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5-Chlorolevulinate Interactions with *Bradyrhizobium japonicum* Porphobilinogen Synthase

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5-Chlorolevulinic acid (5-CLA) is an α-chloroketone analog of the tetrapyrrole precursor 5-aminolevulinate (ALA). As such it serves as an excellent probe of the mechanisms of enzymes which form or utilize ALA. The enzyme porphobilinogen synthase (PBGS) catalyzes the condensation of two molecules of ALA to form the monopyrrole porphobilinogen. In principle, 5-CLA could bind at either or both of the two different ALA binding sites of PBGS. In the presence of an active-site nucleophile, 5-CLA might chemically modify the protein with concomitant inactivation. Such inactivation has been shown for mammalian and *Escherichia coli* PBGS, each of which has reactive cysteines at the active site. In contrast, we report here that 5-CLA does not readily inactivate *Bradyrhizobium japonicum* PBGS (*Bj*PBGS) in either the presence or the absence of ALA. At neutral pH 5-CLA is a reversible inhibitor of *Bj*PBGS (K_i = 47 μM) and affects both the K_m for ALA (0.8 mM) and the V_{max} of the reaction. *Bj*PBGS does not use 5-CLA as an alternative substrate to form the 11-chloro analog of porphobilinogen in a mixed condensation with ALA. ^{13}C NMR was used to observe 5-chloro-[1,4-^{13}C]levulinate bound at the *Bj*PBGS active site. The ^{13}C NMR chemical shifts indicate that 5-CLA preferentially binds to the first ALA binding site and undergoes formation of an analog of the first Schiff base intermediate which normally occurs in the PBGS-catalyzed reaction. These data contribute to a growing body of mechanistic work which supports the proposition that carbon–nitrogen bond formation precedes carbon–carbon bond formation in the PBGS-catalyzed biosynthesis of porphobilinogen.

INTRODUCTION

Porphobilinogen synthase (PBGS) catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA,[1] Fig. 1A) to form porphobilinogen, the monopyrrole precursor of all biological tetrapyrroles. By nature of the keto moiety at C_4 of ALA, this substrate lends itself to mimicry by the α-chloroketone 5-chlorolevulinic acid (5-CLA, Fig. 1B). 5-CLA can act as an active site-directed chemical modification reagent (suicide substrate) of PBGS (*1–3*). In the case of bovine PBGS, 5-CLA preferentially modifies the cysteine residue necessary for the binding of a catalytic zinc (Zn_A) (*2*). In the case of *Escherichia coli* PBGS, 5-CLA modifies two other cysteines which appear to bind a second active-site zinc (Zn_B)

[1] Abbreviations used: ALA, 5-aminolevulinate; *Bj*PBGS, *Bradyrhizobium japonicum* PBGS; 5-CLA, 5-chlorolevulinate; [1,4-^{13}C]CLA, 5-chloro-[1,4-^{13}C]levulinate; βME, 2-mercaptoethanol; PBGS, porphobilinogen synthase.

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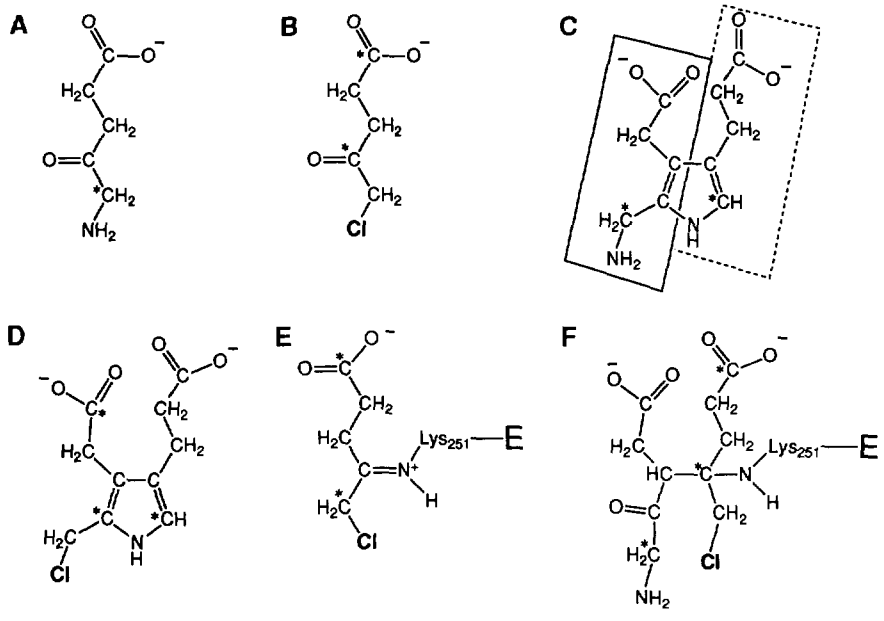

FIG. 1. The * indicates the position of ^{13}C label in all structures illustrated. (A) ALA is the substrate for PBGS. (B) 5-CLA is an α-chloroketone analog of the natural substrate ALA. (C) Porphobilinogen is the natural product of the PBGS-catalyzed condensation of two molecules of ALA. The fate of A-side ALA is depicted by the solid trapezoid. The fate of P-side ALA is depicted by the tapezoid outlined by a dashed line. (D) 5-[Chloromethyl]-4-[carboxymethyl]-1H-pyrrole-3-propionic acid is a possible PBGS-catalyzed condensation product of A-side 5-CLA and P-side ALA. (E) A possible structure for 5-CLA participating in the formation of a Schiff base intermediate at the P-side ALA binding pocket. The stereochemistry and protonation states are as previously determined by ^{13}C and ^{15}N NMR for ALA bound as the P-side Schiff base (*12*). (F) A possible dead-end intermediate complex, arising from carbon–carbon bond formation between A-side ALA and 5-CLA bound as the P-side Schiff base intermediate.

(*3*). *Bradyrhizobium japonicum* PBGS (*Bj*PBGS) does not contain cysteines at positions analogous to those modified in either bovine or *E. coli* PBGS. This is related to the fact that *Bj*PBGS uses metal ions differently from the aforementioned PBGS (*4*). *Bj*PBGS may use magnesium in place of one or both of the two zinc ions. Nevertheless, *Bj*PBGS may contain alternative active-site nucleophiles and modification of *Bj*PBGS by 5-CLA would provide additional information about the active-site structure. In addition to structural information, the interaction of 5-CLA and *Bj*PBGS may provide information about the reaction mechanism. One outstanding question about the PBGS-catalyzed reaction mechanism is which inter-ALA bond forms first, the carbon–nitrogen or the carbon–carbon bond (*5*). Possible *Bj*PBGS-catalyzed reactions of 5-CLA with ALA may provide insight into this aspect of the general enzyme-catalyzed reaction mechanism.

The two substrates of the PBGS-catalyzed reaction can be differentiated by their fate in the product porphobilinogen (see Fig. 1C). A-side ALA forms the *a*cetyl

half of the product; the free *a*mino group remains intact. P-side ALA forms the *p*ropionyl half of the product; the amino nitrogen is incorporated into the *p*yrrole ring. 5-CLA might bind at either or both of the two substrate binding sites. If an appropriate nucleophile is present on the protein, 5-CLA can chemically modify the protein with elimination of HCl as is seen with both bovine and *E. coli* PBGS (*1–3*). If a nucleophile is not present, 5-CLA might act as a reversible inhibitor. The nature of the inhibition might depend on whether 5-CLA binds in place of the ALA molecule which determines the K_m or in place of the ALA molecule which has a much lower K_d value and whose binding does not contribute to the K_m. In both mammalian and *E. coli* PBGS A-side ALA binding determines its K_m at neutral pH (*6, 7*).

In the absence of a reactive active-site nucleophile, other *Bj*PBGS-catalyzed reactions of 5-CLA with the protein or between 5-CLA and ALA may occur. For instance, if 5-CLA binds to the A-side ALA binding pocket, it might participate in a dehydration/condensation reaction with P-side ALA to form a product analog containing a chloro moiety in place of the amino group of porphobilinogen (see Fig. 1D). On the other hand, if 5-CLA binds at the P-side ALA binding pocket, it could participate in formation of the P-side Schiff base intermediate as illustrated in Fig. 1E. Further chemistry might occur between 5-CLA as the P-side Schiff base intermediate and A-side ALA. It would not be possible to form a pyrrole structure because the pyrrole nitrogen is absent. However, it might be possible to form an intermediate involving carbon–carbon bond formation between C_3 of A-side ALA and C_4 of 5-CLA bound as the Schiff base (see Fig. 1F). This would support the notion that carbon–carbon bond formation can precede carbon–nitrogen bond formation. Identification of these intermediates or products would provide information about the normal chemical mechanism of porphobilinogen formation.

This work describes the interaction of 5-CLA with *Bj*PBGS. We show that 5-CLA does not readily modify *Bj*PBGS. We investigate the inhibition of *Bj*PBGS by 5-CLA and probe for any enzyme-catalyzed reactions of 5-CLA or between 5-CLA and ALA. 5-Chloro-[1,4-^{13}C]levulinic acid ([1,4-^{13}C]CLA) is used as an NMR probe of the interactions of 5-CLA at the *Bj*PBGS active site.

METHOD

Materials

5-CLA and [1,4-^{13}C]CLA were synthesized in house by Cynthia Myers as previously described (*2, 3*). [5-^{13}C]ALA was custom synthesized by Merck Isotopes (now C/D/N Isotopes Inc., Canada). ALA-HCl, KH_2PO_4, and D_2O (98%) were purchased from Sigma. 2-Mercaptoethanol (βME) was purchased from Fluka and vacuum-distilled prior to use. All other chemicals were reagent grade or better. Spin columns (P6 resin) were obtained from Bio-Rad. PBGS was purified from the *E. coli* strain BL21(De3)pLysS:pET3cBjhemB, a kind gift from Professor Mark O'Brian of SUNY Buffalo. This strain contains the gene for *Bj*PBGS (the *hem*B gene) in a plasmid (pET3C) as an inducible T7 polymerase expression system in

the host *E. coli* BL21. BL21(DE3)pLysS:pET3cBjhemB expresses *Bj*PBGS to the level of >20% of the soluble protein. Cell growth conditions and protein purification will be reported elsewhere (*8*).

Enzyme Assays

All assays were done in 1-ml volumes containing 0.1 M KPi, pH 7, 10 mM βME, 10 μM $ZnCl_2$, 10 mM $MgCl_2$. The βME and $ZnCl_2$ are not required for activity (*8*). Except where otherwise indicated, *Bj*PBGS (30–40 $\mu g/ml^{-1}$) was preincubated for 10 min in assay buffer and the reaction was started by the addition of ALA-HCl to a final concentration of 10 mM. After 5 min at 37°C the reaction was stopped by the addition of 0.5 ml STOP reagent (20% TCA in 100 mM $HgCl_2$). Pyrrole formation was detected at 555 nm ~10 min after the addition of an equal volume of modified Ehrlich's reagent to the STOPped reaction mixture. Where necessary, the STOPped reaction mixture was diluted with a 2:1 buffer/STOP mixture prior to addition of Ehrlich's reagent to ensure that the absorbance at 555 nm was ≤1. This complex assay procedure is required because *Bj*PBGS activity is adversely affected below ~25 $\mu g/ml^{-1}$ protein. The specific activity of homogeneous *Bj*PBGS under these conditions is ~35 μmol of PBG formed per hour per milligram (*8*).

Inactivation Studies with 5-CLA

*Bj*PBGS at 5 mg/ml was preincubated for 10 min in 0.1 M KPi, pH 7, 1.5 mM βME, 10 μM Zn(II), 1 mM Mg(II) after which time 5-CLA (buffered at pH 7) was added to a final concentration of 20 mM. At set times of 1, 5, 30, and 60 min, 100-μl aliquots were removed, quenched by the addition of βME to 30 mM, and spun through a spin column which had been equilibrated with 0.1 M KPi, pH 7, 10 mM βME, 10 μM Zn(II), 1 mM Mg(II). Standard 5-min assays were carried out.

Inactivation Studies with 5-CLA and ALA

To determine if 5-CLA together with ALA acts to inactivate *Bj*PBGS, 3.4 mg/ml protein was incubated for 10 min in 0.1 M KPi, pH 7, 10 μM Zn(II), 10 mM Mg(II) under the following three conditions: 1 mM ALA alone, 1 mM ALA plus 1 mM 5-CLA, and 1 mM 5-CLA alone. The protein was then separated from the small molecules by two passages through a spin column. Standard 5-min assays were carried out on the protein eluant.

Inhibition Studies

To estimate the effectiveness of 5-CLA as a reversible inhibitor, 5-CLA at concentrations varying from 0.5 to 20 mM was included in a preincubation mixture with *Bj*PBGS for 10 min at 37°C. βME was omitted. The assay was started by addition of ALA-HCl to a final concentration of 10 mM. To determine the K_m for ALA and V_{max} values for *Bj*PBGS in the presence of various concentrations of 5-CLA, ALA-HCl was varied from 0.1 to 5 mM at fixed concentrations of 5-CLA (0, 25, 100, 500 μM). To maintain constant pH, total ALA-HCl and HCl added were kept constant at 5 mM.

^{13}C NMR to Probe for Formation of the 11-Chloro Analog of Porphobilinogen

^{13}C NMR was used to probe for *Bj*PBGS-catalyzed formation of the 11-chloro analog of porphobilinogen (Fig. 1D). A 1.75-ml reaction mixture contained 0.1 M KPi, pH 7, 10 mM [1,4-^{13}C]CLA, 10 mM [5-^{13}C]ALA, 10 μM Zn, 10 mM Mg, 20% D_2O. The reaction was started by the addition of 1 mg *Bj*PBGS and was monitored by ^{13}C NMR for 20 h at 37°C. A control reaction mixture omitted the protein. ^{13}C NMR spectra were acquired on a Bruker AM300 spectrometer at 75.4 MHz as previously reported (9) using low-power broad-band proton decoupling and a 2-s recycle time. Data were processed with a 2 Hz Lorenzian line broadening function.

^{13}C NMR Observation of the Direct Interactions between 5-CLA and BjPBGS

^{13}C NMR was used to probe the active site structure of [1,4-^{13}C]CLA bound to *Bj*PBGS. The 1.6-ml sample contained 106 mg/ml^{-1} *Bj*PBGS (a total of 4.3 μmol enzyme subunits) in 0.1 M KPi, pH 7, 10 mM Mg, 10 μM Zn in 20% D_2O upon which a background spectrum was obtained. [1,4-^{13}C]CLA was added (1.2 μmol) and the ^{13}C NMR spectrum was monitored for ~20 h at 37°C, collecting nine blocks of data at 2.3 h per block. Data were processed using a 20 Hz Lorenzian line broadening function.

RESULTS

Inactivation Studies

*Bj*PBGS is not readily inactivated by 5-CLA alone. Incubation of *Bj*PBGS in phosphate buffer at pH 7.0 with 20 mM 5-CLA for 0, 1, 5, 30, and 60 min results in 100, 108, 100, 107, and 86% residual activity, respectively. It is possible that a mixed reaction of 5-CLA and ALA could inactivate *Bj*PBGS through formation of the species illustrated in Fig. 1F. To test this hypothesis, *Bj*PBGS was incubated for 10 min with substrate alone, with 5-CLA alone, and with both substrate and 5-CLA together. After separation from the reactants, the resulting proteins were all fully active.

Inhibition Studies

A preliminary determination of *Bj*PBGS activity as a function of 5-CLA concentration of 0.5, 1, 3, 10, and 20 mM showed activities of 20, 12, 6, 3, and 2.5%, respectively. The data suggest that the K_i for 5-CLA is considerably tighter than 0.5 mM under these conditions. Because 5-CLA does not readily inactivate *Bj*PBGS, the inhibition is defined as reversible and may be competitive, noncompetitive, or mixed. The results of determining K_m and V_{max} for ALA at various concentrations of 5-CLA are presented graphically in Fig. 2A and show a mixed inhibition pattern. A replot of the slope and intercept data, presented in Fig. 2B, indicates that K_i = 47 μM, $\beta = 0$, and $\alpha = 2.66$. β is a measure of the propensity of the enzyme–inhibitor complex to proceed to product (see *10*). α is a measure of the cooperative nature