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# 5-Chlorolevulinate Modification of Porphobilinogen Synthase Identifies a Potential Role for the Catalytic Zinc†

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ABSTRACT: Porphobilinogen synthase (PBGS) is a Zn(II) metalloenzyme which catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA). The nitrogen of the first substrate ends up in the pyrrole ring of product (P-side ALA); by contrast, the nitrogen of the second substrate molecule remains an amino group (A-side ALA). A reactive mimic of the substrate molecules, 5-chlorolevulinate (5-CLA), has been prepared and used as an active site directed irreversible inhibitor of PBGS. Native octameric PBGS binds eight substrate molecules and eight Zn(II) ions, with two types of sites for each ligand. As originally demonstrated by Seehra and Jordan [(1981) *Eur. J. Biochem. 113*, 435–446], 5-CLA inactivates the enzyme at the site where one of the two substrate molecules binds, and modification at four sites per octamer (one per active site) affords near-total inactivation. Here we report that 5-CLA-modified PBGS (5-CLA-PBGS) can bind up to four substrate molecules and four Zn(II) ions. Contrary to the conclusion of Seehra and Jordan, we find that the preferential site of 5-CLA inactivation is the A-side ALA binding site. On the basis of the dissociation constants, the metal ion binding sites lost upon 5-CLA modification are assigned to the four catalytic Zn(II) sites. 5-CLA-PBGS is shown to be modified at cysteine-223 on half of the subunits. We conclude that cysteine-223 is near the amino group of A-side ALA and propose that this cysteine is a ligand to the catalytic Zn(II). The vacant substrate binding site on 5-CLA-PBGS is that of P-side ALA. We have used $^{13}C$ and $^{15}N$ NMR to view [4-$^{13}C$]ALA and [$^{15}N$]ALA bound to 5-CLA-PBGS. The NMR results are nearly identical to those obtained previously for the enzyme-bound P-side Schiff base intermediate [Jaffe et al. (1990) *Biochemistry 29*, 8345–8350]. It appears that, in the absence of the catalytic Zn(II), 5-CLA-PBGS does not catalyze the condensation of the amino group of the P-side Schiff base intermediate with the $C_4$ carbonyl derived from 5-CLA. On this basis we propose that Zn(II) plays an essential role in formation of the first bond between the two substrate molecules.

Porphobilinogen synthase (PBGS)[1] catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) to form porphobilinogen (PBG), the monopyrrole precursor of porphyrin, chlorophyll, and other naturally occurring tetrapyrroles (Shemin & Russell, 1953). PBGS is a homo-octameric protein with four active sites, four catalytic Zn(II), and four additional Zn(II). Each active site contains two ALA binding sites. The two substrate molecules (ALA) are of course identical; they are differentiated for convenience in terms of their ultimate location in PBG as A-side ALA and P-side ALA.[2] This is illustrated in Figure 1. The enzyme-catalyzed reaction includes formation of a substrate–enzyme Schiff base between $C_4$ of P-side ALA and an active-site lysine (Nandi & Shemin, 1968; Jordan & Seehra, 1980). The stereochemistry and protonation states of the

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[1] Abbreviations: ALA, 5-aminolevulinate; βME, 2-mercaptoethanol; 5-CLA, 5-chlorolevulinate; 5-CLA-PBGS, 5-CLA-modified PBGS; MMTS, methyl methanethiosulfonate; PBG, porphobilinogen; PBGS, porphobilinogen synthase.

[2] A-side ALA is the substrate which contributes the amino group and the acetyl side chain to PBG. P-side ALA is the substrate which contributes the pyrrole nitrogen and the propionyl side chain to PBG.

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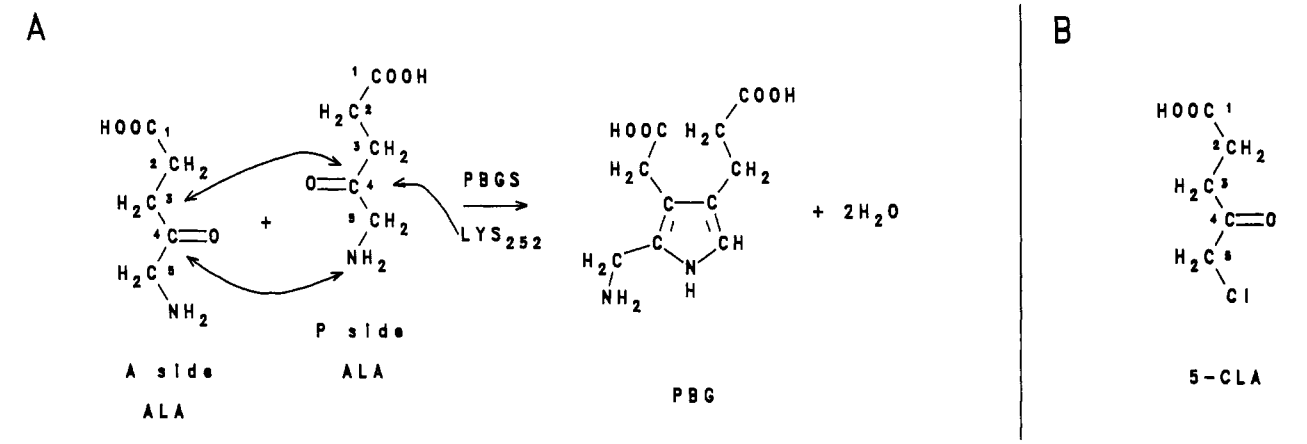


FIGURE 1: (A) PBGS-catalyzed reaction. P-side ALA is known to bind first and form a Schiff base intermediate to Lys-252. The next step in the reaction is proposed to be formation of a second Schiff base between the amino group of P-side ALA and the ketone moiety of A-side ALA. The two points of intersubstrate bond formation are indicated by arrows. (B) 5-Chlorolevulinic acid (5-CLA), a reactive $\alpha$-chloroketone analogue of ALA, has the potential to bind and react at either substrate binding site.

Schiff base intermediate have been determined (Jaffe et al., 1990); these data support the proposal that the enzyme-catalyzed reaction mechanism includes formation of a second Schiff base, in this case between two substrate molecules. This second Schiff base is proposed to form between the nitrogen of P-side ALA and $C_4$ of A-side ALA, as originally suggested by Jordan and Seehra (1980). Other details about the reaction mechanism, including the potential role of the essential Zn(II), remain less clear. PBGS is a somewhat atypical Zn(II) metalloenzyme in that it is highly susceptible to inhibition by the environmental toxin lead.

5-Chlorolevulinate (5-CLA; see Figure 1), is an active-site-directed irreversible inhibitor of PBGS which was first characterized by Seehra and Jordan (1981). The alkylation of active-site nucleophiles by $\alpha$-haloketones is well documented (Schoellmann & Shaw, 1963; Means & Feeney, 1990). Seehra and Jordan demonstrated (1) that 5-CLA binds preferentially at the P-side ALA binding pocket, (2) that 5-CLA can form the P-side Schiff base intermediate, (3) that 5-CLA alkylates the protein at a cysteine residue, and (4) that alkylation at a stoichiometry of four per octamer (one per active site) affords near-total inactivation. What they did not prove was where the *inactivation* occurred. An investigation of this forms part of the present paper.

Because 5-CLA is a structural analogue for both A-side and P-side ALA, the demonstration of preferential *binding* at one site (P-side) does not exclude *binding and reaction* at the other site (A-side). We noted this uncertainly because inactivation of PBGS by 5-CLA occurs only at 5-CLA concentrations much higher than the $K_i$ of 5-CLA when it acts as a reversible competitive inhibitor (11 $\mu$M; Seehra & Jordan, 1981). First, to confirm that 5-CLA inactivates at the binding site of one of the two substrate molecules, we compared the binding of ALA to PBGS and 5-CLA-modified PBGS (5-CLA-PBGS). Having demonstrated that 5-CLA-PBGS retains only half the number of substrate binding sites found in the unmodified enzyme, we demonstrate that the P-side substrate binding pocket is vacant; this was shown by using $NaBH_4$, which trapped radiolabeled substrate bound to 5-CLA-PBGS as the P-side Schiff base intermediate.

5-CLA modification of PBGS was originally reported to occur at a cysteine residue, perhaps contained in a tryptic peptide of composition CY (Seehra & Jordan, 1981). To confirm the location of the 5-CLA-susceptible cysteine, we identified the 5-CLA-modified peptide in an Asp-N protease digest and determined its amino acid sequence. This was done without using radioactivity and used the third position of 5-CLA-modified somatostatin as a standard for 5-CLA-modified cysteine.

PBGS contains more than one catalytically essential cysteine residue (Barnard et al., 1977), and cysteines have been identified as ligands to both the four catalytic and the four other Zn(II) ions (Dent et al., 1990). Therefore we have looked at the effect of 5-CLA modification on the affinity and stoichiometry of PBGS for Zn(II). This approach has implicated the 5-CLA-modified cysteine as a ligand to the catalytic Zn(II). The ultimate aim of this study is to observe $^{13}C$- and/or $^{15}N$-labeled ALA bound to 5-CLA-PBGS in order to determine if any partial reactions of ALA can occur at the modified active sites. Our NMR characterization of [4-$^{13}C$]ALA and [$^{15}N$]ALA bound to 5-CLA-PBGS suggests a mechanistic role for the catalytic Zn(II).

## Experimental Procedures

*Materials.* Aminolevulinic acid hydrochloride (ALA), potassium phosphate monobasic ($KP_i$), TES, cyanogen bromide, methyl methanethiosulfonate (MMTS), $NaBH_4$, *p*-(dimethylamino)benzaldehyde, and somatostatin were purchased from Sigma Chemical Co. 2-Mercaptoethanol ($\beta$ME) was purchased from Fluka Chemical Corp. and distilled under vacuum prior to use. High-purity KOH and Zn atomic absorption standards were purchased from Aldrich Chemical Co. 5-Amino-[4-$^{14}C$]levulinic acid ([4-$^{14}C$]ALA), 46 $\mu$Ci/$\mu$mol, was purchased from Research Products International. [4-$^{13}C$]ALA and [$^{15}N$]ALA were purchased from Cambridge Isotopes, Inc. Ecoscint scintillation fluid was purchased from National Diagnostics. Centrifree and Centriprep ultrafiltration devices were purchased from Amicon Corp. E. Merck PEI TLC plates were purchased from Thomas Scientific. Trifluoroacetic acid was purchased from Pierce. House distilled water was further purified by passage through a Barnstead HN Ultrapure mixed-bed cartridge. Solvents used for peptide mapping were all HPLC grade. Asp-N protease was purchased from Boehringer Mannheim. All other chemicals were reagent grade.

*Preparation of 5-Chlorolevulinic Acid.* 5-CLA was prepared following a combination of published procedures (Casen, 1955; deBoer & Backer, 1963; Bloxham & Chalkley, 1976) with the following modifications. Methylcyclohexane was used to crystallize the monomethyl ester of succinic acid (mp 55–56 °C), and carbon tetrachloride was used to crystallize the product 5-CLA (mp 71–72 °C). 5-CLA was stored dry at

−20 °C and handled with gloves, as it was found to be a potent skin irritant.

*Enzyme Preparation.* Purification of bovine liver PBGS was carried out as previously described (Jaffe et al., 1984). All purification and storage buffers contain 10 mM βME and 10 μM Zn(II). When isolated and stored under these conditions, PBGS contains eight Zn per octamer and is remarkably stable (Jaffe et al., 1984). 5-CLA-PBGS was prepared by reacting PBGS at 0.2–10 mg/mL with 20 mM 5-CLA in 0.1 M $KP_i$, pH 7.0, and 10 μM $ZnCl_2$ at 37 °C. Reaction times varied from 10 min to 3 h. The reaction was quenched with 30 mM βME, which reacts quickly with 5-CLA (as determined by $^1H$ NMR). 5-CLA-PBGS was isolated from the reaction mixture by gel-filtration chromatography (e.g., 200 mg of protein at 10 mg/mL was run on an 800-mL Sephadex G-25 column and eluted with 0.1 M $KP_i$, pH 7.0, 10 mM βME, and 10 μM $ZnCl_2$ at 1.8 mL/min). Unless otherwise stated, the term 5-CLA-PBGS refers to enzyme which was modified for 1 h. The Schiff base which might form either within 5-CLA-PBGS or between the substrate ALA and 5-CLA-PBGS was trapped by reduction with $NaBH_4$ as previously described (Jaffe & Hanes, 1986). MMTS-modified PBGS was prepared as previously described (Jaffe et al., 1984). In order to determine if MMTS modification of PBGS or the presence of the Zn(II) chelator 1,10-phenanthroline (5 mM) protected the enzyme from inactivation by 5-CLA, the enzyme was incubated at 37 °C in the presence of 18 mM 5-CLA. In each case, after 10 min, the reaction mixture was placed on ice for about 2 min and then the protein was separated from the 5-CLA on a 50-mL Sephadex G-25 column which had been equilibrated with 0.1 M $KP_i$, pH 7, 10 μM $ZnCl_2$, and 10 mM βME. The protein-containing fractions were pooled, concentrated, and assayed for catalytic activity.

*PBGS Assays.* Native PBGS was assayed as described previously (15 μg of enzyme/mL of assay mix for 5 min at 37 °C) (Jaffe et al., 1984). 5-CLA-PBGS was assayed for up to 5 h to determine residual activity (80–90 μg of enzyme/mL of assay mix). Assay mixtures lacking enzyme were monitored in parallel to determine nonenzymatic formation of Ehrlich's positive (pyrrole) condensation products. Native PBGS assays, done in the presence of 5 mM 1,10-phenanthroline, contained up to 100 μg/mL PBGS and were carried out for 30 min. A unit is defined as the production of 1 μmol of PBG/h at 37 °C.

For 5-CLA-PBGS, product formation was also determined in a 1:1 enzyme–substrate mixture. The stoichiometric assay was carried out at 2 mM enzyme active sites (140 mg/mL) and 2 mM ALA (one ALA per active site); PBG formation was monitored for 24 h by the colorimetric determination of PBG with Ehrlich's reagent (Granick & Mauzerall, 1958).

*[4-$^{14}$C]ALA Binding to PBGS and 5-CLA-PBGS.* The binding of ALA to native PBGS and to 5-CLA-PBGS was determined using [4-$^{14}$C]ALA (≤46 μCi/μmol) and Amicon Centrifree ultrafiltration devices, which are designed to separate free from protein-bound ligand. Glycerol was washed from the Centrifree membranes before their use with approximately 3 mL of $H_2O$ and then 1 mL of 0.1 M $KP_i$, pH 7.0, by centrifugation at 1500*g*. For the binding studies, the upper chamber of a device contained 200 μL of 25 μM octameric PBGS (7 mg/mL) in 0.1 M $KP_i$, pH 7.0, 10 mM βME, and 10 μM $ZnCl_2$ to which had been added 0.02–4.0 mM ALA (specific radioactivity 46–0.4 μCi/μmol). The Zn(II) chelator 1,10-phenanthroline, when present, was included at a concentration of 5 mM. The reaction mixture was equilibrated at room temperature for 15 min, and the device was centrifuged at 1500*g* for 30 s. The first aliquot passing into the lower chamber was discarded because it is diluted by residual buffer from the washes, and the centrifugation was repeated. A total of no more than 50 μL of solution passed into the lower chamber of the device. The upper chamber retained at least 150 μL of protein solution. Three 5-μL aliquots from each chamber of the device were counted for radioactivity, which indicated concentration of free ALA in the bottom chamber and total ALA in the top chamber. Protein concentration in the top chamber was determined by the method of either Bradford or Lowry using bovine serum albumin as a standard. PBGS is 13% and 15% more sensitive than bovine serum albumin for these two protein assays, respectively. Bound ALA was then calculated as (total ALA − free ALA)/octamer. ALA was shown neither to bind to the walls of the device nor to stick to the membranes by mimicking this procedure with a protein-free [4-$^{14}$C]ALA solution.

*[3,5-$^{14}$C]PBG Binding to 5-CLA-PBGS.* [3,5-$^{14}$C]Porphobilinogen was produced on the day of use from [4-$^{14}$C]ALA, 0.4 and 20 μCi/μmol, 1.3 and 9.7 mM, respectively using 0.06 unit of PBGS/mM ALA at 37 °C. PBG production was monitored by removing aliquots and determining PBG concentration using Ehrlich's reagent. The PBG concentration leveled off after 1 and 3 h for the 1.3 and 9.7 mM solutions, respectively, after which time the reaction mixtures were spun through Centrifree units for 10 min at 1500*g* to remove the enzyme. Final concentrations of PBG were determined using Ehrlich's reagent. The amount of unreacted ALA was determined by thin-layer chromatography (20 cm plastic-backed PEI-cellulose, 2.5–3 μL/lane, prewashing and developing with 0.1 M TES-KOH, pH 7.2). After thorough drying, each lane was cut into 0.5-cm strips, and the radioactivity was determined by scintillation counting. The Centrifree binding procedure described above was used to determine [3,5-$^{14}$C]-PBG binding to 5-CLA-PBGS, taking into account the binding of the unreacted [4-$^{14}$C]ALA.

*Limited Proteolysis and Peptide Mapping of Native and Modified PBGS.* Following denaturation with 6 M urea, reduction with βME, and alkylation of the cysteines with iodoacetate, cyanogen bromide was used to cleave 5 mg each of PBGS, 5-CLA-PBGS, and 5-CLA-PBGS which had been treated with $NaBH_4$. The resulting peptides were separated by reverse-phase chromatography on a Vydac C-18 protein and peptide column (4.6 × 250 mm) using a Waters 600E pump, a Waters 994 diode array detector, and a $H_2O$(0.06% TFA)/acetonitrile(0.056% TFA) gradient (0–63 min, 2–30% 63–95 min, 30–60% acetonitrile; 95–105 min, 60–78% acetonitrile). In each case, 1 mg of protein was used for the preparative peptide maps. The chromatogram was monitored at 214 and 280 nm, and spectra were collected for each of the peaks. From 0 to 60 min, the resulting peptide maps were identical. To simplify the peptide map between 60 and 92 min, this region was pooled and further digested with Asp-N protease, using a 1:200 weight ratio of protease to PBGS CNBr peptides. The peptides resulting from the Asp-N protease digest were rechromatographed as described above.

*5-CLA Modification of Somatostatin.* 5-CLA was used to modify somatostatin as a control to determine how 5-CLA-modified cysteines behave when subjected to amino acid analysis and sequencing. The sequence of somatostatin is AGCKNFFWKTFTSC, with an internal disulfide bridge between positions 3 and 14. In a 1-mL reaction mixture containing 0.5 mg of somatostatin and 6 M guanidine hydrochloride, the disulfide bond was reduced by the addition of 2 mM DTT followed by incubation at 60 °C for 2.5 h. The

reduced somatostatin was made 10 mM in 5-CLA and allowed to react for 15 min at 60 °C; the reaction was quenched by the addition of an excess of βME at 60 °C for 10 min. A 0.3-mg aliquot of 5-CLA-modified somatostatin was further reduced by the addition of 0.1 mL of 1 M $NaBH_4$. This reaction mixture was kept on ice with stirring and the pH was maintained at 7.0 by the addition of 1 M acetic acid. After 10 min the $NaBH_4$-reduced 5-CLA-modified somatostatin was frozen to await purification. The modified peptides were purified by HPLC as described above. The gradient was $H_2O$(0.06% TFA)/acetonitrile(0.056% TFA) (0–5 min, 2% acetonitrile; 5–65 min, 2–30% acetonitrile; 65–85 min, 30% acetonitrile). Under these conditions somatostatin elutes at 64.4 min, DTT-reduced somatostatin elutes at 66.9 min, 5-CLA-modified somatostatin elutes at 64.4 min, and $NaBH_4$-reduced 5-CLA-modified somatostatin elutes at 61.9 min. To ensure that 5-CLA-modified somatostatin was free of all native somatostatin, with which it coelutes, the mixture of these two forms was further reduced with DTT prior to preparative purification. Somatostatin, 5-CLA-modified somatostatin, and $NaBH_4$-reduced 5-CLA-modified somatostatin were subjected to sequencing and amino acid compositional analysis.

*Peptide Sequencing and Amino Acid Compositional Analysis.* Protein sequencing and analysis of amino acid composition was carried out in the Protein Analytical Lab of the Research Center for Oral Biology, University of Pennsylvania School of Dental Medicine.

Peptides to be sequenced were judged to be chemically pure and had an $A_{214}$ of at least 0.1. Peptides were covalently attached to either an acrylamine or diisothiocyanate (Sequelon) membrane via their free carboxy or amino groups, respectively, for solid-phase sequencing (Penning et al., 1991; Pappin et al., 1990). Automated Edman degradation was performed on a Milligen/Biosearch 6600 Prosequencer (Burlington, MA). After each cycle, the phenylthiohydantoin-(PTH-) amino acid derivatives liberated were analyzed by reverse-phase HPLC on a Milligen-Sequetag column (3.9 × 300 mm) using a 35 mM ammonium acetate, pH 4.8/acetonitrile gradient. PTH-amino acids in the eluent were detected at 269 and 313 nM and quantified by comparison to 100 pmol of standard PTH-amino acids. Analysis utilized the Maxima 820 program (version 3.1; Dynamic Solutions, Ventura, CA) modified with a dynamic subtraction routine (Milligen Corp.) to calculate retention times and integrate areas. Subtracted chromatograms were reviewed manually to ascertain accuracy.

For amino acid analyses, portions of the peptides were transferred to 50 × 6 mm acid-washed hydrolysis tubes, and vapor-phase hydrolysis was performed in 6 N HCl containing 1% phenol (v/v) at 110 °C for 20–24 h. The hydrolyzed samples were dried in vacuum, neutralized with triethylamine, redried, and derivatized at room temperature with phenyl isothiocyanate (PITC). The resulting phenylthiocarbamyl-amino acids were separated by reverse-phase HPLC using a Waters Picotag column (3.9 × 150 mm) and detected by UV absorbance at 254 nm (Bidlinmeyer et al., 1984). The derivatized amino acids in the hydrolysates were identified and quantified by comparing their retention times and peak areas, respectively, with those obtained for a freshly prepared mixture of standards.

*Equilibrium Dialysis Was Used To Determine Zn(II) Binding to PBGS and 5-CLA-PBGS.* The enzyme sample (2 mL) at approximately 0.7 μM octamer (0.2 mg/mL) was dialyzed against 200 mL of 0.1 M $KP_i$, pH 7.0, and 10 mM βME using Spectra/por 2 dialysis tubing. The dialysis buffer also contained 0–25 μM $ZnCl_2$, and dialysis proceeded with gentle agitation for 16–20 h at 20–22 °C in polypropylene containers. The zinc concentration of the protein solution and dialysate were determined using a Perkin-Elmer 560 atomic absorption spectrophotometer. Protein concentration was determined by the method of either Bradford or Lowry.

*NMR Data Acquisition.* $^{13}C$ NMR spectra were obtained at 75.45 MHz on a Bruker AM300 spectrometer using acquisition parameters identical with those reported previously for our studies with [4-$^{13}C$]ALA (Jaffe & Markham, 1987). Spectra were acquired with a 45° pulse and a 4-s repetition rate and were digitized at a resolution of 1.2 Hz/point. All spectra were obtained at 37 °C. Protein spectra were processed with a 35-Hz Lorentzian line-broadening function. The $^{13}C$ NMR sample contained 3.36 μmol of 5-CLA-PBGS active sites (235 mg) in 1.5 mL of 0.1 M $KP_i$, pH 7, 10 mM βME, 0.5 mM 1,10-phenanthroline, 20% $D_2O$, and 0.5% ethanol. The 5-CLA-PBGS spectrum was acquired with 10000 scans, after which 2.91 μmol of [4-$^{13}C$]ALA was added. After 3756 scans, a second 2.91-μmol aliquot of [4-$^{13}C$]ALA was added to the sample. Data acquisition proceeded for an additional 6000 scans. The protein spectrum was subtracted from the sum of spectra following the first addition of [4-$^{13}C$]ALA to obtain the difference spectra.

$^{15}N$ NMR spectra of enzyme-bound complexes were obtained at 60.8 MHz on a Bruker AM600 spectrometer, using acquisition parameters identical with those previously reported (Jaffe et al., 1990). Protein spectra (32K data points) were processed with 10-Hz Lorentzian line broadening. The spectra of the $^{15}N$ protein complex required 56 h of data acquisition. The $^{15}N$ NMR sample contained the identical 5-CLA-PBGS which was used for the $^{13}C$ NMR sample. Following $^{13}C$ data acquisition, the [3,5-$^{13}C$]PBG was removed by dilution to 15 mL with 0.1 M $KP_i$ and 10 mM βME and reconcentration to 1 mL using an Amicon Centriprep-30 device. This procedure was repeated twice. The protein sample was then exchanged into $D_2O$ by a similar dilution/reconcentration with 15 mL of $D_2O$ containing 0.1 M $KP_i$, pH 6.6, 10 mM βME, and 5 mM 1,10-phenanthroline. The final volume was 1.5 mL. For the $^{15}N$ protein complex, the higher 1,10-phenanthroline concentration was used to ensure minimal product formation during the lengthy data acquisition. Buffered $D_2O$ was used as solvent in order to obtain sharp spectral lines (Jaffe et al., 1990).

## RESULTS

*Inactivation of PBGS by 5-CLA.* Inactivation of PBGS by 5-CLA is potentially possible through reaction of 5-CLA at both the A-side and P-side ALA binding pockets. On the basis of the work of Seehra and Jordan (1981), a 5-CLA concentration of 20 mM was chosen for inactivation; this is expected to give less than 1% residual activity if incubated with PBGS for more than 20 min. Before modification, PBGS had a specific activity of 25.6 units $mg^{-1}$. After 1 h of treatment with 5-CLA, the activity was reduced to 0.07 units $mg^{-1}$ (0.3% of the original activity) and activity was found to be constant for 5 h. Longer treatment with 5-CLA further reduced the catalytic activity but resulted in a form of 5-CLA-PBGS which bound less than one molecule of substrate per octamer (see below). Since we intended to use 5-CLA-PBGS as a tool for NMR observation of bound substrate, the bulk of our efforts was directed toward 5-CLA-PBGS that had been modified for 1 h because this preparation retains the ability to bind approximately one ALA per active site (four per octamer).

In addition to the assay of 5-CLA-PBGS under $V_{max}$ conditions, we measured PBG formation in a 1:1 complex of ALA

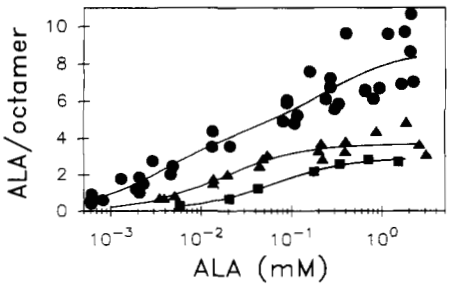


FIGURE 2: ALA binding to PBGS and 5-CLA-PBGS. ALA binding was determined using [4-$^{14}$C]ALA and an ultrafiltration technique. For native PBGS (●), there are two classes of ALA binding sites, each with a stoichiometry of four per octamer; the two dissociation constants are 3.8 $\mu$M and 242 $\mu$M, which correspond to the P-side and the A-side ALA, respectively. For 5-CLA-PBGS (■), there is one class of ALA binding sites ($n$ = 3), $K_d$ = 60 $\mu$M. For 5-CLA-PBGS (▲) in the presence of 5 mM 1,10-phenanthroline, ALA binding is even tighter: $n$ = 4.0, $K_d$ = 20 $\mu$M.

and 5-CLA-PBGS, the situation present in our NMR samples. In both assays product formation is presumed to be due to catalysis at a small percentage of active sites which are not 5-CLA modified (~0.3%). These sites can bind the two substrate molecules necessary for PBG formation. In the 1:1 ALA·5-CLA-PBGS complex, the substrate for PBG formation must derive from free ALA in equilibrium with ALA bound at the modified enzyme active sites. The slow production of 0.5 equiv of free product indicates that the interaction of ALA with 5-CLA-PBGS is readily reversible. This interaction may include formation of covalent bonds between ALA and 5-CLA-PBGS (see below). The initial rate of PBG formation is about 3-fold slower than $V_{max}$ observed in the catalytic assay described above.

*[4-$^{14}$C]ALA Binding to PBGS and 5-ALA-PBGS.* [4-$^{14}$C]ALA binding to PBGS shows the binding of eight ALA molecules per octamer, in agreement with our $^{13}$C and $^{15}$N NMR studies which show free PBG after saturation of four active sites per octamer of PBGS (Jaffe & Markham, 1987). When the data are presented as a semilogarithmic binding curve (Figure 2), it is clear that ALA binding to PBGS falls into two classes, approximately equal in stoichiometry, and with dissociation constants which differ by about 2 orders of magnitude. This is not unexpected, as Jordan and co-workers have already established that the binding of ALA to PBGS is ordered with P-side ALA binding first (Jordan & Seehra, 1980). When fit to a two-site model, where $n_1$ and $n_2$ are constrained to be equivalent, the total binding of ALA per octamer is found to be 8.8 ± 0.9, $k_1$ = 3.8 $\mu$M, and $k_2$ = 242 $\mu$M. The binding constants $k_1$ and $k_2$ are presumed to represent the binding of P-side and A-side ALA, respectively; the weaker of the two binding constants is equivalent to the apparent $K_M$ for the PBGS-catalyzed reaction. Interpretation of [4-$^{14}$C]ALA binding to PBGS is compromised by the fact that a 2:1 complex of ALA and PBGS active sites is ≥80% enzyme–product complex (Jaffe & Markham, 1987).

5-CLA-PBGS that is modified for 1 h in the presence of 20 mM 5-CLA binds ALA tighter than expected for binding at the A-side ALA binding pocket (3.0 ± 0.1 ALA/octamer with a dissociation constant of 60 $\mu$M). In this case, the binding data show good agreement to a single-site model (Figure 2). The tight binding of ALA to 5-CLA-PBGS was our first indication that 5-CLA might not be modifying PBGS at the P-side ALA binding pocket as Seehra and Jordan had concluded. Had 5-CLA inactivated at the P-side ALA binding pocket, we would have expected ALA to bind with a dissociation constant akin to that for A-side ALA, ~250 $\mu$M.

The importance of Zn(II) to ALA binding was interesting because our past work suggested that it is essential to A-side ALA binding. We evaluated the effect of 1,10-phenanthroline on the binding of [4-$^{14}$C]ALA to 5-CLA-PBGS. Under conditions where 1,10-phenanthroline abolishes the activity of native PBGS, it does not compromise the ability of 5-CLA-PBGS to bind ALA. Several substrate binding determinations in the presence of 1,10-phenanthroline indicate a stoichiometry of 4.0 ± 0.4 ALA/octamer with a $k_d$ = 20 $\mu$M. In this case the dissociation constant even more closely mimics that of P-side ALA, thus supporting our suspicion that 5-CLA inactivates at the A-side ALA binding pocket.

[4-$^{14}$C]ALA binding to 5-CLA-PBGS is a function of the time that PBGS is allowed to interact with 20 mM 5-CLA. After a 3-h incubation with 5-CLA, 5-CLA-PBGS can bind less than one ALA per octamer. These results suggest that 5-CLA can bind and react at both the A-side and P-side ALA binding pockets. No attempt was made to further characterize the doubly modified 5-CLA-PBGS.

*[3,5-$^{14}$C]PBG Binding to 5-CLA-PBGS.* The affinity of product for 5-CLA-PBGS provides information about the shape of the active site. If 5-CLA-PBGS contains a covalently bound 5-(*S*-cysteinyl)-4-oxopentanoic acid side chain in place of one of the two substrate molecules, then steric exclusion in the half-filled active sites should prevent product binding. In support of this hypothesis, we have determined that, within experimental error, 5-CLA-PBGS does not bind [3,5-$^{14}$C]PBG.

*$NaBH_4$ Treatment of 5-CLA-PBGS in the Presence of [4-$^{14}$C]ALA.* Our ALA binding data suggest that the P-side ALA binding pocket of 5-CLA-PBGS is available to substrate. This is in contrast to the work of Seehra and Jordan (1981), who concluded, but did not prove, that the P-side ALA binding pocket is the site of inactivation by 5-CLA. If 5-CLA inactivates PBGS at the P-side ALA binding pocket, then the resultant 5-(*S*-cysteinyl)-4-oxopentanoic acid side chain would occupy the P-side ALA binding pocket and we would expect ALA to be excluded from the P-side ALA binding pocket. To resolve this dilemma, we can ask if the ALA which binds to 5-CLA-PBGS can form the P-side Schiff base intermediate. If [4-$^{14}$C]ALA can bind at the P-side ALA binding pocket and form the P-side Schiff base intermediate, then treatment with $NaBH_4$ is expected to trap [4-$^{14}$C]ALA at the active site of 5-CLA-PBGS. Radiolabeling would indicate that the P-side ALA binding pocket is available for substrate binding and for P-side Schiff base formation. If radiolabeling is not observed, this might be because the P-side ALA binding is blocked or because binding is allowed and Schiff base formation is blocked.

$NaBH_4$ treatment of 5-CLA-PBGS in the presence of [4-$^{14}$C]ALA results in radiolabeling of 5-CLA-PBGS at a stoichiometry of 3.4 ± 0.7 ALA/octamer. Radiolabeling was dependent upon the presence of $NaBH_4$. The stoichiometry of radiolabeling is identical to that which we observe when this experiment is performed with native or MMTS-modified PBGS (Jaffe & Hanes, 1986). From this result, we conclude that the P-side ALA binding pocket of 5-CLA-PBGS is available for ALA binding and for P-side Schiff base formation. Therefore the ALA which bind to 5-CLA-PBGS at a stoichiometry of four per octamer bind at the P-side ALA binding pockets. The four A-side ALA binding pockets are the ones which are not available for substrate binding; these are the sites of inactivation by 5-CLA-PBGS.

*Zn(II) Binding to PBGS And 5-CLA-PBGS.* Building on Seehra and Jordan's proof that 5-CLA modifies a cysteine residue and a wealth of evidence suggesting that cysteines are

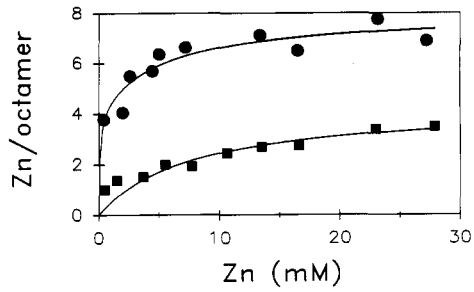


FIGURE 3: Zn(II) binding to PBGS and 5-CLA-PBGS. Zn(II) binding was determined by equilibrium dialysis analyzed by atomic absorption spectroscopy. For native PBGS (●), there are two classes of Zn(II) binding sites, each with a stoichiometry of four per octamer; the two dissociation constants ae ≤0.1 μM and 5.1 μM, which correspond to the catalytic and structural Zn(II), respectively. For 5-CLA-PBGS (■), the majority of the Zn(II) appears to bind to the lower affinity site: $K_d$ = 5.0 μM ($n$ = 3.8).

Zn(II) ligands in PBGS, we asked if this cysteine modification perturbs Zn(II) binding to PBGS. When purified in the presence of 10 μM Zn(II), PBGS contains eight Zn per octamer, though only four Zn per octamer are required for full activity (Bevan et al., 1980; Jaffe et al., 1984). On this basis, the equilibrium binding data of Zn(II) to PBGS is interpreted for a two-site model where $n_1$ and $n_2$ are constrained to be equivalent. Figure 3 illustrates the binding of Zn(II) to PBGS. The curve represents a nonlinear least squares best fit wherein $k_1 \leq 0.1$ μM, $k_2 = 5.1$ μM, and $n_1 = n_2 = 4.0 \pm 0.2$ per octamer. Zn(II) binding to 5-CLA-PBGS, when fitted to a single-site model, shows $n = 3.8 \pm 0.2$ and $k = 5.0$ μM. On the basis of the dissociation constants, it appears that 5-CLA-PBGS retains only the lower affinity half of the Zn(II) binding sites. Kinetic data on native and apoPBGS suggest that it is the higher affinity sites which are required for catalytic activity. Therefore, our data show that 5-CLA modification of PBGS interferes with the binding of the catalytic Zn(II).

*Effect of MMTS on the Inactivation of PBGS by 5-CLA.* As described above, we have identified the A-side ALA binding site as the site of PBGS inactivation by 5-CLA. Therefore, one might propose that agents which interfere with A-side substrate binding should interfere with inactivation of PBGS by 5-CLA. MMTS is a reversible sulfhydryl modifier (Smith et al., 1975). MMTS-modified PBGS is (1) modified at a stoichiometry of three cysteines per octamer (Jaffe et al., 1984) and (2) cannot bind A-side ALA (Jaffe & Markham, 1987). These are *two* related reasons why prior modification with MMTS might protect PBGS from inactivation by 5-CLA. MMTS-modified PBGS also contains no enzyme-bound Zn-(II), which suggested that either Zn(II) or SH groups are essential for A-side ALA binding. Therefore, to further probe the A-side ALA binding pocket, we have investigated the effect of MMTS on the inactivation of PBGS by 5-CLA. The results are presented in Table I. Here, the 5-CLA inactivation reaction was allowed to proceed for 10 min at 37 °C and was terminated by separating the enzyme from the reaction mixture by gel filtration in buffer containing Zn(II) and βME. The control experiment, with 5-CLA omitted, shows 0–15% loss of activity, as has been seen before when PBGS is subjected to elevated temperature in the absence of βME. By contrast, when 5-CLA was included in the reaction mixture, the purified protein lost >98% activity.

When the enzyme is first modified by MMTS and then treated with 5-CLA, at least 75% activity can be recovered in the presence of βME and Zn(II). In this case it is unavoidable that the reaction mixture will at some point contain a mixture of MMTS-modified PBGS, 5-CLA, and βME (see Experimental Procedures). Under these conditions there are three competing reactions: (1) the inactivation of 5-CLA by βME, (2) the reactivation of MMTS-modified PBGS by βME, and (3) the inactivation of PBGS by 5-CLA. Because of the latter reaction, one would not expect total protection by prior modification with MMTS.

Table I: Does either MMTS Modification or 1,10-Phenanthroline (*o*-phe) Protect against 5-CLA Inactivation of PBGS?

| reaction conditions 10 min at 37 °C | % activity |
|---|---|
| PBGS without βME | 85–100 |
| PBGS with 20 mM 5-CLA and 2 mM βME | 1–2 |
| MMTS-modified PBGS with 18 mM 5-CLA | 75–80 |
| PBGS with 5 mM *o*-phe, without βME | 85–100 |
| PBGS with 20 mM 5-CLA, 2 mM βME, and 5 mM *o*-phe | 15–18 |

*Effect of 1,10-Phenanthroline on the Inactivation of PBGS by 5-CLA.* Our previous work on MMTS-modified PBGS led us to propose that Zn(II) may play a role in substrate binding at the A-side ALA binding pocket. If this is so, it may follow that Zn(II) plays a role in 5-CLA binding at the A-side ALA binding pocket and that a Zn(II) chelator might interfere with this binding and the subsequent enzyme inactivation. Therefore, using the techniques described above, we looked at the effect of the Zn(II) chelator 1,10-phenanthroline on the inactivation of PBGS by 5-CLA. The results are included in Table I. A control experiment was done to investigate the stability of PBGS in the presence of 1,10-phenanthroline without any 5-CLA present. This experiment measures the irreversible oxidation of apo-PBGS which has been observed previously by us and others. In this case, PBGS retains 85–100% activity. When 20 mM 5-CLA is included in the 1,10-phenanthroline-containing reaction mixture, the residual activity of the purified enzyme is reduced to 15–18%. This is an order of magnitude more activity than is observed without 1,10-phenanthroline. Thus, the Zn(II) chelator, 1,10-phenanthroline provides some protection against inactivation of PBGS by 5-CLA; this is consistent with reduced 5-CLA binding at the A-side ALA binding pocket.

*Identification and Sequence Determination of 5-CLA-Modified Peptide.* To facilitate identification of the peptide containing the 5-CLA-modified cysteine, three forms of PBGS were subjected to proteolytic digestion: PBGS, 5-CLA-PBGS, and $NaBH_4$-reduced 5-CLA-PBGS. The modified peptide derived from the $NaBH_4$-reduced protein was expected to contain a carbinol rather than a keto group at the $C_4$ derived from 5-CLA. In all cases, the proteins were treated with iodoacetate prior to proteolysis. The reverse-phase peptide maps of the Asp-N protease digests (see Experimental Procedures) were inspected by overlaying the chromatograms, which were remarkably similar. The notable exception occurred in the region of 47–50 min. Because PBGS is a homooctameric protein and 5-CLA modification removes only four of the ALA binding sites, only four of the subunits are expected to be modified. Table II illustrates the intensities ($A_{214}$) and amino acid sequences of the peptides which were identified by inspection for this pattern. In each case the chromatogram represents digestion of 0.7–0.9 mg of protein. For the native protein, a major peptide elutes at 48.3 min, heretofore called peptide 9A. Normalized to the other peptides in the digest, this was twice as intense as peptide 9A derived from 5-CLA-PBGS. The 5-CLA-PBGS digest also contained a new peptide which eluted 0.5 min later, called peptide 9B, which was equal in intensity to peptide 9A. The Asp-N protease digest derived from $NaBH_4$-reduced 5-CLA-PBGS

Table II: Chromatographic and Sequence Data for Peptides Derived from PBGS

| sample | peptide identification | intensity at 214 nm | sequence |
|---|---|---|---|
| PBGS | 9A | 0.65 | DRRCYQLPPGARGLALRAVDR |
| 5-CLA-PBGS | 9A | 0.44 | DRRCYQLPP... |
| | 9B | 0.43 | DRR-YQ... |
| $NaBH_4$-reduced 5-CLA-PBGS | 9A | 0.41 | DRRCYQ... |
| | 9C | 0.30 | DRRC'YQLPPG... |

also contained the parent peptide 9A, which was followed by a second peptide, 9C, 0.8 min later. In this case the second peptide was slightly reduced in intensity. The sequences of these five peptides are shown in Table II and described below.

Amino acid sequencing proved that all five peptides were identical to or derived from the parent peptide 9A. The amino acid sequence of the parent peptide was DRRCYQLPPGARGLALRAVDR where the fourth position appeared as (carboxymethyl)cysteine. This sequence is identical to amino acids 220–240 of human PBGS (Wetmer et al., 1986). The other peptides were each sequenced for at least 6–10 cycles so that complete identity was obtained. In both the cases of 5-CLA-PBGS and $NaBH_4$-reduced 5-CLA-PBGS, peptide 9A showed (carboxymethyl)cysteine in the fourth sequence cycle. For the peptide 9B, derived from 5-CLA-PBGS, the fourth cycle was nearly void of signal. There was an extremely low yield of (carboxymethyl)cysteine which probably derived from a small contamination by peptide 9A. For peptide 9C, derived from $NaBH_4$-reduced 5-CLA-PBGS, the fourth sequence cycle gave excellent yield of a new PTH-amino acid which we denote as C'. The third, fourth, and fifth sequence cycles of the C'-containing peptide are illustrated in Figure 4A and clearly show that C' is distinct from any of the PTH-amino acid standards.

To confirm that C' derives from a reduced form of 5-CLA modified cysteine, we prepared 5-CLA-modified somatostatin and reduced a portion of that peptide with $NaBH_4$ (see Experimental Procedures). Native reduced somatostatin and 5-CLA-modified somatostatin each showed the amino acid sequence AG-KN, where the third cycle was devoid of signal at 269 nm as might be expected for an unmodified cysteine. These peptides did show a significant peak at 313 nm in the third cycle due to PTH-dehydroalanine, which results from loss of $H_2S$ from cysteine during cleavage. However, for $NaBH_4$-reduced 5-CLA-modified somatostatin, the third sequence cycle revealed a significant peak of C' eluting prior to the PTH-arginine standard (see Figure 4B). This demonstrates that C' derives from 5-CLA-modified cysteine. Because the retention time of C' on reverse-phase chromatography is significantly later than the acidic PTH residues, we propose that C' is the lactone form of the 5-(*S*-cysteinyl)-4-hydroxypentanoic acid side chain, whose proposed formation is illustrated in Figure 4C.

Inspection of the peptide maps revealed another peptide unique to 5-CLA-PBGS and suggested that His-277 may also be susceptible to modification by 5-CLA. The sequence of this peptide is DIVREVKNKHPELPLAVYHVSG; His-277, the second histidine in this peptide, was present in very low yield. Position 277 is a histidine in human, bovine, and yeast PBGS; it is a glutamine in mouse, rat, and *Escherichia coli* PBGS (Wetmer et al., 1987; Myers et al., 1987; Bishop et al., 1986, 1989; Echeland et al., 1988; Li et al., 1989).

*NMR of [4-$^{13}$C]ALA and [$^{15}$N]ALA Bound to 5-CLA-PBGS.* The $^{13}$C and $^{15}$N NMR spectra of isotopically labeled ALA bound to 5-CLA-PBGS should allow one to determine

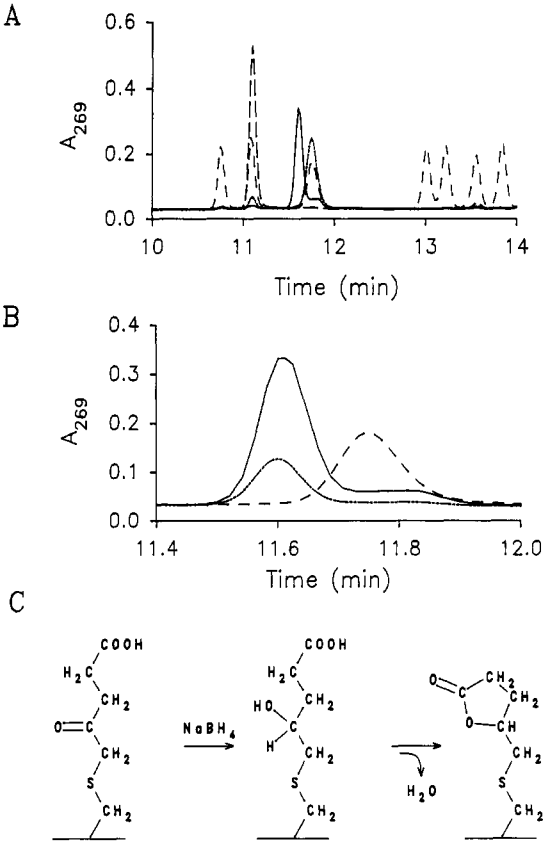


FIGURE 4: Identification of the 5-CLA-modified cysteine. (A) Sequence cycles 3 (—·—·—), 4 (—), and 5 (– – –) for the modified peptide of $NaBH_4$-reduced 5-CLA-PBGS. The PTH-amino acid standards (100 pmol) are included for reference (· · ·). (B) Superposition of sequence cycle 4 of the modified peptide from $NaBH_4$-reduced 5-CLA-PBGS (—), cycle 3 of $NaBH_4$-reduced 5-CLA-modified somatostatin (—· · —), and 100 pmol of PTH-tagged arginine (· · ·). The minor component which coelutes with PTH-arginine has significant absorbance at 313 nm, with PTH-arginine does not, and is believed to be a breakdown product of C'. (C) When 5-CLA-modified cysteine is reduced by $NaBH_4$, the resulting γ-hydroxy acid is expected to condense to form a stable lactone. The lactone is the proposed structure of C'.

the reactions which can occur when P-side ALA is bound to 5-CLA-PBGS. The difference spectrum between the [4-$^{13}$C]ALA·5-CLA-PBGS complex and 5-CLA-PBGS is illustrated in Figure 5A. The spectrum contains signals from three species: (1) free [4-$^{13}$C]ALA (205.9 ppm, line width 32 Hz), (2) the enzyme-bound intermediate (165.4 ppm, line width 100 Hz), and (3) free [3,5-$^{13}$C]PBG (121.0 and 123.1 ppm, line widths 13 and 17 Hz, respectively; data not shown). The ratio of ALA:intermediate:product, 1.0:3.2:7.0, is a time-averaged value and does not reflect an equilibrium condition. The free [3,5-$^{13}$C]PBG derives from the trace activity of 5-CLA-PBGS and its concentration increases with time. Since the signal for ALA is considerably broadened, we can say that free ALA is in exchange with the bound species, but the nonequilibrium condition precludes calculation of exchange rates. However, the important observation is that the chemical shift of the bound species is almost indistinguishable from the P-side Schiff base we have already described (166.5 ppm; Jaffe & Markham, 1987).

The near identity of the $^{13}$C NMR spectrum of [4-$^{13}$C]ALA bound to 5-CLA-PBGS with that of [4-$^{13}$C]ALA bound to MMTS-modified PBGS suggests that the $C_4$ of P-side ALA is in an imine linkage to Lys-252 of 5-CLA-PBGS. However, unlike MMTS-modified PBGS, which is vacant at the A-side ALA binding site, 5-CLA-PBGS contains a covalent 4-oxo-

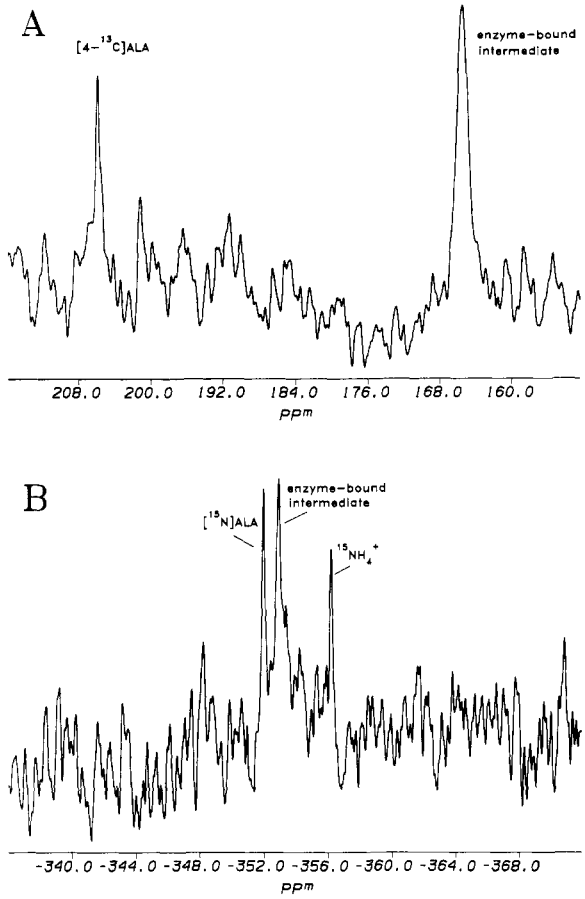


FIGURE 5: NMR spectra. (A) $^{13}C$ NMR difference spectrum of [4-$^{13}C$]ALA bound to 5-CLA-PBGS. The chemical shift of the P-side Schiff base intermediate, 165.4 ppm, indicates that $C_4$ of P-side ALA is in an imine linkage to the protein. (B) $^{15}N$ NMR spectrum of [$^{15}N$]ALA bound to 5-CLA-PBGS shows that no bond is formed between the nitrogen of P-side ALA and $C_4$ derived from ALA.

pentanoic acid moiety in place of A-side ALA (Figure 6A). The formation of a second imine between $C_4$ of A-side ALA and N of P-side ALA has been proposed as the next step in the PBGS-catalyzed reaction. The proposed di-Schiff base intermediate for 5-CLA-PBGS is illustrated in Figure 6B. We know of no model compound which could be used to predict the effect of the second Schiff base on the chemical shift of the first Schiff base. Therefore, to investigate whether the nitrogen of P-side ALA was involved in a second Schiff base linkage, we observed [$^{15}N$]ALA bound to 5-CLA-PBGS. In this case, where the experiment was expected to take several days, we raised the concentration of 1,10-phenanthroline, which completely inhibited product formation.

The spectrum of [$^{15}N$]ALA bound to 5-CLA-PBGS is illustrated in Figure 5B. Again, the results are virtually indistinguishable from that of the P-side Schiff base bound to MMTS-modified PBGS (Jaffe et al., 1990). The spectrum shows three main signals: (1) free [$^{15}N$]ALA (−352.0 ppm, line width 10 Hz), (2) enzyme-bound mono-Schiff base intermediate (−353.0 ppm, line width 21 Hz), and (3) $^{15}NH_4^+$ (−356.2 ppm, line width 8 Hz). The ammonia derives from a contaminant in [$^{15}N$]ALA. The amino group of the P-side Schiff base is partially deprotonated, but it is *not* involved in an imine linkage to the $C_4$ derived from 5-CLA (see Figure 6A).

*Investigations into the Cross-Linking of PBGS.* As an independent probe for di-Schiff base formation in the complex of 5-CLA-PBGS and ALA, we treated this complex with $NaBH_4$. The reduced enzyme complex contains at least two covalent linkages which are not present in the native octameric protein structure. These are $C_5$ derived from 5-CLA covalently attached through Cys-223 at the A-side ALA binding pocket and $C_4$ of P-side ALA trapped as the reduced Schiff base to Lys-252. Figure 6C illustrates one possible form which contains an additional new covalent linkage where the $C_5$ amino group of P-side ALA is trapped as a reduced Schiff base to the $C_4$ of 5-CLA. If this form exists, it must cross-link the active site. Since there is one active site per two subunits, the cross-linked protein might contain a cross-link between subunits. The twice-modified protein was evaluated by SDS–PAGE. The electrophoretic analysis did not provide evidence for cross-linked subunits. It is possible that cross-linking was not observed because the PBGS active sites are each contained in a single subunit. Our X-ray crystallographic studies should shed light on the location of the active sites. However, on the basis of our NMR data and our sequencing studies, Figure 6D is a likely representation of the $NaBH_4$-reduced form of the ALA·5-CLA-PBGS complex.

## Discussion

The study presented here was made possible by the preliminary work of Seehra and Jordan (1981), who first synthesized several $\alpha$-haloketone analogues of ALA and characterized the interactions of these compounds with PBGS. They established that 5-CLA preferentially binds at the P-side ALA binding pocket ($K_i$ = 11 $\mu$M) and can form the P-side Schiff base intermediate. They concluded that 5-CLA inactivates at the P-side ALA binding pocket even though *inactivation* is only observed at much higher concentrations of 5-CLA (5–20 mM). The work presented here shows that 5-CLA-PBGS retains the ability to bind up to four substrate molecules with a dissociation constant much tighter than expected for A-side ALA. When the four substrate binding sites of 5-CLA-PBGS are occupied by [4-$^{14}C$]ALA, it is possible to covalently attach up to all four by treatment with $NaBH_4$, as is the case for P-side ALA. These two observations indicate that the P-side binding site on 5-CLA-PBGS is available for substrate binding. The corollary conclusion is that the occupied substrate binding site is the A-side ALA binding pocket and this is the preferential site of *inactivation* by 5-CLA. We do not dispute the previous finding that 5-CLA also binds in place of P-side ALA (Seehra & Jordan, 1981). In fact, the loss of all substrate binding ability upon long incubation with 5-CLA suggests that this reagent can bind and react at both ALA binding sites.

Initial characterization of 5-CLA-PBGS showed modification at a cysteine residue, which by analogy to the work of Barnard et al. (1977) was suggested to occur in a trypsin-generated dipeptide of composition CY (Seehra & Jordan, 1981). We have demonstrated that 5-CLA-PBGS is modified on half of the subunits at Cys-223 (DRR*C*YQLPPGARGLALRAVDR). This cysteine corresponds to the cysteine which is modified by iodoacetamide and is different from the cysteine modified by iodoacetate, although both modifications result in total loss of enzymic activity when present at a stoichiometry of four per octamer (Barnard et al., 1977).

To further support the conclusions that 5-CLA inactivation occurs at the A-side ALA binding pocket through modification of a cysteine, we have demonstrated that prior modification with MMTS provides substantial protection against inactivation by 5-CLA. In the case of MMTS-modified PBGS, "inactivation" by 5-CLA refers to the loss of the ability to reconstitute to full activity with $\beta$ME and Zn(II) (Jaffe et al., 1984). We cannot say whether prior modification by MMTS protects PBGS against inactivation by 5-CLA because it cannot bind A-side ALA or because one of the MMTS-

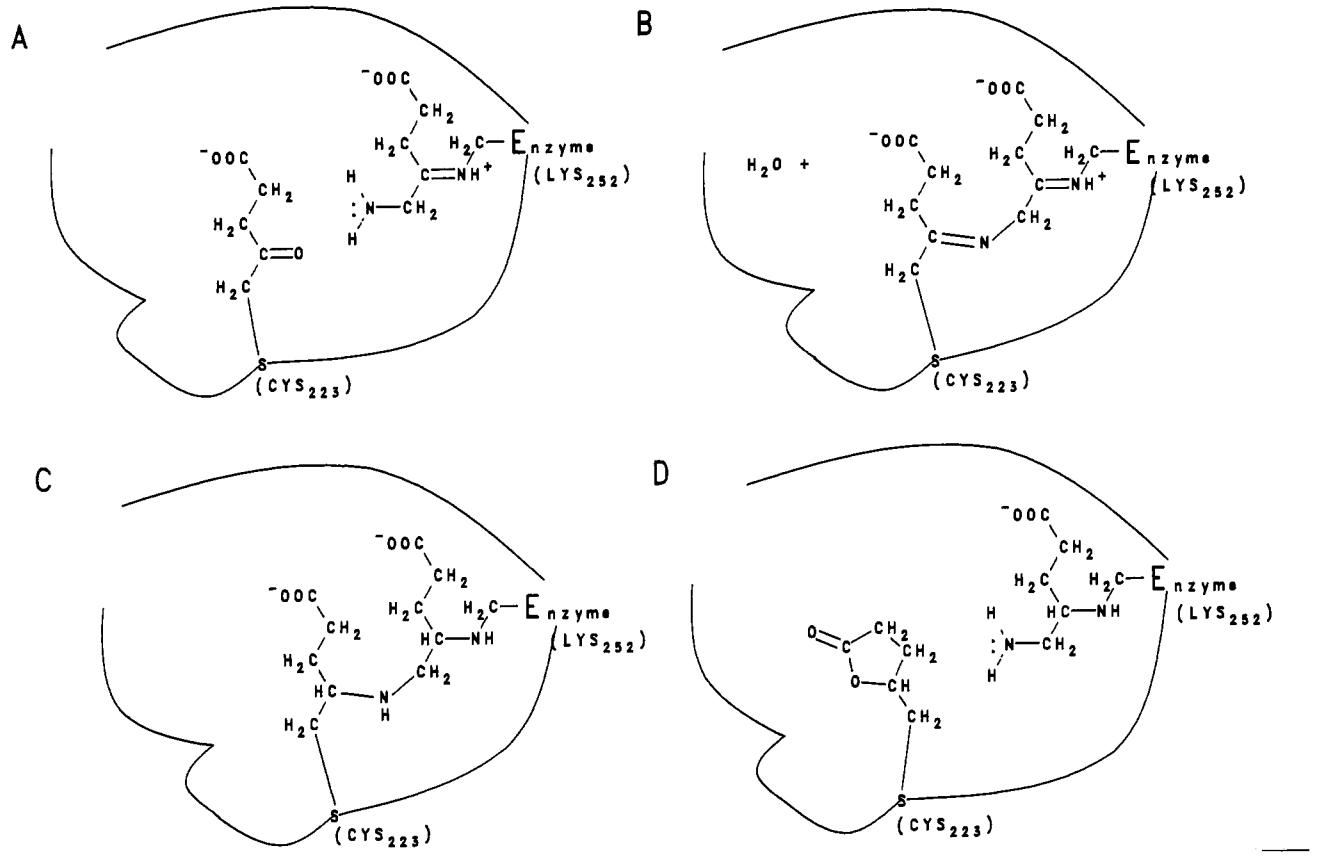


FIGURE 6: (A) ALA bound to 5-CLA-PBGS as the P-side Schiff base intermediate. (B) Proposed di-Schiff base intermediate of the ALA·5-CLA-PBGS complex. (C) $NaBH_4$-reduced form of the complex illustrated in (B). This form would cross-link the active site. (D) $NaBH_4$-reduced form of the complex illustrated in (A). All evidence supports structures (A) and (D) as the ALA·5-CLA-PBGS complex and its $NaBH_4$-reduced form.

modified cysteines is coincident with the 5-CLA-modified cysteine. Nevertheless, protection from 5-CLA inactivation of PBGS by prior modification with MMTS is consistent with our conclusion that 5-CLA inactivation occurs at the A-side ALA binding pocket. Supported by the fact that MMTS-modified PBGS binds neither the catalytic nor the structural Zn(II), it is also suggested that Cys-223 is one of the sites of modification by MMTS.

It has previously been concluded that either Zn(II) and/or sulfhydryl groups are required for the binding of A-side ALA (Jaffe & Markham, 1987). Since binding is a necessary prerequisite to inactivation, and we have demonstrated that 5-CLA inactivates in place of A-side ALA, we asked if a Zn(II) chelator will prevent 5-CLA inactivation of PBGS. The chelator of choice is 1,10-phenanthroline (vide infra), which provides significant protection against PBGS inactivation by 5-CLA. This protection can be taken as evidence that Zn(II), and perhaps not sulfhydryl groups, are necessary for A-side ALA binding. However, if the role of the requisite sulfhydryl group is to bind the necessary Zn(II), this point may be a matter of semantics.

PBGS is well characterized as an Zn(II) metalloenzyme. In support of our two-site model for Zn(II) binding is the recently published extended X-ray absorption fine structure (EXAFS) study of Dent et al. (1990) that suggests two different classes of Zn(II) binding sites on PBGS, each at a stoichiometry of four per octamer. In the EXAFS analysis it is proposed that there is one cysteine ligand to each of the four catalytic Zn(II) and that there are four cysteine ligands to each of the four other Zn(II). This model agrees with the generalizations drawn by Vallee and Auld (1990) on the probable ligands to catalytic versus structural Zn(II) in metalloproteins. 5-CLA-PBGS contains only one of the two classes of Zn(II) sites. The relatively low affinity of Zn(II) for 5-CLA-PBGS suggests that the modified enzyme retains only the looser-binding Zn(II) sites, which appear to play no role in catalysis. The fact that binding sites for the catalytic Zn(II) are removed by 5-CLA modification of PBGS suggests that Cys-223 is the cysteine ligand to the catalytic Zn(II). Since we conclude that 5-CLA inactivation occurs at the A-side ALA binding pocket, this places the catalytic Zn(II) very near the $C_5$ of A-side ALA (see Figure 7). Based on $^{13}C$ and $^{15}N$ NMR data, we have previously proposed that the amino group of PBG which derives from A-side ALA may be a ligand to the active-site Zn(II) (Jaffe et al., 1990).

Our ultimate objective in the production and characterization of 5-CLA-PBGS was to use the modified enzyme as a tool to probe the structure of enzyme-bound substrate[3] or reaction intermediates by $^{13}C$ and $^{15}N$ NMR. Because PBG does not bind to 5-CLA-PBGS, the slow formation of product in a stoichiometric ALA·5-CLA-PBGS mixture would result in an NMR spectrum dominated by free PBG. In the presence of 1,10-phenanthroline, 5-CLA-PBGS has the requisite $<10^{-4}$ activity necessary for NMR characterization of the enzyme-bound species. The resulting spectra are a valid representation of labeled ALA bound to 5-CLA-PBGS only if the trace activity of 5-CLA-PBGS derives from unmodified active sites which retain the ability to bind the catalytic Zn(II). Although this cannot be proven, all data support this view. In this case, we have determined which partial reactions can occur at the

[3] Enzyme-bound ALA may exist as one of the six possible tautomers of ALA. These are the ketone, the hydrate, two $C_3$–$C_4$ enol stereoisomers, and two $C_4$–$C_5$ enol stereoisomers (Jaffe & Rajagopalan, 1990).

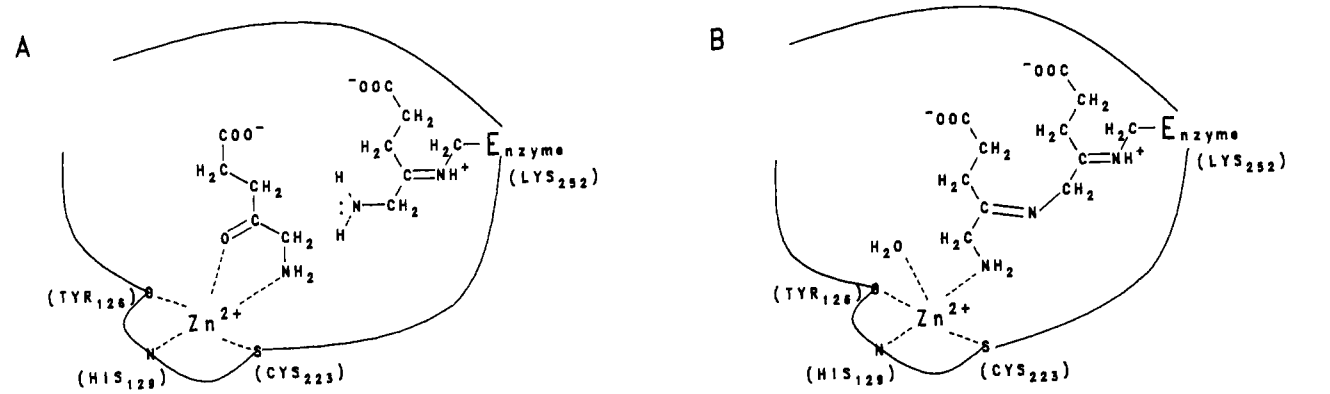


FIGURE 7: Model for the ternary complex of PBGS and two ALA molecules (A) which is proposed to precede the formation of the di-Schiff base intermediate (B).

P-side of the active site in the presence of a covalently bound levulinic acid analogue at the A-side.

The NMR studies presented here unequivocally demonstrate that P-side ALA bound to 5-CLA-PBGS cannot proceed past the first Schiff base intermediate (see Figure 6A). Our peptide sequencing studies show that the A-side levulinic acid moiety of 5-CLA-PBGS remains intact. Therefore, the missing catalytic Zn(II) must be responsible for the failure to a form a bond (or bonds) between the two substrate molecules. The role of the Zn(II) might be (1) to orient A-side ALA properly, (2) to polarize the $C_4$ carbonyl of A-side ALA in order to facilitate formation of the proposed di-Schiff base intermediate, or (3) to remove the hydroxyl group from a carbinolamine precursor to the di-Schiff base. Since we see no evidence in the NMR spectra for accumulation of a carbinolamine intermediate, we favor the former two suggestions. On this basis, we present a model for the ternary complex of PBGS with two ALA molecules as illustrated in Figure 7. Our previous NMR studies on the enzyme–product complex led to the proposal that the A-side amino group is bound to the catalytic Zn(II). Here we propose that the A-side $C_4$ carbonyl is bound to the catalytic Zn(II). This forms a five-membered chelate of Zn(II) and A-side ALA which mimics the X-ray structure of glycyl-L-tyrosine bound to the Zn(II) of carboxypeptidase A (Christianson & Lipscomb, 1986).

In conclusion, the data presented above uniformly support a model for the A-side ALA binding pocket where $C_5$ is near Cys-223, the proposed cysteine ligand to the four catalytic Zn(II) of PBGS. It is interesting to note that this cysteine is not in the cysteine-rich region previously proposed as the Zn(II) binding region of PBGS (Wetmer et al., 1986). In bovine PBGS we have determined that the sequence for the cysteine rich region is LLVACDVCLCPYTSHGHCGLL (positions 115–135). It is probable that this region provides some of the cysteine ligands to the four structural Zn(II). The generalizations of Vallee and Auld (1990) suggest that two of the ligands to the catalytic Zn(II) will be separated by one to three amino acids in the primary structure of the protein. The EXAFS data suggest that the other ligands to the catalytic Zn(II) are two tyrosine and two histidine residues. Since there are no tyrosine or histidine residues in the neighborhood of Cys-223, it is assumed that the pair of neighboring ligands is either $H(X)_nH$, $H(X)_nY$, $Y(X)_nH$, or $Y(X)_nY$. On the basis of the known sequences for PBGS and the absolute conservation of the YTSHGH sequence (positions 126–131), we propose that Tyr126 and His129 are ligands to the catalytic Zn(II). The use of amino acids 126 and 129 on the subunits which bind the catalytic Zn(II) is likely to preclude the use of the nearby cysteines for a structural Zn(II) on the same subunit. This may help to explain the mutually exclusive population of either the catalytic or structural Zn(II) sites on each of the eight PBGS subunits.

## Added in Proof

Following submission of this manuscript, the sequence for pea PBGS was shown to lack the histidine analogous to His129 (Boese et al., 1991). The pea PBGS sequence contains aspartic acid residues in place of Cys124, His129, and Cys132 of mammalian PBGS. The data strongly support the proposal that Mg(II) replaces at least one of the two types of Zn(II) present in mammalian PBGS. It remains to be proven if Mg(II) replaces the catalytic Zn(II). However, the histidine analogous to His131 is present in all species, as well as five PBGS sequences from photosynthetic organisms (M. P. Timko, personal communication). His131 remains the only conserved histidine in PBGS and may prove to be the histidine ligand to the catalytic Zn(II).

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# Kinetic Method for Differentiating Mechanisms for Ligand Exchange Reactions: Application To Test for Substrate Channeling in Glycolysis

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ABSTRACT: We have derived analytical expressions for the kinetics of the two mechanisms involved in ligand substitution reactions. These mechanisms are (i) a dissociative mechanism in which the leaving ligand is first dissociated prior to the binding of the incoming ligand and (ii) an associative mechanism where a ternary complex is formed between the incoming ligand and the complex containing the leaving ligand. The equations obtained provide the theoretical basis for differentiating these two mechanisms on the basis of their kinetic patterns of the displacement reactions. Analysis of these equations shows that an associative mechanism can only generate an increasing kinetic pattern for the observed pseudo-first-ordered rate constants as a function of increasing concentration of the incoming ligand and plateaus, in most cases, at a value higher than the off-rate constant of the leaving ligand. However, a dissociative mechanism can generate either an increasing or a decreasing ($k_{app}$ decreases with increasing concentrations of the incoming ligand) kinetic pattern, depending on the magnitudes of the individual rate constants involved, and, in either case, it will plateau at $k_{app}$ equal to the $k_{off}$ of the leaving ligand. Therefore, the decreasing kinetic pattern is a hallmark for a dissociative mechanism. This general method was used to settle the dispute of whether NADH is transferred directly via the enzyme–enzyme complex between glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) and L-lactate dehydrogenase (LDH; EC 1.1.1.27). Evidence for direct transfer of this metabolite between these two enzymes has been claimed and used to support substrate channeling in glycolysis [Srivastava, D. K., & Bernhard, S. A. (1987) *Biochemistry 26*, 1240-1246; Srivastava, D. K., Smolen, P., Betts, G. F., Fukushima, T., Spivey, H. O., & Bernhard, S. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6464-6468]. Reexamination of this evidence reveals that the conclusion is based on misinterpretation of the kinetics of ligand exchange [Chock, P. B., & Gutfreund, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8870-8874]. Application of this method to analyze the displacement data from our laboratory and those reported by Srivastava et al. (1989) confirms that NADH transfer between its complex with GPDH and with LDH proceeds via a dissociative mechanism. This is consistent with the transient kinetic and sedimentation equilibrium data reported by Wu et al. [Wu, X., Gutfreund, H., Lokatos, S., & Chock, P. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 497-501].

Substrate channeling, where the product of the reaction catalyzed by one enzyme is directly transferred via a multienzyme complex to a second enzyme to be used as its substrate, has been proposed for the glycolytic pathway (Srivastava & Bernhard, 1986a,b). Among the supporting evidence claimed is the kinetic data showing that NADH is transferred directly between its complexes with glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) and with lactate de-

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