Kinetics, Stoichiometry, and Identification of the Reactive Thiolate in the Inactivation of UDP-GlcNAc Enolpyruvoyl Transferase by the Antibiotic Fosfomycin†

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ABSTRACT: Fosfomycin [(1*R*,2*S*)-1,2-epoxypropylphosphonic acid] has been shown to exert its antibiotic effect through the inhibition of UDP-GlcNAc enolpyruvoyl transferase [Kahan, F. M., *et al.* (1974) *Ann. N.Y. Acad. Sci. 235*, 364], the enzyme responsible for catalyzing the first committed step in bacterial cell wall biosynthesis. Time-dependent inactivation of MurZ by fosfomycin was found to be greatly accelerated by the presence of cosubstrate UDP-GlcNAc but could also be speeded appreciably by the unreactive substrate analog 3-deoxy-UDP-GlcNAc. These results argue against a reaction-based participation of the cosubstrate and suggest that UDP-GlcNAc has a role in influencing active site conformation critical to the inactivation event. A study of the influence of UDP-GlcNAc and fosfomycin on the kinetics of inactivation allowed the determination of dissociation constants for fosfomycin (K_F = 8.6 μM) and UDP-GlcNAc (K_S = 14 μM), in addition to a limiting inactivation rate constant (k_{inact} = 7.4 min^{-1}) at saturating UDP-GlcNAc and fosfomycin concentrations. Mass spectrometry of inactivated MurZ demonstrated an increase in molecular weight of 138, consistent with the covalent addition of a molar equivalent of fosfomycin (136 kDa). Titration of MurZ with fosfomycin revealed a stoichiometry of 1 molecule of inhibitor per active site when assessed using either enzyme activity or mass spectrometry as an index of modification. Peptide mapping of tryptic digests of fosfomycin-inactivated MurZ revealed modification of a unique 41-mer, the sequence of which revealed that Cys115 was the site of attachment of fosfomycin.

Since reports of the discovery (Hendlin *et al.*, 1969) and synthesis (Christensen *et al.*, 1969) of the natural antibiotic fosfomycin [Fos,[1] (1*R*,2*S*)-1,2-epoxypropylphosphonic acid], it has been shown to exert its antibiotic effect through the inhibition of UDP-GlcNAc enolpyruvoyl transferase (Kahan *et al.*, 1974). The susceptible transferase catalyzes enolpyruvoyl transfer from phosphoenolpyruvate to the 3-OH of UDP-*N*-acetylglucosamine (UDP-GlcNAc) in the first committed step in bacterial cell wall biosynthesis (Gunetileke & Anwar, 1968). The gene encoding the transferase activity, *murZ*, was recently cloned from an *Escherichia coli* gene library by screening for fosfomycin resistance resulting from overexpression of the gene on a multicopy plasmid (Marquardt *et al.*, 1993a). The gene encoding the transferase from *Enterobacter cloacae* has also been cloned (Wanke *et al.*, 1992).

Several years ago, using small quantities of impure transferase, Kahan *et al.* (1974) demonstrated that fosfomycin inactivation was dependent on the presence of UDP-GlcNAc and were able to isolate a [^{3}H]fosfomycin–cysteine thioether from an exhaustively proteolyzed digest of the inactivated transferase. In addition, mechanistic studies suggested the existence of covalent enolpyruvoyl (Zemell & Anwar, 1975) or phospholactoyl (Cassidy & Kahan, 1973) enzyme intermediates on the basis of the tight binding of radiolabeled PEP to partially purified transferase. Those findings, together with the fact that phosphoenolpyruvate binding could compete with inactivation of enzyme by fosfomycin (Kahan *et al.*, 1974), implicated a nucleophilic cysteine in the active site of the transferase.

More recently, a substantial structural homology (18.3% identity) has been noted in comparing the protein sequences of MurZ and (enolpyruvoyl)shikimate 3-phosphate (EPSP) synthase (Marquardt *et al.*, 1993a), an enzyme which catalyzes enolpyruvoyl transfer from PEP to shikimate 3-phosphate (Anderson & Johnson, 1990). Despite the similarities in structure and function, EPSP synthase is not inactivated by fosfomycin (Steinrucken & Amrhein, 1984), and pre-steady-state kinetic studies suggest that these enzymes operate by different mechanisms. EPSP synthase has been rigorously analyzed for mechanism and is known to catalyze the direct attack of the 5-hydroxyl of shikimate 3-phosphate on C-2 of PEP to form a (phospholactoyl)shikimate 3-phosphate intermediate (Anderson & Johnson, 1990) without the formation of an enzyme–PEP adduct. In the case of MurZ, we have recently reported the detection and isolation of the analogous intermediate (phospholactoyl-UDP-GlcNAc) (Marquardt *et al.*, 1993b) and have, in addition, provided strong evidence in

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[1] Abbreviations: Fosfomycin, or Fos, (1*R*,2*S*)-1,2-epoxypropylphosphonic acid; 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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the accompanying paper (Brown *et al.*, 1994) that the phospholactoyl-UDP-GlcNAc intermediate is preceded on the reaction pathway by a C-2-substituted phospholactoyl enzyme adduct.

An understanding of the susceptibility of MurZ to inactivation by the antibiotic fosfomycin clearly has implications for a complete mechanistic understanding of the enzyme. In the study reported here, we have exploited the large quantities of transferase available to us from overexpression of *E. coli* MurZ to detail the covalent nature, stoichiometry, and kinetics of the modification by fosfomycin, in addition to mapping the site of attachment to an active site thiolate. These findings are particularly interesting in the context of our recent characterization, in the accompanying paper (Brown *et al.*, 1994), of a kinetically competent covalent phospholactoyl enzyme intermediate.

MATERIALS AND METHODS

Chemicals. Sequencing grade trypsin was from Boehringer Mannheim. UDP-GlcNAc, PEP, and fosfomycin were purchased from Sigma. The purity of fosfomycin was determined to be 60% based on integration of the ^{1}H NMR spectrum against an internal standard of dimethyl sulfoxide. The remaining 40% was assumed to be inorganic salts, and all fosfomycin concentrations have been accordingly corrected. 3-Deoxy-UDP-GlcNAc was synthesized as described previously (Srivastava *et al.*, 1990). Triton X-100 was from Calbiochem. MurB was purified as described previously (Benson *et al.*, 1993).

Enzyme Purification. E. coli MurZ was isolated as described in the accompanying paper (Brown *et al.*, 1994). That study details the presence of 1 equiv of covalently bound PEP in enzyme as isolated and details methodology for the production of PEP-free enzyme, which involved chasing with cosubstrate UDP-GlcNAc, urea denaturation to remove noncovalently bound substrates and finally renaturation on Pharmacia Sephadex G-25 column equilibrated in 50 mM Tris·HCl, pH 8.0, and 5 mM DTT. PEP-free enzyme was found routinely to have a specific acitivity which was 60% that of the starting material. 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 PEP-free enzyme molecules were active. The concentration of total MurZ protein was routinely calculated using the extinction coefficient of 0.82 cm^2 mg^{-1} at 280 nm (Brown *et al.*, 1994).

Kinetics of Fosfomycin Inactivation. MurZ was inactivated in a 3.0-mL incubation mixture containing enzyme (1.3 μM), UDP-GlcNAc (2.5, 5.0, 7.5, 10.0, 15.0, or 20.0 μM), and fosfomycin (2.4, 4.8, 7.2, or 9.6 μM) in 100 mM Tris·HCl, pH 8.0, and 5 mM DTT at 25 °C. At regular time intervals, assay of the residual activity of MurZ was initiated by the addition of 200-μL aliquots of the incubation mixture to 800 μL of an assay mixture such that the final concentrations were 50 mM Tris·HCl, pH 8.0, 1 mM PEP, 1 mM UDP-GlcNAc, 150 μM NADPH, 20 mM KCl and 5 μg/mL of the enzyme MurB. The transferase activity was coupled to the oxidation of NADPH as described in in the accompanying article (Brown *et al.*, 1994). Initial rates were the residual activities at the instant of dilution into the assay mixture since the very high concentration of PEP in the assay competitively blocked any time dependent inactivation of MurZ during the course of the assay. Inactivation studies using 3-deoxy-UDP-GlcNAc were similarly performed, substituting 3-deoxy-UDP-GlcNAc for UDP-GlcNAc during the inactivation.

Stoichiometry of Inactivation of MurZ with Fosfomycin. Untreated MurZ (not PEP-free) was inactivated at 25 °C for 1h in 200-μL incubation mixtures containing 14.4 μM MurZ, 133 μM UDP-GlcNAc, and fosfomycin (0, 5.0, 10.0, or 15.0 μM), corresponding to inactivator:enzyme stoichiometries of 0, 35, 70, and 105%. Subsequent to the inactivation, the incubation mixtures were tested for residual activity using the coupled assay as described above and were subjected to liquid chromatography–electrospray ionization mass spectrometry. The chromatography was achieved using a POROS R/H 320 μm × 10 cm column (LC Packings, Amsterdam, The Netherlands). Samples were eluted at a flow rate of 40 μL/min using a 5-min gradient from 0.075% TFA in water to 0.05% TFA in 10% water and 90% acetonitrile. The separation was monitored at 215 nm prior to on-line electrospray mass spectrometry. The spectrometer was a Sciex API III triple quadrupole instrument (Perkin-Elmer-Sciex Instruments, Thornhill, Ontario, Canada). Quadrupole 1 was scanned over a mass range of *m/z* 500–2400 with a step of 0.3 Da and a dwell time of 0.5 ms, giving an overall scan time of 3.54 s. The orifice potential was set at 90 V, and the ion spray voltage was set at 5600 V.

Peptide Mapping, Mass Spectrometry, and Sequencing of the Fosfomycin-Labeled Peptide of Inactivated MurZ. Prior to peptide mapping, a 200-μL incubation mixture containing 90 μM MurZ in 50 mM Tris·HCl, pH 8.0, 5 mM DTT, 180 μM fosfomycin, and 200 μM UDP-GlcNAc was incubated at 25°C for 1 h, spin-desalted (Penefsky, 1977) using Sephadex G-25 preequilibrated in MilliQ-treated water, and quick frozen in liquid nitrogen. A control incubation mixture (no fosfomycin) was similarly treated but was not exposed to fosfomycin.

Control and fosfomycin-inactivated MurZ were brought to 100 mM NH_4HCO_3/10% acetonitrile/1% hydrogenated Triton X-100. Trypsin was added to these solutions to maintain a substrate to enzyme ratio of 25:1 (w/w), and the mixture was incubated at 37 °C for 16–20 h. The resultant peptide mixtures were frozen at –20 °C until separation by reverse-phase HPLC. Tryptic peptides were separated by reverse-phase HPLC on a Hewlett-Packard 1090 chromatograph equipped with a 1040 diode array detector, using a Vydac 2.1 mm × 150 mm C18 column. Briefly, the gradient employed was 5% buffer B (95% buffer A) from 0 to 10 min, 33% buffer B (67% buffer A) at 73 min, 60% buffer B (40% buffer A) at 105 min, and 80% buffer B (20% buffer A) at 115 min with a flow rate of 150 μL/min, where buffer A was 0.06% trifluoroacetic acid/H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile. While absorbance was monitored at 210 nm, fractions were manually collected by peak into 1.5-mL microfuge tubes and immediately stored without drying at –20 °C in preparation for peptide sequence analysis.

Difference peaks, identified in the peptide maps of control and fosfomycin-inactivated MurZ, were subjected to mass analysis using a Finnigan MAT (San Jose, CA) TSQ700 triple-quadrupole mass spectrometer equipped with a Finnigan API electrospray ionization source. Samples in 25% acetonitrile/0.1% trifluoroacetic acid were directly infused, at a flow rate of 3 μL/min, into the electrospray needle operating at a voltage differential of 4.5 kV. Spectra were recorded by scanning the appropriate *m/z* range in 3 s and averaging 16–32 scans in profile mode.

Subsequent to the confirmation of labeling of tryptic peptides by fosfomycin using mass spectrometry, difference peaks in the peptide maps of fosfomycin-inactivated and control MurZ were subjected to amino-terminal peptide sequence analysis.

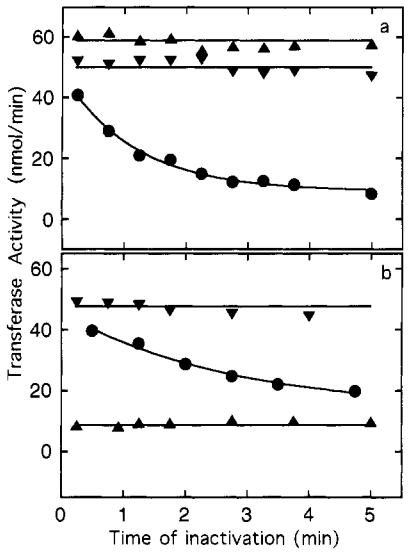


FIGURE 1: Influence of UDP-GlcNAc and its analog 3-deoxy-UDP-GlcNAc on the time-dependent inactivation of MurZ by fosfomycin. Panel a shows a plot of transferase activity remaining after incubation of 1.3 μM MurZ enzyme in the presence of 4 μM UDP-GlcNAc (▲), 2.4 μM fosfomycin (▼), and 4 μM UDP-GlcNAc and 2.4 μM fosfomycin (●). The transferase activity of the incubation mixtures in panel a was assayed as described in Materials and Methods. Panel b shows the transferase activity remaining after incubation of 27 μM MurZ in the presence of 60 μM fosfomycin (▼), 60 μM fosfomycin and 1 mM 3-deoxy-UDP-GlcNAc (●), or 60 μM phosphomycin and 1 mM UDP-GlcNAc (▲). Data in panel b were collected by assaying 15-μL volumes of the incubation mixtures using the coupled assay as described in Materials and Methods. Curves are nonlinear first-order fits of the data to the integrated rate equation (eq 9) as described in the text.

Fractions were applied directly to a polybrene precycled glass fiber filter placed in a reduced-volume reaction microcartridge. The sample was subjected to automated Edman degradation on an ABI Model 477A protein sequencer using the manufacturer's recommendations for faster cycle time (30 min). Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (Lane, 1991).

RESULTS

UDP-GlcNAc Accelerates the Inactivation of MurZ by Fosfomycin. Kahan and co-workers (1974) reported previously that the presence of UDP-GlcNAc was necessary to achieve inactivation of impure *E. cloacae* transferase by fosfomycin. We demonstrate here also (Figure 1a) that in the absence of UDP-GlcNAc no detectable inactivation of MurZ (1.3 μM) occurred after a 5-min exposure to fosfomycin (2.4 μM), compared to the control, while almost complete inactivation (8% residual) occurred in the presence of UDP-GlcNAc (4 μM). Figure 1b depicts the time-dependent inactivation of MurZ (27 μM) at a higher concentration of fosfomycin (60 μM) and a saturating concentration of nucleotide (1 mM), where inactivation in the presence of UDP-GlcNAc was essentially complete before the first time point (15 s) could be recorded. In an effort to understand the importance of the reactive 3-OH of UDP-GlcNAc to the inactivation process, we synthesized the 3-deoxy analog of UDP-GlcNAc and confirmed that it was a competitive inhibitor (K_i = 55 μM) of MurZ with respect to UDP-GlcNAc (data not shown). Inactivation in the presence of 3-deoxy-UDP-GlcNAc was slower than with the authentic nucleotide but was greatly accelerated over the control without either compound.

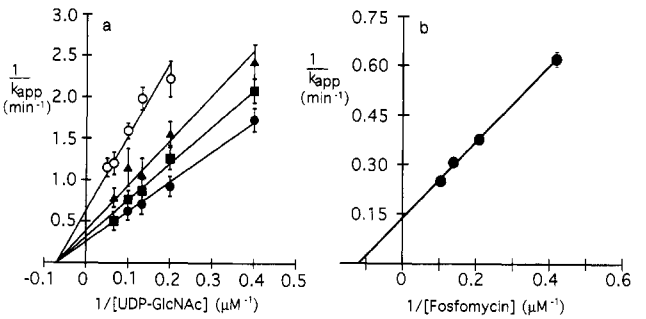


FIGURE 2: Determination of the limiting rate of inactivation (k_{inact}) of MurZ by fosfomycin. Panel a shows the influence of UDP-GlcNAc concentration on the rate of inactivation in a double-reciprocal plot. First-order rate constants for fosfomycin inactivation were obtained at UDP-GlcNAc concentrations of 2.5, 5.0, 7.5, 10, 15, and 20 μM and fosfomycin concentrations of 2.4 (○), 4.8 (▲), 7.2 (■), 9.6 μM (●). Panel b shows a plot of the inactivation rates at infinite UDP-GlcNAc concentration as a function of fosfomycin concentration. The limiting rate of inactivation, k_{inact}, was the reciprocal of the intercept at infinite fosfomycin concentration (7.4 min^{-1}).

Kinetics of Inactivation of MurZ by Fosfomycin. Figure 2 depicts results of a systematic study of the concentration dependence of the rate of inactivation on UDP-GlcNAc and fosfomycin concentrations according to the following model:

$$E + S \overset{K_S}{\rightleftharpoons} ES + F \overset{K_F}{\rightleftharpoons} ESF \overset{k_{inact}}{\rightarrow} IE \quad (1)$$

where MurZ (E) reversibly binds UDP-GlcNAc (S) to form the complex(ES), which then binds fosfomycin (F) to form a second complex of enzyme, substrate, and antibiotic (ESF), which irreversibly produces covalently modified, inactivated MurZ (IE). Assuming the rate of formation of inactivated enzyme is directly proportional to the concentration of enzyme in the ESF complex,

$$\frac{d[IE]}{dt} = k_{inact}[ESF] \quad (2)$$

where k_{inact} is the first-order rate constant of inactivation. With the total amount of enzyme fixed (E_{tot}),

$$[E_{tot}] = [E] + [ES] + [ESF] + [IE] \quad (3)$$

and

$$[E_{tot}] = [ESF]\,(1 + (K_F/[F])(1 + K_S/[S])) + [IE] \quad (4)$$

where

$$K_S = [E][S]/[ES] \quad \text{and} \quad K_F = [ES][F]/[ESF] \quad (5)$$

From eqs 2 and 3,

$$\frac{d([E_{tot}] - [IE])}{dt} = -k_{inact}([E_{tot}] - [IE])/(1 + (K_F/[F])(1 + K_S/[S])) \quad (6)$$

and the rate of inactivation of enzyme can be expressed as a fraction of total enzyme according to

$$\frac{d([E_{tot}] - [IE])}{dt} = -k_{app}([E_{tot}] - [IE]) \quad (7)$$

where

$$k_{app} = k_{inact}/(1 + (K_F/[F])(1 + K_S/[S])) \quad (8)$$

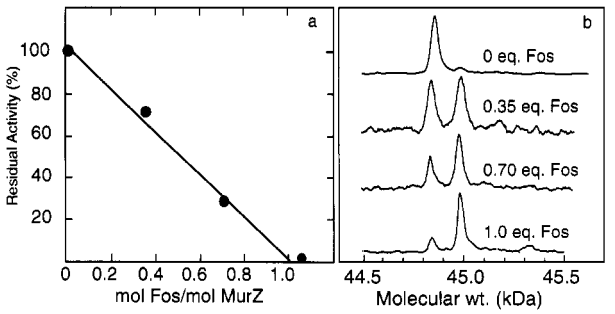


FIGURE 3: Determination of the stoichiometry of inactivation by fosfomycin in the presence of saturating UDP-GlcNAc. Untreated (not PEP-free) MurZ (14.4 μM) was incubated for 1 h at 25 °C with saturating UDP-GlcNAc (133 μM) and four concentrations of fosfomycin, 0, 5, 10, and 15 μM, corresponding to 0, 0.35, 0.70, and 1.05 equiv of Fos. Panel a shows the residual activity of those incubation mixtures as a function of equivalents added. Panel b shows LC–ESI mass spectra of those incubation mixtures.

The rate constants (k_{app}) were therefore determined at a number of fosfomycin and UDP-GlcNAc concentrations and were defined by the integrated rate equation

$$\frac{([E_{tot}] - [IE])}{[E_{tot}]} = e^{-k_{app}t} \quad (9)$$

where $([E_{tot}] - [IE])/[E_{tot}]$ was proportional to the activity of MurZ at a time t of incubation, relative to the activity at time zero.

Figure 2a shows a double-reciprocal plot of the influence of UDP-GlcNAc concentration on the rate of inactivation where the common intercept reflects a K_S of 14 μM for the substrate UDP-GlcNAc, in close agreement with its K_m of 15 μM (data not shown). A plot illustrating the influence of fosfomycin on the rate of inactivation at infinite UDP-GlcNAc concentration is shown in Figure 2b and allowed the determination of a binding constant for fosfomycin (K_F = 8.6 μM) in addition to the limiting inactivation rate constant (k_{inact} = 7.4 min^{-1}) at saturating UDP-GlcNAc and fosfomycin concentrations.

Stoichiometry of Fosfomycin Inactivation. We tested the stoichiometry of inactivation of MurZ by fosfomycin in the presence of saturating UDP-GlcNAc using enzyme activity and mass spectra as indices of the modification at four different concentrations of antibiotic. Figure 3a demonstrates a linear dependence of enzyme activity on the amount of antibiotic added which, at zero enzyme activity, demonstrates an intercept of one molecule of Fos added per molecule of inactivated enzyme. Figure 3b shows the deconvoluted mass spectra of MurZ titrated with fosfomycin. In the absence of added fosfomycin, the enzyme demonstrated a clean spectrum of molecular weight 44 834, which corresponds to that predicted by the gene sequence of *murZ*, 44 823 (Marquardt, 1993a). MurZ exposed to 0.35, 0.70, and 1.05 equiv of fosfomycin demonstrated increasing amounts of material of molecular weight 44 972. The difference of 138 Da between the two species is consistent with the molecular mass of fosfomycin ($C_3H_5O_4P$; 136 Da). While the sample titrated with 1 equiv of Fos was completely inactivated (see Figure 3a), a small amount of native enzyme (44 834 Da) remained, presumably because this small fraction was inactive and unreactive with fosfomycin. We note here also that covalent interaction is strongly suggested in this experiment by the conditions of LC–ESI-MS and because the same shift in mass has been demonstrated with MurZ which has been inactivated with fosfomycin, exposed to 7 M urea and spin-desalted (Penefsky, 1977) to remove small molecules (data not shown).

Site of Fosfomycin Inactivation. Given the covalent nature of the fosfomycin interaction and the stability of the MurZ–Fos complex to the conditions of mass spectrometry, we undertook to determine the site of attachment of the antibiotic to MurZ. Tryptic digests of native MurZ and MurZ treated with fosfomycin produced nearly identical peptide maps when separated by reverse-phase HPLC (Figure 4). The single difference in the peptide maps as a result of inactivation by fosfomycin was the disappearance of a peak at 75.6 min (■) and the appearance of a peak at 74.8 min (●). Mass spectral analysis of the material under those peaks revealed masses of 4239.6 and 4378.9 Da (data not shown), corresponding to a difference in mass of 139.3 Da, in close agreement with the molecular mass of fosfomycin (136 Da). Forty-one cycles of high-confidence amino-terminal sequence analysis provided the entire sequence of the peptide collected from the Fos-inactivated sample. The determined sequence matched exactly the protein sequence 104–144 predicted by the *E. coli* gene *murZ* (Marquardt *et al.*, 1993a) excepting cycle 12, which corresponds to position Cys115 in the protein sequence, in which no PTH-labeled amino acid was observed. Five cycles of the control peptide (peak at 75.6 min) confirmed that it also corresponded to the 41-mer of sequence 104–144. We note that these peptides were not carboxymethylated, and therefore we would expect that the cysteine in the 41-mer at position 115 would be invisible in our sequence analysis regardless of modification by fosfomycin. The discovery by Kahan *et al.* (1974) of a [^{3}H]fosfomycin–cysteine thioether and the conservation of this cysteine among the bacterial UDP-GlcNAc enolpyruvoyl transferases strongly implicate the residue as the site of fosfomycin attachment. Moreover, unlike the native enzyme, C115A and C115S variants of MurZ confer no resistance to fosfomycin when overexpressed in *E. coli* to levels comparable to that of wild type (Marquardt, 1993). Finally, our results are in agreement with Wanke and Amrhein (1993) who, during the preparation of this paper, reported localizing, by mass spectrometry and amino acid sequencing, the site of attachment of fosfomycin to a 17-mer of the transferase from *E. cloacae*, which contained the conserved Cys115.

DISCUSSION

We report here that fosfomycin is a time- and UDP-GlcNAc-dependent stoichiometric inactivator of UDP-GlcNAc enolpyruvoyl transferase which captures a nucleophilic cysteine at the active site of the enzyme. We note also that during the preparation of this paper a report mapping the site of fosfomycin inactivation of the transferase from *E. cloacae* was published by Wanke and Amrhein (1993) which is in agreement with the results of this study.

Kahan *et al.* (1974) previously demonstrated that UDP-GlcNAc potentiated the inactivation of crudely purified transferase preparations. In the accompanying paper (Brown *et al.*, 1994), we report the finding that, as purified, the transferase MurZ contains 1 equiv of tightly bound PEP which is chemically competent for reaction with UDP-GlcNAc to produce 1 equiv of product. We reasoned that if PEP and fosfomycin react with the same active site nucleophile, then UDP-GlcNAc might facilitate inactivation simply by chasing PEP to product and freeing up the reactive nucleophile. In this work, we therefore used purified enzyme which was free of bound PEP and confirmed that, even in the absence of bound PEP, UDP-GlcNAc was required for rapid inactivation of MurZ (Figure 1). In order to probe the possibility that UDP-GlcNAc was somehow participating in the chemistry

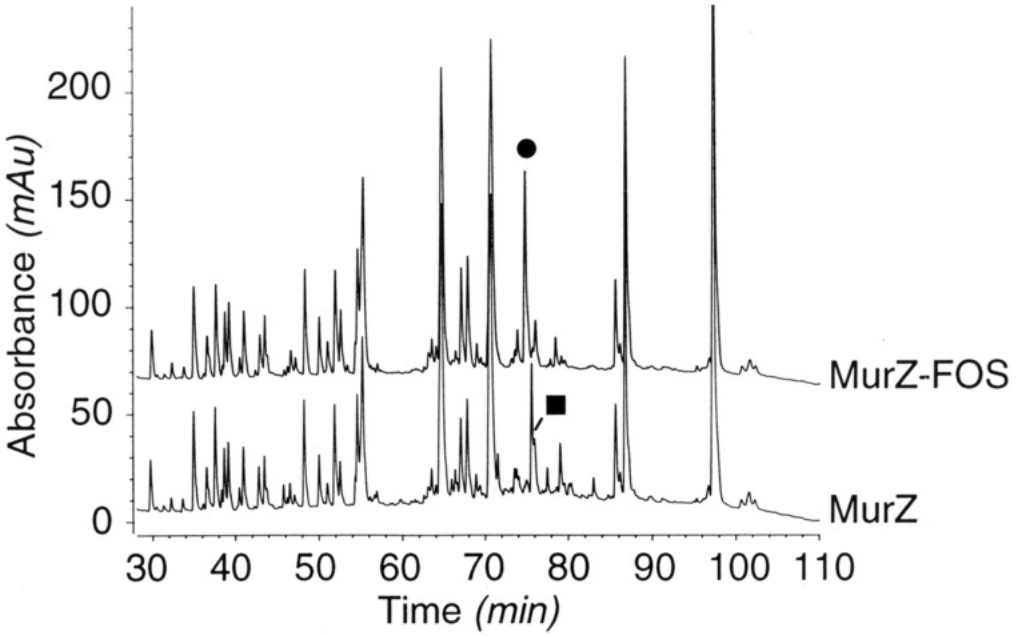
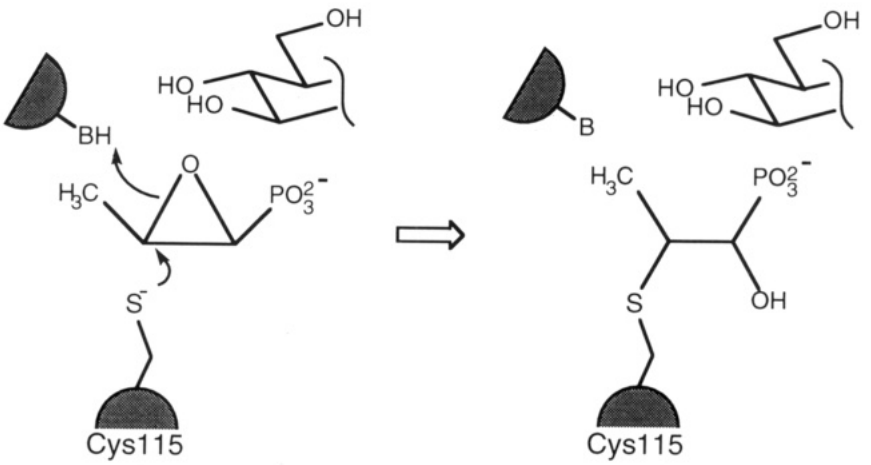


FIGURE 4: Tryptic peptide maps of MurZ and MurZ inactivated with fosfomycin in the presence of saturating UDP-GlcNAc (1 mM). The plot shows an overlay of the HPLC reverse-phase separations of the tryptic maps of MurZ and MurZ which has been inactivated by fosfomycin as described in Materials and Methods. Difference peaks in the two peptide maps are indicated by the symbols ■ at 75.6 min and ● at 74.8 min.

Scheme 1



of the inactivation in a process analogous to its reaction with PEP, we synthesized and tested the unreactive 3-deoxy analog of UDP-GlcNAc for its effect on the inactivation reaction. Although its effect was not as potent as that of authentic UDP-GlcNAc, the unreactive analog was also capable of greatly accelerating the inactivation (Figure 1), suggesting that the nucleotide potentiates inactivation by promoting an active site conformation which is critical to the inactivation event (Scheme 1). Through a systematic study of the UDP-GlcNAc dependence of the reaction, we demonstrated the limiting k_{inact} for that process to be 7.4 min^{-1} and determined the dissociation constants for UDP-GlcNAc and fosfomycin to be 14 and 8.6 μM, respectively (Figure 2). As the k_{cat} for normal turnover of the enzyme is 3 s^{-1}, we calculate that the enzyme-activated nucleophilic ring opening of the epoxide group in fosfomycin occurs at 4% of the normal reation rate.

To determine the stoichiometry of the inactivation, we followed the modification of MurZ by fosfomycin using enzyme activity and mass spectral analysis as indices of covalent attachment (Figure 3). A fosfomycin titration of enzyme activity revealed that inactivation was directly proportional to fosfomycin added and demonstrated that 1 equiv of antibiotic was capable of completely inactivating MurZ. Mass spectral analysis demonstrated an increase in molecular mass of 138 mass units on exposure to fosfomycin, in close agreement with a molecular weight of 136 for the antibiotic. Titration with fosfomycin resulted in an increase in the amount of the higher molecular weight species and was in qualitative agreement with titration of enzyme activity, confirming that inactivation was due the covalent attachment of 1 equiv of fosfomycin.

Peptide mapping and mass spectral studies of this work suggest that Cys115 in the sequence of *E. coli* MurZ is the site of covalent attachment by fosfomycin (Scheme 1) and are in agreement with independent studies by Wanke *et al.* (1993) on the transferase from *E. cloacae*. Previously Kahan *et al.* (1974) were successful in isolating a [^{3}H]fosfomycin–cysteine thioether from an exhaustive digest of partially purified, [^{3}H]fosfomycin-treated transferase preparations. In addition, they observed that the thioether cochromatographed and cocrystallized with authentic 2-((*S*)-L-cysteinyl)-1-hydroxypropylphosphonic acid. That result was intriguing in the context of observations that an active site nucleophile was capable of capturing radiolabeled PEP as an enolpyruvoyl

(Zemell & Anwar, 1975) or a phospholactoyl (Cassidy & Kahan, 1973) adduct. Indeed, in the accompanying paper (Brown *et al.*, 1994), we report the characterization of a kinetically competent phospholactoyl enzyme intermediate in the reaction catalyzed by MurZ and provide evidence that the intermediate is in thioether linkage with an active site cysteine. Furthermore, given that PEP binding is competitive with the inactivation of UDP-GlcNAc enolpyruvoyl transferases by fosfomycin (Kahan *et al.*, 1974) and that C115S and C115A variants of the transferases have no detectable transferase activity (Marquardt, 1993; Wanke *et al.*, 1993), Cys115 is strongly implicated as a catalytic residue.

A complete understanding of the mechanism of UDP-GlcNAc-accelerated, enzyme-assisted inactivation of UDP-GlcNAc enolpyruvoyl transferase by fosfomycin will be invaluable to the design of other antibacterial agents targeting this first step in peptidoglycan biosynthesis. Currently, further mechanistic studies are underway to unambiguously identify the stereochemistry and site of attack of Cys115 on the reactive epoxide of fosfomycin.

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