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Porphobilinogen Synthase: An equilibrium of different assemblies in human health

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Abstract

Porphobilinogen synthase (PBGS) is an essential enzyme that catalyzes an early step in heme biosynthesis. An unexpected human PBGS quaternary structure dynamic drove the definition of morpheins, which are protein multimers that dissociate, change shape, and re-assemble differently with functional consequences. Each PBGS monomer has two domains that can reposition through a hinge motion. Human PBGS exists in an equilibrium among high activity octamer, low activity hexamer, and low mole-fraction dimer in which the hinge motion occurs. The dimer conformation dictates the multimer architecture. An octamer-specific inter-subunit interaction responds to pH, resulting in a pH-dependence to the octamer-hexamer equilibrium. An inborn error of metabolism, ALAD porphyria, is caused by single amino acid substitutions that stabilize the hexamer relative to octamer. Drugs that stabilize the PBGS hexamer result in a drug side effect that can exacerbate porphyria. PBGS is essential for all organisms that require respiration, photosynthesis, or methanogenesis. Consequently, phylogenetic variation in PBGS multimerization equilibria provides insight into how Nature has harnessed oligomeric variation in the control of protein function. The dynamic multimerization of PBGS revealed the morphein mechanism for allostery, a structural basis for inborn errors of metabolism, a quaternary structure focus for drug discovery and/or drug side effects, and a pathway toward new antibiotics or herbicides. The fortuitous discovery of PBGS quaternary structure dynamics arose from characterization of a low-activity single amino acid variant that dramatically stabilized the hexamer, whose existence had previously gone unnoticed.

Keywords

Porphobilinogen synthase; aminolevulinic acid dehydratase; morphein; porphyria

Porphobilinogen Synthase – Background

Porphobilinogen synthase (PBGS, EC 4.2.1.24) catalyzes the first common reaction in the biosynthesis of all the tetrapyrrole pigments (e.g. heme, chlorophyll, B₁₂, etc.) and thus is an ancient enzyme. PBGS has experienced an evolution in the control of its multimerization, which for the human protein involves octamer, hexamer, and a conformationally flexible dimer (1). The PBGS catalyzed reaction is an asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to yield the monopyrrole porphobilinogen and two water

molecules (Fig 1A)(2). The substrates are called A-side ALA and P-side ALA based on whether they contribute the acetyl/amino portion of porphobilinogen or the propionyl/pyrrole-nitrogen portion of the product. P-side ALA binds first and A-side ALA is the K_M -determining substrate (3). Although the enzyme catalyzed reaction mechanism has been fodder for debate for decades (e.g. (4–7)), it is well established to involve Schiff base formation between at least one active site lysine residue and at least one substrate molecule. For historical reasons, much of the medical literature refers to the enzyme as ALAD (aminolevulinic acid dehydratase), though the IUPAC name change was recommended in the 1970s.

There is phylogenetic variation in the use of an active site catalytic metal ion as well as an allosteric metal ion distant from the PBGS active site (8). Human PBGS activity requires an active site zinc ion, whose presence or absence contributes to the position of the quaternary structure equilibrium (Fig 1B) (1). Requirement for an active site zinc is a property that is shared by the PBGS of all animals, fungi, archaea and some bacteria, but not by PBGS of plants and some other bacteria (8). This means that some aspects of the PBGS catalytic mechanism are not phylogenetically conserved. Human PBGS does not use an allosteric magnesium ion, distant from the active site, which is utilized by the PBGS of all archaea, plants, and nearly all bacteria (8). Yet structural insights drawn from the allosteric magnesium of PBGS from these species helped inform our understanding of the quaternary structure dynamics of human PBGS as it relates to human health (9). For those PBGS that do not employ the allosteric magnesium, in its place is the positive charge of a conserved arginine residue (Arg240 in human PBGS) (10). Most significantly, the human PBGS quaternary structure equilibrium went unnoticed for many decades until the fortuitous appearance of a single amino acid variant, F12L, which sufficiently stabilized the human PBGS hexamer to allow it to emerge from the shadows (9,11).

The Structures of PBGS

The PBGS subunit

Each human PBGS subunit contains 330 amino acids. The C-terminal ~300 amino acids of all PBGS exists as an $\alpha\beta$ -barrel where the enzyme active site is in the center of the $\alpha\beta$ -barrel and there is a mobile element (active site lid) that governs active site access (12). The N-terminal region has significant phylogenetic variation in both sequence and length, and is ~23 amino acids long in human PBGS. On the level of a single subunit, this N-terminal “arm” is not near the enzyme active site, but interacts with the $\alpha\beta$ -barrel of two neighboring subunits in the multimer (Fig 1C). Nevertheless, when the human PBGS variant F12L was first discovered to have insignificant activity, this appeared to be a long-distance effect. A clue that the effect was on quaternary structure arose from the techniques of native PAGE, which suggested a difference in the size or shape of the F12L multimer relative to the wild type protein (11), and ion exchange chromatography, which revealed a difference in surface charge (9). In the end (spoiler alert!) we came to realize that PBGS activity is all about modulation of active site access, the control of which requires specific inter-subunit interactions involving the N-terminal arm of one subunit and the active site lid of a neighboring subunit (1). Here we document studies that established the unexpected structure

of F12L and revealed this structure to be a physiologically relevant component of the native protein's multimeric equilibrium. Interestingly, Phe12 is among a small number of residues on one subunit that are in direct contact with residues from two other subunits of the PBGS octamer. However, we lack a rational explanation of why the F12L variant so dramatically perturbs the PBGS quaternary structure equilibrium.

The PBGS octamer

Classic protein sizing methods and early electron microscopy images of an animal (bovine) PBGS suggested that the PBGS multimer is an octamer (e.g. (13)). A few papers suggested that PBGS from other sources might be hexameric (14,15). At the time, a soluble protein existing as both octamer and hexamer was unprecedented. The first crystal structure of PBGS, from a fungal source, detailed the octamer and illustrated how each N-terminal arm was essential to multimerization by interacting with two neighboring subunits (12). In one interaction the arm lay across the inner surface of a neighboring $\alpha\beta$ -barrel, blocking a rear entrance to the enzyme active site. In human PBGS, Trp19 penetrates and plugs this rear entrance (10). In the second interaction, the arm "hugged" the $\alpha\beta$ -barrel of a different neighboring subunit (12). Each of these interactions is reciprocal. Thus, the PBGS octamer can be thought of as comprised of four hugging dimers (black/cyan in Fig 1C) or four diagonal dimers (black/magenta in Fig. 1C). The hugging dimer has a large intersubunit interface and contains many water molecules whose positions were revealed to be identical when comparing crystal structures of PBGS from diverse organisms (16). Octamer dissociation along these water-rich interfaces results in four diagonal dimers, which we propose as physiologically relevant. Within each diagonal dimer the interface containing the penetrating Trp19 is relatively small and less hydrophilic (17).

The first human PBGS octamer crystal structure (PDB: 1E51) showed product bound at half-of-the-sites (Fig 1C), consistent with prior biochemical studies suggesting half-of-the-sites reactivity (e.g. (3,18)). In the human PBGS octamer structure, the hemisphere with enzyme-bound product contains active site lids that are closed, ordered, and held in place through ion pair interactions with the carboxylate that derived from A-side ALA (Fig 1B). The other hemisphere does not contain enzyme-bound product and the active site lid residues are disordered. In the product-containing subunits with a closed active site lid, one sees a stabilizing interaction between the C-terminal end of the active site lid (sulfur of Cys223) and the hugging arm of a neighboring subunit (backbone carbonyl of Phe12). Each of these interactions is included in Fig 1B.

The PBGS hexamer

The crystal structure of human PBGS variant F12L (PDB: 1PV8) revealed a hexameric assembly that conserved the intersubunit interaction that plugged the back of the active site with Trp19, but had lost the water-rich arm-hugging-adjacent barrel interaction seen in the octamer (9). Monomers in the hexamer have experienced a change in the positioning of the $\alpha\beta$ -barrel relative to the N-terminal arm. This conformational change cannot occur at the level of octamer or hexamer, but can at the level of the dimer (Fig 2). In the hexamer crystal structure, one hemisphere contains a Schiff base intermediate at the position of the P-side ALA, again showing the half-of-the-sites phenomenon (Fig 1C). Without the interactions

between the A-side ALA carboxylates and the active site lid (shown in Fig 1B), all of the active site lids are disordered. We have posited that the low activity of the F12L variant is an inability to isolate the enzyme active site from the environment. Schiff base formation requires deprotonation of an active site lysine (Lys252 in Fig 1B), which in a neutral pH milieu requires isolation of the active site from the external environment. Without the ability to gate solvent access to the active site, the PBGS hexamer is a poor catalyst at neutral pH.

The PBGS octamer $\rightleftharpoons$ hexamer equilibrium

PBGS octamer and hexamer are metastable components of a dissociative quaternary structure equilibrium illustrated in Fig 2. An understanding of this equilibrium and the factors that promote multimer equilibration arose from observations described below. A stable conformational difference between wild type human PBGS (WT) and F12L was first revealed during the enzyme isolation process. Had we employed a purification tag, these differences would have remained masked.

Ion Exchange Chromatography separates the PBGS octamer and hexamer.

A classic PBGS purification procedure (cell lysis, ammonium sulfate fractionation, hydrophobic chromatography, ion exchange chromatography, size exclusion chromatography) revealed that the F12L variant eluted significantly earlier than WT from an anion exchange column (similar to Fig 3A). This taught us that F12L, though a neutral substitution, is a conformational isoform with a different surface charge. Shortly thereafter we realized that WT also contained this same conformational isoform, at about 5% (see also Fig 3B), but we had ignored it because it had no observable activity under standard, neutral pH, assay conditions. This brings to question the classic enzyme purification practice of discarding all but column fractions of high (constant) specific activity. For more than 20 years we had been tossing away low-activity, but physiologically relevant, quaternary structure isoforms of PBGS from various sources.

Native PAGE allows quantification of the octamer and hexamer.

Native PAGE can be used to determine relative size of proteins and protein assemblies that have the same charge/mass ratio. Thus, native PAGE is a tool for routine analysis of the PBGS quaternary structure equilibrium. Fig 3B shows the native PAGE separation for F12L and WT.

The pH rate profile reflects a pH dependence to the quaternary structure equilibrium.

Kinetic constants were obtained for both WT and F12L at pH 7 and at pH 9. WT exhibits a rather typical bell shaped pH rate profile (obtained at 10 mM ALA; Fig 3C). Optimal activity occurs at neutral pH and is characterized by a K_M that reflects the physiological concentration of the substrate (~ 0.2 mM) and a turnover number of about 0.6 sec^{-1} . The acidic arm of the profile reflects occupancy of the catalytic zinc site and the basic arm reflects accumulation of hexamer, with an apparent pK_a of $\sim \text{pH } 8$. At pH 9 WT reveals biphasic kinetics with K_M values reflecting both octamer (15 μM) and hexamer (4.5 mM) (Table 1). In contrast, F12L has negligible activity at neutral pH, with a K_M of ~ 18 mM. As the pH rises, the activity rises and at pH 9 the kinetic parameters are nearly indistinguishable

from the high K_M component of the WT protein. Thus, it appears that the hexameric structure of F12L is pH independent, while WT transitions from octamer to hexamer as the pH rises. At pH 9, WT is predominantly hexameric. Without the N-terminal arm providing stabilization for a closed active site lid, hexamer activity requires a high pH essential for Schiff base formation. Without a stable active site lid to help bind A-side ALA, the hexamer has a very high K_M .

Co-expression of WT and F12L from the same plasmid revealed subunit disproportionation.

WT and F12L were expressed together from a bicistronic plasmid from which the purification revealed the expression of both octamer and hexamer (see Fig 2A), each of which were shown by mass spectroscopy to contain both Phe and Leu at position 12 (9). These “WT+F12L” hetero-multimers revealed themselves to be metastable (19). Figure 3D shows the pH-dependent and turnover-dependent transition of WT+F12L hetero-hexamer to hetero-octamer. During this process, the WT chains accumulated in the octamer while the F12L chains accumulated in the hexamer (19). The disproportionation of chains definitively established the dissociative nature of the transition.

Phylogenetic variation teaches about the control of human PBGS quaternary structure

Characterization of WT, F12L, and the WT+F12L hetero-multimers followed earlier studies of the microbial and plant PBGS proteins, whose behaviors informed our interpretation of data on human PBGS. For example, the *E. coli* protein requires zinc at the enzyme active site but also experiences allosteric activation by addition of magnesium, which binds allosterically (20). In the absence of magnesium, *E. coli* PBGS has a high K_M value (>1 mM), which drops significantly in the presence of magnesium (~ 0.2 mM). When purified in the presence of zinc, but not magnesium, homogeneous *E. coli* PBGS resolved into multiple bands on native PAGE (Fig 4A & B). Addition of magnesium (with or without substrate) stabilized a larger molecular mass assembly, presumed to be the octamer (Fig 4 B). Addition of both magnesium and substrate results in nearly total conversion to the octamer. Two dimensional native PAGE revealed that octamer stabilization during turnover conditions can occur within the gel matrix (Fig 4C) (20). The importance of magnesium in the octameric assembly of plant PBGS is illustrated in Fig 4D wherein addition of the magnesium-chelator EDTA promotes the transition from octamer to hexamer in PBGS from the green plant pea. A few years later a PBGS crystal structure from a bacterial species revealed the binding site for the allosteric magnesium as the octamer-specific arm-to-barrel interaction described above as promoting stabilization of the active site lid which in turn promotes binding of the K_M -determining substrate (21). The allosteric magnesium of these bacterial PBGS is isosteric with the guanidinium group of an arginine residue that is conserved in all PBGS that do not have the sequence determinants for the allosteric magnesium (Fig 5A) (10). In human PBGS, this is Arg240.

Single amino acid substitutions can dramatically alter the human PBGS quaternary structure equilibrium.

The identification of Arg240 as a probable ionizable residue intimately involved in the pH dependence of the WT quaternary structure raised the possibility that a single amino acid change might dramatically alter the human PBGS quaternary structure equilibrium in a predictable fashion, unlike the naturally occurring F12L variant. To first test this hypothesis, we characterized the R240A variant, designed to destabilize the octamer-specific arm-to-barrel interface (10). A second variant, W19A, was designed to destabilize the penetrating arm-to-back-of-barrel interaction essential to assembly of both octamer and hexamer (10).

R240A purifies as a metastable hexamer that can be drawn toward octamer by addition of substrate.

Expression and purification of R240A yielded protein in a 20:80 octamer:hexamer ratio as determined by native PAGE and by assaying ion exchange fractions at both pH 7 and pH 9 (10). Interestingly, after a few days, the separated octamer and hexamer pools had re-equilibrated back toward the original 20:80 ratio. Dialysis of R240A against assay buffer containing substrate caused the equilibrium to shift toward octamer in a pH dependent fashion. Dialysis back into substrate-free buffer allowed the quaternary structure equilibrium to relax back to favor the hexamer. Why WT, R240A, and the WT+F12L heteromers can have their quaternary structure equilibrium manipulated by the presence of substrate, while the homo-hexameric F12L assembly cannot remains a mystery. Puzzlingly, extended incubation of F12L with divalent lead resulted in accumulation of measurable amounts of octamer.

W19A is a dimer, even in the presence of substrate.

A second variant, W19A, lacks the tryptophan of one subunit that penetrates the back of the $\alpha\beta$ -barrel of a neighboring subunit in the diagonal dimer. This subunit-subunit interaction is shared by both octamer and hexamer. Thus, W19A was designed to destabilize the arm-to-back-of-barrel interaction essential to assembly of both octamer and hexamer. W19A purifies predominantly as dimer, which has kinetic parameters at pH 9 that are indistinguishable from the F12L hexamer (10). A second IEC pool was of lower activity and appeared to be a dimer/tetramer mixture. Table 1 contains the kinetic parameters of WT, F12L, R240A, and W19A both at pH 7 and pH 9. The double hyperbolic behavior of WT at pH 9 is indicative of a slowly exchanging mixture of both octamer and hexamer (22).

Disease-associated single amino acid variants of human PBGS are hexamer-stabilizing.

Having established that single amino acid variants like F12L, R240A, and W19A can dramatically shift the human PBGS quaternary structure equilibrium away from the high activity octamer, we asked if this was also characteristic of any of the variants associated with ALAD porphyria.

ALAD porphyria

ALAD porphyria is the inborn error of metabolism caused by severely diminished PBGS activity, which is a very rare and highly heterogeneous recessive disease in patients who are compound heterozygotes for a small number of disease-associated single-amino acid variants (23). Each of the single amino acid substitution variants were expressed and characterized *in vitro* in order to determine if they altered the PBGS quaternary structure equilibrium (24). Table 2 contains the data showing that each disease-associated variant favors the hexameric assembly more than WT. Evidence for an increase in the hexamer component of the PBGS octamer-hexamer equilibrium came from the behavior of these variants on ion exchange resin, native PAGE, and the appearance of double hyperbolic kinetics at neutral pH (24). As shown in Table 2, the individual variants perturb the PBGS quaternary structure equilibrium to varying degrees; none have been studied *in vitro* as the mixed heteromers that exist in patients. The disproportionation behavior of the WT+F12L construct that we studied *in vitro* teaches us that the composition of the heteromultimers that function in patients will likely vary with time, in response to enzyme turnover.

Some aspects of lead poisoning are like ALAD porphyria. Interestingly, most instances of severely diminished PBGS activity is not attributed to the inborn error of metabolism. Rather, it is due to lead poisoning (25). Unlike most other zinc metalloenzymes, the zinc at the active site of human PBGS is in an environment that is well adapted to being displaced by divalent lead (26), which has been introduced into the environment by man over centuries, most notably in modern times through the use of lead based paints, leaded gasoline, and lead pipes (e.g. (27)).

Practical and health-related applications of small molecule perturbation of the PBGS quaternary structure equilibrium.

An equilibrium of alternate multimers with alternate functions can potentially be harnessed to control biological systems (e.g. (28)). For PBGS, both the octamer and hexamer have multimer-specific surface cavities that might serve as small molecule binding sites (Fig 6). Because these surface cavities are not at the enzyme active site, we find they are phylogenetically variable. Thus, small molecules that perturb the equilibrium may be species-specific. One can imagine four such applications related to human health. First, there is the small molecule that could stabilize the octameric assembly and act therapeutically for patients with ALAD porphyria. To date, there is no progress on this front (unpublished). Second are the potentially detrimental health effects of molecules that stabilize the human PBGS hexamer and inhibit this essential activity (29–31). Progress on this front has led to the identification of a mechanism for porphyria promoting drug side effects. The most striking example is the OTC analgesic diclofenac, which is contraindicated for patients experiencing porphyria (30). The third and fourth applications were unexpected because tetrapyrrole biosynthesis is universally required. Thus PBGS might be considered an unlikely target for the development of antibiotics and/or herbicides. However, the arm-pit of the PBGS hexamer is in a region of the protein that is phylogenetically variable. Hence, a third application is stabilizing the PBGS hexamer of a human pathogen as a new approach to antibiotic discovery (32–35). In this case, we have learned that many pathogens such a

Pseudomonas aeruginosa, *Wolbachia endobacteria*, and *Toxoplasma gondii* have evolved their PBGS to avoid formation of the susceptible hexameric assembly. The fourth application is as a herbicide. Here, preliminary success was achieved in stabilizing a plant PBGS hexamer, while leaving the human protein unscathed (36). This result has potential for further development into a herbicide.

Concluding remarks

The unexpected discovery of the PBGS quaternary structure equilibrium started with characterization of a disease-associated human variant (9). However understanding the phenomenon as a structural basis for allostery, rather than a one-off artifact, relied on our knowledge of PBGS across broad phylogenetic space (e.g. (8)). We now know that PBGS from many species can equilibrate among octamer, hexamer, and dimer wherein the domain orientation in the dimer dictates the architecture of functionally distinct multimers. In the PBGS example, control of this equilibrium is by pH, the availability of substrate or metal ions, single amino acid substitutions, or small molecules that bind to multimer-specific surface cavities (1,37). Such an equilibrium can be harnessed for drug discovery or serve to explain drug side effects (28). However, many of the described aspects of the interchange of PBGS quaternary structure isoforms, when first observed, were unexpected and met with skepticism. They defied a long-accepted one sequence/one structure/one function dogma in a way that had not previously been observed. The assemblies, though interchangeable, were long-lived (e.g. metastable). In fact, we had studied PBGS for decades, seeing hints of this behavior without understanding. Only the unexpectedly hexameric crystal structure of the F12L variant (9), and knowledge of the role and location of magnesium in a bacterial PBGS (8,38), allowed the puzzle pieces to fall into place. In 2005 we generalized these observations in describing the morpheein model of protein allostery and predicted that such dissociative allosteric phenomena may be common, but go unnoticed because of common presumptions that we (and others) routinely use to interpret protein behaviors (39). It is now satisfying to see documentation of other homomeric protein assemblies that dissociate, change conformation in the dissociated state, and reassemble differently with functional consequences. A few examples include Ebola virus VP40 (40), ribonucleotide reductase (41–43), retroviral integrase (44,45), and pyruvate kinase (46). We have proposed that the morpheein model of allostery may also apply to phenylalanine hydroxylase (47), whose dysfunction causes the most common inborn error of amino acid metabolism, phenylketonuria. Whether in controlling activity level (as in PBGS), or discriminating among alternate “moonlighting” functions (as in VP40), a dissociative equilibrium of architecturally distinct assemblies is a powerful framework for understanding disease and designing therapeutics.

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Figure 1. The PBGS catalyzed reaction in the context of the PBGS assemblies.

A. The PBGS-catalyzed asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to form the monopyrrole, porphobilinogen. Porphobilinogen is a precursor to all the tetrapyrrole pigments of life. **B.** Elements within and outside the human PBGS active site that impinge upon the equilibrium of PBGS assemblies. Each of the illustrated interactions stabilizes the human PBGS octamer. The substrate molecules are in green. The active site Zn is essential for binding A-side ALA. Interaction between A-side ALA and lid residues Arg209 and Arg221 provide a low K_M and stabilize the closed conformation of the active site lid. Amino acids from a neighboring subunit are shown in blue. Interaction between Arg240 of one subunit and Ser5 of a neighboring subunit stabilizes an octamer-specific intersubunit interaction. The guanidinium group of Arg240 is spatially equivalent to the allosteric magnesium of plant and most bacterial PBGS. Interaction between Cys223, which is at the C-terminal end of the active site lid, and Phe12 is an octamer-specific interaction. **C.** Subunit organization in the PBGS octamer (PDB: 1E51, top) and the PBGS hexamer (PDB: 1PV8, bottom). In both cases, the crystallographic asymmetric unit is shown in black and cyan; the N-terminal arm is shown in cartoon. In the octamer, this is a “hugging dimer” where the arm of one subunit hugs the barrel of an adjacent subunit. The “diagonal dimer” for each assembly is shown in black and magenta. In both illustrations, the remaining subunits of the assembly are shown in white space filling representation. The half-of-the-sites bound ligands are shown and colored cpk. In the octamer, this is enzyme-bound

product. In the hexamer, it is a Schiff base intermediate with P-side ALA (shown in part B).
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Figure 2. The equilibrium of human PBGS octamer and hexamer proceeds through a conformationally flexible dimer.

The interconversion between octamer and hexamer requires multimer dissociation to the level of a dimer. Based on analysis of high resolution crystal structures this dissociation likely occurs along a protein-protein interface that is extensively hydrated at phylogenetically conserved residues (17). The dimer can undergo a hinge motion, which has been shown to be the rate determining step in the interconversion (17). The hinge motion occurs between the $\alpha\beta$ -barrel and the N-terminal arm. The pro-octamer conformation of the dimer assembles to the octamer; the pro-hexamer conformation of the dimer assembles to the hexamer.

Figure 3. Behaviors of human PBGS octamer and hexamer.

A. Ion exchange chromatography can resolve hexamer from octamer due to a difference in surface properties. **B.** Native PAGE shows mobility difference between the F12L hexamer and the WT octamer and revealed the hexameric component of the WT protein. **C.** pH rate profiles of WT (red squares) and F12L (blue circles) obtained using 5 min assays at 37 °C and 10 mM ALA. K_M values determined at pH 7 and pH 9 are indicated (see Table 1). **D.** Native PAGE is used as an analytical tool to demonstrate that the WT+F12L hetero-hexamer is metastable. Transition to the hetero-octamer occurs during catalysis (37 °C at an initial

[ALA] of 10 mM, when present). During this transition, the Phe12 containing chains accumulate in the octamer while the Leu12 containing chains accumulate in the hexamer (19).

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Figure 4. Quaternary structure behaviors of microbial and plant PBGS.

A. Coomassie stained SDS PAGE shows purified *E. coli* PBGS as a single band. **B.** Coomassie stained native PAGE of *E. coli* PBGS (purified in the presence of Zn, but not Mg) shows multiple bands. From top to bottom, these bands have relative mobility consistent with octamer, hexamer, tetramer, and dimer. Additions of EDTA, Mg, ALA, or Mg + ALA show dramatic effects on the distribution of multimers. Both Mg and substrate are required to fully stabilize the highest order multimer, presumably the octamer. **C.** Two dimensional native PAGE on *E. coli* PBGS visualized using an activity stain. The first dimension is run without any additions (like the first lane in the gel in part **B**). The gel is then removed from the PhastGel apparatus and soaked in a complete assay mixture (+Zn, +Mg, +ALA). Native PAGE is then run in the 2nd dimension prior to the activity stain. This gel shows that nearly all of the various multimers that separate in the first dimension can transition to the octamer within the gel during the assay conditions soak. **D.** Plant PBGS (*P. sativum*, pea), purified in the presence of Mg, is shown to transition from octamer to hexamer when incubated with EDTA. Sequence and biochemical/biophysical characterization suggests that the plant protein may use magnesium both at the enzyme active site and at the allosteric site (48).

Figure 5. The spatial equivalence of the allosteric magnesium of plant and bacterial PBGS with a highly conserved arginine of metazoan (animal) and fungal PBGS.

Shown left is the *E. coli* PBGS octamer (PDB: 1L6S, top). Below is an enlargement showing the allosteric magnesium securing the “hugging dimer” interface. The human PBGS octamer (PDB: 1E51, top) is shown in the middle. The enlargement below shows the guanidinium group of Arg240 securing the octamer-specific hugging-dimer interface. Shown right is the human PBGS hexamer (F12L, PDB: 1PV8), which lacks this inter-subunit interface.

Figure 6. The PBGS assemblies contain multimer-specific small molecule binding sites that can be used for the design/discovery of allosteric regulators.

Multimer-specific sites are shown with a white circles. On top are two orientations of the octamer. The left image highlights the phylogenetically variable (Mg or Arg) site that stabilizes the octamer (see also Fig 5). The right image shows the central cavity, which could bind an octamer-stabilizing therapeutic for ALAD porphyria. Shown on bottom are two orientations of the hexamer. Shown left is the “arm pit” that was successfully used to computationally screen for species specific PBGS inhibitors (31,36). Shown right is the central cavity, which has not yet been identified as the binding site for a specific inhibitor.

TABLE 1:

Human PBGS kinetic parameters for WT and variants that perturb the quaternary structure equilibrium. Data derived from 5 min stopped time assays carried out at 37 °C.

Parameter	Assay pH	Wild-type (pH dependent octamer/hexamer equilibrium)	F12L (hexamer)	R240A (pH dependent octamer/hexamer equilibrium)	W19A	
					dimer	dimer/tetramer mix
K_M^1	7	0.25 ± 0.01				
V_{MAX}^1	7	55.5 ± 0.2				
K_M^2	7		17.7 ± 1.1	6.92 ± 0.35		
V_{MAX}^2	7		1.14 ± 0.05	3.56 ± 0.09		
K_M^1	9	0.015 ± 0.01				
V_{MAX}^1	9	8.16 ± 0.13				
K_M^2	9	4.46 ± 0.8	4.6 ± 0.1	3.8 ± 0.1	4.36 ± 0.27	5.02 ± 0.28
V_{MAX}^2	9	6.67 ± 0.36	18.2 ± 0.2	10.2 ± 0.1	18.3 ± 0.52	9.76 ± 0.25

TABLE 2:

Characteristics of human PBGS and variants associated with ALAD porphyria.

Variant	% Activity ¹	octamer:hexamer	Best Fit K _M values (mM) at pH 7 ²	
			Octameric K _M	Hexameric K _M
WT (Asn59) ³	100.0	95:5	0.25	-
F12L	0.2	0:100	-	17.0
E89K	64.0	69:31	0.16	6.6
C132R ⁴	0.3	64:36	0.13	20.0
G133R	12.0	52:48	0.19	8.9
V275M	83.0	94:6	0.11	1.4
R240W	4.8	21:80	0.07	35.0
A274T	60.0	86:14	0.18	4.0
V153M	38.0	95:5	0.10	3.1

¹ As purified from a heterologous expression in *E. coli* and assayed at 10 mM ALA, pH. 7.0.

² Two K_M values are reported when the data fit to a double hyperbolic equation, indicative of the presence of both octamer and hexamer.

³ Asn59 and Lys59 are the two most common alleles in the human population.

⁴ Cys132 is a ligand to the catalytic Zn (see Fig 1B).