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Predicting the Zn(II) Ligands in Metalloproteins: Case Study, Porphobilinogen Synthase

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Predicting the Zn(II) Ligands in Metalloproteins: Case Study, Porphobilinogen Synthase

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Mammalian porphobilinogen synthase (PBGS) is a Zn(II) metalloenzyme containing both catalytic and non-catalytic Zn(II). There are sufficient physical and chemical data to establish the types of amino acid which serve as ligands to each of the metal ions. Chemical modification data allow specific sequence assignment of some of the Zn(II) ligands. This Comment attempts the prediction of the remaining amino acids which act as Zn(II) ligands. The predictions are based on published Extended X-ray Absorption Fine Structure data, the sequences of PBGS from eleven different species, and an analysis of twenty-four known X-ray and NMR determined structures for proteins which contain Zn(II).

The analysis of the known structures follows the precedent set by B. L. Vallee and D. S. Auld (Biochemistry **29**, 5647–5659 (1990)), with the current dataset being ca. 50% larger. Non-catalytic Zn(II) sites are all found to contain a tetrahedral coordination of at least two, and often four, cysteine ligands. In all cases, two of the cysteines are separated by fewer than four intervening amino acids. All six of the structures analyzed show the non-catalytic Zn(II) ligands to be derived from a continuous sequence of up to 35 amino acids. Contrary to the original conclusions of Vallee and Auld, the nineteen catalytic Zn(II) are found to be both tetrahedral and pentacoordinate, and need not contain water as a ligand. The most common catalytic Zn(II) ligand is histidine, and the ligand preference series appears to be H > E = D > C = Y = K = backbone carbonyl. In all cases, except alcohol dehydrogenase, two of the catalytic Zn(II) ligands are separated by no more than three intervening amino acids.

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On the basis of these analyses, the amino acid side chains predicted to coordinate the non-catalytic Zn(II) of mammalian PBGS are Cys119, Cys122, Cys124, and Cys132. The amino acid side chains predicted to coordinate the catalytic Zn(II) arise from Asp220, Cys223, Tyr224, His131 and the substrate or product. Because each homo-octameric PBGS contains only four catalytic Zn(II), the four amino-acid-derived ligands need not all be on the same subunit.

PBGS from other phyla appear to use Mg(II) in addition to or in place of the two different Zn(II) of mammalian PBGS. With this in mind, a comparison of the eleven known PBGS sequences reveals three potential divalent metal ion binding sites and suggests an intriguing evolution of this protein.

Key Words: *porphobilinogen synthase, zinc ligands, Zn(II)-metalloproteins, structure predictions*

INTRODUCTION

There are numerous roles for zinc in the structure and function of proteins. Each of the six classes of enzymes contains examples of Zn(II) metalloproteins. Zn(II) is also essential to the structure of many hormones, a variety of nucleic acid binding proteins and countless structural proteins. As we refine our analytical tools, the ubiquitous role of Zn(II) in protein structure and function is becoming ever more apparent. However, detailed molecular structures of Zn(II) metalloproteins deduced from X-ray crystallography or NMR spectroscopy are still relatively few in number. Nevertheless, protein sequence information is often available, and one would like to be able to use sequence as a tool for identifying Zn(II) ligands.

In a landmark publication, Vallee and Auld (1990) analyzed the X-ray crystal structures of eleven enzymes containing a catalytic Zn(II) and two enzymes containing a non-catalytic Zn(II).[1] These structures were used to devise preliminary "rules" to help determine the amino acids that are most likely to serve as Zn(II) ligands. In this report, an expanded Zn(II)-metalloprotein structural database is analyzed in an attempt refine the rules which govern Zn(II) ligation and apply these rules to the prediction of the Zn(II) ligands of the Zn(II) metalloprotein porphobilinogen synthase (PBGS). Following the lead of Vallee and Auld, this analysis is limited to those proteins whose Zn(II) binding sites have been definitively established using X-ray crystallography or NMR spectroscopy. The current data set contains eighteen X-ray crystal

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structures of enzymes with one or more catalytic Zn(II) and six X-ray or NMR determined structures for proteins with non-catalytic Zn(II). A catalytic Zn(II) is defined as a Zn(II) which is directly involved in catalysis. For the purpose of this analysis, all others are called non-catalytic Zn(II). This paper first presents the known data regarding Zn(II) and PBGS, then updates the "rules" of Vallee and Auld, and applies these updated rules to predicting the Zn(II) ligands of PBGS.

PORPHOBILINOGEN SYNTHASE (PBGS)

PBGS (also known as 5-aminolevulinate dehydratase) catalyzes the first common step in tetrapyrrole biosynthesis (see Fig. 1). It catalyzes a complex chemical reaction and plays a central role in the respiration of all organisms. In this paper, the fundamental catalytic mechanism of PBGS is posited to be the same for all organisms; substantial sequence data supports this proposition.[2-11] Depending on the species, the substrate aminolevulinic acid (ALA) can be synthesized from the five carbons of glutamic acid and/or from succinyl-CoA and glycine.[12-16] In all organisms, PBGS condenses two molecules of ALA asymmetrically to form the monopyrrole porphobilinogen (PBG) with the concomitant production of two water molecules.[17] The chemistry of the PBGS catalyzed reaction is illustrated in Fig. 2 and described in the legend.[18-23] PBG serves as both substrate and cofactor for the enzyme porphobilinogen deaminase which synthesizes the linear tetrapyrrole hydroxymethylbilane.[24] The formation of uroporphyrinogen III is the final step of the common pathway. A myriad of enzymes transform the cyclic tetrapyrrole into the porphyrins (e.g., heme and cytochromes), corrins (e.g., vitamin B_{12}), chlorins (e.g., chlorophylls) and other cofactors (e.g., F_{430}, phycobilins).[25,26]

KNOWN METAL ION INTERACTIONS WITH PBGS FROM VARIOUS PHYLA

PBGS, as isolated from mammalian tissues, yeast, and *E. coli* is a Zn(II) containing protein for which caution must be taken to

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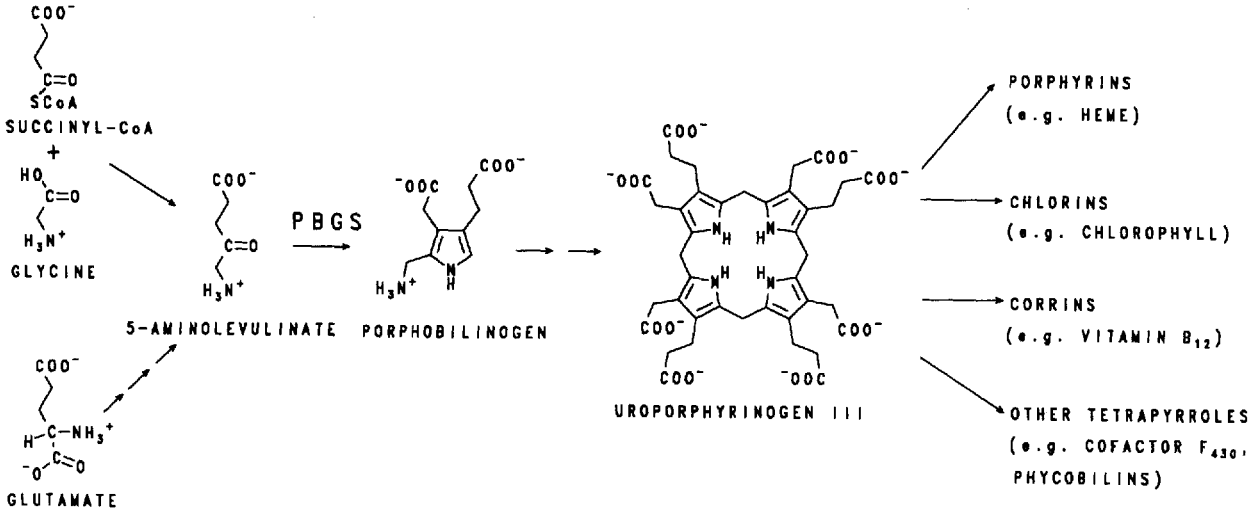

FIGURE 1 PBGS is the first common step in the biosynthesis of the tetrapyrroles (see text).

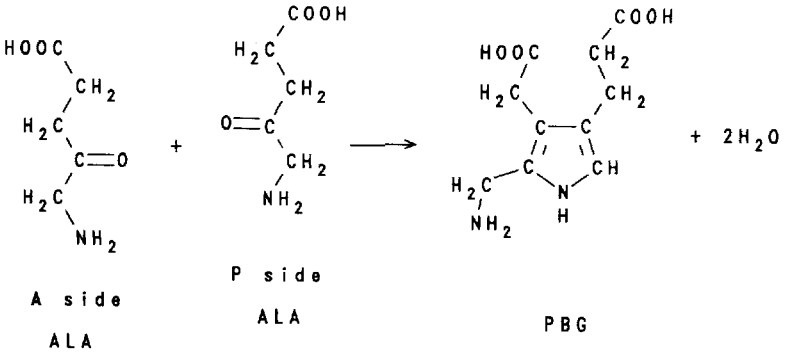

FIGURE 2 PBGS catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) (Ref. 17). P-side ALA, which forms the propionyl containing half of PBG, binds first and forms a Schiff base intermediate to Lys252 (Refs. 18–21). Zn(II) is required for the binding of A-side ALA which forms the acetyl-containing half of PBG (Refs. 22 and 23). The sequence of chemical events which follows the formation of the ternary ES_2 complex, as well as the catalytic role(s) of Zn(II), remains a topic for speculation.

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prevent Zn(II) depletion during purification.[27–32] In these organisms, it has been shown that Zn(II) is *required* for PBGS activity. The bulk of our detailed information on the interactions of Zn(II) with PBGS comes from the study of bovine PBGS, an octameric protein which contains four catalytic Zn(II) and four non-catalytic Zn(II).[23,33] The >95% sequence identity between bovine, mouse, rat, and human PBGS indicates that our detailed knowledge of bovine PBGS may be extrapolated to all mammalian PBGS.[2,4,5,23,34,35]

Unlike most Zn(II)-metalloenzymes, PBGS is sensitive to inhibition by micromolar concentrations of Pb(II). Lead inhibition of PBGS contributes to the neurotoxicity of lead, particularly devastating to the neurologic development of children.[36] The structural similarity between the accumulated substrate ALA and the neurotransmitter gamma amino butyric acid is believed to be one reason for the severe neurotoxicity of low levels of Pb(II).[37] We and others have shown that Pb(II) inhibits PBGS through direct replacement of the catalytic Zn(II).[30,38] In a recent review,[39] Jordan cited unpublished work indicating that complete Pb(II) inhibition of PBGS occurs with only half the enzyme-bound Zn being

displaced, a result consistent with the substantial data indicating two types of Zn(II) in PBGS. The inhibition of plant PBGS by Pb(II) suggests that this PBGS is also a Zn(II) metalloenzyme,[40,41] though this remains to be proven.

The early literature is checkered with conflicting data on the metalloprotein nature of PBGS.[42] Several reports suggest that PBGS from non-mammalian species are responsive to monovalent cations or Mg(II).[43,44] Plant PBGS is generally believed to use Mg(II) in place of the Zn(II) of mammalian PBGS, although the published data show a *stimulation* by Mg(II) rather than an absolute requirement for the metal.[41] We have recently established that *E. coli* PBGS *requires* Zn(II) for catalytic activity and is further *stimulated* by the addition of Mg(II), thus indicating that the roles of Zn(II) and Mg(II) are not interchangeable.[29] It would be appropriate to test whether the plane enzyme actually behaves similarly to *E. coli* PBGS. It is now widely accepted that mammalian PBGS is a Zn(II)-metalloenzyme and unresponsive to Mg(II).[29,41,45] Although it is clearly established that Zn(II) is absolutely required for mammalian PBGS activity,[27,32,46] the bulk of the literature supports only a structural role for the required Zn(II). Even the most recent reviews on PBGS fail to incorporate a role for Zn(II) in the proposed catalytic reaction mechanisms.[39,47] However, we have presented evidence that Zn(II) plays a role in substrate/product binding as well as catalysis.[22,23] This article focuses primarily on the identification of the Zn(II) ligands of mammalian PBGS, but it also addresses the Zn(II) and Mg(II) of other PBGS.

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WHAT IS APPARENTLY KNOWN ABOUT THE CATALYTIC AND NON-CATALYTIC Zn(II) OF MAMMALIAN PBGS?

PBGS is a homo-octamer of 35 kDa subunits. When purified in the presence of 10 μM Zn(II), mammalian PBGS contains eight Zn(II) per octamer.[27,30,46] In the absence of Zn(II), the protein is catalytically inactive, but its quaternary structure remains intact.[27] Each octamer requires four catalytic Zn(II) for maximum activity.[27,46] The K_d for the catalytic Zn(II) is much less than 0.1 μM (see Fig. 3) and too tight to quantify by atomic absorption spec-

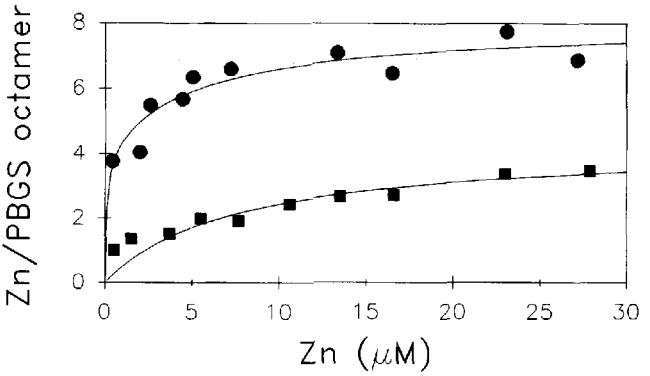

FIGURE 3 Equilibrium dialysis depicting two distinct classes of Zn(II) binding sites on mammalian PBGS (•). The tighter site is the catalytic Zn(II) site, K_d < 0.1 μM. The looser site is called the non-catalytic Zn(II) site, K_d = 5 μM. When Cys223 is modified by 5-chlorolevulinic acid, only the looser binding site remains (■), thus identifying Cys223 as a ligand to the catalytic Zn(II) (Ref. 23). The binding data were determined by atomic absorption spectroscopy (Ref. 23).

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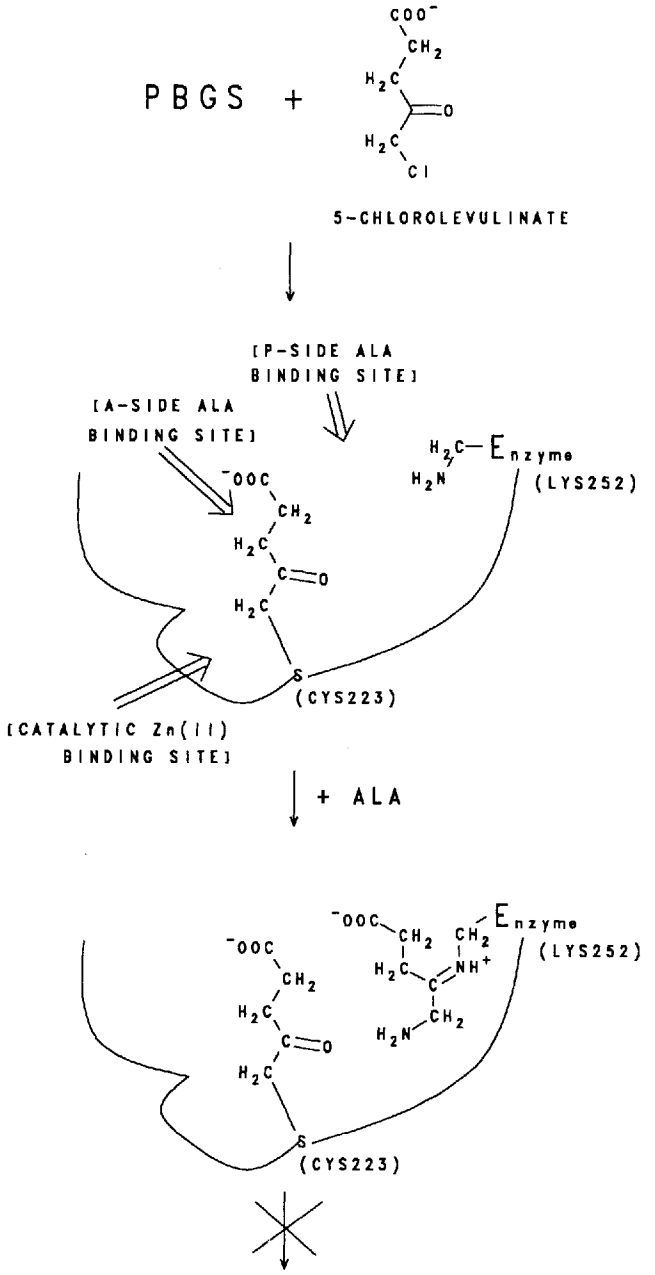
troscopy.[23] The Extended X-ray Absorption Fine Structure (EXAFS) analysis of Dent *et al.* (1990) on the Zn_4:PBGS complex suggests that each catalytic Zn(II) contains five ligands, only one of which is a cysteine; the other ligands are one or more histidines, at least one tyrosine, and one aspartic acid or a water molecule.[33]

Each octamer of PBGS also contains in addition four other Zn(II) which, for lack of a better name, are called the non-catalytic Zn(II). The K_d for the non-catalytic Zn(II) is 5 μM (see Fig. 3).[23] The difference EXAFS data (Zn_8:PBGS–Zn_4:PBGS) strongly suggest that the structural Zn(II) are each coordinated to four cysteines.[33] It is interesting and perhaps perplexing to note that the pentacoordinate Zn(II) with only one sulfur ligand is bound at least two orders of magnitude more tightly than the tetrahedrally coordinated Zn(II) with four sulfur ligands. Mammalian PBGS does not require the non-catalytic Zn(II) for maximal catalytic activity in the presence of 2-mercaptoethanol. However, in the absence of 2-mercaptoethanol and exogenous Zn(II), PBGS is prone to disulfide formation, Zn(II) loss, and a concomitant reversible inactivation. It has been suggested that one role for Zn(II) in PBGS is to prevent disulfide formation between catalytically essential SH groups,[30] and this may be the role of the non-catalytic



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Zn(II). A Zn(II) filling this role is not directly involved in catalysis, but is nevertheless implied to be near the active site.

Early in our investigations of PBGS, we found that modification with methyl methanethiosulfonate (MMTS) at a stoichiometry of three per subunit (24 per octamer, 3/8 of the total cysteines) caused the enzyme to lose all of the intrinsic Zn(II), thus implicating cysteines in binding all the Zn(II) of PBGS.[27] The EXAFS study of Dent *et al.* (1990) confirmed this conclusion and suggested that one and four cysteines were involved in binding the catalytic Zn(II) and non-catalytic Zn(II), respectively. Identification of specific cysteine(s) involved in binding particular metal ions derives predominantly from chemical modification studies, some of which are described below.[23,27,35,49]

The reactive substrate analog 5-chlorolevulinic acid (5-CLA) modifies bovine PBGS at the binding site for A-side ALA (see Figs. 2 and 4).[23] 5-CLA specifically modifies Cys223 on one-half the subunits and this modification removes the catalytic Zn(II) binding site (see Fig. 3). Modification of Cys223 at a stoichiometry of four per octamer displaces the four catalytic Zn(II) and implicates Cys223 as a catalytic Zn(II) ligand. Consistent with the EXAFS data,[33] Cys223 is the sole cysteine ligand to the catalytic Zn(II). The 5-CLA modification data also suggest that the catalytic Zn(II) is very near A-side ALA and we have presented a model where A-side ALA is a bidentate ligand to the catalytic Zn(II) (see Fig. 5).[23] When P-side ALA is bound to 5-CLA modified PBGS, it binds as the Schiff base and no further reaction is observed (see Fig. 4).[23] Because the 5-CLA modified PBGS:P-side Schiff base complex contains all the atoms of substrate necessary to proceed to product but is lacking the catalytic Zn(II), one conclusion is that Zn(II) is necessary for formation of the first intersubstrate bond.

Several reviews have cited the ^{113}Cd NMR data of Sommer and Beyersmann[31] as indicating no interaction between substrate and

FIGURE 4 5-Chlorolevulinate modifies bovine PBGS at the binding site for A-side ALA (Ref. 23). This is contrary to the conclusions of an earlier work (Ref. 49). When ALA is added it binds as the P-side Schiff base and no further reaction occurs (Ref. 23). The stereochemistry and protonation states of the P-side Schiff base were determined using ^{13}C and ^{15}N NMR (Refs. 50–52).

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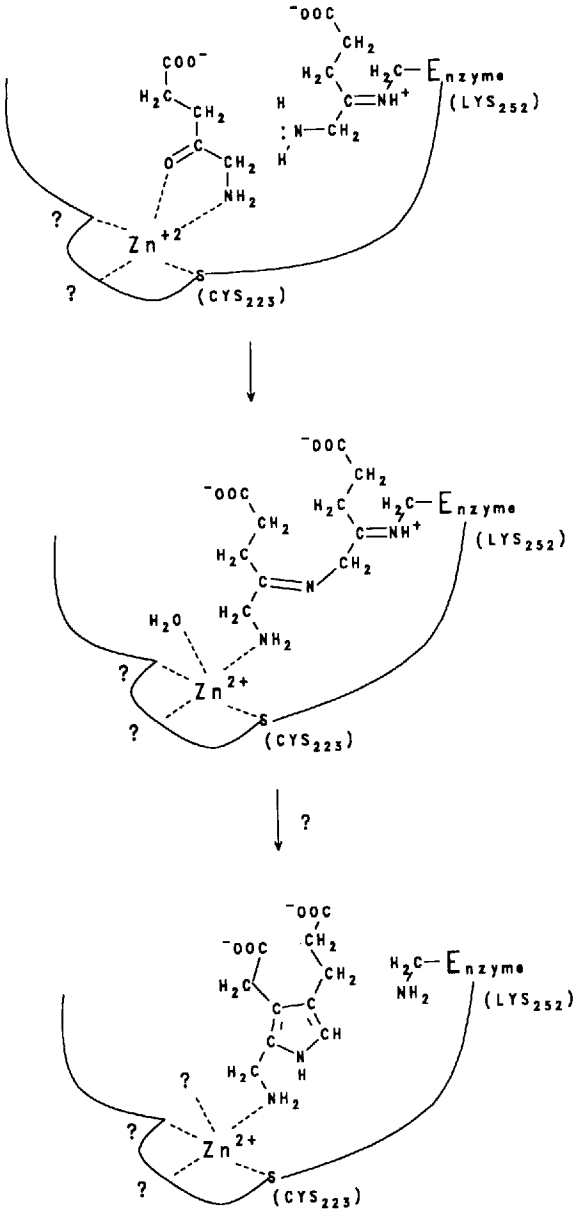

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metal.[39,47] In these cases the reviewer was unaware of a later work by Beyersmann's group which reported that the observed ^{113}Cd spectrum was derived from ^{113}Cd:EDTA, rather than from ^{113}Cd:PBGS.[53] A variety of other attempts to observe ^{113}Cd NMR spectra of ^{113}Cd:PBGS have failed, presumably limited by ligand exchange.[27,50] However, two additional lines of evidence support substrate and product serving as Zn(II) ligands. First, our ^{13}C and ^{15}N NMR data suggest that Zn(II) is bound to the amino group of the product PBG.[52] This amino group derives from A-side ALA. Second, MMTS-modified PBGS, which is free of Zn(II), cannot bind A-side ALA.[50] On the basis of these data, we have proposed a mechanism in which Zn(II) is essential to binding both A-side ALA and product and to the chemistry of intersubstrate bond formation (see Fig. 5).[23]

PBGS has also been modified with a spin label derivative of MMTS (SL-MMTS) at a stoichiometry of three per subunit.[35] Like MMTS modification, SL-MMTS modification removes both the catalytic and non-catalytic Zn(II) binding sites. The modified residues are Cys223, Cys119, and a third as yet unidentified cysteine.[35] Because Cys223 is already identified as the cysteine ligand to the catalytic Zn(II), this result implicates Cys119 and/or the unidentified cysteine as a ligand to the non-catalytic Zn(II). We have proposed that this unidentified cysteine is likely near Cys119 in the primary structure of the protein for the following reason. When SL-MMTS modifies PBGS cysteines, the spin labels are attached through stable disulfide linkages. Denaturing SL-MMTS-modified PBGS results in a disulfide exchange reaction leading to loss of spin label from Cys119 and the third cysteine. This disulfide exchange reaction also results in two spin labels becoming disulfide-linked to each other and magnetically coupled (not enzyme-bound).[35]

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FIGURE 5 The proposed role of the catalytic Zn(II) of PBGS is to bind A-side ALA and facilitate the formation of the first bond between the two ALA molecules (Ref. 23). It has been proposed that this first bond is in the form of a second Schiff base between the C_4 of A-side ALA and the nitrogen of P-side ALA as shown. Alternatively, the first bond between the two ALA molecules may be between C_3 of A-side ALA and C_4 of the P-side Schiff base (not shown). The enzyme-product complex is proposed to involve direct coordination of the catalytic Zn(II) to the amino group of PBG (Ref. 52).

IDENTIFYING THE LIGANDS TO THE NONCATALYTIC Zn(II) OF MAMMALIAN PBGS

The SL-MMTS modification data led us to examine the sequences near Cys119 to identify potential non-catalytic Zn(II) ligands. Sequences are available for six PBGS enzymes which have been *proven* to contain or require Zn(II): bovine, human, rat, mouse, yeast, and *E. coli* PBGS.[2-6,23,27-29,34,35] Quantitative Zn(II) binding curves have been published only for bovine PBGS (see Fig. 3).[23,31] On the basis of the >95% sequence identity of all known mammalian PBGS sequences, the existence of two types of Zn(II) is conservatively extrapolated to all the mammalian PBGS. While yeast and *E. coli* PBGS contain at least a catalytic Zn(II), the existence of a non-catalytic Zn(II) binding site in yeast and *E. coli* PBGS is currently conjecture.

Figure 6A depicts the sequence near Cys119 as a cysteine- and histidine-rich region in six PBGS sequences. On the basis of sequence alone, this region was first cited by Wetmur *et al.* (1986) as *the* "zinc binding site" of mammalian PBGS.[2] Despite the fact that the literature already indicated the existence of two kinds of Zn(II) binding sites on PBGS,[27,47] the proposed $CX_{2-4}CX_{4-12}HX_{3-5}C$ zinc-finger-like binding motif did not discriminate between a catalytic and a non-catalytic Zn(II). On the basis of the more recent EXAFS data which suggest that the non-catalytic Zn(II) contains four cysteine ligands, it is appealing to identify the four cysteines emphasized in Fig. 6B (CX_2CXCX_7C) as the non-catalytic Zn(II) ligands. However, mammalian PBGS contains eight cysteines, all of which are conserved in mammals. Therefore, to collect additional data supporting the four cysteines of this domain as the non-catalytic Zn(II) ligands, we analyze proteins of proven structure which contain non-catalytic Zn(II).

The analysis of non-catalytic Zn(II) ligands follows that of Vallee and Auld[1] in simply identifying the nature of the amino acid ligand and the number of intervening amino acids between ligands. The data for six X-ray or NMR determined structures are presented in Table I. Two additional proteins for which there is excellent physical/chemical data indicating the non-catalytic Zn(II) ligands are also discussed. At the time of the 1990 publication of Vallee and Auld,[1] only the first two entries in Table I were available. These

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A

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                    119
                    *
Bovine      SLLVACDVCLCPYTSHGHCGLLSEN
Human       NLLVACDVCLCPYTSHGHCGLLSEN
Rat         TLLVACDVCLCPYTSHGHCGLLSEN
Mouse       SLLVACDVCLCPYTSHGHCGLLSEN
Yeast       ELYIICDVCLCEYTSHGHCGVLYDD
E. coli     EMIVMSDTCFCEYTSHGHCGVLCEH
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B

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                    119
                    *
Bovine      SLLVACDVCLCPYTSHGHCGLLSEN
Human       NLLVACDVCLCPYTSHGHCGLLSEN
Rat         TLLVACDVCLCPYTSHGHCGLLSEN
Mouse       SLLVACDVCLCPYTSHGHCGLLSEN
Yeast       ELYIICDVCLCEYTSHGHCGVLYDD

E. coli     EMIVMSDTCFCEYTSHGHCGVLCEH
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C

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                    119
                    *
bovine      SLLVACDVCLCPYTSHGHCGLLSEN
Human       NLLVACDVCLCPYTSHGHCGLLSEN
Rat         TLLVACDVCLCPYTSHGHCGLLSEN
Mouse       SLLVACDVCLCPYTSHGHCGLLSEN
Yeast       ELYIICDVCLCEYTSHGHCGVLYDD
E. coli     EMIVMSDTCFCEYTSHGHCGVLCEH
B. subtilis EMVVVADTCLCEYTDHGHCGLVKDG
M. sociabili LVVI TDVCLCQYTEHGHCGIVKNK
Moss        DLVIYTDVALDPYSSDGHDGIVRED
Spinach     DLIIYTDVALDPYYYDGHDGIVTQH
Pea         DLIIYTDVALDPYSSDGHDGIVRED
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FIGURE 6 (A) The cysteine- and histidine-rich region of PBGS which was initially cited as the "zinc binding site" of PBGS (Ref. 2). The cysteines and histidines are highlighted. (B) The four-cysteine cluster, CX_2CXCX_7C, which is herein proposed as the binding site for the non-catalytic Zn(II) of mammalian, and yeast, PBGS. If *E. coli* PBGS also contains a non-catalytic Zn(II), its binding site is proposed to be $CXCX_7CX_3C$, as highlighted. (C) The sequences from plant PBGS do not contain the cysteines proposed herein to bind the non-catalytic Zn(II) of mammalian, fungal, and bacterial PBGS (Ref. 7). The large number of aspartic acids in this region of the plant sequences suggests that plant PBGS may use Mg(II) in place of the non-catalytic Zn(II) of mammalian PBGS. This potential Mg(II) binding site is *not* responsible for binding the stimulatory Mg(II) which is found in both bacterial and plant PBGS (see Fig. 8) (Ref. 29).

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TABLE I

Non-catalytic Zn(II) ligands in proteins of established structure.

	Ligand	# of Intervening Amino Acids	Ligand	# of Intervening Amino Acids	Ligand	# of Intervening Amino Acids	Ligand
Aspartate Transcarbamoylase[1]	C	4	C	22	C	2	C
Alcohol Dehydrogenase[1]	C	2	C	2	C	7	C
Zif268[55]	C	2–4	C	12	H	3–4	H
Glucocorticoid receptor[56]	C	2	C	13*	C	2	C
	C	5	C	9	C	2	C
GAL4 transcription factor[57]	C	2	C	6	*C*	9	*C*[s]
	C	6	C	2	*C*[s]	6	C
Retroviral gag polyprotein[58]	C	4	C	4	H	4	C

Note: Italics are used for structures which were not available at the first analysis of Vallee and Auld (Ref. 1).
*Contains an intervening cysteine.
[s]Shared.

authors have since dealt quite eloquently with the different Zn(II) protein structures involved in nucleic acid binding and the reader is referred to that work.[54] The most commonly cited non-catalytic Zn(II) binding motif is the zinc finger which often contains histidine as a zinc ligand. However, as Vallee *et al.* have pointed out, there is no one tertiary structure which defines a non-catalytic Zn(II) bound to a protein and the general term "zinc finger" has become grossly overused.[54] Other nucleic acid binding structures have been called the zinc twist, exemplified by the glucocorticoid receptor and the zinc cluster, exemplified *GAL4* and are reminiscent of metallothionein. The metallothionein structure itself, where many Zn(II) may be bound to a multitude of shared cysteine residues, is not included in this analysis because the protein *function* appears to be heavy metal binding.

In spite of differences between the proteins in Table I, a striking common characteristic of their non-catalytic Zn(II) is that cysteines are ligands. Therefore, the first derived "rule" is that non-catalytic Zn(II) have at least two cysteine ligands. Most non-catalytic Zn(II) have four cysteine ligands, in a tetrahedral geometry. In *all* cases at least two of the cysteine ligands are separated by 2–4 amino acids. The second derived "rule" is that non-catalytic Zn(II) ligands are contained in a continuous stretch of 15–35 amino acids. This "rule" may be of limited applicability only insofar as the bulk of X-ray crystal structures are on homomeric proteins and all the NMR determined structures are on relatively small proteins.

The preparation of Table I revealed some interesting caveats. First, proteins containing non-catalytic Zn(II) are not all involved in gene expression and regulation. This may seem too obvious to state. However, the popularity of zinc fingers has misled several investigators who reveal a cysteine-rich or a cysteine- and histidine-rich sequence to conclude that the region is a Zn(II) binding site and that the protein is involved in nucleic acid binding. Second, even purified proteins known to contain both Zn(II) and a sequence homologous to a proven Zn(II) binding site do not necessarily have the Zn(II) bound at the indicated site. For instance, methionine tRNA synthetase contains one Zn(II) and the sequence $CX_2CX_9CX_2C$. The X-ray crystal data show that the Zn(II) is at the active site and the sequence $CX_2CX_9CX_2C$ is not anywhere near the Zn(II).[59] Also, the glucocorticoid receptor contains ad-

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ditional cysteines, between and beyond those used as Zn(II) ligands, which are not coordinated to the Zn(II).[56]

The adenylate kinase from the thermophile *Bacillus stereothermophyles* contains a non-catalytic zinc that appears to enhance the thermostability of the protein.[60] The previously characterized adenylate kinases do not appear to contain Zn(II). Physical-chemical data suggest that the Zn(II) binding motif of adenylate kinase is $CX_2CX_{16}CX_2C$, which is consistent with the "rules" derived from Table I but which remains to be verified by X-ray crystallography. Another protein containing a non-catalytic Zn(II), but not included in Table I, is the *E. coli* Ada protein. The Zn(II) binding motif of Ada is proposed to be the sequence $CX_3CX_{26}CX_2C$. The third cysteine of this motif (Cys69) is demonstrably the nucleophile which is methylated by methyl phosphotriesters. Upon methylation of Cys69, Ada becomes a transcription factor specific for methylation repair genes. Thus, in the case of *E. coli* Ada, ligand exchange at a non-catalytic zinc may be involved in a genetic switch.[61]

On the basis of the analysis of known non-catalytic Zn(II) sites, the non-catalytic Zn(II) site in mammalian PBGS is proposed to be the sequence CX_2CXCX_7C which is also present in yeast PBGS (see Fig. 6B). This site contains four cysteines, at least two of which are within four residues of each other, and falls in a continuous sequence of <35 amino acids. If there is a structural Zn(II) in *E. coli* PBGS, it is proposed to be in the related sequence $CXCX_7CX_3C$ (see Fig. 6B).

PREDICTION OF THE LIGANDS TO THE CATALYTIC Zn(II) OF PBGS

Ligands to the catalytic Zn(II) of PBGS may be predicted on the basis of the following three considerations. The EXAFS data of Dent *et al.* (1990) indicate that the most probable constellation of catalytic Zn(II) ligands contains one cysteine, at least one histidine, at least one tyrosine, and one acidic residue like aspartic acid or a water molecule[33]; Cys223 has been identified as one of the catalytic Zn(II) ligands[23] (see above); and eighteen crystal structures of proteins with catalytic Zn(II) are analyzed to update the "rules" for catalytic Zn(II) ligands.

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In 1990, Vallee and Auld used the first eleven structures in Table II to set three oft-cited "rules" regarding ligands to catalytic Zn(II).[1] On the basis of the eighteen structures cited in Table II, the "rules" can be updated as follows:

(1) It has been proposed that catalytic Zn(II) are tetrahedrally coordinated to three amino acids and one water molecule. At this point there are several examples where catalytic Zn(II) are shown instead to be pentacoordinate and other examples where water is not a ligand. One interesting example is the endopeptidase astacin, which contains the HEXXH motif considered diagnostic for Zn(II) endoproteases.[67] Thermolysin and *B. cereus* neutral protease bind a tetrahedral Zn(II) through the two histidines of this motif. For astacin, the two histidines of HEXXH are also Zn(II) ligands, but the Zn(II) is pentacoordinate with a trigonal bipyramid geometry. The E is bound as a second-sphere ligand through an intervening water molecule. The diversity shown in Table II suggests that the chameleon nature of the geometry of Zn(II) complexes contributes to its widespread use as a catalytic metal ion in biology.

(2) The proposition that probable ligands to a catalytic Zn(II) are H > E > D = C is often interpreted to imply that catalytic Zn(II) are limited to these ligands. It is clear from Table II that histidine is by far the most probable ligand to a catalytic Zn(II). To date there is only one example of a catalytic Zn(II) which does not contain a histidine ligand, and that is the unique two-Zn(II) center of leucine aminopeptidase.[66] Table II also shows that the potential catalytic Zn(II) ligands are expanded to include lysine, tyrosine, and a backbone carbonyl oxygen; the preference appears to be H > E = D > C = Y = K = backbone carbonyl. However, the dataset is still quite small and it remains possible that any backbone and/or side-chain oxygen, nitrogen, and/or sulfur atom can serve as a catalytic Zn(II) ligand. Further protein structure determination will reveal the true variation in Zn(II) ligation patterns.

(3) It was proposed that in non-coenzyme dependent Zn(II) enzymes, a short spacer of 1–3 amino acids exists between two of the ligands. Table II shows that all the proteins, except the coenzyme-dependent alcohol dehydrogenase, follow this rule that two of the catalytic Zn(II) ligands are separated by 1–3 amino acids. In the case of alkaline phosphatase, the most recent of the two

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TABLE II

Catalytic Zn(II) ligands in proteins of established structure.

	Ligand	# of Intervening Amino Acids	Ligand	# of Intervening Amino Acids	Ligand	# of Intervening Amino Acids	Ligand	# of Intervening Amino Acids	Ligand
Alcohol dehydrogense[1]			C	20	H	106	C		H_2O
Carboxypeptidase A[1]			H	2	E	123	H		H_2O
Carboxypeptidase B[1]			H	2	E	123	H		H_2O
Thermolysin[1]			H	3	H	19	E		H_2O
B. cereus neutral protease[1]			H	3	H	19	E		H_2O
DD carboxypeptidase[1]			H	2	H	40	H		H_2O
β-lactamase[1]			H	1	H	121	H		H_2O
Phospholipase C[1]			H	3	E	13	H		H_2O
Alkaline phosphatase[1]			D	3	H	80	H		H_2O
Carbonic anhydrase I[1]			H	1	H	22	H		H_2O
Carbonic anhydrase II[1]			H	1	H	22	H		H_2O
Leucotriene A4 hydrolase[62]			H	3	H	18	E		H_2O
Angiotensin converting enzyme[63]			H_{cat}	39	H	1	H		
Adenosine deaminase[64]	H	1	H	196	H	80	D	&	substrate
Superoxide dismutase (Cu/Zn)[65]			H^S	7	H	8	H	2	D
Leucine aminopeptidase (Zn/Zn)[66]			D	76	$D_{\&\ \text{its backbone}}$		carbonyl	1	E^S
			K	23	D	60	E^S		
Astacin (endopeptidase)[67]	H_2O		H_E	3	H	5	H	46	Y
Alkaline phosphatase[68] (*Zn/Zn/Mg*)*	D	same	D	3	H	80	H		PO_4
			D	318	D	0	H		PO_4

Note: Italics indicate structures not available at the time of Vallee and Auld (Ref. 1).

*Of the two Zn in alkaline phosphatase, one is tetrahedral and one is penta coordinate. The Mg is octahedral.

[S]Ligand shared between two metal ions.

structures shows that adjacent amino acids are ligands.[68] For both leucine aminopeptidase and alkaline phosphatase, one residue is seen to ligate Zn(II) in a bidentate fashion.[66,68] Therefore, of the rules established by Vallee and Auld, only the third "near neighbor" rule holds true for catalytic Zn(II) ligands.

The contention that all species of PBGS contain a catalytic Zn(II) with similar (if not identical) ligands is central to predicting such ligands. This contention is based on the inhibition by Pb(II) of all PBGS (which have been tested) and the binding of Pb(II) to the catalytic Zn(II) site. Based on EXAFS identification of histidine, tyrosine, and aspartic acid as probable Zn(II) ligands, the eleven known PBGS sequences were searched for these residues in absolutely conserved positions. Using the sequence of human PBGS as an example, the potential catalytic Zn(II) ligands are highlighted in Fig. 7. If we impose the additional constraint that two potential ligands must be separated by 1–3 amino acids, only one pair meets this criteria; Asp220 and Tyr224 are thus predicted to be likely catalytic Zn(II) ligands. We note that the ligand pair Asp220 and Tyr224 flank Cys223, which we have also identified as a catalytic Zn(II) ligand in mammalian PBGS. Since adjacent amino acids can be ligands to the same Zn(II), the constellation of catalytic Zn(II) ligands may be DX_2CY. However, because the catalytic Zn(II) is present at a stoichiometry of four per octamer, the Asp220 and Tyr224 ligand pair need not be on the same subunit as the Cys223 ligand.

On the basis of the high probability that histidine is a ligand to the catalytic Zn(II), and the chemical modification data suggesting an essential histidine,[48] His131 is cited as the only absolutely conserved histidine and thus is a likely catalytic Zn(II) ligand. His131 does lie within the non-catalytic Zn(II) binding region. Thus, we have described this region as being important for binding both the non-catalytic and the catalytic Zn(II). We have previously proposed that this may help explain the mutually exclusive population of either the four non-catalytic or four catalytic Zn(II) on each of the eight subunits.[23] This presents a model in which two subunits come together in an asymmetric fashion to form one active site. The close association of two monomers is supported by Small Angle X-ray scattering data.[69] The structure of HIV1 reverse transcriptase is cited as an example of an asymmetric dimer in which

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Met Gln Pro Gln Ser Val Leu His Ser Gly Tyr Phe His Pro Leu Leu Arg Ala Trp Gln 20

Thr Ala Thr Thr Thr Leu Asn Ala Ser Asn Leu Ilu Tyr Pro Ilu Phe Val Thr Asp Val 40

Pro Asp Asp Ilu Gln Pro Ilu Thr Ser Leu Pro Gly Val Ala Arg Tyr Gly Val Lys Arg 60

Leu Glu Glu Met Leu Arg Pro Leu Val Glu Glu Gly Leu Arg Cys Val Leu Ilu Phe Gly 80

Val Pro Ser Arg Val Pro Lys Asp Glu Arg Gly Ser Ala Ala Asp Ser Glu Glu Ser Pro 100

Ala Ilu Glu Ala Ilu His Leu Leu Arg Lys Thr Phe Pro Asn Leu Leu Val Ala Cys **ASP** 120

Val Cys Leu Cys Pro **TYR** Thr Ser His Gly **HIS** Cys Gly Leu Leu Ser Glu Asn Gly Ala 140

Phe Arg Ala Glu Glu Ser Arg Gln Arg Leu Ala Glu Val Ala Leu Ala Tyr Ala Lys Ala 160

Gly Cys Gln Val Val Ala Pro Ser Asp Met Met Asp Gly Arg Val Glu Ala Ilu Lys Glu 180

Ala Leu Met Ala His Gly Leu Gly Asn Arg Val Ser Val Met Ser **TYR** Ser Ala Lys Phe 200

Ala Ser Cys Phe **TYR** Gly Pro Phe Arg Asp Ala Ala Lys Ser Ser Pro Ala Phe Gly **ASP** 220

Arg Arg Cys **TYR** Gln Leu Pro Pre Gly Ala Arg Gly Leu Ala Leu Arg Ala Val Asp Arg 240

ASP Val Arg Glu Gly Ala **ASP** Met Leu Met Val Lys Pro Gly Met Pro **TYR** Leu Asp Ilu 260

Val Arg Glu Val Lys Asp Lys His Pro Asp Leu Pro Leu Ala Val **TYR** His Val Ser Gly 280

Glu Phe Ala Met Leu Trp His Gly Ala Gln Ala Gly Ala Phe Asp Leu Lys Ala Ala Val 300

Leu Glu Ala Met Thr Ala Phe Arg Arg Ala Gly Ala Asp Ilu Ilu Ilu Thr Tyr Tyr Thr 320

Pro Gln Leu Leu Gln Trp Leu Lys Glu Glu 330

FIGURE 7 The sequence of human PBGS, highlighting those histidines, tyrosines, and aspartic acids which are conserved in all eleven known PBGS sequences. The highlighted residues are candidates for the catalytic Zn(II) ligands. His131 is the only conserved histidine. Asp220 and Tyr224 are the only highlighted residues which are spaced by 1–3 intervening amino acids. Thus, His131, Asp220, and Tyr224 are proposed to contribute ligands to the catalytic Zn(II). It is noted, however, that there are three Asp (172, 259, and 313) residues and one Tyr (318) residue which are apparently conserved in all but one of the eleven sequences.

there exist identical primary structures, highly similar secondary structure, but vastly different tertiary structures for the two subunits.[70] The functional unit of PBGS may prove to be another example of an asymmetric dimer.

In conclusion, we predict that His131, Asp220, Cys223, and Tyr224 contribute to the catalytic Zn(II) ligands of mammalian PBGS. In the presence of substrate, we have proposed that the keto oxygen and the amino nitrogen of A-side ALA serve as Zn(II) ligands (see Fig. 5).[23] For this to be the case, one of the four proposed protein-derived ligands would have to be displaced by the binding of A-side ALA. We propose that in the presence of product the amino group of PBG is the fifth catalytic Zn(II) ligand. The proposed model for catalytic and non-catalytic Zn(II) ligands awaits the support of or dismissal by site-directed mutagenesis studies and/or an X-ray crystal structure of PBGS.

It is interesting to note that there are no absolutely conserved cysteines in all species of PBGS. This demands an EXAFS investigation of one of the forms of PBGS which does not contain Cys223 (e.g., bacterial or plant PBGS which all contain serine or threonine in this position). To reconcile the fact that Cys223 is not absolutely conserved, one can argue that Ser223 and/or Thr223 are also potential Zn(II) ligands, if currently unprecedented. Further structural studies, and/or mutagenesis may resolve this question.

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WHAT IS THE RELATIONSHIP BETWEEN Zn(II) AND Mg(II) OF PBGS?

Decades of research in the laboratories of Shemin, Neilands, Batlle, Jordan, Beyersmann, Jaffe and others have contributed to our current understanding of the complex relationship between Zn(II) and mammalian PBGS. Prior to the work of Dent *et al.*, few labs except our own considered that the eight Zn(II) of PBGS were not uniform in ligation pattern. This uniform Zn(II) theory, coupled with Wetmur's elucidation of the zinc-finger-like human sequence CX_2CXCX_4HXHC, has caused the majority of investigators to interpret all PBGS metal ion interactions in terms of this one putative metal binding sequence.[7,39,47] Our recent characterization of *E. coli* PBGS suggests that there are not one but *three*

different types of interactions between divalent metals and PBGS.[29] A confirmatory report by Spencer and Jordan (1993) interpreted similar data in terms of two metal binding sites, each at a stoichiometry of one per subunit.[71] However, their data clearly indicate that the tightest bound Zn(II) is present at a stoichiometry of 0.5 per subunit.

Mammalian PBGS contains two different types of Zn(II) and is insensitive to the presence of Mg(II). Herein we have proposed the binding sites for these two types of Zn(II) and find that *E. coli* PBGS contains both of these sites. It was recently demonstrated that *E. coli* PBGS activity is also stimulated by the addition of Mg(II), suggesting that Mg(II) binds to a *third* metal binding site.[29] The sequences for plant PBGS, which are also stimulated by Mg(II), are lacking in the cysteine-rich region proposed to bind the non-catalytic Zn(II). In place of this region is an aspartic acid-rich domain which has been proposed as a Mg(II) binding site for plant PBGS (see Fig. 6C).[7] Because of the uniform Zn(II) theory, the original proposal contended that this sequence substitution explained how plant PBGS is a Mg(II)-metalloenzyme rather than a Zn(II)-metalloenzyme.[7] It was implied that Mg(II) substitutes for the catalytic Zn(II) of mammalian PBGS. Because the altered amino acids in this region of the sequence are implicated in binding the non-catalytic rather than catalytic Zn(II), it is hereby proposed that plant PBGS does not contain a non-catalytic Zn(II). Plant PBGS might contain an analogous Mg(II) which binds to the aspartic acid residues illustrated in Fig. 6C.

To determine where the stimulatory Mg(II) binds, it is appropriate to look for similarities between those PBGS sequences which are responsive vs. not responsive to Mg(II). When comparing the sequences of *E. coli*, plant, and mammalian PBGS, a region was cited in *E. coli* and plant PBGS that is rich in oxygen-containing ligands (ligands likely to bind Mg(II)) and quite dissimilar from mammalian PBGS. This region, from Asp220–Asp247, is illustrated in Fig. 8 and has been proposed to contribute ligands to the stimulatory Mg(II).[29] In contrast, the mammalian sequence contains more positively charged amino acids in this region.

The identification of the ligands to the catalytic Zn(II) of PBGS depends heavily on the assumption that all PBGS contain a catalytic Zn(II), an assumption which remains to be proven for the

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	225
Human	QLPPGARGLALRAVDRDVRE
Bovine	QLPPGARGLALRAVDRD???
Mouse	QLPPGARGLALRAVARDIQE
Rat	QLPPGARGLALRAVARDIQE
Yeast	QLPPAGRGLARRALERDMSE
E. coil	QMNPMNRAEGIAEYLLDEAQ
B. subtilis	QMDPANRMEALREAQSDVEE
M. sociabilis	QMDPPNSLGALRGVKLDIDE
Moss	QMNPANYREALLEVHADESE
Spinach	QMNPANYREALIETQEDESE
Pea	QMNPANYREALTEMREDESE

FIGURE 8 This is the proposed binding region for the stimulatory Mg(II) numbered according to human PBGS (Ref. 29). The Mg(II) responsive sequences of plant and bacterial PBGS contain a large number of oxygen-rich side chains most of which are not present in mammalian PBGS. In contrast the mammalian sequences contain more positively charged residues in this region. The ? are used where no sequence information is available.

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plant enzymes. Regardless of the reality of the Zn(II) metalloenzyme nature of the plant enzyme, PBGS is an intriguing example of protein evolution where the metal ion usage of one enzyme, clearly of common ancestry, has evolved in an unusual way. If we name the metal ion sites as A, B, and C, mammalian PBGS contains Zn(II) in sites A (catalytic) and B (non-catalytic) and contains positively charged amino acids in place of site C; bacterial PBGS contains Zn(II) in sites A and B and Mg(II) in site C; plant PBGS probably contains Zn(II) in site A and Mg(II) in sites B and C. For enzymes obviously so closely related by sequence homology, it is interesting to ponder what evolutionary pressures might have led to the diversion in use of metal ions. For instance, because Mg(II) is required to complete the chlorophyll structure, one might suggest that in photosynthetic organisms it makes sense for Mg(II) to up-regulate the activity of an early step in tetrapyrrole biosynthesis. However, *E. coli* is not photosynthetic and *E. coli* PBGS is responsive to Mg(II). Other bacterial PBGS, those of *M. sociabilis* and *B. subtilis* share the putative Mg(II) responsive sequence motif. The missing link in our PBGS evolutionary tree is the photosynthetic bacteria, for which no PBGS sequence is currently

available. Sequence information from this class of organisms may cause us to further refine our unifying three-metal ion model for PBGS.

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