5-Chloro[1,4-^{13}C]levulinic Acid Modification of Mammalian and Bacterial Porphobilinogen Synthase Suggests an Active Site Containing Two Zn(II)†

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Received March 2, 1994; Revised Manuscript Received July 14, 1994®

ABSTRACT: 5-Chloro[1,4-^{13}C]levulinic acid ([1,4-^{13}C]CLA) is an active site-directed inactivator of porphobilinogen synthase (PBGS). PBGS asymmetrically condenses two molecules of 5-aminolevulinic acid (ALA) which are called A-side ALA and P-side ALA in reference to their fates as the acetyl and propionyl halves of the product. [1,4-^{13}C]CLA modifies bovine PBGS at the A-side ALA binding site. The C_4 chemical shift indicates an intact keto moiety; the C_1 chemical shift indicates a deprotonated carboxyl group. In contrast, [1,4-^{13}C]CLA modification of *Escherichia coli* PBGS is heterogeneous and occurs preferentially at the P-side ALA binding site. The C_1 chemical shifts indicate substantially deprotonated carboxylic acid groups. For one of four observed forms of [1,4-^{13}C]CLA-modified *E. coli* PBGS, an analog of the P-side Schiff base is found. Bovine and *E. coli* PBGS contain two different zincs, Zn_A and Zn_B. Past results placed Zn_A near A-side ALA. [1,4-^{13}C]CLA modifies *E. coli* PBGS at Cys119 or Cys129, which is part of a four-cysteine cluster implicated in binding Zn_B. This result places Zn_B near P-side ALA. *E. coli* PBGS binds a third type of divalent metal, Mg_C or Mn_C, which is found to have no significant effect on the ^{13}C NMR spectrum of the [1,4-^{13}C]CLA-modified protein.

Porphobilinogen synthase (PBGS)[1] is a Zn(II) metalloenzyme that catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA) to give porphobilinogen (PBG) (see Figure 1). The two substrates are denoted as A-side ALA and P-side ALA, referring to their respective fates as the acetyl and propionyl halves of PBG. 5-Chlorolevulinic acid (5-CLA) is a reactive ALA analog that might bind to and/or react with PBGS at either or both ALA binding sites. 5-CLA has already been characterized as an inactivator of bovine PBGS ($PBGS_{bovine}$)(Jaffe et al., 1992). Herein 5-CLA is characterized as an inactivator of *Escherichia coli* PBGS ($PBGS_{E.\ coli}$), and 5-chloro[1,4-^{13}C]-levulinic acid ([1,4-^{13}C]CLA) is used as a ^{13}C NMR probe of the active sites of both $PBGS_{bovine}$ and $PBGS_{E.\ coli}$.

Mammalian PBGS contains two types of Zn(II) (Zn_A and Zn_B), each at a stoichiometry of four per octamer. Zn_A binds most tightly ($K_d < 0.1\ \mu M$) and is essential to catalysis. Zn_B binds less tightly ($K_d = 5\ \mu M$), and a possible role is discussed herein. $PBGS_{E.\ coli}$ is a Zn(II) metalloenzyme (Mitchell & Jaffe, 1993; Spencer & Jordan, 1993). The sequence of $PBGS_{E.\ coli}$ contains the putative binding sites for Zn_A and Zn_B as illustrated in Table 1 (Jaffe, 1993). Extended X-ray absorption fine structure (EXAFS) analysis shows that the Zn_8 form of $PBGS_{E.\ coli}$ and $PBGS_{bovine}$ are very similar (Dent et al., 1990; S. Hasnain, R. Strange, L. W. Mitchell, and E. K. Jaffe, unpublished results).

Table 1: Bovine and *E. coli* Sequences of Putative Metal Binding Regions of PBGS[a]

	122	132
Bovine	V A C D V C L C P Y T S H G H C G L L S E	
E. coli	V M S D T C F C E Y T S H G H C G V L C E	
	119	129
	223	
Bovine	D R R C Y Q L P P G A R G L A L R A V D R D	
E. coli	D R K S Y Q M N P M N R A E G I A E Y L L D	
	217	
	252	
Bovine	M V *K P G M P Y L D I V R E	
E. coli	M V *K P A G A Y L D I V R E	
	246	

X = possible Zn_A ligands

X = putative Zn_B ligands

X = putative Mg_C ligands

[a] *K = active site lysine.

† This publication was made possible by Grant ES03654 from the National Institute of Environmental Health Sciences, NIH; by NIH Grants CA06927 and RR05539 (ICR); and by an appropriation from the Commonwealth of Pennsylvania. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: ALA, 5-aminolevulinate; βME, 2-mercaptoethanol; 5-CLA, 5-chlorolevulinate; [1,4-^{13}C]CLA, 5-chloro[1,4-^{13}C]levulinic acid; [1,4-^{13}C]CLA-$PBGS_{bovine}$, [1,4-^{13}C]CLA-modified bovine PBGS; [1,4-^{13}C]CLA-$PBGS_{E.coli}$, [1,4-^{13}C]CLA-modified *E. coli* PBGS; PBG, porphobilinogen; PBGS, porphobilinogen synthase; $PBGS_{bovine}$, bovine PBGS; $PBGS_{E.coli}$, *E. coli* PBGS; Pi, inorganic phosphate.

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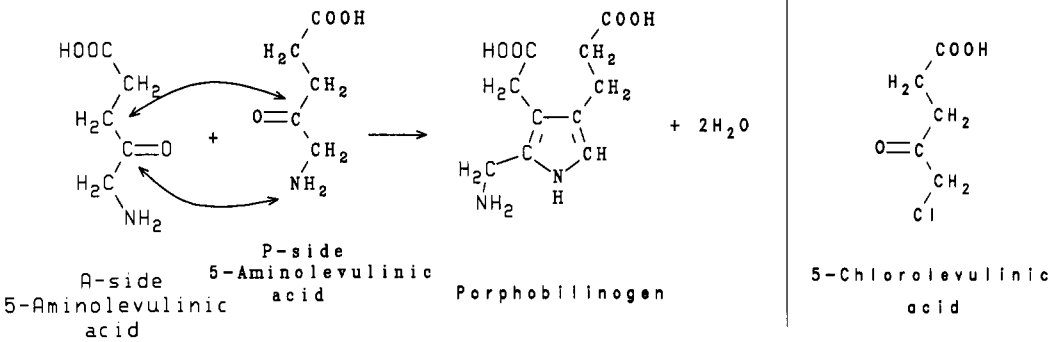

FIGURE 1: The PBGS-catalyzed reaction is the asymmetric condensation of two molecules of ALA. P-side ALA forms the propionyl containing half of PBG; its nitrogen is incorporated into the pyrrole ring. P-side ALA binds first and forms a Schiff base intermediate of known stereochemistry and protonation states to Lys252 of mammalian PBGS (Lys246 of *E. coli* PBGS). A-side ALA forms the acetyl containing half of PBG; its nitrogen remains an amino group. Zn(II) is required for binding A-side ALA and appears to be involved in the formation of the first bond between the two ALA moieties. The sequence of events which follows the formation of the ternary ES_2 complex remains a topic for speculation. The structure of 5-CLA is included on the right.

5-CLA modifies $PBGS_{bovine}$ at the A-side ALA binding site. Cys223 is modified at a stoichiometry of four per octamer, and the binding site for Zn_A is lost upon modification. Consistent with the EXAFS analysis, Cys223 is proposed as the single cysteine ligand to Zn_A. 5-CLA-modified $PBGS_{bovine}$ can bind [4-^{13}C]ALA as the P-side Schiff base intermediate. ^{13}C NMR analysis of the ternary complex suggests that no further reaction occurs. Figure 2A illustrates the active site model which arose from the characterization of 5-CLA-modified $PBGS_{bovine}$ and earlier work (Jaffe et al., 1992). Zn_A is proposed to be essential to binding A-side ALA and to the formation of the first bond between the two ALA molecules. Here [1,4-^{13}C]CLA is used to confirm the proposed active site model.

There is a high degree of conservation among all known PBGS sequences. Cys223 is common to mammalian and yeast PBGS (Wetmur et al., 1986; Bishop et al., 1986, 1989; Myers et al., 1987; Jaffe et al., 1992). The analogous amino acid in bacterial and plant PBGS is either a serine or a threonine. Each of these amino acids can serve as a Zn(II) ligand. However, unless the pK_a of the serine or threonine is significantly depressed by the active site environment, only cysteine is expected to react readily with an α-halo ketone. Consequently, $PBGS_{E.\ coli}$ with its analogous Ser217 is an interesting target for investigation with 5-CLA (see Table 1).

Here, we report that $PBGS_{E.\ coli}$ is rapidly inactivated by 5-CLA. The modified residues are part of a four-cysteine cluster that is implicated in binding Zn_B (Jaffe, 1993; Table 1). Based on ^{13}C NMR characterization of [1,4-^{13}C]CLA-modified $PBGS_{E.\ coli}$ ([1,4-^{13}C]CLA-$PBGS_{E.\ coli}$) and the effects of 1,10-phenanthroline, the site of modification is deduced to be the P-side ALA binding site. Consequently, a model is presented where both Zn_A and Zn_B of mammalian and bacterial PBGS are at or near the active site.

EXPERIMENTAL PROCEDURES

Materials. Aminolevulinic acid hydrochloride (ALA·HCl), potassium phosphate monobasic (KPi), $NaBH_4$, and *p*-(dimethylamino)benzaldehyde were purchased from Sigma Chemical Co. 2-Mercaptoethanol (βME) was purchased from Fluka Chemical Corp. and was distilled under vacuum prior to use. High-purity KOH and ultrapure $MgCl_2$ were purchased from Aldrich Chemical Co. Centriprep ultrafiltration devices were purchased from Amicon Corp. Spin columns were obtained from Bio-Rad. HPLC-grade trifluoroacetic acid was purchased from Pierce. House-distilled water was further purified by passage through a Milli-Q water purification system (Millipore). Solvents used for peptide mapping were all HPLC grade. V8 protease was purchased from Pierce. [1,4-^{13}C]Succinic acid was purchased from Isotec, Inc., Miamisburg, OH (99% ^{13}C). [5-^{13}C]ALA was custom-synthesized by MSD (now CDN) isotopes, and [5,5-^{2}H; 5-^{13}C]ALA was prepared through enolization in D_2O as previously described (Jaffe & Markham, 1988). All other chemicals were reagent grade.

Preparation of 5-Chloro[1,4-^{13}C]levulinic Acid. [1,4-^{13}C]-CLA was synthesized from [1,4-^{13}C]succinic acid in five steps. [1,4-^{13}C]Succinic acid was converted to [1,4-^{13}C]succinic anhydride in 86% yield (Aspelund & Petander, 1944). [1,4-^{13}C]β-Carbomethoxy propionyl chloride was prepared in 70% yield from [1,4-^{13}C]succinic anhydride by the method of Cason (1955). 5-Chloro[1,4-^{13}C]levulinic acid was obtained in turn in 34% yield by the method of Bloxham and Chalkley (1976). Alterations in the purification steps were as for unlabeled 5-CLA as previously described (Jaffe et al., 1992). Purity was determined by melting point, ^{13}C NMR, ^{1}H NMR, and thin-layer chromatography, using unlabeled 5-CLA as the standard.

Enzyme Purification and Assays. Purification of $PBGS_{bovine}$ carried out as previously described (Jaffe et al., 1984). All purification and storage buffers contained 10 mM βME and 10 μM Zn(II). Purification of $PBGS_{E.\ coli}$ was as previously described (Mitchell & Jaffe, 1993) with the exception that 10 μM Mg(II) was added to all purification buffers. PBGS assays uniformly included a 10-min preincubation in 0.1 M KPi, pH 7, 10 mM βME, and Zn(II) at 10 μM for $PBGS_{bovine}$ or 30 μM for $PBGS_{E.\ coli}$. PBG production was started by the addition of ALA·HCl to 10 mM, which brought the pH to 6.8. PBG formation was quantified by the pink complex formed with Ehrlich's reagent as previously described (Jaffe et al., 1984).

Preparation of [1,4-^{13}C]CLA-$PBGS_{bovine}$. Two preparations of [1,4-^{13}C]CLA-$PBGS_{bovine}$ were carried out. The first sample was prepared by a 1-h 37 °C incubation of 240 mg of $PBGS_{bovine}$ (3.5 μmol of active sites) with 41 μmol of βME, and 341 μmol of [1,4-^{13}C]CLA in 15 mL of 0.1 M KPi, pH 7.0, and 10 μM $ZnCl_2$. The reaction was quenched by the addition of βME to a concentration of 30 mM followed by a 10-min incubation at 37 °C. The protein was immediately purified on an 800-mL Sephadex G-25 column, which had

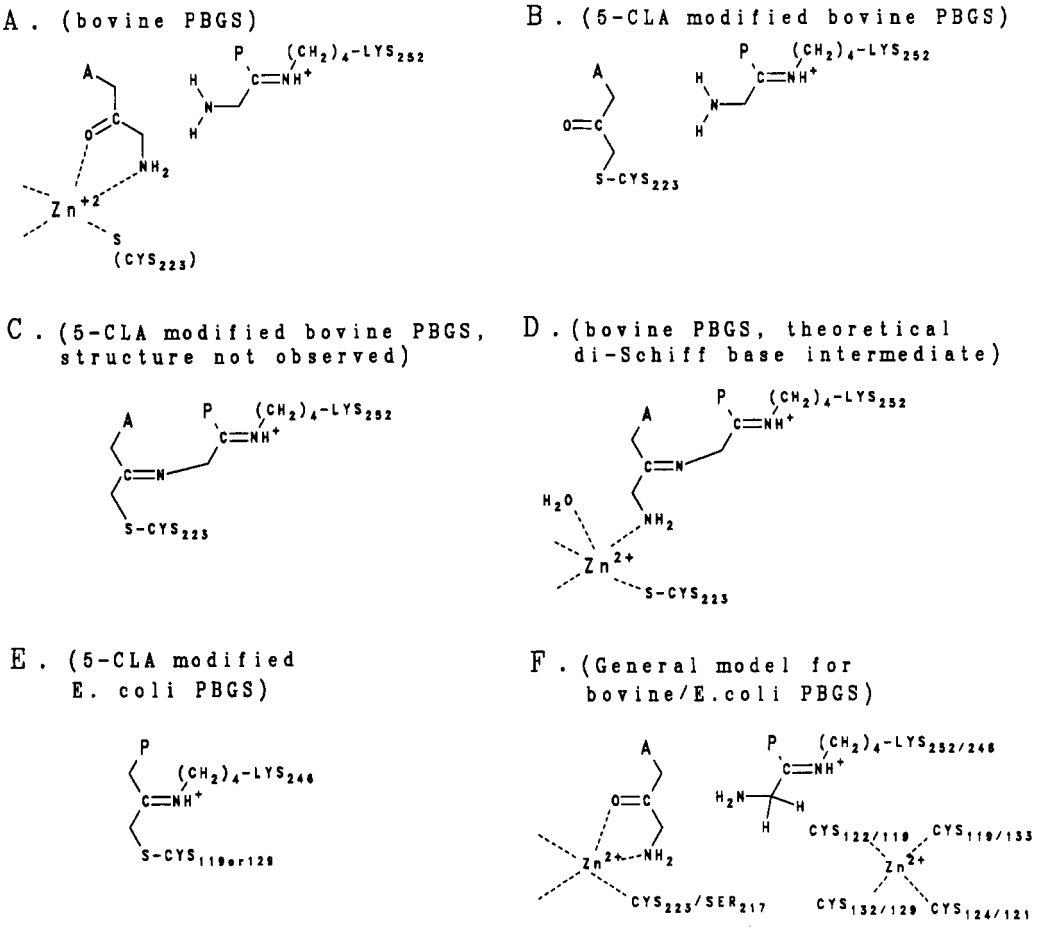

FIGURE 2: Models of the active site of PBGS using A for acetyl and P for propionyl and not labeling most methylene groups. Part A is a model for the transient ternary ES_2 complex of PBGS based on prior work. The Zn(II) bound to A-side ALA is Zn_A, and residue numbers correspond to $PBGS_{bovine}$. The two unlabeled Zn_A ligands are predicted to be near neighbors (Jaffe, 1993). Part B is our original model for 5-CLA-$PBGS_{bovine}$ including 5-CLA modification at the A-side ALA binding pocket, no catalytic Zn(II), and ALA bound as the P-side Schiff base intermediate. The stereochemistry and protonation state of the intermediate are illustrated as was determined by ^{13}C and ^{15}N NMR (Jaffe et al., 1990). Part C illustrates the theoretical di-Schiff base intermediate which has been proposed to be involved in the normal pathway (Jordan & Seehra, 1980). Alternatively, carbon–carbon bond formation could precede carbon–nitrogen bond formation (Jordan, 1991). Part E illustrates one form of 5-CLA-$PBGS_{E.\ coli}$ including modification of the P-side ALA binding site with Schiff base formation (see text). Residue numbers correspond to $PBGS_{E.\ coli}$. Part F illustrates a general model for the transient ES_2 complex of PBGS based on current work. The Zn(II) bound to A-side ALA is Zn_A, the Zn(II) coordinated to four cysteines near P-side ALA is Zn_B. Residue numbers correspond to $PBGS_{bovine}/PBGS_{E.\ coli}$. One cysteine is proposed to be the acceptor for a C_5 proton derived from P-side ALA as the final step in PBG formation. The pro-*R* proton is removed (Chauddhry & Jordan, 1976). In the case of plant and some bacterial PBGS, Zn_B appears to be replaced by a Mg_B which is coordinated to aspartic acid residues. The current work also indicates that the acetyl and propionyl groups are deprotonated.

been equilibrated with 0.1 M KPi, pH 7.0, 10 mM βME, and 10 μM $ZnCl_2$ at a flow rate of 3 mL min^{-1}. The bulk of [1,4-^{13}C]CLA-$PBGS_{bovine}$ eluted at 300–390 mL and was concentrated to 1.25 mL using Amicon Centriprep-10 concentrators. The overall yield was 130 mg of purified [1,4-^{13}C]CLA-$PBGS_{bovine}$ with a specific activity of 0.14 μmol h^{-1} mg, which corresponds to ~0.5% of the original activity. This preparation mimicked that of the unlabeled 5-CLA-$PBGS_{bovine}$ that we had previously characterized (Jaffe et al., 1992).

The second preparation of [1,4-^{13}C]CLA-$PBGS_{bovine}$ was designed to mimimize nonspecific labeling (see below). In this case, the modification reaction mixture contained 200 mg of $PBGS_{bovine}$, 50 μmol of βME, and 180 μmol of [1,4-^{13}C]CLA in 6 mL of 0.1 M KPi, pH 7.0, and 10 μM $ZnCl_2$. The reaction proceeded for 3 min at 37 °C, after which time it was quenched by the addition of 5 mL of ice-cold buffer containing 30 mM βME. Immediate purification was as above, and the modified protein contained approximately 10% residual activity.

Preparation of [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ [1,4-^{13}C]CLA-$PBGS_{E.coli}$ was prepared from 181 mg of protein, which was first preincubated for 10 min at 37 °C in 0.5 mL containing 0.1 M KPi, pH 7.0, 10 mM βME, 10 μM Zn(II), and 10 μM Mg(II). [1,4-^{13}C]CLA, 0.9 mL of 0.2 M, was added and allowed to react for 10 min at 37 °C. The reaction was quenched with 5 mL of ice-cold 0.1 M KPi, pH 7, and 30 mM βME. Purification was as above using a 800-mL Bio-Rad P-6 column. The peak of protein contained 130 mg of [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ and exhibited 9% residual activity.

[1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ was also prepared in the presence of 5 mM 1,10-phenanthroline. In this case, the enzyme (190 mg) was preincubated (5 min, 37 °C) with 0.1 M KPi, pH 7.0, 10 mM βME, 10 μM Zn(II), and 10 μM Mg(II). 1,-10-Phenanthroline (1 M in ethanol) was added to a final concentration of 5 mM, and [1,4-^{13}C]CLA (0.2 M, neutralized with KOH) was added to a final concentration of 30 mM. After 2 min at 37 °C, the reaction was quenched by a 2-fold dilution with ice-cold 0.1 M KPi and 50 mM βME. Purification was as above. The peak of protein contained 116 mg of [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$, and the enzyme had 12% residual activity.

Time Course for Inactivation of $PBGS_{bovine}$ and $PBGS_{E.\ coli}$ with 5-CLA. Protein (100 μL) at 1 mg/mL in 0.1 M KPi, pH 7, 2 mM βME, and 3 μM Zn(II) [10 μM Zn(II) for

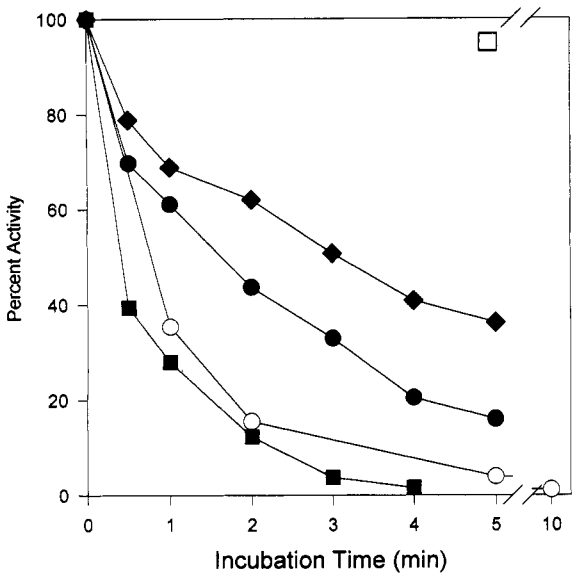

FIGURE 3: Time course for 5-CLA (20 mM) inactivation of $PBGS_{bovine}$ [○], $PBGS_{E.\ coli}$ [●], $PBGS_{E.\ coli}$ in the presence of 5 mM 1,10-phenanthroline [■], and $PBGS_{E.\ coli}$ in the presence of 10 mM ALA [◆]. [□] indicates a control incubation of $PBGS_{E.\ coli}$ with 5 mM 1,10-phenanthroline, without 5-CLA. See Experimental Procedures for inactivation and assay protocols.

$PBGS_{bovine}$] was exposed to 22 mM 5-CLA for times varying from 30 s to 5 min at 37 °C. The reactions were quenched by the addition of 1 M βME to a final concentration of 32 mM, and 100-μL aliquots were immediately purified on spin columns which had been previously equilibrated with the above buffer containing 10 mM βME. Following purification, the protein samples were immediately assayed for residual activity. The effects of either 10 mM ALA or 5 mM 1,10-phenanthroline on the inactivation kinetics included these reagents in the reaction buffers but not in the purification buffers. 1,10-Phenanthroline, at 5 mM, is known to fully inhibit $PBGS_{E.\ coli}$, presumably by chelating the essential Zn(II) (Mitchell & Jaffe, 1993).

Limited Proteolysis and Peptide Mapping of Native and 5-CLA-Modified PBGS. $PBGS_{E.\ coli}$ (2 mg) was inactivated with 5-CLA in the presence of Zn(II) under conditions which result in 90% inactivation (see Figure 3). Unmodified $PBGS_{E.\ coli}$ was used as a control. The 5-CLA modified and unmodified proteins were reduced with $NaBH_4$ as described previously and precipitated with ammonium sulfate (Jaffe et al., 1992). The $NaBH_4$ reduction of CLA-modified cysteine results in the formation of a lactone, which is stable to the conditions of automated protein sequence determination (Jaffe et al., 1992). The isolated modified proteins (0.5 mg) were dissolved in 0.1 M KPi, pH 7.5, 10 mM βME, and 10 μM Zn(II). Solid urea was added to a final concentration of 8 M, and the sample was boiled for 30 s. The denatured protein solutions were each passed through a spin column which had been equilibrated with 8 M urea in water. The resultant protein was diluted to 2 M urea with 0.1 M ammonium bicarbonate, pH 7.8. V8 protease (2.3 units) was added, and the digestion was allowed to proceed for 20 h at 37 °C with vigorous shaking. The proteolysis mixture was filtered through a 0.2 μm filter prior to peptide mapping by reverse-phase HPLC as previously described (Jaffe et al., 1992).

Peptide Sequencing. Protein sequencing was carried out in the Protein Analytical Laboratory of the Research Center for Oral Biology, University of Pennsylvania School of Dental Medicine, using procedures we have previously described (Jaffe et al., 1992). The cysteine residues of $NaBH_4$-reduced, 5-CLA-modified somatostatin served as the standard for identification of the modified cysteines of PBGS, as previously described (Jaffe et al., 1992). During sequence determination, PTH-derived amino acids were monitored at 269 and 313 nm to monitor the dehydro products derived from serine, cysteine, and threonine. When more than one peptide was present, residue assignments were initially made by comparison to the expected cDNA-derived sequence and confirmed on the basis of yield.

NMR Data Acquisition. ^{13}C NMR spectra were obtained on a Bruker AM300 spectrometer as previously described (Jaffe & Markham, 1987, 1988). Each spectrum required *ca.* 12 h of data acquisition. Spectra were processed using the program Felix 2.0 (Hare, Inc.). Either a 25-HZ or a 15-Hz exponential line-broadening function was used. Fortunately, because both C_1 and C_4 are quaternary carbons, we are assured relatively sharp lines for the enzyme-bound species, even for a 280 000-Da protein (Jaffe & Markham, 1987). In spectra utilizing [5-^{13}C]ALA as substrate, deuteration at ^{13}C ensured narrow lines for enzyme-bound species (Jaffe & Markham, 1988).

RESULTS

Inactivation Kinetics of $PBGS_{bovine}$ and $PBGS_{E.\ coli}$ with 5-CLA. $PBGS_{bovine}$ was first shown sensitive to 5-CLA-induced inactivation by Seehra and Jordan (1981). We later demonstrated that the inactivation of $PBGS_{bovine}$ was due to modification of Cys223 in the A-side ALA binding pocket (Jaffe et al., 1992). In keeping with these results, Figure 3 shows that the activity of $PBGS_{bovine}$ drops to *ca.* 5% within the first 5 min of exposure to 20 mM 5-CLA. Despite the presence of the analogous Ser217 in $PBGS_{E.\ coli}$, Figure 3 indicates that $PBGS_{E.\ coli}$ is only slightly less susceptible than $PBGS_{bovine}$; after 5 min with 20 mM 5-CLA, the *E. coli* protein retains nearly 20% activity. Therefore, the 5-CLA susceptible residue of $PBGS_{E.\ coli}$ is less reactive and/or less accessible than Cys223 of $PBGS_{bovine}$. As expected for an active site directed inactivation, Figure 3 also shows that the substrate ALA protects $PBGS_{E.\ coli}$ from 5-CLA inactivation.

Active site chemistry, be it modification or catalysis, is minimally a two-step process of binding followed by a chemical transformation. Presentation of the effects of 1,10-phenanthroline on 5-CLA modification of PBGS requires discrimination between these two steps. If Zn(II) is required for binding 5-CLA, then the Zn(II) chelator 1,10-phenanthroline is expected to *protect* PBGS from 5-CLA inactivation. This was shown to be the case for $PBGS_{bovine}$ (Jaffe et al., 1992) and was consistent with a previous demonstration that Zn(II) is required for A-side ALA binding. 1,10-Phenanthroline causes quite the opposite effect on 5-CLA inactivation of $PBGS_{E.\ coli}$ (see Figure 3). Thus, Zn(II) is clearly not required for 5-CLA binding. This difference between $PBGS_{bovine}$ and $PBGS_{E.\ coli}$ is most likely due to respective 5-CLA modification at the A-side ALA binding pocket vs at the P-side ALA binding pocket. Figure 3 illustrates that 1,10-phenanthroline *potentiates* the inactivation of $PBGS_{E.\ coli}$ by 5-CLA. Thus, removal of Zn(II) (Zn_A and Zn_B) makes the 5-CLA susceptible residues more exposed or more reactive as would be expected if these residues were normally Zn(II) ligands. The effect of 1,10-phenanthroline on 5-CLA inactivation of $PBGS_{E.\ coli}$ is the first datum which suggests that the P-side ALA binding pocket is near one of the Zn(II) of PBGS.

Identification of 5-CLA-Modified Residues of $PBGS_{E.\ coli}$ and Clarification of Sequence of $PBGS_{E.\ coli}$. Identification

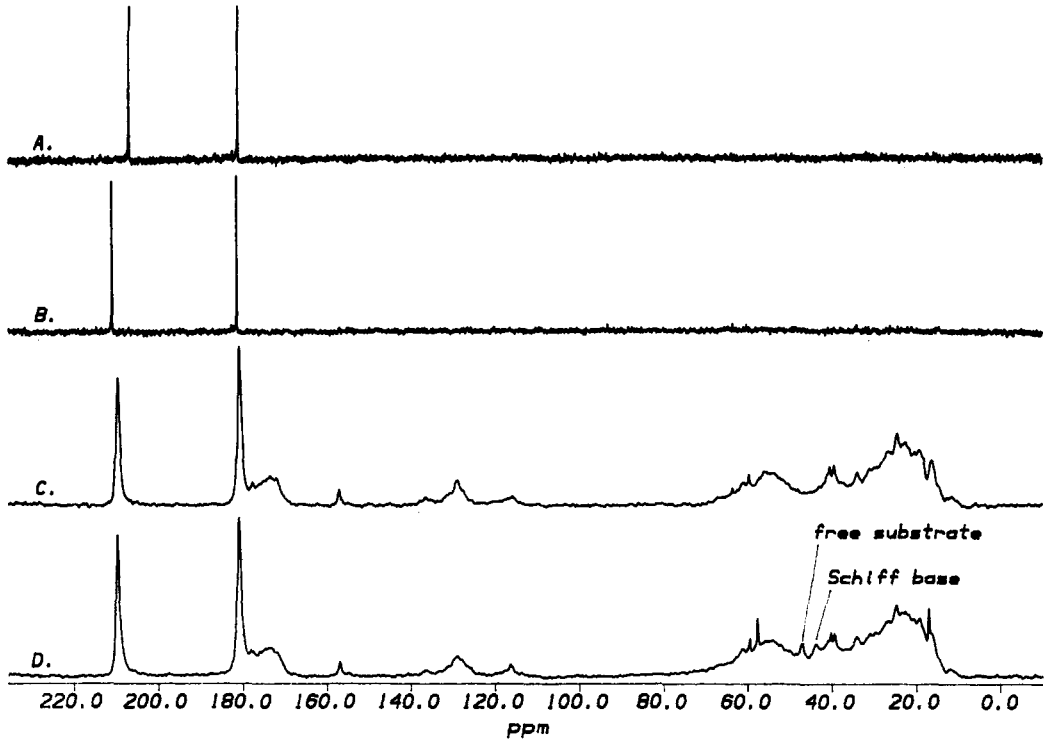

FIGURE 4: ^{13}C NMR spectra of (A) [1,4-^{13}C]CLA, 10 mM, 2000 scans, 2-s replication rate, processed with a 5-Hz exponential line-broadening function. (B) [1,4-^{13}C]-5-(2-mercapto-1-hydroxyethyl)-4-oxo-1-pentanoic acid prepared from [1,4-^{13}C]CLA and 2-mercaptoethanol; conditions and parameters as in part A. (C) [1,4-^{13}C]CLA-$PBGS_{bovine}$ in 0.1 M KPi and D_2O, pD 6.6 (1-h modification protocol) (125 mg), 36000 scans, 2-s repetition rate with a 25-Hz line-broadening function. (D) Spectrum obtained after 7.5 μmol of 1,10-phenanthroline and 3.6 μmol of [5,5-^2H; 5-^{13}C]ALA is added to part C. All spectra were obtained at 37 °C.

of the 5-CLA-modified peptide of a V8 protease digest of $NaBH_4$-reduced 5-CLA-modified $PBGS_{E. coli}$ was done by inspection of the peptide map as compared with a V8 digest of $NaBH_4$-reduced $PBGS_{E. coli}$. The three suspect peptides, named peaks 12, 13, and 54, were selected for sequence analysis. Peak 54 was at the end of the acetonitrile gradient and was very broad. Sequencer malfunctions caused the early cycles for peaks 12 and 54 to be lost. Cycles 8–23 of peak 54 revealed the sequence *CFCEYTSHGH*CGVLCE where *C is identified as a 5-CLA-modified $NaBH_4$-reduced cysteine. The *C standard derived from the cysteines of 5-CLA-modified $NaBH_4$ reduced somatostatin. The C of peak 54 is deduced from a blank cycle at 269 nm and a dehydroalanine peak at 313 nm as is expected for an unmodified cysteine. This sequence confirms both proposed sequences and identifies both Cys119 and Cys129 to be susceptible to 5-CLA modification. The cycles containing *C also contained dehydroalanine, which is consistent with modification at Cys119 or Cys129 on any one peptide molecule.

The literature contains two DNA sequences for $PBGS_{E. coli}$ (Li et al., 1989; Echelard et al., 1988). They contain several discrepancies, some of which have been previously resolved through peptide sequencing (Spencer & Jordan, 1993). The N-terminal protein sequence is herein found to be TDLIQR-PRR, which establishes the start codon as 30 nucleotides downstream from that proposed by Li et al. (1989) and confirms that the N-terminal methionine is cleaved (Spencer & Jordan, 1993). Peptide peak 12 clarifies the N-terminal region. From cycle five onward, two sequences were identified in peak 12 as TTLSLNDLVLPIFVEEEIDDYKAVE and GSxAWRE. The former sequence agrees with the N-terminal sequence of Li et al. (1989) and disproves the cysteine-rich N-terminal sequence proposed by Echelard et al. (1988). The blank cycle *x* in the latter peptide is consistent with an aspartic acid, common to both published sequences, because the peptide was immobilized on an arylamine-derivatized membrane. Peptide peak 13 also revealed two sequences KVVLE and IDDYKAVE, both of which agree with V8 protease peptides predicted from the sequence of Li et al. (1989).

^{13}C NMR Characterization of [1,4-^{13}C]CLA, Its Adducts, and Other Model Compounds. The use of [1,4-^{13}C]CLA as a protein modification reagent provides information both on potential chemistry occurring at C_4 (*e.g.*, keto vs imine) as well as information on the protonation state of the enzyme-bound carboxylic acid moieties. Fortunately, the acid and keto resonances are readily assigned on the basis of chemical shift, and both C_1 and C_4 are quarternary carbons negating concerns about deuteration. For interpretation of the enzyme-bound signals, spectral information is presented for the following model compounds: (1) free [1,4-^{13}C]CLA both as the acid and the neutral potassium salt; the latter is illustrated in Figure 4A; (2) the reaction product of [1,4-^{13}C]CLA and βME (as a model for modification at cysteine) at both acidic and neutral pH: the latter is illustrated in Figure 4B; (3) the reaction product of the [1,4-^{13}C]CLA·βME adduct with hydrazine (as a model for a Schiff base of the cysteine acylation product), at neutral and basic pH (spectra not shown); and (4) the reaction product of [1,4-^{13}C]CLA and hydrazine (spectra not shown). The chemical shifts of both C_1 and C_4 for each of these compounds are presented in Table 2 as are the chemical shifts of the C_1 and C_4 of [1-^{13}C]ALA and [4-^{13}C]-ALA, respectively.

Some generalities can be drawn from the chemical shifts of the model compounds presented in Table 2. For the ketones, the chemical shift of the protonated carboxylic acid (C_1) is at ~177.5 ppm irrespective of whether the substituent at C_5 is a chloro group, an amino group, or a thioether. Deprotonation of the carboxylic acid causes a chemical shift change to ~181.3 ppm as is also seen for the Schiff base adducts with hydrazine at neutral pH. The hydrazones cannot be char-

Table 2: ^{13}C NMR Chemical Shifts of [1,4-^{13}C]CLA and Related Model Compounds[a]

compound	pH	chemical shift (ppm) C_1	chemical shift (ppm) C_4	$J_{C_1C_4}$ (Hz)
[1,4-^{13}C]CLA	2	177.3	205.9	
	7	181.2	206.8	2.2
	6.6 in D_2O	181.1	206.8	
[1,4-^{13}C]CLA·βME adduct	2	177.5	209.6	
	7	181.3	210.8	
	6.6 in D_2O	181.3	210.9	
[1,4-^{13}C]CLA·βME·hydrazone	7.4	181.4	155.2	
	9.3	171.1	156.9	6.6
[1,4-^{13}C]CLA·hydrazone	7	182.2	154.0	
	9	181.3	154.8	
[1-^{13}C]ALA	2	177.7		
	7	181.1		
[4-^{13}C]ALA	7		205.9	
[4-^{13}C]ALA·hydrazone	7		154.0	

[a] Except where otherwise noted, all solutions are 26% D_2O.

acterized at acid pH because of their acid lability. In contrast to C_1, the chemical shift of the C_4 keto moiety is sensitive to the substituent at C_5. For ALA, the amino substituent at C_5 results in a chemical shift of 205.9 ppm; for 5-CLA, the chloro substituent results in a chemical shift of 206.8 ppm; and the thioether adduct has a chemical shift of 210.8. In each of these cases, protonation at C_1 causes less than a 1 ppm downfield shift in the keto resonance. The C_4 chemical shifts for each of the hydrazones derived from [1,4-^{13}C]CLA fall in the range of 155.5 ± 1.5 ppm. The C_4 chemical shift of the hydrazone of ALA is 154.0 ppm.

An interesting spectral change was observed upon deprotonation of the hydrazone of the 5-CLA·βME reaction product [5-(2-mercapto-1-hydroxyethyl)-4-hydrazono-1-pentanoic acid]. In this case, the C_1 resonance appears at 171.1 ppm, the C_4 resonance does not change dramatically (Table 2), and the coupling constant between C_1 and C_4 increases from ~2 to ~6 Hz. The increased coupling constant and the C_1 chemical shift are suggestive of the formation of a rotationally hindered ring structure, presumably a lactam (Marshall, 1983). The formation of the ring is not observed with the hydrazone of 5-CLA. The structure of the cyclic product was not pursued.

^{13}C NMR Characterization of [1,4-^{13}C]CLA-PBGS$_{bovine}$. Our previous characterization of 5-CLA-PBGS$_{bovine}$ indicated that a 1-h modification protocol resulted in protein that was preferentially modified at the A-side ALA binding pocket (Jaffe et al., 1992). ^{13}C NMR studies of 5-CLA-PBGS$_{bovine}$ using the substrate [4-^{13}C]ALA confirmed that the substrate could bind and form the P-side Schiff base intermediate, as illustrated in Figure 2B, and suggested that no further reaction occurred. However, we could not be certain where the [4-^{13}C]-ALA-derived resonance would occur for the postulated di-Schiff base intermediate illustrated in Figure 2C, particularly since enzyme-bound ligands may be in strained configurations and bond torsion angles play a significant role in determining ^{13}C chemical shifts.

To address the question of di-Schiff base formation, PBGS$_{bovine}$ modified with [1,4-^{13}C]CLA is described (see Table 3). The observed C_4 chemical shift, 209.5 ppm, indicates that the A-side 5-CLA-derived carbon is a ketone. The chemical shift of C_1, 180.9 ppm, indicates that the A-side carboxylic acid group is substantially deprotonated, a molecular detail we had not previously addressed. The results of a 1-h modification reaction show unexpectedly intense signals from the keto and acid moieties derived from [1,4-^{13}C]CLA, suggesting additional nonspecific modification of the protein,

Table 3: ^{13}C NMR Chemical Shifts of [1,4-^{13}C]CLA-Modified PBGS

species	reaction conditions	chemical shift (ppm) C_1	chemical shift (ppm) C_4
bovine	1 h	180.9	209.5
bovine	10 min	180.8	209.3
E. coli	with Zn(II) (four forms)	181.0	209.8
		180.6	209.1
		180.4	207.0
		180.1	173.1
E. coli	with 1,10-phenanthroline (two forms)	181.0	209.7
		180.1	173.1

but the spectra do not differ significantly from that of a 10-min modification. In either case there is no indication of the loss of the keto functionality. To further differentiate between the models illustrated in Figure 2B and 2C, the substrate is added as [5,5-^{2}H; 5-^{13}C]ALA. Deuteration at the ^{13}C-labeled methylene group ensures a narrow ^{13}C resonance (Jaffe & Markham, 1988). Prior to substrate addition, it is necessary to exchange the protein into buffered D_2O (to prevent loss of the C_5 deuterons) and to add 5 mM 1,10-phenanthroline (to prevent product formation at any unmodified active sites). Neither exchange into D_2O (Figure 4C) nor addition of 1,-10-phenanthroline alters the ^{13}C NMR spectra of [1,4-^{13}C]-CLA-PBGS$_{bovine}$. The first addition of [5,5-^{2}H; 5-^{13}C]ALA results in a small signal at 43.7 ppm, almost indistinguishable from a resonance we had previously assigned to the P-side Schiff base intermediate formed from [5,5-^{2}H; 5-^{13}C]ALA (Jaffe & Markham, 1988). A second addition results in an increase in the intensity of the 43.7 ppm Schiff base signal as well as the appearance of a signal at 47.0 ppm which derives from free [5,5-^{2}H; 5-^{13}C]ALA (Figure 4D). The addition of [5,5-^{2}H; 5-^{13}C]ALA does not cause a significant change in the signals derived from [1,4-^{13}C]CLA-PBGS$_{bovine}$. These reuslts confirm that there is no formation of the di-Schiff base analog illustrated in Figure 2C and supports our proposal that Zn_A is essential to formation of the first inter-ALA bond.

^{13}C NMR Characterization of [1,4-^{13}C]CLA-PBGS$_{E.\ coli}$. The ^{13}C NMR spectra of [1,4-^{13}C]CLA-PBGS$_{E.\ coli}$ at neutral pH indicates heterogeneous protein modification as expected from the sequencing results which showed that at least two different cysteines are modified. When modification is carried out in the presence of Zn(II), the difference spectrum ([1,4-^{13}C]CLA-PBGS$_{E.\ coli}$ − PBGS$_{E.\ coli}$, Figure 5A) exhibits three distinct keto resonances at 209.8, 209.1, and 207.0 ppm; at least three carboxylic acid resonances at 181.0, 180.6, and 180.1 ppm; and a resonance at 173.1 ppm which is consistent with that expected for C_4 of the thioether analog of the P-side Schiff base intermediate (Breitmaier & Voelter, 1987). As was previously observed with [4-^{13}C]ALA, the P-side Schiff base signal is very broad (Jaffe & Markham, 1987). The chemical shifts of the two upfield keto resonances are consistent with modification at cysteine while the 207.0 ppm resonance might suggest modification at a nitrogen by analogy to ALA. Although the acid resonances indicate slightly different chemical environments, in each case the carboxylates are substantially deprotonated. The chemical shift of the Schiff base signal (173.1 ppm), which is attributed to the C_4 derived from [1,4-^{13}C]CLA (see Figure 2E), differs considerably from the P-side ALA-Schiff base (165.4 ppm) illustrated in Figure 2A. The chemical shift differs presumably because the molecular geometry of the species with two covalent bonds to the enzyme is significantly different than the normal Schiff base. The chemical shifts of the model Schiff bases 5-CLA·βME·hydrazone (155.2 ppm, see Table 2) and [4-^{13}C]-

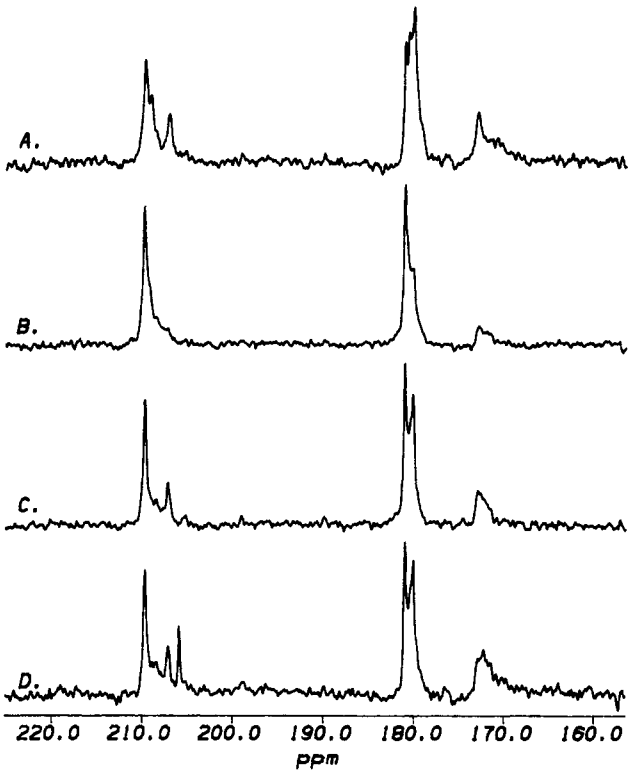

FIGURE 5: ^{13}C NMR difference spectra of (A) [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ (140 mg) prepared in the presence of Zn(II), 32000 scans, 2-s repetition rate, 15-Hz line-broadening minus the ^{13}C NMR spectra of $PBGS_{E.\ coli}$ under similar conditions and identical processing parameters. (B) [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ (120 mg) prepared in the presence of 1,10-phenanthroline; 32000 scan minus the ^{13}C NMR spectra of $PBGS_{E.\ coli}$ under similar conditions and identical processing. (C) Difference spectrum 5A plus 5 mM 1,10-phenanthroline. (D) Difference spectrum 5C plus 1 μmol of [4-^{13}C]ALA, using a 4-s recycle time. All spectra were obtained at 37 °C.

ALA·hydrazone (154 ppm) are both quite different from that observed for the analogous enzyme-bound Schiff base (164 ppm), again presumably due to the hindered rotations of the species which are tethered to the enzyme. An alternative assignment for the 173.1 ppm resonance derived from [1,4-^{13}C]CLA comes from a possible Favorski rearrangement, which would create an enzyme-bound [1,5-^{13}C]thioester (March, 1977). The analogous ^{13}C resonance of a model compound thioacetic acid 5-methyl ester falls at 195 ppm (Breitmaier & Voelter, 1987). Thus, the Favorski product is not proposed.

Assignment of the complex mixture of [1,4-^{13}C]CLA-derived signals illustrated in Figure 5A is simplified by consideration of a sample of [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ prepared in the presence of 1,10-phenanthroline (Figure 5B). Inclusion of 1,10-phenanthroline in the modification reaction mixture reduces the number of species to two which are assigned on the basis of signal intensity (see Table 3). The resonances at 181.0 and 209.8 ppm are assigned to the same species which retains the keto functionality at C_4; the 180.1 and 173.1 ppm resonances are assigned to the Schiff base intermediate formed from [1,4-^{13}C]CLA. Because Zn_A is generally believed to be essential for A-side ALA binding, both of these species are assigned to the P-side ALA binding pocket. We suggest that 5-CLA has modified two cysteines, and in one case the distance between the modified cysteine and lysine 246 is too far to allow Schiff base formation while modification at the other cysteine allows Schiff base formation. The remaining signals seen in Figure 5A, which are absent from Figure 5B, are less abundant species and are suggested to represent a small amount of modification at the A-side ALA binding pocket.

Further assignments can be made based on manipulations to the sample for which the spectrum is illustrated in Figure 5A. Before the substrate can be added to further probe potential active site chemistry, it is necessary to add 1,10-phenanthroline in order to prevent product formation at the unmodified active sites (~10%). Addition of 1,10-phenanthroline causes the 209.1 and 180.6 ppm resonances to collapse respectively into the 209.8 and 181.0 ppm resonances which increase in intensity (Figure 5C). In addition, a previously masked carboxylate resonance at 180.4 ppm is revealed. On the basis of this observation, the 209.1 and 180.6 ppm resonances are assigned to the same minor species which is proposed to be directly coordinated to Zn(II) at the A-side ALA binding pocket. The remaining pair of minor resonances at 180.4 and 207 ppm are also assigned to the A-side ALA binding pocket and may represent modification at histidine or a backbone nitrogen. During acquisition of the spectrum illustrated as a difference spectra in Figure 5C, some protein precipitation occurred (see below). Addition of [4-^{13}C]ALA to the NMR sample at a stoichiometry of 0.6 per active site yields the difference spectra illustrated in Figure 5D. The predominant new signal corresponds to free [4-^{13}C]ALA at 205.9 ppm; no significant changes in the signals derived from [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ are observed. Thus, in contrast to 5-CLA-modified $PBGS_{bovine}$, 5-CLA-modified $PBGS_{E.\ coli}$ does not bind the substrate in the presence of 1,10-phenanthroline. Unfortunately, it is not possible to do the experiment in the absence of 1,10-phenanthroline. Because we have previously established that Zn(II) is required for A-side ALA binding and reactivity, the lack of substrate binding in the presence of 1,10-phenanthroline is consistent with the proposal that the P-side ALA binding pocket is not available; *e.g.*, 5-CLA modifies $PBGS_{E.\ coli}$ at the P-side ALA binding pocket.

Spectrum 5D also reveals several minor enzyme-bound species in the region of 120–150 ppm (data not shown). Based on other spectra of $PBGS_{E.\ coli}$ in the presence of 1,10-phenanthroline and [4-^{13}C]ALA, these unassigned resonances are attributed to reactions occurring at 10% of the active sites which are not 5-CLA-modified. Following dialysis of the [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ sample back into 0.1 M KPi, pH 7, and 30 μM Zn, the resultant spectrum is indistinguishable from that of Figure 5A with the exception that the peaks at 209.1 and 180.6 ppm are missing; these resonances may correspond to the protein which precipitated.

The activity of $PBGS_{E.\ coli}$ is stimulated by Mg(II) (Mitchell & Jaffe, 1993; Spencer & Jordan, 1993), a trait not exhibited by $PBGS_{bovine}$. The stimulatory Mg, denoted Mg_C, is also present in plant PBGS (Prasad et al., 1988). Since Mg(II) is known to bind often to carboxylic acid groups in proteins (Glusker, 1991), addition of Mg(II) to [1,4-^{13}C]CLA-$PBGS_{E.\ coli}$ might effect the C_1-derived resonances. Addition of 1 mM Mg causes no significant spectral change, suggesting either that the Mg(II) does not interact with the carboxyl groups derived from [1,4-^{13}C]CLA or that Mg(II) does not bind to the modified protein. Since the paramagnetic Mn(II) ion mimicks the effect of Mg(II) on the activity of PBGS *E. coli*, addition of Mn(II) might cause line broadening. Several 0.15-μmol additions of Mn(II) cause a very subtle line broadening for the C_1 signals, suggesting that the carboxyl groups are available to solvent but not in direct contact with the Mn(II). Mn(II) EPR data suggests that Mn(II) does not bind to 5-CLA-modified $PBGS_{E.\ coli}$ (data not shown).

DISCUSSION

Characterization of [1,4-^{13}C]CLA-PBGS$_{E. coli}$ demonstrates heterogeneous modification, predominantly at the P-side ALA binding pocket, with modification at Cys119 and Cys129. These cysteines are the first and third cysteines of the sequence CFCEYTSHGHCGVLCE, which is putatively a four-cysteine cluster operative in binding the Zn_B of PBGS$_{E. coli}$ (see Table 1). The EXAFS analysis of Dent et al. (1990) indicates that Zn_B of PBGS$_{bovine}$ has four cysteine ligands and the EXAFS spectrum of the Zn_8 form of PBGS$_{E. coli}$ is very similar to that of PBGS$_{bovine}$ (S. Hasnain, R. Strange, L. W. Mitchell, and E. K. Jaffe, unpublished results). PBGS$_{E. coli}$ appears to contain three metal ion binding sites, Zn_A, Zn_B, and Mg_C (Mitchell & Jaffe, 1993; Jaffe, 1993; see Table 1). The current work places Zn_B near P-side ALA and extends our active site model to include both Zn_A and Zn_B as illustrated in Figure 2F. Previous work with PBGS$_{bovine}$ showed that extended treatment with 5-CLA results in modification at both substrate binding sites. To test the model illustrated in Figure 2F, we must confirm that doubly modified PBGS$_{bovine}$ is modified at the four-cysteine cluster. The four cysteines which chelate Zn_B are proposed to arise from a single subunit while the ligands to Zn_A may arise from adjacent subunits (Jaffe, 1993).

The active site model illustrated in Figure 2F differs considerably from past models of the active site or chemical mechanisms proposed for PBGS. Various well-cited mechanistic schemes do not include a role for Zn(II) in the PBGS-catalyzed reaction (Nandi & Shemin, 1968; Jordan & Seehra, 1980; Jordan, 1991). One proposed model depicts the active site at the interface of two subunits with Zn(II) securing the subunits by forming a chelation bridge between two cysteines from different subunits (Batlle & Stella, 1978). It has been proposed that one role for Zn(II) is to prevent disulfide formation between essential sulfhydryls (Tsukamoto et al., 1979). Another active site model places one Zn(II) near the active site (Schlösser & Beyersmann, 1987); this Zn(II) is proposed to be ligated to residues adjacent to those which are important to catalysis, *e.g.*, it contributes to the structure but not the function of the active site. This proposal proceeded the discrimination of Zn_A and Zn_B by EXAFS (Dent et al., 1990). Our earlier work placed Zn_A at the active site (see Figure 2A) and proposed that Zn_A plays a role in A-side ALA binding (Jaffe & Hanes, 1986), in inter-ALA bond formation (Jaffe et al., 1992), and in product binding (Jaffe et al., 1990). Most recently Spencer and Jordan (1994) independently conclude that Zn(II) is involved in catalysis at A-side ALA. The current work implicates Zn_B as an active site component, although we have no direct evidence that Zn_B plays a role in catalysis. Because the ligands to Zn_B are all cysteines, which is unprecedented for a catalytic Zn(II) (Jaffe, 1993), there is no similarity between the model presented in Figure 2F and the co-catalytic zinc motif recently defined by Vallee and Auld (1993).

The question arises as to the role of Zn_B at the active site. Could it be that Zn_B is that Zn(II) previously proposed to prevent disulfide formation between essential sulfhydryl groups? If these sulfhydryl groups are essential, then what is their role in catalysis? Consider that the chloro group of 5-CLA is on C_5, that 5-CLA modifies the four-cysteine cluster, and that this cluster is implicated in binding Zn_B. Therefore, Zn_B is placed near the C_5 of P-side ALA as illustrated in Figure 2F. The C_5 of P-side ALA is a carbon which must dispose of one proton during the catalytic cycle, and loss of this proton is often proposed to be the final step in PBG formation. Here we propose that one or more of the cysteines of the four-cysteine cluster act(s) as the acceptor for the C_5 proton of P-side ALA. Why then is Zn_B not seen necessary for the activity of PBGS$_{bovine}$? The reason appears to be that PBGS assays are done in the presence of copious amounts of βME, a situation somewhat different from that which would be found in tissues. If the role of Zn_B is to keep the cysteines reduced, and the four-cysteine cluster is readily accessible to solvent, then βME could serve the role of Zn_B, and we would not see a requirement for Zn_B for PBGS activity. This is particularly true if the role of Zn_B is not part of a significantly rate-determining step. It should be pointed out that the PBGS turnover number is $\sim 1/s$.

The four-cysteine cluster of mammalian, yeast, and bacterial PBGS is replaced by an aspartic acid-rich region in plant PBGS which has been proposed as a Mg(II) binding site (Boese et al., 1991). The role of a C_5 proton acceptor can be played by the aspartic acid residues at metal site B coordinated to a Mg(II). In this case, exogenous reagents like βME would not mask a requirement for a B site Mg(II). Thus, we propose that plant PBGS requires both Zn_A and Mg_B for catalysis, a hypothesis which awaits testing. The Mg_C of plant and bacterial PBGS does not appear to be essential to catalysis, and its mechanism for stimulating the activity of these proteins is the topic of another paper (Jaffe et al., 1994).

In conclusion, we have shown that 5-CLA modifies PBGS$_{E. coli}$ in a different way than was observed for PBGS$_{bovine}$. PBGS$_{E. coli}$ is modified by 5-CLA at either Cys119 or Cys129, and we concluded that modification is predominantly at the P-side ALA binding pocket. Because Cys119 and Cys129 are implicated in binding Zn_B, we propose that Zn_B is near P-side ALA.

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