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Mini-review

The porphobilinogen synthase catalyzed reaction mechanism

Eileen K. Jaffe

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

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Abstract

Porphobilinogen synthase (PBGS) catalyzes the first common reaction in the biosynthesis of the tetrapyrroles, the asymmetric condensation of two molecules of δ-aminolevulinic acid to form porphobilinogen. There is a variable requirement for an essential active site zinc that necessitates consideration of PBGS as an enzyme that may exhibit phylogenetic diversity in its chemical reaction mechanism. Recent crystal structures suggest reaction mechanisms that involve two covalent Schiff base linkages between adjacent active site lysine residues and each of the two substrate molecules. The reaction appears to stall at a covalently bound almost-product intermediate that is poised for breakdown to product upon binding of a substrate molecule to an adjacent active site and a subsequent conformational change.

Keywords: Porphobilinogen synthase

1. Introduction

Porphobilinogen synthase (PBGS), which is also known as δ-aminolevulinic acid dehydratase (ALAD), catalyzes a unique asymmetric condensation of two molecules of δ-aminolevulinic acid (ALA) to form the monopyrrole porphobilinogen. ALA and porphobilinogen are the biosynthetic precursors to all the tetrapyrrole pigments (e.g., porphyrin, chlorin, and corrin), which are essential to most life forms. In the course

E-mail address: ek_jaffe@fccc.edu.

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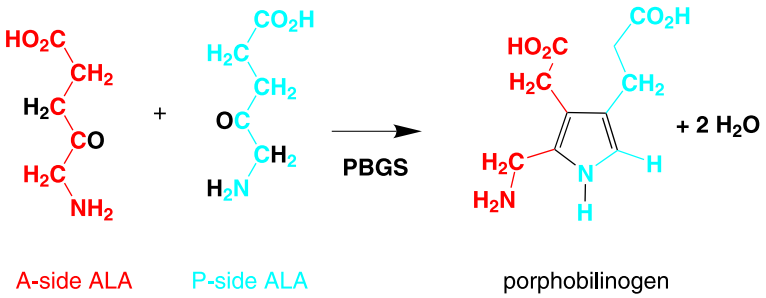

Fig. 1. The porphobilinogen synthase catalyzed asymmetric condensation of two molecules of δ-aminolevulinic acid. P-side ALA (cyan) becomes the half of porphobilinogen that contains the **p**ropionyl side chain and the **p**yrrole nitrogen. A-side ALA (red) becomes the half of porphobilinogen that contains the **a**cetyl side chain and the **a**mino nitrogen. The atoms that are formally lost as water are shown in black.

of the PBGS catalyzed reaction, illustrated in Fig. 1, at least eight bonds are made or broken. The order and details of these chemical events was the subject of investigation for three decades prior to solution of PBGS crystal structures; key references are cited [1–9]. Crystal structures of PBGS began to appear in 1997 [10] and shortly thereafter structures appeared with enzyme-bound active site ligands [11]. There are now many different active-site ligand-containing structures of wild-type and mutant PBGS from the diverse species human, yeast, *Escherichia coli*, and *Pseudomonas aeruginosa*; these structures have been interpreted differently with regard to the PBGS catalyzed reaction mechanism [11–20]. Several novel mechanistic schemes, which are based on observations of substrates, substrate analogs, putative reaction intermediates, and product bound at the enzyme active site, have appeared in the recent literature [17,20–22].

2. Sequence and structure

PBGS is highly conserved in both sequence and structure. The most phylogenetically distant PBGS sequences are ~35% identical. The PBGS active site lies in the center of a triose phosphate isomerase-like αβ barrel [10]; there is a mobile section of the protein that serves as an active site lid that gates access to solvent. Wild-type protein crystal structures show a homo-octameric oligomer with the active site lids on the solvent exposed surface. With the exception of the amino acids that directly interact with the catalytic zinc, which is present only in PBGS from some species (see below), and some residues of the active site lid, the amino acids that make up the PBGS active site are phylogenetically invariant. Despite this sequence conservation, there is a dichotomy set up by the variable requirement for an essential active site zinc: this dichotomy necessitates consideration of PBGS as an enzyme that may exhibit phylogenetic diversity in its chemical reaction mechanism.

3. Required cofactors

Before discussion of PBGS reaction mechanism details, evidence for required cofactors must be considered. Organic cofactors are not required, but early studies on PBGS suggested an unusual diversity in the use of metal ions [23]. At least some of this diversity has since been attributed to the presence or absence of a cysteine rich zinc-binding sequence DX<u>C</u>X<u>C</u>X(Y/F)X_3G(H/Q)<u>C</u>G [24,25]. The crystal structure of *E. coli* PBGS, which contains both an active site zinc ion and an allosteric magnesium ion [16], helped clarify a disparate literature and allowed a better understanding of how different PBGS differ in their use of these two different metal ion-binding sites [25]. It is now clear that only a subset of species, about half of those in the current databases, contain PBGS with catalytically essential active site zinc. These are animals (metazoa), fungi, archaea, and some bacteria. In the zinc-containing PBGS, the catalytic zinc has been shown to be crucial for the binding and reactivity of the second substrate molecule [26], which is the ALA that becomes the acetyl-containing half of porphobilinogen (A-side ALA, see Fig. 1); this is the ALA that determines the K_m value. Among those PBGS that do not use the active site zinc, some appear to use a monovalent cation at the active site [17], some may use magnesium at the active site [27], and others may not use any metal ions at all [25]. On the other hand, there is an allosteric magnesium-binding site that is present in ~90% of the documented PBGS sequences, which encompass all but metazoa, fungi, and the bacterial genus *Rhodobacter* [25]. The allosteric magnesium does not directly participate in the PBGS catalyzed reaction mechanism and is proposed to modulate activity by affecting the mobility of the active site lid.

4. The PBGS catalyzed reaction mechanism: experimental limitations

A primary limitation to experimental studies of the PBGS catalyzed reaction mechanism is the chemical identity of the two substrate molecules. For example, one cannot simply add one or the other substrate and "see how much happens," and interpretation of isotope effects is always ambiguous. A second limitation is the functional irreversibility of the reaction where the thermodynamic driving force is both enthalpic and entropic. Third, when considering mechanistic alternatives for the PBGS catalyzed reaction, it is important to consider that the overall turnover is slow, on the order of one per second. Turnover appears to be limited by protein motion or product release rather than the bonding changes involved in the complex transmogrification of ALA to porphobilinogen. Consequently, phylogenetically different PBGS that may use different chemical reaction mechanisms may not exhibit dramatic differences in measurable kinetic parameters. Finally, crystal structures show that much of the active site serves the mission of bringing the two substrates into a proper proximity and orientation. Residues poised to stereospecifically pluck protons are ambiguous at best. Nevertheless, the early steps in the reaction are well established.

5. P-side Schiff base formation happens first

One accepted element of the PBGS catalyzed reaction mechanism is the formation of at least one Schiff base linkage between at least one of the two substrate molecules and at least one of two active site lysine residues. The identity of the substrate that is involved in the first Schiff base has been the subject of some debate [1,6,8]; very appealing, but disproven, mechanisms are present in outdated biochemistry texts. It is now well established that the first interaction between PBGS and its substrates is the binding of the ALA that becomes the propionyl-containing half of porphobilinogen, which is P-side ALA (Fig. 1). C4 of P-side ALA forms a Schiff base with the enzyme (Fig. 2A). The PBGS structure shows that the P-side ALA-binding site, which is phylogenetically conserved, is deeper into the active site than is the A-side ALA-binding site. Also, unlike A-side ALA, P-side ALA does not interact with the active site lid. This lid is disordered in many PBGS crystal structures that contain only the P-side Schiff base. The carboxyl group of the P-side Schiff base intermediate can be hydrogen bonded to the hydroxyl group of a conserved tyrosine or the backbone carbonyl oxygen of a conserved serine (Fig. 2A). C2–C5 of the P-side Schiff base intermediate are bathed in a hydrophobic pocket (not shown) and the first product water molecule appears to have been released to solvent prior to binding the second ALA molecule [18]. Based on crystal structures of the P-side Schiff base intermediate on PBGS forms that either do or do not use the catalytic zinc, these aspects of the PBGS catalyzed reaction mechanism appear to be phylogenetically conserved. It is interesting that one elegant reaction mechanism proposed for PBGS invokes participation of an active site zinc ion in P-side Schiff base formation [22] despite significant data supporting the metal ion independence of P-side Schiff base formation [7,26].

6. A-side ALA binding happens next

The next step in the PBGS catalyzed reaction is the binding of A-side ALA, and it is in the binding and reactivity of this substrate molecule where phylogenetic variation must come into play. For those PBGS that require a catalytic zinc, the metal is essential to the binding of A-side ALA [26] and this Zn–ALA complex has been modeled as a bidentate ligation of the zinc ion through the C4 carbonyl oxygen and the C5 amino nitrogen atoms (Fig. 3). Various PBGS crystal structures support this model by showing the active site zinc either as ligated to a water molecule positioned to have derived from C4 of A-side ALA (Fig. 2B) [16] or showing the active site zinc directly coordinated to the amino group of porphobilinogen that derives from the amino group of A-side ALA [20,21,28] (Fig. 2C). For those PBGS that do not use a catalytic zinc, there are no biochemical data indicating a requirement for alternative monovalent or divalent ions for A-side ALA binding and reactivity. There is however an intriguing crystal structure for *P. aeruginosa* PBGS that contains density consistent with the monovalent cation sodium in the vicinity the ALA analog inhibitor, 5-fluorolevulinic acid, which is bound at both

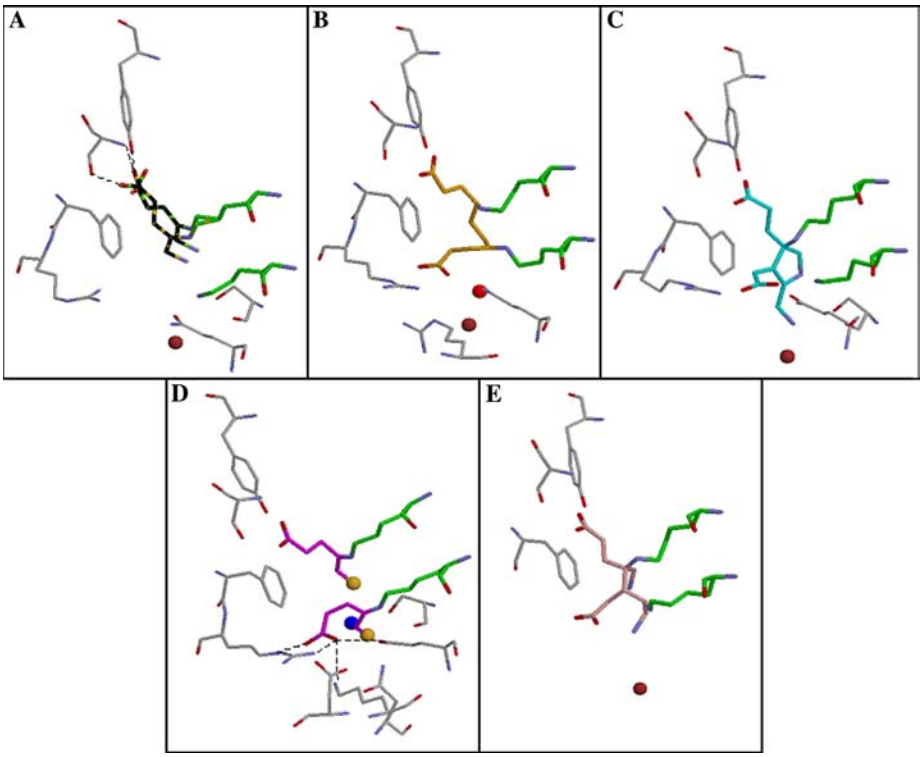

Fig. 2. Representations of PBGS active site ligands from selected X-ray crystal structures. In all views, the active site residues are colored CPK, except for the carbons of the active site lysine residues, which are shown in green. Zinc, when present is shown as a dark red ball. (A) The natural substrate ALA is bound as the P-side Schiff base to yeast PBGS (PDB code 1H7O [14]). The carbons of ALA are shown mottled black and two different orientations for this intermediate are present. Hydrogen-bonding is shown for the P-side ALA carboxyl group. (B) The disubstrate analog 4,7-dioxosebacic acid (carbons in gold) bound through two Schiff base linkages to *E. coli* PBGS (PDB code 1L6S [18]). 4,7-Dioxosebacic acid (HOOC-CH_2-CH_2-CO-CH_2-CH_2-CO-CH_2-CH_2-COOH) is a structural analog of a PBGS catalyzed reaction intermediate where C–N bond formation has occurred, but C–C bond formation has not [29]. (C) A product-like late reaction intermediate (carbons in cyan) is shown bound to yeast PBGS (PDB code 1OHL [20]). Here the pyrrole-like ring is complete but remains covalently bound to an active site lysine. The amino group of the porphobilinogen-like intermediate serves as a ligand to the zinc ion. (D) Two molecules of 5-fluorolevuinic acid (carbons in magenta) bound through two Schiff base linkages to inactive *P. aeruginosa* PBGS variant D139N. The blue ball represents a sodium ion, which is coordinated to both the fluorine atoms (gold balls). Hydrogen-bonding is shown for the A-side carboxylate. (E) Two molecules of the natural substrate ALA (carbon atoms in pink), each bound through a Schiff base linkage, to the rare F12L allele of human PBGS (PDB code 1PV8 [19]). In this case, the inter-substrate carbon–carbon bond has formed between the two substrate molecules, the amino groups have not reacted, and most of the residues that define the A-side ALA-binding site are disordered, including the zinc ligands [19].

substrate-binding sites as illustrated in Fig. 2D [17]. One cannot ascertain whether or not the fluoro moieties, both of which appear to be coordinated to the monovalent cation, dictate the location of the ion.

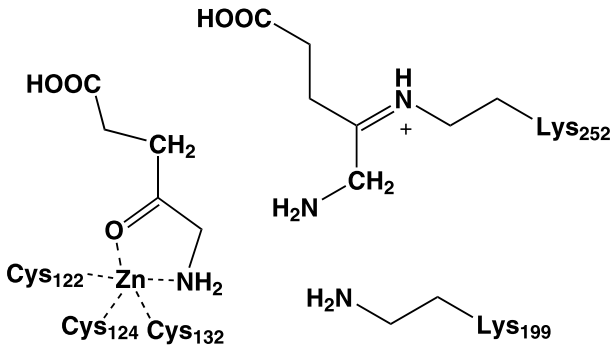

Fig. 3. The putative terniary complex for those PBGS that use the active site zinc. P-side ALA is bound as the Schiff base of shown tautomeric and protonation states. A-side ALA is bound as a bidentate ligand to the active site zinc. Amino acids are numbered according to human PBGS.

7. A-side ALA binding invokes closing of the active site lid

A-side ALA binding stabilizes the closed lid configuration due to the A-side carboxyl group forming hydrogen bonds to residues on the active site lid. These residues include one invariant arginine, one invariant glutamine, and one basic residue which is either lysine or arginine depending, respectively, on whether or not the PBGS uses an active site zinc [16,25]. Some of the interactions between the lid and A-side ALA appear to be transient, and some are illustrated in Fig. 2D. A progression of hydrogen bonding patterns between lid residues and the A-side carboxylate group allows the acetyl side chain its necessary motion as hybridization changes occur at C3 of A-side ALA as it loses both of its protons during the reaction [18].

8. Once both substrates are bound: what happens next?

At the point in the reaction where both substrates are bound, shown schematically in Fig. 3, porphobilinogen formation is poised to happen. P-side ALA is bound as the Schiff base, A-side ALA is bound, presumably as the zinc chelate, and the active site lid is closed, which isolates the proximate groups from the effects of bulk solvent. For those PBGS that do not use the active site zinc, the coordination chemistry aspect of A-side ALA must be different. Subsequent to formation of the terniary complex, there are many possible alternative schemes for the order of events, and much attention has been given to whether intersubstrate carbon–carbon bond formation precedes or follows the intersubstrate carbon–nitrogen bond formation. Historically, the Knorr-type mechanism was preferred for schemes where P-side ALA forms the Schiff base to the protein (e.g. [6,7]) and the aldol-type condensation was preferred for schemes where A-side ALA forms the Schiff base to the enzyme [1]. Stereochemical arguments have also been put forth for the aldol-type mechanism along with a P-side Schiff base [9]. A detailed ^{13}C and ^{15}N NMR characterization of a chemically

modified from of bovine PBGS correctly discerned the tautomerization state and stereochemistry of the P-side Schiff base intermediate and suggested that the amino group destined to be incorporated into the pyrrole was deprotonated [7], which would be the perfect set-up for the Knorr-type reaction. Also in support of the sequence in which inter-substrate C–N bond formation precedes C–C bond formation (at least for the PBGS with an active site zinc) is the high affinity of 4,7-dioxosebacic acid and 4-oxosebacic acid, which mimic the intermediate that contains the C–N bond, but not the C–C bond [16,29]. Intermediates that mimic the aldol condensed intermediate were found not to be potent inhibitors [29]. Hence, at the cusp of the new millennium a consensus was building that supported the Knorr mechanism.

9. Mechanisms involving two Schiff base linkages

Now however, there is a growing consensus is for mechanisms unforeseen prior to crystal structures. These mechanisms include two active site lysine residues simultaneously participating in Schiff bases with both substrates. For clarity in the following descriptions, the atoms of P-side ALA are described in normal font while those of A-side ALA are described in italic font. The structures that prompt these mechanistic proposals are illustrated in Figs. 2B, D, and E. The former two contain substrate analog or intermediate analog bound through two Schiff base linkages. The latter is a most recent structure of human PBGS variant F12L, which shows the natural substrate ALA bound in this fashion. This F12L structure contains the *C3*–C4 carbon–carbon bond, which is the strongest evidence yet that this is the first bond to form between the two substrate molecules.

On the basis of the structures illustrated in Fig. 2, the order of the PBGS catalyzed reaction is best considered to be as follows: first P-side ALA binds and forms a Schiff base intermediate between C4 and one active site lysine (Lys252 of human PBGS). This formally generates a water molecule, which is free to leave the open active site. Fig. 2A shows two possible orientations for the P-side Schiff base, and two possible hydrogen-bonding interactions through the substrate's carboxylate. A-side ALA binds next and forms a Schiff base intermediate between *C4* and the adjacent active site lysine (Lys199 of human PBGS). Because chemical trapping studies have never captured the second Schiff base [6,26,30], lid closure probably occurs before A-side Schiff base formation. The *C4* derived oxygen atom is seen in Fig. 2B as a ligand to the active site zinc, consistent with Fig. 3. The next step in the reaction appears to be the formation of a bond between *C3* of A-side ALA and C4 of P-side ALA, accompanied by the loss of one proton from *C3* of A-side ALA. Stereochemical studies suggest that this is the pro-R proton [2]. This carbon–carbon bond formation does not obliterate the covalent linkage between C4 of P-side ALA and its associated lysine residue; at this point in the reaction C4 of P-side ALA is an sp^3 carbon (Fig. 2E). To complete formation of the pyrrole ring, the penultimate step in the reaction is a transfer of *C4* of A-side ALA from its Schiff base with the active site lysine to a chemically comparable linkage with the C5-amino moiety of P-side ALA. This generates an almost-pyrrole intermediate shown in Fig. 2C. A tightly bound "product-like"

molecule, which retains a covalent linkage to one active site lysine, has been seen in crystal structures of yeast PBGS (Fig. 2B), human PBGS [28], and *E. coli* PBGS (H.L. Carrell, unpublished results). This molecule has been found to purify with the protein from human blood [28], is consistent with the "tightly bound product" referred to in our NMR studies [31], single turnover experiments with *E. coli* PBGS [32], and is found in human, bovine, and *E. coli* PBGS at a stoichiometry of one per dimer (half-of-the-sites stoichiometry) [28,31,32]. An unpublished experiment shows that the enzyme "almost-product" complex made with [4-^{14}C]ALA, does not show loss of radioactivity upon extensive dialysis. However, addition of unlabelled substrate to the dialysis medium provokes release of the radioactivity from the enzyme sample. Hence, we propose that the final step in the PBGS catalyzed reaction is the binding of P-side ALA to an adjacent active site of the octamer, which is then accompanied by conformational changes that open the neighboring active site lid, which is accompanied by the breakdown of the covalent "almost-product" complex and release of porphobilinogen. This is consistent with a reciprocating motion model as was recently supported by studies with PBGS from *Drosophila melanogaster* [33].

It is intentional that the above mechanistic description is not accompanied by a reaction schematic involving curly arrows. The reason is simple: a lack of definitive data implicating one or another active site residue as being involved in the multiple protonation and deprotonation reactions that must accompany the double dehydration encompassed in conversion of ALA to porphobilinogen. Clearly the active site lysine residues mitigate the removal of the protons from P-side ALA, but exactly how this occurs is unknown. Indeed, there is a remarkable dearth of active site ligands poised to accept the two protons that must be lost from *C3* of A-side ALA. Instead there is a small bath of water molecules which are seen in several different structures, and one of which is associated with the active site zinc, at least for those PBGS that use the active site zinc [18]. The past few years have seen the publication of several different detailed reaction schematics for double Schiff base mechanisms [17,20–22], and the reader is referred to one especially thoughtful discussion of the almost-product complex shown in Fig. 2C [20]. However, existing reaction schematics do not critically evaluate the role of the active site metal ion (or lack thereof), nor do they include the important role of protein motion.

10. What is the role of the active site zinc and how does the enzyme work when this essential metal ion is not involved?

The zinc-binding site illustrated in Fig. 3 is quite unusual for a catalytic zinc, as most catalytic zinc ligands are not rich in cysteines. The unusual arrangement of three cysteine ligands all to one side of the metal ion is what makes PBGS a primary target for inhibition by the environmental toxin lead, which has unusual stereochemical coordination preferences [34,35]. Each of the cysteine ligands is important as mutation of any one of them dramatically affects the kinetic characteristics of the protein. The individual mutations C122A, C124A, and C132A in human PBGS result in specific activities that are less than 1% the wild-type value. Removal of all three cysteines by

mutagenesis from the human enzyme results in a protein whose activity is reduced by six orders of magnitude and whose K_m value is huge [34].

The unusual chemistry of the active site zinc may lie in part in its lability. Early reports showed metal ion (and activity) loss during purification and this could be circumvented by inclusion of zinc (10 μM) in the purification buffers. Crystal structures show the zinc chelated to the amino moiety of almost-porphobilinogen [20,28] and cited biochemical results suggest that the enzyme is purified with product bound [28,31]. Consistent with this notion, our most recent studies with *D. melanogaster* PBGS suggest that the zinc may dissociate from the protein with each turnover, as if this is an integral part of the process of product release [33]. Of course, none of these zinc related concerns come into play when considering the PBGS that do not use the catalytic zinc, and these PBGS appear to have evolved a variety of alternative solutions.

11. Caveats and conclusion

It is appealing to deduce probable enzyme catalyzed reaction mechanisms from enzyme-bound active site ligands seen in X-ray crystal structures as has been done above. But, at least in the case of PBGS, there are caveats to consider, the most important of which is that the structures illustrated in Figs. 2B, D, and E represent what *can* happen at the PBGS active site under unusual conditions; they do not necessarily represent what *does* happen at the PBGS active site for wild-type proteins under physiological conditions. Also important is that some of the structures seen are specific either to the PBGS that do or that do not use the active site zinc ion. For instance, Fig. 2B shows a very slow tight binding inhibitor, 4,7-dioxosebacic acid, which is only effective against the PBGS that contain an active site zinc [16]. Fig. 2D shows a highly electronegative fluorine-containing ALA analog bound to an inactive mutant of PBGS [17] that does not use the active site zinc. Fig. 2E shows the natural substrate ALA bound to a rare human PBGS variant that exhibits an entirely different quaternary structure than the wild-type human, yeast, *P. aeruginosa*, or *E. coli* PBGS structures and shows extraordinary disorder in the solvent accessible half of the active site [19], including the zinc-binding residues. In conclusion, it remains possible that the conserved aspects of the PBGS catalyzed reaction mechanism are to bind P-side ALA as the Schiff base, to bind A-side ALA in a geometry that will support the highly exothermic condensation reaction, and to close off access to bulk solvent. The less conserved aspects may include how the protein achieves positioning and activation of A-side ALA, the order of events that follow formation of the ternary enzyme–substrate complex, and the details that accompany almost-product breakdown and release.

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