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Mechanistic Diversity in a Metalloenzyme Superfamily†

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ABSTRACT: It is now appreciated that the relationships of proteins, particularly enzymes, within a protein superfamily can be understood not only in terms of their sequence similarities and three-dimensional structures but also by chemical threads that relate their functional attributes. The mechanistic ties among superfamily members can often be traced to a common transition state for the rate-limiting step of the reactions being catalyzed. This paper presents an analysis of a metalloenzyme superfamily, the members of which catalyze a very diverse set of reactions with unrelated transition states but a more general common mechanistic imperative. The vicinal oxygen chelate (VOC) superfamily is composed of structurally related proteins with paired $\beta\alpha\beta\beta\beta$ motifs that provide a metal coordination environment with two or three open or readily accessible coordination sites to promote direct electrophilic participation of the metal ion in catalysis. The known types of reactions that are catalyzed include isomerizations (glyoxalase I), epimerizations (methylmalonyl-CoA epimerase), oxidative cleavage of C−C bonds (extradiol dioxygenase), and nucleophilic substitutions (fosfomycin resistance proteins). The remarkable access to mechanism space that is provided by the VOC superfamily appears to derive from a simple, pseudosymmetric structural fold that maximizes the catalytic versatility of the metal center.

Understanding the correlated evolution of protein structure and function is the essence of elucidating the design principles utilized by nature in developing and improving the molecular toolbox of life. It is now widely appreciated that proteins can be organized into superfamilies of structurally related molecules with very similar or radically diverse functions. Members of protein superfamilies can often be detected through pairwise or multiple sequence alignments and even more convincingly via similarities in their three-dimensional structures. Although it is fair to say that the revolution in genomics and the beginnings of structural genomics are driven in large measure by gene sequence and three-dimensional structural information, consideration of the functional attributes of gene products provides unique and often underappreciated insight into the process of evolutionary diversification of enzymic catalysis.

A significant, though perhaps obvious, conceptual advance in defining the relationships in protein structure and function was the realization that enzyme superfamilies can also be understood and organized by the chemical requirements of the reaction being catalyzed (*1*, *2*). Perhaps the most extensively scrutinized example from a functional standpoint is the enolase superfamily (*2*, *3*) where the mechanistic imperative is abstraction of a proton α to a carboxylate. Although the types of reactions catalyzed by members of the enolase superfamily are quite different and include racemizations, epimerizations, and β-eliminations, they are all related by the obligatory formation of an enolic interme-

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Table 1: Functionally Distinct Members of the VOC Superfamily

member	metal ion	reaction type	structure[a]
bleomycin resistance protein	none	none, sequestration	1BYL[b]
fosfomycin resistance proteins	Mn^{2+}, Mg^{2+}	nucleophilic opening of epoxide	–
extradiol dioxygenases	Fe^{2+}, Mn^{2+}	oxidative cleavage of C−C bond	1HAN[c]
glyoxalase I	Zn^{2+}, Ni^{2+}	isomerization	1FRO[d]
methylmalonyl-CoA epimerase	Co^{2+}	epimerization	–

[a] Protein Data Bank file names for original structure reports. [b] See ref *12*. Two additional structures of bleomycin resistance proteins from other organisms have been deposited in the PDB under file names 1QTO (*13*) and 1ECS. [c] See ref *14*. A lower-resolution structure of the related catechol 2,3-dioxygenase is also available under file name 1MPY (*15*). [d] See ref *16*. Of additional importance is the structure of this same enzyme in complex with a transition state analogue, PDB file name 1QIP (*17*). Glyoxalase I from *E. coli* is a Ni^{2+}-dependent enzyme (*18*).

diate. The structural platforms that support the chemistry are quite similar and consist of a TIM barrel preceded by a $\beta_3\alpha_4$ domain. Similarly, members of the α/β-hydrolase fold superfamily (*4*−*6*) share look-alike structures and catalyze related, two-step hydrolytic reactions even though the substrates can be quite different, e.g., amides, esters, epoxides, and haloalkanes. The fact that members of enzyme superfamilies share common structures and mechanistic imperatives suggests, but does not prove, that divergent evolution from a common ancestor is responsible for their functional diversification.

Although the chemical threads that tie superfamily members together mechanistically can often be traced to a common transition state for the rate-limiting step as in the two examples above, this is not always the case. In this paper, a somewhat different perspective is provided by the analysis of a metalloenzyme superfamily, the members of which catalyze a very diverse set of reactions with unrelated transition states (*7*). The members of the vicinal oxygen chelate (VOC)[1] superfamily are structurally related proteins that provide a metal coordination environment with two or three open or readily accessible coordination sites to promote direct electrophilic participation of the metal ion in catalysis. In addition, metal ion coordination may have played a role in the evolution of the protein fold (*8*).

Members of the VOC Superfamily. Sequence alignments, three-dimensional structures, and functional analysis reveal that there are at least five functionally distinct members of the VOC superfamily (Table 1). The family was originally described on the basis of a mechanistic analysis of the fosfomycin resistance protein (FosA). FosA was found to be a Mn^{2+}-dependent enzyme with regions of sequence that are similar to those of two other classes of metalloenzymes, glyoxalase I and the extradiol dioxygenases (*7*). This group of enzymes was coined the vicinal oxygen chelate superfamily on the basis of the hypothesis that the mechanistic imperative relating the three charter members was inner sphere coordination of substrates or intermediates to the metal center via vicinal oxygen ligands (*2*, *9*). The structural and functional evidence for this hypothesis is discussed in more detail below.

Two additional members that belie the superfamily name are methylmalonyl-CoA epimerase (*10*, *11*) and the bleomycin resistance proteins (*8*, *12*). The former bears a clear mechanistic similarity to the charter members of the family but, as discussed later, obviously does not function by chelation of vicinal oxygen ligands (*11*). The latter is neither an enzyme nor a metalloprotein (Table 1). The metal binding ability of the progenitor of the bleomycin resistance proteins may have been shed in favor of a more hydrophobic cavity to accommodate the antibiotic.

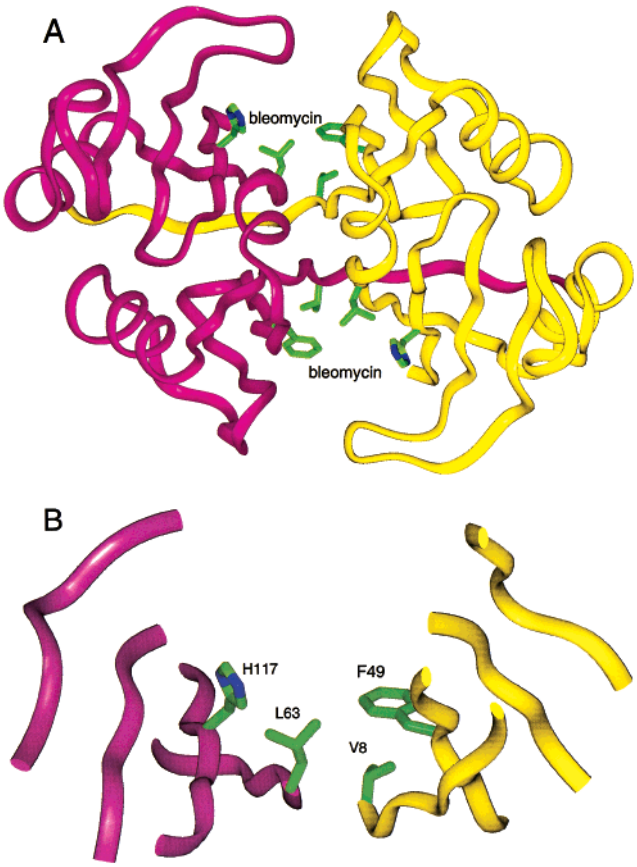

FIGURE 1: Ribbon representation of the structure of the bleomycin resistance protein: (A) view of the homodimer and (B) closeup view of the cavity formed by the paired $\beta\alpha\beta\beta\beta$ motifs. The side chains shown are at the positions occupied by the metal ligands of other VOC superfamily members. The representations were constructed with PDB coordinate file 1BYL (*12*).

Paired $\beta\alpha\beta\beta\beta$ Motifs Form the Basic VOC Superfamily Structure. Three-dimensional structures are available for three of the five distinct VOC superfamily members now known (Table 1). An extensive and very lucid analysis of these structures has appeared recently (*8*). The fundamental structural unit of the VOC superfamily is the $\beta\alpha\beta\beta\beta$ motif first described in the structure of the bleomycin resistance protein (*12*) and illustrated in Figure 1. This protein is a homodimer where each subunit consists of two tandem motifs, $(\beta\alpha\beta\beta\beta)_1$ and $(\beta\alpha\beta\beta\beta)_2$. The two bleomycin binding sites in the dimer are formed at the subunit interface by an edge-to-edge interaction of $(\beta\alpha\beta\beta\beta)_1$ of one subunit with

[1] Abbreviations: GSH, glutathione; VOC, vicinal oxygen chelate; EPR, electron paramagnetic resonance.

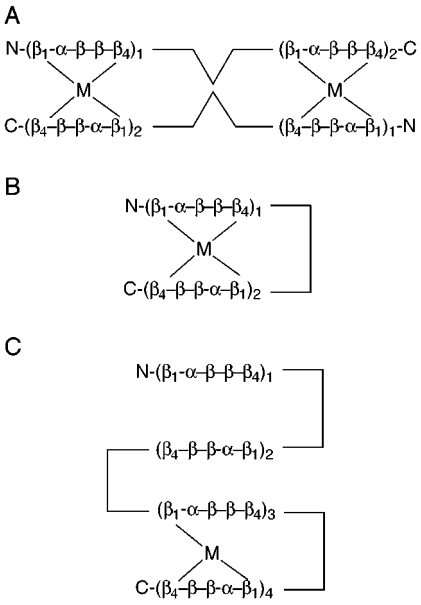

FIGURE 2: Examples of alternate arrangements of the metal binding sites composed of paired $\beta\alpha\beta\beta\beta$ motifs in the VOC superfamily: (A) human glyoxalase I dimer, (B) domain-swapped glyoxalase I monomer from *P. putida*, and (C) four-motif subunit of the extradiol dioxygenase from *B. cepacia*. The metal ligands in each case are contributed by residues from β-strands 1 and 4 of each motif.

$(\beta\alpha\beta\beta\beta)_2$ of the other (Figure 1). The paired $\beta\alpha\beta\beta\beta$ motifs form a U-shaped pocket lined with the strands of the two β-sheets. The pairing of the first β-strand of each motif forms the bottom of the pocket. As a consequence, alternating side chains of each strand converge in the interior of the pocket to provide specific interactions with the drug. The structure of a bleomycin resistance protein−bleomycin complex has not been determined, so the details of these interactions are not known.

Paired $\beta\alpha\beta\beta\beta$ motifs in the VOC superfamily members can be arranged in alternative configurations by three-dimensional domain swapping. For example, human glyoxalase I has an arrangement like that observed in the crystal structure of the bleomycin resistance protein as illustrated in Figure 2A. However glyoxalase I from *Pseudomonas putida* can exist either as a dimer, as does the human enzyme, or as a monomer in which the tandem $\beta\alpha\beta\beta\beta$ motifs of a single subunit are paired to form an active enzyme as illustrated in Figure 2B (*19*). The extradiol dioxygenase of *Burkholderia cepacia* provides another example of an active site composed of tandem $\beta\alpha\beta\beta\beta$ motifs derived from a single subunit (Figure 2C) (*14*). In this instance, each polypeptide contains four motifs that fold into two domains, only the second of which binds metal and functions in catalysis. The unoccupied cavity in the N-terminal domain may be a vestigial active site that lost the ability to bind metal to refine or enhance the catalytic function of the C-terminal domain (*8*).

The convergent functional groups in the interior of the paired $\beta\alpha\beta\beta\beta$ motifs are ideal for formation of a ligand geometry that is appropriate for metal chelation with cis-configured, open, or solvent-occupied coordination sites. The U-shaped cavity and pseudo *C*2 symmetry of the two β-sheets provide an effective platform for up to four metal ligands protruding from the bottom of the cavity as illustrated in Figure 3 for the structure of glyoxalase I. In this instance,

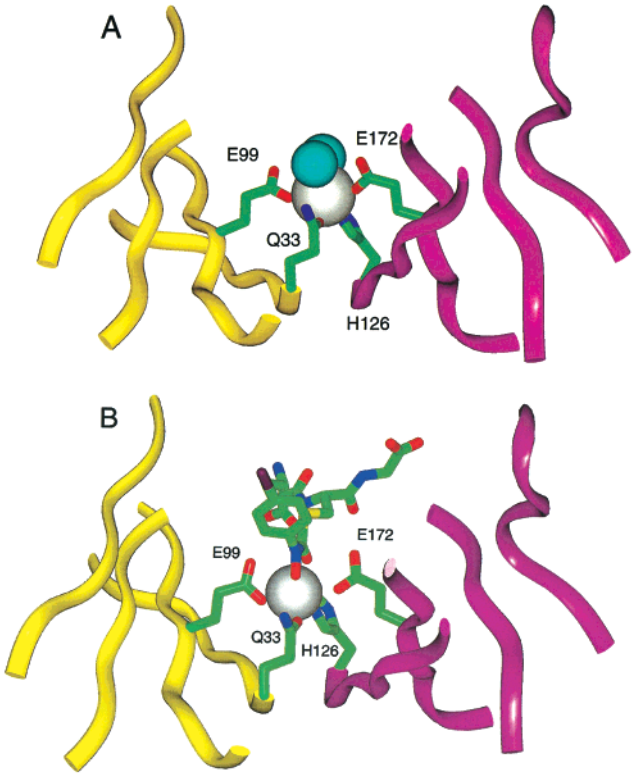

FIGURE 3: Representations of the Zn^{2+} binding site of human glyoxalase I. The β-sheets from the $\beta\alpha\beta\beta\beta$ motifs contributed by each subunit are shown in purple and yellow. The α-helices and loops connecting the β-strands have been eliminated for clarity. The four metal ion ligands are shown in stick representation, while the zinc ion and associated water molecules are shown as white and blue spheres, respectively. (A) Structure of the *S*-(benzyl)-glutathione complex from PDB file 1FRO (*16*). The *S*-(benzyl)-glutathione molecule is not shown to provide a clear view of the metal center. (B) Structure of the enzyme complexed with the endiolate analogue, *S*-(*N*-hydroxy-*N*-*p*-iodophenylcarbamoyl)glutathione, from PDB file 1QIP (*17*). The analogue is shown in stick representation.

each motif supplies two of the four protein ligands: one from the first residue in β-strand 1 and one from the central residue of β-strand 4 of each β-sheet. The pseudo *C*2 symmetry of the two motifs imposes a trans configuration on ligands derived from the opposing β-strands 1 on one hand and β-strands 4 of each motif on the other (Figure 3A). Thus, the two solvent-occupied coordination sites are cis to one another. One crucial difference between glyoxalase I and the extradiol dioxygenases is the absence of one of the four ligands from the protein. This appears to be true of FosA as well. The residue corresponding to E99 in human glyoxalase I is an alanine in the extradiol dioxygenase. Thus, a third solvent-occupied coordination site is available for substrate binding. The mechanistic implications of this are discussed in more detail below.

A Common Mechanistic Imperative for Diverse Reactions. The metalloenzyme members of the VOC superfamily catalyze quite diverse types of reactions. However, it is apparent that the reactions known thus far share a very basic mechanistic imperative that can be satisfied by a mononuclear metal ion site with two or more open or solvent-occupied coordination sites (Figure 3A). The designation vicinal oxygen chelate (VOC) superfamily derives from the hypothesis that the transition states for the reactions benefit

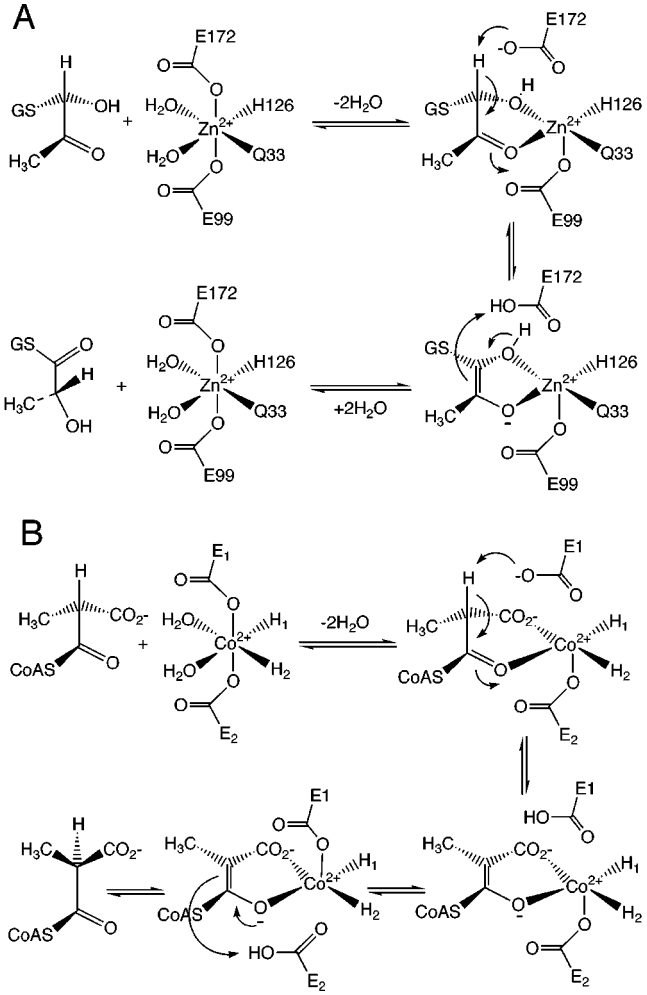

FIGURE 4: Proposed mechanisms for (A) human glyoxalase I with the *S*-diastereomer of the thiohemiacetal adduct of methylglyoxal and GSH (*17*) and (B) methylmalonyl-CoA epimerase (*11*). In panel B, the ligands to the metal are inferred from sequence alignments.

from inner-sphere coordination of vicinal oxygen atoms of a substrate or intermediate to the metal site. The strength of the experimental evidence for this proposition varies but is quite strong for the charter members of the superfamily. As will become apparent, the VOC designation is a bit of a misnomer since it is clear that at least one superfamily member, methylmalonyl-CoA epimerase, would involve chelation of oxygen atoms with a different geometric relationship. Nevertheless, all of the reactions can obviously benefit from the electrophilic assistance that is available from a metal center.

Perhaps the most compelling case for vicinal oxygen chelation is that of glyoxalase I. The Zn^{2+}-dependent human enzyme catalyzes conversion of either diastereomeric thiohemiacetal adduct of an α-keto aldehyde such as methylglyoxal and GSH to *S*-(D-lactoyl)glutathione by stereospecific protonation of a *cis*-enediol intermediate as illustrated in Figure 4A (*20*, *21*). For quite some time, it was thought that the enzyme functioned by an outer-sphere mechanism (*20*, *22*) perhaps with the participation of metal-coordinated water molecules of which there are two (Figure 3A). This is in contrast to the mechanistic imperative proposed for the VOC superfamily (*7*). However, the recently determined crystal structure of human glyoxalase I (*17*) in complex with *S*-(*N*-hydroxy-*N*-*p*-iodophenylcarbamoyl)glutathione, a *cis*-enediol intermediate analogue of the type originally conceived by Creighton and co-workers (*23*), reveals that the inhibitor forms an inner-sphere coordination complex with the Zn^{2+} in the active site. A view of that structure is shown in Figure 3B.

The analogue displaces the two Zn^{2+}-bound water molecules found in product analogue complexes (Figure 3A). Interestingly, the intermediate analogue forms a five-coordinate complex in the active site where the carboxylate of E172 moves out of the inner coordination sphere (Figure 3B). On this basis, it has been proposed that E172 acts as the base to abstract a proton from C1 of the metal-coordinated substrate to form the enediolate intermediate and subsequently delivers a proton to C2 to complete the stereospecific transformation to product. This mechanism works well for the *S*-diastereomer of the thiohemiacetal. However, it is much more difficult to envision how the *R*-diastereomer could also be processed using E172 alone to accomplish the acid−base catalysis. Cameron et al. have pointed out that the carboxylate of E99 which is trans to E172 in the enzyme−metal complex is appropriately positioned to abstract the proton from the bound *R*-diastereomer. Although there are no data to directly support this proposition, it is a reasonable one. If true, it represents an interesting example of how the pseudo *C*2 symmetry of the protein scaffold has been exploited by nature to facilitate dichotomous stereochemical behavior of a single active site. It still remains unclear as to how the proton abstracted from the *R*-diastereomer by E99 could be delivered, without exchange, to the opposite face enediol intermediate. Results from a structural survey of various metals bound to the *Escherichia coli* glyoxalase I, a Ni^{2+}-dependent enzyme, have been interpreted to suggest that one of the metal-bound water molecules is important in catalysis (*18*). On the basis of this consideration, it has been suggested that the intermediate occupies only a single coordination site on the metal leaving one metal coordinated water molecule to participate in acid−base chemistry. There clearly remains some uncertainty with respect to the details of the catalytic mechanism.

The most recently discovered member of the VOC superfamily is the enzyme methylmalonyl-CoA epimerase. Its lineage and identity were deduced from sequence similarities to other VOC superfamily members (*10*) and the fact that the epimerase genes are located in operons for succinyl-CoA biosynthesis or ones containing methylmalonyl-CoA decarboxylase in the organisms *Bacillus subtilis*, *Archaeoglobus flugidus*, and *Veillomella parvala*.[2] The enzyme is reported to use Co^{2+}, but there is no direct evidence concerning the coordination environment of the metal (*24*). Like glyoxalase I, this enzyme catalyzes an isomerization reaction involving an enolic intermediate. A possible role for the metal in the epimerization reaction is illustrated in Figure 4B where the metal acts as an electrophile to stabilize the intermediate. Interestingly, sequence alignments suggest that the metal may be bound in a tetradentate fashion with two trans-configured glutamate residues, one supplied by β-strand 4 of each βαβββ motif as is observed in glyoxalase I. The mechanistic implication is, again, that either one or the other of the two glutamate residues may disengage from the metal and act as a base to

[2] J. A. Gerlt, personal communication.

abstract the proton. The fact that the two glutamates are related by a pseudo *C*2 symmetry provides a means by which the enzyme can abstract the proton from either the *R*- or *S*-diastereomer of methylmalonyl-CoA. The most problematic aspect of this mechanism is the place from where the proton comes in the reprotonation of the intermediate. If the opposing carboxylate ligand (E_2 in Figure 4B) is to act as the acid, then an isomerization is required to reversibly move the proton from E_1 to E_2 in the intermediate. To be taken more seriously, this proposition requires both structural and mechanistic experimental support.

Oxidative Cleavage of Carbon−Carbon Bonds. The extradiol dioxygenases compose a group of metalloenzymes that typically catalyze the oxidative meta ring cleavage of catechol or similar substrates to give α-hydroxymuconic semialdehyde or related products (*25*). The enzymes have been classified into three types (I, II, and III) on the basis of their domain composition and structure. The type I and II extradiol dioxygenases are composed of paired $\beta\alpha\beta\beta\beta$ motifs, while the type III enzymes have quite different three-dimensional structures (*26*, *27*). The enzyme requires Fe^{2+}, though Mn^{2+}-dependent versions have also been identified (*28*, *29*). The crystal structure of the enzyme in the absence of substrates reveals that the protein supplies only three ligands to the catalytic metal as illustrated in Figure 5A. The position that is structurally equivalent to E99 in glyoxalase I is replaced with an alanine residue in the dioxygenase, thus opening up another coordination site for chemistry. Three ligands from the protein are probably the minimum compliment necessary for tight coordination of the metal to the active site.

The tridentate coordination leaves three open or solvent-occupied coordination sites at the metal center for substrate binding, two to accommodate the catechol and one for the binding and activation of dioxygen as illustrated in Figure 6A. It is clear that from spectroscopic studies and a crystal structure of the substrate complex that the vicinal oxygen atoms of the catechol bind to the Fe^{2+} center leaving one open coordination site for dioxygen (*29*, *30*). The substrate binds as the catecholate monoanion and activates the metal center for binding O_2. A mechanism for the oxygen atom transfer reaction involving nucleophilic attack of a metal-bound superoxide anion on the substrate followed by a Criegee-type ring insertion reaction and rearrangement to form the lactone has been proposed (*29*) and is shown in Figure 6A.

Antibiotic Resistance. The fosfomycin resistance protein, FosA, catalyzes yet another type of reaction, the nucleophilic addition of glutathione to C1 of the oxirane ring of the antibiotic fosfomycin as illustrated in Figure 6B (*7*, *31*, *32*). FosA is a plasmid-encoded protein identified in clinical isolates of microbial populations resistant to the antibiotic (*33*). The enzyme requires Mn^{2+} for catalysis but will use other divalent cations as well. In addition, the enzyme requires the monovalent cation K^+ for optimum catalytic activity (*10*). Although the structure of FosA has not yet been determined, sequence alignments, spectroscopic evidence, and mutagenesis studies suggest that the metal coordination is tridentate and very similar to that observed in the extradiol dioxygenases (Figure 5B). Superhyperfine coupling of the Mn^{2+} EPR signals to $H_2{}^{17}O$ indicates that all three open coordination sites are occupied by water in the absence of

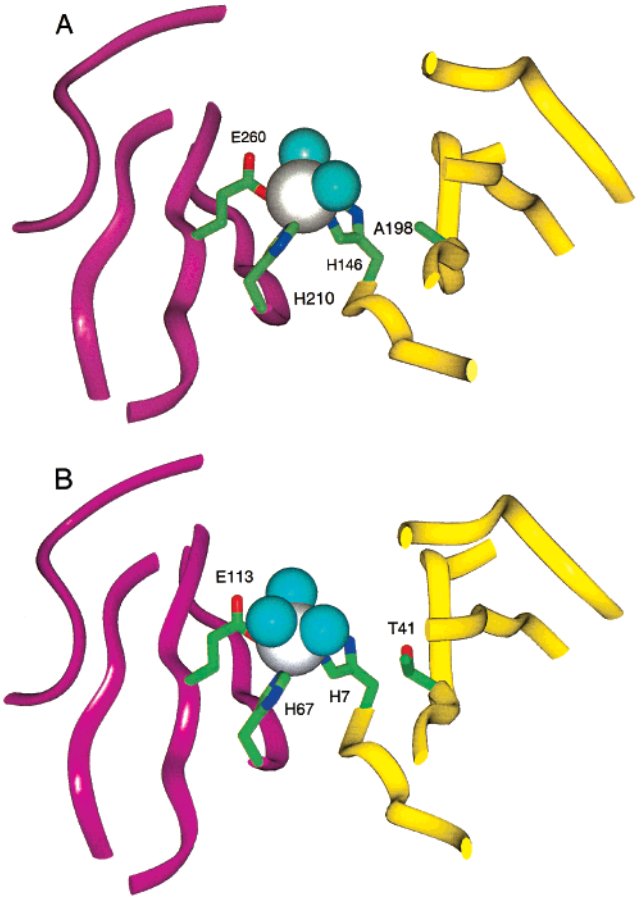

FIGURE 5: (A) Representation of the structure of the active site of the extradiol dioxygenase from *B. cepacia* in the absence of substrates derived from PDB file 1HAN (*14*). (B) A model of the active site of FosA based on sequence alignments, EPR spectroscopy, and the structure of the extradiol dioxygenase. The β-sheets contributed by the two $\beta\alpha\beta\beta\beta$ motifs are shown in purple and yellow. The α-helices and loops connecting the β-strands have been eliminated for clarity. The metal ion ligands are shown in stick representation, while the metal ion and associated water molecules are shown as white and blue spheres, respectively.

substrates as indicated in Figure 5B. The very dramatic alterations in the Mn^{2+} EPR signal seen in the presence of fosfomycin (but not in the presence of GSH) suggest that fosfomycin forms an inner sphere complex with the enzyme-bound metal (*7*). The formation of a quaternary complex of enzyme, Mn^{2+}, fosfomycin, and K^+ can also be detected by EPR spectroscopy (*10*).

At first glance, one might not suspect the necessity for a metal to promote nucleophilic addition of a thiolate to an oxirane. However, the oxirane ring of fosfomycin is exceedingly stable toward nucleophilic attack with a bimolecular rate constant for addition of GSH (pH 8, 25 °C) of about 10^{-8} M^{-1} s^{-1} (*10*). Part of the difficulty with this reaction is the addition of an anionic nucleophile to an anionic substrate, a situation that could be mitigated by a Lewis acid such as Mn^{2+} or another divalent cation. A mechanistic hypothesis consistent with the above-mentioned spectroscopic observations has been proposed in which the binding of fosfomycin to the Mn^{2+} center results in recruitment of a K^+ ion and activation of the oxirane ring for nucleophilic attack by GS^- (Figure 6B). The details of these interactions remain to be elucidated. Whether the activation involves bidentate or tridentate coordination of fosfomycin is not clear. Even

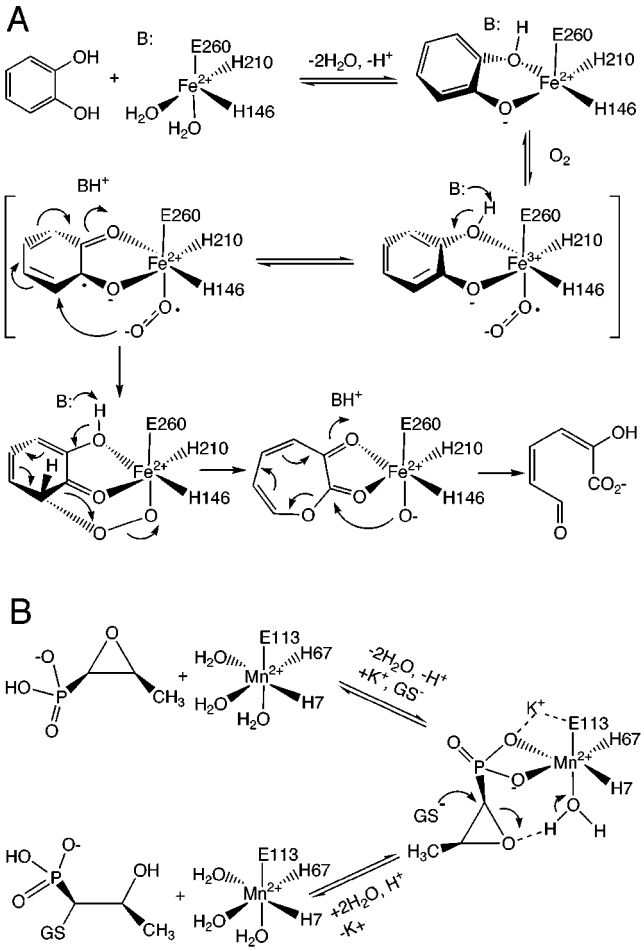

FIGURE 6: (A) Proposed mechanism of the extradiol dioxygenase as determined by Lipscomb, Que, and co-workers (*29*). (B) One mechanism for the fosfomycin resistance protein, FosA (*7*, *10*). Alternate coordination schemes in this mechanism are possible.

though there is no evidence to suggest that GSH interacts directly with the metal center, a transient interaction along the reaction coordinate cannot be ruled out. The Mn^{2+} and K^+ ions in this reaction appear to be necessary to provide a charge-neutral transition state for the nucleophilic addition.

Several genes, termed *fosB*, encoding close relatives of FosA that may also confer resistance to fosfomycin have been identified in the sequence databases. Although one is a plasmid-borne gene found in *Staphylococcus epidermidis*, the others are found in the genomes of *B. subtilis*, *Bacillus anthracis*, and *Staphylococcus aureus*. The genomically encoded FosB protein of *B. subtilis* does confer resistance to fosfomycin, a fact made all the more interesting because this particular Gram-positive microorganism does not make GSH. Sequence alignments with FosA indicate a conservation of the metal binding ligands. A preliminary characterization of the protein suggests that it may function by catalyzing the addition of L-cysteine to fosfomycin.[3] The enzyme activity is dependent on Mg^{2+} rather than Mn^{2+}, and the efficiency of the reaction with L-Cys ($k_{cat}/K_M^{L-Cys} = 170\ M^{-1}\ s^{-1}$) is far below that of FosA with its optimal substrate, GSH ($k_{cat}/K_M^{GSH} = 1.7 \times 10^5\ M^{-1}\ s^{-1}$). It is unclear whether this rather anemic catalytic activity is sufficient to support antibiotic resistance or if the protein functions by a different mechanism such as sequestration or transport.

Metal Ion Selectivity of the VOC Superfamily. It is evident from Table 1 that the members of the VOC superfamily utilize different divalent cations to support catalysis. For example, human glyoxalase I is a Zn^{2+}-dependent enzyme, whereas the homologue in *E. coli* is completely inactive with Zn^{2+} and utilizes Ni^{2+} (*18*, *34*). Although individual enzymes exhibit a preference for a particular metal some, such as glyoxalase I and the fosfomycin resistance proteins (*7*), function perfectly well with a variety of metal ions. This is not too surprising for reactions that simply require an electrophilic center to stabilize an intermediate or transition state. It is a consequence of the fundamental nature of the mechanistic imperative relating the reactions. In contrast, the requirements of redox chemistry are considerably more stringent. The redox potential of the metal center needs to be matched with that of the substrates, a fact that requires a greater degree of active site tuning. Thus, an Fe^{2+}-dependent extradiol dioxygenase will not function with Mn^{2+}.

Evolution of the VOC Superfamily. Bolin and colleagues (*8*) have proposed a provocative evolutionary pathway for the structural scaffolding of this superfamily based on the generally accepted processes of gene duplication, fusion, and modification. A modified and abbreviated version of this pathway is illustrated in Figure 7. The progenitor of the superfamily is proposed to be a minigene-encoded single $\beta\alpha\beta\beta\beta$ motif. It is easy to imagine a simple and functional *C*2 symmetric metalloprotein dimer arising from $\beta\alpha\beta\beta\beta$ motif monomers having appropriately positioned side chains to act as metal ligands. Gene duplication and fusion leading to a more robust two-motif pseudosymmetric metallomonomer could enhance the utility of the dimer. This step amounts to conversion of a relatively simple bidentate chelate to a more stable and selective tetradentate system that is presumably able to function at lower metal ion concentrations. Additional gene duplication−fusion events (not shown) can give four motif monomers as is eventually observed in some of the extradiol dioxygenases (Figure 2) and yeast glyoxalase I (*35*). Three-dimensional domain swapping and further gene modifications can yield metallodimers with enhanced stability and new catalytic properties. For example, the loss of one of the four ligands about each metal results in further functional diversification by opening an additional coordination site to support chemistry.

It is not possible to determine which of the known VOC superfamily members appeared first in biology. If the hypothetical evolutionary pathway in Figure 7 is accurate, then it can be argued that enzymes with four protein ligands (e.g., glyoxalase I or perhaps methylmalonyl-CoA epimerase) may have preceded the tridendate enzymes such as FosA and the extradiol dioxygenases that catalyze more complex bisubstrate reactions. Speculation about smaller evolutionary steps may be more accurate. For example, the relatively low catalytic activity of the genomically encoded fosfomycin resistance protein, FosB, suggests that its enzymatic activity may be a nascent one and an evolutionary precursor to the much more efficient plasmid-encoded FosA enzyme.

The evolution of the bleomycin resistance protein clearly deserves comment since this particular family member is unique in that it does not possess a metal binding site and is

[3] M. Cao, B. A. Bernat, Z. Wang, J. Qiu, R. N. Armstrong, and J. D. Helmann, unpublished results.

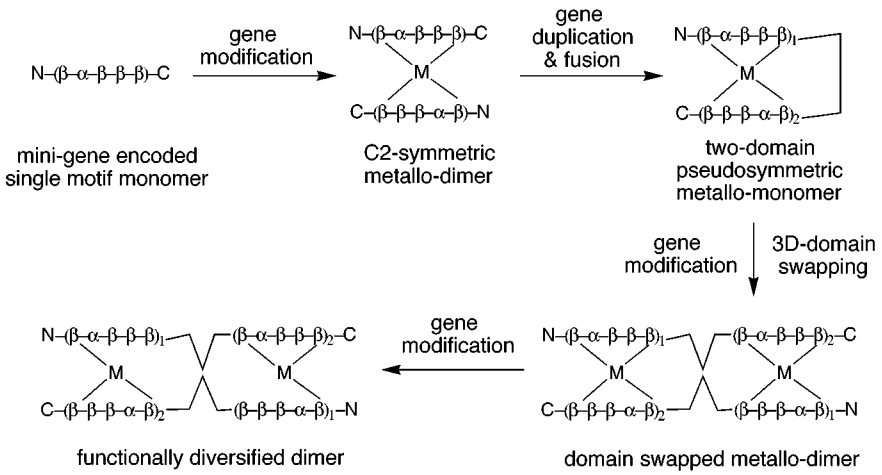

FIGURE 7: Proposed evolutionary pathway for the VOC superfamily. The pathway is a modification of the one originally proposed by Bolin and co-workers (*8*).

not an enzyme. If it is accepted that early evolution of the VOC superfamily involved optimization of an emergent metal binding motif, then the bleomycin resistance protein represents a situation where the metal binding ability of the progenitor has atrophied in the process of developing a protein with a cavity suitable for sequestering the antibiotic. Although the identity and function of the progenitor are unclear at this point, there is every reason to believe that it diverged from VOC ancestors (*8*). The complete loss of one protein function to provide for another is not at all unusual and does not diminish the mechanistic imperative hypothesis. It is simply the imposition of a new biological imperative on an extant protein fold.

Symmetry: Use It or Lose It. The issue of symmetry in the evolution of the structure and function of VOC superfamily members is an informative one to consider. Bolin and colleagues have suggested that the evolution of the progenitor of this superfamily, a metal-binding two-module dimer (Figure 7), was facilitated by the symmetry inherent in the paired $\beta\alpha\beta\beta\beta$ motifs. Fewer mutations are required to create a metal ion binding site possessing multiple ligands. In contrast, the functional diversification of the superfamily requires a reduction of symmetry at the metal site to accommodate more complex chemistry.

As suggested above, the residual symmetry in the active site of glyoxalase I and perhaps in methylmalonyl-CoA epimerase may, in fact, be the crucial structural feature that permits the abstraction of stereochemically distinct protons required in the isomerization and epimerization reactions catalyzed by these enzymes. The opposing carboxylate ligands in the coordination sphere of the metal are appropriately positioned to participate in the stereospecific abstraction of one proton or another of the metal-bound substrate. This presupposes that each carboxylate can disengage the metal ion in the transition state as is observed for E172 in the crystal structure of glyoxalase I (*17*). The charges about the metal ion in the transition states for these reactions are neutral. Thus, the local electrostatic field would not be expected to impair the ability of either carboxylate to act as a base. From the standpoint of molecular symmetry, then, it can again be argued that glyoxalase I and methylmalonyl-CoA epimerase may be the more ancient members of the VOC superfamily.

From Sequence Space to Mechanism Space. Protein structures and families are often discussed in terms of sequence space, the linear arrangement of amino acid residues which specifies the conformation space occupied by the folded, three-dimensional structure of the molecule. Sequence/conformation space is vast. For example, Dill has estimated by lattice simulations that there are as many as 10^{100} different sequences that would specify the native backbone conformation of a small, simple protein such as ribonuclease A (*36*). Obviously, only a fraction of these sequences could occupy the precise conformation space that is needed to specify ribonuclease A function. Yet others can specify different catalytic and biological activities (e.g., angiogenin in the ribonuclease superfamily).

It is now apparent that enzyme superfamilies can be discussed not only with respect to the sequence and conformation space they occupy but also in terms of the mechanism space to which they provide access. On first consideration, the concept of mechanism space may seem to be too impalpable to be useful. However, the mechanisms of chemical reactions are described with real collections of atoms in molecules arranged in ground state and transition state structures with definable internuclear distances and potential energy surfaces. If a common chemical or physical thread that characterizes a particular aspect of mechanism space that is available to a given superfamily can be defined, then that thread can often be used either to reveal the function of an unknown protein or to identify the mechanism of a protein of known function. In some instances, the extent of known mechanism space accessed by a particular superfamily can be defined by a single rate-limiting step or transition state as in the case of the enolase superfamily (*3*).

Perhaps the most remarkable aspect of the VOC superfamily is the breadth of mechanism space that it covers. The impressive mechanistic diversity is clearly related to the fact that the catalytically active members of the superfamily are metalloenzymes. Metal centers are usually quite versatile, being able to provide electrophilic assistance as well as electron-transfer capabilities that are necessary for redox chemistry. The simple, pseudosymmetric structural fold of paired $\beta\alpha\beta\beta\beta$ motifs maximizes the catalytic versatility of the metal center. The full extent of the catalytic diversity of the VOC superfamily is not yet known. Moreover, it remains

to be seen if metalloenzyme superfamilies, in general, provide enhanced access to mechanism space as is apparent in the particular case examined here.

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