The Phylogenetically Conserved Histidines of Escherichia coli Porphobilinogen Synthase Are Not Required for Catalysis*

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Porphobilinogen synthase (PBGS) is a metalloenzyme that catalyzes the first common step of tetrapyrrole biosynthesis, the asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to form porphobilinogen. Chemical modification data implicate histidine as a catalytic residue of PBGS from both plants and mammals. Histidine may participate in the abstraction of two non-ionizable protons from each substrate molecule at the active site. Only one histidine is species-invariant among 17 known sequences of PBGS which have high overall sequence similarity. In Escherichia coli PBGS, this histidine is His128. We performed site-directed mutagenesis on His128, replacing it with alanine, and both His126 and His128 simultaneously to alanine. All mutant proteins are catalytically competent; the V_{max} values for H128A (44 units/mg), H126A (75 units/mg), and H126/128A (61 units/mg) were similar to wild type PBGS (50 units/mg) in the presence of saturating concentrations of metal ions. The apparent K_{d} for Zn(II) binding to H128A is about an order of magnitude higher than for the wild type protein. E. coli PBGS also contains His126 which is conserved through the mammalian, fungal, and some bacterial PBGS. We mutated His126 to alanine, and both His126 and His128 simultaneously to alanine. All mutant proteins are catalytically competent; the V_{max} values for H128A (44 units/mg), H126A (75 units/mg), and H126/128A (61 units/mg) were similar to wild type PBGS (50 units/mg) in the presence of saturating concentrations of metal ions. The apparent K_{d} for Zn(II) of H126A and H126/128A is not appreciably different from wild type. The activity of wild type and mutant proteins are all stimulated by an allosteric Mg(II); the mutant proteins all have a reduced affinity for Mg(II). We observe a pK_{a} of 7.5 in the wild type PBGS K_{cat}/K_{m} pH profile as well as in those of H128A and H126/128A, suggesting that this pK_{a} is not the result of protonation/deprotonation of one of these histidines. H128A and H126/128A have a significantly increased K_{m} value for the substrate ALA. This is consistent with a role for one or both of these histidines as a ligand to the required Zn(II) of E. coli PBGS, which is known to participate in substrate binding. Past chemical modification may have inactivated the PBGS by blocking Zn(II) and ALA binding. In addition, the decreased K_{m} for E. coli PBGS at basic pH allows for the quantitation of active sites at four per octamer.

Porphobilinogen synthase (PBGS) catalyzes the asymmetric condensation of two molecules of ALA to form porphobilinogen. This reaction is the first common step in the tetrapyrrole biosynthetic pathway, which is responsible for the formation of porphyrins, chlorins, corphins, and other essential cofactors (1). There is high sequence similarity among the 17 documented sequences of PBGS from a phylogenetically diverse selection of prokaryotes and eukaryotes (2-14, 16-20). A review of data on the PBGS mechanism has been published recently (21). All PBGS are metalloenzymes, and there is an intriguing phylogenetic variation in the use of metal ions.

Under physiologic conditions, Escherichia coli PBGS contains Zn(II) and Mg(II), each at a stoichiometry of eight per octamer (or two per active site) (22, 23). The Zn(II) are believed to be analogous to the eight Zn(II) of mammalian PBGS; four Zn_{A} participate in catalysis and four Zn_{B} appear to bind essential cysteine residues (24-26). Zn_{A} and Zn_{B} are both at the active site (27). For E. coli PBGS, it is difficult to differentiate between the two types of bound Zn(II), and Zn_{A} and Zn_{B} is used to denote enzyme-bound Zn(II) in both sites of E. coli PBGS. The eight equivalent Mg(II) (termed Mg_{C}) act as allosteric activators and have no counterpart in the mammalian protein (23), but appear to be common to bacterial and plant PBGS (21, 28). Although not at the active site, Mg_{C} causes a 50-fold increase in k_{cat}/K_{m}, affecting both parameters favorably. Mg_{C} increases the affinity of the protein for Zn_{A}Zn_{C} (23), but Mg_{C} cannot bind to PBGS unless Zn(II) is bound (22).

It has been proposed that both Zn_{A} and Zn_{B} draw ligands from a putative metal binding domain in a mutually exclusive fashion and that Mg_{C} binds elsewhere in the sequence (21, 25, 26, 28). Fig. 1 shows the sequence of the proposed metal binding domain. The technique of extended x-ray absorption fine structure spectroscopy discriminated between Zn_{A} and Zn_{B} on the basis of the chemical nature of their ligands (26). The conserved tyrosine, aspartate, and histidines of the metal binding domain are candidates for some of the predominantly non-sulfur ligands to a pentacoordinate Zn_{A}. The four cysteines of this region are proposed to tetrahedrally coordinate Zn_{B}. In those plant and bacterial species where the cysteines are not present, there are many aspartic acid residues, and Mg_{B} is proposed to functionally replace Zn_{B} (17, 21).

The PBGS-catalyzed reaction (shown in Fig. 2) involves the overall loss of two water molecules. Various proposed mecha-
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![Diagram of the PBGS-catalyzed reaction.](image)

**Fig. 1.** The putative metal binding region of PBGS. The region from amino acid 110 to 135 in E. coli PBGS is shown along with the homologous region from 16 other sequences. Proposed Zn(II) ligands are from amino acid 110 to 135 in Zn(II) ligand. Extended x-ray absorption fine structure spectroscopy (EXAFS) of isolated ZnA showed that the modified histidine is a Zn(II) ligand (32). His126 in the E. coli sequence and shown in Fig. 1 is the only species invariant histidine in the PBGS sequence and thus is an attractive candidate for an essential histidine.

Histidine at the active site of PBGS might also serve as a Zn(II) ligand. Extended x-ray absorption fine structure spectroscopy studies of bovine PBGS indicate that ZnA (which binds directly to the substrate (25)) contains at least one imidazole ligand (26). Diethylpyrocarbonate inactivation of porcine PBGS can be protected against by the addition of Zn(II), suggesting that the modified histidine is a Zn(II) ligand (32). His126 in the E. coli sequence has previously been proposed to be a ligand to ZnA (25). His126 and His128 may form the nucleation site for binding ZnA. Recently His126 was proposed to be the key "switch" in determining a preference for binding Zn(II) (mammals and some bacteria) or Mg(II) (plants and some bacteria) to the region illustrated in Fig. 1.

To investigate whether His126 or His128 are involved in catalysis and/or ZnA ligation, we performed site-directed mutagenesis on E. coli PBGS to replace His126 and His128 with alanine, both in turn and simultaneously. Alanine was selected because the side chain methyl group of alanine is unable to serve either as a general base or as a Zn(II) ligand. A small side chain was chosen to have a minimal effect on protein functioning. We report here the kinetic and other physical properties of the mutant proteins relative to wild type E. coli PBGS. These results also provide significant new information about wild type E. coli PBGS.

**MATERIALS AND METHODS**

Materials—ALA-HCl, potassium Pi, pH 7.0, and 10 mM MgCl2 (ultrapure), and high purity KOH were purchased from Aldrich. [4,14C]ALA was custom-synthesized by C/D/N Isotopes. Centrifree and Centricon ultrafiltration devices were purchased from Amicon Corp. House distilled water was further purified by passage through a Milli-Q water purification system (Millipore). DNA plasmid purification kits were purchased from Qiagen. Oligonucleotides were synthesized in house by the Fox Chase Cancer Center Oligonucleotide Synthesis Facility. All other chemicals were reagent grade.

Assay, Kinetic Characterization, and pH Rate Profile Determination—PBGS activity assays as well as Km and Vmax determinations were as described previously (28). Most assay buffers contained 0.1 mM potassium Pi, pH 7.0, and 10 mM MgCl2. Except where noted, the assay buffer also contained 10 μM Zn(II) and 1 mM MgCl2. In most cases, the assay was started by the addition of ALA-HCl to a final concentration of 10 mM, which lowered the pH to 6.8. For determination of Km and Vmax, ALA was varied from 0.1 to 10 mM, and the final pH of the assay was controlled by adding HCl such that the total ALA-HCl plus HCl was equivalent to 10 mM. The buffer bis-tris propane was used for the pH profiles. Protein to be assayed in the absence of Mg(II) was prepared by passing twice through a Bio-Rad P6 spin column previously equilibrated with 0.1 mM potassium Pi, pH 7.0, 10 mM MgCl2, 10 μM Zn(II) as per the manufacturer’s directions. This procedure removes free Mg(II), but less than 1 equivalent of bound Mg(II) is retained. A 100–500-fold dilution in an assay mixture followed by a 10-min incubation releases this Mg(II) and results in free Mg(II) of 0.5–1 μM. A unit of activity is defined as the production of 1 μmol of porphobilinogen/h.

Construction of Plasmid pCR261f1 and Site-directed Mutagenesis—The plasmid pCR261 was a kind gift of Dr. C. Roessner of Texas A&M, College Station, TX. This plasmid is pUC19 with a 1.6-kilobase HemB, thr1, leuB6, thi1, lacY1, tonA21, supE44, M. A unit of activity is defined as the production of 1 μmol of porphobilinogen/h.

Expression and Purification of Wild Type and Mutant PBGS—The E. coli strain CR261 was used to generate wild type E. coli PBGS as reported previously (28). The mutant plasmids were transformed into HU1000 (hemB, thr1, leuB6, thi1, lacY1, tonA21, supE44, λ−, and F−) (a
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Table I
Primers used for site-directed mutagenesis

| Protein       | Mutagenic primer mismatch          | Amino acid change | New restriction site |
|---------------|-----------------------------------|-------------------|----------------------|
| H126A         | CACGGTT → GCCGGG                  | HG → AG           | NglM                 |
| H128A         | CAC → GCA                         | H → A             | Sphl                 |
| H126/128A     | CACGGT/CAC → GCCGGTGCA            | HGH → AGA         | Sphl                 |

Table II
Purification of PBGS mutant H126A from 10 g of cell paste

| Step                     | Volume (ml) | Total units | Protein (mg/ml) | Specific activity (units/mg) | Yield (%) | -Fold purification |
|--------------------------|-------------|-------------|-----------------|-----------------------------|-----------|-------------------|
| Crude extract            | 60          | 1488        | 8.4             | 3                           | 100       |                   |
| DEAE sample              | 31          | 840         | 11.2            | 2.4                         | 56        | 0.8               |
| Phenyl-Sepharose sample  | 140         | 742         | 0.4             | 13.3                        | 50        | 4.4               |
| Sephacryl sample         | 10          | 312         | 1.4             | 22.3                        | 21        | 7.4               |
| Concentrated Sephacryl pool | 1.2      | 193         | 4.7             | 34.3                        | 13        | 11.4              |

* Assayed carried out at 10 μM Zn(II), 1 mM Mg(II).

kind gift of Dr. Charlotte Russell of City College New York), an E. coli strain that does not have a functioning PBGS, requires hemin for normal growth and is heme-permeable. After initial growth on LB + hemin, complementation was determined by streaking individual colonies in parallel on LB plates with and without hemin (4 μg/ml). In the absence of hemin, HU1000 cells grow as microcolonies at 37 °C which appear ~36 h after plating. Under the same conditions, HU1000 cells transformed with a plasmid containing a functional copy of hemB grow as normal sized colonies at 37 °C which appear ~18 h after plating. Purification of the mutant proteins was as described previously for wild type E. coli PBGS (28) with the addition of 1 mM Mg(II) to all purification buffers. Yields of ~0.5 mg of protein/g of cells were obtained from an expression level of ~3% total soluble protein. A phenyl-Sepharose column was added prior to the final Sephacryl S-300 column as described below. The protein pool from the DEAE column (in 30 mM potassium Pi, pH 7.5, 0.4 M KCl, 30 μM Zn(II), 1 mM Mg(II), 10 mM βME, 0.1 mM phenylmethylsulfonyl fluoride) was loaded directly onto a 200-ml phenyl-Sepharose column equilibrated in 20 mM potassium Pi, pH 6.8, 30 μM Zn(II), 1 mM Mg(II), 0.25 M KCl, 10 mM βME. The column was washed with 200 ml of starting buffer followed by 200 ml of 20 mM potassium Pi, pH 6.8, 30 μM Zn(II), 1 mM Mg(II), 0.1 M KCl, 10 mM βME. Wild type PBGS was eluted with 200 ml of 2 mM potassium Pi, pH 6.8, 30 μM Zn(II), 1 mM Mg(II), 0.1 M KCl, 10 mM βME. Elution of the mutant proteins H126A and H126/128A required a wash of 200 ml of wild type elution buffer containing 5% ethylene glycol. The pooled protein was concentrated and applied to a Sephacryl S-300 column as described previously (28). Amino acid analysis to confirm the conversion of histidine to alanine was carried out by Dr. William Abrams of the University of Pennsylvania School of Dental Medicine.

13C NMR Spectroscopy to Quantify the Active Site Stoichiometry—
[4-13C]ALA was added incrementally to a 1.6-ml solution containing 4.8 μmol of E. coli PBGS subunits in 0.1 M bis-tris propane, pH 8, 10 mM βME, 10 μM ZnCl2, and 1 mM MgCl2. The stock [4-13C]ALA was standardized by rapid quantitative PBGS-catalyzed conversion to [3,5-13C]porphobilinogen which was quantified using Ehrlich's reagent. 13C NMR spectra were obtained on a Bruker AM300 spectrometer as described previously (28) and processed with a 20-Hz Lorentzian line broadening function. F is defined as the integral of a well resolved signal at 123 ppm arising from C-3 of free [3,5-13C]porphobilinogen, indicative of one carbon of free porphobilinogen. The C-5 signal of free [3,5-13C]porphobilinogen (120.5 ppm) and the C-3 signal of bound [3,5-13C]porphobilinogen (121 ppm) are not fully resolved, but together indicate one carbon of free plus one carbon of bound. The integral of this signal is denoted as T. T − F = bound. Integrals were quantified by the cut and weigh method which is valid because C-3 and C-5 are both quaternary carbons with equivalent relaxation times and because free and bound porphobilinogen are in fast exchange (35). Because there is no measure of F when F ≤ T/10, the data could not be fitted reliably to a binding curve.

RESULTS

General Characterization of the Mutants H126A, H128A, and H126/128A—Site-directed mutagenesis was used to generate the E. coli PBGS mutant proteins H126A, H128A, and H126/128A (see Fig. 1). All proteins (mutant and wild type) were able to complement the hemB− strain HU1000, as evidenced by normal growth of the transformed strains in media without supplementation by hemin. The mutant proteins are soluble and have the same quaternary structure as wild type PBGS, as evidenced by their behavior on a Sephacryl column. A summary of the protein purification of H126A is presented in
Table II. The purification characteristics of all mutants were similar. Only the low overall yield of activity (15%) was different from purification of wild type where our yield is typically 40–50% (28). SDS-polyacrylamide gel electrophoresis of all proteins showed the purified mutant proteins to be homogenous and to migrate identically to wild type E. coli PBGS (data not shown). Total amino acid analysis was used to confirm the histidine content; the results are 6.15 ± 0.15 for wild type, 5.0 ± 0 for H128A, and 3.9 ± 0.2 for H126/128A, each an average value of two independent preparations and very close to the expected values of 6, 5, and 4. Therefore, the observed catalytic activity is unlikely to be due to wild type protein present as the result of a recombination event during expression. H126A was not subjected to total amino acid analysis.

The Response of Wild Type and Mutant PBGS to Zn(II) and Mg(II)—The specific activities of H128A, H126A, and H126/128A measured at 10 μM Zn(II) and 1 mM Mg(II) were 56.4, 59.4, and 38.9, respectively. To test whether the conserved histidines are involved in Zn(II) binding, the response of enzyme activity to Zn(II) and Mg(II) were measured. Fig. 3A shows the effect of added Zn(II) on the activity of wild type and mutant proteins in the absence and presence of MgC. Only the mutant protein H128A shows a significantly increased K_d for Zn_A/Zn_B relative to wild type PBGS. Under the conditions of Fig. 3A, in the absence of MgC, all the mutant proteins show reduced activity relative to wild type PBGS. However, when Zn(II) is added in the presence of MgC, the activities of the mutant proteins are close to or exceed (H126A and H128A) that of wild type.

We previously established that Mg_c is an allosteric activator of wild type PBGS which increases the V_max, reduces the K_m, and stabilizes the quaternary structure, and reduces the K_d for Zn_A/Zn_B (23). The multiple functions of Mg_c are retained in the mutant proteins but the magnitudes of the effects vary. The kinetic parameters for mutant and wild type PBGS (determined at 10 μM Zn(II), 1 mM Mg(II)) are shown in Table III. Mg_c causes V_max to increase by 3.4–13.2-fold and K_m to decrease by 5–40-fold for the mutant proteins. Native polyacrylamide gel electrophoresis reveals similar stabilization of the quaternary structure of the mutant proteins by Mg_c. Fig. 3B shows the effect of added Mg(II) on the activity of wild type and mutant PBGS and can be used to estimate the K_d for Mg_c. H126A, H128A, and H126/128A all have a higher K_d for Mg_c (0.4, 0.3, and 0.5 mM, respectively) than wild type PBGS (<0.1 mM).

pH Activity Relationships of H128A, H126/128A, and Wild Type PBGS—Histidine residues are frequently implicated to be responsible for near-neutral p_Ka values of enzymes. We determined the kinetic parameters K_m and V_max at various pH values for H128A, H126/128A, and wild type PBGS in the presence of 10 μM Zn(II) and 1 mM Mg(II). The results are illustrated in Fig. 4. The catalytic efficiency (k_cat/K_m; Fig. 4A)
shows a pK\textsubscript{a} of -7.5 which is maintained in H128A and H126/128A. This pK\textsubscript{a} arises from the pH dependence of the K\textsubscript{m} (Fig. 4B) and is the same in all proteins studied. In contrast to the pK\textsubscript{a} effect, the K\textsubscript{m} values for the mutant proteins differ by more than an order of magnitude from wild type PBGS; V\textsubscript{max} for H128A is reduced about 30% from wild type PBGS at neutral pH, and the V\textsubscript{max} for H126/128A is reduced about 50% from wild type at alkaline pH (Fig. 4C). These results indicate that neither His\textsuperscript{126} nor His\textsuperscript{128} are involved in the rate-determining step of the enzyme-catalyzed reaction, but implies that them in substrate binding.

The stoichiometry of E. coli PBGS Active Sites—The high K\textsubscript{m} of ALA for E. coli PBGS at neutral pH in the absence of Mg\textsuperscript{2+} (-3 mM) previously prevented our quantifying the stoichiometry of enzyme active sites (28). Although it is well established that mammalian PBGS has four active sites per octamer, the stoichiometry of active sites on E. coli PBGS is a matter of some controversy (36). Herein we document that the K\textsubscript{m} for ALA at pH 8 in the presence of saturating Zn(II) and Mg(II) is tight enough (-50 \mu M) that \textsuperscript{13}C NMR spectroscopy can be used to quantify the active site stoichiometry. Fig. 5 illustrates the integrated signal area of free and bound [3,5-\textsuperscript{13}C]porphobilinogen which is from the addition of [4-\textsuperscript{13}C]ALA to a solution containing 4.8 \mu mol of E. coli PBGS subunits. Free [3,5-\textsuperscript{13}C]porphobilinogen (chemical shifts at 123 and 120.5 ppm) is not observed until after the addition of -4 \mu mol of ALA. Further additions of [4-\textsuperscript{13}C]ALA cause signals from both free and bound porphobilinogen to increase until the bound signal saturates at a stoichiometry of 4.0 per enzyme octamer, indicating four active sites.

Temperature Dependence of H126/128A and Wild Type PBGS—We previously reported an E\textsubscript{\textsuperscript{a}} (energy of activation) of 64.2 kJ mol\textsuperscript{-1} for E. coli PBGS in the absence of Mg\textsuperscript{2+} (28). This is similar to the bovine PBGS E\textsubscript{\textsuperscript{a}} of 77.1 kJ mol\textsuperscript{-1} (37); bovine PBGS is unresponsive to Mg(II). Fig. 6 shows Arrhenius plots of the temperature dependence for H126/128A and wild type PBGS, obtained in the presence of 1 mM Mg(II). The Arrhenius plot of H126/128A in the presence of Mg(II) is clearly biphasic. Below 35 °C E\textsubscript{\textsuperscript{a}} is 26.5 kJ mol\textsuperscript{-1} and above 35 °C E\textsubscript{\textsuperscript{a}} is 2.1 kJ mol\textsuperscript{-1}, 13-fold less than E\textsubscript{\textsuperscript{a}} at lower temperatures. This result implies a change in the rate-determining step at 35 °C or a change in the K\textsubscript{m} for Zn(II) or Mg(II). The biphasic character of the data for wild type E. coli PBGS is less pronounced. Below 35 °C E\textsubscript{\textsuperscript{a}} is 19.7 kJ mol\textsuperscript{-1}, above 35 °C E\textsubscript{\textsuperscript{a}} is 2.1 kJ mol\textsuperscript{-1}. The 50 °C data point for both plots is not included in the calculation of E\textsubscript{\textsuperscript{a}} and may reflect a second transition. One surprising observation is that at 37 °C Mg\textsuperscript{2+} causes an 11-fold decrease in E\textsubscript{\textsuperscript{a}} for wild type PBGS; the magnitude of this effect is in sharp contrast to the Mg\textsuperscript{2+} induced 2-fold increase in V\textsubscript{\textsuperscript{max}}. This suggests that Mg\textsuperscript{2+} causes a change in the rate-determining step of the reaction.

**DISCUSSION**

Site-directed mutagenesis has been used to probe the roles of one species-invariant histidine His\textsuperscript{126} of E. coli PBGS and its partially conserved neighbor His\textsuperscript{128}. Previous chemical modification data had suggested an essential histidine (32, 33). The most striking result is the retained ability of the mutant proteins H126A, H128A, and H126/128A to catalyze the formation of porphobilinogen, indicating that these histidines are not essential for catalysis. Under approximately physiologic conditions the V\textsubscript{\textsuperscript{max}} and K\textsubscript{m} values for the mutant proteins vary little from wild type E. coli PBGS, suggesting that the rate-determining steps of the reaction are unaltered by the mutation. Thus, one may ask why these histidines are so highly conserved. Perhaps these residues are involved in a step in catalysis whose rate is several orders of magnitude faster than the rate-determining step; the overall rate of PBGS is slow (k\textsubscript{cat} is \textasciitilde 1 s\textsuperscript{-1}). The PBGS-catalyzed reaction includes several essential steps. P-side ALA (see Fig. 2) must bind and form a Schiff base to Lys\textsuperscript{246}; Zn\textsuperscript{2+} must bind to facilitate binding of A-side ALA. Beyond catalyzing formation of these early complexes, the protein may play a passive role, simply providing the scaffolding for binding substrates and metal ions. The conserved residues may contribute only secondary interactions to catalysis.

Mutation of His\textsuperscript{126} and/or His\textsuperscript{128} to alanine perturbs the K\textsubscript{m} for ALA; the K\textsubscript{m} is determined by the more loosely bound of the two identical substrates, the ALA known to require ZnA in order to bind at the active site (25, 38). This increased K\textsubscript{m} is consistent with our active site model because the increase may be due to altered affinity for ZnA or to alteration of the A-side ALA binding pocket. Despite the value of K\textsubscript{m} for the mutant proteins, they maintain the pK\textsubscript{a} of 7.5 in the K\textsubscript{cat}/K\textsubscript{m} profile. This pK\textsubscript{a} cannot be due to His\textsuperscript{126} nor His\textsuperscript{128}; the possibility that this pK\textsubscript{a} arises from another histidine is diminished by the fact that some PBGS (e.g. Bradyrhizobium japonicum PBGS) contain only these two histidines. Since this pK\textsubscript{a} must be related to
Thus, care must be taken in evaluating the metal ion requirements for PBGS from all species.

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