Detection of the Covalent Intermediate of UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase by Solution-State and Time-Resolved Solid-State NMR Spectroscopy†

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ABSTRACT: Uridine diphosphate-*N*-acetylglucosamine (UDP-NAG) enolpyruvyl transferase from *Enterobacter cloacae* catalyzes the transfer of an enolpyruvyl moiety from phosphoenolpyruvate (PEP) to the 3-hydroxyl of UDP-NAG to form enolpyruvyl UDP-NAG and inorganic phosphate. Indirect evidence for the involvement of a covalent intermediate, in which the C-2 of *O*-phosphothioketal moiety is attached to Cys-115, in the reaction catalyzed by UDP-NAG enolpyruvyl transferase has been reported by Wanke and Amrhein [Wanke, C., & Amrhein, N. (1993) *Eur. J. Biochem. 218*, 861−870]. In the enzyme from *Escherichia coli*, a noncovalent tetrahedral intermediate in which the C-2 of PEP is attached to the 3-OH of UDP-NAG via an ether linkage has been isolated by Marquardt et al. [Marquardt, J. L., Brown, E. D., Walsh, C. T., & Anderson, K. S. (1993) *J. Am. Chem. Soc. 115*, 10398−10399]. In this study, we provide direct evidence for the formation of a covalent *O*-phosphothioketal enzyme intermediate from UDP-NAG enolpyruvyl transferase of *E. cloacae* overexpressed in *E. coli*. The intermediate was obtained by incubation of the enzyme with [2,3-$^{13}C_2$]PEP and UDP-NAG and was characterized by solution-state 1D ^{13}C and ^{31}P NMR, ^{13}C DEPT NMR, and $^{1}H\{^{13}C\}$2D HMQC NMR spectroscopy. The ^{13}C NMR spectra showed two coupled resonances at 29.3 and 88.7 ppm which were assigned to the C-3 and C-2 of the covalent intermediate, and the ^{13}C DEPT confirmed that C-3 was a methyl group and C-2 was quaternary. The ^{31}P NMR spectra showed a resonance at −0.18 ppm, which indicated the presence of a phosphate group in the intermediate, and these resonances disappeared when the intermediate was incubated with excess UDP-NAG. Direct characterization of the covalent intermediate was carried out using time-resolved solid-state NMR spectroscopy. ^{13}C TOSS CP-MAS solid-state NMR spectra of the rapidly frozen solution showed two resonances that are consistent with the C-2 and C-3 resonances of the covalent enzyme intermediate as obtained by ^{13}C NMR solution-state spectroscopy, as well as resonances that are consistent with the C-3 and C-2 of the phosphoketal moiety of a noncovalently bound intermediate.

Uridine diphosphate-*N*-acetylglucosamine (UDP-NAG)[1] enolpyruvyl transferase catalyzes the first committed step in the synthesis of bacterial cell wall peptidoglycan (Rogers et al., 1980). The enzyme is involved in the unusual transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 3′-hydroxyl group of the glucosamine moiety of UDP-NAG to form enolpyruvyl UDP-NAG (UDP-NAG-EP) and inorganic phosphate. Another enzyme that catalyzes the transfer of the enolpyruvyl moiety of PEP is 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. There is some similarity in the amino acid sequences of EPSP synthase from *Escherichia coli* and UDP-NAG enolpyruvyl transferase from *Enterobacter cloacae* (25% sequence identity and 47% sequence similarity), but their mechanistic similarity remains in question.

The mechanism of EPSP synthase has been investigated more extensively than UDP-NAG enolpyruvyl transferase. The EPSP synthase reaction pathway involves a tetrahedral enzyme intermediate arising from nucleophilic attack of the 5-hydroxyl group of shikimate-3-phosphate on C-2 of PEP, with concomitant protonation of C-3 of PEP (Anderson et al., 1988a,b; Barlow et al., 1989; Evans, 1992). In contrast, the mechanism of UDP-NAG enolpyruvyl transferase remains unclear in the light of two different lines of evidence published recently. The involvement of a noncovalent tetrahedral intermediate (**2** in Scheme 1) in the UDP-NAG enolpyruvyl transferase from *E. coli* has been reported recently by Marquardt et al. (1993), and they concluded that the mechanisms of EPSP synthase and UDP-NAG enolpyruvyl transferase are similar. The NMR assignments presented in this paper only weakly support the structure proposed for the species, since the majority of the resonances in the ^{1}H NMR spectrum of the "purified" species were unassigned (actually only one resonance was assigned), with a single ^{13}C chemical shift, which showed a possible $^{2}J_{CP}$ coupling, but no $^{2}J_{PC}$ coupling in the ^{31}P spectrum, as the only evidence for the proposed structure. This highlights one of the

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[1] Abbreviations: CP-MAS, cross-polarization magic angle spinning; DEPT, distortionless enhancement by polarization transfer; DTT, dithiothreitol; E-I, enzyme intermediate; EPSP, 5-enolpyruvylshikimate 3-phosphate; FPLC, fast protein liquid chromatography; HMQC, heteronuclear multiple-quantum coherence; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; TOSS, total suppression of sidebands; UDP-NAG, uridine-*N*-acetylglucosamine; UDP-NAG EP, uridine-*N*-acetylglucosamine enolpyruvate.

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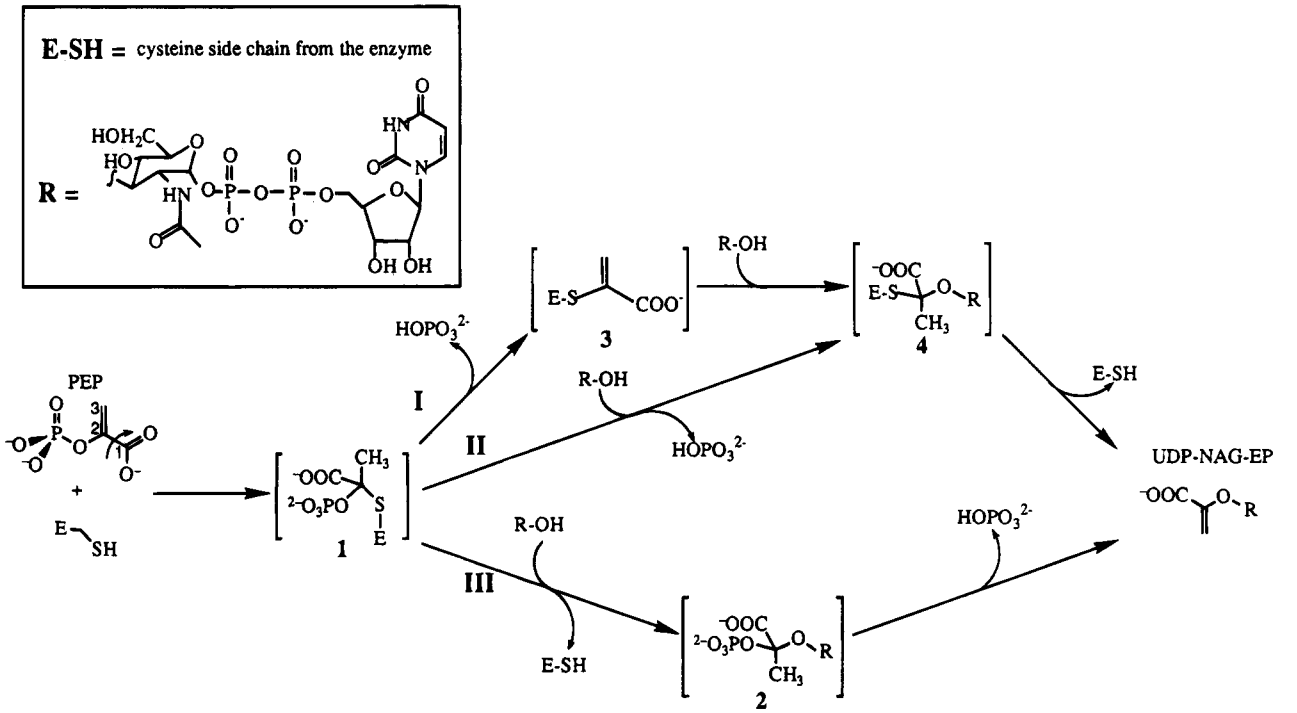
Scheme 1: Mechanism of UDP-NAG Enolpyruvyl Transferase[a]



[a] All the intermediates are covalently bound to the enzyme except **2**, which is a noncovalently bound intermediate. Enzyme intermediate **1** is the proposed structure for the isolated covalent enzyme intermediate from *E. cloacae* UDP-NAG enolpyruvyl transferase, which *only* forms in the presence of UDP-NAG. Intermediate **2** was reported to have been isolated from *E. coli* UDP-NAG enolpyruvyl transferase.

weaknesses of the chemical quench approach, which is that even when rigorous NMR assignments and structural determination have been carried out, there is always the possibility that the species isolated might represent an artefact of isolation.

Wanke and Amrhein (1993), however, showed indirect evidence from ^{32}P and ^{14}C labeling for the involvement of a covalent intermediate (**1** in Scheme 1) for the enzyme from *E. cloacae* UDP-NAG enolpyruvyl transferase. It was proposed that the intermediate is an *O*-phosphothioketal of pyruvic acid bound to Cys-115 of the enzyme. This was based on identification of tryptic fragments containing a radioactive C_3 unit and confirmation by site-directed mutagenesis of C115S, which resulted in inactive (<0.05%) enzyme. The intermediate forms from PEP *only* when the enzyme is incubated with UDP-NAG. On the basis of these observations and quantum chemistry calculations from the Evans laboratory (Li and Evans, unpublished results), a possible mechanism for the reaction of UDP-NAG enolpyruvyl transferase involves either the covalent intermediate or, conceivably, both noncovalent and covalent enzyme intermediates as illustrated in Scheme 1. Of the three possible mechanisms shown, mechanism I has been discounted because intermediate **3** has an inordinately high energy, mechanism II cannot be discounted at this stage, although there is no evidence to date for the covalent intermediate **4**, and mechanism III is the most likely, although the evidence for intermediate **2** is weak at present.

Evans and co-workers have applied the new method of time-resolved solid-state NMR spectroscopy (Evans, 1995) to detect the transient enzyme intermediate complex of EPSP synthase. The kinetic competence of the intermediate was demonstrated both under pre-steady-state and steady-state conditions (Appleyard et al., 1994; Evans et al., 1992, 1993). In this paper we report the direct detection of the enzyme intermediate of UDP-NAG enolpyruvyl transferase by time-resolved solid-state NMR spectroscopy as well as by solution-state NMR spectroscopy.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were purchased from Sigma (St. Louis, MO) except [2,3-$^{13}C_2$]PEP which was obtained from MSD Isotopes (Canada).

Purification of UDP-NAG Enolpyruvyl Transferase. The enzyme was purified from *E. cloacae* according to the method previously reported (Wanke & Amrhein, 1993). All manipulations were carried out at 4 °C.

Enzyme Activity Assay and Protein Determination. UDP-NAG enolpyruvyl transferase activity was determined by measuring the rate of release of inorganic phosphate (Lanzetta et al., 1979). Protein was determined by the method of Bradford (1976).

Modification of UDP-NAG Enolpyruvyl Transferase. The covalent enzyme intermediate was prepared by incubating 0.01 mM 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, and 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 Tris buffer, pH 7.4. Fractions corresponding to the enzyme intermediate were pooled, washed extensively with Tris buffer, and concentrated to about 200 μL by ultrafiltration.

Solution-State NMR Spectroscopy. Solution ^{13}C and ^{31}P NMR data were obtained on a Varian VXR-500 spectrometer operating at a ^{13}C frequency of 125.697 MHz and a ^{31}P frequency of 202.334 MHz. The ^{13}C NMR data were

acquired at 277 K using a 90° excitation pulse, 2.543 s recycle time, 32 K points, and a spectral width of 30 kHz. ^{31}P NMR data were acquired at 277 K using a 90° excitation pulse, 1.749 s recycle time, 16 K points, and a spectral width of 12.6 kHz. The spectra were acquired over the course of 2−15 h. Two-dimensional ^{1}H{^{13}C}HMQC (with GARP decoupling) was performed on a Bruker AMX-300 spectrometer using the standard HMQC pulse sequence (Bax et al., 1983) at a ^{13}C frequency of 75.47 MHz and a ^{1}H frequency of 300.13 MHz, with a spectral width of 2.5 and 5.7 kHz in the proton and carbon domains, respectively, with accumulations for 10 h at 277 K. DEPT data were acquired on a Bruker AMX-300 using the standard DEPT135 pulse sequence at a ^{13}C frequency of 75.47 MHz and a ^{1}H frequency of 300.13 MHz with a spectral width of 9.1 kHz, maintained at a temperature of 277 K. The temperature was maintained with a Bruker VT-1000E variable-temperature control unit, using boil-off liquid nitrogen. Data were processed off-line on a Silicon Graphics 4D25TG computer using FELIX (Biosym).

Rapid Freeze−Quench Experiments. The fast-frozen samples were prepared at a mixing time of 50 ms according to the method previously described (Appleyard & Evans, 1993; Appleyard et al., 1994). The enzyme to substrate ratio, UDP-NAG enolpyruvyl transferase/UDP-NAG/[2,3-$^{13}C_2$]-PEP, was 1:2:2. The rapidly frozen solution was kept at 177 K prior to acquisition of data by solid-state NMR spectroscopy.

Solid-State NMR Spectroscopy. ^{13}C TOSS CP-MAS spectra were obtained on the enzyme−substrate mixture under pre-steady-state and steady-state conditions using a Chemagnetics CMX-400 solid-state NMR spectrometer, operating at 400.1 MHz for ^{1}H and 100.6 MHz for ^{13}C. The spectra were acquired at 223 K using a 4 μs pulse width, a 0.5 s contact time, and a 2 s relaxation delay. The data were also processed off-line using FELIX (Biosym).

RESULTS

Solution-State ^{13}C NMR Spectroscopy. Figure 1C shows the ^{1}H-decoupled ^{13}C NMR difference spectrum of the enzyme modified with [2,3-$^{13}C_2$]PEP (see Materials and Methods). The appearance of four new resonances at 29.3 ($^{1}J_{CC}$ = 37.9 ± 0.2 Hz), 30.5 ($^{1}J_{CC}$ = 39.4 ± 0.2 Hz), 88.7 ($^{1}J_{CC}$ = 37.9 ± 0.2 Hz, $^{2}J_{CP}$ = 7.0 ± 0.2 Hz), and 161.6 ($^{1}J_{CC}$ = 79.3 ± 0.2 Hz) ppm in the covalently modified enzyme is clearly evident in the difference spectrum. The coupling constants for the resonances at 29.3, 30.5, and 88.7 ppm are characteristic of carbon atoms that are sp^3 hybridized. In contrast, the coupling constant for the sp^2 carbons in [2,3-$^{13}C_2$]PEP and the UDP-NAG enolpyruvate product are $^{1}J_{CC}$ = 80.7 ± 0.2 and 81.2 ± 0.2 Hz, respectively. The resonance at 30.5 ppm, which has a line width of 224 Hz, is assigned to C-3 of bound pyruvate, close to that previously reported by this laboratory (Barlow et al., 1989) for free pyruvate (29.4 ppm). The resonance at 29.3 ppm, which has a line width of 170 Hz, is assigned to C-3 of the *O*-phosphothioketal moiety of the covalent enzyme intermediate. The C-2 of the enolpyruvyl moiety of the tetrahedral intermediate of EPSP synthase has a chemical shift of 107 ppm (Barlow et al., 1989), which is consistent with a carbon atom in a diether linkage. The resonance at 88.7 ppm, which has a line width of 172 Hz, has an upfield shift

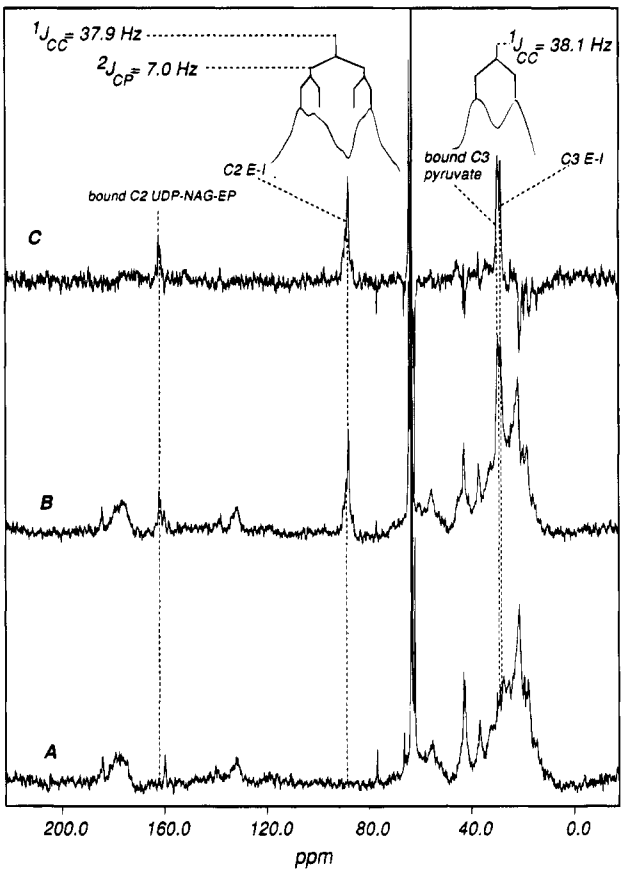


FIGURE 1: ^{1}H-decoupled ^{13}C solution-state NMR spectra (at 125.7 MHz) of (A) free UDP-NAG enolpyruvyl transferase, (B) UDP-NAG enolpyruvyl transferase covalently modified with [2,3-$^{13}C_2$]-PEP (in the presence of UDP-NAG and purified by FPLC), and (C) the difference spectrum (B − A). The spectra were acquired using 2.2 mM enzyme in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 12 h. The insets show line narrowed expansions of the resonances at 29.3 and 88.7 ppm, displaying the couplings referred to in the text.

of ~18 ppm relative to the C-2 of the enolpyruvyl moiety of the intermediate of EPSP synthase. This difference in chemical shift is consistent with the difference (24 ppm) in the chemical shifts of the model compounds 1,3-dioxolane (the chemical shift of the carbon atom in a diether linkage is 95.4 ppm) and 1,3-oxathiane (the chemical shift of the carbon atom in a thioether linkage is 71.2 ppm) (Levy et al., 1980). The resonance at 88.7 ppm is therefore consistent with a carbon atom in a thioether linkage, and the additional $^{2}J_{CP}$ coupling implies the presence of a phosphate group. The resonance at 161.6 ppm, which has a line width of 213 Hz, is tentatively assigned to C-2 of the enolpyruvyl moiety of bound product, UDP-NAG EP, although it is not clear why the corresponding resonance due to C-3 of bound product is not detectable. The insets in Figure 1 display the characteristic $^{1}J_{CC}$ couplings for the resonances assigned to the covalent intermediate. In turnover experiments, shown in Figures 2 and 3, the loss of the resonances assigned to covalent intermediate is demonstrated. In Figure 2C, these resonances have disappeared after addition of excess UDP-NAG to the covalently modified enzyme and two different resonances corresponding to the product, UDP-NAG-EP, with the C-3 of the enolpyruvyl moiety at 97.3 ppm and the C-2 at 158.6 ppm, have replaced them (refer to structures shown in Scheme 1). Figure 3A shows the ^{13}C NMR spectrum of the enzyme with a 10-fold excess UDP-NAG.

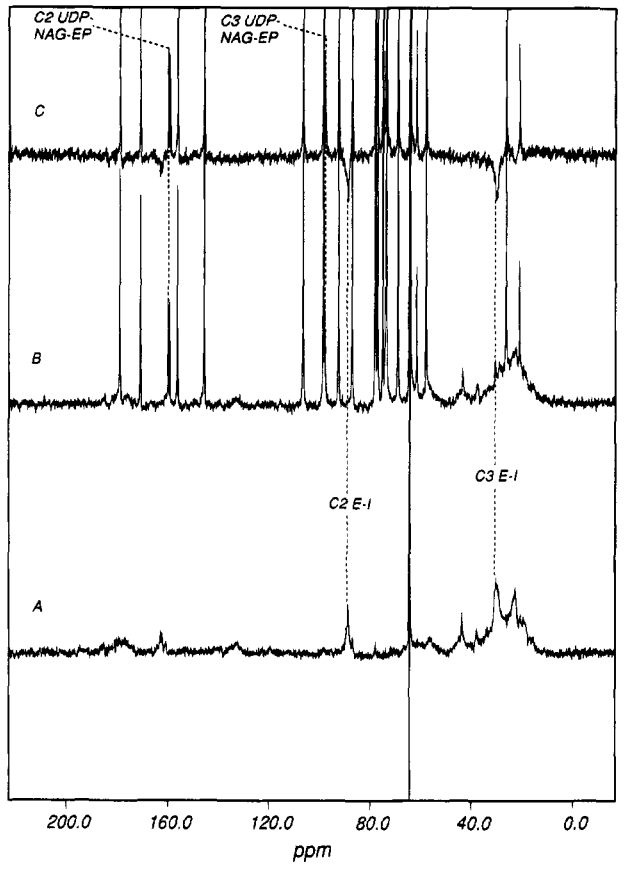


FIGURE 2: ^{1}H-decoupled ^{13}C solution-state NMR spectra (at 125.7 MHz) of (A) the UDP-NAG enolpyruvyl transferase modified with [2,3-$^{13}C_2$]PEP (see Materials and Methods), (B) the same modified enzyme *plus* UDP-NAG (43.6 mM), and (C) the difference spectrum (B − A). The spectra were acquired using 2.3 mM enzyme in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 3 h.

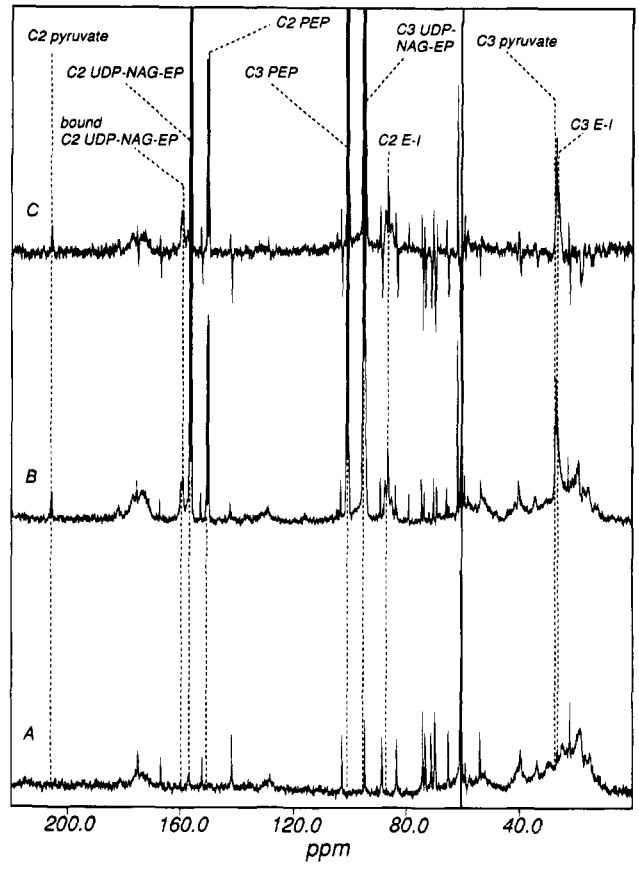


FIGURE 3: ^{1}H-decoupled ^{13}C solution-state NMR spectra (at 125.7 MHz) of (A) UDP-NAG enolpyruvyl transferase (1.9 mM) plus excess UDP-NAG (18.2 mM), (B) UDP-NAG enolpyruvyl transferase (2.0 mM) plus excess UDP-NAG (18.2 mM) + excess [2,3-$^{13}C_2$]PEP (18.3 mM), and (C) the difference spectrum (B − A). The data were acquired in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 12 h.

Only the resonances for the free enzyme and UDP-NAG at natural abundance are evident. Upon addition of [2,3-$^{13}C_2$]-PEP, seven major resonances with chemical shifts of 29.3, 88.7, 97.3, 103.2, 152.5, and 158.6 ppm appeared. The resonances at 29.3 and 88.7 ppm clearly correspond to those that were observed in the ^{13}C NMR spectrum of the isolated, covalently modified enzyme as shown in Figure 1C. The resonances at 103.2 and 152.5 ppm are assigned to C-3 and C-2 of [2,3-$^{13}C_2$]PEP (Barlow et al., 1989) and those at 97.3 and 158.6 are the C-3 and C-2 of the enolpyruvyl moiety of the product. The resonance at 29.3 ppm is also assigned to C-3 pyruvate since there is a resonance at 207.8 ppm which is consistent with the carbonyl C-2 of pyruvate (Barlow et al., 1989). The minor resonance at 161.6 ppm may correspond to bound C-2 of the enolpyruvyl moiety of the product, and possibly the resonance due to C-3 of bound product is underneath that of free product at 97.3 ppm.

Solution-State ^{31}P NMR Spectroscopy. There are four major resonances in the ^{1}H-decoupled ^{31}P spectrum of the modified enzyme shown in Figure 4A. The resonance at 2.75 ppm is assigned to inorganic phosphate (P_i) and the one at −0.83 ppm corresponds to the phosphorus of PEP. The resonance at −0.18 ppm, which disappears upon addition of excess UDP-NAG to the modified enzyme, is assigned to the phosphorus of the intermediate (although no $^{2}J_{PC}$ was resolved for this resonance). There is a precedent for phosphomonoesters at a tetrahedral carbon being upfield of the inorganic phosphate resonance, for example, the tetrahedral intermediate of EPSP synthase (Anderson et al., 1988b; Y. Li, R. J. Appleyard, and J. N. S. Evans, unpublished results). The broad resonance at −9.85 ppm which is present even after the addition of the other substrate and after subjecting the modified enzyme to ultrafiltration and FPLC corresponds to tightly bound UDP-NAG since the same peak appears in the ^{31}P spectrum of the free enzyme with 0.5 equiv of UDP-NAG (Figure 5B). This peak is not present in the ^{31}P spectrum of the free enzyme plus 10 equiv of PEP (Figure 5A). The reason that the α and β phosphorus resonances are not resolved is that the line width of the bound species is 264 Hz, which is almost as large as the separation between the two resonances (340 Hz in free UDP-NAG). After addition of UDP-NAG to the modified enzyme (see Figure 4B), two major resonances appear at −11.1 and −12.7 ppm, corresponding to the α and β phosphorus atoms of the UDP-NAG moiety of the product, together with a minor resonance at 1.54 ppm, consistent with bound P_i. The observation of the peak at −0.18 ppm which disappears upon addition of excess UDP-NAG to the modified enzyme is consistent with the intermediate being an *O*-phosphothioketal moiety rather than a thioketal moiety.

2D $^{1}H\{^{13}C\}$HMQC NMR Spectroscopy. Figure 6 shows the HMQC spectrum of the covalently modified enzyme. The resonances in the proton domain occur at 1.06, 1.27, 1.52,

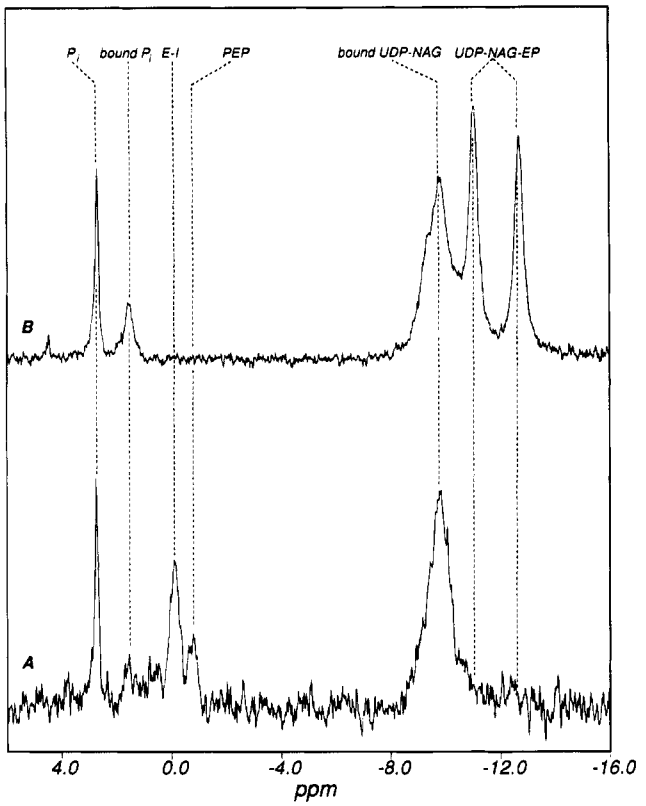


FIGURE 4: ^{1}H-decoupled ^{31}P solution-state NMR spectra (at 202.3 MHz) of (A) the UDP-NAG enolpyruvyl transferase (2.4 mM) covalently modified with [2,3-$^{13}C_2$]PEP (in the presence of UDP-NAG and purified by FPLC), and (B) the modified enzyme *plus* UDP-NAG (4.9 mM). The data were acquired in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 70 min.

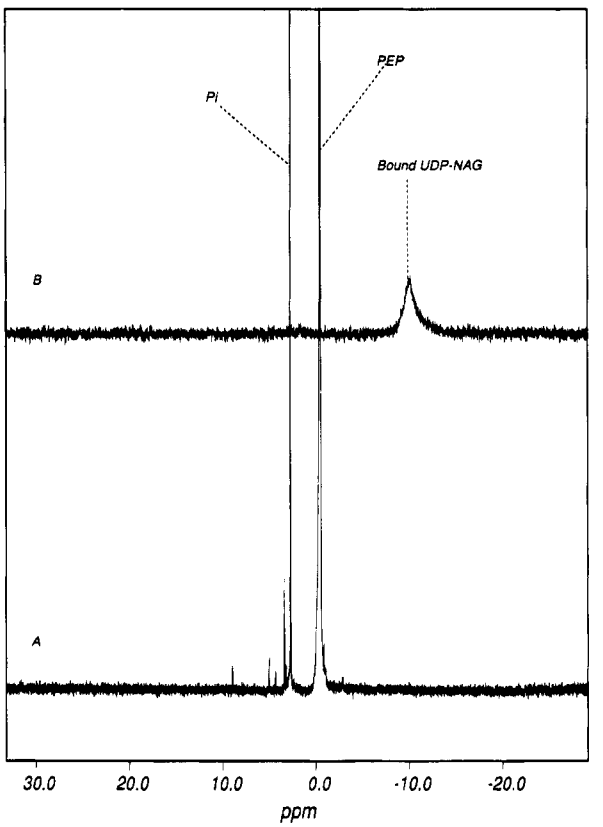


FIGURE 5: ^{1}H-decoupled ^{31}P solution-state NMR spectra (at 202.3 MHz) of (A) UDP-NAG enolpyruvyl transferase (1.8 mM) plus 10 equiv of [2,3-$^{13}C_2$]PEP. (B) UDP-NAG enolpyruvyl transferase plus 0.5 equiv of UDP-NAG. The data were acquired in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 6 h.

1.95, 2.28, and 2.59 ppm. The correlations with ^{13}C resonances are 28.5, 29.3, 28.5, 29.4, 29.9, and 42.2 ppm, respectively. The proton resonances at 1.06 and 1.95 ppm are assigned to the C-3 methyl protons of hydrated pyruvate and pyruvate, respectively, on the basis of our previous HMQC analysis of a PEP-utilizing enzyme (Barlow et al., 1989). The resonance at 1.27 ppm is assigned to the C-3 methyl protons of the *O*-phosphothioketal moiety of the intermediate. The broad resonances at 1.52 and 2.59 ppm presumably correspond to protein background, and the resonance at 2.28 probably corresponds to DTT.

^{13}C DEPT NMR Spectroscopy. The DEPT135 spectrum of the covalently modified enzyme shows a negative peak for the methylene carbons of Tris (spectrum not shown). An expansion of the upfield region (Figure 7) shows one broad major positive peak at 29.4 ppm and a minor resonance at 28.3 ppm. The negative resonance at 28.9 ppm is presumably due to the methylene resonances of the protein natural abundance background. This result indicates that these resonances correspond to either methyl or methine carbon atoms. That they represent methine carbons can be ruled out from the HMQC data (*vide supra*). There is no resonance detectable at 88−100 ppm, which indicates that the resonances at 88.7 and 99 ppm corresponding to C-2 of the *O*-phosphothioketal moiety of the intermediate and C-2 of hydrated pyruvate, respectively, are quaternary carbon atoms. Thus, the carbon atoms at 28.3 and 29.4 ppm are methyl carbon atoms, and their assignments are indicated on Figure 7.

Time-Resolved Solid-State ^{13}C NMR Spectroscopy. Figure 8 shows the ^{13}C TOSS CP-MAS difference spectra, with the free enzyme subtracted, of UDP-NAG enolpyruvyl transferase and UDP-NAG rapidly mixed under nominal single-turnover conditions (at an enzyme/UDP-NAG/PEP ratio of 1:2:2) with [2,3-^{13}C]PEP and freeze-quenched under pre-steady-state conditions. At a 50 ms reaction time, there is no product observed (Figure 8A). The resonances due to C-2 (152 ppm) and C-3 (99.1 ppm) of PEP are clearly apparent. The C-3 of the intermediate (C3 E-I) is clearly visible at 26 ppm while C-2 of the intermediate appears as a partially resolved resonance (83.2 ppm) on a broad peak centered at ~85 ppm. There are also major resonances at 21 and 104 ppm. The resonance at 104 ppm is consistent with the C-2 of the tetrahedral intermediate of EPSP synthase (Evans et al., 1993). The resonance at 21 ppm is consistent with C-3 of the tetrahedral intermediate of EPSP synthase on the basis of the chemical shift (25.2 ppm) of the C-3 of the tetrahedral intermediate of EPSP synthase obtained from solution-state NMR (Barlow et al., 1989) and on the basis of the relationship between the ^{13}C solution-state and ^{13}C CP-MASS solid-state chemical shifts (Appleyard et al., 1994). These resonances are therefore tentatively assigned to a noncovalent tetrahedral intermediate of UDP-NAG enolpyruvyl transferase.

Another ^{13}C TOSS CP-MAS difference spectra was acquired (Figure 8B) on the same sample used to obtain Figure 8A after 30 min of equilibration at room temperature of the rapidly frozen sample, followed by refreezing in the probe. The intensity of the resonance at 26 ppm increased

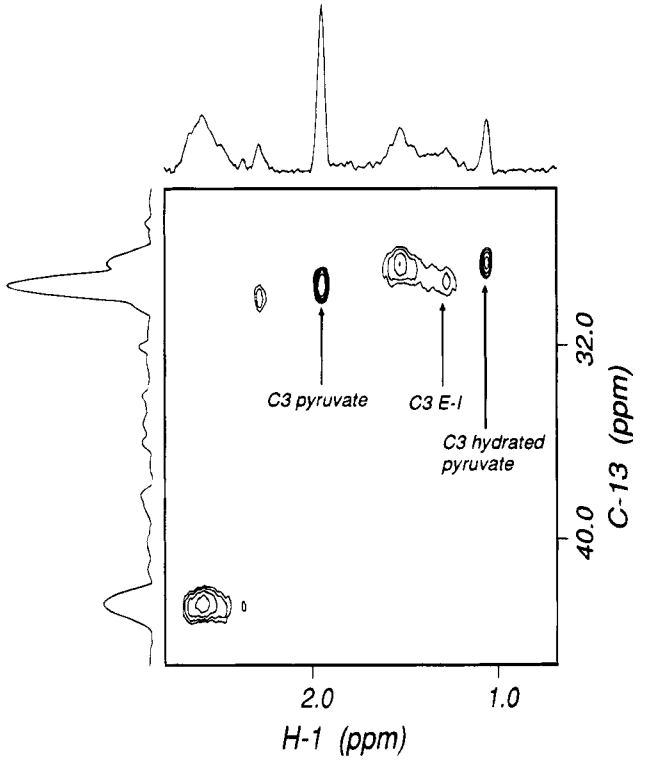


FIGURE 6: $^{1}H\{^{13}C\}$2D HMQC (with ^{13}C decoupling, at 300.13 MHz ^{1}H, 75.5 MHz ^{13}C) of UDP-NAG enolpyruvyl transferase covalently modified with [2,3-$^{13}C_2$]PEP (in the presence of UDP-NAG and purified by FPLC). The spectra were acquired using 3.7 mM enzyme in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K in about 10 h.

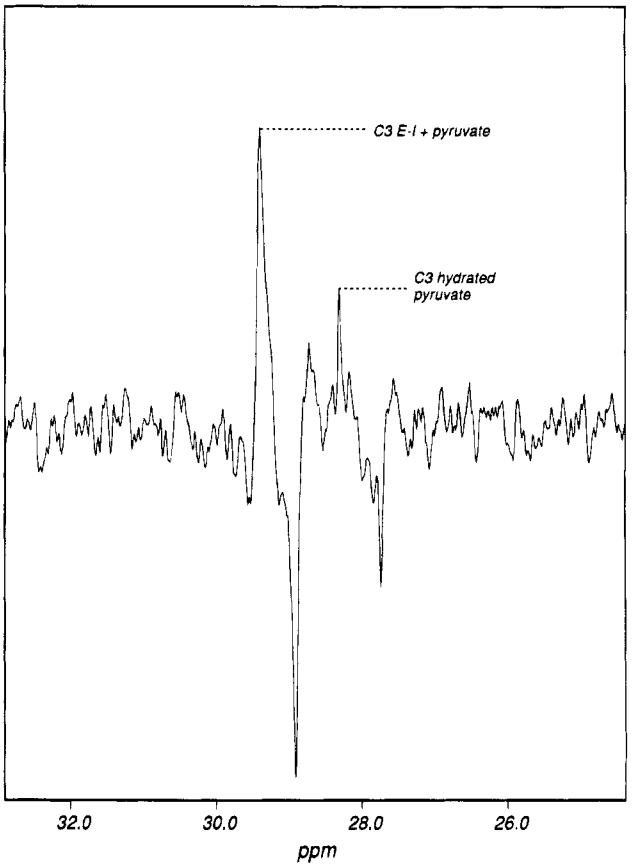


FIGURE 7: ^{1}H-decoupled ^{13}C DEPT spectrum (at 75.5 MHz) of UDP-NAG enolpyruvyl transferase covalently modified with [2,3-$^{13}C_2$]PEP (in the presence of UDP-NAG and purified by FPLC). The spectrum was acquired using 2.8 mM enzyme in 50 mM Tris-HCl (pH 7.8), 1 mM DTT, and 15% D_2O at 277 K for a total of 18 h.

significantly. Another resonance with a chemical shift of 96.5 ppm appears as a major resonance in Figure 8B. These resonances correspond to the C-3 and C-2 of hydrated pyruvate, respectively, as confirmed by solution-state NMR of the equilibrated sample (data not shown). The resonances assigned to either the C2 of PEP or the enolpyruvyl moiety of the covalent intermediate or of the noncovalent intermediate disappear or are reduced in intensity in the spectrum of the equilibrated sample. A new resonance close to the C2 of the enolpyruvyl moiety of the covalent intermediate appears, which is unassigned (and marked with a "?" on Figure 8). Although the resonances due to product are not detectable in Figure 8B, presumably this is due to inefficient cross-polarization for these species, since with the same batch of active enzyme at higher enzyme to substrate ratios (for example, enzyme/UDP-NAG/PEP = 1:10:10), the resonances due to product are clearly detectable (Figure 8C).

DISCUSSION

Recent cloning of the gene coding for UDP-NAG enolpyruvyl transferase in *E. cloacae* and overexpression of the enzyme in *E. coli* has allowed the isolation of large amounts of purified enzyme, approximately 900 mg from 20 g of cells of fresh bacteria (Wanke & Amrhein, 1993). The availability of gram quantities of the enzyme has enabled the direct observation of the enzyme intermediate both by solution-state and time-resolved solid-state NMR spectroscopy.

The free enzyme was modified in the presence of equimolar amount of UDP-NAG and a 10-fold molar excess of [2,3-$^{13}C_2$]PEP. The structure of the isolated intermediate was determined by solution-state NMR spectroscopy. ^{13}C NMR difference spectra of the modified UDP-NAG enolpyruvyl transferase, minus the free enzyme, showed new resonances at 29.3 and 88.7 ppm which indicate that the enzyme was covalently modified. These resonances displayed characteristic $^{1}J_{CC}$ couplings for sp^3 carbons, and the resonance at 88.7 ppm also displayed $^{2}J_{CP}$ coupling, suggesting the presence of a phosphate group. By analogy with our work using $^{1}H\{^{13}C\}$HMQC on EPSP synthase (Barlow et al., 1989), use of the same approach with UDP-NAG enolpyruvyl transferase showed that the ^{13}C resonance at 29.3 ppm correlates with a proton resonance at 1.27 ppm, which indicates that this resonance corresponds to a methyl carbon. Further evidence to show that the resonance at 29.3 ppm is a methyl carbon was obtained from the DEPT spectrum which gave the same resonance with the opposite phase to that of the methylene carbon of Tris. Since neither the 2D HMQC spectrum nor the DEPT spectra showed a resonance at 88.7 ppm, this resonance corresponds to a quaternary carbon atom. These results therefore suggest that the methylene carbon of PEP at the C-3 position was protonated to a methyl carbon and C-2 is covalently bound to the enzyme, presumably via a cysteine residue. It has been shown previously that binding of PEP to the enzyme does not occur in the absence of the other substrate UDP-NAG (Cassidy & Kahan, 1974). Observation of a tightly bound UDP-NAG to UDP-NAG-EP transferase indicates that the role of UDP-NAG in the formation of the covalent enzyme intermediate is indeed in effecting a conformational change

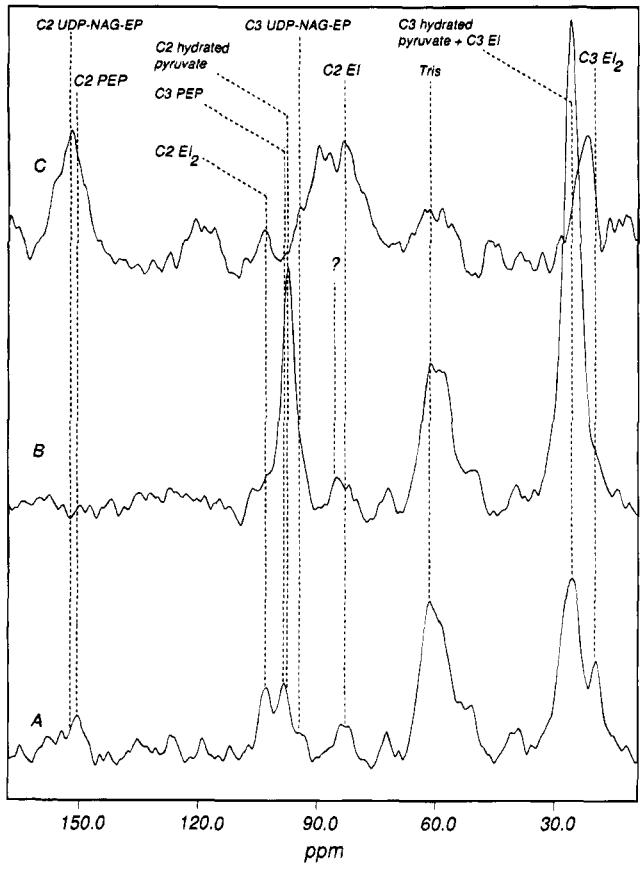


FIGURE 8: Time-resolved solid-state NMR study (at 100.6 MHz) of UDP-NAG enolpyruvyl transferase and UDP-NAG *plus* [2,3-^{13}C]PEP under nominal single-turnover conditions (enzyme/UDP-NAG/PEP ratio of 1:2:2). Solid-state ^{13}C TOSS CP-MAS difference spectra (with the free enzyme plus UDP-NAG subtracted) of UDP-NAG enolpyruvyl transferase (4 mM final concentration) and UDP-NAG (8 mM final concentration) *plus* [2,3-^{13}C]PEP (8 mM final concentration) after (A) 50 ms rapid freeze-quench and (B) 30 min equilibration at room temperature and slow freezing. In panel C, enzyme/UDP-NAG/PEP rapidly mixed and frozen in the ratio of 1:10:10, after 30 min equilibration at room temperature, and slow freezing is shown obtained under conditions identical to those in panels A and B. Data were acquired at 223 K for 36 h.

in the enzyme that favors PEP binding. Comparison of the ^{31}P NMR spectra of the covalently modified enzyme with excess UDP-NAG and the covalently modified enzyme alone show that the disappearance of a resonance at −0.18 ppm parallels the appearance of two resonances at −11.2 and −12.7 ppm. These results indicate that the covalent enzyme intermediate has a phosphothioketal and not a thioketal or enolpyruvyl moiety.

The results of the turnover experiments (Figures 2 and 3) imply that the covalent intermediate formed from the reaction of PEP and UDP-NAG is a true intermediate. ^{13}C NMR spectra of the modified enzyme alone and the modified enzyme incubated with excess UDP-NAG show that the disappearance of the resonances at 29.3 and 88.7 ppm (C-3 and C-2 of the *O*-phosphothioketal moiety of the intermediate) parallel the appearance of resonances at 97.3 and 158.3 ppm (C-2 and C-3 of the enolpyruvyl moiety of the product). ^{31}P NMR spectrum of the covalently modified enzyme plus excess UDP-NAG shows resonances at −11.1 and −12.7 ppm (β and α phosphoruses of the UDP moiety of the product) which are not present in the spectrum of the covalently modified enzyme alone. The resonance at −0.18 ppm (phosphorus of *O*-phosphothioketal moiety of the intermediate) in the ^{31}P spectrum of the covalently modified enzyme alone disappeared upon its incubation with excess UDP-NAG.

The covalent intermediate was also characterized by time-resolved solid-state NMR spectroscopy. ^{13}C CP-MAS TOSS NMR difference spectra of a mixture of enzyme and substrates under nominal single turnover-conditions (at an enzyme/UDP-NAG/PEP ratio of 1:2:2) with a mixing time of 50 ms show resonances at 26 and 83.2 ppm that correspond to the *O*-phosphothioketal moiety of the covalent enzyme intermediate. Additional resonances at 21 and 104 ppm, which have chemical shifts similar to those of the C-9 and C-8 of the tetrahedral intermediate of EPSP synthase, suggest that these resonances might correspond to the C-3 and C-2 of the phosphoketal moiety of a tetrahedral, noncovalently bound enzyme intermediate of UDP-NAG enolpyruvyl transferase. The spectra of the equilibrated sample shows a decrease in intensity for the resonances assigned to C-2 of the enolpyruvyl moiety of both the covalent and noncovalent intermediates, and PEP, and concomitant increase in intensity of the resonances due to hydrated pyruvate. Although the resonances due to product were not evident in the spectrum of the equilibrated sample, presumably this is due to inefficient cross-polarization for these species, enzyme at higher enzyme to substrate ratios (enzyme/UDP-NAG/PEP = 1:10:10) shows clearly detectable resonances due to product. This therefore demonstrates kinetic competence for the intermediates detected.

The formation of pyruvate and/or pyruvate hydrate is clearly observed in solution over a short period of time at 277 K even at a slightly basic pH (pH 7.8). It is also likely that pyruvate can be formed in the pre-steady-state reaction of UDP-NAG enolpyruvate transferase when warmed to room temperature for 30 min followed by slow-freezing at 223 K, as indicated by the large increase in the intensity of the resonance at 26 ppm (assigned to the C-3 intermediate and C-3 hydrated pyruvate). However, although the broad resonance at 26 ppm in the ^{13}C TOSS CP-MAS spectrum of a mixture of enzyme and substrates was clearly resolved into a single peak with a chemical shift of 28.2 ppm (C-3 hydrated pyruvate) in the ^{13}C NMR spectrum of the equilibrated sample in solution (data not shown), it is unlikely that hydrated pyruvate forms under time-resolved single-turnover conditions. Presumably the formation of pyruvate and/or hydrated pyruvate is an artifact resulting from nonenzymatic breakdown of the *O*-phosphothioketal intermediate.

It has previously been suggested that Cys-115 is the amino acid residue to which the C-2 of the *O*-phosphothioketal moiety of the intermediate is bound (Wanke & Amrhein, 1993) on the basis of the finding that only the C115S mutant lost enzymatic activity and from the observation that even in the denatured enzyme intermediate the thiol group of Cys-115 is not available for modification by Ellman's reagent. The resonance at 88.7 ppm (by solution-state ^{13}C NMR) and 86.0 ppm (by solid-state ^{13}C NMR) corresponding to C-2 intermediate is characteristic of a carbon in thioether linkage as compared to one with an oxyether bond (107 ppm in C-2 of EPSP synthase noncovalent intermediate). This result provides supporting evidence for the notion that a Cys residue serves as the nucleophile that attacks C-2 of PEP to form the covalent intermediate. It is highly likely that this residue is Cys-115 as previously suggested (Wanke & Amrhein, 1993).

Observation of the covalent enzyme intermediate of UDP-NAG enolpyruvyl transferase by solution-state and time-resolved solid-state NMR spectroscopy not only settles the issue of the involvement of a covalent intermediate in the reaction pathway of the enzyme, suggested in the 1970s (Cassidy & Kahan, 1973), but also demonstrates further the applicability of the time-resolved method in the study of enzyme reaction mechanisms. The advantage of time-resolved solid-state NMR spectroscopy over solution-state NMR spectroscopy in delineating the enzymatic reaction mechanism is clearly illustrated in this study. Both the formation of the covalent intermediate of UDP-NAG enolpyruvyl transferase and its kinetic competence were shown using this method alone. Although solution-state NMR spectroscopy has also been used in both the characterization of a stable covalent intermediate, and demonstration of its kinetic competence, time-resolved solid-state NMR spectroscopy has also been able to detect a putative second intermediate. Experiments in these laboratories are in progress to use this approach to delineate the pre-steady-state kinetic pathway.

In recent work reported while this paper was being revised, Kim et al. (1994) provided 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 (Walker et al., 1992) and results in accumulation of fluorinated intermediates, which are unable to proceed to product. Although a broad resonance was observed 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 noncovalent 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 artifacts can be generated, this does not provide particularly compelling evidence for the covalent intermediate. Better evidence, however, has recently been obtained by the same laboratory and again reported while this paper was being revised. Brown et al. (1994) used [2-^{13}C]PEP in a manner similar to that reported here, together with some preliminary pre-steady-state kinetic studies, which provide support for a covalent intermediate. The ^{13}C NMR spectra show the same resonance observed here and assigned to C2 of the phosphothioketal together with additional resonances arising from the substrate, [2-^{13}C]PEP, a small amount of the product, [^{13}C]UDP-NAG EP, and C3 of PEP at natural abundance (showing characteristic $^1J_{CC}$ coupling), although the latter was not assigned in the paper. The preliminary pre-steady-state kinetics appear to suggest that the covalent species is turned over at rates consistent with being on the reaction pathway. These rates were calculated from computer simulations of a kinetic model containing 12 unknowns, and the rationale is unclear for the choice of values for individual kinetic constants, aside from those few obtained experimentally. Indeed, some of the values bear a remarkable similarity to those reported by Anderson et al. (1988a) for the mechanistically dissimilar EPSP synthase. It is also asserted by Brown et al. (1994), contrary to our findings, that the covalent intermediate forms in the absence of UDP-NAG. This is a surprising assertion, given that the NMR spectrum presented showed evidence for the formation of the product supposedly in the absence of UDP-NAG, strongly suggesting that UDP-NAG is already present in their preparations. Finally in an accompanying paper, Marquardt et al. (1994) provide confirmation of the earlier observation (Wanke & Amrhein, 1993) that fosfomycin inactivates UDP-NAG enolpyruvyl transferase by specifically modifying Cys 115.

Since the evidence presented in this paper is conclusive for the involvement of a covalent enzyme intermediate **1** and provides evidence for a noncovalently bound tetrahedral intermediate **2** in the *E. cloacae* UDP-NAG enolpyruvyl transferase, we propose that mechanism III in Scheme 1 is operating. This is similar to that proposed by Walsh's group (*vide supra*) for the *E. coli* enzyme. The conversion of **1** to **2** presumably occurs by an S_N2 mechanism in which attack of the 3′-OH of UDP-NAG at C-2 of the phosphothioketal moiety of **1** leads to the formation of the free enzyme and **2**. This mechanism is analogous to that proposed for the formation of the EPSP ketal in the side-reaction catalyzed by EPSP synthase (Leo et al., 1990). In this mechanism, nucleophilic attack of the 2′-OH of the shikimate moiety occurs at the tetrahedral center, although it does result in expulsion of the phosphate leaving group, whereas in mechanism III the phosphate group is retained. Alternatively, a second covalent intermediate **4** might form (mechanism II), which would be difficult to distinguish from intermediate **1** on the basis of chemical shift, if both are present at the same time. It still remains a puzzle why UDP-NAG enolpyruvyl transferase and EPSP synthase, which catalyze the same kind of reaction, should differ so dramatically in their reaction mechanisms. It is conceivable that the advantage of having PEP covalently bound to UDP-NAG enolpyruvyl transferase is that it confers stability to the enzyme. For another enzyme of the shikimate pathway, DAHP synthase, the addition of PEP to the purified enzyme is required to increase the stability of the enzyme (McCandliss et al., 1978). Further studies are underway in our laboratories to complete the mechanistic details of this intriguing enzyme.

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