A Structural Basis for Half-of-the-sites Metal Binding Revealed in Drosophila melanogaster Porphobilinogen Synthase*

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Porphobilinogen synthase (PBGS) proteins fall into several distinct groups with different metal ion requirements. Drosophila melanogaster porphobilinogen synthase (DmPBGS) is the first non-mammalian metazoan PBGS to be characterized. The sequence shows the determinants for two zinc binding sites known to be present in both mammalian and yeast PBGS, proteins that differ in the exhibition of half-of-the-sites metal binding. The pH-dependent activity of DmPBGS is uniquely affected by zinc. A tight binding catalytic zinc binds at 0.5/subunit with a K_d well below μM. A second inhibitory zinc exhibits a K_d of −5 μM and appears to bind at a stoichiometry of 1/subunit. A molecular model of DmPBGS suggests that the inhibitory zinc is located at a subunit interface using Cys-219 and His-10 as ligands. Zinc binding to this previously unknown inhibitory site is proposed to inhibit opening of the active site lid. As predicted, the DmPBGS mutant H10F is active but not inhibited by zinc. H10F binds a catalytic zinc at 0.5/subunit and binds a second nonessential and noninhibitory zinc at 0.5/subunit. This result reveals a structural basis for half-of-the-sites metal binding that is consistent with a reciprocating motion model for function of oligomeric PBGS.

The porphobilinogen synthases (PBGS) are a family of highly homologous homo-octameric proteins responsible for catalyzing the first common step in the biosynthesis of a broad range of tetrapyrrole pigments such as heme, vitamin B12, chlorophyll, and cofactor F430 of the methanogenic bacteria (1). PBGS is also known as 5-aminolevulinate dehydratase or ALAD. The most significant phylogenetic difference among PBGS proteins is in the constellation of metal ions at catalytic and allosteric sites (2, 3). Yeast and mammalian PBGS share the sequence determinants for two zinc binding sites, one of which is absent from the PBGS of any archaea, bacteria, protist, or photosynthetic eucarya (3). The human and yeast PBGS both contain the sequence determinants for a catalytic active site zinc (also known as ZnB), as well as a second non-essential zinc (also known as ZnA) (4), but they differ in metal binding stoichiometry. For instance, in the case of human PBGS, the catalytic zinc shows half-of-the-sites binding at a stoichiometry of 4/homo-octamer (4) (PDB accession number 1E51), whereas the fungal enzyme binds the catalytic zinc at 8/octamer (6) (PDB accession number 1AW5). Some bacterial PBGS show half-of-the-sites metal binding at catalytic and/or allosteric sites (e.g. Bradyrhizobium japonicum and Pseudomonas aeruginosa), whereas others (e.g. Escherichia coli) do not.

The current sequence databases contain PBGS sequences from ~130 different organisms. Of these, the PBGS of Drosophila melanogaster (DmPBGS) is the only complete non-mammalian and non-fungal PBGS sequence that shows the sequence determinants for the two zinc binding sites found in yeast and mammalian PBGS. The gene encoding PBGS is absent from the completed genome of Caenorhabditis elegans and not yet verified in other incomplete non-mammalian metazoan genomes such as that of Danio rerio. Hence, to help deduce the molecular determinants for expression of the half-of-the-sites metal binding phenomenon, we obtained the expressed sequence tag encoding DmPBGS and cloned, expressed, purified, and characterized the protein, as described herein. Unexpectedly, DmPBGS was found to interact with zinc somewhat differently from either mammalian or yeast PBGS. Investigation of these differences provides novel insight into the structural basis for the half-of-the-sites metal binding phenomenon.

Characterization of DmPBGS is also significant because insects are among the most abundant metazoan species on earth and can act as agricultural pests or human disease vectors. For many insects, flight is essential and depends upon aerobic respiration; consequently, tetrapyrroles (hemes) play an important role. Hence, species-specific inhibition of tetrapyrrole biosynthesis might prove useful in control of insects and insect-borne diseases. Because PBGS has recently been identified to have species-specific sensitivity to certain active site-directed inhibitors (7, 8), we have characterized DmPBGS with these inhibitors.

EXPERIMENTAL PROCEDURES

Cloning and Expression of DmPBGS—The expressed sequence tag containing the gene for DmPBGS (catalogue number 98002) was purchased from ResGen. The gene was amplified by PCR with the addition of the Ndel and BamHI sites, using the primers 5'-GCTAAGGGAGCATTGGGAGATCCCTCGG-3' and 5'-GCATATCTACGATCACAT- GGTATTACGATCGG-3', where the restriction sites are underlined. The amplified gene was digested with Ndel and BamHI and ligated directly into pET17b, which had also been digested with Ndel and BamHI.

The sequences of several resulting plasmids were determined...
throughout the gene in both directions using a series of internal and external primers. An error-free plasmid was denoted pMPBGGS and was used for further study. pMPBGGS was transformed into E. coli strain BLR(DE3) and the CodonPlus variant BL21(DE3)-RIL for expression using the procedure we had previously used for pMVh (9). The Dm PBGS mutant H10F was prepared by the QuikChange method (Stratagene) using the sense strand primer 5'-GCAGCTGGAATGTCGAATCCGGCTGCGGC-3'. All oligonucleotides were synthesized in the Fannie E. Rippel Biochemistry and Biotechnology Facility at Fox Chase Cancer Center (FCCC). DNA sequence determination was in the FCCC DNA Sequencing Core Facility.

Dm PBGS purification initially followed the protocol for human PBGS expressed from pMVh (9). The protocol was altered as differences were observed. The final purification protocol followed the published procedures up to the ammonium sulfate cut, which was altered to use the protein that precipitated between 30 and 50% ammonium sulfate. This protein was dissolved in 30 mM KPi, pH 7.5, 10 mM Zn²⁺, 10 mM 2-mercaptoethanol (β-ME), 0.1 mM phenylmethylsulfonyl fluoride, and 10% ammonium sulfate, adsorbed to a 100-ml phenyl-sepharose column, and eluted in 2 mM KPi, pH 7.5, 10 mM Zn²⁺, 10 mM β-ME, 0.1 mM phenylmethylsulfonyl fluoride following an 800-ml linear gradient to the final buffer.

Dm PBGS was pooled and applied directly to a 100-ml DEAE Biogel A column that was equilibrated in 2 mM KPi, pH 7.5, 10 mM Zn²⁺, 10 mM β-ME, 0.1 mM phenylmethylsulfonyl fluoride. The protein was eluted with a 1-liter linear gradient from 0 to 0.3 M KCl in 30 mM KPi, pH 7.5, 10 mM Zn²⁺, 10 mM β-ME, 0.1 mM phenylmethylsulfonyl fluoride. Dm PBGS elutes in the first third of the gradient and is baseline separated from chromosomally encoded E. coli PBGS activity that elutes in the second half of the gradient. Dm PBGS was pooled, concentrated to ~10 mg/ml, and further purified on a 1-meter long 270-ml Sephacryl S300 column in 0.1 mM KPi, pH 7.0, 10 mM Zn²⁺, 10 mM β-ME. Dry weight analysis was used to determine the extinction coefficient of Dm PBGS as previously described (10).

Building a Model of Dm PBGS—Two models were prepared to approximate the structure of octameric Dm PBGS. These were based on the crystal structures of yeast PBGS with aminolevulinic acid bound (PDB accession number 1H7P, 1.67 Å resolution; Ref. 11) and human PBGS with the porphobilinogen bound (PDB accession number 1K51, 2.83 Å resolution). The unit cell of the former is a monomer and dimer, respectively. Sequence alignments were performed with the BLAST program (12) with some manual adjustment upon examination of the sequences in light of the structural differences. All insertions and deletions in the alignment occurred in coil regions located between regular secondary structure segments. For both models, loop modeling in insertion and deletion regions in the alignment was performed with the Modeller program (version 6) (13), and side chain conformations were predicted with the program SCWRL (version 2.95) (14–16). Side chains for residues that were identical in the Dm PBGS sequence and the template proteins were kept fixed in their crystallographic Cartesian coordinates. The octamers were built using the symmetry transformations provided in the PDB entries with in-house custom software. The side chain calculations were repeated following formation of the octamer from the fundamental asymmetric units. Again, conserved amino acids were kept in their original positions.

**PBGS Activity Assays**—Determinations of PBGS activity used a fixed-time assay at 37°C in 0.1 M bis-tris-propane-HCl, 10 mM β-ME, and the substrate 5-aminolevulinic acid (ALA), with variations in assay pH, concentrations of metal ions such as Zn²⁺, Mg²⁺, K⁺, and the Zn²⁺-chelator, 1,10-phenanthroline (o-phen). Procedures for pH versus activity profiles, Kₐ, and Vₘₐₓ determinations, and inhibition by 4,7-dioxosobasic acid and 4-oxosobasic acid were as previously described (4, 7).

**RESULTS**

**Protein Expression and Purification**—The expression of Dm PBGS from pMPBGGS was attempted in E. coli strains BLR(DE3) and BL21-CodonPlus-RIL. Based on SDS gels following a small scale growth, the CodonPlus host did not appear to greatly influence expression. However, because the native gene encoding Dm PBGS contains clusters of rare codons, the CodonPlus strain was selected for expression in order to minimize the possibility of translational errors. From each 1-liter growth we obtained ~10 g of cells, from which ~45 mg of Dm PBGS was purified to homogeneity. The final purification buffer was 0.1 M potassium phosphate, pH 7, containing 10 mM β-ME, and 10 mM Zn²⁺, which was originally selected because it corresponds to optimal assay conditions for mammalian PBGS. UC in the final purification buffer was demonstrated to be optimal for Dm PBGS under these conditions. A specific activity of ~2.5 μmol h⁻¹ mg⁻¹, which is an order of magnitude lower than human PBGS. Under optimal conditions for Dm PBGS (see below), its specific activity is ~17 μmol h⁻¹ mg⁻¹. Freshly purified Dm PBGS contains 1.7 ± 0.2 zinc/subunit as determined by atomic absorption spectroscopy. Based on a dry weight analysis, Dm PBGS has a 16% asparagine or aspartate.

**Kinetic and Metal Binding Properties of Dm PBGS**—Dm PBGS activity varies as a function of pH as illustrated in Fig. 1 and quantified in Table I. The maximal activity is observed with no added metals and peaks at ~pH 8. Under these conditions, the pH activity profile shows a good fit to a simple bell curve with pKₐ = 7.2, pKᵦ = 9.1 and a maximal velocity of 21 μmol h⁻¹ mg⁻¹. The addition of 0.1 M K⁺ or 1 mM Mg²⁺, which are required for, or stimulate, the activity of most other PBGS, has little effect on the pKᵦ, and pKᵦ values, though Mg²⁺ appears to affect some inhibition based on Vₘₐₓ (see Table I). The addition of 10 μM Zn²⁺, however, dramatically alters the profile to reveal two pKᵦ values of 6.1 and 10.0, both of which fit best to a two-proton model. The net effect of 10 μM Zn²⁺ at pH values below the optimal pH of 8 is a dramatic inhibition. For comparison, at its optimal pH of ~7, human PBGS is not inhibited by Zn²⁺, at concentrations below ~30 μM (9, 17). The pH activity profile of human PBGS at 10 μM Zn²⁺ is included in Fig. 1 and Table I.

The Kᵦ for ALA was determined for Dm PBGS at pH 8 with no added metal ions and found to be 108 ± 12 μM, which is in the range of all other PBGS at their conditions of optimal pH and metal ions (e.g. 4). The Vₘₐₓ was found to be 16.8 ± 0.3
μmol h⁻¹ mg⁻¹. The kinetic parameters \(K_m\) and \(V_{max}\) were not quantified under other conditions of pH or buffer metal ion concentrations.

Fig. 2 quantifies the effect of adding Zn²⁺ on the activity of DmPBGS at pH 8 and at 10 mM ALA. The data show inhibition to about 10% of maximal activity, which fit well to Equation 1.

Velocity = \(V_{max} + (V_{max} - V_{min})/1 + (\text{Zn}²⁺/\text{IC}_{50})^{1/10}\) (Eq. 1)

The resulting IC₅₀ = 4.24 ± 0.49 μM Zn²⁺, and the Hill coefficient is 1.51 ± 0.23. It is interesting to note that the best fit to \(V_{min}\) is 1.89 ± 0.61 μmol h⁻¹ mg⁻¹, in excellent agreement with the plateau rate observed between pH 6.5 and 7.5 at 10 μM Zn²⁺ (see Fig. 1 and \(V_{max}\) in Table I).

To quantitatively correlate the inhibitory effect of zinc on enzyme activity with the zinc content of the protein, equilibrium dialysis studies were carried out under assay conditions in the presence and absence of 1 mM ALA in the dialysis buffer. These data are shown in Fig. 3A and quantified in Table II. The data fit well to the following simple binding in Equation 2,

\[ Z_{\text{Btot}} = \text{Zn}_{\text{tot}} + (\text{Zn}_{\text{tot}} - \text{Zn}^2+)(\text{K}_{\text{ZnInh}} + [\text{Zn}^2+]) \] (Eq. 2)

where \(\text{Zn}_{\text{tot}}\) is total bound zinc, \(\text{Zn}_{\text{sat}}\) is the stoichiometry of a required zinc, \(\text{Zn}_{\text{inh}}\) is the stoichiometry of an inhibitory zinc, \([\text{Zn}^2+]\) is the free Zn²⁺ concentration, and \(\text{K}_{\text{ZnInh}}\) is the dissociation constant for the inhibitory zinc. Correlation of Fig. 2 with Fig. 3 indicates that under conditions where zinc does not inhibit, there are −0.5−0.7 zinc/subunit, which is consistent with half-of-the-sites metal binding. Under conditions where zinc shows significant inhibition of DmPBGS, the protein contains −1.5 zinc/subunit, suggesting that the inhibitory zinc has a stoichiometry of 1/subunit. The \(\text{K}_{\text{ZnInh}}\) value for binding the inhibitory zinc in the presence of substrate, −7 μM Zn²⁺, is in good agreement with the kinetically determined IC₅₀ of −4 μM Zn²⁺ (see Fig. 2).

To demonstrate that DmPBGS requires at least some of the tightly bound zinc that is found to co-purify with the protein, DmPBGS activity was assessed as a function of the zinc chelator, o-phe, as illustrated in Fig. 2. Maximal activity is observed at o-phe concentrations below 0.1 mM, and the inhibition profile fits best to a cooperative model where IC₅₀ = 0.84 ± 0.03 mM o-phe with a Hill coefficient of 1.9 ± 0.1. To determine enzyme-bound Zn²⁺ as a function of o-phe equilibrium dialysis, experiments were carried out in the presence and absence of 1 mM ALA as illustrated in Fig. 3A and quantified in Table II. At low o-phe the maximal catalytic activity seen in Fig. 2 is again associated in Fig. 3A with Zn²⁺ bound at a stoichiometry of 0.5/subunit, confirming half-of-the-sites binding of the catalytic Zn²⁺. In this case, the binding data fit best to a cooperative model for o-phe inhibition (Table II). Based on the IC₅₀ of o-phe (the amount of o-phe required to remove one-half of the catalytic zinc) in the presence and absence of the substrate ALA (1 mM versus 0.3 mM o-phe, respectively), the catalytic zinc appears to be more tightly bound in the presence of ALA than in its absence. This is consistent with a model where ALA provides some of the ligands to this required zinc ion. It is reassuring that the apparent IC₅₀ of o-phe (1 mM) in the presence of ALA is in excellent agreement with the kinetically determined IC₅₀ of o-phe (−0.8 mM).

The data presented in Figs. 2 and 3 show that DmPBGS binds Zn²⁺ differently from either human or yeast PBGS. Like the ZnB of human PBGS (4), the catalytic Zn²⁺ of DmPBGS shows half-of-the-sites metal binding. However, Fig. 3A suggests that the inhibitory Zn²⁺ of DmPBGS does not show the half-of-the-sites binding that has been seen for the non-essential ZnA of human PBGS (4). In support of the notion that the inhibitory zinc of DmPBGS is somehow related to ZnA of yeast and mammalian PBGS, the \(\text{K}_{\text{ZnInh}}\) of DmPBGS is of comparable magnitude to the \(K_c\) for ZnA of mammalian PBGS (−5 μM), which binds at 0.5 zinc/subunit (4, 9, 17).

The DmPBGS Mutant H10F—Cys-219 of DmPBGS is analogous to the cysteine that has been shown to bind the non-inhibitory ZnA of human (4) and yeast (6) PBGS. In our model of DmPBGS, shown in Fig. 4A, Cys-219 lies very near to His-10 of a neighboring subunit, such that Cys-219 and His-10 could form part of a previously uncharacterized zinc binding site. Various PBGS crystal structures suggest that the position of Cys-219 is dependent on whether the active site lid is opened or closed. Hence, zinc binding to this site in the closed lid conformation shown in Fig. 4A could inhibit lid opening and thus inhibit catalysis. To test the hypothesis that His-10 is involved in binding the inhibitory zinc, this residue was altered to Phe, which is found in the analogous position of human PBGS. The behavior of DmPBGS mutant H10F during purification was indistinguishable from wild type DmPBGS. The yield was 75 mg from 17 g of cell paste. The specific activity of H10F at pH 8 is seen to be −12 μmol h⁻¹ mg⁻¹, only marginally lower than wild type, regardless of
whether or not 10 μM Zn2+ was added to the assay mixture. The pH rate profile of H10F does not exhibit the zinc inhibition phenomenon but is otherwise the same as the wild type protein (see Fig. 1). The fitted pH rate profile data is detailed in Table I; however, for clarity of presentation only the combined fit line is illustrated in Fig. 1 ($R^2 = 0.96$). The behavior of H10F supports the hypothesis that inhibition by zinc is because of zinc binding through His-10 of DmPBGS.

DmPBGS mutant H10F provides an independent tool for determining the stoichiometry of the inhibitory zinc of DmPBGS. To do so, the H10F protein was evaluated for its ability to bind zinc in an equilibrium dialysis experiment, the results of which are illustrated in Fig. 3B. In this case the lesser binding zinc of H10F retains the $K_q$ of $-5 \mu M$ but binds at a reduced stoichiometry of $0.5/subunit$. Thus, H10F has lost the inhibitory zinc, which appears to have bound at a stoichiometry of 0.5/subunit. The conclusion is that DmPBGS contains the catalytic Zn2+ (akin to ZnB) at 4/octamer, contains the non-essential Zn2+ (akin to ZnA) at 4/octamer, and contains an inhibitory Zn2+ at 4/octamer. Because Cys-219 is implicated in binding both ZnA and the inhibitory zinc, the binding of these two metal ions must be mutually exclusive (see “Discussion”).

**Discussion**

DmPBGS is the first insect PBGS for which a sequence became available and the first to be purified and characterized. The DmPBGS sequence shows the binding determinants for two zinc ions that have been previously characterized with both mammalian and yeast PBGS (2). Like mammalian PBGS, but unlike yeast PBGS, DmPBGS shows half-of-the-sites binding for the catalytically essential zinc at 0.5/subunit (4/octamer). Catalytic zinc binding to DmPBGS is presumed to be to the
cysteine residues of the sequence DVCICPYSSHG at the active site. Half-of-the-sites binding to this site is seen in the crystal structure of human PBGS (PDB accession number 1E51). Yeast PBGS structures show the catalytically essential zinc bound to the corresponding three-cysteine site, but there is no evidence for half-of-the-sites binding (6). Mutagenesis studies on human PBGS complement the crystal structures in asserting the essential nature of the zinc ion bound to this (ZnB) site (4). Both human and yeast PBGS bind a second zinc ion, which for human PBGS is at a stoichiometry of 0.5/subunit (4). Chemical modification studies on bovine PBGS implicate Cys-223 of human PBGS in binding this second (ZnA) zinc ion (19). As noted above, the analogous residue is Cys-219 of DmPBGS.

The ZnA binding site is confirmed by a crystal structure of yeast PBGS that shows weak binding of zinc to the analogous cysteine with a second ligation through the italicized His residue of the cysteine-rich catalytic zinc-binding sequence.

### Table II

| PBGS          | Dialysis conditions | Zinc/subunit corresponding to full activity | Zinc/subunit at maximum binding | $K_d$ for loose binding zinc | $IC_{50}$ for o-pha $^a$ | Hill coefficient for zinc |
|---------------|---------------------|---------------------------------------------|--------------------------------|-----------------------------|--------------------------|---------------------------|
| DmPBGS [Zn$^{2+}$]  |                     | 0.47 ± 0.03                                | 1.92 ± 0.33                    | 25.4 ± 10.8                 |                          |                           |
| DmPBGS [Zn$^{2+}$] + ALA |                     | 0.69 ± 0.02                                | 1.45 ± 0.06                    | 6.79 ± 1.54                 |                          |                           |
| DmPBGS [o-pha] + ALA |                     | 0.50 ± 0.04                                | 0.00 ± 0.04                    | 0.27 ± 0.06                 | 0.14 ± 0.06               | 6.47 ± 3.72               |
| H10F [Zn$^{2+}$]  |                     | 0.52 ± 0.06                                | 1.18 ± 0.14                    | 1.03 ± 0.02                 | 0.12 ± 0.09               | 5.72 ± 4.06               |
| H10F [o-pha] + ALA |                     | 0.54 ± 0.06                                | 1.01 ± 0.12                    | 1.03 ± 0.02                 | 0.12 ± 0.09               | 5.72 ± 4.06               |

$^a$ [o-pha] required for 50% loss of the catalytic zinc.
$^b$ Apparent $K_d$ for inhibitory zinc.
$^c$ Apparent $K_d$ for nonessential zinc.

Fig. 4. PBGS structures. A, a stereoview homology model of a dimer of DmPBGS based on the high resolution crystal structure of yeast PBGS, PDB accession number 1H7P. The subunits are magenta and green. The active site lid of both subunits is cyan. Shown in spacefill representation (atoms colored Corey-Pauling-Koltun) are the cluster of cysteine residues that serve as catalytic zinc ligands (Cys-120, -122, and -130), the putative inhibitory zinc binding site (Cys-219 of one subunit and His-10 of the other subunit), and His-129, which has been implicated in binding the non-essential ZnA. B, the $\alpha\beta_8$ barrel domain of yeast PBGS structure 1AW5 showing the ZnA binding site where a spacefill representation is used for the cysteine ligand (Cys-243, analogous to Cys-219 of DmPBGS) and the histidine ligand (His-142, analogous to His-129 of DmPBGS). In this structure the bulk of the active site lid is disordered. The orientation of the yeast PBGS is the same as the model for DmPBGS; hence, the view of sulfur of Cys-243 is partially blocked.

Fig. 5. Inhibition of DmPBGS by species-selective inhibitors. A, PBGS inhibition by 4,7-DOSA showing a 100-min incubation of the inhibitor prior to addition of substrate. The species represented are human (○), D. melanogaster (▼), E. coli (■), B. japonicum (▲), P. aeruginosa (●), and P. sativum (□). The lines represent a non-linear best fit to the Copeland approximation as previously described (18). The species depicted in black contain the active site zinc; the species depicted in gray do not. Each of these species has a unique stoichiometry of active site and allosteric metal ions (3, 4, 5, 10). B, inhibition of DmPBGS (▼) and E. coli PBGS (■) by 4-OSA depicting a 16-h preincubation with the inhibitor.
scribed above. A mutation to these ligands in human PBGS results in an active protein, which binds only the catalytic zinc at 0.5/subunit and which has a somewhat mildly elevated \( K_m \) for ALA (4).

The data presented here suggest that for \( Dm \) PBGS zinc also binds to a site that results in an incomplete inhibition of activity. The possibility that this inhibitory site is spatially equivalent to the previously described ZnA site was considered. Like other TIM-barrel enzymes, PBGS contains an active site lid, which is a continuous mobile stretch of amino acids that serves to gate access to the active site. Based on other PBGS structures, the \( Dm \) PBGS lid is comprised of amino acids 205–223, as illustrated in Fig. 4A, and the putative ZnA ligand Cys-219 lies within this lid. As a part of the mobile lid, this cysteine has been seen in more than one location in various PBGS crystal structures. In yeast PBGS structure 1AW5, where much of the lid is disordered, the analogous cysteine is seen pointing in toward the active site and is ligated to an atom of ZnA, at least part of the time, as illustrated in Fig. 4B. The second ZnA ligand is seen in structure 1AW5 as a histidine, analogous to His-129 of \( Dm \) PBGS, which is italicized within the catalytic zinc binding sequence above and is included in Fig. 4A. The ZnA seen in structure 1AW5 resides at the periphery of the active site, about 7 Å from ZnB and about 9 Å from the nearest active site catalytic lysine. Crystal structures of yeast PBGS containing heavy metals such as Hg and Pb (PDB accession numbers 1QM and 1QMV; Ref. 20) also show the cysteine pointed in toward the barrel near this histidine, and in these structures much of the active site lid is also disordered. On the other hand, there are many other yeast PBGS structures (e.g. PDB accession number 1H7F) where the lid is closed and the lid cysteine is pointing away from the active site, as illustrated in the \( Dm \) PBGS model in Fig. 4A. In these cases, the cysteine in question is in close contact with the N-terminal arm of an adjacent subunit. In \( Dm \) PBGS (Fig. 4A), the nearest neighbor is His-10, potentially forming an inhibitory zinc site wherein zinc binding to this site would stabilize the closed lid conformation, thus inhibiting active site lid opening for product release. To test this hypothesis, we prepared the H10F variant of \( Dm \) PBGS, choosing to alter the putative Zn\(^{2+}\) ligand His-10 to the cognate found in human PBGS, which is Phe-12. The behavior of H10F, which is very similar to \( Dm \) PBGS but is not inhibited by zinc, supports this hypothesis. Hence the characterization of \( Dm \) PBGS and H10F reveals the location of a previously unknown inhibitory zinc binding site. Although tetrapyrrole biosynthesis is generally tightly regulated, the physiologic significance of the regulation of \( Dm \) PBGS activity by zinc remains unclear.

As would be expected, removal of zinc inhibition is found to correlate with a loss of binding of the inhibitory zinc. The interesting result is the stoichiometry of that inhibitory zinc; H10F binds 0.5 zinc/subunit less than the wild type \( Dm \) PBGS, which suggests that binding through Cys-219 and His-10 is at a stoichiometry of 0.5/subunit. In addition to the catalytic zinc, H10F retains the binding of 0.5 zinc/subunit, which does not inhibit activity and which has a comparable dissociation constant of \( \sim 5 \mu M \). Thus, zinc binding to \( Dm \) PBGS mutant H10F is akin to the binding of zinc to human PBGS, which binds the catalytic ZnB at 4/octamer and the nonessential ZnA at 4/octamer (4). \( Dm \) PBGS appears to bind these two zinc ions at a half-of-the-sites stoichiometry and also binds an inhibitory zinc at a half-of-the-sites stoichiometry. One attractive model is that both the inhibitory zinc and ZnA are bound through Cys-219 but that the spatial location of this zinc is a function of whether or not the active site lid is opened or closed. When the lid is closed, the ligands are Cys-219 and His-10, as in Fig. 4A; when the lid is open, the ligands are Cys-219 and His-129, analogous to the yeast PBGS conformation illustrated in Fig. 4B. The fact that both can be bound at the same time dictates that for the PBGS octamer, half of the lids must be open and half of the lids must be closed, as is seen in the asymmetric crystal structures of \( P. aeruginosa \) PBGS (PDB accession number 1B4R) and human PBGS (PDB accession number 1E51).

The stoichiometry of zinc binding to \( Dm \) PBGS is in keeping with a reciprocating motion model for the function of oligomeric PBGS. When the active site lid is closed, the inhibitory zinc is bound to the same subunit as the catalytic zinc. The other subunit of the asymmetric dimer would contain zinc bound at the ZnA site. With every catalytic turnover the lid would open and the metal ions would move to the positions seen in the adjacent monomer. The reciprocating motion model provides a structural basis for half-of-the-sites metal binding and half-of-the-sites reactivity for homo-octameric PBGS. Multiple studies show that mammalian PBGS is fully active when zinc is present at a total stoichiometry of 0.5/subunit (9, 17, 21). When this is put in the context of the reciprocating motion model, it dictates that with every turnover the zinc ion must dissociate from one subunit and reassociate to the adjacent subunit or move between subunits along the protein surface. Because turnover for PBGS is generally quite slow, on the order of 1/s, this is entirely feasible. The outstanding question remains as to how events at the active site of one subunit are communicated to the other subunit in the dimer pair.

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