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New Perspective on Zinc Biochemistry: Cocatalytic Sites in Multi-Zinc Enzymes†

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Zinc is an integral component of a large number and variety of proteins involved in a multiplicity of vital processes accounting for its essentiality in metabolism, transmission of the genetic message, growth, and development. The chemically stable but stereochemically flexible, nontoxic nature of zinc combined with its amphoteric properties is the basis for the biochemical organization of a series of zinc binding motifs critical to life and its perpetuation. Our first perspective on zinc binding sites in biology focused on the zinc coordination, function, and structure of mono-zinc enzymes and the existing limited knowledge of zinc in the control of gene expression (Vallee & Auld, 1990a). Since that time systematic examination of zinc enzymes containing two or more zinc atoms has become available and has led to the definition of a new zinc binding motif in zinc enzymes, the *cocatalytic or coactive* site. The present perspective focuses on the properties of this group of multi-zinc enzymes.

Cocatalytic Zinc Enzyme Families

As in our past surveys and perspectives, computer and literature searches have ascertained protein sequences and the functional characteristics of *cocatalytic* or *coactive* zinc enzyme families based on the X-ray structure of a protein that serves as a standard of reference (Vallee & Auld, 1990a,b, 1992a). A "family" is defined as a group of proteins with identical or very similar functions and related by common ancestry as revealed by their sequence homology. In order to compare amino acid sequences in a family that abut a specific ligand to a particular zinc site, the nomenclature N_n---N_2-N_1-L_n-C_1-C_2---C_n is used. Here, L_n is an amino acid ligand (n = 1–4), and N_1 to N_n and C_1 to C_n refer to the amino acids abutting the N- and C-terminal sides, respectively; numbering begins with the amino acid closest to the ligand.

Cocatalytic zinc binding sites occur in enzymes that contain two or more zinc atoms in close proximity to one another (Vallee & Auld, 1993). Together they operate as a catalytic unit. X-ray studies reveal that in such zinc sites a single amino acid residue, either Asp or Glu, simultaneously binds to two metal atoms to form a bridge between two of the participating metal atoms. Asp, in particular, provides a bridge between **Zn*** and **$\bar{\text{Z}}$n** atoms or between **Zn*** and a magnesium atom.[1] The nomenclature chosen to identify, systematize, and verify the description of cocatalytic sites will use the following conventions: The catalytic zinc atom will be "**catalytic Zn**". The zinc atom physically closest to it by X-ray diffraction is designated "**Zn***". The zinc atom furthest from the catalytic Zn will be called "**$\bar{\text{Z}}$n**". The placement of one molecule of H_2O as a ligand to a metal site is cited in a generic sense, not as a topological detail. The physical contiguity of these metal atoms provides the structural rationale for their biological interaction.

Three of the four cocatalytic zinc enzymes whose structures have been determined thus far contain three metal atoms: in two of these all three are zinc, and in the other, two are zinc and one is magnesium. The fourth—leucine aminopeptidase of bovine lens—contains only two zinc atoms.

Cocatalytic (or Coactive) Zinc Sites

Alkaline phosphatase (*Escherichia coli*), a nonspecific phosphomonoesterase (Kim & Wyckoff, 1991); phospholipase C (*Bacillus cereus*), a phosphatidylcholineesterase (Hough et al., 1989); and P1 nuclease (*Penicillium citrinum*), a phosphodiesterase (Volbeda et al., 1991), all catalyze the hydrolysis of phosphate esters. All three contain one catalytic zinc atom plus an additional pair of metals bridged by an amino acid. Thus, alkaline phosphatase contains two zinc

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[1] The literature has referred to such an involvement of zinc—or other metal atoms—as "modulating" or "regulatory", owing to these seemingly indirect functional effects (Vallee, 1983; Vallee & Auld, 1992b).

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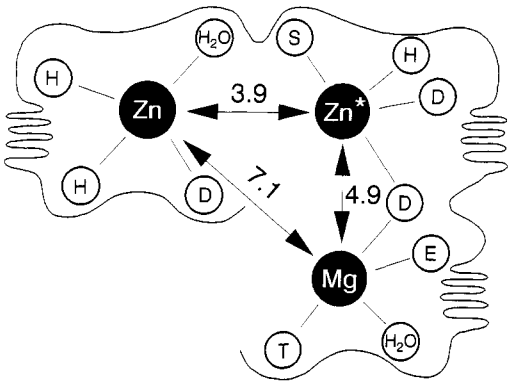

FIGURE 1: Schematic of the cocatalytic zinc binding site of *E. coli* alkaline phosphatase.

atoms and one magnesium atom, whereas the other two each contain three zinc atoms. In all of these one zinc atom is catalytic, and the **Zn*** and **Zn**/Mg are linked by a bridging amino acid. In all of these the three metal atoms are in close proximity to one another. Further, the three-dimensional structures of these enzymes, their cocatalytic metal binding sites, and their mechanistic features are quite similar. The existent kinetic, chemical, and structural information on alkaline phosphatase far exceeds that on the others. As a consequence, detailed comparison of their mechanisms of action would not be profitable at this junction but will be given further attention as pertinent knowledge becomes more extensive.

(*A*) *Alkaline phosphatase* of *E. coli* (EC 3.1.3.1) contains 4 mol of zinc plus 2 mol of magnesium per 80 000 MW (Plocke et al., 1962; Anderson et al., 1975; Bosron et al., 1977). It catalyzes the nonspecific hydrolysis of oxyphosphate monoesters and related phosphoryl derivatives, i.e., thiophosphate, phosphoramidates, and *S*-phosphorothioates, and is both a transferase and a hydrolase (Coleman, 1992). The crystalline enzyme is a dimer of 2-fold symmetry whose active sites are at the surface separated by about 30 Å (Kim & Wyckoff, 1991). Each monomer has an α/β topology with a central 10-stranded β-sheet flanked by 15 helices of varying lengths.

The three metal atoms, **catalytic Zn**, **Zn***, and Mg form the apices of each of the two nonequilateral triangles (Figure 1). **Catalytic Zn** and **Zn*** are separated by 3.94 Å, **Zn*** and Mg by 4.88 Å, and **catalytic Zn** and Mg by 7.09 Å (Kim & Wyckoff, 1991). The structure of the **catalytic Zn** site closely resembles those of catalytic sites in mono-zinc enzymes, but the participation of Asp as a ligand is indicative of an environmental difference. The **catalytic Zn** site is comprised of Asp-327 and His-331, separated by a short 3 amino acid spacer, and a third ligand, His-412, separated from His-331 by a long spacer of 80 amino acids (Figure 2).

The **catalytic Zn** is coordinated to an imidazole nitrogen of both His-331 and His-412 and to both carboxylate oxygens of Asp-327. Minimally, one water molecule completes the coordination, as implied by a positive peak 2.2 Å from the zinc site in the electron density map of the inhibitor-free form of the enzyme. The results of both water relaxation and Cl^- NMR studies suggest that this coordination site may contain two water molecules (Gettins et al., 1984; Schulz et al., 1989). In the enzyme·phosphate complex, one of the phosphoryl oxygens of the inhibitory phosphate is a ligand, likely the result of displacement of the water. In the A and B subunits the details of the resultant coordination complex differ in terms of the distances of the two carboxylate oxygens from the **catalytic Zn** atom: Oδ1/Oδ2 = 2.00/2.30 and 2.26/2.56 Å, respectively.

	327							331				412							
E. coli	G	A	S	I	D	K	Q	D	H	A	A	N	P	D	S	Q	E	H	T	G	S	Q
E. fergusonii	G	A	S	I	D	K	Q	D	H	A	A	N	P	D	S	Q	E	H	T	G	S	Q
S. marcescens	G	A	S	I	D	K	Q	D	H	A	A	N	P	E	S	Q	G	H	T	G	T	Q
S. griseus	G	A	S	I	D	D	R	A	H	E	A	D	P	E	T	Q	E	H	T	G	V	P
B. subtilis III	G	S	Q	I	D	W	A	G	H	D	N	D	I	W	T	G	G	H	T	G	E	D
B. subtilis IV	G	S	Q	I	D	W	A	A	H	D	N	D	R	T	S	T	D	H	T	G	E	E
S. cerevisiae	G	S	R	I	D	H	A	G	H	Q	N	D	P	T	T	H	G	H	S	A	V	D
B. licheniformis	G	S	K	P	D	W	A	A	H	Q	N	D	P									
Placental 1	G	G	R	I	D	H	G	H	H	E	S	R	A	D	E	E	T	H	A	G	E	D
Placental 2	G	G	R	I	D	H	G	H	H	E	G	K	A	R	H	E	T	H	G	G	E	D
Intestinal 1	G	G	R	I	D	H	G	H	H	E	G	V	A	S	S	E	T	H	G	G	E	D
Intestinal 2	G	G	R	I	D	R	G	H	H	L	G	T	A	K	S	E	T	H	G	G	E	D
Intestinal 3	G	G	R	I	D	Q	G	H	H	A	G	T	A	S	S	E	T	H	G	G	E	D
Embryonic 1	G	G	R	I	D	H	G	H	H	E	T	V	A	S	S	E	T	H	S	G	E	D
Osteosarcoma 3	G	G	R	I	D	H	G	H	H	E	G	K	A	R	H	E	T	H	G	G	E	D
Carcinoma 1	G	G	R	I	D	H	G	H	H	E	S	R	A	D	G	E	T	H	A	G	E	D
Liver 1	G	G	R	I	D	H	G	H	H	E	G	K	A	R	H	E	T	H	G	G	E	D
Liver 3	G	G	R	I	D	H	G	H	H	E	G	K	A	R	H	E	T	H	G	G	E	D
Renal 4	G	G	R	I	D	H	G	H	H	E	G	K	A	R	H	E	T	H	G	G	E	D
Membrane 5	G	G	R	I	D	H	A	H	H	D	N	Y	A	D	S	E	T	H	G	G	D	D

FIGURE 2: Sequences abutting the **catalytic Zn** ligands of alkaline phosphatase. Lightly stippled boxes denote the enzyme X-ray standard of reference for each family. The numbers indicated are the ligand positions for the reference enzyme. Black vertical columns indicate the proposed metal binding ligands based on the structure of the standard of reference. Key: 1, human; 2, mouse; 3, rat; 4, bovine; 5, silk moth.

Both Asp-327 (L_1) and His-331 (L_2) as well as the short spacer, KQD, between them are part of an α-helix encompassing residues 325–334. The 80 amino acid long spacer that separates His-412 from His-331 projects from the N-terminus of the only antiparallel β-sheet, formed by residues 412–424. Removal of zinc from the catalytic site distorts the α-helix of the short spacer region. Moreover, the secondary structure of the peptide segments that provide the ligands for the **catalytic Zn** may undergo conformational changes during catalysis. Indeed, zinc might modulate this change in conformation, thereby reflecting an entatic state favorable to catalysis (Vallee & Williams, 1968).

Jointly, **Zn*** plus magnesium complete the cocatalytic site of alkaline phosphatase, whose coordination features have not been seen previously in metalloenzyme chemistry (Figure 1). **Zn*** is tetracoordinated to one carboxylate oxygen each of Asp-51 and Asp-369, the imidazole nitrogen of His-370, and the hydroxyl group of Ser-102, known to be a nucleophile in *E. coli* phosphatase action (Kim & Wyckoff, 1991). The very participation of Ser-102 as a ligand gives special meaning to the designation "cocatalytic". Structural studies on phosphoserine-102 of cadmium alkaline phosphatase indicate that the ester oxygen of the phosphoserine is coordinated to **Cd*** while a second phosphate oxygen bridges the **catalytic Cd** and **Cd*** (Kim & Wyckoff, 1991). Since the phosphate oxygen is coordinated, at least temporarily, to both the **catalytic Zn** and **Zn*** during catalysis, this is indeed a *cocatalytic site*. This further suggests a search for analogous mechanistic implications in other instances. In addition in the enzyme·phosphate complex one of the phosphate oxygens bridges the **catalytic Zn** and **Zn***, while a second interacts with a Mg-coordinated water molecule. Thus, *phosphate interacts with all three metal atoms*, and phosphate release from the enzyme becomes the rate-determining step in catalysis. Alteration in the Asp-51 bridge could either lengthen, shorten, or even transiently break the **Zn*** and Mg bonds while profoundly affecting this rate as well as those reflecting other steps in catalysis.

Magnesium, the third metal of this site, is linked to this process through the Asp-51 bridge while coordinated to one of the oxygens of Glu-322 and the hydroxyl of Thr-155 plus three water molecules (Figure 2, Table I). This calls further attention to the importance of the number and mode of activation of water molecules in this catalytic process.

Table I: Zinc Ligands and Their Spacing in Amino Acid Bridging Zinc–Metal Sites[a]

enzyme	metal	L_1	X	L_2	Y	L_3	Z	L_4
E. coli alkaline phosphatase	**Zn***	Asp_β	0	His_β	317	***Asp***$_\beta$(N)	50	Ser
	Mg	***Asp***$_\beta$	103	Thr_β	166	Glu_β(C)		H_2O[b]
B. cereus phospholipase C	**Zn***	Trp[c]	12	His_α	107	***Asp***$_\alpha$(C)		H_2O
	$\bar{Z}$n	His_α	3	***Asp***$_\alpha$	48	His(N)	13	Asp_α(N)[d]
P. citrinum nuclease P1	**Zn***	Trp[c]	5	His_α	113	***Asp***$_\alpha$(C)		H_2O
	$\bar{Z}$n	His	3	***Asp***$_\alpha$	55	His_α(N)	14	Asp_α(N)[d]
bovine lens aminopeptidase	**Zn**	Asp[e]	1	***Glu***$_\alpha$	76	Asp_β(N)		H_2O[f]
	Zn*	Lys_β	22	Asp	60	***Glu***$_\alpha$(C)		

[a] The amino acid spacer between ligands L_1 and L_2 is X, that between L_3 and the nearest ligand L_1 or L_2 is Y, and that between ligands L_3 and L_4 is Z. The symbols N and C indicate that L_3 is located on the amino (N) or carboxyl (C) site of L_2. The Greek subscripts identify the secondary structural support for the ligand. The amino acid residues which bridge the two metal sites are shown in italic bold face. [b] Three water molecules complete this hexacoordinate site. [c] Ligands are the α-amino and carbonyl group of the N-terminal Trp. [d] A bridging water molecule or hydroxide ion from Zn* completes this pentacoordinate site. [e] The Asp is coordinated to **catalytic Zn** through both the carboxyl group and amide carbonyl. [f] A water molecule is observed at 3.2 Å.

Asp-369 and His-370, both **Zn*** ligands, are part of the β-strand stretching from residue 367 to residue 380 (Figure 3, Table I). On the other hand, all ligands of magnesium, Asp-51, Glu-322, and Thr-155, originate from three parallel β-sheets.

The sequences of a number of other alkaline phosphatases from other phyla have been identified recently, all of which are much longer than those of bacteria. Their alignment generates two major groups of phosphatases in which the length of the long spacer for the catalytic zinc atom is the most striking difference. *E. coli* alkaline phosphatase has an 80 amino acid spacer that separates the second ligand, His-331, from the third, His-412, and which is virtually the same length in all other bacterial enzymes, i.e., those from *E. fergusonii*, *Serratia marcescens*, *Streptomyces griseus*, and *Bacillus subtilis* III and IV. However, the long spacer in human, mouse, rat, bovine, and silk moth contains an insertion of ~30–34 amino acids that elongates it to ~110–114 residues; this structure differs significantly from that of the bacterial enzymes.[2]

The residues surrounding Asp-327 and His-331, comprising the short spacer of the mammalian alkaline phosphatase, are highly conserved (Figure 2). In the phosphatase family the N-terminal side of Asp-327 exhibits the greatest number of identities, perhaps because this region contains Glu-322, which is a **Zn*** ligand.

The amino acid residues bordering on the amino acid metal bridge are well conserved in this family of 20 enzymes, encompassing bacteria, insects, and mammals (Figure 3). This would seem to underline the functional importance of this double ligand. Such conservation seems remarkable considering that the residues arise from nearly the entire sequence of the protein, extending from Asp-51 to His-370.

(*B*) *Phospholipase C* (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) cleaves the bond between the hydrophobic moiety and the polar head of specific phospholipids both in microorganisms and in mammals (Clark et al., 1986). The enzymes from *B. cereus* (Little, 1981) and *Clostridium perfringens* (Krug & Kent, 1984) are zinc metalloenzymes, the former containing 1.89 mol of zinc per MW 23 000 (Little & Otnäss, 1975) and the latter 2.3 mol of zinc per MW 43 000 (Krug & Kent, 1984). In guanidine hydrochloride removal of zinc followed by its readdition reversibly unfolds and refolds the enzyme (Little & Johansen, 1979). Therefore, the structural stability of the *B. cereus* enzyme is linked to zinc, whose addition to assay mixtures increases activity about 2-fold, which implies that additional sites in the enzyme may bind zinc weakly.

The *B. cereus* enzyme recrystallized from a solution containing 10 μM Zn^{2+} ± phosphate (Hough et al., 1989) binds three zinc atoms which are in close proximity with interatomic zinc distances of 3.3–5.8 Å. The resultant motif of three zinc atoms resembles the two zinc plus one magnesium site in alkaline phosphatase (Figure 4A).

A short α-helix extending from residue 140 to residue 153 provides both His-142 and Glu-146 as ligands to the **catalytic Zn** site. A 13 amino acid spacer separates His-142 from His-128, the third ligand. Two water molecules complete a pentacoordinate site.

Asp-122 connects **Zn*** and **$\bar{Z}$n** atoms through a carboxylate bridge similar to that in alkaline phosphatase (Table I). However, in phospholipase C this ligand is located within the short spacer for the **$\bar{Z}$n** atom. Both His-118 and Asp-122, ligands to **$\bar{Z}$n**, originate from an α-helix which extends from residue 105 to residue 125, Asp-55, His-69, and a H_2O molecule complete this pentacoordinated zinc site.

Zn* is bound to the remaining oxygen of the carboxylate of Asp-122 and to a water—or hydroxide—which also bridges to **$\bar{Z}$n**. His-14 and the amino and carbonyl groups of Trp-1 are additional ligands. All three zinc atoms reside in an approximately trigonal-bipyramidal coordination sphere (Hough et al., 1989).

The phosphate of the phosphate·PLC complex is again closely associated with all three metal atoms (Hough et al., 1989). As might be expected for a cocatalytic site in which all three metal atoms act in concert to bring about catalysis, one of the phosphate oxygens replaces one of the water molecules coordinated to the **catalytic Zn** and a second replaces the water molecule that bridges **$\bar{Z}$n** and **Zn***, somewhat reminiscent of *E. coli* phosphatase. Molecular modeling has indicated that all three zinc atoms are involved in catalysis, with Glu-4 acting as the general base that activates a water bound to the **catalytic Zn** site (Byberg et al., 1992).

Comparison of the *C. perfringens* (Titball et al., 1989) and *Clostridium bifermentans* (Tso & Siebel, 1989) phospholipase C sequences to that of *B. cereus* phospholipase C (Johansen et al., 1988; Gilmore et al., 1989) localizes the catalytic domain in the *Clostridium* enzymes to the N-terminal sequence consisting of 245 amino acids; the varying sizes of the primary structure (370 vs 245 amino acids) may account for the differences in their enzymatic specificities. In this regard, the turnover number of phospholipase C from *C. perfringens* (i.e., α-toxin) is higher toward both phosphatidylcholine and sphingomyelin, key components of eukaryotic cell membranes (Titball et al., 1989). α-Toxin also readily hydrolyzes phospholipids in intact cell membranes. On the other hand, the *B. cereus* enzyme is nontoxic and hydrolyzes neither sphingomyelin nor intact cell membranes (Little, 1981). In addition, a different sequence and distinct *B. cereus* sphingomyelinase activity have been reported (Gilmore et al., 1989).

The ligand spacers and vicinal amino acids of the *B. cereus* enzyme also indicate the likely location of the metal binding

[2] At present it is more difficult to predict the third ligand for *Bacillus licheniformis* and *Saccharomyces cerevisiae*; the full sequence for the former is not available, and for the latter there exists more than one possibility. Alignment of *S. cerevisiae* with the *B. subtilis* enzymes suggests that His-484 is the third ligand (THGHSAV) (Hulett et al., 1991). However, on the basis of the similarity of the residues surrounding this ligand in the family, His-419 (VHEHKGA) would also be a possibility.

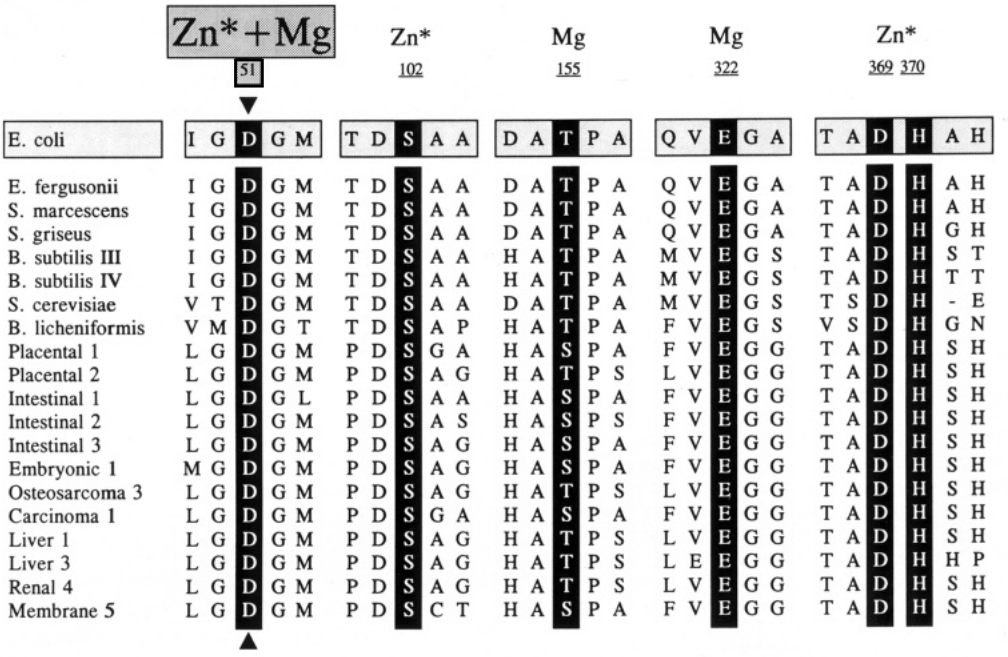

FIGURE 3: Sequences abutting the **Zn*** and Mg ligands of the bridging amino acid site of the alkaline phosphatase family. For key to figure and references, see caption of Figure 2. The column indicated by black arrows (▼ ▲) indicates the aspartate bridge.

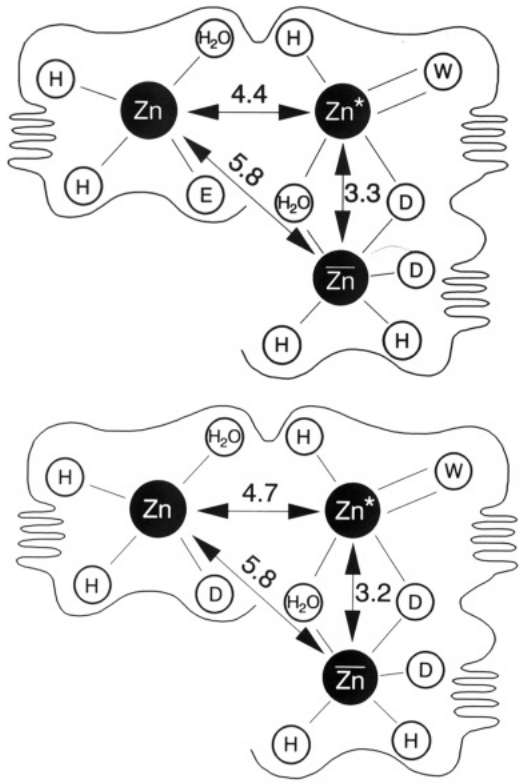

FIGURE 4: Schematic of the cocatalytic zinc site of (A, top) phospholipase C and (B, bottom) nuclease P1.

sites in the *C. perfringens* and *C. bifermentans* enzymes. The size of the short spacer of the **catalytic Zn** atom in the *Clostridium* phospholipases C is identical to that in the *B. cereus* enzyme while that of their long spacer is 11 instead of 13 amino acids (Figure 5A). In this region about 70% of the aligned amino acids are either identical or conservative replacements.

The short three amino acid spacer of the **$\bar{Z}$n** site is conserved along with the residues bordering on it (Figure 5B). Examination of these sequences also reveals that the ligands to **Zn*** and **$\bar{Z}$n** in the *Clostridium* enzymes are likely the same as those in the *B. cereus* enzyme. A glycine is conserved at the N1 position of the bridging amino acid Asp-122.

(*C*) *Nuclease P1* (EC 3.1.3.6) from *Penicillium citrinum* is both a phosphodiesterase, forming 5′ nucleotides from single-stranded ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and a phosphomonoesterase, hydrolyzing 3′ monophosphonucleotides to give a nucleoside plus phosphate (Fujimoto et al., 1974). Nuclease S1 and nuclease PA3, both glycosylated zinc enzymes with similar specificities, have been purified from *Aspergillus oryzae* (Vogt, 1973) and *Penicillium sp.* (Kazama et al., 1990), respectively. Nuclease P1 and nuclease PA3 contain 2.77 mol of Zn/mol of MW 44 000 protein (Fujimoto et al., 1975a) and 2.0 mol of Zn/mol of MW 32 000 protein (Kazama et al., 1990), respectively, while nuclease S1 is about 3 mol of Zn/mol of MW 32 000 protein (Shishido & Habuka, 1986).

EDTA inactivates both nucleases P1 and PA3 in a time- and concentration-dependent fashion (Rokugawa et al., 1980; Kazama et al., 1990). The changes in enzymatic activity, circular dichroism at 200–240 nm, and thermal stability of the enzyme that accompany depletion of one, two, or three zinc atoms have all been interpreted to demonstrate that the first two metals are not involved in secondary structure while removal of the third causes a large change in the α-helical structure, reflected in the CD spectrum (Fujimoto et al., 1975b; Rokugawa et al., 1980). The enzyme depleted of one or two zinc atoms can fully recover activity, but when all three have been removed and are then added back, the restoration of activity is only partial. This has been interpreted to denote that the first two zinc atoms may participate only in function while the third might have a structural role (Rokugawa et al., 1980).

Nuclease P1 has been crystallized from a solution containing 1 mM zinc acetate (Lahm et al., 1990; Volbeda et al., 1991), and its structure has been determined by X-ray crystallography. The cocatalytic zinc site of P1 is seen at the bottom of the substrate binding cleft consisting of both a relatively inaccessible di-zinc and a more exposed mono-zinc site (Volbeda et al., 1991). The three zinc atoms are arranged in a triangle and are separated by 3.2–5.8 Å (Figure 4B). The mono-zinc site closely resembles that of a catalytic zinc motif

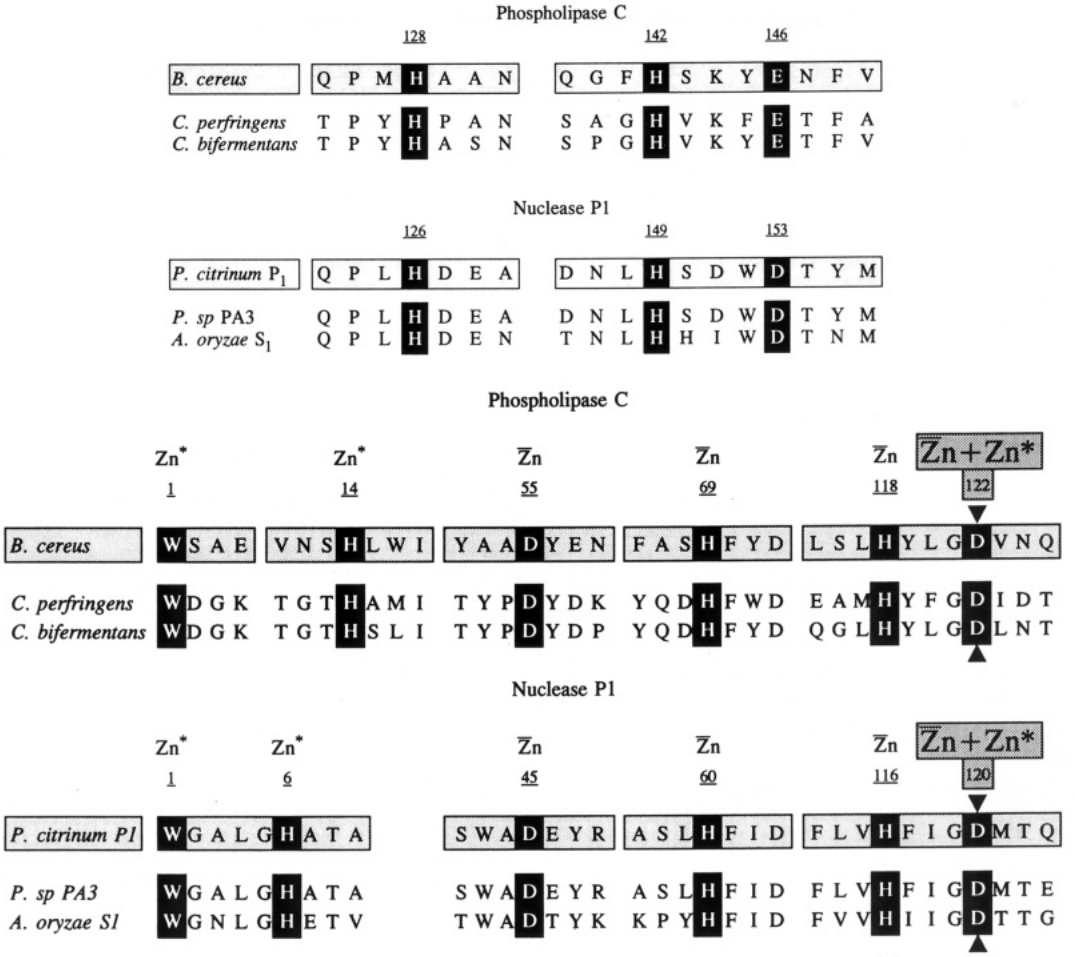

FIGURE 5: (A, top) Ligands to the catalytic Zn sites of the phospholipase C and nuclease P1 families. See caption of Figure 2 for key to figure. The corresponding residue positions of the ligands for the *Clostridium* phospholipases are 136, 148, and 152. In the case of nuclease S1 the positions are 125, 148, and 152. (B, bottom) Sequences abutting the ligands to the **Zn*** and $\bar{\mathbf{Z}}\mathbf{n}$ sites of the phospholipase C and nuclease P1 enzymes. The columns indicated by black arrows (▲ ▼) represent the aspartate bridge. The corresponding residue positions of the ligands for the *Clostridium* phospholipases are 1, 11, 56, 68, 126, and 130. For nuclease S1, they differ from the nuclease P1 only in the last two, 115 and 119.

(Vallee & Auld, 1990a,b). A short spacer of 3 amino acids separates His-149 and Asp-153, a long spacer of 12 amino acids separates His-126 and His-149, and 2 water molecules bind to the zinc atom. Both His-149 and Asp-153 are part of an α-helix, which has turned out to be a frequent feature of such sites (Vallee & Auld, 1992a).

The di-zinc site of nuclease P1 comprise both a bridging amino acid, Asp-120, and a bridging water molecule (Figure 5B, Table I). All but one of the amino acid residues that supply the ligands to the **Zn*** and $\bar{\mathbf{Z}}\mathbf{n}$ sites, Trp-1, His-6, His-45, Asp-60, His-116, and Asp-120, arise from four different α-helical regions of the protein (Figure 5B). Since the X-ray structure of the apoenzyme has not been determined as yet, the extent to which **Zn*** and/or $\bar{\mathbf{Z}}\mathbf{n}$ and the α-helices mutually affect one another is still unknown. However, if this zinc atoms were to promote the formation of helices in specific regions throughout the 120 amino acid segment, then the removal of these atoms could cause the large change in CD spectrum between 200 and 240 nm that has been reported (Fujimoto et al., 1975b).

Phosphate binds in the center of the three zinc atoms (Volbeda et al., 1991). Since phosphate is the product of monoesterase hydrolysis, all three metal atoms could be involved in the 3′-nucleotidase reaction catalyzed by these enzymes much as is observed for alkaline phosphatase.

In addition to the primary structure of nuclease P1, those of nucleases PA3 and S1 are also known (Figure 5A). Nucleases P1 and PA3 differ by only one amino acid, Thr/Ile 190; the sequence of nuclease S1 is about 50% identical with both. All three zinc binding sites in nuclease P1 are highly conserved in both the S1 and PA3 enzymes (Figure 5).

(*D*) *Structural Similarities between the Nuclease P1 and Phospholipase C Zinc Binding Sites.* Inspection of the spacing between zinc ligands and their adjoining sequences in the nuclease P1 and phospholipase C enzyme families also reveals some remarkable identities (Figure 5A). In the **catalytic Zn** site the short spacers consist of 3 amino acids while the long spacers are from 11 to 13 amino acids. In both families a Pro is in the N2 locus of the third ligand, and either Gln or Asn is generally in the N3 and C3 positions. Hydrophobic amino acids are usually found at the N1 locus of all three ligands. The replacement of an Asp for a Glu ligand in the short spacer region of the phospholipase C family is a significant difference.

The strong similarities extend to the site of the amino acid bridge (Figure 5B). In both instances Trp-1 and a histidine, separated by 4–12 amino acids, are ligands to **Zn***. The C_1 to C_3 positions of His-69 in phospholipase C and His-60 in nuclease P1 are nearly identical. In both families an Asp (either 122 or 120) is the bridging ligand between the **Zn*** and $\bar{\mathbf{Z}}\mathbf{n}$. As in all 20 members of the alkaline phosphatase family, a Gly abuts the bridging Asp in all these enzymes (Figures 4 and 6B).

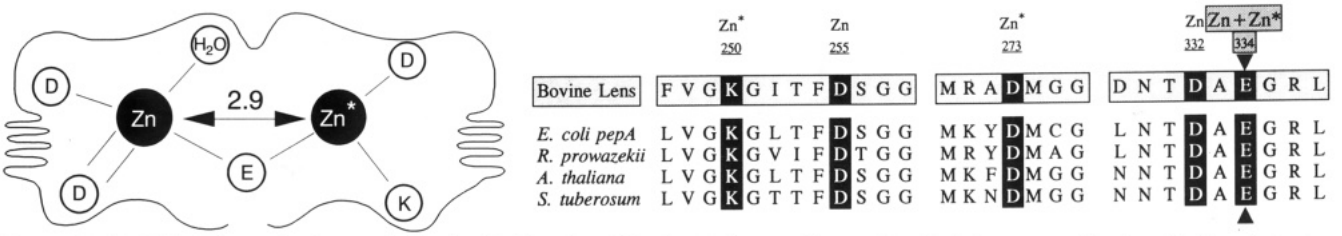

FIGURE 6: (A, left) Schematic of cocatalytic zinc binding site of leucine aminopeptidase. (B, right) Sequences abutting the ligands to the coactive zinc site of aminopeptidases. See caption of Figure 2 for key to figure. The column indicated by black arrows (▲ ▼) represents the glutamate bridge. References: (1) Cuypers et al., 1982; (2) Stirling et al., 1989; (3) D. O. Wood, R. R. Speed, and M. Solomon, translated from GenBank, 1991; (4) Bartling & Weiler, 1992; (5) Hildmann et al., 1992. The corresponding residue positions for the other aminopeptidases are *E. coli*, 270, 275, 293, 352, and 354; *R. prowazekii*, 265, 270, 288, 347, and 349; *A. thaliana*, 288, 293, 313, 373, and 375; and *S. tuberosum*, 323, 328, 348, 408, and 410.

(*E*) *Two Metal Atom Cocatalytic Site.* Thus far, leucine aminopeptidase of bovine lens is the only two-zinc enzyme whose cocatalytic motif has been identified structurally,[3] though yet additional, seemingly similar members of this family have been isolated from other sources.

"*Leucine*" *aminopeptidases* (EC 3.4.11.1) catalyze the hydrolysis of amino-terminal peptide bonds and are presumed to be involved in the processing and turnover of intracellular proteins. The enzyme from bovine lens (LAP) has been examined most extensively. The hexamer contains 2 mol of zinc per subunit of MW 54 000 (Carpenter & Vahl, 1973). The zinc in site 1 exchanges rapidly while that in site 2 exchanges slowly. LAP is active only when zinc or cobalt occupies site 2, which has been named the "specificity site", in contrast to site 1, which is the "activation site" (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b). Zinc fully restores activity to the apoenzyme, but copper, calcium, magnesium, and manganese do not, nor do they bind stoichiometrically. For the sake of uniformity we here refer to site 2 as "**catalytic Zn**" and site 1 as **Zn***, in accord with our terminology.

When a zinc atom binds to the **catalytic Zn** site of LAP, zinc, magnesium, or cobalt can then bind to the **Zn*** site also, but zinc is much preferred as is seen from the relative dissociation constants at that site; i.e., ${}^1K_{Zn}/{}^1K_{Mg}$ is 20 700 at pH 8.16 and ${}^1K_{Zn}/{}^1K_{Co}$ is 115 at pH 7.5 (Thompson & Carpenter, 1976a,b). Quite the same, catalytic efficiencies toward leucine *p*-nitroanilide and leucine amide are increased in derivatives where magnesium or cobalt replaces zinc, as reflected in the k_{cat} and k_{cat}/K_m values (MgZn > CoZn > ZnZn) (Allen et al., 1983).

Porcine kidney leucine aminopeptidase, also a hexamer, contains 1 mol of zinc per subunit of MW 53 000 (Van Wart & Lin, 1981). Incubation of this enzyme with either magnesium or manganese yields an enzyme containing 1 mol of Mg or Mn *in addition to 1 mol of Zn per subunit*; the product is 10-fold more active in hydrolyzing leucine *p*-nitroanilide. However, there are no data demonstrating a second zinc atom (and/or Mg or Mn) in the native enzyme. Copper and nickel also bind stoichiometrically to this Mg/Mn site, but the catalytic activities of those species are reduced by about 50%.

There also are two nonidentical but interacting metal binding sites in *Aeromonas* aminopeptidase (Prescott et al., 1985) which contains 1.6 mol of zinc/mol of a MW 30 000 protein (Prescott et al., 1971). Addition of zinc, cobalt, copper, and nickel to the apoenzyme all significantly increase the activities of the resultant metalloenzyme whose magnitude(s) depend-(s)on the sequence and order of addition. When copper is added prior to zinc, activity increases 21 times more than when the reverse order is followed. More remarkable yet, *both* these enzymes are from *3* to *59* times more active than the native one. In the absence and presence of 1-butylboronic acid, a transition-state analog inhibitor, cobalt has been assigned to a tetrahedral-like site.

The compositional, functional, and structural data available for the multi-zinc leucine aminopeptidase family are incomplete. The lens, kidney, and *Aeromonas* enzymes all contain at least one zinc atom required for activity, but the bovine lens enzyme is the only one known to contain two zinc atoms. The other two additionally bind various metal atoms. These have marked and different effects on catalytic activities and their magnitudes, both as a function of the identity and sequence of addition of metal(s). In the absence of three-dimensional structures for all three enzymes valid comparisons cannot be made at this time. It is quite possible that the identity of ligands at the zinc binding site, their three-dimensional arrangement, and motifs may differ. Hence, an assignment of these enzymes to a common, single family could be premature.

The three-dimensional structure of the 487 amino acid bovine lens enzyme has been determined in the absence and presence of the slow binding inhibitor bestatin (Burley et al., 1992). The 327 amino acid C-terminal domain supplies the ligands for the two zinc binding sites which are within 2.9 Å of each other (Figure 6A). The hexamer is formed from an upper and a lower trimer, in each of which the cocatalytic zinc sites are both at the center of this complex and at the apices of roughly equilateral triangles. Both α-helix and β-sheet structures supply the ligands to the cocatalytic zinc site which resides within a relatively short primary sequence between residues 250 and 334 (Table I).

The **catalytic Zn** site exchanges metals more slowly than **Zn***, which led to the assignment of the **catalytic Zn** as the one with the lower crystallographic temperature factor (Table I, Figure 6A) (Burley et al., 1992). A single amino acid separates Asp-332 and the bridging Glu-334, each of which is coordinated to the **catalytic Zn** through one of their carboxylate oxygens. The other two catalytic zinc ligands are the carbonyl oxygen to Asp-332 and one Oδ of Asp-255, which are separated by a 76 amino acid long spacer (Table I). Further, a weakly bound H_2O molecule at 3.2 Å and **Zn*** at 2.91 Å are also believed to be involved in the **catalytic Zn** coordination site.

One of the carboxylate oxygens of Asp-273 and one from the bridging carboxylate of Glu-334 as well as the side-chain

[3] Zinc and magnesium have also been implicated in the 3–5-exonuclease activity of *E. coli* DNA polymerase I (Beese & Steitz, 1991; Derbyshire et al., 1991). The Cu/Zn superoxide dismutases from bovine (Tainer et al., 1982; Djinovic et al., 1992a), human (Parge et al., 1992), spinach (Kitagawa et al., 1991), and yeast (Djinovic et al., 1992b) all contain a bridging metal ligand, His-61. Structurally it resembles Asp and Glu in binding two metal atoms, but since Cu clearly is the catalytic metal in superoxide dismutase (Bannister et al., 1987), this enzyme is not discussed here.

primary amine of Lys-250 are coordinated to **Zn*** and thought to constitute the activation site. Neither a H_2O molecule nor other anions are found near the metal. The **catalytic Zn** is presumed to complete the coordination to this site, though the manner in which it would participate has not been detailed (Burley et al., 1992). The α-amino and hydroxyl groups of bestatin bind to **Zn*** of lens aminopeptidase. Further, the amino group is also hydrogen bonded to the carboxylate oxygen of Asp-273 which is not bound to **Zn***. Thus, in this enzyme·inhibitor complex **Zn*** becomes five-coordinate.

The cocatalytic zinc site for LAP differs markedly both from that of the mono-zinc aminopeptidases and from that of LTA_4 hydrolase. Two histidines separated by a 3 amino acid short spacer, a glutamate separated by an 18 amino acid long spacer, and an H_2O molecule are the ligands to the **catalytic Zn** of these enzymes (Vallee & Auld, 1990a).

A number of aminopeptidases in the evolutionary tree are homologous to the bovine lens enzyme. As in the case of LTA_4 hydrolase some of these enzymes have been described to have metabolic functions, quite different from that of peptidases. Their homology to the bovine lens enzyme led to the recognition of their aminopeptidase activity. Thus, *xerB*, an *E. coli* gene, is required for the stability of the plasmid ColE1 and dissociates unstable plasmid multimers into their monomeric form (Summers & Sherratt, 1984). Remarkably, the C-terminal region of the XerB protein sequence (residues 230–470) is 52% identical to that for the analogous sequence of the bovine lens enzyme (residues 210–451) (Cuypers et al., 1982), while the overall sequence identity is 31% (Stirling et al., 1989). Moreover, this gene appears to be the *E. coli* counterpart of the *pepA* gene in *Salmonella typhimurium* that encodes aminopeptidase A. In fact, the protein encoded by *xerB* has aminopeptidase activity toward leucine *p*-nitroanilide; manganese activates and EDTA inhibits it.

Manganese and magnesium activate whereas 1,10-phenanthroline and EDTA inhibit an aminopeptidase from *Arabidopsis thaliana* (Bartling & Weiler, 1992). Their C-terminal regions reveal 45% sequence identity with the bovine enzyme. Finally, the sequences for a purported aminopeptidase A from *Rickettsia prowazekii* (D. O. Wood, R. R. Speed, and M. Solomon, translated from GenBank, 1991) and that from *Solanum tuberosum* (Hildmann et al., 1992) similarly are 47% and 46% identical respectively to the corresponding region of the bovine enzyme. The cocatalytic zinc binding sites of all these enzymes are highly conserved on both the N3 and C3 sides of the proposed ligands (Figure 6B). A Gly and an Ala are conserved at the C1 and N1 positions of Glu-334, the bridging amino acid.

The discovery of additional lens-like aminopeptidases and the availability of their sequences foreshadow much additional information to be expected in the near future, considering the recent developments in the zinc motifs of proteins.

Chemical Features of Cocatalytic Zinc Sites

Cocatalytic sites of multi-zinc enzymes reveal a new structural role for aspartic and glutamic acids. The two electronegative oxygens of their carboxylate groups serve as zinc–zinc or zinc–magnesium bridges in enzymes with cocatalytic zinc sites. Alteration in the bond lengths or dissociation of either or both of these atoms from their respective metals during substrate interaction with the enzyme or formation of intermediates could profoundly affect catalysis due to the change in charge imposed upon the metals. In this manner these residues become pivotal components of the resultant functional template which consists of a network of closely spaced metal atoms and their ligands.

Leaving aside this motif, there are yet other features that set the coordination of their cocatalytic components (**Zn*** and $\bar{\mathbf{Z}}\mathbf{n}$/Mg) apart from that of **catalytic Zn** sites. In the multi-zinc enzymes here considered, Ser, Thr, Lys, and Trp turn out to be ligands to **Zn*** or $\bar{\mathbf{Z}}\mathbf{n}$/Mg sites. While His and Cys predominate as ligands of catalytic and structural zinc atoms, respectively (Vallee & Auld, 1990b, 1992a), Asp predominates in cocatalytic zinc sites where the binding frequency is Asp > His ≫ Glu.

Coordinated H_2O molecules are abundant throughout the three metal coactive sites. One or more of these is coordinated to the **catalytic Zn** atoms, **Zn***, and $\bar{\mathbf{Z}}\mathbf{n}$/Mg sites (Figures 2 and 5). In fact, in two of these instances H_2O bridges the **Zn*** and $\bar{\mathbf{Z}}\mathbf{n}$ atoms, implying its significant role in such cocatalytic templates. Thus, substrate binding might displace this H_2O from one or both of the sites during catalysis. If H_2O remains in place or is converted to OH^- during this step of the reaction, it could subsequently serve as a nucleophile.

The existence of five-coordinate zinc is well established in both model systems and at zinc active sites, particularly in substrate intermediates and transition states during catalysis (Vallee & Galdes, 1984; Auld & Vallee, 1987; Vallee & Auld, 1990a). The structural identification of H_2O in these zinc coordination sites suggests the formation of pentacoordinate substrate intermediates involving the displacement of these water molecules. It is already known that in all the phosphate ester hydrolyzing enzymes the phosphate product interacts with all three metal atoms. Such participation of all metal atoms in substrate interactions clearly makes these sites cocatalytic.

The ionizable groups of Ser, Thr, and Lys have high pK_a values. Hence, at neutrality their affinity for binding zinc is relatively lower than that of the nitrogen, oxygen, and sulfur donor groups of catalytic—or other—sites. Moreover, the amide carbonyl ligands should also coordinate zinc weakly. Thus, when substrate enters the coordination sphere, ligand displacement reactions at the moment of catalysis would be plausible and consistent with a *cocatalytic* mechanism. In the absence of substrate the coordination numbers of mono-zinc and those of cocatalytic zinc enzymes would seem to be different, i.e., tetra- versus penta- and hexacoordinate, respectively.

All of the above properties are likely to be important for a group of metal atoms that are in close proximity and which act in concert to bring about catalysis. Thus, individual metal atoms and their associated ligands may have different roles in the different steps of catalysis. Thus, for the phosphate ester hydrolyzing enzymes interaction of the substrate with their cocatalytic zinc site may involve initial contact with all three metals, as is observed in their final phosphate product complex. One of the zinc atoms may then polarize a nucleophile, e.g., either water or Ser-102 in the case of alkaline phosphatase, to attack an amide or a phosphoester bond. A second zinc atom could act transiently as the receptor for the alcohol or amine leaving group. In subsequent steps the role of these zinc atoms may be reversed. The second zinc atom might activate H_2O to hydrolyze an acyl intermediate formed, while the first zinc atom may polarize a phosphooxygen or carbonyl bond for the attack of water promoted by the second zinc atom. The bridging Asp and H_2O ligands could have critical roles in this process. Thus, their dissociation from either or both metal atoms during catalysis will change the charge on the metal, promoting the metal's action as a Lewis acid or allowing interaction with an electronegative atom of the substrate. Alternatively, the bridging ligand may par-

ticipate transiently in the reaction as a nucleophile or general acid/base catalyst. In this manner the metal atoms and their associated ligands would play specific roles in each step of the reaction that works in concert to bring about catalysis.

It is apparent that the formation of cocatalytic sites in zinc metalloenzymes likely reflects sophistication of catalytic mechanisms brought about by innovations in chelate chemistry, structural design of coordinating sites, and modification/modulation of mechanistic arrangements. It would seem to represent an unusual biological utilization of zinc chemistry directed toward different ends of biological needs. It will be of particular interest to examine the structure/function relationships of small peptides that incorporate the essential features of these sites. The discernment of the structure/function relationships in *cocatalytic* or *coactive* sites likely heralds yet other motifs to be discerned.

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