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Similar active sites in lysostaphins and D-Ala-D-Ala metallopeptidases

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Abstract

Specific peptidases exist for nearly every amide linkage in peptidoglycan. In several cases, families of peptidoglycan hydrolases with different specificities turned out to be related. Here we show that lysostaphin-type peptidases and D-Ala-D-Ala metallopeptidases have similar active sites and share a core folding motif in otherwise highly divergent folds. The central Zn$^{2+}$ is tetrahedrally coordinated by two histidines, an aspartate, and a water molecule. The Zn$^{2+}$ chelating residues occur in the order histidine, aspartate, histidine in all sequences and contact the metal via the N$^{9255}$/H, the O$^{9254}$/H, and the N$^{9254}$/H, respectively. The identity of the other active-site residues varies, but in all enzymes of known structure except for VanX, a conserved histidine is present two residues upstream of the second histidine ligand to the Zn$^{2+}$. As the same arrangement of active-site residues is also found in the N-terminal, cryptic peptidase domain of sonic hedgehog, we propose that this arrangement of active-site residues be called the “LAS” arrangement, because it is present in lysostaphin-type enzymes, D-Ala-D-Ala metallopeptidases, and in the cryptic peptidase in the N-domain of sonic hedgehog.

Keywords: active site; D-Ala-D-Ala; metallopeptidase; LAS enzymes; lysostaphin; Lyt M

Most species of bacteria are surrounded by a rigid cell wall that consists predominantly of peptidoglycan. Alternating units of N-acetyl-glucosamine and N-acetyl-muramic acid that are linked through 1,4-glycosidic bonds form the backbone of this structure. The muramyl residues serve as anchors for short polypeptides that contain both L- and D-amino acids and typically have two D-alanine residues at the C terminus. These peptidic “tails” of the sugar “backbone” can be crosslinked by transpeptidation, leading to a loss of the terminal D-alanine and to a strengthening of the bacterial cell wall. The crosslinks are typically directed in gram-negative species and indirect via a linker, often a glycine-rich sequence, in some, but not all gram-positive bacteria (Schleifer and Kandler 1972).

In recent years, it has become clear that specific peptidases exist for nearly every amide bond that occurs in peptidoglycan, and that in some cases even several structurally and mechanistically distinct peptidase families have evolved to target the same amide bond (Smith et al. 2000). Although these observations suggest that a bewildering number of peptidase families should be present, it has also become clear that many peptidase families with different specificities are related. Several authors have described a large group of peptidoglycan hydrolases with similarity to the prototypical cysteine protease papain that can cleave N-acetyl-muramyl-L-alanine, D-$^{9253}$/H-glutamyl-meso-diamino-pimelate, and D-alanyl-glycine amide linkages (Anantharaman and Aravind 2003; Bateman and Rawlings 2003; Rigden et al. 2003).

In this communication, we combine information from the first structure of a lysostaphin-type peptidase (Odintsov et al. 2004) with prior information about the similarity between different families of metallopeptidases with specificity for D-Ala-D-Ala (Bussiere et al. 1998) to show that all of these enzymes have highly similar active sites that are formed in the context of a common folding motif, an anti-
parallel, four-stranded β-sheet with identical segment order and connectivity.

Lysostaphin-type peptidases occur mostly in bacteria and bacteriophages (Hooper 1994). In vitro, most well characterized family members from gram-positive bacteria are specific for glycine-rich sequences (Vessillier et al. 2001), and many family members are believed to be glycyl-glycine endopeptidases (Sugai et al. 1997; Ramadurai et al. 1999). In vivo, these enzymes are believed to cleave the glycine-rich crosslinks that strengthen the peptidoglycan in some, but not all species of gram-positive bacteria. Intriguingly, characterized family members from gram-positive bacteria are bacteriophages (Hooper 1994). In vitro, most well characterized family members from gram-positive bacteria are specific for glycine-rich sequences (Vessillier et al. 2001), and many family members are believed to be glycyl-glycine endopeptidases (Sugai et al. 1997; Ramadurai et al. 1999). In vivo, these enzymes are believed to cleave the glycine-rich crosslinks that strengthen the peptidoglycan in some, but not all species of gram-positive bacteria. Intriguingly, lysostaphin-type enzymes are also present (Rawlings et al. 2002) in gram-positive bacteria such as Bacillus subtilis that lack glycyl-glycine peptide linkages and in gram-negative bacteria that are typically crosslinked directly (Schleifer and Kandler 1972), suggesting that these enzymes are either not directed against peptidoglycan or that they have different specificities. An example of the latter situation is the lysostaphin-type enzyme LytH (YunA) from B. subtilis that was recently shown to be specific for L-Ala-D-Glu linkages in peptidoglycan (Horsburgh et al. 2003). Although the predominant physiological role of lysostaphin-type peptidases appears to be in peptidoglycan hydrolysis, some enzymes have been reported to have weak protease activity as well, at least in vitro (Peters et al. 1992; Park et al. 1995).

The first structure of a lysostaphin-type glycyl-glycine endopeptidase was recently solved in our laboratory, although in a latent form. We crystallized full-length LytM from Staphylococcus aureus, including the inhibitory N-terminal part that occludes the active site (Odintsov et al. 2004). In this structure (PDB accession code 1QWY), the Zn\(^{2+}\) is tetrahedrally coordinated by four amino acid ligands. An asparagine side chain from the N-terminal inhibitory part takes the place that should be occupied by a solvent molecule in active protease. This latency mechanism is analogous to the “cysteine switch” mechanism in matrix metalloproteases (Van Wart and Birkedal-Hansen 1990) and has therefore been termed “asparagine switch” (Odintsov et al. 2004). In silico removal of the N-terminal inhibitory fragment of the LytM structure and introduction of a water molecule as the fourth zinc ligand generated the first atomic model of an active, lysostaphin-type peptidase.

VanX-type D-Ala-D-Ala (aminodipeptidases have come into the spotlight because of their role in vancomycin resistance (Lessard and Walsh 1999). They deplete the cellular pool of D-Ala-D-Ala dipeptide and thus assist bacterial cells in the switch from vancomycin-susceptible D-Ala-D-Ala-containing peptidoglycan precursors to vancomycin-resistant D-Ala-D-Lac-based precursors (Lessard and Walsh 1999). The structures of Enterococcus faecium VanX alone and in complex with phosphinate and phosphonate transition state analogs have been reported (Bussiere 1998), but it seems that no coordinates have been deposited in the PDB.

VanY-type enzymes are similar in sequence to VanX-type enzymes and share with them the characteristic ExxH motif (Arthur et al. 1992). Unlike VanX-type enzymes, which are believed to be specific for the D-Ala-D-Ala dipeptide, VanY-type enzymes are D-Ala-D-Ala carboxypeptidases and cleave D-Ala-D-Ala in the peptidoglycan context (Arthur et al. 1992; Wright et al. 1992). No structure of a VanY-type metallopeptidase is available, but based on sequence similarity, the structure is expected to be closely related to the VanX structure.

Although thought to be a carboxypeptidase as well, the metallopeptidase with specificity for D-Ala-D-Ala from Streptomyces albus G shows only very weak sequence similarity to both VanX- and VanY-type peptidases, and contains an H-x-H motif instead of the E-x-x-H motif in VanX and VanY (Rawlings et al. 2002). The crystal structure was solved many years ago at moderate resolution and before the full sequence of the enzyme became available (Dideberg et al. 1982). The original structure, which assigned three histidines as Zn\(^{2+}\) ligands, has since been revised. The current version (at high resolution and with good statistics) that was used in the present study shows the Zn\(^{2+}\) bound to two histidines and an aspartate (P. Wery, Ph.D. thesis), and is available from the PDB under accession code 1LBU. In the following text, the term “D-Ala-D-Ala carboxypeptidase” refers to this structure.

The occurrence of an H-x-H motif in both lysostaphins and the D-Ala-D-Ala carboxypeptidase from S. albus G was noted earlier by Rawlings and Barrett (1995). In light of the structural data available at the time (Dideberg et al. 1982), the motif was believed to contain two zinc ligands to the catalytic zinc ion, a conclusion that now appears to require revision. Here we reinterpret the original observation by Rawlings and Barrett in light of the new structural data (P. Wery, Ph.D. thesis; Odintsov et al. 2004) and extend it to show that D-Ala-D-Ala carboxypeptidase from S. albus G and lysostaphins have very similar active sites altogether and also share a core folding motif around the active site. A similarity between the D-Ala-D-Ala carboxypeptidase from S. albus G and VanX-type D-Ala-D-Ala aminopeptidases had been suggested previously by McCafferty and colleagues (1997), even though the latter lack the characteristic H-x-H motif. The predicted similarity between D-Ala-D-Ala carboxypeptidase and VanX-type enzymes was subsequently confirmed by the VanX crystal structure from the Abbott laboratories (Bussiere 1998). Even before the structural similarity between the different D-Ala-D-Ala metallopeptidases became apparent, Murzin (1996) had noted a similarity between the D-Ala-D-Ala carboxypeptidase from S. albus G and N-terminal domain of sonic hedgehog (PDB accession code 1VHH). Although the N-domain of sonic hedgehog, an important molecule for patterning in embryogenesis (Porter et al. 1995), looks like a peptidase (Hall et al. 1995), it has no known substrates (Fuse et al. 1999).
Here we compare the structure of the lysostaphin-type enzyme LytM with the structures of the various D-Ala-D-Ala metallopeptidases and with the structure of the N-domain of sonic hedgehog, and we show that the similarity among all of these enzymes extends beyond the active-site architecture, to a β-sheet core folding motif with identical segment order and connectivity that anchors the active-site residues. As this arrangement is currently known to occur in lysostaphin-type enzymes, D-Ala-D-Ala metallopeptidases and sonic hedgehog, we propose the term “LAS,” derived from the initials of the major protein families that share this arrangement, to describe this disposition of active-site residues. We also refer to lysostaphin-type enzymes, D-Ala-D-Ala metallopeptidases, and the N-domain of sonic hedgehog collectively as LAS proteins or enzymes, assuming that a substrate for the cryptic peptidase in the N-domain of sonic hedgehog will eventually be found. We avoid the term “superfamily” for LAS proteins or enzymes because we do not want to prejudice the discussion regarding whether the similarity between LAS proteins is the result of convergent or divergent evolution.

Results

The starting point for this report was our observation that crystal structure of LytM shared the tetrahedral metal coordination geometry and the set of zinc ligands, two histidines and an aspartate, with MEROPS clan MD metallopeptases (Rawlings et al. 2002). Superposition of the LytM structure with 1LBU then demonstrated that the similarity went beyond the zinc coordination sphere and included an H-x-H motif that is present in nearly all lysostaphin-type peptidases and in D-Ala-D-Ala carboxypeptidase from S. albus G. Although the H-x-H motif is not present in VanX-type peptidases, a similarity between VanX-type peptidases and the carboxypeptidase from S. albus G had been noted before (Bussiere 1998). A systematic search of the PDB (Berman et al. 2000) with PINTS (Stark et al. 2003) using the spatial arrangement of the three zinc ligands either with or without the first histidine of the H-x-H pattern expanded the set of related structures to include the N-terminal domain of sonic hedgehog (Hall et al. 1995) and, with a somewhat lower score, endonuclease IV (Hosfield et al. 1998). The endonuclease was excluded from the set, both because it cleaves phosphodiester rather than amide bonds and because it contains a trinuclear rather than a mononuclear Zn$^{2+}$ center (Hosfield et al. 1998).

Fold

The degree of similarity of the other enzymes is striking, especially in the context of otherwise highly divergent folds. As can be seen in Figure 1, all active-site residues are located on or around a β-sheet scaffold. The core of this central β-sheet consists of four antiparallel β-strands that are arranged in the same order in all enzymes (continuous lines in Fig. 1). Disregarding β-strands (broken lines in Fig. 1) outside this core region, the arrangement can be described as topologically equivalent to two β-hairpins that are connected by a +2x crossover connection in Richardson nomenclature (Richardson 1977).

Without this simplification, the situation is more complex. In all structures, the core folding unit of four antiparallel β-strands is part of a larger five- or six-stranded β-sheet that is either antiparallel (Fig. 1A) as in LytM or mixed as in the other structures (Fig. 1B,C). In the LytM structure, the two additional strands outside the core motif are located at the bottom of the sheet (in the orientation of Fig. 1) and consist of residues that are inserted in sequence between the first and second β-hairpin of the core. This insertion is unique for LytM and lysostaphin-type peptidases. In all other available structures, additional β-strands that extend the core motif are located either upstream or downstream (Fig. 1B,C). In D-Ala-D-Ala carboxypeptidase, residues downstream of the core motif contribute one β-strand that runs parallel to strand 3 (Fig. 1B). In contrast, the β-strand that extends the core motif in VanX is on the N-terminal side of the core motif and would be located at the top of the sheet in the Figure 1 orientation (VanX not shown). In sonic hedgehog, two β-strands that are both located upstream of the core motif in sequence extend the four-stranded antiparallel β-sheet to a six-stranded mixed β-sheet with one strand at the “top” running parallel to strand 1 and one strand at the “bottom” running antiparallel to strand 3.

Zn$^{2+}$ ligands

The active sites of LAS enzymes are shown in Figure 2 and summarized in Table 1. In all structures, the central Zn$^{2+}$ is tetrahedrally coordinated by two histidines, an aspartate and a fourth ligand. In D-Ala-D-Ala carboxypeptidase, VanX, and sonic hedgehog, this fourth ligand is the usual water molecule. In the case of LytM, a latent form of the enzyme that includes the inhibitory N-terminal region was crystallized. In the resulting structure, an asparagine fills the fourth Zn$^{2+}$ coordination site. We presume that this asparagine displaces the water molecule that would be present in the active enzyme, although formal proof will require the structure of the active form of LytM.

The three amino-acid ligands to the Zn$^{2+}$ occur in the order histidine, aspartate, histidine. The first histidine (“1” in Fig. 2) is always anchored in the loop that connects strands 1 and 2. Although the orientation of the main chain varies between the structures, the orientation of the imidazole ring relative to the Zn$^{2+}$ is extremely well conserved: In all structures, it is the NE of the imidazole ring that contacts the metal ion directly. The NO is therefore protonated, and donates a hydrogen bond to a carbonyl oxygen of the main
In all structures, the second ligand, an aspartate, is anchored on strand 2 and follows the first ligand, a histidine, rather closely in sequence (“2” in Fig. 2). In LytM and other lysostaphin-type peptidases, the spacer consists of three residues only, whereas in the other structures, it is longer and comprises six residues. Both the histidine and the aspartate are largely conserved throughout the lysostaphin family. Nevertheless, the alignment in MEROPS for family M23/37 (Rawlings et al. 2002) contains a number of sequences that lack either one or both ligands, suggesting that these family members are either inactive or recruit other metal ligands, possibly from different regions in the sequence.

In all protein families, there is a large gap in sequence between the second and third Zn$^{2+}$ ligand that is a histidine in all structures (“3” in Fig. 2). This histidine is anchored on strand 4 and donates its N$^\alpha$ free electron pair to the zinc ion. The N$^\alpha$ is therefore protonated and acts as the hydrogen-bond donor. In contrast to the first histidine ligand, the corresponding hydrogen-bond acceptor is a side-chain atom, not a main-chain carbonyl oxygen, although the lack of coordinates for VanX again precludes conclusions for this enzyme. The details of the arrangement vary (“5” in Fig. 2). In LytM, the acceptor residue is a glutamine that is located just two residues downstream of the H-x-H motif. In D-Ala-D-Ala carboxypeptidase and sonic hedgehog, the acceptor is a glutamate that is located further upstream in the sequence. This charged variant of the elec-His-Zn motif (Alberts et al. 1998) with a carboxylate as the “elec” is

Figure 1. Stereo representations of LAS proteins. The four β-strands that make up the core folding motif are presented as continuous lines; the rest of the Cα-trace as dotted lines. Side chains of functionally important residues are shown in black. The structures of (A) the active form of LytM (residues 183–314), (B) D-Ala-D-Ala carboxypeptidase, and (C) sonic hedgehog are shown. Black spheres mark the position of the Zn$^{2+}$ in all three panels.
thought to be particularly favorable, because the secondary interaction of the imidazole ring with a carboxylate enhances its basicity and thus its ligand strength (Christianson and Alexander 1989).

Other active-site residues

Two residues upstream of the histidine Zn\(^{2+}\) ligand, all structures with the exception of VanX contain a second conserved histidine residue (“7” in Fig. 2). The location and orientation of the imidazole ring relative to the metal center are extremely well conserved, suggesting a catalytic role for this residue. This interpretation is consistent with experimental data indicating that substitution of this residue in LasA (Gustin et al. 1996) and LytM abolishes activity. The importance of this residue is also underscored by its presence at the end of the previously described signature motif for lysostaphins Y-x-H-x(11)-V-x(12/20)-G-x(5-6)-H (Sugai et al. 1997; Ramadurai et al. 1999), which would be better defined as Y-x-H-x(11)-V-x(12/20)-G-x(5-6)-H-x-H to include the histidine zinc ligand.

The very first histidine of the motif has spatial and functional equivalents in the other structures as well (“6” in Fig. 2). In D-Ala-D-Ala carboxypeptidase, there is a histidine in the same spatial location, whereas this space is filled with a glutamate in sonic hedgehog. Although the active-site geometry is different in sonic hedgehog and thermolysin, the analogy with thermolysin prompted Hall et al. (1995) to suggest a role for this residue as general base in the activation of the nucleophilic water molecule. According to their mechanism, the glutamate activates the incoming nucleophilic water molecule, and a (protonated) histidine that is located across the hypothetical position of the substrate together with the Zn\(^{2+}\) polarizes the substrate for nucleophilic attack (Hall et al. 1995). It is remarkable and supports their mechanism that a proton donor is present in exactly this position in the other structures as well, arginine in D-Ala-D-Ala carboxypeptidase and tyrosine in LytM (not shown in Fig. 2). Unfortunately, the lack of coordinates for VanX precludes a precise comparison of its active-site residues with those in the other structures, but from the published results it is clear that the catalytic base in this structure is a glutamate that is “assisted” by an arginine residue.

Discussion

A mechanistic role for the first histidine of the H-x-H motif?

On theoretical grounds, several authors have assigned the residue marked “6” in Figure 2 as the catalytic base that activates the water molecule (Bussiere 1998; Lessard and Walsh 1999). If this is correct, then it is not clear what the role of the residue marked “7” in Figure 2 would be in the catalytic mechanisms. Experimentally, it has been shown that this residue is required for activity, at least in the lysostaphin-type peptidase LasA (Gustin 1996) and in LytM (Odintsov et al. 2004). At present, several interpretations are possible. In lysostaphin-type peptidases, both histidines could be required for water activation. Alternatively, histidine “7” could act as the proton-donor for the leaving group nitrogen. To prove or disprove this hypothesis, a cocrystal structure with a substrate analog will be required.

A similar active site in MepA?

LAS enzymes are very dissimilar outside a small, catalytic core region. This has precluded the recognition of similarity
between lysostaphin-type peptidases and D-Ala-D-Ala-specific peptidases on the sequence level, and it makes it difficult to identify additional peptidase families with similar active sites. Interestingly, a scan of MEROPS alignments shows that the conserved H-x(3-6)-D and H-x-H motifs are present in the correct order in MepA-like peptidases that are listed in several databases as peptidases of unknown catalytic type (Bateman et al. 2002; Rawlings et al. 2002; Mulder et al. 2003). MepA from Escherichia coli has been characterized biochemically and is believed to cleave D-alanylmeso-2,6-diamino-pimelyl peptide bonds in E. coli peptidoglycan. Although these properties suggest that MepA may be a metallopeptidase with LAS active-site architecture, we caution that MepA sequences are highly similar, and short, conserved sequence motifs may therefore occur by chance. Clearly, the idea requires further experimental testing.

A group of peptidoglycan amidases?

It is remarkable that most enzymes that share active sites with LAS architecture are specific for amide bonds in peptidoglycan, peptidoglycan precursors, or peptidoglycan degradation products. The specificities vary: Most lysostaphin-type enzymes are believed to cleave D-alanyl-meso-2,6-diamino-pimelyl peptide bonds in E. coli peptidoglycan. Although these properties suggest that MepA may be a metallopeptidase with LAS active-site architecture, we caution that MepA sequences are highly similar, and short, conserved sequence motifs may therefore occur by chance. Clearly, the idea requires further experimental testing.

Other metallopeptidases with specificity for peptidoglycan

Although it could be true that most LAS metallopeptidases are specific for peptidoglycan, the reciprocal statement that peptidoglycan-specific metallopeptidases are LAS enzymes is clearly incorrect. AmpD from Citrobacter freundii, a peptidoglycan amidase with specificity for the amide bond between N-acetylmuramic acid and L-alanine, coordinates the catalytic metal via two histidines and an aspartate, but the architecture of the active site is clearly different, and the LAS core folding motif is not present (Liepinsh et al. 2003). Instead, AmpD is similar in fold to the catalytic domain of T7 lysozyme, an enzyme with the same specificity that was reported to resemble carboxypeptidase A in some aspects (Cheng et al. 1994). Based on sequence data, a similarity with carboxypeptidase A is also very likely for an amidase from Bacillus sphaericus that was shown to be specific for γ-D-glutamyl-(L)-meso-diaminopimelic acid amide bonds in peptidoglycan (Hourdou et al. 1993).

Other metallopeptidases with an H-x-H motif

A conserved H-x-H motif together with a conserved aspartate and glutamate have recently led to the definition of a new family of isopeptide hydrolases that are present in two large protein complexes, the 26S proteasome and the signalosome. In proteasomes, subunit Rpn11/POH1 is responsible for the ubiquitin-hydrolase activity of the 19S cap (Verma et al. 2002; Yao and Cohen 2002). In signalosome, Jab1/Csn5 was found to underlie the particle’s NedD iso-peptidase activity (Cope et al. 2002). The very first structure

### Table 1. Active-site residues in LAS enzymes

<table>
<thead>
<tr>
<th>Role</th>
<th>Nr</th>
<th>LytM (1QWY) Fig. IA</th>
<th>DDC (1LBU) Fig. 1B</th>
<th>VanX (—)</th>
<th>Sonic hedgehog (1VHH) Fig. 1C</th>
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A common catalytic core

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of a Jab1/MPN domain recently became available (Tran et al. 2003). At first sight, the structure shows superficial similarity with LAS enzymes: The fold of the Jab1/MPn domain is organized around a central β-sheet that includes the H-x-H motif in extended conformation, and the catalytic Zn²⁺ is liganded by two histidines and an aspartate. However, in contrast to lysostaphins, both histidine ligands to the Zn²⁺ in Jab1/MPn domain proteins are from the H-x-H motif, and the connectivities of the central β-sheet in LAS enzymes and Jab1/MPn domain proteins are different, clearly showing that the Jab1/MPn domain and LAS enzymes are unrelated.

The H-x-H motif and a conserved aspartate are also present in the peptidases that belong to the superfAMILY of “amidohydrolases related to urease” originally defined by Holm and Sander (1997). Despite this similarity, the amidohydrolases related to urease are clearly not related to LAS enzymes including GSP amidase and peptidoglycan hydrolases. Trends Biochem. Sci. 28: 234–237.

Materials and methods

The MEROPS (Rawlings et al. 2002) database was essential for the initial recognition of the similarity between the C-terminal part of LytM and D-Ala-D-Ala carboxypeptidase. Once this similarity was established, the program PINTS (Stark et al. 2003) was used to extend the family to include other family members with local similarity around the active site. Structure superpositions were done with Isqakb (Kabsch 1976), which is part of the CCP4 (Collaborative Computational Project 1994) suite of programs, and secondary structures were assigned with STRIDE (Frishman and Argos 1995). All figures were drawn with MOLSCRIPT (Kraulis 1991).

Acknowledgments

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The H-x-H motif and a conserved aspartate are also present in the peptidases that belong to the superfAMILY of “amidohydrolases related to urease” originally defined by Holm and Sander (1997). Despite this similarity, the amidohydrolases related to urease are clearly not related to LAS enzymes. Firstly, the scaffold of the urease-type amidohydrolases is a β6β-barrel, that is, a structure with parallel β-strands (Holm and Sander 1997). Secondly, in these hydrolases, both histidines of the H-x-H motif are ligands to the zinc (Holm and Sander 1997). Finally, the peptidase members of known structure, human renal dipeptidase (Nitanai et al. 2002) and a β-aspartyl dipeptidase (Thoden et al. 2003), have binuclear rather than mononuclear centers.

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