Regiochemical and Stereochemical Course of the Reaction Catalyzed by the Fosfomycin Resistance Protein, FosA

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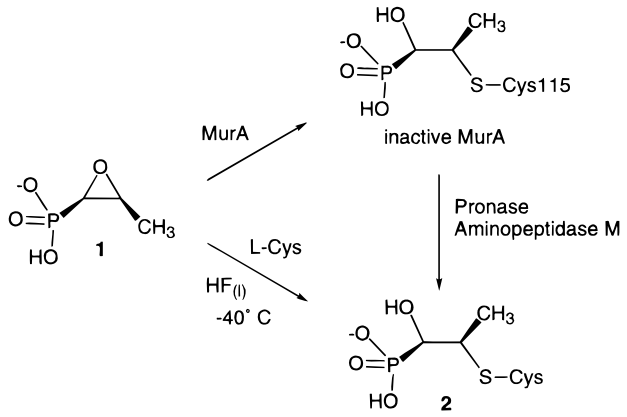
Received January 5, 1998

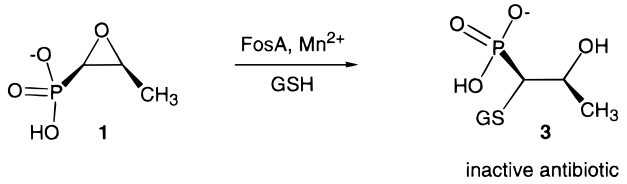
Fosfomycin [(1*R*,2*S*)-epoxypropylphosphonic acid], **1**, is a broad spectrum antibiotic effective against both Gram-positive and Gram-negative microorganisms.[1] The antibiotic irreversibly inactivates the enzyme, uridine-5′-diphospho-*N*-acetylglucosamine-3-enolpyruvyltransferase (MurA), which catalyzes the first committed step in cell wall biosynthesis.[2] The inactivation occurs through alkylation of a cysteine residue (Cys115 of the enzyme from *Escherichia coli*) in the active site of MurA, as illustrated in Scheme 1, and requires the presence of the substrate, UDPGlcNAc, or substrate analogue.[2,3] That the alkylation involves attack of the thiol at C2 of the antibiotic was originally established[2] by comparison of the cysteine adduct excised from inactivated enzyme with a synthetic standard of *threo*-2-(*S*-L-cysteinyl)-1-hydroxypropylphosphonate, **2**, as illustrated in Scheme 1 and was recently confirmed in the crystal structure of the fosfomycin-inactivated MurA enzyme.[4]

Plasmid-mediated resistance to the antibiotic was demonstrated a number of years ago and was subsequently found to depend on the expression of a fosfomycin-specific metalloglutathione transferase called FosA.[5,6] The reaction catalyzed by FosA was proposed to involve addition of the thiol of glutathione to C1 of the antibiotic as illustrated in Scheme 2. This regiochemistry is somewhat surprising in that nucleophilic substitutions α to phosphonates are generally difficult due to both steric and electronic factors.[7] Although the previously published NMR properties of the product, **3**, are consistent with addition of the nucleophile at C1, the regiochemistry has not been rigorously established.[6a] Furthermore, the stereochemical course of the addition,

Scheme 1



Scheme 2



which is presumed to occur as a single displacement with inversion of configuration at C1, has not been elucidated.

In this paper we show that L-cysteine can act as an alternative thiol substrate for FosA. The regiochemistry of the reaction is the same as that exhibited with normal substrate, glutathione, with addition of the nucleophile at the most hindered carbon, C1. The stereochemical course of the reaction is shown to occur with inversion of configuration at C1 of the antibiotic, strongly suggesting that the enzyme catalyzes the reaction via an S_N2 or borderline S_N2 mechanism and argues against a double displacement mechanism involving initial attack by an enzymic nucleophile.

The purified enzyme[6b] was used for a preparative scale synthesis of both the GSH and cysteine adducts, **3** and **4**. The previously proposed regiochemistry of **3** (Scheme 2) was first examined by heteronuclear multiple-bond correlation (HMBC) spectroscopy as illustrated in Figure 1. The cross-peaks between the ^{13}C resonances of C1 of the antibiotic fragment (split by coupling to ^{31}P, ^1J = 127.0 Hz) and the two diastereotopic Cβ-protons of the cysteinyl residue of the peptide establish that the thioether linkage indeed involves C1 of the hydroxypropylphosphonate.

In contrast to previous reports we find that FosA will catalyze the addition of alternative thiol substrates such as L-cysteine to **1**, albeit with reduced efficiency. The rate constants for the enzyme-catalyzed addition of GSH and L-cysteine are compared in Table 1. The efficiency is reduced by factors of about 8 and >400 in k_{cat} and k_{cat}/K_m, respectively. Nevertheless, the reaction proceeds with the same regiochemistry as the enzyme-catalyzed addition of GSH. One advantage of the cysteine adduct is that it was easily crystallized for structure determination by X-ray crystallography. The absolute configuration at C1 (Scheme 3) was deduced from the crystal structure of **4** from its relationship to the known config-

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S0022-3263(98)00014-0 CCC: $15.00
Published on Web 04/30/1998

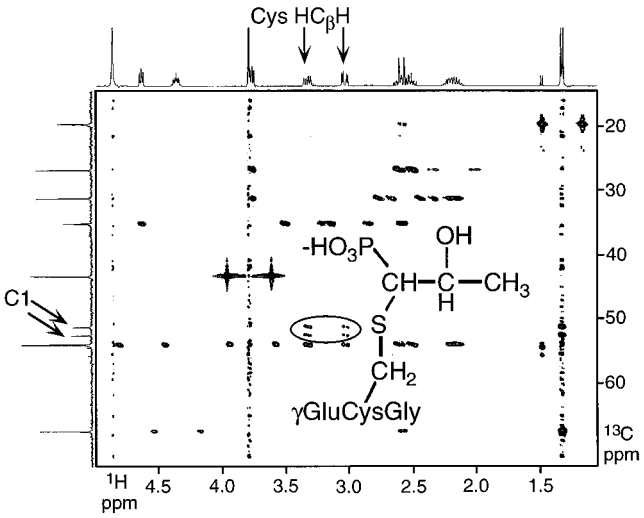


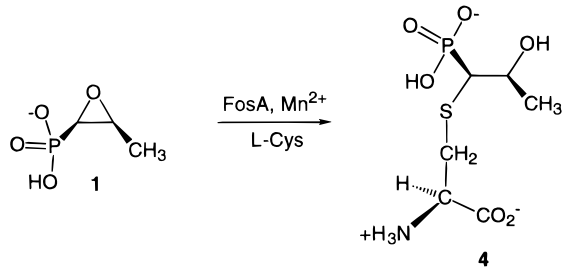
Figure 1. Portion of the HMBC NMR spectrum of **3**. The cross-peaks between C1 and the two diastereotopic protons at Cβ of the cysteine residue are circled. A complete list of resonance assignments appears in the Supporting Information.

Table 1. Comparison of Kinetic Constants for the Enzyme-Catalyzed Addition of GSH and L-Cysteine to Fosfomycin, 1[a]

substrate	k_{cat} (s^{-1})	k_{cat}/K_m^{thiol} (M^{-1} s^{-1})	K_m^{thiol} (mM)
GSH	1,070 ± 50	173,000 ± 27,000	6.2 ± 0.7
L-Cys	134 ± 5	419 ± 43	320 ± 20

[a] Reactions were carried out as described in the Experimental Section with a fixed concentration of **1** (0.5 mM) while the concentration of the thiol substrate was varied.

Scheme 3



uration at the α-carbon of the L-cysteinyl group. The product **4** is established as (1*R*,2*S*)-1-(S-L-cysteinyl)-2-hydroxypropylphosphonate. Thus, the stereochemical course of the enzyme-catalyzed reaction results in inversion of configuration at C1 (Scheme 3), a good indication that the enzyme proceeds by direct S_N2 or borderline S_N2 attack of the nucleophile at the oxirane carbon of the activated substrate. A double displacement reaction at C1 involving an enzymic nucleophile would result in retention of configuration.

Under normal physiologic conditions, fosfomycin is refractory to the addition of nucleophiles due to the presence of the anionic phosphonate group. The facile enzyme-catalyzed addition of GSH or L-Cys to the antibiotic is particularly surprising with respect to the regiochemistry of the reaction. Nucleophilic addition α to the phosphonate is difficult due to both steric and electrostatic factors. The phosphonyl group is roughly equivalent in size to a neopentyl group. In addition, it is ionized at physiologic pH, a fact that does not encourage approach of anionic nucleophiles. Clearly the enzyme, presumably with direct participation of the metal center,[6a] is able to activate C1 sufficiently for efficient addition of the nucleophile. Although a role for the metal in neutralizing the negative charge on the phosphonate is easy to envision, how it might mitigate the steric effect is less obvious. That acid-catalyzed addition of cysteine to **1** occurs at the least hindered carbon, C2 (Scheme 1) suggests that FosA and the associated metal do more than simply act as a Lewis acid to activate the oxirane. Exactly how the enzyme promotes nucleophilic addition at the more hindered position is under active investigation.

Experimental Section

Assay of the Enzyme. The assay of the enzyme with GSH and L-cysteine as substrates was carried out by the fluorescence-detected HPLC procedure using the Waters AQC [6-(aminoquinolyl)-*N*-hydroxysuccinimidyl carbamate] reagent as previously described.[6b] All reactions were carried out in 100 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES)/KOH buffer (pH 8.0) at 25 °C. The K^+ concentration was 60 mM. The dependence of the kinetics on the concentration of thiol was studied with a fixed (0.5 mM) concentration of the tetramethylammonium salt of fosfomycin, which is the K_m for fosfomycin when [GSH] = 50 mM. It should be noted that the K_m for fosfomycin varies with the identity and increases with the concentration of the thiol substrate. Reactions with cysteine as substrate were carried out with 30 nM FosA, 200 μM $MnCl_2$, 0.5 mM fosfomycin, 80, 100, 133, 200, 400, and 760 mM L-Cys for 90 s. Reactions with GSH were carried out with 2 nM FosA, 200 μM $MnCl_2$, 0.5 mM fosfomycin, and 3, 4, 5, 6.7, 10, 20, 50 mM GSH incubated for 45 s. The tetramethylammonium salt of fosfomycin was prepared by loading 1 gm of fosfomycin sodium salt dissolved in 10 mL of water onto a 1.8 × 10 cm Dowex AG-1-X2 column (200−400 mesh, hydroxide form). The resin was washed with 85 mL of water, and the fosfomycin was eluted with a 200 mL of 0−500 mM gradient of tetramethylammonium chloride.

Enzymatic Synthesis of 3 and 4. For the synthesis of **4** a 100 mL solution of 200 mM fosfomycin, 200 mM L-cysteine, 5 μM FosA, 10 μM $MnCl_2$ at pH 8.0 was incubated for 18 h at 22 °C. The completion of the reaction was determined by HPLC with Waters AQC reagent as described above (retention times: **4** = 9.7 min, cysteine = 13.3 min). No product was detected in a control sample in the absence of FosA. Protein was precipitated with 10% trichloroacetic acid and removed by centrifugation. The product solution was extracted with 7 equal volumes of ethyl ether until the pH = 4.5 and dried. The product was resuspended in water, and the pH was adjusted to 8.5 with ammonium acetate and loaded onto a 2.5 × 15 cm bed of Dowex AG1-X2 resin equilibrated with 10 mM ammonium bicarbonate, pH 8.0. The resin was washed with 200 mL of equilibration buffer, and the product was eluted by a 1 L linear gradient of 10 mM to 2 M ammonium bicarbonate (pH 8). The product was detected by placing a drop of each fraction on a silica plate and staining with ninhydrin. The fractions containing product were pooled, and the excess ammonium bicarbonate was removed by repeated lyophilization. ^{1}H NMR (400.13 MHz, D_2O, pD 8.4) δ 1.19 (d, 3H, J = 6.2 Hz), 2.35 (dd, 1H, J = 2.0 and 15.1 Hz), 2.9−3.0 (m, 2H), 3.72 (dd, 1H, J = 4.7 and 7.8 Hz), 4.22 (t, 1H, J = 6.0 Hz); ^{13}C NMR (75.469 MHz, D_2O, pD 8.4), δ 19.24 (d, J = 40.5 Hz), 33.63 (s), 49.91 (d, J = 128.8 Hz), 52.69 (s), 66.45 (s), 172.47 (s); ^{31}P NMR (121.496 MHz, D_2O, pD 8.4) δ 17.74 (dd J = 8.5 and 16.9 Hz). Complete NMR assignments appear in Supporting Information. Crystals of the diammonium salt monohydrate (see below) gave the following elemental analysis consistent with the crystal structure. Anal. Calcd for $C_6H_{12}NO_6PS•2NH_4^+•H_2O$: C, 23.15; H, 7.12; N, 13.50; P, 9.95; S, 10.30. Found: C, 23.35; H, 7.10; N, 13.27; P, 9.58; S, 10.05.

The synthesis of **3** followed a similar procedure. The product was purified by a procedure similar to that described for **4** with the following modifications. The protein was removed by an Amicon YM-10 membrane instead of trichloroacetic acid precipitation. Fractions from the AG1-X2 column containing **3** were dried, resuspended in 15 mL of water. The pH of the solution

was adjusted to 4.5 with HCl and was loaded onto a 2.5 × 15 cm bed of Dowex AG50-X8 (200−400 mesh, H^+ form) equilibrated with water. The column was washed with water and the free acid of **3** eluted after 150 mL. The final product exhibited a single peak on HPLC. The product was fully characterized by ^1H, ^{13}C, ^{31}P and heteronuclear multiple-bond correlation (HMBC) NMR spectroscopy. ^1H NMR (400.13 MHz, D_2O, pD 8.4) δ 1.33 (d, 3H, J = 6.4 Hz) 2.15−2.21 (m, 2H) 2.51−2.60 (m, 2H) 2.58 (dd, 1H, J = 2.6 and 16.2 Hz) 3.03 (dd, 1H, J = 4.4 and 13.9 Hz) 3.32 (dd, 1H, J = 4.4 and 13.8 Hz) 3.76 (dd, 1H, J = 5.1 and 7.2 Hz) 3.79 (s, 1H) 4.3−4.4 (m, 1H) 4.62 (dd, 1H, J = 4.4 and 6.6 Hz): ^{13}C NMR (100.614 MHz, D_2O, pD 8.4) δ 19.80 (d, J = 9.7) 26.91 (s) 31.36 (s) 35.29 (d, J = 2.8 Hz) 43.50 (s) 52.00 (d, J = 127.0 Hz) 54.14 (s) 54.15 (s) 67.64 (s) ^{31}P NMR (121.496 MHz, D_2O, pD 8.4) δ 17.21 (dd, J = 9.2 and 16.6 Hz). Anal. Calcd. for $C_{13}H_{24}N_3O_{10}PS\bullet H_2O$: C, 33.70; H, 5.66; N, 9.07; P, 6.68; S, 6.92. Found: C, 33.38; H, 5.57; N, 8.83; P, 6.65; S, 7.27.

Crystal Structure of 4. Crystals were grown by the liquid diffusion method.[8] The product was dissolved in water (75 mg/mL) and added to a glass tube (500 μL), and crystals were grown by layering acetone:methanol (10:1) on the aqueous layer containing the product. The tube was stoppered and allowed to sit, undisturbed, for 2 days. Crystals of the diammonium salt monohydrate grew at the solvent interface. The structure determination was performed by Dr. Paul D. Boyle, Department of Chemistry, North Carolina State University, Raleigh. The crystals were of the triclinic space group $P1$ with Z = 2. The final structure had an R factor = 0.023 for all 2411 reflections. The crystallographic data has been deposited with the Cambridge Crystallographic Data Centre.

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Acknowledgment. The initial part of this work was supported in part by National Institutes of Health Grants R01 GM30910, P30 ES00267, and T32 ES07028. Subsequent support was derived from R01-AI42756. L.T.L. was supported by NRSA postdoctoral fellowship F32 GM18296. We thank Marcus Voehler for his assistance in obtaining the NMR spectra, Dr. Kris Tesh for his assistance in crystallization experiments, and Dr. Christopher Halkides for his comments on substitutions α to phosphonates.

Supporting Information Available: NMR assignments for **3** and **4** (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO980014B