Mechanistic Implications of Mutations to the Active Site Lysine of Porphobilinogen Synthase*

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Porphobilinogen synthase (PBGS) is a homo-octameric protein that catalyzes the complex asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA). The only characterized intermediate in the PBGS-catalyzed reaction is a Schiff base that forms between the first ALA that binds and a conserved lysine, which in Escherichia coli PBGS is Lys-246 and in human PBGS is Lys-252. In this study, E. coli PBGS mutants K246H, K246M, K246W, K246N, and K246G and human PBGS mutant K252G were characterized. Alterations to this lysine result in a disabled but not totally inactive protein suggesting an alternate mechanism in which proximity and orientation are major catalytic devices. This lysine to ALA Schiff base is not required to initiate the PBGS-catalyzed reaction is a Schiff base that forms between this lysine and ALA and eight active sites. A unifying hypothesis is that formation of the Schiff base between this lysine and ALA triggers a conformational change that results in asymmetry. Product binding studies with wild-type E. coli PBGS and K246G demonstrate that both bind porphobilinogen at four per octamer although the latter cannot form the Schiff base from substrate. Thus, formation of the lysine to ALA Schiff base is not required to initiate the asymmetry that results in half-site reactivity.

Porphobilinogen synthase (PBGS, 1 also known as 5-aminolevulinate dehydratase) is a metalloenzyme that catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to form porphobilinogen, the monopyrrole precursor of tetrapyrroles (see Fig. 1). Although PBGS proteins from different organisms differ in their use of metal ions, there is remarkable sequence conservation between the PBGS from euacteria, archaea, and eucaryotes implying a commonality in the overall protein architecture and reaction mechanism (1). The two PBGS studied herein, those of Escherichia coli and human, both require a catalytic Zn(II), neither require monovalent cations, and the E. coli PBGS activity is stimulated by an allosteric Mg(II) (2–5). The overall protein sequence identity between E. coli and human PBGS is 42%, and the active site residues are significantly more conserved.

Considerable effort has been applied to the characterization of PBGS proteins from various organisms, and several crystal structures are now available (6–10). Yet much of the chemical reaction mechanism remains speculative (7). Only one intermediate, an enzyme-substrate Schiff base, has been identified (11). Several x-ray crystal structures contain levulinic acid bound in a fashion analogous to the Schiff base (Protein Data-bases 1B4K, 1YLV, and 1B4E), and the actual Schiff base involving ALA has been observed by 13C and 15N NMR for a chemically modified form of bovine PBGS (12, 13). This work describes the catalytic and physical properties of PBGS variants with mutations to the Schiff base forming lysine, which for E. coli and human PBGS are Lys-246 and Lys-252, respectively. An appealing aspect of these variants is their expected inability to catalyze porphobilinogen formation and thus there exists the potential of observing new enzyme-bound reaction intermediates using 13C NMR. Although the proteins were found not to be sufficiently inactive for that purpose, the 13C NMR spectra of E. coli Lys-246 mutants with 13C-labeled product bound at the active site are presented.

The number of functional active sites reported for the PBGS homo-octamer varies between four and eight, and it remains unclear if there is a unifying thread that will resolve this apparent discrepancy. For instance, data on E. coli PBGS have been interpreted to support both four and eight active sites/ octamer (3, 15–16), yet the data on mammalian PBGS uniformly supports four functional active sites (17–20). The present active site lysine mutants further probed this apparent anomaly.

EXPERIMENTAL PROCEDURES

Materials—ALA-HCl, KPᵢ, Bis-tris propane, and p-(dimethylamino)benzaldehyde were from Sigma; 2-Mercaptoethanol (βME) was from Fluka (Ronkonkoma, NY) and distilled under vacuum prior to use. HgCl₂, ZnCl₂, MgCl₂ (ultrapure), and high purity KOH were from Aldrich. [4-13C]ALA was custom-synthesized by C/D/N Isotopes (Pointe-Claire, Quebec, Canada). [4-13C]ALA (50 μCi/mmol) and [2H]ALA (1.6 Ci/mmol) were from Amersham Pharmacia Biotech. Centrifree and Centriprep ultrafiltration devices were from Amicon Corp. (Danvers, MA). Slide-A-Lyser devices were from Pierce. House distilled water was further purified by passage through a Milli-Q water purification system (Millipore, Bedford, MA). DNA plasmid purification kits were from Qiagen (Valencia, CA). Oligonucleotides were synthesized in-house by the Fannie Ripple Biotechnology Center. All other chemicals were reagent grade.

PBGS Activity Assays and Kinetic Characterization—PBGS activity assays were as described previously for E. coli (2) and human PBGS (4).
The standard assay buffer contained 0.1 m KPO₄, pH 7.0, 10 mM βME, 10 μM Zn(II), and ~5–15 μg ml⁻¹ PBGS. E. coli PBGS assays also contained 1 mM Mg(II). The standard assay is started by the addition of ALA-HCl to a final concentration of 10 mM, which lowers the pH to 6.8. After 5 min the reaction is quenched, and porphobilinogen is determined using Ehrlich’s reagent. Up to 1 mg ml⁻¹ of purified PBGS and a 15-min incubation were used for mutant proteins.

Site-directed Mutagenesis of E. coli PBGS and Human PBGS—The plasmid pHemB containing the E. coli hemB gene for PBGS, was a kind gift of Dr. C. Rossema of Texas A & M College Station, TX (21). A 1400-base pair fragment containing the 1100-base pair hemB gene was excised from the EcoRI and BamHI site of pCR261 and inserted into the multiple cloning site of pUC119 to form pLM1228. The Muta-Gene Kit from Biol-Rad was used for site-directed mutagenesis on multiple cloning site of pUC119 to form pLM1228. The Muta-Gene kit and no second site mutations. The sequence agreed with the sequence derived from the 6'-8' region of the E. coli genome (GenBank™ accession number U73857). There are significant differences between these and earlier published hemB gene sequences (22, 23).

Mutations to human PBGS were obtained using the QwikChange technique by W. R. Abrams in the Protein Analytical Laboratory at the University of Pennsylvania School of Dental Medicine.

Expression and Purification of PBGS—The constructs HB101-(pLM1228), HB101(pK246N), HB101(pK246G), HB101(pK246H), HB101(pK246M), and HB101(pK246W) gave excellent constitutive expression of mutant or wild-type E. coli PBGS at 15–30% the total soluble cellular protein. In an unsuccessful attempt to obtain inactive recombinant PBGS from these constructs, HB101 at a level of 0.02–0.1%. Human PBGS corresponding to the K59 allele and the K252G variant were purified as described previously where the DEAE column provided a separation of the Mg(II)-responsive and chromosomally encoded wild-type PBGS from Mg(II)-insensitive human PBGS (4).

Peptide Mapping and Mass Spectral Analysis of Wild-type and Mutant E. coli PBGS—E. coli PBGS and all the Lys-246 variants (~200 μg) were digested overnight using 2 μg of AspN protease after cysteine residues were modified by iodoacetamide. The resultant peptides were mapped by reverse phase high pressure liquid chromatography as described previously (20). Peaks were collected manually. “Matching” peaks were identified by inspection of the peptide map. All matching peaks as well as major “additional peaks” were characterized by MALDI-TOF mass spectral analysis using a Perseptive Voyager DE instrument. The program PAWS (R. C. Beavis, New York University) was used to help identify the various peptides.

For identification of the Schiff base containing peptide, high specific radioactivity [H⁺]ALA and NaBH₄ were used as above to label the reactive lysine; then the Schiff base-modified peptide was identified following AspN protease digestion. The amino acid sequence of the radiolabeled peptides was obtained using Edman degradation techniques by W. R. Abrams in the Protein Analytical Laboratory at the University of Pennsylvania School of Dental Medicine.

Atomic Absorption Analysis of Metal Content— Atomic absorption analysis of E. coli PBGS for Zn(II) and Mg(II) were done on a PerkinElmer Life Sciences AAnalyst 100 spectrometer using an air/ acetylene flame. Slide-A-Lysers containing each PBGS variant, ~25–35 mg ml⁻¹, were dialyzed together against 2 liters of 0.1 mM Bis-tris propane, pH 8, containing 10 mM βME, 1 mM Mg(II), and either 10 μM or 30 μM Zn(II). The dialysate was analyzed for Mg(II) directly without dilution. The dialysate was diluted 50-fold prior to analysis for Mg(II) to be within the linear range of the instrument. The protein samples were each diluted ~50-fold prior to analysis for Zn(II), Mg(II), and protein.

Equilibrium Dialysis to Determine Porphobilinogen Binding Stoichiometries—E. coli PBGS samples, at concentrations of both 7 mg ml⁻¹ (0.2 mM subunits) and 25–35 mg ml⁻¹ (~1 mM subunits) in ~250-μl volumes, were placed in Slide-A-Lysers (0.5 ml maximum volume) and equilibrated by dialysis at room temperature against 40 ml of 0.1 mM Bis-tris propane, pH 8, 10 mM βME, 10 mM Zn(II), 1 mM Mg(II), and various concentrations of porphobilinogen ranging from 5 μM to 1.4 mM.
The concentration of porphobilinogen inside and outside the Slide-A-Lyser was determined using Ehrlich’s reagent. Protein concentrations were determined by quantitative total amino acid analyses using vapor phase hydrolysis in 6 N HCl, 0.1% phenol, at 110 °C for 22 h followed by phenylisothiocyanate pre-column derivatization and high pressure liquid chromatography analysis.

\[13C\text{NMR Spectral Acquisition and Sample Preparation—Spectral parameters were as described previously (12). The } E.\ coli \text{ protein samples uniformly contained 100–150 mg of PBGS in 1.8 ml (~1 mm active sites) of buffer containing 20% D}_2\text{O. The buffers were 0.1 M KP, or Bis-tris propane-HCl, 10 mM pME, 1 mM Mg(II), 10 mM Zn(II) at pH 6, 7, or 8. In all cases, natural abundance } 13C\text{NMR spectra of the proteins were obtained (12,000–36,000 scans) prior to the addition of [4-13C]ALA. Following addition of [4-13C]ALA, all samples catalyzed the stoichiometric conversion to [3,5-13C]porphobilinogen. Dialysis at pH 6 was used to remove enzyme-bound product after the enzyme-product spectra were obtained. The chemical shifts of [3,5-13C]porphobilinogen (both free and bound) are insensitive to these changes in buffer or pH. However, the K_s for the enzyme-product complex is highly pH-dependent (15). Spectra obtained after dialysis at pH 6 confirmed the dissociation of enzyme-bound product.}

\textbf{RESULTS}

\textbf{Recombinant PBGS Expression and Activity—The } E.\ coli \text{ PBGS proteins K246M, K246W, K246H, K246G, and K246N as purified from the } E.\ coli \text{ host strain HB101 have specific activities ranging from 0.01 to 0.1 μmol h}^{-1}\text{ mg}^{-1}\text{, compared with the wild-type enzyme value of } \approx 50 \text{ μmol h}^{-1}\text{ mg}^{-1}\text{ (Table I). Thus, these mutants are remarkably impaired in their catalytic potency. Some proportion of the activities observed in these mutants was determined to be due to variable levels of the chromosomally encoded wild-type PBGS because this activity can be reduced significantly by treatment with ALA and NaBH}_4\text{ (see below). The mutant protein K246G was designed to contain a “hole” at the active site into which the lysine side chain surrogate ethylamine might bind and restore activity. Addition of 50 mM ethylamine had no effect on the specific activity of the K246G preparation or wild-type PBGS and did not alter the chemical shifts of [3,5-13C]porphobilinogen bound to K246G (see below). The purification properties of all the mutant proteins did not differ appreciably from wild-type } E.\ coli \text{ PBGS protein. All were stable, highly soluble, octameric proteins. Attempts to obtain expression of inactive Lys-246 mutants in the } hemB \text{ hosts, RP523 and HU1000, either failed or resulted in recombination events that regenerated wild-type protein.}

Human PBGS and the K252G mutant were purified from a BLR(DE3) host } E.\ coli \text{ strain (4). Fig. 2 shows that the chromosomally encoded } E.\ coli \text{ PBGS activity separates from the relatively inactive human PBGS K252G variant during the DEAE chromatography step used during purification. Whether the residual activity is due to } E.\ coli \text{ PBGS can be shown by its ability to be activated by Mg(II). The low activity of the final purified K252G from the S-300 column showed no activation upon addition of Mg(II). The purified human PBGS K252G variant showed a K_m of 5.6 mM and a V_max of 0.026 μmol h}^{-1}\text{ mg}^{-1}\text{ relative to the wild-type values for K59 of 0.1 mM and 44 μmol h}^{-1}\text{ mg}^{-1}\text{ for } K_m \text{ and } V_{max} \text{, respectively, giving a } 10^3\text{-fold reduction in } V/K. \text{ In this case, the low level of activity was not sensitive to inactivation through treatment with ALA and NaBH}_4\text{ (see below). Thus, we can exclude the possibility that the activity is due to either 1) translational error or 2) incorporation of } E.\ coli \text{ PBGS monomers into the homo-octameric human PBGS. Human PBGS K252G activity is also sensitive to inhibition by Pb(II) which indicates that the alternative Lys-252 Schiff base independent mechanism depends on the catalytic Zn(II) (25).}

\textbf{Atomic Absorption Analysis of the } E.\ coli \text{ Lys-246 Variants—The binding of Zn(II) and Mg(II) to } E.\ coli \text{ PBGS is cooperative (5, 13). To address the role of Lys-246 in this cation binding...}
process, we analyzed the ability of the Lys-246 variants to bind Zn(II) and Mg(II). For all five mutants, atomic absorption analysis showed binding of Zn(II) and Mg(II) at the same stoichiometry and affinity as wild-type PBGS (see Table I). Thus, Lys-246 is not functional in the cooperativity of Zn(II) and Mg(II) binding. Because the mutants are not defective in metal binding, this supports an alternative E. coli PBGS reaction mechanism involving the catalytic Zn(II) but lacking the Schiff base to Lys-246.

Schiff Base Trapping Studies—All the E. coli proteins in this study were subjected to Schiff base trapping using radiolabeled ALA and NaBH₄. Controls were carried out in the absence of ALA or NaBH₄ and both reagents. The results are presented in Table II. For wild-type E. coli PBGS, the control samples, which included all the physical manipulations to the protein, retained complete catalytic activity. In contrast, when treated with both [4-14C]ALA and NaBH₄, three samples of wild-type PBGS each showed ~80% inactivation and 14C labeling at 0.42–0.49 ALA/subunit. This stoichiometry is consistent with four functional active sites per octamer. In mutants for which activity was above detection levels, the trace activities were significantly reduced, indicating that the activity of these preparations derives from contamination by chromosomally encoded wild-type E. coli PBGS.

Human PBGS K59 and K252G were subjected to Schiff base trapping studies using unlabeled ALA. In this case, 83% inactivation was observed for K59, and no inactivation was observed for the K252G variant, which indicates that the activity observed for the latter does not depend upon Schiff base formation between ALA and the protein. Prior studies on mammalian PBGS uniformly show >85% inactivation at stoichiometries approaching 0.5 NaBH₄ trapped [14C]ALA per subunit (11, 26).

Mass Spectral Analysis of Peptides from Wild-type E. coli PBGS, Mutant E. coli PBGS, and NaBH₄/ALA-treated E. coli PBGS—AspN protease digestion followed by peptide mapping was carried out for all the E. coli proteins under study. In cases without radioactivity, the identity and sequence of the peptides were deduced from the masses determined by MALDI-TOF analysis. Some peptides were found to result from N-terminal cleavage at glutamic acid. The Lys-246 containing peptide (retention time, ~52 min, see “Experimental Procedures”) was confirmed by sequencing. In the case of K246M and K246W, the retention time of this peak moved in a predictable fashion as calculated by the program PEPTIDESORT of the GCG package (+10.3 min and −20 min, respectively). For K246G, K246H, and K246N, the match between the predicted and observed variation in retention time was less precise. The observed changes were +9.4 min, −0.4 min, and −4.9 min (predicted times were +2.7, +4.0, and 1.6 min, respectively). As illustrated in Table I, the peptide masses confirmed the identity of the mutation. For wild-type E. coli PBGS treated with tritiated ALA and NaBH₄, the radioactivity was found in a cluster of peptide peaks in the 50–54-min range. The multiple peaks are presumably due to proline isomerization, incomplete modification of the cysteine, and partial labeling at Lys-246. These peptides were pooled and subjected to conventional sequencing which revealed a lysine at position 7 of some proportion of the isolated peptides as well as another compound that was common to wild-type PBGS samples. This compound is presumably the reduced Schiff base between ALA and lysine.

Equilibrium Dialysis to Determine the Active Site Stoichiometry for E. coli PBGS—A stoichiometry of four active sites per octamer is consistent with previous biochemical studies of bovine, human, E. coli, Bradyrhizobium japonicum, and Pseudomonas aeruginosa PBGS (2, 4, 12, 13, 15, 26–29) and is seen in the crystal structure of P. aeruginosa PBGS (8), but is not consistent with crystal structures of yeast or E. coli PBGS (6, 7). We propose that any PBGS can exist as either a symmetric or asymmetric octamer depending upon the presence or absence of certain active site ligands, as has been shown for human PBGS with regard to Zn(II) binding (4). A difference in active site stoichiometry between wild-type PBGS and the Lys-246 mutants would suggest that formation of the Schiff base at Lys-246 triggers the asymmetry in an otherwise symmetric protein. Active site stoichiometry was determined for wild-type E. coli PBGS and K246G using equilibrium dialysis techniques. The data presented in Fig. 3 show that K246G binds phorbolilogen (Kᵢ = 18 μM) about 2-fold tighter than wild type (Kᵢ = 35 μM), but the stoichiometry is unchanged at four per octamer. Data obtained at subunit concentrations of 0.2 mM for wild-type and K246G at 1 mM for wild-type, K246G, and K246N are all consistent with four active sites per octamer. Thus, Schiff base formation with ALA is not responsible for a conformational change that dictates half-site reactivity. This is consistent with crystallographic data that show Schiff base formation to levulinic acid does not cause protein asymmetry (7, 9). It remains possible that binding of the C-5 amino group of P-side ALA, which is the ALA that forms the Schiff base, is essential for the evolution of the asymmetric structure.

13C NMR Studies of Mutant and Wild-type PBGS Using [4-13C]ALA as Substrate—All E. coli PBGS proteins were readily brought to ~2 mM subunit concentration. There were no
significant differences between the natural abundance $^{13}$C NMR spectra of the proteins and no buffer dependence of the spectra between potassium phosphate at pH 7 and Bis-tris propane at pH 8. As before, line width considerations for $\sim$300-kDa protein caused us to use [4-$^{13}$C]ALA as substrate because observation of quaternary carbons avoids both dipolar broadening by directly attached protons and the need to deuterate the observed carbon (12). In all cases the [4-$^{13}$C]ALA was added in incremental amounts of 0.5–1.0 per subunit. Residual enzymatic activity caused the stoichiometric conversion of ALA to porphobilinogen in less than 1 h. Free [4-$^{13}$C]ALA has a chemical shift of 206.8 ppm, and free [3,5-$^{13}$C]porphobilinogen has chemical shifts of 121.0 and 123.1 ppm for C-5 and C-3, respectively (12, 30). In the case of K246W and K246M, there were varying amounts of a degradation product of unknown structure (see Table I). The marginal catalytic activity for the E. coli PBGS variants K246G, K246N, K246W, K246M, and K246H and human PBGS variant K252G support the essential nature of this lysine. Nevertheless, the undeniable and Pb(ii)-sensitive residual activity of K252G indicates an alternative mechanism for this mutant that utilizes the catalytic Zn(II) and takes advantage of the orientation of the substrates bound to PBGS.

The essential role of divalent metal ions has been established for almost all PBGS (1). Human and E. coli PBGS share a requirement for an essential catalytic Zn(II) whose ligation sphere is rich in cysteine residues and whose role in catalysis is only beginning to be understood. This Zn(II) has been proposed to be essential for A-side ALA binding but is not required for P-side ALA binding or Schiff base formation (see Fig. 1) (26). Conversely, this work shows the Schiff base forming lysine is not required for metal binding. These two mechanistic elements are distinct, with the lysine impacting P-side ALA and the Zn(II) impacting A-side ALA. The current study shows a 10$^5$-fold reduction in V/K when the essential lysine is obscured; concurrent studies show a 10$^6$-fold reduction in V/K when the catalytic Zn(II) is obscured (25). It would appear that in PBGS the catalytic importance of metal ion-based catalysis is comparable to that of covalent catalysis.

Orientation of ALA bound to PBGS is identified in this study as an important mechanistic factor. Although mutation of the Schiff base forming lysine might reasonably be expected to yield a totally inactive protein, such was not the case. Clearly other residues in the active site orient the P-side ALA such that porphobilinogen formation is able to take place in the absence of electron-withdrawing effect of the Schiff base. This is significant because at least eight bonds are made or broken in the course of the PBGS-catalyzed reaction (see Fig. 1). Consider that the thermodynamic driving force toward product is so large that a characterization of the enzyme-bound reaction equilibrium reveals only product (12). The mutation of the Schiff base forming lysine is not sufficient to overcome thermodynamics; so long as PBGS can bind both ALA molecules, catalysis is able to occur.

Whether the number of functional active sites is the same for all PBGS remains controversial. For E. coli PBGS, data presented here supporting four active sites are consistent with studies of mammalian PBGS (5, 20, 26, 27, 32), the crystal structure of P. aeruginosa PBGS (8), and prior $^{13}$C NMR data on E. coli PBGS (22) but is inconsistent with one published crystal structure of E. coli PBGS (7) and some other biochem-

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2 In the case of K246W and K246M, there were varying amounts of a contaminating enzyme that slowly transformed [3,5-$^{13}$C]porphobilinogen into another compound with chemical shifts of 118.3 and 126.6 ppm.
The 13C NMR resonances of [3,5-13C]porphobilinogen (structure shown upper right) falls in the region of the aromatic residues of PBGS, and only this region of the spectra is shown. Free porphobilinogen resonances fall at 123.0 and 121.0 for C-3 and C-5, respectively, and are marked with parallel dashed lines (2). For wild-type PBGS, the resonances from enzyme-bound [3,5-13C]porphobilinogen fall at 121.4 and 127.2 ppm and are labeled as C-3 and C-5, respectively (7, 17). Free porphobilinogen is also observed. Similar complexes of K246N, K246H, K246M, and K246G are labeled as such. K246W (spectra not shown) revealed only free porphobilinogen. The K246M spectra also contains a set of sharp resonances at 118.3 and 126.6 ppm (marked with asterisks) that are known to derive from a degradation of porphobilinogen, the formation of which is catalyzed by a contaminating activity.

The reconciliation of existing data may lie in the notion that the apo-PBGS structure, with no active site ligands such as metals, substrate, or product, is a strictly symmetric octamer with the potential to become asymmetric upon binding of one or more determining factors such as metal, substrate, or product. One proposal that Schiff base formation triggers the asymmetry of the half-site reactivity phenomenon. Future crystallographic results promise to reveal the molecular nature of the half-site reactivity phenomenon.

Conclusion—The activity of the mutants in this study indicates that the active site lysine although important is not absolutely essential for catalysis. The residual activity in all PBGS variants at the Schiff base forming lysine precluded the observation of enzyme-bound reaction intermediates by 13C NMR. To observe enzyme-bound intermediates to PBGS by 13C NMR, the enzyme must be reduced in velocity by a factor of at least 10^6, whereas substrate/intermediate binding remains substantial at millimolar concentrations of enzyme and ligand. Our reasoning in preparing the human Lys-252 variants was the ability to remove chromatographically the wild-type E. coli PBGS activity in the hope that the activity of K252G would be low enough to directly observe new intermediates. Although we were unable to observe any additional enzyme-bound intermediates, this work does establish the importance of orientation and proximity in the overall mechanism of PBGS. Although unable to form the Schiff base to the K252G, P-side ALA binds, and since A-side ALA is presumably unaffected by these mutations, the reaction is able to proceed. It is unclear if one can make a PBGS mutant that will still bind two ALA but be inactive enough to view an enzyme-bound intermediate via NMR.

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