and mutagenesis data on EPSP synthase suggesting that there are at least three lysine and two arginine residues which are both highly conserved and appear to be located in the enzyme active site, consistent also with the X-ray structure of the enzyme. However, the interactions of substrates, intermediates and products with arginine appear to be in an end-on arrangement (Scheme 2).

Fig. 1B shows that for the complex with bound UDP-NAG, there are strong interactions to the backbone amides and interactions only with the internal arginine nitrogens. Although not quantified in this study, the strength of the interaction is determined by the resonance intensities in the difference spectra, which are directly related to distance. Fig. 1C shows that both 3-*Z*-fluoro-PEP and the fluorinated intermediate that is formed [23,26] interact with the enzyme active site in a different manner compared to the other complexes, an observation also true for EPSP synthase [14]. In particular, there appear to be additional interactions to arginine, and fewer interactions with backbone amide residues. Fig. 1D shows that in the complex with the covalent intermediate, there are strong interactions to both backbone amide groups and to internal and external nitrogens in arginine residues. Finally, in the complex trapped under time-resolved conditions (Fig. 1E), there appear to be different interactions with the amide backbones residues compared to the other complexes. This result is perhaps surprising, as one might have anticipated that similar amide interactions to those seen in the complex with UDP-NAG and 3-*Z*-fluoro-PEP (Fig. 1C), or interactions representing the sum of Fig. 1B and D. Clearly, there is some conformational change associated with the formation of the covalent and non-covalent intermediates in the presence of UDP-NAG that is not present in the complex with either UDP-NAG alone or purified EPT covalently modified with the *O*-phosphothioketal. Additional interactions are also observed with arginine residues, that probably involve both internal and external nitrogens, and with lysine residues.

Time-resolved solid-state NMR spectroscopy used in conjunction with heteronuclear solid-state REDOR NMR methods for distance measurements, as outlined here, is clearly an important method for the study of enzymatic reaction mechanisms. Experiments are currently underway to measure intermolecular and intramolecular distances quantitatively.

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