Accelerated Publications

Fosfomycin Resistance Protein (FosA) Is a Manganese Metalloglutathione Transferase Related to Glyoxalase I and the Extradiol Dioxygenases[†]

Bryan A. Bernat, L. Timothy Laughlin, and Richard N. Armstrong*

Department of Biochemistry and the Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

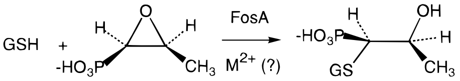
Received December 27, 1996; Revised Manuscript Received February 4, 1997[⊗]

ABSTRACT: The enzyme conferring resistance to the antibiotic fosfomycin [(1*R*,2*S*)-1,2-epoxypropylphosphonic acid] originally reported by Suarez and co-workers [Arca, P., Hardisson, C., & Suarez, J. E. (1990) *Antimicrob. Agents Chemother. 34*, 844−848] is demonstrated in this study to be a metalloglutathione transferase. The apoenzyme is a dimer of 16 kDa subunits. Electron paramagnetic resonance spectroscopy and water proton nuclear magnetic resonance longitudinal relaxation rates suggest that each subunit contains a mononuclear Mn^{2+} center that interacts strongly with the substrate fosfomycin ($K_d = 17\ \mu M$) more weakly with the product ($K_d = 1.1$ mM) and very weakly or not at all with GSH. Inhomogeneous broadening of the EPR signals of enzyme-bound Mn^{2+} in the presence of $H_2{}^{17}O$ indicates that three of the coordination sites on the metal are occupied by water. Sequence alignments, three-dimensional structures, and mechanistic considerations suggest that FosA is related to at least two other metalloenzymes, glyoxalase I and the Mn^{2+}- or Fe^{2+}-containing extradiol dioxygenases. The mechanistic imperative driving the evolution of this previously unidentified superfamily of metalloenzymes is proposed to be bidentate coordination of a substrate or intermediate to the metal center in the enzyme-catalyzed reactions.

In 1969, Merck reported the biological properties, synthesis, and structure of a potent, broad-spectrum antibiotic, (1*R*,2*S*)-epoxypropylphosphonic acid (phosphonomycin, subsequently called fosfomycin), synthesized by certain strains of *Streptomyces* (Hendlin et al., 1969; Christensen et al., 1969). The mechanism of action of the antibiotic was determined to be the irreversible inactivation of the enzyme catalyzing the first committed step in peptidoglycan biosynthesis, UDPGlcNAc-3-enolpyruvyltransferase[1] or MurA. Analysis of the inactivated enzyme revealed the antibiotic alkylated a cysteine residue (Kahan et al., 1974) subsequently identified as Cys115 in the active site (Marquardt et al., 1994). Acquired resistance to fosfomycin was observed early in the microbiological studies and was associated with chromosomal mutations affecting transport of the antibiotic into the cytosol (Kadner & Winkler, 1973; Tsuruoka & Yamamda, 1975) and with mutations in the target, MurA (Venkateswaran & Wu, 1972).

Some years later Garcia-Lobo and Ortiz (1982) described a plasmid from clinical isolates that also conferred resistance to the antibiotic. Subsequent investigations by Suarez and co-workers (Arca et al., 1988, 1990) established that the resistance gene encoded a 16 kDa polypeptide (FosA) which catalyzed the addition of GSH to the antibiotic, as shown in Scheme 1, rendering it inactive.

Scheme 1



In addition to establishing the regiochemistry of the reaction, the enzymatic activity was shown to be stimulated to a small degree (2−3-fold) by the addition of divalent cations specifically, Mn^{2+}, Co^{2+}, and Fe^{2+}. Although the original data were not sufficient to establish FosA as a metalloenzyme, the remarkable stability of the oxirane ring (Kahan et al., 1974) suggested to us a specific role for a metal ion acting as an electrophilic catalyst for activation of fosfomycin toward nucleophilic attack.

In this paper, we describe the expression, purification, and characterization of apo-FosA and its activation by several divalent cations. Evidence is presented that the active site of the native enzyme contains a mononuclear Mn^{2+} metal center that is involved in the activation of the oxirane ring of fosfomycin for nucleophilic attack. Electron spin and nuclear magnetic resonance experiments suggest that each 16 kDa subunit contains a single metal-ion binding site. The coordination environment of the metal is altered upon binding fosfomycin and the product 1-(*S*-glutathionyl)-2-hydroxypropylphosphonic acid (GS-fos) but not GSH. Sequence similarities, three-dimensional structures, and mechanistic considerations suggest that FosA is one member of a diverse

---

[†] Supported by National Institutes of Health Grants R01 GM30910, P30 ES00267, and T32 ES07028. L. T. L. is supported by NRSA postdoctoral fellowship F32 GM18296.

* Address correspondence to this author. Phone: 615 343-2920. FAX: 615 343-2921. Email: armstrong@toxicology.mc.vanderbilt.edu.

[⊗] Abstract published in *Advance ACS Abstracts*, March 1, 1997.

[1] Abbreviations used are UDP-GlcNAc, uridine-5′-diphospho-*N*-acetyl-D-glucosamine; GSH, glutathione; Tris, Tris-(hydroxymethyl)-amino methane; MES, 2-(*N*-morpholino)ethanesulfonic acid; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; PRR, proton relaxation rate.

S0006-2960(96)03172-8 CCC: $14.00

superfamily of divalent metal-ion-dependent enzymes, including glyoxalase I and the extradiol dioxygenases. The mechanistic basis for the evolutionary relationship of these enzymes is proposed to be bidentate coordination of a substrate or intermediate in the enzyme-catalyzed reactions.

EXPERIMENTAL PROCEDURES

Materials. Fosfomycin disodium salt and MES were purchased from Fluka. Tris-(hydroxymethyl)aminomethane hydrochloride (Tris) and GSH were obtained from Sigma (St. Louis, MO). AQC was purchased from Waters (Milford, MA). All metal salts were of Puratronic grade except for $FeCl_2$, which was ultra dry, and were purchased from Alfa Inorganics (Ward Hill, MA). Water, enriched in ^{17}O (74.5 mol % ^{17}O) was from Isotec, Inc. *Escherichia coli* BL21 cells and pET20 plasmid were purchased from Novagen (Madison, WI). The pGEM plasmid was purchased from Promega (Madison, WI). Vent DNA polymerase and all restriction enzymes were purchased from New England Biolabs (Beverly, MA). *E. coli* DH5α cells were purchased from Gibco-BRL (Bethesda, MD). The oligonucleotides used for the gene synthesis were obtained from the Molecular Physiology and Biophysics Core Lab at Vanderbilt University, and those used for mutagenesis were from Gibco-BRL. *E. coli* K12 strains AB 1157 and 821 were obtained from the *E. coli* Genetic Stock Center, Yale University.

Synthesis of the Gene and Construction of the Expression Plasmid. The gene encoding FosA, flanked by *Nde*I and *Eco*RI restriction sites, was synthesized based on the sequence reported by Navis et al. (1990). The convergent synthesis using 13 synthetic oligonucleotides is described in detail in the Supporting Information. The oligonucleotides were designed for optimal codon usage based on the sequence of the soluble GSH transferase from *E. coli* (Nishida et al., 1994). The phosphorylated synthetic product was ligated into the pGEM vector that had been digested with *Sma*I. The resulting plasmid was used to transform DH5α cells, and colonies harboring the cloning plasmid were selected for ampicillin resistance. The coding region was cut out of the cloning vector with *Nde*I and *Eco*RI, ligated into a pET20 expression vector, and transformed in DH5α cells as above. Sequencing of both strands identified two deletions and one three base missense region introduced in the gene synthesis. The errors were corrected using the Kunkel (1985) method of site-directed mutagenesis, and the resulting plasmid pFRP1 was used for expression of the protein.

Expression and Purification of the Apoenzyme. The enzyme was expressed in *E. coli* BL21-(DE3) cells transformed with pFRP1 and grown at 37 °C in the presence of 100 μg/mL ampicillin and induced with IPTG. The enzyme was purified from harvested cells by chromatography on DEAE-cellulose and hydroxylapatite. Details of the purification of the enzyme are given in the Supporting Information. Apoenzyme was prepared by dialysis against 2 L of 20 mM MES (pH 6.0), containing 5 mM EDTA and 1 mM DTT. The EDTA was removed by dialysis against 2 L of 20 mM MES, 1 mM DTT (pH 6.0). The protein was then passed through a 1 × 25 cm column of 100−200 mesh Chelex 100 (H^+-form) equilibrated with 20 mM MES, 1 mM DTT (pH 6.0), to remove any remaining metal. Protein concentration was determined using the extinction coefficient of $\epsilon_{280} = 31\,300\ M^{-1}\ cm^{-1}$ calculated from the tryptophan and tyrosine content (Demchenko, 1986).

Standard Enzyme Assay and Metal-Ion Activation. A typical assay contained 150 nM enzyme, 10 μM $MnCl_2$, 10 mM fosfomycin, 10 mM GSH, and 250 mM sodium borate (pH 8.0) in a total volume of 100 μL. Samples were incubated for 5 min or less at 25 °C. The reaction was stopped by the addition of 200 μL of 5% trichloroacetic acid and neutralized with 100 μL of 0.8 M NaOH. Valine (100 μL of a 1.25 mM solution) was added as an internal standard. The derivatization was completed according to the protocol provided by Waters. Samples were injected onto a 4.6 mm × 25 cm Beckman C-18 Ultrasphere column equilibrated with 140 mM sodium acetate, 12.5 mM triethylamine (with the pH adjusted to 5.05 with phosphoric acid), and separated by a gradient of 0−35% CH_3CN in 15 min at a flow rate of 1.0 mL/min. The HPLC assay clearly separated the product from the reactants. The *N*-(6-aminoquinolyl carbamate) derivatives of GS-fos, GSSG, GSH, and the valine internal standard had retention times of 9.2, 10.9, 11.1, and 15 min, respectively.

EPR Spectroscopy. EPR spectra were recorded at 35 GHz with a Varian E109Q spectrometer in which the temperature of the sample cavity was maintained at 0 °C. EPR spectra at 9 GHz were recorded with a Varian E3 spectrometer. The temperature was controlled at 20 °C. All samples were contained in quartz capillary tubes. Both spectrometers were interfaced with an IBM AT computer for data acquisition and analysis. The number of coordinated water molecules in the $E{\cdot}Mn^{2+}$ complex was determined from the extent of inhomogeneous line broadening of the Mn^{2+} EPR signal arising from the ^{17}O nuclear superhyperfine interaction in the presence of $H_2{}^{17}O$. The spectral subtraction and resolution enhancement methods used to determine the number of coordinated waters have been previously described (Reed & Leigh, 1980; Latwesen et al., 1992 and references therein).

Water Proton Relaxation Rate Measurements. The longitudinal PRR of the bulk solvent water was determined at 20 °C and a radio frequency of 24.3 MHz using a 180°−τ−90° NMR pulse sequence (Mildvan & Engle, 1972). τ was adjusted for each sample until the resulting NMR signal was nulled. The influence of substrates or product on the relaxation rates was determined by titration of a constant concentration of the $E{\cdot}Mn^{2+}$ complex with either fosfomycin, GSH, or GS-fos. Dissociation constants (K_d) of the substrate or product from $E{\cdot}Mn^{2+}$ were determined by fitting the titration data to eq 1 where $1/T_{1pf}$ is the observed longitudinal water PRR in the presence of $E{\cdot}Mn^{2+}$, and $1/T_{1pb}$ is the observed longitudinal relaxation rate in the presence of $E{\cdot}Mn^{2+}{\cdot}S$.

$$1/T_{1p} = \frac{\left([E{\cdot}Mn^{2+}]\frac{1}{T_{1p_f}} + [E{\cdot}Mn^{2+}{\cdot}S]\frac{1}{T_{1p_b}}\right)}{[E]t} \qquad (1)$$

where

$$[E{\cdot}Mn^{2+}{\cdot}S] = \frac{b - \sqrt{b^2 - [4([E{\cdot}Mn^{2+}]_t)([S]_t)]}}{2}$$

$$b = K_d + [S]_t + [E{\cdot}Mn^{2+}]_t \quad \text{and}$$

$$[E{\cdot}Mn^{2+}] = [E{\cdot}Mn^{2+}]_t - [E{\cdot}Mn^{2+}{\cdot}S]$$

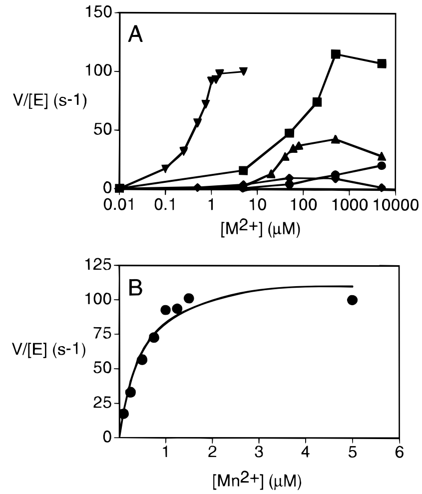


FIGURE 1: (A) Metal ion activation of FosA by Mn^{2+} (▼), Fe^{2+} (■), Co^{2+} (▲), Zn^{2+} (◆), and Mg^{2+} (●). (B) Activation of FosA by Mn^{2+}. The solid line is a fit of the data to a hyperbola with $K_a = 0.4 \pm 0.05$ μM.

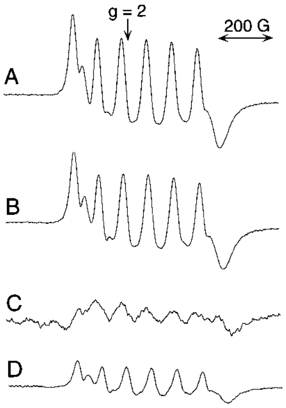


FIGURE 2: EPR spectra (35 GHz) of the E·Mn^{2+} complex in the presence of substrates and product. (A) E·Mn^{2+}. (B) E·Mn^{2+} + GSH. (C) E·Mn^{2+}·fos. (D) E·Mn^{2+}·GS-fos. Spectrum C was obtained with a gain setting 10-fold greater than the others. All spectra were obtained in the presence of excess enzyme [E] = 4.0 mM, Mn^{2+} = 2.3 mM. The concentrations of GSH, fosfomycin, and GS-fos in spectra B, C, and D were 10 mM.

RESULTS AND DISCUSSION

Microbiological Properties of the Expression Plasmid. When *E. coli* K12 strain AB 1157, normally susceptible to fosfomycin, is transformed with the plasmid pFRP1, it becomes highly resistant to the antibiotic, surviving concentrations > 10 mg/mL. The sister K12 strain 821, which differs from AB 1157 in that the gene encoding γ-glutamylcysteine synthetase is not functional such that the cells are completely lacking in GSH (Apontoweil & Berends, 1975), remains susceptible to fosfomycin even after transformation with pFRP1. The microbiological results clearly suggest that the synthetic plasmid encodes a protein that confers GSH-dependent resistance to fosfomycin.

Purification and Properties of the Apoenzyme. The enzyme was expressed at high levels (>50% of the soluble protein) in *E. coli* BL21 cells transformed with the pFRP1 plasmid. Purification yielded about 200 mg of enzyme/L of culture. The polypeptide had the expected molecular mass of 16 kDa as determined by SDS−PAGE. Gel filtration of the native apoenzyme on Superdex 200 (Pharmacia) gave a Stokes radius consistent with a dimer of 16 kDa subunits. The enzyme exhibited an isoelectric point of 4.2 consistent with the preponderance of acidic amino acids in the sequence. The apoenzyme had very low catalytic activity under the standard assay conditions in the absence of divalent metal ions with an apparent turnover number, k_{cat}(app) ≤ 0.2 s^{-1}.

Activation of the Apoenzyme by Divalent Cations. Addition of low to moderate concentrations of particular divalent metal ions results in a several hundred-fold activation of the apoenzyme as illustrated in Figure 1. Low concentrations of Mn^{2+} (1.5−5 μM) result in a 500-fold enhancement of catalytic activity. The activation constant, K_a, for Mn^{2+} is 0.4 μM (Figure 1B). Although addition of Fe^{2+} and Co^{2+} also enhanced catalytic activity nearly as well as Mn^{2+} (580- and 220-fold, respectively), maximal activation required much higher divalent cation concentrations (ca. 500 μM for both). Activations by Zn^{2+} (45-fold at 50 μM) and Mg^{2+} (100-fold at 5 mM) were not as dramatic. Although the activation data do not prove Mn^{2+} is the physiologic metal, the fact that FosA is activated at metal concentrations well below that typically found in microorganisms strongly suggests that Mn^{2+} is the preferred metal *in vivo*. In addition, purified enzyme that had been dialyzed against EDTA but not treated with Chelex 100 retained about 1% of the maximal activity and exhibited a six-line EPR signal intensity consistent with 0.5−1 mol % Mn^{2+} per subunit (data not shown). The enzyme is the first example of a metalloglutathione transferase (Armstrong, 1997).

EPR Spectra of Mn^{2+} Complexes of FosA. The Q-Band EPR spectrum of Mn^{2+} in the presence of excess enzyme is shown in Figure 2. That Mn^{2+} is bound to the protein is evident from the line broadening and decreased amplitude of the EPR signals as compared to free Mn^{2+}. The appearance of pronounced EPR signals for free Mn^{2+} at $[Mn^{2+}]/[E] \geq 1$ in X-band spectra of the enzyme with increasing concentrations of Mn^{2+} is consistent with a stoichiometry of 1 metal per 16 kDa subunit (data not shown) and a $K_d < 5$ μM. The estimated upper limit on the dissociation constant of the Mn^{2+} is consistent with the observed K_a of 0.4 μM (Figure 1B). Addition of GSH has little effect on the 35 GHz spectrum of the E·Mn^{2+} complex (Figure 2B). In marked contrast, addition of fosfomycin to the E·Mn^{2+} complex virtually obliterates the EPR spectrum (Figure 2C), suggesting a significant change or electronic distortion of the coordination environment of the enzyme-bound metal. This effect on the EPR spectrum is reminiscent of that observed in Mn^{2+} complexes of pyruvate kinase in the presence of phosphoenol pyruvate (Reed & Cohn, 1973). Addition of fosfomycin to free Mn^{2+} has no effect on the EPR spectrum (data not shown). Finally, the signals of enzyme-bound Mn^{2+} in the presence of enzymatically generated product, GS-fos, are qualitatively similar but of smaller amplitude than those of the E·Mn^{2+} complex (Figure 2D).

The EPR signals of enzyme-bound Mn^{2+} are diminished in amplitude and significantly broadened in the presence of $H_2{}^{17}O$ (Figure 3A), suggesting that some of the coordination sites on the metal are occupied by water. Resolution enhanced difference spectra (Reed & Leigh, 1980; Latwesen et al., 1992) reveal that there are a minimum of three water molecules in the inner coordination sphere of the metal (Figure 3B). A similar set of experiments with the E·Mn^{2+}·GS-fos complex indicates three metal-coordinated water molecules in the product complex as well (see

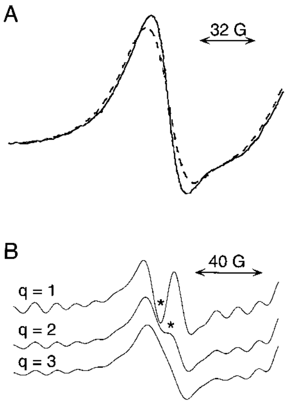


FIGURE 3: Comparison of the low-field EPR signals for the E•Mn^{2+} complex in normal water and water enriched in ^{17}O. (A) Before resolution enhancement in normal water (—) and 37.3 mol % $H_2{}^{17}O$ (- - -). (B) Resolution-enhanced difference spectra corresponding to one ($F_n = 0.63$), two ($F_n = 0.40$), and three ($F_n = 0.25$) coordinated water molecules. F_n is the fractional contribution to the EPR signal from Mn^{2+} exclusively coordinated to the nonmagnetic isotopes ^{16}O and ^{18}O. The parameters used for the resolution enhancement were $L = 0.1\ G^{-1}$ and $\sigma = 15$ G. The anomalies in the difference spectra with $q = 1$ and $q = 2$ are denoted by an asterisk (*).

Supporting Information). It therefore seems likely that product is not in the inner coordination sphere of the metal. Unfortunately, the absence of sufficiently intense, narrow EPR signals (Figure 2C) precludes a similar determination of the number of coordinated waters in the substrate complex (E•Mn^{2+}•fos).

Binding of Substrates and Products to E•Mn^{2+}. The exchangeable water molecules in the inner coordination sphere of the protein-bound metal results in a significant enhancement of the paramagnetic contribution to the dipolar nuclear magnetic resonance relaxation rate of the water protons, $1/T_{1p}$ (eq 2) where q is the water coordination number, T_{1M} is the relaxation rate of the coordinated ligand, τ_M is the residence time of the water in the inner coordination sphere, $1/T_{os}$ is the outer-sphere contribution to the observed relaxation rate, and $p = [Mn^{2+}]/[H_2O]$.

$$1/T_{1p} = \frac{pq}{T_{1M} + \tau_M} + \frac{p}{T_{os}} \qquad (2)$$

The observed $1/T_{1p}$ for the E•Mn^{2+} complex is diminished by a factor of 12 on addition of increasing concentrations of fosfomycin as illustrated in Figure 4 providing a dissociation constant for fosfomycin of 17 μM. Since the rotational correlation time of the E•Mn^{2+} complex is unlikely to be changed by the binding of substrate, the large decrease in $1/T_{1p}$ is probably due to a decrease in q, the number of water molecules in the inner coordination sphere of the metal and/or an increase in τ_M, the residence time in the inner coordination sphere. Binding of the product GS-fos to E•Mn^{2+} has a much more modest influence on the observed $1/T_{1p}$ decreasing it only 2-fold. The product also binds much less tightly ($K_d = 1.1$ mM) than the substrate. Inasmuch as the EPR spectra indicate that q is the same for E•Mn^{2+} and E•Mn^{2+}•GS-fos, the small decrease in $1/T_{1p}$ in the presence of the product is probably due to a decrease in the exchange rate of the inner-sphere water ligands. Addition of up to 50 mM GSH to E•Mn^{2+} had no significant effect on the observed $1/T_{1p}$. The dramatic differences in the dissociation constants and $1/T_{1p}$ values for the fosfomycin and product complexes are consistent with inner-sphere coordination of fosfomycin and a weaker, outer-sphere interaction with the product.

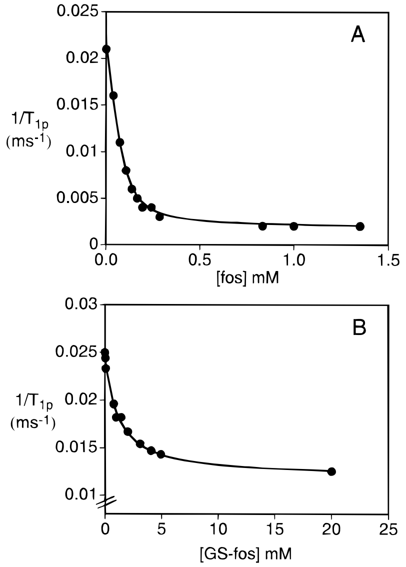


FIGURE 4: Titration of observed water proton relaxation rates ($1/T_{1p}$) of the E•Mn^{2+} complex with (A) fosfomycin and (B) GS-fos. The solid lines are fits of the experimental data to eq 1 for fosfomycin with $K_d = 17 \pm 2\ \mu M$, $1/T_{1pf} = 21.8 \pm 0.3\ \mu s^{-1}$, and $1/T_{1pb} = 1.8 \pm 0.2\ \mu s^{-1}$ and for GS-fos with $K_d = 1.1 \pm 0.1$ mM, $1/T_{1pf} = 24.6 \pm 0.3\ \mu s^{-1}$, and $1/T_{1pb} = 11.9 \pm 0.4\ \mu s^{-1}$.

Taken together, the PRR titrations and the changes in the EPR spectra of enzyme-bound metal suggest that each subunit contains a mononuclear Mn^{2+} center that interacts strongly with the substrate fosfomycin perhaps displacing one or more of the three water molecules in the inner coordination sphere of the metal. The metal center interacts much more weakly with the product and very weakly or not at all with GSH. The most reasonable mechanistic role for the metal consistent with these facts is electrophilic activation of the very unreactive oxirane ring of fosfomycin for attack of an anionic nucleophile. This could be most directly accomplished by inner-sphere coordination of the substrate to the enzyme-bound metal as discussed in detail below.

Mechanistic and Structural Relatives of FosA. A BLAST search (Altschul et al., 1990) of protein and gene sequence databases reveals two relatives of FosA, glyoxalase I, and the bacterial extradiol dioxygenases that have very interesting structural and mechanistic implications (Figure 5). The relationship to glyoxalase I is particularly compelling since it is a glutathione-dependent metalloenzyme as is FosA. Glyoxalase I catalyzes conversion of either diastereomeric thiohemiacetal adduct of an α-ketoaldehyde such as methylglyoxal and GSH to *S*-(D-lactoyl)glutathione by stereospecific protonation of a cis-enediol intermediate as illustrated in Figure 6B (Landro et al., 1992; Sellin et al., 1982). The second set of relatives is the extradiol dioxygenases, a group of metalloenzymes generally utilizing either Fe^{2+} or Mn^{2+} to activate molecular oxygen and a vicinal diol substrate for meta ring cleavage as illustrated in Figure 6C (Shu et al., 1995; Whiting et al., 1996). It is interesting to note that the Mn^{2+}-dependent extradiol dioxygenase, DHPD(AG) (Figure 5), exhibits a higher sequence similarity with FosA near the Glu113 ligand than does the Fe^{2+}-containing enzyme, DHBP-(PC).

The mechanism proposed for FosA involving electrophilic activation of the oxirane ring by the active site metal is

```
FOSA         5 LNHLTLAVSDLQKSVTFWHELLGLTL
               •||•| •||•• ||• |• ••||  •
FOSB(SE)     5 INHITYSVSNIAKSIEFYRDILGADI
               • |    | •• •||•||  •||  •
GLXI(EC)     3 LLHTMLRVGDLQRSIDFYTKVLGMKL
                 |||||| |•••| ||||•||| ||
GLXI(PP)    26 FNHTMLRVKDIEKSLDFYTRVLGFKL
                 | •  | | •|•| ||| ||||•|
DHBD(PC)   144 LGHFVRCVPDSDKALAFYTDVLGFQL
               | || •  ||  •  |•  |•
DHPD(AG)   153 LDHFNQVTPDVPRGRAYLEDL
                 ↑
                 metal ligands
                      ↓
FOSA       106 DPDGHKLEL
               | |||||||
FOSB(SE)   108 DLDGHKLEL
               | ||•|•||
GLXI(EC)   115 DPDGYKIEL
               |||||||||
GLXI(PP)   115 DPDGYKIEL
                | | ••|
DHBP(PC)   253 TPSGVEVEY
                | |  •|
DHPD(AG)   259 DPDGHRIEI
```

FIGURE 5: Examples of sequence similarities between FosA and FosB [a related putative GSH transferase (Garcia et al., 1995)] from *Staphylococcus epidermidis,* FOSB(SE), bacterial glyoxalase I from *E. coli,* GLXI(EC), and *Pseudomonas putida,* GLXI(PP), and the bacterial extradiol dioxygenases 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Pseudomonas cepacia,* DHBD(PC), and 3,4-dihydroxyphenylacetate-2,3-dioxygenase from *Arthrobacter globiformis,* DHPD(AG). Protein ligands to the metal are deduced from the crystal structure of DHBD (Han et al., 1995).

illustrated in Figure 6A. We postulate that the metal center of FosA may serve two roles in facilitating attack of the nucleophile, neutralization of the negative charge on the phosphonate, and polarization of the carbon−oxygen bond in the oxirane ring. Although the exact coordination geometry of the E•Mn^{2+}•fos complex cannot be ascertained from the data presented here, three possibilities that are consistent with the distorted electronic environment apparent from the 35 GHz EPR spectrum (Figure 2C) include bidentate coordination to the oxirane oxygen and the phosphonate, bidentate coordination to the phosphonate alone or tridentate coordination to the oxirane and the phosphonate. The latter two schemata would provide the greatest distortion of the electronic symmetry of the metal.

FosA, glyoxalase I, and the extradiol dioxygenases utilize different metals and clearly catalyze very different chemical reactions. Yet, the mechanistic feature relating these enzymes is not as cryptic as might be expected from the obvious differences in chemistry. Each of the enzyme catalyzed reactions appears to utilize a metal center with octahedral coordination geometry for electrophilic activation of a substrate or stabilization of an intermediate. In each instance two or three of the coordination sites in the E•M^{2+} complex are occupied by water. We further propose that two or more of these water molecules may be displaced upon inner-sphere coordination of a substrate or intermediate. This is clearly the case with the extradiol dioxygenases where the two coordinated water molecules (Han et al., 1995) are displaced from the iron by substrate (Shu et al., 1995). This may also be true with glyoxalase I. Although the best evidence to date suggests that the product in the glyoxalase I reaction forms an outer-sphere complex with the metal where the interaction is mediated by coordinated water (Sellin et al., 1982; Rosevear et al., 1983), it is not clear if this is also true for the bound intermediate. The evidence presented here suggests that the catalytic mechanism of FosA may also involve displacement of inner-sphere water ligands to give an activated substrate with bidentate (or tridentate) coordination to the metal.

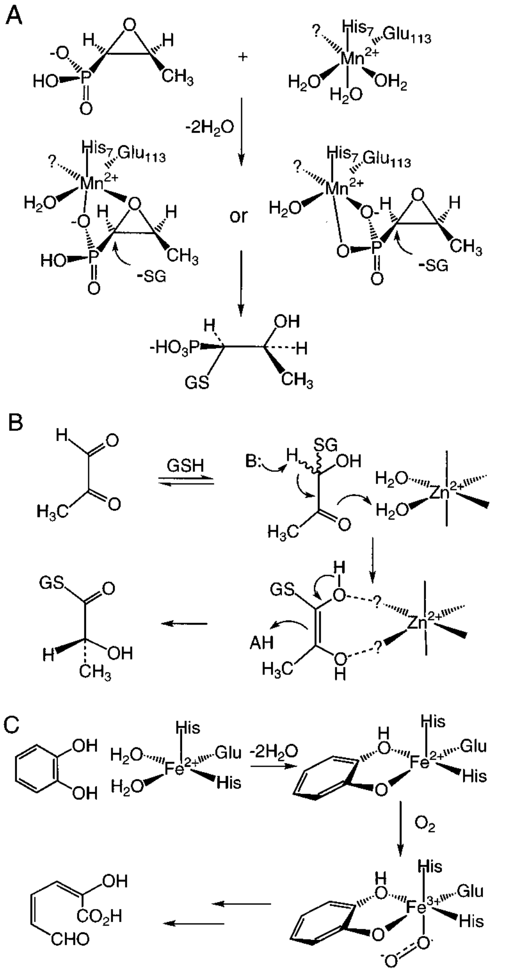


FIGURE 6: Proposed catalytic mechanisms for FosA (this work), glyoxalase I (Landro et al., 1992; Sellin et al., 1982), and catechol-2,3-dioxygenase (Shu et al., 1995). Two possible bidentate coordination geometries are illustrated for FosA. Tridentate coordination to the phosphonate and oxirane oxygens (not shown) cannot be excluded. Whether the coordination of the enediol intermediate in the glyoxalase I-catalyzed reaction is inner sphere or outer sphere is not known.

In the aggregate, these considerations suggest that FosA, glyoxalase I, and the bacterial extradiol dioxygenases are all members of the same metalloenzyme superfamily having similar metal ion sites, namely an octahedral coordination geometry with two or three coordination sites occupied by displaceable water ligands. We propose that the mechanistic imperative for conscription of the protein fold providing such a metal site is bidentate coordination of a substrate or intermediate for electrophilic catalysis. This superfamily is another example where basic mechanistic requirements appear to direct the evolution of enzymes catalyzing different chemical reactions as has been elegantly demonstrated in the recent analysis of the enolase superfamily (Babbitt et al., 1996). Additional structural and mechanistic investigations of this proposal are underway.

ACKNOWLEDGMENT

We thank Prof. George Reed for helpful discussions, advice, and the use of the EPR and NMR instrumentation at the Enzyme Institute, University of Wisconsin, Madison and

Dr. Russell Poyner and Vahe Bandarian in the Reed laboratory for assistance in analyzing the EPR spectra.

SUPPORTING INFORMATION AVAILABLE

Details of the synthesis of the gene encoding FosA, the expression and purification of the enzyme, and resolution-enhanced EPR spectra of the product complex including one table and three figures (6 pages). Ordering information is given on any current masthead page.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.

Apontoweil, P., & Berends, W. (1975) *Biochim. Biophys. Acta 399*, 10−22.

Arca, P., Rico, M., Brana, A. F., Villar, C. J., Hardisson, C., & Suarez, J. E. (1988) *Antimicrob. Agents Chemother. 32*, 1552−1556.

Arca, P., Hardisson, C., & Suarez, J. E. (1990) *Antimicrob. Agents Chemother. 34*, 844−848.

Armstrong, R. N. (1997) *Chem. Res. Toxicol. 10*, 2−18.

Babbitt, P. C., Hasson, M. S., Wedekind, J. E., Palmer, D. R. J., Barrett, W. C., Reed, G. H., Rayment, I., Ringe, D., Kenyon, G. L., & Gerlt, J. A. (1996) *Biochemistry 35*, 16489−16501.

Christensen, B. G., Leanza, W. J., Beattie, T. R., Patchett, A. A., Arison, B. H., Ormond, R. E., Kuehl, F. A., Albers-Schonberg, G., & Jardetzky, O. (1969) *Science 166*, 123−124.

Demchenko, A. P. (1986) in *Ultraviolet Spectroscopy of Proteins*, p 222, Springer-Verlag, Berlin.

Garcia, P., Arca, P., & Suarez, J. (1995) *Antimicrob. Agents Chemother. 39*, 1569−1573

Garcia-Lobo, J. M., & Ortiz, J. M. (1982) *J. Bacteriol. 151*, 477−479.

Han, S., Eltis, L. D., Timmis, K. N., Muchmore, S. W., & Bolin, J. T. (1995) *Science 270*, 976−980.

Hendlin, D., Stapley, E. O., Jackson, M., Wallick, H., Miller, A. K., Wolf, F. J., Miller, T. W., Chaiet, L., Kahan, F. M., Flotz, E. L., Woodruff, H. B., Mata, J. M., Hernandez, S., & Mochales, S. (1969) *Science 166*, 122−123.

Kadner, R. J., & Winkler, H. H. (1973) *J. Bacteriol. 113*, 895−900.

Kahan, F. M., Kahan, J. S., Cassidy, P. J., & Kroop, H. (1974) *Ann. NY Acad Sci. 235*, 364−385.

Kunkel, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Landro, J. A., Brush, E. J., & Kozarich, J. W. (1992) *Biochemistry 31*, 6069−6077.

Latwesen, D. G., Poe, M., Leigh, J. S., & Reed, G. H. (1992) *Biochemistry 31*, 4946−4050.

Marquardt, J. L., Brown, E. D., Lane, W. S., Haley, T. M., Ichskawa, Y., Wong, C.-H., & Walsh, C. T. (1994) *Biochemistry 33*, 10646−10651.

Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26C*, 654−682.

Navis, J., Leon, J., Arroyo, M., & Garcia-Lobo, J. M. (1990) *Antimicrob. Agents Chemother. 34*, 2016−2018.

Nishida, M., Kong, K.-H., Inoue, H., & Takahashi, K. (1994) *J. Biol. Chem. 289*, 32536−32541.

Reed, G. H., & Cohn, M. (1973) *J. Biol. Chem. 248*, 6436−6442.

Reed, G. H., & Leigh, J. S. (1980) *Biochemistry 19*, 5472−5480.

Rosevear, P. R., Chari, R. V. J., Kozarich, J. W., Sellin, S., Mannervik, B., & Mildvan, A. S. (1983) *J. Biol. Chem. 258*, 6823−6826.

Sellin, S., Eriksson, L. E. G., & Mannervik, B. (1982) *Biochemistry 21*, 4850−4857.

Shu, L., Chiou, Y.-M., Orville, A. M., Miller, M. A., Lipscomb, J. D., & Que, L. (1995) *Biochemistry 34*, 6649−6659.

Tsuruoka, T., & Yamamda, Y. (1975) *J. Antibiot. 28*, 906−911.

Venkateswaran, P., & Wu, H. (1972) *J. Bacteriol. 110*, 935−944.

Whiting, A. K., Boldt, Y. R., Hendrich, M. P., Wackett, L. P., & Que, L., Jr. (1996) *Biochemistry 35*, 160−171.

BI963172A