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Protective Effects of Covalent Cross-Linking on Proteolysis of Human Coproporphyrinogen Oxidase and Implications for Porphyria

Ahmed W. Jafri, Jason R. Stephenson, Justin B. Morgenthaler,
Jon A. Friesen and Marjorie A. Jones
Department of Chemistry, Illinois State University, Normal, IL 61790-4160

Abstract: The effects of covalent cross-linkers on the enzyme, coproporphyrinogen oxidase, had been previously studied but their role in protecting the enzyme from protease cleavage has not been evaluated. Therefore, we examined how the cross-linker bis (sulfosuccinimidyl) suberate (BS^3) affects the ability of trypsin to digest purified, wild type recombinant human coproporphyrinogen oxidase and selected mutants. Following incubation, the apparent molecular weights of peptides were evaluated by SDS-PAGE and enzymatic activity was assessed by spectroscopy following HPLC. For both wild type and mutants, the results indicated that the cross-linker was indeed able to protect against trypsin digestion relative to the enzyme incubated with trypsin in the absence of the cross-linker. These data have implications for the episodic nature of porphyria.

Key words: Coproporphyrinogen oxidase, BS^3, cross-linking, trypsin, porphyria

INTRODUCTION

Porphyrias, a series of clinical problems involved with heme biosynthesis, have been correlated with specific amino acid changes in the primary protein structure and at least 19 single amino acid changes have been reported[1-4]. However, induced porphyrias, which appear to be triggered by a variety of factors such as lead, mercury synthetic estrogens and progestins, are not well understood[5,6] Although enzymes involved in porphyria have been isolated, characterized and evaluated for their substrate specificity, only a few studies have evaluated the stability of the enzymes relative to protease digestion and three dimensional conformations. Arnould *et al*.[7] reported that acylation stabilized a protease resistant conformation of protoporphyrinogen oxidase. Jaffe and Stith[8] indicated that ALAD porphyria, caused by a deficiency in porphobilinogen synthase activity, was related to formation of low activity hexamer forms rather than the normal high activity octamer quaternary structure assemblies. Herrmann *et al*.[9] indicated that ultraviolet irradiation and uroporphyrin treatment of fibroblasts increased the synthesis of matrix metalloproteinases suggesting a role for these enzymes in the accelerated photoaging in porphyria cutanea tarda patients. Using purified coproporphyrinogen oxidase from bovine liver, Kohno *et al*.[10] reported that trypsin digestion resulted in 7 peptides. The stability of the enzymes of heme biosynthesis is clearly understudied at this time. Here we report the sensitivity of the coproporphyrinogen oxidase human recombinant enzyme to trypsin digestion *in vitro* and the modification of this sensitivity by covalent cross linking.

Heme is the classic example of a functional porphyrin. In the presence of the globin proteins, it forms the hemoglobin complex necessary to transport oxygen in the blood. Coproporphyrinogen oxidase (CO, EC, 1.3.3.3), the sixth enzyme in the heme biosynthetic pathway, catalyzes the sequential oxidative decarboxylation of coproporphyrinogen-III (C-III) to protoporphyrinogen-IX (reviewed by Jones and Lash, 2004[11]). Two porphyrinogen products are sequentially produced by this enzymatic activity: the monovinyl intermediate (harderoporphyrinogen) followed by the divinyl product (protoporphyrinogen-IX). Although there has been controversy in the past, current literature[10,12,13] supports a dimeric active form of mammalian coproporphyrinogen oxidase. The amino acid sequence for this enzyme has been reported for a number of species and the human enzyme has a total of 44 lysine and arginine residues in the primary sequence and there are 42 potential trypsin cleavage sites which can be estimated using ExPASy Peptide Cutter[14] software.

Medlock and Dailey[12] purified recombinant human coproporphyrinogen oxidase and used SDS-PAGE to determine its molecular weight as 37,000 Da. Kohno *et al*. [10]also determined the enzyme to have a molecular mass of 37 kDa using SDS-PAGE.

Corresponding Author: Marjorie A. Jones, Department of Chemistry, Illinois State University, Normal, IL 61790-4160

Stephenson *et al*.[15] showed that when human recombinant coproporphyrinogen oxidase was incubated with selected cross-linkers, higher molecular weight bands of approximately 65, 86 and 107 kDa were detectable by SDS-PAGE relative to the enzyme not incubated with the cross-linkers. This work indicated that two or more monomeric units could be readily cross linked. Although Arnould *et al*.[7] studied the effects of trypsin on protoporphyrinogen oxidase the seventh enzyme in the heme biosynthetic pathway, effects of trypsin specifically on coproporphyrinogen oxidase in the presence of covalent cross-linkers have not been evaluated. We have now extended these studies. Experiments using a selected protein covalent cross-linker were carried out to determine subunit interaction in solution and how they are affected by the protease trypsin. We now report both the apparent weight and the catalytic activity of coproporphyrinogen oxidase with trypsin only, with cross-linker only and then with both cross-linker and trypsin together (with both orders of incubation). Following incubations with trypsin and/or cross-linkers, the catalytic ability of the enzyme was also evaluated. Determination of the apparent molecular weights of the bands on the SDS-PAGE gels was used to evaluate if the enzyme was a monomer, a dimer, a trimer, or even a tetramer. We have also evaluated how trypsin interacts, in the presence of the covalent cross-linker, with two recombinant coproporphyrinogen oxidase mutants, which were generated by site-directed mutagenesis. These mutants R401A and S244A were selected since it has been reported that arginine (R) 401 is directly involved in the active site[3,4,16] and that serine (S) 244 is indirectly implicated in substrate binding[4].

MATERIALS AND METHODS

A bifunctional cross-linker molecule, BS^3 bis-sulfosuccinimidyl- suberate purchased from Pierce and trypsin, a serine protease, purchased from Sigma Chemical Company were used in these studies. BS^3 forms covalent adducts with primary amines of a protein that are within a distance of 11.4Å [15]. Following incubation of highly purified, cloned human enzyme with or without the cross-linker, the reactions were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE[15]. Assessment of enzymatic activity was performed by the micro method of[17]. High Performance Liquid Chromatography HPLC with a Beckman normal phase 4.6 mm × 25 cm 5 μsilica column using a solvent of 35/65, v v^{-1} ethyl acetate/cyclohexane was used to separate substrate and products which were evaluated at A404 nm. The original plasmid pHHCPO for wild type enzyme was a generous gift from Harry Dailey, University of Georgia. For the mutants, site-directed mutagenesis was performed using the PCR-mediated overlap extension method of[18] with Platinum Pfx DNA Polymerase from Invitrogen. This method employed three universal oligonucleotides and a selected mutagenic nucleotide. In each case, the conserved amino acid serine 244 or arginine 401 was changed to alanine thus indicated as S244A or R401A, respectively. Recombinant mutants were screened by the use of the iLAP plate methodology[19]. Once cultures of *E. coli* expressing 6x-his-tagged human recombinant wildtype or mutant enzyme were grown in liter cultures at 37°C, they were then harvested by centrifugation at 4,420 xg for 5 min. The *E. coli* cells were lysed using a French Hydrolytic Press at 20,000 psi followed by centrifugation at 27,000 xg, 4°C, for 30 min. The supernatant was then applied to a nickel affinity column Qiagen in order to bind the 6x-his tagged coproporphyrinogen oxidase which was eluted with 250mM imidazole buffer, pH 7.0 following the method of[12].

BS^3 was freshly prepared in dimethyl sulfoxide DMSO in stock solutions of 100 mM. Trypsin was prepared as stock solutions of 1 mg 1 mL^{-1} in de-ionized water. For each incubation, 0.13 mg of coproporphyrinogen oxidase wild type or mutant was added to each polypropylene micro-centrifuge tube. Once the enzyme in the 250 mL imidazole buffer, pH 7, was added, only the order of addition of the cross-linker and trypsin was varied. The final volumes in each polypropylene 2 mL micro-centrifuge tube were 100 μL. The incubation time at 37°C for the enzyme with or without the BS^3 or the protease was 10 min. After incubation with both BS^3 and protease, 30 μL of the reaction was evaluated for an enzyme activity using the method of Jones *et al*.[17] while 30 μL from each tube was used for SDS-PAGE analysis[15]. The BlueRanger®Prestained Protein Molecular Weight Marker Kit (Pierce) was used as molecular weight markers. For the enzyme assay, 270 μL of 250 mM Imidazole buffer, pH 7 was added followed by addition of freshly prepared coproporphyrinogen-III at a final concentration of 0.5 to 1 μM as a substrate. Reactions were allowed to incubate at 37°C for 5 min before the reaction was stopped by addition of 3/7 v/v acetic acid/ethyl acetate. Following extraction, porphyrins were methyl esterified overnight prior to HPLC analysis[17]. For comparison purposes, enzyme data are

reported as percent product formed[11]. Incubations of enzyme with no BS^3, no trypsin, with neither BS^3 nor trypsin and BS^3 and trypsin with no enzyme were used as controls. The cross-linker did not affect the substrate and the DMSO did not perturb the enzyme data not shown.

RESULTS AND DISCUSSION

To determine the apparent molecular weights of the enzymes via SDS-PAGE, (12%) acrylamide was used[15]. Using the molecular weight markers, the apparent molecular weight of the purified recombinant wild type enzyme was evaluated as 37.5 kDa in Fig. 1a. Incubation of coproporphyrinogen oxidase with only trypsin yielded three new well defined bands at lower molecular weights of 37.1, 28.5 and 9.9 kDa, indicating that the trypsin cleaved select amide bonds of the enzyme to yield smaller peptides in Fig. 1a. The enzyme incubated with only trypsin retained approximately (20%) of the activity detected in the absence of trypsin. Of the total product detected, (22%) was the divinyl product and (19%) was monovinyl intermediate resulting in about a 1:1 ratio of the two products. Wildtype enzyme incubated without trypsin has a much different ratio of product formation with about a 7:1 of divinyl to monovinyl ratio. Incubation of coproporphyrinogen oxidase with the BS^3 cross-linker only yielded three bands at higher molecular weights 126, 79 and 40 kDa with marked band width broadening, clearly implying inter- and intra-molecular cross-linking as in Fig. 1b. It is not surprising that the migration patterns of cross-linked proteins in the gels were affected by the covalent adducts since the proteins were not able to be denatured completely prior to electrophoresis. Since the SDS detergent influences globular structure of proteins, all proteins may not have migrated in the gel only as a function of molecular weight. Hence, it is not possible to determine the exact molecular weights of the cross-linked subunits with this method. Nevertheless, the marked increase in molecular weight relative to enzyme not incubated with covalent cross-linkers clearly indicates subunit interaction. Enzyme incubated in the presence of BS^3 was previously shown to retain approximately (50%) of its activity with the divinyl product being the major product detected[15].

When the wild type coproporphyrinogen oxidase was incubated with BS^3 first followed by addition of trypsin in Fig. 2a, the catalytic activity detected was more than twice compared to experiments with trypsin added first followed by the addition of BS^3 cross-linker in Fig. 3a. This indicates that the cross-linker was

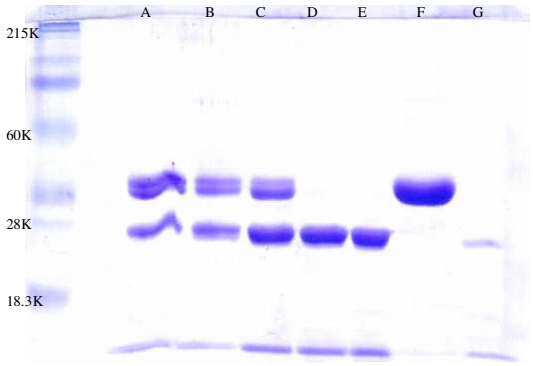

Fig. 1a: SDS-PAGE of trypsin digestion of 0.13 mg wild type C.O. Lane A+ 5 ug trypsin, B: 10 ug trypsin, C: 15 ug trypsin, D: 20 µg trypsin, E: 30 µg trypsin, F: no trypsin. Molecular weight markers are in the far left lane

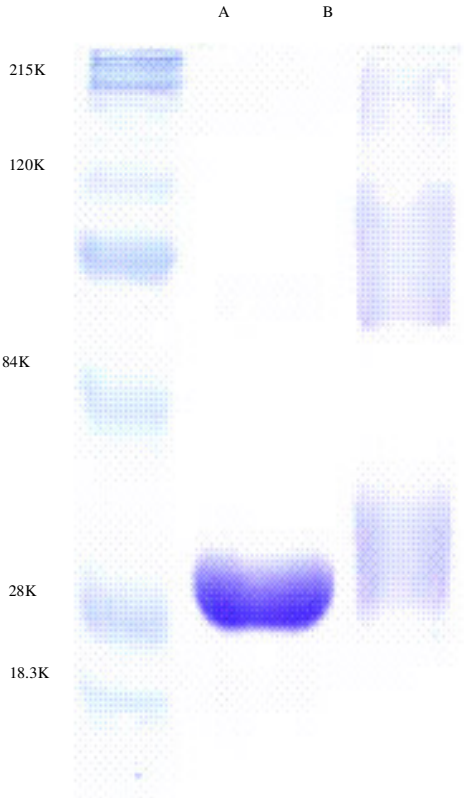

Fig. 1b: SDS-PAGE of BS^3 incubated with coproporphyrinogen oxidase (C.O.) Molecular weight markers are in the far left lane. Lane A, no BS^3, B, with 285 µg BS^3

indeed protecting the enzyme from possible cleavage by trypsin and loss of catalytic activity. The ratios of

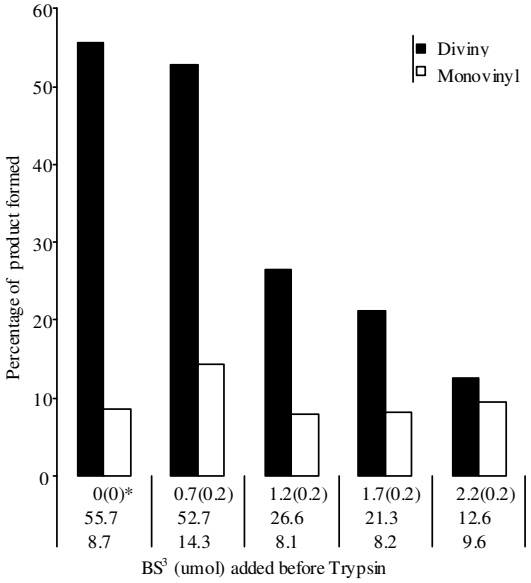

Fig. 2a: Catalytic activity data based on HPLC for wild type coproporphyrinogen oxidase when incubated with BS^3 followed by trypsin. a(b)* where a = µmol BS^3 + µmol of trypsin and b = µmol of trypsin only

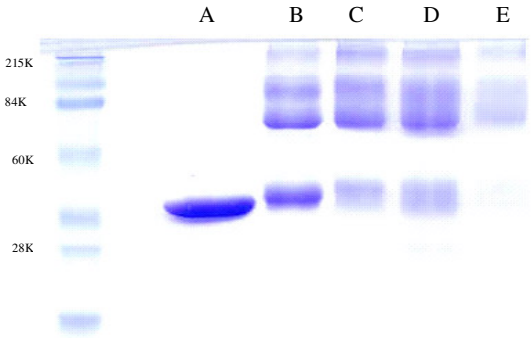

Fig. 2b: SDS-PAGE of wild type coproporphyrinogen oxidase (CO) with BS^3 Added Before trypsin. Lane A: CO only, B: CO with 285 µg BS^3 and 25 µg trypsin, C: CO+570 µg BS^3+25 µg trypsin, D: CO+855 µg BS^3+25 µg trypsin, E: CO+1140 µg BS^3+25 µg trypsin. Molecular weight markers are in the far left lane

the divinyl product to the monovinyl product detected varied with the order in which the BS^3 and trypsin were incubated with the enzyme in Table. 1. When trypsin addition was followed by BS^3 addition, the ratios of divinyl product to the monovinyl product were about two fold higher relative to those for which trypsin was added before BS^3. This confirms that the BS^3 was

Table 1: Percent product formed during incubations with the coproporphyrinogen-III substrate only

Enzyme	Divinyl (%)	Monovinyl (%)	Total product (%)
Wild type	55.7	8.7	64.4
S244A	23.9	9.1	33.0
R401A	27.4	34.2	61.6

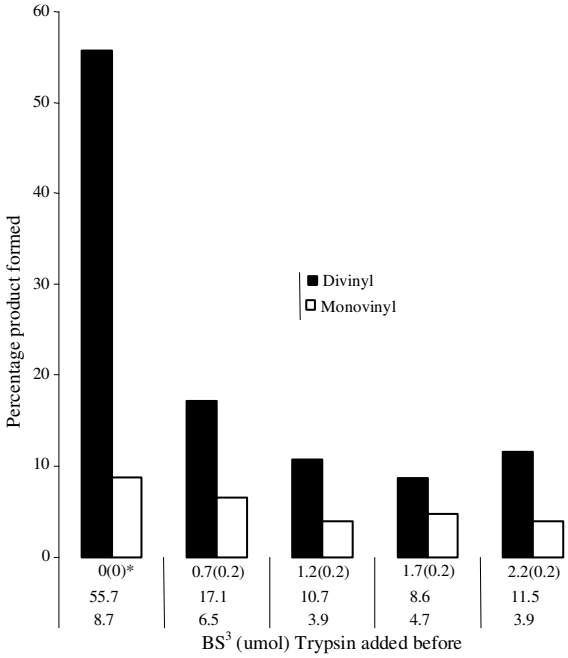

Fig. 3a: Catalytic activity of wild type coproporphyrinogen oxidase when incubated with trypsin followed by incubation with BS^3. a(b)* where a = µmol of trypsin + µmol BS^3 and b = µmol of trypsin only

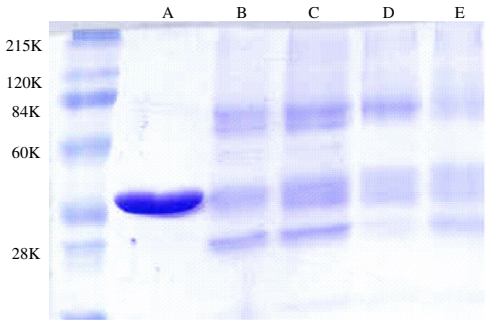

Fig. 3b: SDS-PAGE of trypsin added before BS^3 to wild type coproporphyrinogen oxidase (CO). Lane A: CO Only, B: CO+285 µg BS^3+25 µg trypsin, C: CO+570 µg BS^3+25 µg trypsin, D: CO+855 µg BS^3+25 µg trypsin, E: CO+1140 µg BS^3+25 µg trypsin. Molecular weight markers are in the far left lane

Table 2: Relative proportions of the formation of divinyl product to monovinyl product using coproporphyrinogen-III as substrate with and without BS^3 or Trypsin additions

	Ratio of divinyl/monovinyl product		
Enzyme	No additions	BS^3 before Trypsin	Trypsin before BS^3
Wild type	6.4:1	3.7:1	2.6:1
S244A	2.6:1	0.9:1	0.8:1
R401A	0.8:1	0.3:1	No products detected

protecting the enzyme structure to retain some catalytic ability. We suggest that the protection could be related to cross-linking subunits at interfaces containing primary amines thereby protecting the enzyme from trypsin digestion. SDS-PAGE analysis for incubation of enzyme with BS^3 and then trypsin showed three major bands with apparent molecular weights of 157, 87.3 and 43.3 kDa as in Fig. 2b. These apparent molecular weights are higher than those found following addition of trypsin only and similar to those found using only BS^3. However, when trypsin was added first, four major bands, with apparent weights of 79, 67, 43 and 31 kDa in Fig. 3b, were observed. Thus when BS^3 was added first, it was successful in covalently cross-linking subunits thus reducing trypsin digestion.

Two mutants with single amino acid changes were selected for these studies. The S244A incubated with authentic substrate had a modest amount approx (50%) of catalytic activity relative to wild type enzyme in Table 1 with the divinyl product being detected in higher concentration than the monovinyl intermediate Table 2. The R401A mutant incubated with authentic substrate exhibited approximately the same total catalytic competence as the wild type enzyme although the ratio of the monovinyl and divinyl products was substantially different than formed by the wild type enzyme Table 1 and 2 which yielded a much higher proportion of divinyl product.

When purified recombinant coproporphyrinogen oxidase mutant S244A was evaluated with the same methods as used for wild type enzyme, it was found that the mutant in the presence of both trypsin and cross-linker together was less active by a factor of more than two compared to that of the wild type enzyme in Fig. 4a and 5a. Incubations with the wild type enzyme generated (56%) divinyl product and (9%) monovinyl, whereas the mutant enzyme formed only (24%) divinyl product and (9%) monovinyl product when using coproporphyrinogen III as the substrate Fig. 5a. This indicated that the mutant was less active than the wild type enzyme under any condition tested. Nevertheless, this part of the experiment confirmed that in the presence of BS^3, the mutant catalytic ability was better

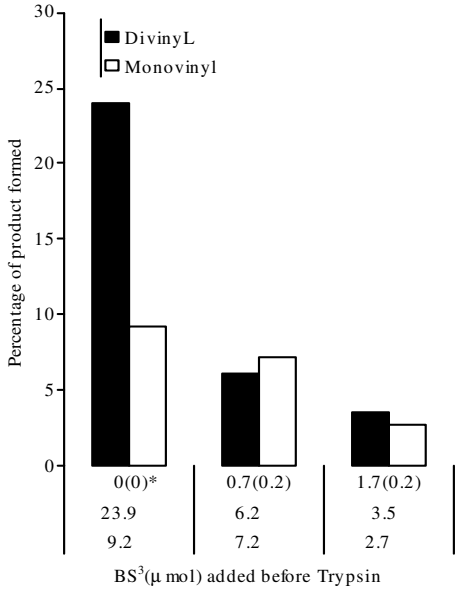

Fig. 4a: Catalytic activity for S244A mutant when incubated with BS^3 followed by trypsin. a(b)* where a = µmol BS^3 + µmol of trypsin and b = µmol of trypsin only

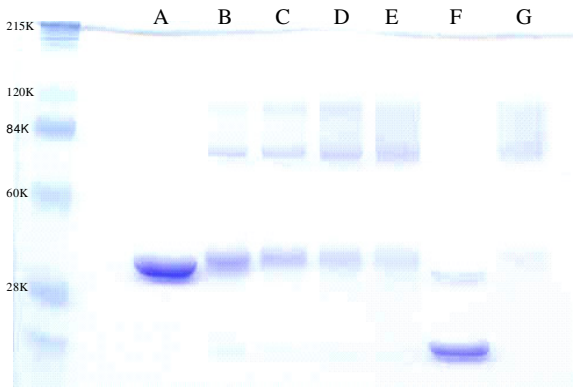

Fig. 4b: SDS-PAGE of BS^3 added before trypsin to S244A mutant. Lane A = S244A only, B = +285 µg BS^3 +25 µg trypsin, C = +570 µg BS^3 +25 µg trypsin, D = +855 µg BS^3 +25 µg trypsin, E = +1140 µg BS^3 +25 µg trypsin, F = +25 µg trypsin, G = +1425 µg BS^3

protected from trypsin digestion as (6%) divinyl product and (7%) monovinyl product were formed while only (3%) divinyl and (3%) monovinyl product were formed under the same conditions and same volumes when BS^3 was added following trypsin digestion in Fig. 4a and 5a. The electrophoresis gels,

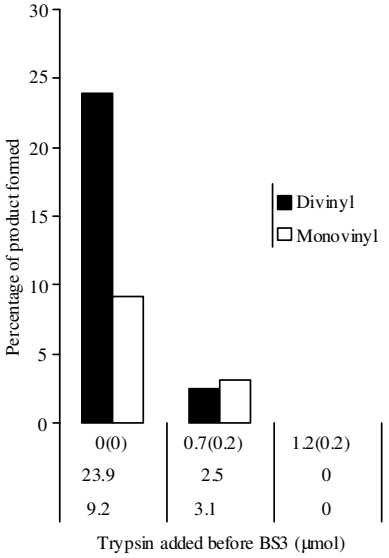

Fig. 5a: Catalytic activity of S244A mutant when incubated with trypsin followed by incubation with BS^3. a(b)* where a = µmol of trypsin + µmol BS^3 and b = µmol of trypsin only

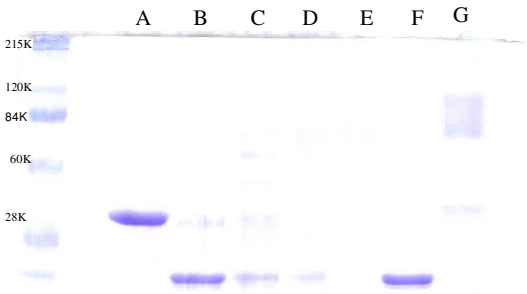

Fig 5b: SDS-PAGE of trypsin added before BS^3 to S244A mutant. Lane A = S244A only, B = +285 µg BS^3 +25 µg trypsin, C = +570 µg BS^3 +25 µg trypsin, D = +855 µg BS^3 +25 µg trypsin, E = +1140 µg BS^3 +25 µg trypsin, F = +25 µg trypsin, G = +1425 µg BS^3

especially when BS^3 was added first, looked nearly identical to those of the wild type enzyme. More specifically, the same numbers of bands were found with similar molecular weights, implying that the S244A mutant had similar 3-dimensional folding as the wild type in Fig. 2b and 4b.

When the mutant R401A enzyme was incubated with BS^3 first followed by trypsin, there were 4 broad bands at 215, 85, 39 and 25 kDa in Fig. 6b. It is interesting that this mutant resulted in the highest molecular weight cross-linked form. When this mutant

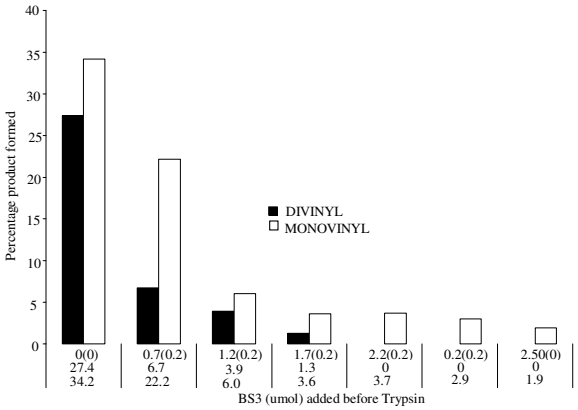

Fig. 6a: Catalytic activity for R401A mutant when incubated with BS^3 followed by trypsin. a(b)* where a = µmol BS^3 +µmol of trypsin and b = µmol of trypsin only

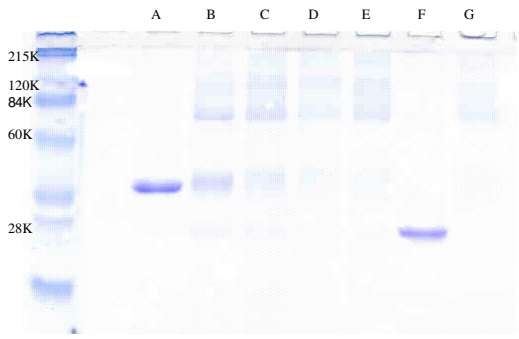

Fig. 6b: SDS-PAGE of BS^3 added before trypsin to R401A mutant CO Lane A = S244A only, B = +285 µg BS^3 +25 µg trypsin, C = +570 µg BS^3 +25 µg trypsin, D = +855 µg BS^3 +25 µg trypsin, E = +1140 µg BS^3 +25 µg trypsin, F = +25 µg trypsin, G = +1425 µg BS^3

was incubated with trypsin first followed by BS^3, the bands were at about the same molecular weights except that the lowest molecular weight band was darker and broader compared to those in which BS^3 was added first in Fig. 7. This appearance of a more prominent band at a lower molecular weight in the incubation with trypsin first clearly indicates that the trypsin cleaved the amide bonds before the cross-linker could link peptides to generate larger complexes. In addition, it was noted that unlike the other mutant and wild type enzyme, the monovinyl product formation by the R401A mutant was predominant relative to the divinyl product when BS^3 was added first indicating that the rate constants for the first and second oxidative decarboxylation have been changed in Fig. 6a and Table 2. More

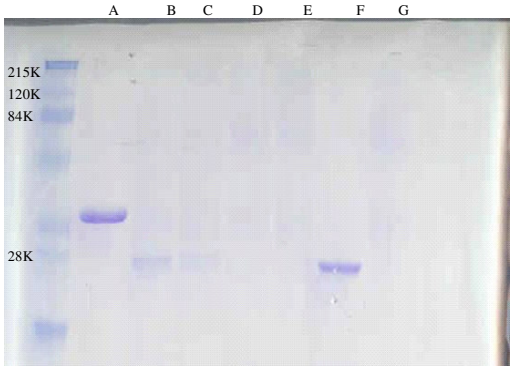

Fig. 7: SDS-PAGE of trypsin added before BS^3 to R401A mutant CO Lane A = S244A only, B = +285 μg BS^3 +25 μg trypsin, C = +570 μg BS^3 +25 μg trypsin, D = +855 μg BS^3 +25 μg trypsin, E = +1140 μg BS^3 +25 μg trypsin, F = +25 μg trypsin, G = +1425 μg BS^3

interestingly, when this mutant was incubated with trypsin first followed by BS^3, HPLC data indicated that there was no catalytic activity at all. The trypsin rendered this mutant enzyme completely non-functional. This also indicates that the 401st amino acid in the polypeptide sequence of coproporphyrinogen oxidase might be a critical for its 3-dimensional structure as well as binding substrate[16].

CONCLUSION

This study provides a systematic study of the stability of coproporphyrinogen oxidase to protease digestion which has been missing from the literature. It also provides more information correlating enzyme conformation with function. While the apparent molecular weight of the wild type enzyme was consistent with literature values[12], the molecular weights of the covalently cross-linked protein were harder to accurately evaluate. The values reported here are similar but not identical to those of[15] which is not too surprising since the width of the bands make the molecular weight estimates difficult and dependent on the conditions used.

It was surprising that trypsin digestion of the enzyme coproporphyrinogen oxidase, in our hands, resulted in only 3 major bands as evidenced by SDS-PAGE. This is in contrast to the report by[10] who reported that enzyme purified from bovine liver treated with trypsin generated at least 7 peptides. However, incubation time and other details were not reported making it difficult to directly compare our experimental procedures. Also, they did not evaluate the enzyme for subsequent catalytic activity following trypsin digestion. Nevertheless, our data strongly suggest that the recombinant wild type human coproporphyrinogen oxidase is fairly resistant to trypsin digestion in spite of the fairly large number of lysine and arginine residues 21 and 23, respectively in the primary sequence of this enzyme which is highly conserved across the phylogenetic tree[12]. Clearly the tertiary structure of the subunit and the interaction of the subunits into the dimer form are masking a number of these potential trypsin digestion sites. Comparing the results for the two mutants with the wild type enzyme, it is apparent that the amide bonds of both of the mutants were more easily accessible to trypsin relative to the wild type enzyme due to different folding patterns. In addition, these single amino acid mutants had reduced catalytic abilities relative to wild type enzyme.

Since both hereditary and induced Coproporphyria and Harderoporphyria are difficult clinical problems[20,21] and we see increases in monovinyl (harderoporphyrinogen) production after trypsin digestion especially for the R401A mutant, these data have implications for an apparent mechanism for the development of episodic porphyria. Proteolytic damage of mutant enzymes that retain some activity before being completely rendered inactive will have the potential to change the normal flux of porphyrins through the heme biosynthetic pathway. For such mutations, accumulation of the monovinyl product could be substantial. As a result, heme synthesis will be disrupted. The condition will manifest itself with the photosensitivity, skin problems and/or neurological complications that accompany porphyria.

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