Published in final edited form as:

Arch Biochem Biophys. 2012 March 15; 519(2): 144–153. doi:10.1016/j.abb.2011.10.010.

Allostery and the dynamic oligomerization of porphobilinogen synthase

Eileen K. Jaffe and Sarah H. Lawrence

Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA, 19111

Abstract

The structural basis for allosteric regulation of porphobilinogen synthase (PBGS) is modulation of a quaternary structure equilibrium between octamer and hexamer (via dimers), which is represented schematically as $8mer \rightleftharpoons 2mer \rightleftharpoons 2mer^* \rightleftharpoons 6mer^*$. The “*” represents a reorientation between two domains of each subunit that occurs in the dissociated state because it is sterically forbidden in the larger multimers. Allosteric effectors of PBGS are both intrinsic and extrinsic and are phylogenetically variable. In some species this equilibrium is modulated intrinsically by magnesium which binds at a site specific to the 8mer. In other species this equilibrium is modulated intrinsically by pH; the guanidinium group of an arginine being spatially equivalent to the allosteric magnesium ion. In humans, disease associated variants all shift the equilibrium toward the 6mer* relative to wild type. The 6mer* has a surface cavity that is not present in the 8mer and is proposed as a small molecule allosteric binding site. *In silico* and *in vitro* approaches have revealed species-specific allosteric PBGS inhibitors that stabilize the 6mer*. Some of these inhibitors are drugs in clinical use leading to the hypothesis that extrinsic allosteric inhibition of human PBGS could be a mechanism for drug side effects.

Porphobilinogen synthase (PBGS) is an ancient metabolic enzyme responsible for the biosynthesis of the fundamental monopyrrole that is the building block for myriad tetrapyrrolic cofactors required for life (porphyrin, chlorophyll, vitamin B12, siroheme, phytobilin, cofactor F430, etc.) [1]. The PBGS catalyzed reaction is an asymmetric condensation of two molecules of the substrate 5-aminolevulinic acid (ALA). All organisms that carry out respiration, photosynthesis, or methanogenesis require PBGS, and the enzyme is remarkably conserved throughout evolution [2]. The metabolic pathway from porphobilinogen to the tetrapyrroles is phylogenetically variable but universally populated by photoreactive intermediates whose accumulation can be toxic [3–4]. Thus, as part of the control of tetrapyrrole biosynthesis, PBGS evolved an allosteric regulation mechanism. Unexpectedly, PBGS allostery involves alternate, functionally distinct multimeric assemblies whose architectures are so different as to require multimer dissociation as part of the interconversion between active and inactive assemblies [5]. Novel aspects of the allosteric regulation of PBGS led us to define a morpheein model of allostery (described elsewhere [6]); proteins that use this allosteric mechanism display properties that are characteristically different from proteins that follow the classic Monod-Wyman-Changeux and Koshland-Nemethy-Filmer models [7]. Herein, we describe the phylogenetically

© 2011 Elsevier Inc. All rights reserved

Corresponding Author: Eileen K. Jaffe Fox Chase Cancer Center 333 Cottman Avenue Philadelphia, PA, 19111 Tel: 215-728-3695 Fax: 215-728-2412 Eileen.jaffe@fccc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

variable allosteric regulation of PBGS; presumably these variations evolved due to the considerably different environments that organisms inhabit and the phylogenetic variation in the subcellular location of PBGS. In most species PBGS is located in the cytosol [8–9]. However, in plants it is in the chloroplast [10], and in apicomplexan parasites it is in the apicoplast [9].

An overview of PBGS structure and allostery

PBGS is encoded by a single gene and each PBGS multimer is composed of multiple copies of the same protein. Each PBGS subunit consists of a ~300 residue $\alpha\beta$ -barrel domain, which houses the enzyme active site in its center, and a >25 residue N-terminal arm domain (Fig. 1) [11]. Allosteric regulation of PBGS can be described in terms of the orientation of the $\alpha\beta$ -barrel domain with respect to the N-terminal arm domain [5]. These two domains undergo a dramatic reorientation around a hinge region in the transition between active and inactive multimers (Fig. 1). Like most $\alpha\beta$ -barrel enzymes, substrate access to the active site is mediated by a mobile loop that serves as an active site lid; the lid of each subunit comes from the same subunit as the rest of the active site. Allosteric regulation of PBGS can be described in terms of intersubunit interactions that assist in stabilizing an ordered conformation of the active site lid [12]. These interactions involve the N-terminal arm domain from a subunit that is different from the subunit contributing the active site. In the active multimeric state, the N-terminal arm of one subunit interacts with the $\alpha\beta$ -barrel domains of two other subunits. Similarly, each $\alpha\beta$ -barrel interacts with the N-terminal arm of two adjacent subunits. One of these interactions helps to stabilize a “closed” conformation of the active site lid; this is the A/B interaction of Figure 2. The other interaction restricts solvent access from the other end of the $\alpha\beta$ -barrel; this is the A/F interaction in Figure 2. In the inactive multimeric state, the N-terminal arm domain is not involved in the lid-stabilizing A/B subunit-subunit interaction. In the crystal structure of the inactive assembly, the active site lid is disordered (see Fig. 1 and Fig. 2B).

As described above, allosteric regulation of PBGS fits a familiar theme; the transition between active and inactive forms involves a conformational change that affects the traffic of ligands into and out of the enzyme active site. The initially unexpected aspect of PBGS allostery is that the active multimer is an octamer (8mer), the inactive multimer is a hexamer (6mer*), and the transition between 8mer and 6mer* requires dissociation to a dimer. The dimer exists in an equilibrium of conformations, one of which can assemble to the 8mer, another of which can assemble to the 6mer*. The shorthand equation for this phenomenon is $8mer \rightleftharpoons 2mer \rightleftharpoons 2mer^* \rightleftharpoons 6mer^*$, where the oligomer-determining conformational change ($2mer \rightleftharpoons 2mer^*$) is a reorientation of the N-terminal arm domain of each subunit with respect to the $\alpha\beta$ -barrel domain of the same subunit (seen most clearly in Fig. 1B). Steric considerations prevent this reorientation from occurring in either the 8mer or 6mer*, thus dissociation is an essential part of the allosteric mechanism.

The allosteric dance of PBGS

Many PBGS proteins can participate in the quaternary structure dynamic equilibrium illustrated in Figure 3A, though the 2mer* and 6mer* assemblies are unavailable to some PBGS (Fig. 3B). The octameric assembly is supported by PBGS crystal structures from metazoa, fungi, bacteria, and alveolata (e.g. [11, 13–16]). The bacterial and alveolata structures all contain an allosteric magnesium ion located at the aforementioned lid-stabilizing arm-to-barrel A/B interface (Fig. 3B and 6B below) [2, 13–15]. An extensive phylogenetic analysis determined that the allosteric magnesium binding site is present in PBGS from nearly all species except metazoa and fungi [2]. A multiple sequence alignment of the PBGS discussed in this review is provided in Figure 4 with key structural features highlighted. Before we appreciated the significance of the smaller alternate PBGS

multimers, native PAGE was used to show that addition of magnesium to *E. coli* PBGS induces assembly to 8mer (Fig. 5A) [17]. The 6mer* assembly is supported by the crystal structure of the human PBGS variant F12L and by analytical ultracentrifuge studies of both human and plant PBGS [5, 18]. In fact, native PAGE (Fig. 5B) and analytical ultracentrifugation have been used to show that removal of magnesium from plant PBGS promotes formation of the hexamer [5, 17]. The existence of dimeric assemblies is supported by size exclusion chromatography (SEC) and native PAGE for PBGS proteins from various species [19–20], but our understanding of the physiologically relevant dimer architecture has evolved in the past decade; furthermore the interconversion pathway may not be phylogenetically conserved.

Figure 3A illustrates two alternate pathways for interconversion of hexamer and octamer; the upper pathway shows the transition through “hugging” and “detached” dimers that are the asymmetric units of human PBGS crystal structures, while the lower pathway shows the transition through the alternate “pro-octamer” and “pro-hexamer” dimers. The identity of the dimeric species, which comprise less than 1% of the total human PBGS in solution [19], remains an unknown but intriguing question. Thermodynamic rationale for favoring one pathway over the other is discussed extensively in [19], and summarized below.

The hugging and detached dimers were originally considered as the solution structures for the PBGS dimers and were depicted as such in many of our earlier publications (e.g. [5–7, 18, 21–24]). Dissociation of an 8mer or 6mer* to hugging or detached dimers (respectively) involves breaking the subunit-subunit interface wherein the N-terminal arm of one subunit excludes solvent access from the back of the $\alpha\beta$ -barrel of another subunit (A/F interface of Fig. 2A). Breaking this arm-to-back-of-barrel interface exposes a hydrophobic patch dominated by Trp19. In support of the importance of Trp19 to multimerization, the human PBGS variant W19A is dimeric and cannot assemble to either 8mer or 6mer* [22]. We note that the residues that participate in this arm-to-back-of-barrel (A/F) interaction are not phylogenetically conserved. For instance, in PBGS sequence alignments (e.g. Fig. 4), Trp19 of human PBGS appears as an insertion in the N-terminal arm domain, which itself has a much lower sequence conservation than the $\alpha\beta$ -barrel domain [15]. The primary conformational change between the hugging and detached dimers is observed as a rotation that changes the orientation of the N-terminal arm (Fig 3A). In the “arm down” conformation of the hugging dimer, the hugging interaction of the arm with the $\alpha\beta$ -barrel of its partner monomer is evident. In the context of the 8mer, additional interactions that may stabilize the secondary structure of the arm also derive from a third subunit (subunit F in Figs. 2A and 3A). The “arm up” conformation of the detached dimer places the arm distal from the $\alpha\beta$ -barrel of its partner monomer, thereby leaving this arm with no protein-protein stabilizing interactions. This lack of stabilizing interactions for the N-terminal arm conformation in the detached dimer, coupled with exposed hydrophobic patches in both hugging and detached dimers lead us to hypothesize that oligomeric interconversion proceeds through the pro-octamer and pro-hexamer dimers as discussed below.

Dissociation via the pro-octamer and pro-hexamer dimers exposes the large A/B subunit-subunit interface, which has two components. One component of the A/B interface is a symmetric barrel-barrel interaction that is highly conserved, dominated by hydrophilic residues and charge-charge interactions, and for which water-containing crystal structures from different species all contain ordered water molecules whose locations are phylogenetically conserved. Components of this interface include reciprocal bonds between the universally conserved ion pair Asp259 and Arg309, and the hydrogen bonded pair Tyr257 and Glu302 (numbering corresponds to human PBGS). For human PBGS the barrel-barrel interface does not contain any aromatic-aromatic intersubunit interactions, nor does it contain a hydrophobic patch like that described above for the arm-to-back-of-barrel

interface. The second component of the A/B interface is the hugging arm-to-barrel interaction that stabilizes the ordered conformation of the active site lid. Although phylogenetically variable, this arm-to-barrel interaction is also hydrophilic, significantly hydrated in structures containing modeled waters, and includes the binding site for the aforementioned allosteric magnesium (see Fig. 3B). Metazoan and fungal PBGS, which do not have the magnesium binding site, contain instead a spatially equivalent arginine guanidinium group (Fig. 6). Removal of this arginine (e.g. human PBGS variants R240A and R240W [22–23]) results in a protein that favors assembly to 6mer* relative to wild type, which favors assembly to 8mer. We have argued that dissociation along the A/B interface is energetically feasible because the dissociating surfaces are hydrophilic and already hydrated in 8mer and 6mer* [19]. The same changes in ϕ/ψ angles that produce the arm-up and arm-down conformations of the hugging and detached dimers are reflected in the pro-octamer and prohexamer dimers as a twist at a hinge between the N-terminal arm and the $\alpha\beta$ -barrel domains. This twist changes the orientation of the two $\alpha\beta$ -barrels relative to each other (best seen in Fig. 3A) and, importantly, maintains the hydrophobic arm-stabilizing arm-to-back-of-barrel interactions of the A/F interface for both the pro-octamer and prohexamer dimers. Further support of the physiologic relevance of the pro-octamer dimer comes from the X-ray crystal structure of PBGS from *Toxoplasma gondii*, as shown in Fig. 3B and discussed below.

As noted above, the N-terminal arm of PBGS, whose position is of pivotal importance to multimer architecture, has significant phylogenetic variability. When first characterizing the oligomeric equilibrium, we found the 8mer $\rightleftharpoons$ 2mer $\rightleftharpoons$ 2mer* $\rightleftharpoons$ 6mer* pathway to be common to PBGS from both humans and plants, even though the N-terminal sequences vary (see Fig. 4). Thus, when we undertook characterization of PBGS from bacterial and eukaryotic pathogens, we were surprised to discover that PBGS from many of these species does not sample the 2mer* $\rightleftharpoons$ 6mer* components of the equilibrium. Two of these species are the human pathogens *Vibrio cholerae* and *Toxoplasma gondii*, where our work with recombinant PBGS provided no evidence for the hexamer as a component of the equilibrium of quaternary structure isoforms [20]. Native PAGE of PBGS from these species shows predominantly octamer, with low levels of dimer present. Sequence alignment shows that these two species of PBGS are among a relatively small number that also includes a short sequence C-terminal to the common $\alpha\beta$ -barrel domain [14]. The recently solved crystal structure for *T. gondii* PBGS revealed that a 13 amino acid C-terminal extension (see Fig. 4) forms a domain-swapped intersubunit β -sheet (Fig. 3B) that wraps around the hinge region of the pro-octamer dimer and prevents rotation to the pro-hexamer dimer, thus preventing hexamer formation (Fig. 3B) [14]. We posit that for other species where PBGS has a C-terminal extension, a similar intersubunit β -sheet prevents population of the hexamer. In PBGS where the pro-octamer dimer is tethered by a β -sheet, it appears that this is the architecture of the dimeric component of the quaternary structure equilibrium. For these species, it may have been evolutionarily advantageous to keep PBGS in an active octameric conformation. Alternatively, allosteric regulation may be achieved through a simple equilibrium between 8-mer and pro-octamer dimer. Although the C-terminal extension provides a rationale for why some species of PBGS do not populate the 2mer* $\rightleftharpoons$ 6mer* portion of the quaternary structure equilibrium, it does not explain why some other PBGS without this extension also fail to form the hexamer. One well characterized example is PBGS from *Pseudomonas aeruginosa*, where we have found small molecules that stabilize a dimeric assembly (see below) [25].

The physiologic relevance of the human PBGS quaternary structure equilibrium

Inborn errors of metabolism often arise from defects in protein structure and function. ALAD-porphyria is a very rare inborn error of metabolism that arises from mutations to the

gene encoding PBGS [26–27]. Wild type levels of PBGS activity are far in excess of that required for heme biosynthesis [8]. Consequently, individuals with clinical ALAD porphyria result from compound heterozygotes who inherited one defective gene from each of two asymptomatic parents. With insufficient PBGS activity, these patients can experience elevated levels of ALA, a structural analog of the neurotransmitter gamma-amino butyric acid, resulting in adverse neurologic consequences. There are eight ALAD-porphyria associated variants that have been documented, one of which is the aforementioned F12L. When heterologously expressed, F12L appears as an obligate hexamer with a pH rate profile that is dramatically different from the wild type protein (Fig. 7A). However coexpression of the wild type and F12L variant leads to production of both 8mer and 6mer* hetero-multimers; the pH rate profile of the hetero-6mer* is included in Figure 7A and highlights its metastable character (see below). The positions of the seven additional ALAD-porphyria associated variants are widely distributed throughout the protein and, like F12L, their positions would not be predicted to affect the quaternary structure equilibrium (Fig. 7B). However, when heterologously expressed in *E. coli*, each of the ALAD-porphyria associated variants exhibited an increased mole fraction of hexamer relative to the wild type protein [23]. This disease association was the first indication of the physiologic relevance of the human PBGS quaternary structure equilibrium. Later studies revealed drug-derived allosteric inhibitors of human PBGS (see below) [28–29].

The allosteric regulators of PBGS

As a nearly universally essential enzyme with a highly conserved active site, PBGS is not a canonical target for the development of antimicrobials and/or herbicides. However, allosteric sites can be much more phylogenetically variable than active sites and present drug development opportunities. Phylogenetic variations in the PBGS quaternary structure equilibrium suggests that we can target allosteric regulation of PBGS in a species-specific manner for development of antimicrobials and herbicides. The classic definition of an enzyme's allosteric regulator is an entity that binds at a site other than the active site and alters the rate of the catalytic reaction. The earliest revealed allosteric regulators were metabolites, which can be considered intrinsic. Alternatively, extrinsic factors are exogenous entities such as drugs or toxins that bind outside the enzyme active site but change the protein structure in such a way as to inhibit activity. Phylogenetic variation in PBGS allostery leads us to frame discussion of PBGS allosteric regulators in terms of intrinsic and extrinsic factors.

Intrinsic allosteric regulation of PBGS

Magnesium—One intrinsic allosteric regulator is the aforementioned allosteric magnesium ion, which is generally present in PBGS from species other than metazoa and/or fungi. The allosteric magnesium ion lies at the highly hydrated (A/B) interface of two prooctamer dimers (Fig. 6A). It has only one amino acid side chain-derived first coordination sphere ligand (always an aspartate; Asp231 in *E. coli* PBGS), with the other five ligands being water molecules. Second coordination sphere ligands include the guanidinium group of an arginine that comes from a different subunit (Arg11 of *E. coli* PBGS), which is in the N-terminal arm of an adjacent pro-octamer dimer. Thus, this magnesium appears to be easily dissociable and we have shown by native PAGE and analytical ultracentrifugation that hexamers accumulate when magnesium is removed *in vitro* (see Fig. 5B) [5, 18]. In plants, where PBGS is located in the chloroplast, the $K_{\text{activation}}$ for the allosteric magnesium is ~ 2.5 mM [30]. We posit that magnesium activation of PBGS in plants is physiologically relevant because one of the earliest events when developing chloroplasts are exposed to light is a change in the magnesium concentration from <1 mM to >10 mM [31]. Accumulation of photoreactive chlorophyll precursors in the dark can be deadly; for instance, application of the PBGS substrate ALA to plants in the evening has been used as a herbicide [32–36]. The

physiologic relevance of the PBGS allosteric magnesium in other species is unclear, particularly in light of the fact that magnesium in the millimolar range is required for many biochemical structures and metabolic processes (e.g. oligonucleotide structure, phosphoryl transfer reactions, ligases).

pH—It is not common to consider protons (or hydronium ions) as allosteric regulators. However, in the case of PBGS, side chain protonation at locations other than the active site have been shown to affect the quaternary structure equilibrium, and thus to affect the rate of the PBGS catalyzed reaction. Here we discuss the arginine residue, Arg240 of human PBGS, whose guanidinium group is spatially and functionally equivalent to the allosteric magnesium (Fig. 6B). This arginine is conserved in PBGS from metazoa and fungi. The importance of Arg240 to the stability of the human PBGS 8mer is illustrated by the behavior of our designed variant R240A and the naturally occurring ALAD-porphyria associated variant R240W [22–23]. Although wild type human PBGS purifies at an 8mer:6mer* ratio of 95:5, this ratio for R240W is 20:80, and for R240A is 10:90. We posit that this shift in quaternary structure equilibrium is related to the role of Arg240 in stabilizing the interaction between two pro-octamer dimers; the guanidinium group of Arg240 forms a hydrogen bond with the carbonyl oxygen of Ser5 of a neighboring pro-octamer dimer. In the obligate 6mer* F12L variant, the displaced N-terminal arm (Fig. 6C) obviously also forbids the intersubunit hydrogen bond. Of course, this single hydrogen bond is not the only determinant for 8mer stability. Rather it is one of a multitude of factors that must all be present to maintain human PBGS as the 8mer as described in the following paragraphs.

The experimentally determined behavior of R240A teaches us about the fragile nature of the human PBGS 8mer, maintenance of which requires a multitude of factors illustrated in Figure 8A. Each of these factors contributes to stabilizing the ordered or “closed” conformation of the active site lid. Figure 8B illustrates an experiment wherein the isolated R240A 6mer* is dialyzed for 24 h against assay buffer containing the substrate ALA, at both pH 7 and pH 9, and samples are removed at various times to determine the 8mer:6mer* ratio by native PAGE [22]. Regardless of the pH, dialysis against substrate draws the equilibrium from 6mer* toward 8mer, though the extent of conversion is pH dependent. Stabilization of the active site lid, which occurs when the carboxyl group of A-side ALA interacts with two lid-derived arginine residues (see Fig. 8A), favors 8mer formation. PBGS crystal structures lacking a carboxyl-containing A-side ligand show a disordered active site lid. Raising the pH of the dialysis buffer from pH 7 to pH 9 causes a partial deprotonation of 8mer-stabilizing side chains, which may include the lid arginine residues, as well as Arg240, and reduces the ALA-induced conversion to 8mer. This pH-dependent decrease in the 8mer:6mer* ratio is also reflected in the basic arm of the human PBGS pH rate profile (see Fig. 7A) [19].

In the second phase of the experiment shown in Figure 8B, the dialysis cassette is transferred to the same buffer in the absence of substrate. Native PAGE demonstrates a time-dependent shift of the equilibrium back toward 6mer*. This slow transition back to 6mer* as the substrate induced active site lid stabilization dissipates illustrates the metastable nature of the human PBGS multimers. Another factor that is essential to binding A-side ALA to human PBGS is the active site zinc ion [37]; exposing the protein to the zinc chelator 1,10-phenanthroline also causes accumulation of the 6mer* (unpublished data). As illustrated in Figure 8A, there is one additional intersubunit interaction that appears essential to maintain the integrity of the 8mer. This is between the side chain of Cys223 and the backbone oxygen of Phe12 on the neighboring pro-octamer dimer. Although we do not yet fully understand why, we believe that the F12L mutation compromises this interaction and thus results in observation of 6mer* as the only higher order multimer.

The observations detailed above depict the human PBGS 8mer as relatively fragile, requiring many different interactions to maintain 8mer structural integrity. We posit that the half-of-the-sites reactivity seen in mammalian PBGS is a consequence of this frailty. Data supporting half-of-the-sites in mammalian PBGS stems from active site ligand trapping studies (e.g. [37–39]), and the stoichiometry of active site metal ion requirement (e.g. [40–42]). In the event that human PBGS were without A-side substrate (or product) bound at the enzyme active site, active 8mer stability is compromised. Therefore, the protein has evolved a half-of-the-sites mechanism where product is not released from one hemisphere of 8mer until the first substrate (P-side ALA) binds to the other hemisphere of 8mer. This ultimate form of negative cooperativity allows the protein to remain in the 8mer state. It is intriguing to hypothesize that maintenance of quaternary structure integrity may be a common theme for proteins that exhibit half-of-the-sites reactivity. This phenomenon could be considered a form of allostery, where binding events at one active site affect the reactivity of a neighboring active site.

Extrinsic allosteric regulation of PBGS

Small molecule hexamer stabilization

Inspection of the PBGS 6mer* reveals a surface cavity that is not present in the 8mer (Fig. 9A). Small molecule binding to this phylogenetically variable cavity was proposed to stabilize 6mer* of the targeted PBGS and consequently inhibit activity. We have called such allosteric regulators morphlocks because they lock PBGS in a specific morpheein form (6mer*). Although we have yet to find intrinsic ligands that act as 6mer*-stabilizing morphlocks, both computational docking and *in vitro* library screening have revealed a significant number of morphlocks as described below.

A computational docking approach was used to find morphlocks for PBGS from plants (*Pisum sativum*, a.k.a. pea), human, and the human pathogen *Yersinia enterocolitica*. The docking was directed to the 6mer*-specific surface cavity (Fig. 9A and 9B) and the small molecule library was a collection of ~300,000 drug like compounds that were reportedly commercially available from Life Chemicals Inc. In the case of pea PBGS and *Y. enterocolitica* PBGS, the docking targets were carefully constructed protein structure models, and in the case of human PBGS it was the F12L crystal structure [24, 28, 43]. Docking was used to refine the large library down to a number that we could afford to purchase and test *in vitro*. In each case 100 compounds were selected from the top scoring 1% of the docked molecules. In this case, docking to a crystal structure yielded a larger number of morphlocks than docking to a protein structure model [24, 28]. One such inhibitor is morphlock-1 (2-oxo-1,2-dihydro-benzo(*cd*)indole-6-sulfonic acid(2-hydroxy-2-[4-nitro-phenyl]-ethyl)-amide), which like most of our 6mer*-stabilizing PBGS inhibitors shows the predicted species specificity, inhibiting its targeted pea PBGS with an IC_{50} of ~1 μ M while no inhibition is seen for human PBGS at a morphlock-1 concentration of 100 μ M. Figure 9C illustrates an activity stained native PAGE of pea PBGS which establishes that morphlock-1 binds to the hexamer, inhibits enzyme activity, and is bound sufficiently tightly to prevent the substrate ALA drawing the equilibrium back to the octamer.

The first set of 6mer*-stabilizing human PBGS morphlocks arose from the computational docking study described above (and depicted in Fig. 9 for pea PBGS). Surprisingly, one of the identified human PBGS morphlocks (5-chloro-7-(diethylaminomethyl)quinolin-8-ol) is a chemical homolog to a drug currently in clinical use (5-chloro-8-hydroxy-7-iodoquinoline, a.k.a. clioquinol); purchase and testing of the drug confirmed that it is also a 6mer*-stabilizing human PBGS morphlock. The literature suggests that side effects of this drug mimic a porphyric attack [44] and we established that 6mer*-stabilizing inhibition of this drug was more potent with ALAD-porphyria associated variants, which are inherently more

prone to form the 6mer [28]. This result suggested significant physiologic relevance to the allosteric regulation of the human PBGS quaternary structure equilibrium by extrinsic factors, such as drugs and/or environmental contaminants. To address this hypothesis, a medium throughput native PAGE mobility shift assay was used to screen the Johns Hopkins Clinical Compound Library and an alarming 1% of the components in that library were found to act as 6mer*-stabilizing inhibitors of human PBGS *in vitro* [29]. One notable example is the widely available nonsteroidal anti-inflammatory diclofenac (a.k.a. Voltaren). Porphyrins in general are highly episodic diseases and the contributing factors are not well understood. One contributing factor is exposure to lead, which inhibits human PBGS by replacing the active site zinc. It is possible that exposure to small molecule 6mer*-stabilizing human PBGS allosteric inhibitors, such as the drugs we have identified, could be another contributing factor.

Small molecule dimer stabilization

Despite our remarkable success in the identification of 6mer*-stabilizing PBGS inhibitors, this approach to PBGS inhibitor discovery is not applicable to all species. Our studies of PBGS from the human pathogens *Pseudomonas aeruginosa*, *Toxoplasma gondii*, and *Vibrio cholerae* indicate that these proteins do not sample the 2mer* and 6mer* components of the equilibrium. Thus, discovery of allosteric regulators of these species of PBGS is directed at small molecule inhibition of 8mer assembly. Again a docking approach was taken targeting a site predicted to prevent 2mers from assembling to 8mers, and some success has been obtained. Unsure of the structure of the physiologically relevant dimer of *P. aeruginosa* PBGS, docking was carried out against the interface between two hugging dimers as well as the interface between two pro-octamer dimers. Small molecules that prevent dimer assembly to 8mer (as determined by native PAGE) were discovered only in the latter case [25]. In this case, however, addition of ALA was sufficient to draw the equilibrium back toward the active 8mer. Thus, none of these oligomerization disabling compounds were found to inhibit the bacterial PBGS catalytic activity. Efforts toward small molecule modulation of the quaternary structure equilibrium of *T. gondii* and *V. cholerae* PBGS are ongoing.

PBGS as the prototype morphoein

A dissociative quaternary structure dynamic can contribute to allostery

The morphoein model of allostery exemplified by PBGS adds an additional layer of understanding to potential mechanisms for regulation of protein function and complements the increased focus that the protein science community is placing on protein structure dynamics. The physiologic importance of phenomena such as alternate protein conformations, alternate oligomeric states, and transient protein-protein interactions are well-established in cell signaling (i.e. oligomerizing cell surface receptors), virology (i.e. the existence of multiple oligomers of identical virus coat proteins), and amyloid disease. The morphoein model of allostery illustrates how the dynamics of these phenomena can be harnessed for allosteric regulation of catalytic activity. The well-characterized PBGS example illustrates how a powerful dissociative allosteric mechanism has been adapted throughout evolution to provide phylogenetically variable control of an essential pathway. Our work on PBGS from various species illustrates how activity can be regulated via its oligomeric equilibria in response to a changing intracellular environment (intrinsic modifiers). Our *in silico* and *in vitro* screening has identified inhibitors that function by shifting the PBGS oligomeric equilibrium, thus highlighting the sensitivity of this allosteric mechanism to small molecules not native to the cell (extrinsic modifiers).

New questions about dissociative mechanisms

The quaternary structure equilibrium of PBGS provides an opportunity to address new aspects of protein folding and assembly. The dissociative nature of the 8mer $\rightleftharpoons$ 2mer $\rightleftharpoons$ 2mer* $\rightleftharpoons$ 6mer* equilibrium raises intriguing questions about the thermodynamics of multimer dissociation. For instance, we have not found rigorously benchmarked resources for determining the thermodynamic value of hydrating newly dissociated protein interfaces that take into account waters that are present prior to dissociation. Furthermore, only some protein crystal structures contain modeled water that can contribute to this calculation. These uncertainties confound theoretical identification of which interfaces are maintained or sacrificed when a higher-order oligomer dissociates. Such structural questions gain importance when searching for allosteric inhibitors designed to interfere with assembly of an inactive PBGS 2mer to the active 8mer. However, we intend to pursue experimental approaches that can help clarify the PBGS dissociative pathway.

REFERENCES

- [1]. Shemin D, Russell CS. J. Amer. Chem. Soc. 1953; 75:4873.
- [2]. Jaffe EK. Chemistry & Biology. 2003; 10:25–34. [PubMed: 12573695]
- [3]. Battersby AR. Nat Prod Rep. 2000; 17:507–526. [PubMed: 11152419]
- [4]. Holliday GL, Thornton JM, Marquet A, Smith AG, Rebeille F, Mendel R, Schubert HL, Lawrence AD, Warren MJ. Nat Prod Rep. 2007; 24:972–987. [PubMed: 17898893]
- [5]. Breinig S, Kervinen J, Stith L, Wasson AS, Fairman R, Wlodawer A, Zdanov A, Jaffe EK. Nature Structural Biology. 2003; 10:757–763.
- [6]. Jaffe EK. Trends in Biochemical Sciences. 2005; 30:490–497. [PubMed: 16023348]
- [7]. Lawrence SH, Jaffe EK. Biochem Mol Biol Educ. 2008; 36:274–283. [PubMed: 19578473]
- [8]. Shemin, D. The Enzymes. Boyer, P., editor. Academic Press; New York: 1972. p. 232-237.
- [9]. Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, Foth BJ, Tonkin CJ, Roos DS, McFadden GI. Nat Rev Microbiol. 2004; 2:203–216. [PubMed: 15083156]
- [10]. Smith AG. Biochemical Journal. 1988; 249:423–428. [PubMed: 3277625]
- [11]. Erskine PT, Senior N, Awan S, Lambert R, Lewis G, Tickle LJ, Sarwar M, Spencer P, Thomas P, Warren MJ, Shoolingin-Jordan PM, Wood SP, Cooper JB. Nature Structural Biology. 1997; 4:1025–1031.
- [12]. Jaffe EK. Bioorganic Chemistry. 2004; 32:316–325. [PubMed: 15381398]
- [13]. Frankenberg N, Erskine PT, Cooper JB, Shoolingin-Jordan PM, Jahn D, Heinz DW. J Mol Biol. 1999; 289:591–602. [PubMed: 10356331]
- [14]. Jaffe EK, Shanmugam D, Gardberg A, Dieterich S, Sankaran B, Stewart LJ, Myler PJ, Roos DS. J Biol Chem. 2011; 286:15298–15307. [PubMed: 21383008]
- [15]. Kervinen J, Jaffe EK, Stauffer F, Neier R, Wlodawer A, Zdanov A. Biochemistry. 2001; 40:8227–8236. [PubMed: 11444968]
- [16]. Mills-Davies, NL. Biochemistry. University of Southampton; Southampton, UK: 2000. Structure of human erythrocyte 5-aminolaevulinic acid dehydratase, the second enzyme in the biosynthesis pathway of haem.
- [17]. Jaffe EK, Ali S, Mitchell LW, Taylor KM, Volin M, Markham GD. Biochemistry. 1995; 34:244–251. [PubMed: 7819203]
- [18]. Kokona B, Rigotti DJ, Wasson AS, Lawrence SH, Jaffe EK, Fairman R. Biochemistry. 2008; 47:10649–10656. [PubMed: 18795796]
- [19]. Selwood T, Tang L, Lawrence SH, Anokhina Y, Jaffe EK. Biochemistry. 2008; 47:3245–3257. [PubMed: 18271513]
- [20]. Shanmugam D, Wu B, Ramirez U, Jaffe EK, Roos DS. Journal of Biological Chemistry. 2010; 285:22122–22131. [PubMed: 20442414]
- [21]. Tang L, Stith L, Jaffe EK. Journal of Biological Chemistry. 2005; 280:15786–15793. [PubMed: 15710608]

- [22]. Tang L, Breinig S, Stith L, Mischel A, Tannir J, Kokona B, Fairman R, Jaffe EK. *Journal of Biological Chemistry*. 2006; 281:6682–6690. [PubMed: 16377642]
- [23]. Jaffe EK, Stith L. *American Journal of Human Genetics*. 2007; 80:329–337. [PubMed: 17236137]
- [24]. Lawrence SH, Ramirez UD, Tang L, Fazliyez F, Kundrat L, Markham GD, Jaffe EK. *Chem Biol*. 2008; 15:586–596. [PubMed: 18559269]
- [25]. Reitz AB, Ramirez UD, Stith L, Du Y, Smith GR, Jaffe EK. *ARKIVOC*. 2010; 2010:175–188. [PubMed: 21643541]
- [26]. Maruno M, Furuyama K, Akagi R, Horie Y, Meguro K, Garbaczewski L, Chiorazzi N, Doss MO, Hassoun A, Mercelis R, Verstraeten L, Harper P, Floderus Y, Thunell S, Sassa S. *Blood*. 2001; 97:2972–2978. [PubMed: 11342419]
- [27]. Sassa S. *Int J Hematol*. 2000; 71:1–17. [PubMed: 10729988]
- [28]. Lawrence SH, Ramirez UD, Selwood T, Stith L, Jaffe EK. *J Biol Chem*. 2009; 284:35807–35817. [PubMed: 19812033]
- [29]. Lawrence SH, Selwood T, Jaffe EK. *ChemMedChem*. 2011; 6:1067–1073. [PubMed: 21506274]
- [30]. Kervinen J, Dunbrack RL, Litwin S, Martins J, Scarrow RC, Volin M, Yeung AT, Yoon E, Jaffe EK. *Biochemistry*. 2000; 39:9018–9029. [PubMed: 10913315]
- [31]. Walker CJ, Weinstein JD. *Biochem J*. 1994; 299(Pt 1):277–284. [PubMed: 8166650]
- [32]. Duke SO, Becerril JM, Sherman TD, Matsumoto H. *Acs Sym Ser*. 1991; 449:371–386.
- [33]. Rebeiz CA, Montazerzouhoor A, Hopen HJ, Wu SM. *Enzyme Microb. Technol*. 1984; 6:390–401.
- [34]. Mascal M, Dutta S. *Green Chem*. 2011; 13:40–41.
- [35]. Fukuda H, Casas A, Batlle A. *Int J Biochem Cell B*. 2005; 37:272–276.
- [36]. Sasikala C, Ramana CV, Rao PR. *Biotechnol Progr*. 1994; 10:451–459.
- [37]. Jaffe EK, Hanes D. *J Biol Chem*. 1986; 261:9348–9353. [PubMed: 3722199]
- [38]. Jaffe EK, Abrams WR, Kaempfen HX, Harris KA. *Biochemistry*. 1992; 31:2113–2123. [PubMed: 1346974]
- [39]. Seehra JS, Jordan PM. *Eur J Biochem*. 1981; 113:435–446. [PubMed: 7215335]
- [40]. Bevan DR, Bodlaender P, Shemin D. *J Biol Chem*. 1980; 255:2030–2035. [PubMed: 7354072]
- [41]. Jaffe EK, Volin M, Bronson-Mullins CR, Dunbrack RL, Kervinen J, Martins J, Quinlan JF, Sazinsky MH, Steinhouse EM, Yeung AT. *Journal of Biological Chemistry*. 2000; 275:2619–2626. [PubMed: 10644722]
- [42]. Sawada N, Nagahara N, Sakai T, Nakajima Y, Minami M, Kawada T. *J Biol Inorg Chem*. 2005; 10:199–207. [PubMed: 15747133]
- [43]. Ramirez UD, Myachina F, Stith L, Jaffe EK. *Adv Exp Med Biol*. 2010; 680:481–488. [PubMed: 20865533]
- [44]. Kauffman RE, Banner W, Blumer JL, Gorman RL, Lambert GH, Snodgrass W, Bennett DR, Cordero JF, Dooley S, Licata SA, Peterson R, Petricciani JC, Troendle G. *Pediatrics*. 1990; 86:797–798. [PubMed: 2146587]

- > Allosteric regulation of PBGS proceeds via a dissociative equilibrium of oligomers.
- > Active 8mers interconvert with inactive 6mers: $8mer \rightleftharpoons 2mer \rightleftharpoons 2mer^* \rightleftharpoons 6mer^*$.
- > The “*” is a rotation around a hinge that is sterically forbidden in 8mer or 6mer*.
- > The position and modifiers of the equilibrium are phylogenetically variable.
- > Intrinsic and extrinsic regulators activate or inhibit by shifting the equilibrium.

Figure 1.

Structural overview of PBGS. **A)** Schematic of PBGS structural features numbered as for human PBGS. The upper bar represents wild-type human PBGS with the $\alpha\beta$ -barrel in purple and the N-terminal arm in salmon. Yellow and red bars highlight an active site metal binding region and the active site lid. Conserved lysine residues interacting with the substrates and an arginine responsible for quaternary structure maintenance are indicated in green and cyan. The lower (gray) bar represents the naturally occurring F12L variant of human PBGS, which has similar tertiary structure, but a dramatically different orientation of the N-terminal arm relative to the $\alpha\beta$ -barrel. Regions of disorder in the F12L structure are highlighted with dashed grey lines. **B)** Overlay of a subunit of wild-type human PBGS (pdb: 1E51) and the F12L variant (pdb: 1PV8) colored as in A. The product porphobilinogen (CPK coloring with the carbons in white) and the active site zinc (colored lime) are shown as spheres. **C)** Top and side views of human PBGS octamer (8mers) in purple and white, and hexamer (6mer*) in gray and white shown as surfaces.

Figure 2.

Front and side views of the A, B and F subunits of the active PBGS octamer (8mer) (pdb: 1E51) and inactive hexamer (6mer*) (pdb: 1PV8) crystal structures. **A)** Subunit A of the PBGS octamer is shown as a dark pink cartoon with porphobilinogen (spheres, colored CPK with carbons in gray) bound in the active site. The active site lid is highlighted by the yellow arrow. Subunits B and F of the octamer are shown as transparent surfaces in gray and light pink, respectively. The lid-stabilizing arm-to-barrel interface (exclusive to the 8mer) is highlighted by the cyan arrow. **B)** Subunit A of the PBGS hexamer is shown as a dark blue cartoon with a product intermediate (spheres, colored CPK with carbons in gray) bound in the active site. The active site lid is disordered in the hexamer structure. Subunits B and F of the hexamer are shown as transparent surfaces in gray and light blue, respectively.

Figure 3.

The oligomeric equilibria of PBGS from human and *T. gondii* [14, 20]. **A)** Subunits A, B and F of wild-type human PBGS (pdb: 1E51) are shown as dark pink, gray and light pink cartoons with the remaining subunits of the 8mer shown as transparent white spheres. Subunits A, B and F of the F12L variant of human PBGS (pdb: 1PV8) are shown as dark blue, gray and light blue cartoons with the remaining subunits of the 6mer* shown as transparent white spheres. The upper pathway illustrates interconversion between 8mer and 6mer* species via "hugging" and "detached" dimers; the lower pathway illustrates interconversion between 8mer and 6mer* species via "pro octamer" and "pro hexamer" dimers as discussed in the text. **B)** Subunits B, A and E (corresponding to subunits A, B and F of the oligomers illustrated in part A) of *T. gondii* PBGS (pdb: 3OBK) are shown as dark teal, gray and light teal cartoons with the remaining subunits of the 8mer shown as transparent white spheres. An allosteric magnesium stabilizing the arm to barrel interface is shown as a magenta sphere and highlighted by a magenta arrow. An intersubunit β -sheet formed by the C-termini of subunits comprising the pro-octamer dimer (highlighted in orange and yellow in the enlarged panel) sterically forbids the conformational change required to form the pro-hexamer dimer.

Figure 4. Multiple sequence alignment of PBGS from *H. sapiens* (human), *E. coli*, *P. sativum* (pea) and *T. gondii*. Residues that are identical or similar among these species are shaded in black or gray. Numbering corresponds to the human PBGS sequence. PBGS structural features are highlighted as in Figure 1 as follows: salmon box – N-terminal arm; yellow box – active site metal binding region; red box – active site lid; green boxes – conserved active site lysines; cyan boxes – residues in a key quaternary structure interaction in human PBGS. Additional highlighted features include lilac boxes around residues that coordinate an allosteric magnesium critical to quaternary structure interactions in some PBGS (see Fig. 6) and an orange box denoting the C-terminal extension of *T. gondii* PBGS (see Fig. 3).

Figure 5.

Native PAGE of *E. coli* and pea PBGS oligomers under varied conditions. **A)** Native PAGE of *E. coli* PBGS that migrates as a single band during SDS PAGE (not shown) [17]. Lane 1 – 8mers and smaller species are observed in untreated *E. coli* PBGS; Lane 2 – incubation with 5 mM EDTA dramatically decreases the percentage of *E. coli* PBGS 8mer; Lane 3 – incubation with 1 mM MgCl₂ increases the percentage of *E. coli* PBGS 8mer; Lane 4 – incubation with 10 mM ALA increases the percentage of *E. coli* PBGS 8mer; Lane 5 – incubation with 1 mM MgCl₂ and 10 mM ALA causes all of the *E. coli* PBGS to migrate as 8mer. **B)** Native PAGE of pea PBGS that migrates as a single band during SDS PAGE (not shown). Lane 1 – purified pea PBGS migrates as a mixture of 8mer and 6mer*; Lane 2 – dialysis versus 2 mM EDTA causes pea PBGS to migrate predominantly as 6mer*; Lane 3 – following subsequent dialysis of the lane 2 sample out of EDTA, pea PBGS remains predominantly 6mer*; Lane 4 – addition of 10 mM MgCl₂ to the lane 3 sample increases the percentage of pea PBGS 8mer; Lane 5 – addition of 10 mM ALA to the lane 3 sample increases the percentage of pea PBGS 8mer; Lane 6 – addition of 10 mM MgCl₂ and 10 mM ALA to the lane 3 sample causes all of the pea PBGS to migrate as 8mer.

Figure 6.

Intrinsic allosteric stabilization of the A/B interface in *E. coli* and human PBGS. For all structures, chains are labeled as in the pdb files. **A)** The *E. coli* PBGS 8mer (pdb: 1L6S) is shown as surfaces with subunits A, B and C shown in green, gray and light green, and the remaining subunits in white; the yellow circle highlights the position of the arm to barrel interface detailed in the enlargement. The labeled subunits comprise A:C and F:B pro-octamer dimers. The 8mer-stabilizing allosteric magnesium is shown as a magenta sphere with the five waters (cyan spheres) that comprise its first coordination sphere ligands, and the side chains of residues from chains A and B that also contribute to magnesium coordination. **B)** The human PBGS 8mer (pdb: 1E51) is shown as surfaces with subunits A, B and F in dark pink, gray and light pink, and the remaining subunits in white; the yellow circle highlights the position of the arm to barrel interface detailed in the enlargement. The labeled subunits comprise A:F and C:B pro-octamer dimers. The side chain of Arg240 forms an 8mer-stabilizing hydrogen bond with the backbone carbonyl of Ser5 from its hugging dimer partner. **C)** The human PBGS 6mer* (pdb: 1PV8) is shown as surfaces with subunits A, B and F in dark blue, gray and light blue, and remaining subunits in white; the yellow circle highlights the displacement of the N-terminal arm and resultant obliteration of the arm to barrel interface in the hexamer. The labeled subunits comprise A:F and C:B pro-hexamer dimers. The enlargement does not show Ser5 of the detached dimer partner because it is spatially distant from Arg240, and the 8mer-stabilizing hydrogen bond can not form.

Figure 7.

ALAD porphyria-associated PBGS. **A**) pH profiles of human PBGS 8mers and 6mer*s are shown in pink and blue with distinct axes. Wild type PBGS 8mers (closed pink circles) display a classic bell-shaped pH-rate profile (solid pink line) with maximum activity at pH 7. F12L PBGS 6mer*s (closed blue circles) display a one-limbed pH rate profile (solid blue line) with very low activity at pH 7 and maximum activity above pH 9. Coexpression of wild type and F12 PBGS yields hetero-8mers and hetero-6mer*s that can be chromatographically separated [5, 21]. PBGS hetero-8mers (open pink circles) display a bell shaped pH rate profile (dashed pink line) similar to wild-type 8mers, but with lower activity. PBGS hetero-6mer*s (open blue circles) display a pH rate profile that reflects a metastable mixture of 6mer*s converting to 8mers upon exposure to substrate. Note that, at pH 9, all of the samples exhibit the same specific activity because all of the species convert to 6mer* at this pH. **B**) The location of ALAD-porphyria associated mutations. A subunit of human PBGS is shown as a transparent white cartoon. Residues whose replacement is associated with ALAD-porphyria are shown as balls and sticks colored CPK with the carbons in blue (Phe12), magenta (Glu89), orange (Cys132), purple (Gly133), black (Val153), cyan (Arg240), green (Ala274), or pink (Val275). Residue labels include the one-letter designation for the native and (*mutated*) amino acid at each position.

Figure 8.

The fragile nature of the human PBGS 8mer. **A)** Active-site lid interactions required for maintenance of the human PBGS 8mer including: the active site zinc-dependent binding of A-side ALA; an intersubunit hydrogen bond between Arg240 and Ser5; lid-stabilizing hydrogen bonds between A-side ALA and Arg209 and Arg221; and an intersubunit interaction between Cys223 and Phe12. Residues originating from a second subunit are indicated with a *. The substrate ALA is shown in both the A-side and P-side locations, though only the K_M -determining A-side ALA contributes directly to 8mer stabilization. **B)** The influence of substrate and pH on the quaternary structure of R240A human PBGS [22]. Equilibrium dialysis was performed starting with predominantly hexameric R240A dialyzed at 37 °C against assay buffer containing 10 mM ALA (*open symbols*) for 24 h, after which time the protein was dialyzed back into buffer without ALA (*closed symbols*). *Circles* represent the experiment at pH 7; *squares* represent the experiment at pH 9. The percent octamer was determined by densitometry measurements of native gels. The *lines* are fits to a simple exponential equation.

Figure 9.

Identification of 6mer*-stabilizing allosteric inhibitors of pea PBGS [24]. **A)** A structural model of the pea PBGS hexamer is shown as surfaces with subunits A, B and F in dark blue, gray and light blue, and the remaining subunits in white. The arrow highlights the position of a 6mer*-specific surface cavity that is the putative small molecule binding site. **B)** Subunits A,B and F are shown as cartoons colored as in A with the docking box superimposed to show the boundaries within which the center of the ligand must be contained (green box), and in which the entire ligand must fit (purple box). The allosteric inhibitor morphlock-1 is shown as balls and sticks (CPK with carbons in green) in its docked location. **C)** Matched Coomassie (left) and PBGS activity (right) stained native PAGE illustrates specific binding of morphlock-1 to the 6mer* assembly. The PBGS activity observed at the 6mer* position in lanes 1–4 results from substrate-induced conversion of the inactive 6mer*s to 8mers during staining. Enzyme-bound morphlock-1 prevents the in-gel transition, and no activity is seen in lanes 5 and 6.