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Mechanistic Diversity of Fosfomycin Resistance in Pathogenic Microorganisms

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Fosfomycin [(1*R*,2*S*)-epoxypropylphosphonic acid, **1**] is a natural product with broad-spectrum antimicrobial activity against Gram-positive and Gram-negative microorganisms.[1] The clinical efficacy of fosfomycin is compromised by both plasmid and genomically encoded resistance proteins that catalyze the addition of thiols to the oxirane ring (Scheme 1).[2]

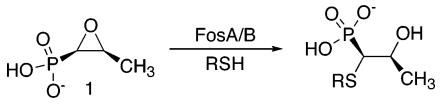
The FosA protein encoded in the genome of in the Gram-positive organism *Pseudomonas aeruginosa* is an Mn(II)-dependent metalloenzyme that catalyzes the addition of glutathione (GSH) to the antibiotic and requires the monovalent cation K^+ for optimal activity.[2a] In contrast, Gram-negative microorganisms, which do not make glutathione, have evolved a related Mg^{2+}-dependent enzyme (FosB) that catalyzes the addition L-cysteine.[2c] Thus, the selective pressure of the antibiotic has resulted in alternate mechanisms of resistance that depend on the biology of the organism. The extent of the mechanistic diversity in resistance to fosfomycin is not known. An understanding of the chemical basis and evolution of resistance in microbial populations is essential for developing strategies to counter resistance in clinical settings.

Interrogation of the microbial genome sequence database with the sequences of the FosA and FosB proteins revealed a related subfamily of enzymes (FosX) sharing 30−35% sequence identity with FosA and FosB in several microorganisms, including *Mesorhizobium loti* and the pathogens *Listeria monocytogenes*, *Brucella melitensis*, and *Clostridium botulinum*. In this paper we present evidence that the FosX proteins from *M. loti* and *Listeria* catalyze the hydration of fosfomycin and represent an alternative, genomically encoded mechanism of resistance.

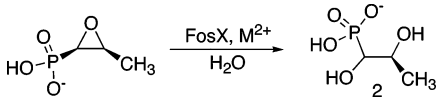
The genes *mlr*3345 and *lmo*1702 were amplified from genomic DNA and ligated into a pET-20b(+) expression plasmid. The proteins, when expressed in *E. coli* BL21(DE3) cells, confer resistance to fosfomycin.[3] Sequence alignments of the *M. loti* FosX with FosA and FosB enzymes showed the absolute conservation of H7, H69, and E118. In FosA, these residues are protein ligands involved in divalent cation binding, which suggests the same would occur in FosX.[2a] Incubation of both the *M. loti*- and *Listeria*-derived FosX proteins in the presence of divalent cations and fosfomycin yielded a previously unobserved product, which was characterized by NMR and mass spectrometry to be 1,2-dihydroxypropylphosphonic acid (**2**) (Scheme 2).[4] The identification was further confirmed by comparison with the diol obtained from acid-catalyzed hydrolysis of **1**.

A survey of the activation of the FosX proteins by divalent cations indicated a clear preference for Mn(II). Unlike FosA, the FosX proteins do not require a monovalent cation for optimum activity. The steady-state kinetic constants for the *M. loti* FosX are not very impressive [$k_{cat} = 0.15 \pm 0.02$ s^{-1} and $k_{cat}/K_m = (5.0 \pm 0.6) \times 10^2$ M^{-1} s^{-1}] and correlate with the fact that the protein confers only modest resistance to the antibiotic (MIC = 0.025 mg/mL)[3] in *E. coli*. In contrast, the *Listeria*-derived protein is a far superior catalyst [$k_{cat} = 34 \pm 2$ s^{-1} and $k_{cat}/K_m = (9 \pm 2) \times 10^4$ M^{-1} s^{-1}] that confers very robust resistance (MIC > 25 mg/mL) to the antibiotic in the biological context of *E. coli*.

Scheme 1



Scheme 2



To better define the mechanistic basis of the hydration reaction, the crystal structure of the FosX from *M. loti* was determined. The protein crystallized at 20 °C from 32% PEG 8000, 100 mM Tris (pH 8.5), and 50 mM Li_2SO_4 in the presence of 1 mM $MnCl_2$ in the space group $P2_12_12$. The structure was solved at a resolution of 1.83 Å by molecular replacement with the FosA model[2a] as the probe.[3] The atomic coordinates and structure factors for the final FosX model ($R_{work} = 0.201$, $R_{free} = 0.243$) appear in the Protein Data Bank as file 1R9C.

The FosX structure (Figure 1) is quite similar to that of the FosA from *Pseudomonas aeruginosa*.[2a] As with FosA and other members of the VOC superfamily, the structure contains paired $\beta\alpha\beta\beta\beta$ motifs that form a cup-shaped metal ion binding site.[2a,5] The subunits are in a 3D-domain swapped arrangement, where H7 of one subunit joins H69 and E118 from the opposite subunit to form the divalent metal binding site. No electron density is observed for residues 99−103 in subunit A or residues 100−102 in subunit B. The equivalent regions in FosA are part of the K^+-binding loop.

An overlay of the FosX and FosA active sites is very informative from a mechanistic perspective. There is a large degree of structural similarity between the two enzymes that extends beyond the metal binding ligands (Figure 2). The similarities include residues involved in substrate recognition, such as the hydroxyl group of T9, which has been proposed to act as an electrophilic catalytic group in concert with Mn(II) in FosA,[2a] and the side chain of R127, which appears to be involved in the binding of fosfomycin.

The most provocative aspect of the comparison of the two active sites is the carboxylate of E44 in FosX, for which the structurally equivalent residue in FosA is a glycine (G37). Assuming that fosfomycin binds in the same orientation in FosA and FosX, the carboxylate of E44 is positioned 5.0 Å from the backside of C1 of the antibiotic. The location of E44 suggests that it could act either as a nucleophile to form an alkyl-enzyme intermediate (a common mechanism for epoxide hydrolases)[7] or as a general base for the direct addition of water to the oxirane carbon. Sequence alignments

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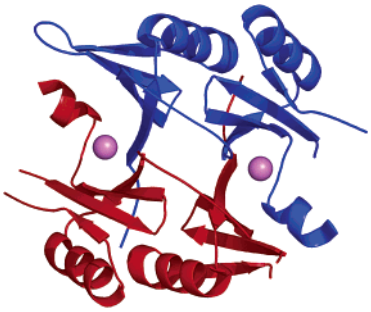

Figure 1. Ribbon diagram of the FosX dimer, with the two subunits illustrated in blue and red. The positions of the Mn(II) ions are shown in magenta. This image was generated using the program PyMOL.[6]

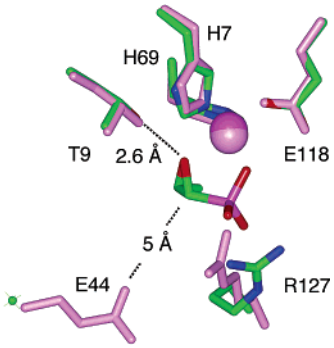

Figure 2. Overlay of the active site residues of FosA, with bound fosfomycin and Mn(II) (1LQP), and FosX (pink) with bound Mn(II). The side chains for the FosX protein are labeled. The equivalent positions in FosA, listed clockwise from lower left, are G37 (green dot), T9, H64, H7, E110, and R118. The carboxylate of E44 of FosX is positioned to play a direct role in the addition of water to C1.

show that this glutamate is fully conserved in this subfamily, including the *Listeria* FosX.

The ^{13}C NMR spectrum of the diol product, obtained by incubation of either the *M. loti* or *Listeria* FosX with substrate in 45% $H_2{}^{18}O$, exhibits C1 signals at 74.09 and 72.21 ppm, with 45% of the signal appearing 0.023 ppm upfield due to the ^{18}O isotopic perturbation of the ^{13}C chemical shift. No ^{18}O perturbation is seen for the C2 signal, indicating that the enzyme is regiospecific in adding water to C1.

The origin of the oxygen atom in the FosX-catalyzed reaction was determined by performing single turnover experiments in 95% $H_2{}^{18}O$. The mass of the product ($[M - H]^- = 156.9$) is consistent with quantitative incorporation of ^{18}O into the diol during the first turnover when compared to the mass of a nonlabeled control.[4a] This result is consistent with a mechanism involving direct addition of water to C1 and not a covalent alkyl-enzyme intermediate, where the oxygen in the first turnover would be derived from the carboxylate group.[7a]

The hypothesis that E44 plays a key role in catalysis was tested by generation of the E44G mutant for both enzymes. The mutation reduced the turnover number of the *M. loti* FosX 700-fold to 0.0002 s^{-1} and essentially abolished the catalytic activity of the *Listeria* enzyme ($k_{cat} < 0.00004$ s^{-1}). Taken together, the results suggest that E44 acts as a general base in the direct addition of water to the antibiotic.

The tepid catalytic activity of the *M. loti* FosX and its inability to confer robust resistance to fosfomycin suggest that it may have a role in biology other than the hydration of **1**. The *mlr*3345 gene resides in what appears to be an operon for phosphonate utilization in rhizobia. Interestingly, when the *M. loti* FosX is incubated in the presence of Mn(II), fosfomycin, and GSH, a substantial amount of the GSH adduct is also formed, albeit at a slower rate ($k_{cat} \approx 0.06$ s^{-1}) than the hydration reaction. In contrast, the much more efficient FosX from *Listeria* is not promiscuous and does not generate any detectable adduct with GSH.

The catalytic promiscuity and low efficiency of the *M. loti* protein suggest that it may be an intermediate in the evolution of fosfomycin resistance proteins such as FosA, FosB, and FosX, derived from a phosphonate utilization pathway.

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Supporting Information Available: Description of the cloning of the gene, purification, characterization of the protein, data collection statistics, refinement protocol, and structure validation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(3) Details of all experimental procedures can be found in the Supporting Information.

(4) (a) $[M - H]^- = 155.03$ for **2**, as observed by negative-ion ESI-MS. (b) 1,2-Dihydroxypropylphosphonic acid: ^1H NMR (300.131 MHz, D_2O) δ 1.19 (d, 3H, $J = 6.6$ Hz), 3.35 (d of d, 1H, $J = 4$ Hz, $J = 10$ Hz), 4.05 (m, 1H); ^{13}C NMR (75.471 MHz, D_2O) δ 18.72 (d, $J = 8.4$ Hz), 68.29 (s), 73.25 (d, $J = 141$ Hz); ^{31}P NMR (121.495 MHz, H_2O, pH 8.0) δ 16.95 (s).

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