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Spatial proximity and sequence localization of the reactive sulfhydryls of porphobilinogen synthase

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Abstract

The zinc metalloenzyme porphobilinogen synthase (PBGS) contains several functionally important, but previously unidentified, reactive sulfhydryl groups. The enzyme has been modified with the reversible sulfhydryl-specific nitroxide spin label derivative of methyl methanethiosulfonate (MMTS), (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate (SL-MMTS) (Berliner, L.J., Grunwald, J., Hankovszky, H.O., & Hideg, K., 1982, *Anal. Biochem. 119*, 450–455). EPR spectra show that SL-MMTS labels three groups per PBGS subunit (24 per octamer), as does MMTS. EPR signals reflecting nitroxides of different mobilities are observed. Two of the three modified cysteines have been identified as Cys-119 and Cys-223 by sequencing peptides produced by an Asp-N protease digest of the modified protein. Because MMTS-reactive thiols have been implicated as ligands to the required Zn(II), EPR spectroscopy has been used to determine the spatial proximity of the modified cysteine residues. A forbidden ($\Delta m = 2$) EPR transition is observed indicating a through-space dipolar interaction between at least two of the nitroxides. The relative intensity of the forbidden and allowed transitions shows that at least two of the unpaired electrons are within at most 7.6 Å of each other.

SL-MMTS-modified PBGS loses all Zn(II) and cannot catalyze product formation. The modified enzyme retains the ability to bind one of the two substrates at each active site. Binding of this substrate has no influence on the EPR spectral properties of the spin-labeled enzyme, or on the rate of release of the nitroxides when 2-mercaptoethanol is added. The results indicate that binding of this substrate does not affect the environment of the majority of the nitroxides. This is consistent with our previous proposal that it is the substrate that binds only to the Zn(II) enzyme that provides ligands to the catalytic Zn(II) (Jaffe, E.K., Abrams, W.R., Kaempfen, K.X., & Harris, K.A., 1992, *Biochemistry 31*, 2113–2123).

SL-MMTS readily forms disulfide-linked dimers. The rate of dimer formation is proportional to the hydroxide ion concentration and is independent of both buffer and SL-MMTS concentrations. This suggests a rate-limiting hydrolysis of the S-S bond of SL-MMTS followed by rapid reaction of the liberated thiol with a second molecule of SL-MMTS. Therefore, substantial care is required in the use of SL-MMTS as a protein modification reagent at even slightly alkaline pH values.

Keywords: porphobilinogen synthase; spin-labeled cysteine; spin label MMTS dimerization

Porphobilinogen synthase catalyzes the condensation of two molecules of ALA to form PBG, the monopyrrole precursor to all naturally occurring tetrapyrroles (e.g., heme, chlorophyll, B_{12}). Bovine PBGS has several cysteines that react with various sulfhydryl reagents leading to inactivation of the enzyme (Barnard et al., 1977; Jaffe et al., 1984). There are eight cysteines per 35-kDa subunit of octameric mammalian PBGS that are uniformly conserved in the sequences deduced from cDNA sequences of human, mouse, and rat (Bishop et al., 1986, 1989; Wetmur et al., 1986). Through peptide sequence analysis of the bovine protein, we have confirmed the presence of these eight cysteines in bovine PBGS. The spatial relationships of these thiols have not been characterized in any case.

MMTS is a sulfhydryl-specific reagent that reversibly modifies proteins by forming a mixed disulfide (Smith

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Abbreviations: ALA, 5-aminolevulinate; EXAFS, extended X-ray absorption fine structure; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; BME, 2-mercaptoethanol; MMTS, methyl methanethiosulfonate; KP_i, potassium phosphate; SL-MMTS, (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate; PBG, porphobilinogen; PBGS, porphobilinogen synthase; PTH, phenylthiohydantoin; SL, 1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline.

et al., 1975). MMTS modifies PBGS with incorporation of three methanethio groups per subunit (24/octamer; 6/active site), however, the sites of modification are not known. Modification results in total loss of the enzyme-bound Zn(II) and the total loss of catalytic activity (Jaffe et al., 1984). The MMTS-modified enzyme can be fully reactivated by addition of BME and Zn(II). There are two distinct classes of Zn(II) binding sites, each with a stoichiometry of 4/octamer (1/active site); only one class of Zn(II) is required for catalytic activity (Bevan et al., 1980; Jaffe et al., 1984). The EXAFS studies of Beyersmann and coworkers (Dent et al., 1990) suggest that the catalytic Zn(II) each possess one sulfur ligand, whereas the other (structural) Zn(II) each contain four sulfur ligands. Because MMTS-modified PBGS contains no Zn(II), the implication is that at least one cysteine ligand per zinc is modified by MMTS. If two or more MMTS-modified cysteines are ligands to either type of Zn(II) then for each active site at least two of the MMTS-reactive thiols should be in close spatial proximity; however, there are no data to support this proposition. To test this proposal, we capitalized on the fact that EPR spectroscopy can be used to measure the distance between two spins in a protein (Eaton & Eaton, 1989). We prepared the nitroxide spin label derivative of MMTS (SL-MMTS) (Berliner et al., 1982) (Fig. 1A) and have characterized its interaction with PBGS. EPR spectroscopy has been used to determine the stoichiometry of SL-MMTS labeling of PBGS and to demonstrate proximity of at least two of the modified cysteine residues to one another. As we had not previously identified the MMTS-modified cysteines, sequences of spin-labeled peptides produced by an Asp-N protease digest of SL-MMTS-modified PBGS have been used to determine the primary sequence location of the modified cysteines.

MMTS-modified PBGS binds only four equivalents of the substrate ALA, half saturating each of the four active sites by formation of a Schiff base intermediate between Lys-252 and the ALA molecule whose nitrogen becomes incorporated into the pyrrole ring of PBG (P-side ALA) (Jaffe & Markham, 1987). Because SL-MMTS-modified cysteines are significantly larger than MMTS-modified cysteines, we determined the ability of SL-MMTS-modified PBGS to bind P-side ALA. Furthermore, because one of the MMTS-modified cysteines is proposed to be a ligand to the catalytically essential Zn, and we have proposed an active site model where one ALA is a ligand to that Zn (Jaffe et al., 1992), we also investigated the effect of P-side ALA binding on the EPR spectra of SL-MMTS-modified PBGS.

Results

Dimerization of SL-MMTS

Initial studies focused on the conditions for use of the SL-MMTS in modification of PBGS. When EPR spectra of a solution of SL-MMTS in neutral pH buffer were obtained, a time-dependent decrease in the intensity of the three hyperfine lines was observed. Concomitantly, two new broader lines appeared between the original three lines, eventually leading to a stable five-line spectrum (Fig. 1). The spectral changes suggested formation of a nitroxide dimer in which the two unpaired electrons interact by spin exchange (cf. Luckhurst, 1976). Addition of excess BME reversed the spectral change, suggesting that disulfide-linked nitroxide dimers were forming from SL-MMTS (see below). An SL-MMTS solution equilibrated at pH 8.5 was chromatographed on a reverse-phase HPLC column, allowing isolation of a compound that displayed the five-line EPR spectrum (see Materials and methods). The five lines were spaced at one-half the apparent hyperfine coupling of monomeric SL-MMTS, which results from the combination of spin exchange coupling of two unpaired electron spins, hyperfine coupling to two equivalent ^{14}N nuclei, and an exchange coupling

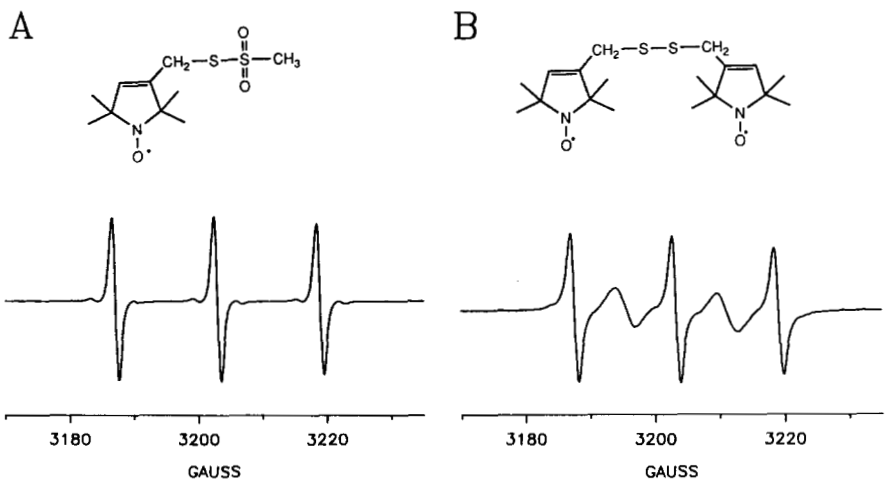

Fig. 1. The structures and EPR spectra of solutions of SL-MMTS (**A**) and its dimer (**B**). Spectra were obtained for 1.0 mM SL-MMTS immediately after dilution into 0.1 M KP_i buffer, pH 7.0, and after incubation of the solution for 24 h at 25 °C. The spectrometer gain for spectrum B was threefold greater than for spectrum A.

constant greater than the hyperfine coupling constant (Luckhurst, 1976). The alternating narrow and broad line widths are characteristic of nitroxide dimers in which the two unpaired electron spins are exchange coupled with coupling constants comparable to molecular reorientation rates (Luckhurst, 1976). EPR spectra shown in Figure 2 for the dimer frozen in a 50% glycerol glass reveal a forbidden transition at half the magnetic field of the allowed transition; the (doubly) integrated intensity of the forbidden transition was 0.004% of the integrated intensity of the allowed transition. The same relative intensity was seen at sixfold higher nitroxide concentration, indicating that the $\Delta m = 2$ transition reflects an intramolecular spin-spin interaction. The intensity of the half-field transition reflects the magnitude of the through-space dipolar interaction between the unpaired electrons and is independent of the presence of exchange coupling (Eaton et al., 1983). Hence the relative intensity of the allowed and forbidden transitions can be used to estimate the distance between the unpaired electrons using the equation:

$$\frac{\text{Forbidden intensity}}{\text{Allowed intensity}} = \frac{A(9.1\ \text{GHz})^2}{r^6 \nu^2},$$

where the constant $A = 19.5$ Å^6, r is the distance between electrons in Angstroms, and ν is the spectrometer frequency in GHz (Eaton et al., 1983). In a point-dipole approximation the observed relative intensity would reflect a separation of 8.6 Å between the unpaired electrons. Molecular models show that the maximal distance between the two electrons of the dimer is 12.8 Å when an extended all-trans conformation is present (assuming localization of the unpaired electron midway between the nitrogen and oxygen atoms of the nitroxide moieties). Because the r^6 dependence of the dipolar interaction heavily weights the forbidden spectral intensities by the fraction of the molecules with shorter distances between electrons, the results indicate that the dimer has a predominantly extended configuration.

The rates of the spectral changes occurring during SL-MMTS dimer formation were monitored under a variety of conditions. In all cases the time course of the spectral alterations followed a single exponential. The rates were directly proportional to the hydroxide ion concentration over the pH range 7.0–9.0 (experiments at 8 pH values, data not shown). At each pH value the rates were the same at 0.1 mM and 1.0 mM SL-MMTS and were independent of buffer (either KP_i at 10 mM or 100 mM, or a mixture of KCl and HEPES/KOH with both components at either 5 mM or 50 mM). The second-order rate constant for dimer formation is 2.4×10^2 M^{-1} [OH$^-$] s^{-1} at 21 °C. The kinetic behavior suggests a rate-limiting reaction of SL-MMTS with OH$^-$ followed by rapid attack of the liberated thiol-bearing nitroxide on a second molecule of SL-MMTS to form the nitroxide dimer:

$$\text{SL-MMTS} + \text{OH}^- \rightarrow \text{SL-CH}_2\text{SH} + \text{CH}_3\text{SO}_3^-$$

$$\text{SL-CH}_2\text{SH} + \text{SL-MMTS} \rightarrow \text{SL-CH}_2\text{S-SCH}_2\text{-SL} + \text{CH}_3\text{SO}_3^- + \text{H}^+.$$

Because reaction of the dimer with cysteine would be expected to have very different kinetics than SL-MMTS, these results indicate that substantial care must be taken with the use of SL-MMTS, and perhaps MMTS itself, as a protein modification reagent at alkaline pH values. Addition of BME to solutions of the purified dimer restored the spectrum to that of a monomeric spin label. The rate of formation of monomer was proportional to the BME concentration with a second-order rate constant of ~60 M^{-1} [BME] s^{-1} at pH 7.0, 21 °C.

SL-MMTS modification of PBGS

When PBGS was treated with SL-MMTS, the enzyme lost all catalytic activity (<0.01% of initial). The activity was completely recovered upon incubation of the modified enzyme with 10 mM BME and 10 μM Zn(II) for 10 min at 37 °C. The stoichiometry of modification was found to be 3.0 ± 0.1 nitroxides per subunit as determined from the doubly-integrated EPR spectrum obtained after incubation of spin-labeled enzyme (0.3 mM subunits) with excess BME (20 mM). Atomic absorption experiments showed that SL-MMTS-modified PBGS contained no Zn(II) (<0.1/subunit). These properties all parallel MMTS-modified PBGS (Jaffe et al., 1984).

Fig. 2. EPR spectra of the purified SL-MMTS dimer (0.5 mM) frozen in 0.1 M KP_i containing 50% glycerol (to facilitate glass formation). The gain for the forbidden transitions (**B**) was 500 times that used for the allowed transitions (**A**), and the microwave power was 100-fold higher. Ten scans were averaged for the forbidden transition and four scans were averaged for the allowed signals.

Substrate binding properties

MMTS-modified PBGS is able to bind one of the two ALA substrate molecules, the one denoted as P-side ALA, and to form the Schiff-base intermediate, which is on the normal reaction pathway (Nandi & Shemin, 1968; Jaffe & Hanes, 1986). In view of the size of the nitroxide derivative of MMTS, the substrate binding properties of the spin-labeled enzyme were determined. [4-^{14}C]ALA was found to bind to SL-MMTS-modified PBGS at a stoichiometry of four equivalents of ALA per octamer with an affinity of 90 μM, as illustrated in Figure 3. ALA binds to MMTS-modified PBGS with a stoichiometry of 4/octamer and a dissociation constant of 150 μM (data not shown). There was no detectable binding of a second equivalent of ALA to SL-MMTS-modified PBGS or the MMTS-modified enzyme. Thus although the nitroxide moieties are vastly larger in size than the methyl group of MMTS, the nitroxide spin label does not affect the stoichiometry or profoundly alter the affinity of P-side ALA binding at the modified enzyme active site. We have previously reported the dissociation constants for ALA binding to native PBGS as 3.8 μM and 240 μM (Jaffe et al., 1992).

EPR spectra of SL-MMTS-modified PBGS

The EPR spectrum of a solution of SL-MMTS-modified PBGS is shown in Figure 4A. Three spectral components can be discerned, reflecting three nitroxides with different degrees of immobilization (see arrows). None of the nitroxides are rigidly immobilized on the protein (expected correlation time ca. 300 ns), which would give a near rigid-limit line shape (compare line shape to a frozen sample, Fig. 4B). The solution EPR spectrum provides no indications of magnetic interactions between the nitroxides. However, the spectrum of a frozen solution of SL-MMTS-modified PBGS contains a weak EPR signal at half the magnetic field of the allowed transition indicating a dipolar interaction between at least two of the enzyme-bound nitroxide groups (Fig. 4C). Double integration showed that the area of the forbidden transition is 0.03% of the area of the allowed transitions. Assuming that only two out of six nitroxides present per active-site equivalent have dipolar interactions, the relative intensity of the forbidden and allowed transitions can be used to calculate a lower limit on the distance between two nitroxides of 5.3 Å. It is possible that pairwise interactions between more than two nitroxides contribute to the intensity of the forbidden transition. On the basis of three interacting nitroxides, an estimated separation between pairs of 7.6 Å can be made based on the r^6 dependence of the interaction and assuming equal distances between all three unpaired electrons. Because the distance between the sulfur of the modified cysteine and the unpaired electron is ~5.6 Å, the distances between the modified sulfhydryls could be small enough so that they could ligate the same Zn(II) in the holoenzyme.

When 10 mM ALA was added to the enzyme no alterations in the EPR spectra were observed for solution or

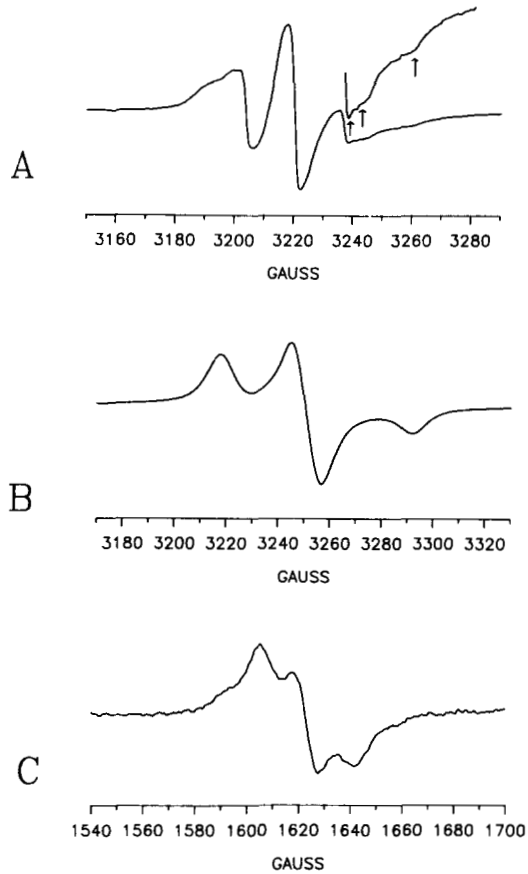

Fig. 4. EPR spectra of SL-MMTS-modified PBGS. **A:** Spectrum of 3.3 mM SL-MMTS-modified PBGS subunits in 0.1 M KP_i, pH 7.0 in solution; four scans were averaged. The inset shows the high field region at fivefold higher gain. The arrows show positions of the high field resonances for three classes of nitroxides with different mobilities. **B, C:** Spectra of 3.3 mM SL-MMTS-modified PBGS in frozen solution (−140 °C). B shows the allowed transitions; four scans were averaged. C shows the forbidden transitions at half the magnetic field of the allowed transitions; these signals were recorded at 200-fold higher gain, 100-fold higher microwave power, and 10 scans were averaged.

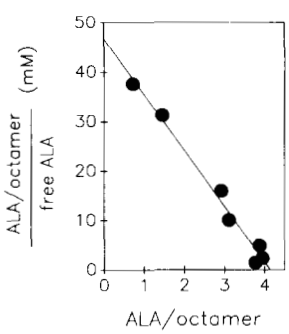

Fig. 3. Scatchard plot for ALA binding to SL-MMTS-modified PBGS. [4-^{14}C]ALA binding was determined using ultrafiltration. SL-MMTS-modified PBGS binds four ALA per octamer (one per active site) with a dissociation constant of 90 μM.

frozen samples. There was no significant (<20%) change in the relative intensity of the forbidden EPR transition, indicating no alteration in the spatial distribution of the dipolar-coupled nitroxide moieties upon ALA binding to SL-MMTS-PBGS.

Rate of reaction of SL-MMTS-modified PBGS with BME

To determine whether the binding of substrate affected the accessibility of the spin-labeled sulfhydryl to bulk solvent, as might be expected if ALA binding induced a conformational change, we examined the rate of reaction of 0.3 mM spin-labeled enzyme with BME in the presence and absence of 10 mM ALA. The increase in the intensity of the EPR spectrum upon formation of the free nitroxide showed a time course consistent with a single exponential for at least two half-times. The rate of nitroxide release from the enzyme was proportional to the BME concentration (five concentrations between 4 and 35 mM) and was not altered by the presence of ALA. The second-order rate constant for release of the nitroxide is 0.11 M^{-1} [BME] s^{-1}, which is ~550-fold slower than the rate of reaction of the SL-MMTS dimer with BME. The large decrease in the rate of reaction of BME with SL-MMTS-modified PBGS compared to the SL-MMTS dimer suggests the inability of BME to readily approach the disulfide linkages of modified PBGS. Consequently none of the modified cysteines are likely to be on the exterior surface of the protein. Although the kinetics of release of the nitroxide are apparently first order, computer simulations showed that two or three different rates could not have been distinguished if the rate constants were of the same order of magnitude.

Identification of the SL-MMTS-modified cysteines

Five chromatography peaks containing spin label were identified from an HPLC chromatogram of an Asp-N protease digest of SL-MMTS-modified PBGS. These peptides, denoted by elution order as peaks #13, #15, #22, #26, and #33 had retention times of 58, 60, 74, 78, and 86 min, respectively. Upon sequence analysis of the spin-labeled peptides, peaks #13 and #15 were both found to correspond to an Asp-N peptide beginning at Asp-220 and ending at either Val-238 or Arg-240. We had previously reported this peptide sequence, which contains only one cysteine, and identified this Cys-223 as the site of PBGS modification by the suicide substrate 5-chlorolevulinic acid (Jaffe et al., 1992). The sequence is identical to positions 220–240 of human PBGS (Wetmur et al., 1986), and it is the human cDNA-derived sequence that we use as the basis for numbering the residues of the bovine PBGS peptides. While sequencing spin-labeled peptide peak #15 it was found to contain a second peptide whose sequence turned out to be DLLMVKPGTPYL, which is analogous, though not identical, to the Asp-247–Leu-258 sequence of the human protein (Wetmur et al., 1986). Lys-252 has been identified as the active-site lysine (Gibbs & Jordan, 1986).

The third and fourth spin-labeled peptides, peaks #22 and #26, contained relatively little spin label per absorbance at 214 nm leading us to suspect the spin-labeled peptide was a minor component. These peptides were subjected to further HPLC purification using 6 mM HCl in place of trifluoroacetic acid. As a consequence of the second purification, the spin label was lost, leaving considerable ambiguity about which of the resulting 214-nm peaks to sequence. Therefore, no sequences of these peptides were determined. The fifth spin-labeled peptide, peak #33, when sequenced was also found to correspond to an Asp-N peptide of native PBGS for which we had previously reported the sequence (Jaffe et al., 1992). This peptide, whose complete sequence is DSPAIEAIRLLRK NFPSLLVAC, corresponds to Glu-98–Cys-119 of human PBGS and differs from the human sequence at positions 98, 106, 111, and 114. Cys-119 defines the N-terminal region of a cysteine- and histidine-rich portion of mammalian PBGS, which has been suggested as a Zn(II) binding region (Wetmur et al., 1986).

Each of the SL-MMTS-modified peptides that were sequenced contained only one cysteine, but it was not uniformly possible to obtain positive evidence for SL-MMTS modification. For peptide peaks #13 and #15, where each peptide was available in excess of 100 pmol, and the modified residue was in the fourth sequencing cycle, the SL-MMTS-modified cysteine yielded a small amount of a unique PTH amino acid, which eluted at 9.2 min. Peptide peak #33, however, contained only ~20 pmol of material, the cysteine was at sequence cycle 22, and the new PTH amino acid was not observed. Therefore, to confirm that the spin label observed in the EPR spectra of these peptides reflected a modified amino acid in the sequence of the major peptide component, we compared the intensity of the spin label signal to the PTH amino acid yield at various sequencing cycles. The data are presented in Table 1. The ratio of the EPR signal intensities for the three peptide peaks #13, #15, and #33 is 100:23:3.5, which is remarkably similar to the ratio of PTH amino acid yields for these same three peptides. This quantitatively supports the conclusion that in each case the EPR signal corresponded to the major peptide and allows identification of Cys-119 of peptide peak #33 as an SL-MMTS-modified cysteine.

The relatively low yield of spin label in peptide peaks #22, #26, and #33 serves as a clue to the identity of the third spin-labeled cysteine (see Table 1). The PBGS sequence of amino acids 119–132 is

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119     122 124                 132
C D V C L C P Y T S H G H C,
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Table 1. *Spin-labeled peptides from an Asp-N protease digest of SL-MMTS-modified PBGS*

Peptide peak number[a]	Retention time (min)	Normalized spin label g = 2 intensity (%)	Phenylthiohydantoin amino acid yield at various sequence cycles (pmol) (X) = sequence cycle			Average pmol (%)
13	58	100	430 (2)	370 (5)	427 (6)	409 (100)
15	60	23	116 (2)	84 (5)	116 (6)	105 (25)
22	~74	~7	ND	ND	ND	ND
26	~78	8.6	ND	ND	ND	ND
33	86	3.5	24 (3)	18 (4)	14 (20)	19 (4.6)

[a] Sequence analysis of the spin-labeled peptides revealed that peptide peaks #13 and #15 both started at Asp-220 (DRRCYQLPPGARGLALRAV(DR)); peptide peak #33 includes positions 98–119 (DSPAIEAIRLLRKNFPS LLVAC); peptides 22 and 26 were not sequenced (see text).

and we have learned through sequencing peptide peak #33 that Cys-119 is modified by SL-MMTS. We suspect that the third modified cysteine is one of the remaining cysteines in this, a putative metal binding region (Wetmur et al., 1986; Jaffe et al., 1992). The low yield is proposed to be a consequence of disulfide exchange between the two spin label-modified cysteines on the denatured protein prior to being cut by the protease at Asp-120. All three of the suspect cysteines are conserved in the human, rat, mouse, yeast, and *Escherichia coli* PBGS sequences and are implicated in binding the structural Zn(II) (see below).

The hypothesis that two SL-MMTS-modified cysteines are subject to disulfide exchange under denaturing conditions was tested. We denatured SL-MMTS-modified PBGS and monitored changes in the EPR spectrum (spin label signal) over 24 h (37 °C). The addition of 8 M urea causes significant sharpening of the EPR signals as the modified cysteines become more mobile. This apparent unfolding process is complete in ~20 min. At this time gel filtration indicates that the spin label is still attached to the denatured protein. Over the course of ~3 h, one can observe formation of a spin-coupled species, hereafter called the spin dimer. After 24 h the spectrum looks like a linear combination of equal parts of spectra 1A and 1B. At this point, gel filtration shows that at least 90% of the spin label is no longer associated with the protein. In this case, the formation of free spin monomer as SL–SH is due to the reaction of modified cysteines with unmodified cysteines:

$$\text{Cys-SH} + \text{Cys-S-S-SL} \rightarrow \text{Cys-S-S-Cys} + \text{HS-SL}.$$

The formation of spin dimer is likely due to disulfide exchange between two modified cysteines:

$$2\ \text{Cys-S-S-SL} \rightarrow \text{Cys-S-S-Cys} + \text{SL-S-S-SL}.$$

The facility of the later reaction in denatured SL-MMTS-modified PBGS supports our hypothesis that two of the modified cysteines are in the same region of the PBGS sequence.

Discussion

SL-MMTS modification of PBGS mimics MMTS modification of PBGS and results in total loss of catalytic activity; full activity is restored after addition of BME and Zn(II). SL-MMTS modifies PBGS with incorporation of six nitroxides per active-site equivalent, which consists of two subunits. Therefore at least one of six SL-MMTS-modified cysteines is probably a ligand to the catalytically essential Zn(II), which is present at a stoichiometry of one per active site. We have previously identified Cys-223, on one-half the subunits, as the cysteine ligand to the catalytic Zn(II) (Jaffe et al., 1992). Here we show that Cys-223 is also modified by SL-MMTS presumably on each subunit, because the Asp-N peptide corresponding to that with unmodified Cys-223 is not observed in peptide maps of digested SL-MMTS-modified PBGS.

EPR spectra unambiguously show that at least two spin labels of SL-MMTS-modified PBGS are very close in space. It is likely that at least two of the six modified cysteines are normally ligands to the structural Zn(II), which is usually bound to PBGS at one equivalent per active site, but which is not required for catalytic activity. We have identified Cys-119 as an SL-MMTS-modified cysteine and propose that Cys-119 of one or more subunits provides one or two of the sulfur ligands to the structural Zn(II). These conclusions are drawn in the context of the model of Dent et al. (1990) who used EXAFS spectroscopy to quantify the differences between the two classes of Zn(II) binding sites present on bovine PBGS. The EXAFS data support a model where the tight binding catalytic Zn(II) each possess one cysteine ligand that we identify as Cys-223, whereas the looser binding structural Zn(II) each contain four cysteine ligands. By analogy to the structural Zn(II) site of alcohol dehydrogenase (Bergman et al., 1992), $C(X)_2C(X)_2C(X)_7C$, the four cysteines at positions 119, 122, 124, and 132 of bovine PBGS (see above)

make an attractive model. However, because the ligands to each structural Zn(II) may come from two subunits, the four cysteine ligands may derive from as few as two different cysteines in the sequence.

EPR spectra indicate that substrate binding has a negligible effect on the environments of the nitroxides in SL-MMTS-modified PBGS. Substrate binds to SL-MMTS-modified PBGS at the P-side ALA binding site. These observations support the conclusion that at least two out of six SL-MMTS-susceptible cysteines are normally ligands to a structural Zn(II), which is spatially distinct from the active site. A third SL-MMTS-susceptible cysteine, proposed to be a ligand to the active-site Zn(II), is also likely to be distant from the P-side ALA, though it may interact with the second ALA substrate molecule, the one that does not bind to SL-MMTS-modified PBGS. We have proposed a model where Cys-223, as well as the C_4 keto oxygen and C_5 amino nitrogen of the second ALA, serve as ligands to the catalytic Zn(II) (Jaffe et al., 1992).

A coincidental finding is the alkaline lability of SL-MMTS. This indicates that substantial care should be taken in the analysis of the rates of protein modification with this reagent if the studies are conducted above neutral pH.

Materials and methods

Materials

The nitroxide from which the synthesis of SL-MMTS was started, 3-carboxy-2,2,5,5-tetramethyl-Δ^3-pyrroline-1-oxyl, was purchased from Aldrich. Other reagents were obtained from commercial sources. [4-^{14}C]ALA (46 μCi/μmol) was purchased from Research Products International.

Synthesis of SL-MMTS

SL-MMTS (Fig. 1A) was prepared essentially as described by Berliner et al. (1982). In a modification of the published protocol, the final product was purified by chromatography on silica gel (60 Å particle size, 70–230 mesh). A 1.7 × 33-cm column was used for a reaction mixture containing 187 mg of the nitroxide. Elution was accomplished using a step gradient of hexane/diethylether mixtures: successive 50-mL portions of solutions ranging from 100% hexane to 50% diethylether/50% hexane, in 10% increments, were used followed by 300 mL of 60% ether/40% hexane. The pure compound was found in fractions 19–31 (~18 mL each) and yielded yellow crystals from diethylether/hexane, with the expected melting point (106–107 °C). The compound gave a single UV-absorbing spot with an R_f of 0.15 on silica thin layer chromatograms developed with 60% diethylether/40% hexane (detected by absorbance at 254 nm).

Isolation of the dimer formed from SL-MMTS

The nitroxide dimer formed from SL-MMTS at alkaline pH values was isolated by HPLC. A 0.2-mL volume of a 0.5-mM solution of SL-MMTS in 50 mM HEPES/KOH, pH 8.5, was incubated for 2 h at 25 °C. The solution was diluted to 1 mL with water and was injected onto a Rainin C-18 HPLC column (4.6 × 250 mm) attached to a Beckman HPLC system. The column was developed isocratically at a flow rate of 0.7 mL/min with 7 mL of 10% isopropanol in water followed by a 21-mL linear gradient to 100% isopropanol. Fluid exiting from the UV detector (229 nm) flowed through ~2 m of teflon tubing (1.8 mm outside diameter, 0.4 mm inside diameter), which passed through the dielectric resonator of an EPR spectrometer. The magnetic field scanning capability of the spectrometer's oscilloscope module was used to repetitively display a 26-G wide spectrum every 28 ms. Therefore, it was possible to observe EPR spectra of compounds as they eluted from the HPLC column. A UV-absorbing compound possessing the characteristic EPR spectrum of the dimer eluted at 21 mL and was collected. Compound(s) with the EPR spectra of monomeric nitroxides eluted as a baseline resolved peak at 18 mL. The dimer was taken to dryness under vacuum and stored at −20 °C where it was stable for at least several months. The concentration of the dimer was determined after reaction with BME and comparison of the EPR signal intensity with a solution of SL-MMTS of a known concentration that had also been reacted with BME. No time-dependent decrease in the intensity of the BME-spin label adduct spectrum was noted, in contrast to the observations of Berliner et al. (1982), who reacted SL-MMTS with dithiothreitol.

Enzyme preparations and assays

Purification of bovine liver PBGS and PBGS activity assays were carried out as previously described (Jaffe et al., 1984). SL-MMTS-modified PBGS was prepared by mixing 28 mg of PBGS (0.8 μmol of subunits, specific activity 24 μmol PBG h^{-1} mg^{-1} at 37 °C) with 21 μmol SL-MMTS in 10 mL 0.1 M KP_i, pH 7, 10 μM $ZnCl_2$, 0.35 mM 2-mercaptoethanol, 1% dimethylformamide (to facilitate dissolving the spin label). The reaction mixture was incubated at 37 °C for 1 h with periodic mixing. Following incubation, SL-MMTS-modified PBGS was purified on an 800-mL G-25 column (4.4 × 48 cm) equilibrated with 0.1 M KP_i, pH 7, at a flow rate of 1.7 mL min^{-1}.

Determination of [4-^{14}C]-ALA binding to MMTS-modified PBGS and SL-MMTS-modified PBGS

The binding of ALA to MMTS-modified PBGS and SL-MMTS-modified PBGS was determined as previously

described by using [4-^{14}C]ALA and Amicon Centrifree ultrafiltration devices, which are designed to separate free from protein-bound ligand (Jaffe et al., 1992).

EPR spectral studies

EPR spectra were recorded on a Varian E-109 spectrometer operating at 9.1 GHz; data acquisition and processing were performed using an IBM AT microcomputer (Dykstra & Markham, 1988). Spectra were digitized at a resolution of 0.2 G/point and the magnetic field was swept at 0.3 G/s. Spectra of solution samples were obtained at 21 °C, using a dielectric resonator in place of a conventional cavity because the resonator provides a substantial increase in signal to noise (Dykstra & Markham, 1986); samples were contained in 0.4-mm inside diameter tubes. Spectra of frozen samples in 4-mm tubes were recorded at −140 °C using a Varian TE-102 cavity. Modulation amplitudes of 1 and 2 G were used for solution and frozen spectra, respectively. In all cases care was taken to avoid power saturation; for frozen samples spectra of allowed transitions were recorded with 1 mW microwave power (10-fold lower power than that giving maximal signal intensity), whereas spectra of the forbidden transitions were recorded with 100 mW microwave power as the latter showed no evidence of saturation at the highest power available. Quantitation of spectral components was obtained by double integration; the estimated accuracy of the double integral is ±20%. Comparison of the areas of forbidden and allowed transitions takes into account the effectively 10-fold higher gain for the forbidden transitions that results from the square root dependence of signal intensity on microwave power in the absence of saturation (Wertz & Bolton, 1986). Due to the variation of the calculated distance with the sixth-root of the relative signal areas, even a twofold error in relative areas would cause only a 12% error in the calculated distances. For determination of the stoichiometry of enzyme modification, areas for BME-treated enzyme were compared with a sample of BME-reacted SL-MMTS of known concentration.

Limited proteolysis and peptide mapping of SL-MMTS-modified PBGS

Following denaturation with 8 M urea, SL-MMTS-modified PBGS (0.25 mg in 1.1 mL 8 M urea, pH 8.5) was treated with 8.7 mg iodoacetate in order to modify the remaining cysteines. The acylated protein was precipitated by the addition of trichloroacetic acid to 25%, and the precipitate was washed with acetone (Matsudiara, 1989). The precipitate was dissolved in 1 mL 8 M urea, diluted to 8 mL with 57 mM sodium phosphate, pH 8, and digested with 2 μg Asp-N protease for 18 h at 37 °C. The resulting peptides were separated by HPLC on a Vydac C-18 protein and peptide column as previously described (Jaffe et al., 1992). The individual peptides were collected manually. Throughout these procedures, BME was scrupulously avoided to ensure that we retained the SL-MMTS modification. All 37 peptide peaks were each dried in vacuo, dissolved in 100 μL water, and analyzed by EPR for nitroxide content. Those peptide peaks that tested positive for spin label were subjected to protein sequencing as previously described (Jaffe et al., 1992). Protein sequencing was carried out by Dr. William R. Abrams in the Protein Analytical Lab of the Research Center for Oral Biology, University of Pennsylvania School of Dental Medicine.

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