# Detection and Characterization of a Phospholactoyl–Enzyme Adduct in the Reaction Catalyzed by UDP-*N*-acetylglucosamine Enolpyruvoyl Transferase, MurZ†

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ABSTRACT: The MurZ enzyme catalyzes enolpyruvoyl transfer from phosphoenolpyruvate to the 3-OH of UDP-*N*-acetylglucosamine (UDP-GlcNAc) in *Escherichia coli* peptidoglycan biosynthesis. The kinetic mechanism of MurZ has been shown to involve the generation of a non-covalently bound tetrahedral phospholactoyl-UDP-GlcNAc intermediate [Marquardt, J. L., *et al.* (1993) *J. Am. Chem. Soc. 115*, 10398–10399]. In the work described here, MurZ overproduced in *E. coli* copurified with 1 equiv of bound PEP. Enzyme free of bound PEP was prepared by incubation of purified MurZ with UDP-GlcNAc followed by removal of small molecules. Addition of either [$^{14}$C]PEP or [$^{32}$P]PEP to PEP-free enzyme led to stoichiometric labeling. The MurZ–PEP complex was stable to 6.7 M urea, and digestion of the [$^{32}$P]PEP-labeled protein followed by SDS–PAGE generated a labeled peptide of molecular weight 5000. Solution NMR of MurZ incubated with [2-$^{13}$C]PEP suggested a tetrahedral phospholactoyl enzyme adduct attached via C-2 to an enzyme nucleophile. The $k_{on}$ for generation of the phospholactoyl enzyme in the absence of UDP-GlcNAc was 0.24 $\mu M^{-1}$ $s^{-1}$, too slow to represent the binding order of the kinetically preferred pathway since the lower limit of the second-order binding consant for PEP ($k_{cat}/K_m$) is 15 $\mu M^{-1}$ $s^{-1}$. Rapid chemical quench analysis under single-turnover conditions using [$^{32}$P]PEP in the presence of UDP-GlcNAc demonstrated that the covalent enzyme–phospholactoyl adduct appeared and decayed on a time scale consistent with catalysis. Substrate-trapping experiments demonstrated that the phospholactoyl enzyme, when incubated with UDP-GlcNAc, was competent to form the non-covalently associated phospholactoyl-UDP-GlcNAc intermediate and ultimately product. These studies are consistent with a mechanism involving the formation of two tetrahedral active site intermediates, where a phospholactoyl enzyme adduct precedes the formation of phospholactoyl-UDP-GlcNAc.

The first committed step in the synthesis of the peptidoglycan component of the bacterial cell wall involves an unusual transfer of the enolpyruvoyl moiety of PEP[1] to the 3-OH of the glucosyl group of UDP-GlcNAc to yield enolpyruvoyl-UDP-GlcNAc (Scheme 1). The vinyl ether formed is subsequently reduced with reducing equivalents from NADPH by the flavoenzyme MurB (Benson *et al.*, 1993) to generate the lactoyl ether of UDP-muramic acid, to which five amino acids are subsequently added one or two at a time to produce UDP-muramoyl pentapeptide in the cytoplasmic phase of peptidoglycan assembly. The involvement of MurZ in bacterial cell wall biosynthesis makes the enzyme a potential target for the development of new antibacterial agents, and indeed, MurZ is the site of action of the antibiotic fosfomycin (Hendlin *et al.*, 1969; Kahan *et al.*, 1974).

The enolpyruvoyl transfer catalyzed by MurZ has analogy with a small number of like utilizations of PEP including those catalyzed by enolpyruvoyl shikimate 3-phosphate (EPSP) synthase (Anderson & Johnson, 1990) and 3-keto-3-deoxyoctulosonate (KDO) 8-phosphate synthase (Ray, 1980). In terms of structural homology, most striking is the similarity of the primary structure of MurZ (Marquardt *et al.*, 1993a) with that of EPSP synthase (18.3% identical), an enzyme whose mechanism has been extensively studied. The EPSP synthase reaction pathway has been shown to involve an intermediate tetrahedral adduct arising from direct attack of the 5-hydroxyl group of shikimate 3-phosphate on C-2 of PEP to yield a C-2-substituted phospholactoyl moiety (Anderson *et al.*, 1988a,b). We have recently reported the detection and NMR characterization of a similar tetrahedral adduct between UDP-GlcNAc and PEP (Scheme 1) which was isolated by rapid chemical quench methodology (Marquardt *et al.*, 1993b). The appearance and disappearance of the tetrahedral intermediate paralleled the decay and formation of substrates and products, respectively, and was consistent with a mechanism analogous to that of EPSP synthase involving the direct attack of UDP-GlcNAc on C-2 of PEP.

Earlier mechanistic studies of UDP-GlcNAc-enolpyruvoyltransferase, however, had suggested the existence of covalent enolpyruvoyl (Zemell & Anwar, 1975) or phospholactoyl (Cassidy & Kahan, 1973) enzyme intermediates, as assessed by tight binding of radiolabeled PEP to small quantities of enzyme purified from wild-type bacteria. In this study, we have exploited the availability of large quantities of the transferase from overexpression of cloned *Escherichia coli murZ* (Marquardt *et al.*, 1993a) to examine further the possibility that a covalent enzyme adduct has a role in catalysis

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[1] Abbreviations: PEP, phosphoenolpyruvate; UDP-GlcNAc, uridine diphospho-*N*-acetyl-D-glucosamine; EP-UDP-GlcNAc, (enolpyruvoyl)-uridine diphospho-*N*-acetyl-D-glucosamine; DTT, dithiothreitol; EPSP, 5-(enolpyruvoyl)shikimate 3-phosphate.

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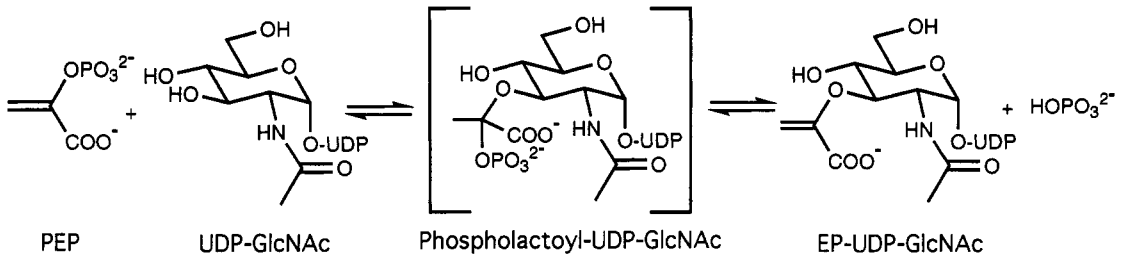
Scheme 1




by MurZ. We report the finding that enzyme, as purified, contains 1 equiv of PEP that can be converted to product by addition of UDP-GlcNAc and that the PEP equivalent is covalently bound in the active site, probably as a phospholactoyl enzyme tethered via C-2 of the phospholactoyl group. In addition, we have investigated the kinetic competence of the phospholactoyl enzyme intermediate using pre-steady-state techniques and have authenticated the role of the adduct in the reaction pathway.

## MATERIALS AND METHODS

*Chemicals.* Enolpyruvoyl-UDP-GlcNAc was synthesized enzymatically with MurZ as described previously (Benson *et al.*, 1993) and purified by anion-exchange HPLC (described below). Synthesis of [$^{32}$P]PEP was enzymatic and involved anion exchange HPLC purification (described below) of a 1-h room temperature incubation mixture (250 $\mu$L) containing 0.5 mM potassium phosphate, pH 8, 1 mCi [$^{32}$P]phosphate (9000 Ci/mmol), 100 mM Tris·HCl, pH 8, 25 mM UDP-GlcNAc-EP, and 0.05 mg/mL PEP-free MurZ (see below). Uridine diphospho-*N*-acetyl-D-[U-$^{14}$C]glucosamine (309 mCi/mmol) and phosphoenol[1-$^{14}$C]pyruvate (23 mCi/mmol) were purchased from Amersham. The [2-$^{13}$C]PEP was obtained from Dr. Jeremy Evans. The [1,2,3-$^{13}$C]PEP was synthesized enzymatically and involved anion exchange HPLC purification (described below) of a 1-h room temperature incubation mixture (1 mL) containing 30 mM potassium phosphate, pH 7, 20 mM [1,2,3-$^{13}$C]pyruvate (Cambridge Isotopes), 20 mM $MgCl_2$, 20 mM $NH_4Cl$, 20 mM ATP, 50 mM Hepes, pH 7.0, 50 $\mu$g/mL inorganic pyrophosphatase (Sigma), and 600 $\mu$g/mL pyruvate phosphate dikinase (a generous gift of Dr. Debra Dunaway-Mariano).

*Enzyme Purification. E. coli* MurZ was isolated from an overproducing strain (JLM16) with the following modifications to the previously published procedure (Marquardt *et al.*, 1993a). Freshly transformed cells(2 L) were grown, induced, harvested, lysed, and subjected to a 70% ammonium sulfate precipitation as described previously (Marquardt *et al.*, 1993a). All enzyme isolation procedures were carried out at 4 °C. Following column desalting of the ammonium sulfate pellet and anion-exchange chromatography on Pharmacia Hi-Load Q as previously described (Marquardt *et al.*, 1993a), pooled fractions were brought to 1.5 M ammonium sulfate and chromatographed on Pharmacia butyl-Sepharose (2.5 × 25 cm) in a 400-mL gradient from 1.5 to 0 M ammonium sulfate in 50 mM Tris·HCl and 5 mM DTT, pH 8. Those fractions were then dialyzed against 50 mM Tris·HCl and 5 mM DTT, pH 8, applied to a Matrex Gel Blue A (Amicon) agarose column (30 × 10 cm), and eluted in a 400-mL gradient from 0 to 1.5 M KCl in 50 mM Tris·HCl and 5 mM DTT, pH 8. Typically the purification yielded 250 mg of homogeneous MurZ of specific activity 4.5 $\mu$mol min$^{-1}$ mg$^{-1}$.

*Removal of Bound PEP from Purified Enzyme.* Purified enzyme was treated approximately 125 mg at a time by first membrane-concentrating the enzyme to a volume of approximately 5 mL and then incubating it with a 2-fold molar excess of UDP-GlcNAc for 10 min to chase covalently bound PEP to product. Subsequently, urea (10 M) was added to a final concentration of 6 M to facilitate the dissociation of any tightly but non-covalently bound substrates. That solution was then desalted on a Pharmacia Sephadex G-25 column (45 × 2.5 cm) preequilibrated in 50 mM Tris·HCl, pH 8, and 5 mM DTT. The eluted enzyme typically had a specific activity of 60% that of the starting material (2.7 $\mu$mol min$^{-1}$ mg$^{-1}$). Purified and PEP-free MurZ was stored at −80 °C. The extinction coefficient of MurZ was determined to be 0.82 cm$^2$ mg$^{-1}$ at 280 nm by quantitative amino acid analysis of enzyme of predetermined $A_{280}$. Unless otherwise indicated, PEP-free enzyme was used in all experiments and molar concentrations of MurZ refer to the concentration of active enzyme, where 60% of the total population of enzyme molecules were active.

*Enzyme Assays.* MurZ was assayed during purification using the phosphate release assay as described previously (Marquardt *et al.*, 1993a). Purified enzyme was assayed in a coupled assay with excess MurB, which reduces the product enolpyruvoyl-UDP-GlcNAc to UDP-muramic acid and couples the transferase activity with NADPH oxidation. Typically, 5 $\mu$L of MurZ solution (5 mg/mL) was added to 995 $\mu$L of coupled assay mix with the following composition: 50 mM Tris·HCl, 5 mM DTT, 20 mM KCl, 15 $\mu$M NADPH, 4 $\mu$g/mL purified MurB [purified as described by Benson *et al.* (1993)], 0.1 mM phosphoenolpyruvate, and 1 mM UDP-GlcNAc. The oxidation of NADPH could be monitored at 340 nm ($\epsilon$ = 6220 mM$^{-1}$ cm$^{-1}$) and was stoichiometric with the production of enolpyruvoyl-UDP-GlcNAc.

*Anion-Exchange HPLC.* Anion-exchange HPLC using MonoQ 5/5 (Pharmacia) was successful in providing baseline resolution of peaks corresponding to all components of the reaction and was used both for preparative and analytical purposes. In all cases, separations were performed at pH 8.0 and employed a flow rate of 2 mL/min with an initial 4-mL wash in 20 mM triethylammonium bicarbonate, followed by a 10-mL gradient of triethylammonium bicarbonate from 20 to 500 mM and 9 mL of 500 mM triethylammonium bicarbonate. Analytical separations were automated with a Waters WISP (Milford, MA) autosampler and employed an in-line radioactivity detector (either Flo-One, Packard Instruments, Tampa, FL, or Beckman 171 radioisotope detector, Beckman Instruments, Fullerton, CA) in which 1 vol of eluent was mixed with 3 vol of liquid scintillation cocktail (National Diagnostic Monoflow 5 or Beckman Ready Flow III) prior to detection.

*Electrophoresis and Quantitation of [$^{32}$P]PEP Bound to MurZ.* MurZ–[$^{32}$P]PEP complexes, formed as a result of incubation of MurZ with [$^{32}$P]PEP, were stable to and were routinely quenched with KOH to a final concentration of 0.2 N. Quenched MurZ–[$^{32}$P]PEP complexes and free [$^{32}$P]PEP

were found to be easily separated by direct electrophoresis, with the addition of only glycerol (final concentration, 5%) and bromophenol blue (0.01%) to facilitate gel loading, in 10–20% acrylamide gels (Bio-Rad) using the standard Laemmli (1970) buffer system. Quantitation of the phosphor images (using either a Bio-Rad molecular imager system, Hercules, CA, or a Molecular Dynamics PhosphorImager, Sunnyvale, CA, and associated software) of gels allowed the determination of the relative amounts of radioactivity associated with MurZ (bound) and the dye front (free). Molar concentrations of each species were then calculated on the basis of the distribution of counts arising from the concentration of [$^{32}$P]PEP present in the incubation mix prior to quenching. Stock [$^{32}$P]PEP was found by anion-exchange HPLC (described above) to be contaminated with 34% [$^{32}$P]-phosphate. Therefore, in calculations of the molar concentrations of each species, that portion of the radioactivity ([$^{32}$P]phosphate) was assumed to have run at the dye front and was accordingly subtracted from the free and total PEP.

*NMR of PEP Bound to MurZ.* All NMR data were acquired on a Varian VXR500S spectrometer operating at a $^{13}$C frequency of 125.697 MHz. The data were acquired at 20 °C using a 30° excitation pulse and a 2.035-s cycle time, and 64K points were acquired with a spectral width of 31421.8 Hz. Data were processed with a line broadening of 3.5 Hz, using VNMR. One-dimensional spectra were acquired for 12–30 h in a 10-mm sample tube. Two-dimensional HMQC spectra (Bax *et al.*, 1983) were acquired in a 5-mm triple-resonance (HCN) probe equipped with pulsed-field gradients. The solvent was presaturated for 1 s using a 100-Hz field and $^{13}$C WALTZ16 decoupling (4500 Hz) as applied during acquisition. Proton and carbon spectral widths were 10 and 100 ppm, respectively. Two thousand forty-eight points in $F_2$ and 128 complex points in $F_1$ were acquired using the States TPPI method (Marion *et al.*, 1989). Data were processed using Gaussian apodization.

*Rapid Quench Experiments.* Transient kinetic experiments were performed using a rapid quench apparatus (KinTek Instruments, University Park, PA) designed by Johnson (Johnson, 1992). Preliminary experiments established that 0.2 N KOH was sufficient to quench the reaction and that all species in the reaction catalyzed by MurZ—PEP, UDP-GlcNAc, enolpyruvoyl-UDP-GlcNAc, phospholactoyl-UDP-GlcNAc, and phospholactoyl-MurZ—were stable to the quench solution. Reactions were initiated by mixing two solutions (enzyme and substrates) of volume 15 μL, quenched with the addition of 60 μL of KOH to a concentration of 0.2 N, and subsequently analyzed by HPLC and electrophoresis.

Rapid quench experiments typically required electrophoresis of part of the quenched reaction mixture (20 μL), as described above, while the remainder (70 μL) was filtered through a membrane of molecular weight cutoff 30 000 before analysis on HPLC as described above. Electrophoresis allowed the determination of the relative amounts of enzyme-bound and soluble radioactivity, while HPLC of the filtrate allowed quantitation of the soluble radioactive fraction distributed among substrate PEP, product inorganic phosphate, and intermediate phospholactoyl-UDP-GlcNAc. Concentrations of each species at the time of quenching were calculated on the basis of the distribution of counts arising from a given concentration of labeled substrate present in the incubation mix prior to quenching. A control sample (enzyme quenched before substrates added) was rountinely run to verify quenching and recovery of total radioactivity in both HPLC and electrophoretic analysis. During HPLC chromatography, a "cold" standard (either PEP or UDP-GlcNAc) was included and its retention was monitored in-line by absorption at 260 nm to confirm retention times and recovery.

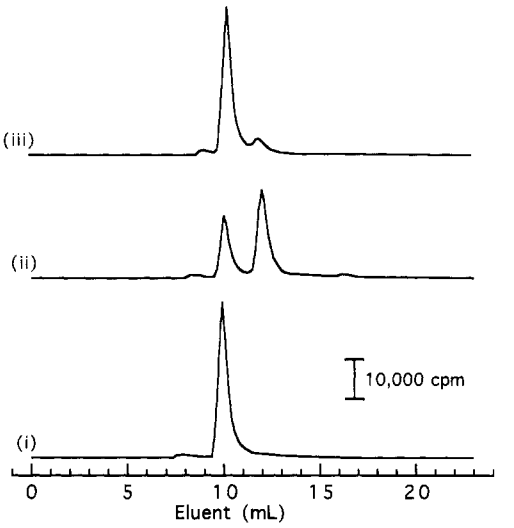



FIGURE 1: Detection of bound PEP associated with purified MurZ. Anion-exchange HPLC was performed as described in Materials and Methods. Chromatogram (i) shows elution of 0.27 nmol of [$^{14}$C]-UDP-GlcNAc. Untreated (ii) and treated (to remove PEP) MurZ (0.17 nmol) (iii) were incubated with 0.27 nmol of [$^{14}$C]UDP-GlcNAc for 2 min, quenched with 0.2 N (final concentration) KOH, and chromatographed. Integration of the areas under the peaks for UDP-GlcNAc (elution volume, 10.2 mL) and product enolpyruvoyl-UDP-GlcNAc (elution volume, 11.9 mL) allowed the determination of bound PEP. The untreated enzyme contained 0.16 nmol of PEP (0.95 equiv), and after removal of PEP, the enzyme contained 0.012 nmol of PEP (0.07 equiv).

Curves were fit to the data from rapid quench experiments using the kinetic simulation program KINSIM, as described previously (Barshop *et al.*, 1983; Anderson *et al.*, 1988a). In this study, simulations were not run with the goal of unambiguously determining the rate constants for each step in the reaction pathway, but were instead intended to provide preliminary fits of data to a first model, prefatory to experiments now underway to describe the complete kinetic pathway of MurZ. The data were fit by a trial and error process with the benefit, in part, of $K_m$ values of 0.2 μM for PEP, 15 μM for UDP-GlcNAc, and 2 mM for $P_i$ (Marquardt, 1993).

## RESULTS

*MurZ Purifies with a Tightly Bound PEP Equivalent.* Previously we reported the development of a rapid, large-scale purification of MurZ overproduced in *E. coli*, yielding *ca.* 125 mg of enzyme from 1 L of bacterial culture (Marquardt *et al.*, 1993a). In the course of examining catalysis by MurZ under single-turnover conditions (near stoichiometric levels of enzyme and substrates), we have consistently noted the surprising observation that incubation of [$^{14}$C]UDP-GlcNAc with pure enzyme yields almost a full equivalent of $^{14}$C-labeled product, enolpyruvoyl-UDP-GlcNAc in the absence of added PEP (Figure 1). From several enzyme preparations, it was concluded that, as purified, MurZ contains about 1 equiv of tightly associated PEP which can be converted to product. In contrast, the complementary experiment, involving the incubation of enzyme as purified with [$^{14}$C]PEP, did not yield product (data not shown), indicating the absence of any bound UDP-GlcNAc equivalents.

The nature of the MurZ–PEP interaction was investigated by devising a procedure to purify the enzyme of bound PEP

Table 1: Binding of PEP to MurZ[a]

| | [$^{14}$C]PEP/MurZ (mol/mol) | [$^{32}$P]PEP/MurZ (mol/mol) |
|---|---|---|
| no denaturation | 1.28 ± 0.36 | 0.87 ± 0.20 |
| denatured in 6.7 M urea | 1.33 ± 0.12 | 0.95 ± 0.35 |

[a] Binding experiments were performed in duplicate and involved a 30-min incubation at 20 °C of MurZ with a 3-fold molar excess of PEP followed by spin desalting (Penefsky, 1977) to remove free PEP. Where denaturation is indicated, the enzyme was denatured by the addition of urea to 6.7 M after incubation and prior to spin desalting. Radioactivity associated with desalted MurZ was quantitated by scintillation counting and blank-corrected on the basis of a similarly treated blank incubation which contained only buffer and radioactive PEP. Stoichiometry is based on the concentration of active MurZ after removal of bound PEP as described in Materials and Methods. Errors are standard deviations of two independent experiments.

through incubation with excess UDP-GlcNAc to chase all bound PEP equivalents to product. Subsequently, the enzyme solution was exposed to 6 M urea to dissociate any non-covalently bound substrates and products, followed by renaturation on a desalting column (G-25 Sephadex, Pharmacia). This purification process typically resulted in enzyme with a specific activity which was 60% that of the starting material and was found to produce MurZ which was free of bound substrates when challenged with either [$^{14}$C]PEP (data not shown) or [$^{14}$C]UDP-GlcNAc and assessed by HPLC analysis for product formation (Figure 1). The $K_m$ for UDP-GlcNAc for the PEP-free enzyme was unchanged from that of the starting material (15 $\mu$M). This PEP-free enzyme preparation was used for all subsequent experiments.

*Titration of MurZ with PEP.* The capacity of pure, PEP-free MurZ to bind PEP was assayed using either [$^{14}$C]PEP or [$^{32}$P]PEP (Table 1). It was observed that, after a 30-min incubation with excess PEP, both [$^{14}$C]PEP and [$^{32}$P]PEP were bound to active MurZ with approximately the same stoichiometry, i.e., approximately 1.3 and 0.9 molecule, respectively, per molecule of MurZ, even after treatment with the denaturant urea (6.7 M). We note here also that the enzyme is completely inactive in 6.7 M urea and demonstrates a midpoint of denaturation of 2 M as judged by protein fluorescence (Marquardt, 1993). Titration of MurZ with [$^{32}$P]-PEP followed by polyacrylamide gel electrophoresis of quenched incubation mixtures revealed saturation behavior with a maximal stoichiometry of about 0.95 equiv of PEP per active site of MurZ (Figure 2).

*Proteolysis of [$^{32}$P]PEP-Labeled MurZ.* To test further that the PEP bound to the active site of MurZ was covalently attached, [$^{32}$P]PEP-labeled MurZ was subjected to a limited proteolysis followed by SDS–PAGE. Digestions with *Staphylococcus aureus* V8 protease, chymotrypsin, or both followed by gel electrophoresis and autoradiography are shown in Figure 3. Covalent attachment of PEP to MurZ is strongly suggested by the generation of radiolabeled fragments as small as *ca.* 5 kDa, which were evident after denaturing electrophoresis of a double digest using both V8 protease and chymotrypsin.

*NMR Analysis of the Covalent Enzyme Adduct Is Consistent with a Phospholactoyl Derivative of PEP.* Given the evidence for stoichiometric and covalent association of PEP with MurZ, NMR spectroscopy was performed on 60 mg of PEP-free MurZ and an excess of [2-$^{13}$C]PEP. Spectrum i of Figure 4 is the $^{13}$C NMR spectrum of 0.5 mM MurZ alone. Spectrum ii shows the result of the addition of 4 equiv of [2-$^{13}$C]PEP with the expected resonance for excess [2-$^{13}$C]-PEP at 152.6 ppm. Most striking is the broad resonance at

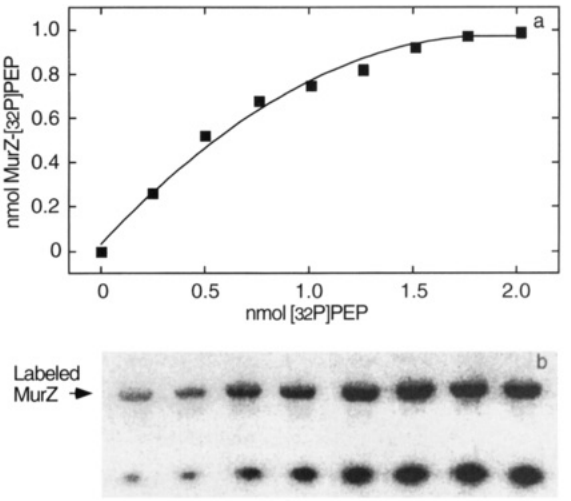



FIGURE 2: Titration of 1.0 nmol of PEP-free MurZ (50 $\mu$M in the incubation mixture) with [$^{32}$P]PEP. MurZ was incubated with [$^{32}$P]-PEP at 20 °C for 45 min prior to being quenched with 0.2 N (final concentration) KOH and electrophoresed (panel b) in a 10–20% acrylamide gel (Bio-Rad) as described in Materials and Methods. From left to right, panel b shows autoradiography of the addition of 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 nmol of [$^{32}$P]PEP, respectively. The titration curve in panel a was determined as described in Materials and Methods.

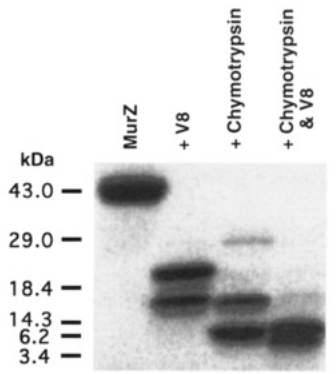



FIGURE 3: Digestion of [$^{32}$P]PEP-labeled MurZ. [$^{32}$P]PEP-labeled MurZ (6 $\mu$g) was proteolyzed with 1 $\mu$g of each protease for 30 min at 20 °C in SDS–PAGE sample buffer (50 mM Tris, pH 8.3, 2% SDS, 5% $\beta$-mercaptoethanol, and 5% glycerol) and electrophoresed without boiling in a 10–20% gradient polyacrylamide gel (Bio-Rad) prior to autoradiography.

87.8 ppm, which disappears after the addition of excess UDP-GlcNAc (spectrum iii, Figure 4). We assign this to a covalent phospholactoyl moiety in which an enzyme nucleophile has attacked the C-2 of bound PEP to yield a tetrahedral phospholactoyl enzyme adduct, with sp$^3$ hybridization at C-2. Also evident after addition of [2-$^{13}$C]PEP is a trace of product enolpyruvoyl-UDP-GlcNAc (163.1 ppm), present due to a small amount of contaminating UDP-GlcNAc left over from the chasing process aimed at removing bound PEP (see Materials and Methods).

To probe the chemical nature of hydrogen and carbon atoms at position 3 of enzyme-bound PEP, we incubated MurZ with [1,2,3-$^{13}$C]PEP and analyzed the enzyme–substrate adduct using two-dimensional NMR (data not shown). A $^1$H{$^{13}$C} heteronuclear mulitple quantum coherence (HMQC) spectrum was acquired, and a single correlation cross peak was observed in the two-dimensional spectrum (data not shown). The carbon chemical shift was 28.0 ppm, consistent with a methyl group at position 3, and that of the protons was 1.89 ppm, which was in agreement with the resonances of the previously characterized methyl protons (1.78 ppm) of the tetrahedral phospholactoyl-UDP-GlcNAc intermediate (Mar-

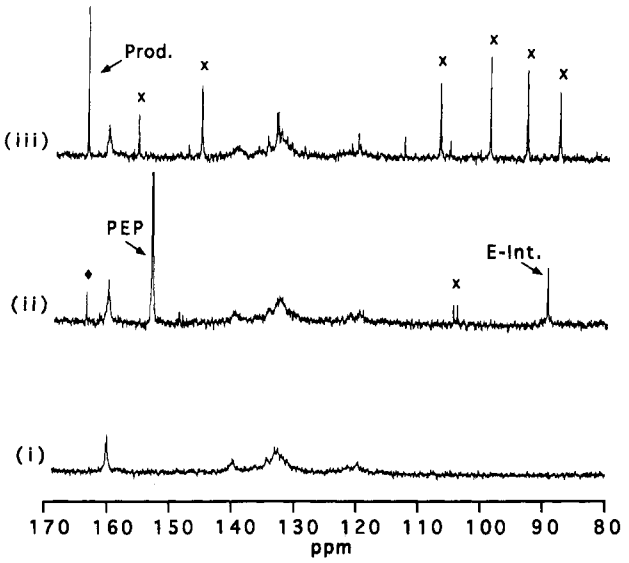



FIGURE 4: $^{13}C$ NMR spectroscopy of [2-$^{13}C$]PEP-labeled MurZ. Spectra were recorded at 20 °C as described in Materials and Methods. Spectrum i was a 30-h accumulation on a sample containing 0.5 mM MurZ in 50 mM Tris, 5 mM DTT, pH 8.0, and 20% $D_2O$. Spectrum ii was collected for 12 h after the addition of [2-$^{13}C$]PEP to a concentration of 2 mM. The resonance designated PEP (152.6 ppm) is that for C-2 of free PEP, and that labeled E-Int. (87.8 ppm) is the presumed phospholactoyl intermediate. A resonance at 163.1 ppm (labeled with a ♦) also appeared on addition of [2-$^{13}C$]PEP and corresponded to [2-$^{13}C$]enolpyruvoyl-UDP-GlcNAc, presumably due to the presence of trace amounts of UDP-GlcNAc in this enzyme preparation. Two other resonances of 102.5 and 103.1 ppm were evident in a "blank" sample containing 2 mM [2-$^{13}C$]PEP in the same buffer and are marked with an "x". Spectrum iii was collected for 12 h after the addition of UDP-GlcNAc to a final concentration of 4 mM. Of note is the absence of a resonance corresponding to E-Int. at 87.8 ppm and the expected appearance of a resonance at 163.1 ppm from enolpyruvoyl-UDP-GlcNAc, designated Prod. Other resonances not in spectrum ii but evident in a blank sample containing 4 mM UDP-GlcNAc in the same buffer are each marked with an x. Those resonances correspond to C-4′, C-1′, C-1, C-5″, C-6″, C-2″, and C-4″ of UDP-GlcNAc at 86, 91.5, 97, 105.5 , 144.5, 155, and 169.5 ppm, respectively.

quardt *et al.*, 1993b). The absence of other cross peaks also confirms that no other protons are directly attached to either C-1 or C-2 in the complex, consistent with the formation of a covalent phospholactoyl adduct via attachment to the enzyme at position 2.

*Kinetics of Formation of Phospholactoyl-MurZ in the Absence of UDP-GlcNAc.* Figure 5 depicts the determination of a bimolecular rate constant for the association ($k_{on}$) of the enzyme with PEP to form a phospholactoyl enzyme.

$$\text{PEP} + \text{MurZ} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{phospholactoyl-MurZ} \qquad (1)$$

The rate of formation of the phospholactoyl derivative is given by

$$\frac{d[\text{phospholactoyl-MurZ}]}{dt} = k[\text{MurZ}][\text{PEP}] \qquad (2)$$

If MurZ is in large excess over PEP, then $k_{MurZ}$ is the pseudo-first-order rate constant for the transformation of PEP and is defined by the integrated rate equation

$$\frac{[\text{phospholactoyl-MurZ}]}{[\text{PEP}]_o} = (1 - e^{-k[\text{MurZ}]t}) \qquad (3)$$

In the above relationship, the ratio [phospholactyl-MurZ]/

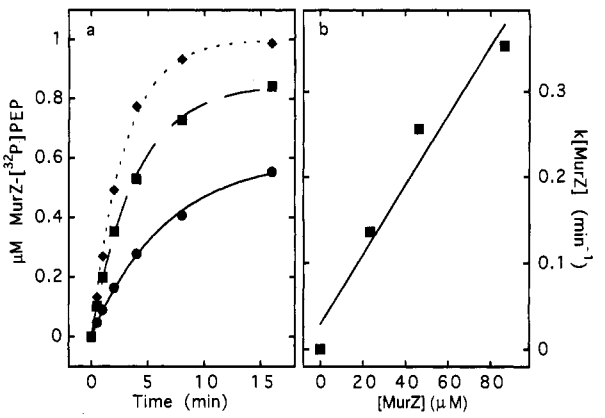



FIGURE 5: Determination of a bimolecular rate constant for the formation of phospholactyl-MurZ. Panel a shows the production [$^{32}P$]-phospholactoyl-MurZ over time. MurZ at concentrations of 22 (●), 43 (■), and 86 μM (◆) was incubated for a given time at 20 °C with 1.1 μM [$^{32}P$]PEP, quenched with KOH to a concentration of 0.2 N, and electrophoresed to determine the extent of labeling as described in Materials and Methods. Curves are nonlinear fits of the data to the first-order integrated rate equation (eq 3) from which the pseudo-first-order rate constants $k_{MurZ}$ were extracted. Panel b plots the influence of MurZ concentration on the constant $k_{MurZ}$. The bimolecular rate constant was determined by linear regression of the data to be 0.24 $\mu M^{-1}$ $s^{-1}$.

$[PEP]_o$ is the fraction of PEP associated with MurZ at a given incubation time and can be calculated from the relative amounts of free and enzyme-bound [$^{32}P$]PEP, quantitated following electrophoresis of a base quench of enzyme incubated with [$^{32}P$]PEP as described in Materials and Methods.

Figure 5a depicts first-order fits (eq 3) of time courses for [$^{32}P$]PEP binding to MurZ at three different concentrations of MurZ. The influence of MurZ concentration on the pseudo-first-order rate constants is roughly linear (Figure 5b) and suggests that the reaction can be described by a simple bimolecular model where the rate constant $k$ is 0.24 $\mu M^{-1}$ $s^{-1}$. It is unlikely that formation of phospholactoyl enzyme in the absence of UDP-GlcNAc is on the kinetically preferred pathway since this rate constant is approximately 60-fold slower than the lower limit of $k_{cat}/K_m$ (15 $\mu M^{-1}$ $s^{-1}$) determined from steady-state kinetics, which represents the second-order binding constant for PEP. To determine the off-rate of PEP from the complex, [$^{32}P$]phospholactoyl-MurZ was incubated with a large excess of unlabeled PEP and the loss of [$^{32}P$]PEP was monitored using the electrophoretic methodology described in Materials and Methods. No significant loss, however, could be detected over 2 h, compared to a parallel incubation without cold PEP. Assuming an error of 5% in detecting the loss of label, we can place upper limits of $7 \times 10^{-6}$ $s^{-1}$ for the unimolecular off rate and $3 \times 10^{-11}$ M for the dissociation constant, $K_d$, in the absence of UDP-GlcNAc.

*Single-Turnover Experiments with [$^{32}P$]PEP.* Given the chemical competence of the covalent phospholactoyl enzyme species in producing product upon incubation with UDP-GlcNAc,we were very interested in determining the kinetic significance of the enzyme adduct. Single-turnover experiments were performed by preincubating the enzyme with excess UDP-GlcNAc, followed by mixing and reaction with [$^{32}P$]-PEP before quenching with 0.2 N KOH. Figure 6 depicts the results of subsequent HPLC and electrophoretic quantitation of reaction substrates, products, and intermediates. Most notable was the formation and decay of the phospholactoyl-MurZ species on a time scale paralleling the disappearance of PEP and the appearance of product inorganic phosphate, indicating that, in the presence of UDP-GlcNAc, the adduct

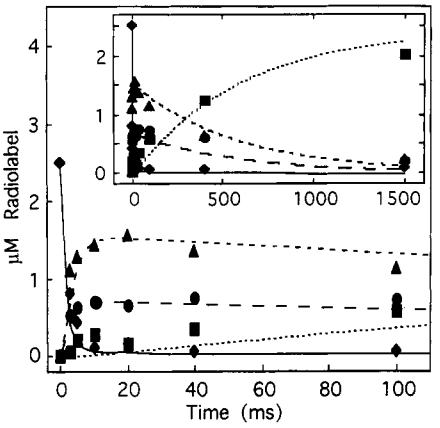



FIGURE 6: A single turnover in the reaction catalyzed by MurZ. A solution of UDP-GlcNAc and enzyme was mixed with a solution of [$^{32}$P]PEP and allowed to react for a given time at 15 °C before quenching with KOH to a concentration of 0.2 N. Final concentrations were 25 μM MurZ, 80 μM UDP-GlcNAc, and 2.5 μM [$^{32}$P]PEP. The amounts of radiolabeled substrate PEP (◆), product $P_i$ (■), phospholactoyl-MurZ (●), and phospholactoyl-UDP-GlcNAc (▲) were monitored by gel electrophoresis and HPLC as described in Materials and Methods. The curves were calculated by numerical integration using the program KINSIM and the following model as described in Materials and Methods:

$$EA + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EAB \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EQP \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EQ + P \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} E + Q$$

where E is enzyme, A is UDP-GlcNAc, B is PEP, $EI_1$ is phospholactoyl-MurZ, $EI_2$ is phospholactoyl-UDP-GlcNAc, Q is enolpyruvoyl-UDP-GlcNAc, and P is phosphate. The following rate constants were employed: $k_1 = 50\ \mu M^{-1}\ s^{-1}$, $k_{-1} = 200\ s^{-1}$, $k_2 = 400\ s^{-1}$, $k_{-2} = 100\ s^{-1}$, $k_3 = 2200\ s^{-1}$, $k_{-3} = 1000\ s^{-1}$, $k_4 = 2.8\ s^{-1}$, $k_{-4} = 2.8\ s^{-1}$, $k_5 = 250\ s^{-1}$, $k_{-5} = 1.0\ \mu M^{-1}\ s^{-1}$, $k_6 = 200\ s^{-1}$, $k_{-6} = 200\ \mu M^{-1}\ s^{-1}$.

represents a kinetically competent intermediate in the reaction. The data also demonstrated that the lifetime of the phospholactoyl-MurZ species paralleled that of the soluble phospholactoyl intermediate. Simulation of the data by numerical integration according to the model proposed in the caption to Figure 6 led to first-order rate constants of 2200 and 1000 $s^{-1}$ describing the forward and back reactions, respectively, for the apparently rapid equilibrium between enzyme-bound and soluble tetrahedral intermediates. Simulation of the data was also consistent with a $k_{on}$ for PEP in the presence of UDP-GlcNAc of 50 $\mu M^{-1}\ s^{-1}$, more than 200-fold that determined in the absence of UDP-GlcNAc (0.24 $\mu M^{-1}\ s^{-1}$), suggesting that prior binding of UDP-GlcNAc represents a kinetically preferred binding order.

As a negative control, single-turnover experiments were also conducted with EPSP synthase. EPSP synthase was preincubated with shikimate 3-phosphate and mixed with [$^{32}$P]-PEP to final concentrations of 20, 100, and 3.5 μM, respectively, before quenching with KOH (final concentration, 0.2 N). HPLC analysis of the filtered fraction demonstrated the anticipated formation and decay of tetrahedral (phospholactoyl)shikimate 3-phosphate intermediate (Anderson *et al.*, 1988a), paralleling the loss of [$^{32}$P]PEP and the formation of $P_i$ however, no radiolabel could be detected in association with EPSP synthase as assessed by electrophoresis and autoradiography of the unfiltered fraction.

*PEP Can Be Trapped at the Active Site of MurZ.* To determine whether the covalent phospholactoyl-MurZ intermediate was capable of giving rise to the phospholactoyl-UDP-GlcNAc intermediate and ultimately product, enzyme and [$^{14}$C]PEP were preincubated for a time sufficient to generate stoichiometric [$^{14}$C]phospholactoyl-MurZ (≈10 min) and mixed with a 50-fold excess of cold PEP and saturating

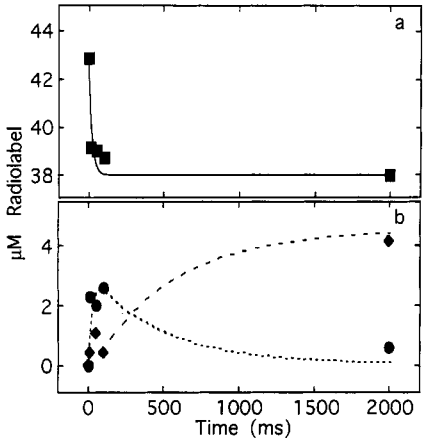


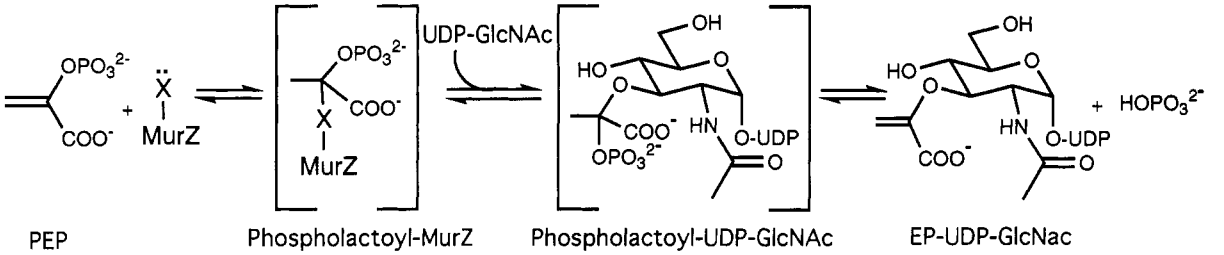

FIGURE 7: Time course of turnover of trapped [$^{14}$C]phospholactoyl-MurZ. A preincubation (≈10 min) mixture of [$^{14}$C]PEP (86 μM) and MurZ (24 μM) was mixed with an equal volume of a solution containing UDP-GlcNAc (1 mM) and unlabeled PEP (4.0 mM), allowed to react for a given time at 15 °C, and quenched with KOH to a concentration of 0.2 N. Concentrations after mixing are shown. Panel a shows the time course for the disappearance of [$^{14}$C]PEP, which was covalently bound to the enzyme. Panel b shows the time course for the appearance of both [$^{14}$C]phospholactoyl-UDP-GlcNAc (●) and product [$^{14}$C]enolpyruvoyl-UDP-GlcNAc (◆). Since, in these experiments, only the soluble fraction (filtrate, as described in Materials and Methods) was analyzed, the concentrations of the soluble species were corrected by accounting for that fraction which can be predicted to be enzyme bound on the basis of the relative abundance of the soluble intermediate and the constant $k_3/k_{-3} = 2.2$, determined for the rapid equilibrium between soluble and enzyme-bound intermediates (see the caption to Figure 6). The curves were calculated by numerical integration using the program KINSIM according to the model and constants described in Figure 6, excepting the first step, which required a reversal of the binding order. In this trapping experiment, UDP-GlcNAc was mixed with phospholactoyl enzyme, EB + A ⇌ EAB, where the constants $k_1 = 0.05\ \mu M^{-1}\ s^{-1}$ and $k_{-1} = 0.05\ s^{-1}$ were the values used for the forward and back reactions, respectively.

UDP-GlcNAc. If the [$^{14}$C]phospholactoyl-MurZ complex were capable of reacting with UDP-GlcNAc, then radiolabeled [$^{14}$C]phospholactoyl-UDP-GlcNAc intermediate and [$^{14}$C]-enolpyruvoyl-UDP-GlcNAc would have been formed. Indeed, the data (Figure 7) showed a rapid rise in phospholactoyl-UDP-GlcNAc intermediate and a slower formation of product on a time scale which was modeled using the rate constants determined in single-turnover experiments (Figure 6). The amplitude of product formation corresponded to 38% of the phospholactoyl-MurZ trapped, far in excess of the amount which could be accounted for by the turnover of radiolabeled PEP had it been diluted into the unlabeled pool prior to reaction (i.e., 2%). The converse experiment, to determine the amount of UDP-GlcNAc which could be trapped, produced a reaction amplitude corresponding to 61% of trapped MurZ-UDP-GlcNAc (data not shown).

## DISCUSSION

MurZ is one of a small group of enzymes that utilize enolpyruvoyl group transfer from phosphoenolpyruvate to an attacking hydroxyl nucleophile on a cosubstrate atom. In a formal sense, MurZ catalyzes an attack on PEP, where C-2 functions as an electrophile. In the cases of EPSP synthase and UDP-GlcNAc enolpyruvoyl transferase, an enzyme-associated, but non-covalently bound, tetrahedral intermediate containing a phospholactoyl moiety has been found to result from attacks of the 5-OH of shikimate 3-phosphate (Anderson *et al.*, 1988a,b) and the 3-OH of UDP-GlcNAc (Marquardt *et al.*, 1993b), in the respective active sites. These intermedi-

Scheme 2




ates have been isolated by rapid chemical quench methods, characterized by NMR, and found to be chemically and kinetically competent in catalysis. This has led us, in the case of MurZ, to the minimal kinetic mechanism described by Scheme 1.

The work described in this paper adds to the mechanistic picture of MurZ catalysis with the unexpected demonstration that, in the absence of UDP-GlcNAc, PEP forms a covalent adduct with the enzyme. Indeed, the adduct accumulates stoichiometrically on the enzyme as isolated and may represent a resting form of the protein in the bacterial cell. Furthermore, the covalent MurZ–PEP complex is capable of forming product enolpyruvoyl-UDP-GlcNAc on exposure to UDP-GlcNAc. The existence of this chemically competent covalent MurZ–PEP species is, not proof, however, that the species is an authentic intermediate along the kinetically preferred catalytic pathway for MurZ in presence of $K_m$ quantities of both substrates. That question has been addressed in this work through single-turnover experiments which have principally substantiated the existence of a covalent MurZ–PEP complex on the mechanistic pathway of the enzyme.

The initial discovery of the covalent enzyme–PEP complex in purified MurZ was made possible by its chemical competence in a single-turnover reaction with added [$^{14}$C]UDP-GlcNAc (Figure 1). Elimination of this bound form of PEP from purified enzyme was accomplished by chasing with excess UDP-GlcNAc followed by removal of non-covalently bound substrate and product under denaturing conditions. Subsequent renaturation of the protein resulted in an enzyme preparation which was free of PEP and allowed the *in vitro* preparation of MurZ, to which stoichiometric amounts of isotopically labeled PEP ([$^{32}$P]PEP or [$^{14}$C]PEP) could be bound (Table 1). The stability of the PEP–enzyme adduct to urea denaturation, SDS–gel electrophoresis, and limited proteolysis strongly suggested covalent association of the PEP backbone (Table 1, Figure 3). During the preparation of this paper, a report by Wanke and Amrhein (1993) was published which demonstrated that incubation of the UDP-GlcNAc enolpyruvoyl transferase from *Enterobacter cloacae* with UDP-GlcNAc, [$^{32}$P]$P_i$, and [$^{14}$C]PEP resulted in the stable association (stable to 8 M urea) of both the $C_3$ and inorganic phosphate components of PEP in apparent stoichiometry with the transferase, leading those researchers to the same conclusion.

In the present study, NMR analysis of MurZ incubated with [2-$^{13}$C]PEP confirmed the suggestion that the interaction of PEP with MurZ was covalent, demonstrating a shift in the $^{13}$C resonance from 152.6 ppm for the sp$^2$-hybridized PEP to 87.8 ppm for the signal in the adduct, consistent with sp$^3$ hybridization (Figure 4). In addition, heteronuclear NMR revealed the formation of a C-3 methyl (proton resonances, 1.8 ppm; carbon resonance, 28 ppm) in MurZ incubated with [1,2,3-$^{13}$C]PEP. Taken together, the NMR data verify the expectation that an enzyme nucleophile attacks PEP in the active site to yield a phospholactoyl group tethered covalently to that nucleophile at C-2 (Scheme 2).

Toward the definitive identification of the enzyme nucleophile we have attempted mass spectrometry of the MurZ–PEP complex but have been unsuccessful, likely due to instability of the complex to the acidic conditions of the analysis (0.1% trifluoroacetic acid), and we have determined molecular weights for the PEP-containing enzyme corresponding to that of the native enzyme only. We therefore also suspect that identification of the nucleophilic residue using classical digestion and acidic reverse-phase separation techniques will prove infeasible. Circumstantially, however, we suggest that the thiolate side chain of Cys115 is a reasonable candidate. In the accompanying paper we document the covalent attachment of this residue to fosfomycin on inactivation of MurZ by that antibiotic (Marquardt *et al.*, 1994). Indeed, the structural (18.3% sequence identity) and functional homologue EPSP synthase does not proceed through an enzyme-bound intermediate, is not inactivated by fosfomycin, and lacks this cysteine, which is conserved among the bacterial UDP-GlcNAc enolpyruvoyl transferases so far sequenced (Marquardt *et al.*, 1993a). Furthermore, C115A and C115S variants of MurZ express in *E. coli* to levels comparable to that of wild-type MurZ but, unlike the native enzyme, confer no resistance to fosfomycin when overproduced and show no detectable enzyme activity either in cell extracts or after purification (Marquardt, 1993). In addition, the recent report on the *E. cloacae* transferase (Wanke & Amrhein, 1993) implicates Cys115 by thiol titration and peptide mapping of PEP-labeled enzyme and also by C115S mutagenesis as noted here. Finally, the upfield shift of 16.2 ppm in the $^{13}$C resonance at C-2 of the phospholactoyl moiety of the enzyme-bound structure relative to that of the phospholactoyl-UDP-GlcNAc intermediate (87.8 and 104.0 ppm, respectively) corresponds to that expected ($\approx$15 ppm) of a quaternary carbon in thioether linkage compared to one with an oxyether bond (Breitmaier & Voelter, 1987). Cys115 is therefore strongly implicated as the active site nucleophile by an assortment of evidence.

Having defined the nature of the MurZ–PEP complex and its chemical competence, we were interested in determining whether the enzyme adduct lay on the normal reaction pathway. Single-turnover experiments with limiting [$^{32}$P]-PEP, quenched on a millisecond time scale allowed the direct examination of phospholactoyl-MurZ by electrophoresis and of soluble reaction species by HPLC. The time course of the single turnover (Figure 6) demonstrated that the phospholactoyl-MurZ species formed and decayed on a time scale paralleling the disappearance of the substrate PEP and the appearance of the product enolpyruvoyl-UDP-GlcNAc. Given the kinetic competence of the phospholactoyl enzyme intermediate, a reasonable chemical model would predict that the covalent enzyme-bound tetrahedral intermediate forms first and then gives rise in the active site to the phospholactoyl-UDP-GlcNAc intermediate as a result of subsequent attack

by the 3-OH of UDP-GlcNAc (Scheme 2). The single-turnover data (Figure 6), however, demonstrate only that the two intermediates form and decay in parallel, providing no direct evidence that one might precede the other. Indeed, it might be argued that the two intermediates lie on two different pathways to product which coincidentally proceed with the same rates of formation and decay. An alternative explanation for the parallel detection of the two intermediates is highlighted by kinetic simulation of the data using the model described in Scheme 2 (and in the caption to Figure 6). Best fits of the data were achieved by setting the forward and reverse rate constants of the proposed equilibrium between phospholactoyl-MurZ and phospholactoyl-UDP-GlcNAc at $2200^{-1}$ and 1000 $s^{-1}$, respectively, suggesting that, on the time scale of the quench, a rapid equilibrium exists between the two species. Further support for the proposed mechanism (Scheme 2) comes from substrate-trapping experiments (Figure 7) which demonstrated that phospholactoyl-MurZ was capable of producing the phospholactoyl-UDP-GlcNAc intermediate, which subsequently decayed to form product on a time scale consistent with turnover. In aggregate, the single-turnover and substrate-trapping experiments of this study provide strong evidence that a phospholactoyl enzyme intermediate precedes the previously characterized (Marquardt *et al.*, 1993b) phospholactoyl-UDP-GlcNAc intermediate in the reaction catalyzed by MurZ.

It is intriguing that MurZ and its homologue EPSP synthase have in common the non-covalently bound tetrahedral phospholactoyl intermediate but differ fundamentally in the route of formation of that species. Also surprising, in the case of MurZ, is the ready formation of the phospholactoyl enzyme species in the absence of UDP-GlcNAc and its lack of propensity to reverse in a 2-h observation or, indeed, throughout our multistep purification protocol. It is conceivable that the enzyme adduct represents an accumulating form of the enzyme when UDP-GlcNAc concentrations are low. While the physiological or mechanistic rationale behind such a resting form awaits understanding, the existence of a covalent adduct at the beginning of a catalytic cycle has precedent in the bacterial enzyme citrate lyase (Srere, 1975). Furthermore the existence of a reactive active site nucleophile may explain the efficacy of fosfomycin as a potent inactivator of MurZ. Clearly, several questions remain concerning this reaction mechanism, and studies are currently underway to provide a complete kinetic and thermodynamic characterization of the reaction pathway catalyzed by MurZ.

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