Glycine reductase selenoprotein A is not a glycoprotein: The positive periodic acid–Schiff reagent test is the result of peptide bond cleavage and carbonyl group generation

(Periodic acid oxidation/glycoprotein analysis)

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ABSTRACT  The complete amino acid sequence of Clostridium sticklandii selenoprotein A, a selenocysteine-containing protein component of the glycine reductase complex, has been established. Both the intact protein and peptide fragments produced by Staphylococcus aureus V8 protease or trypsin were purified by reversed-phase high-performance liquid chromatography and subjected to electrospray ionization mass spectrometric analysis and standard Edman degradation. Selenoprotein A consists of 157 amino acids with a chemical molecular weight of 17,011, in reasonable agreement with the observed molecular weight (17,022.7) determined from its ionization mass spectrum. The sequence of the amino-terminal region of the isolated native protein is Ser-Arg-Phe-Thr-Gly-Lys-Lys-Ile-Val-Ile-Ile-Gly-Asp-Arg-Asp.- An N-terminal methionine residue deduced from the gene sequence was not present. Although selenoprotein A reacted positively in a glycoprotein stain when using either the periodic acid–Schiff reagent procedure or a commercial glycan detection kit, no saccharide was detected by carbohydrate analyses after acid hydrolysis or methanolysis. Identity of the amino acid sequence determined by analysis with that deduced from the gene sequence is further evidence of the absence of bound carbohydrates.

Selenoprotein A is a low molecular weight, acidic, heat-stable protein component of the clostridial glycine reductase complex (1, 2). In earlier studies with pure selenoprotein A isolated from Clostridium sticklandii (3), a strongly positive periodic acid–Schiff reagent test exhibited by protein bands in native acrylamide gels was interpreted as evidence of the presence of bound glycosyl groups. This was further suggested by the detection of reducing substances in acid hydrolysate fractions of the protein. However, a preliminary attempt made subsequently to identify individual neutral or amino sugars in hydrolysates was unsuccessful. Selenoprotein A isolated from Eubacterium acidaminophilum and from Clostridium littoralis by Andreesen and associates (4) also was concluded to be glycosylated based on positive tests with the periodic acid–Schiff reagent, a commercial glycan detection kit, and a thymol–sulfuric acid procedure. Furthermore, certain neutral sugars were stated to be present based on qualitative analysis by gas chromatography, but no data were presented.

Based on recently reported structures of N-linked and O-linked oligosaccharides of some bacterial glycoproteins (5–7) an N-glycosylation consensus sequence [Asn-Xaa-(Thr or Ser)] was identified. The absence of this consensus sequence in the deduced amino acid sequences of selenoprotein A from C. sticklandii (8) and from Clostridium purinolyticum (9) suggested that N-linked glycosidic groups would not be expected. The sequence of an internal selenocysteine-containing

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MATERIALS AND METHODS

Enzyme Purification. Crude acidic protein fractions that had been separated from sonic extracts of C. sticklandii by large-scale DEAE-cellulose chromatography (11) in 1991 and 1993 and stored at −80°C were used as the selenoprotein A source. Bio-Gel P-30 permeation chromatography of this material, ammonium sulfate precipitation, and dialysis using Spectra/Port tubing (3, 11, 12) was followed by rechromatography on DEAE-Trisacyl (LKB) using a linear gradient of 0–0.3 M NaCl in 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM dithiothreitol. Radioactive selenoprotein A isolated from extracts of 75Se-labeled cells as described (1, 13) was added prior to DEAE-Trisacyl chromatography, and fractions were monitored for 75Se with a Beckman 5000 γ counter. Fractions containing a protein peak (280-nm absorbency) coincident with the peak of radioactivity were pooled and further purified by HPLC with a Bio-Gel TSK DEAE-5PW column (7.5 × 75 mm) and a Hewlett–Packard 1090 liquid chromatograph system with a built-in diode array detector (13).

Gel Electrophoresis. SDS/PAGE (14) was performed under reducing conditions with 18% acrylamide gels (Novex, San Diego).

Carbohydrate Analysis. Selenoprotein A (10 nmol) was hydrolyzed with 4.2 M CF3COOH at 100°C for 4 hr. After removal of CF3COOH under a stream of nitrogen, the dried residue was taken up in 100 μl of H2O. Aliquots (2 and 10 μl) were analyzed by high-pH anion-exchange chromatography (HPAEC) using a Dionex Bio-LC system equipped with a pulsed amperometric detector. Isocratic elution with 15 mM NaOH was used to determine monosaccharides in the acid hydrolysate. Carbohydrate analysis after methanolysis (1.5 M methanolic HCl) and derivatization with trimethylsilyl reagent (Pierce) was carried out by gas–liquid chromatography with a Hewlett–Packard GC-MS system (15). The periodic acid–
Schiff reagent was used to treat proteins in gels after SDS/PAGE (16). A glycan detection kit also was used as instructed by the manufacturer (Oxford GlycoSystems, Abingdon, Oxford, U.K.).

Generation and Purification of Peptides. Selenoprotein A was reduced and alkylated with iodoacetic acid prior to enzymic digestion (10). The Se- and S-carboxymethylated protein (2 mg) dissolved in 0.1 M NH₄HCO₃ was digested with *Staphylococcus aureus* V8 protease (Miles; 1:50, wt/wt) at 37°C for 6 hr. For tryptic digestion, a 1-mg sample was incubated with TPCK (1-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma; 1:50, wt/wt) for 6 hr at 37°C. Peptide mixtures were fractionated by HPLC with a Hewlett-Packard 1090 liquid chromatographic system as described in the figure legends.

Amino Acid and N-Terminal Sequence Analyses. Samples were hydrolyzed in 5.7 M HCl containing 0.02% 2-mercaptoethanol under an argon atmosphere at 150°C for 45 min. The amino acids were analyzed in a Hewlett-Packard 1090 liquid chromatographic system after derivatization with phenyl isothiocyanate (PTC), as described by Heinruckson and Meredith (17). The amino acid sequences were determined by automatic Edman degradation using an Applied Biosystems model 477A sequencer or a gas-phase protein sequencer, PSQ-1 (Shimadzu).

RESULTS AND DISCUSSION

After the initial isolation steps outlined in *Materials and Methods*, final purification of selenoprotein A was easily achieved by using a DEAE-5PW HPLC column and the biphasic gradient elution system of NaCl in potassium phosphate buffer (pH 7.2) described earlier (11, 13). The protein in the radioactive peak from this column exhibited the characteristic electronic absorption spectrum previously reported for selenoprotein A (3, 11) and migrated as a single protein band with an apparent molecular mass of about 20 kDa on SDS/polyacrylamide gels under reducing conditions (Fig. 1). This larger apparent molecular mass had been considered previously (3, 8, 11) to be due to both SDS-binding capacity and glycosylation. However, as shown in lane 3 of Fig. 1, migration of the protein was unchanged after incubation in 0.2 M NaOH at 30°C for 16 hr, conditions that easily liberate oligosaccharide in O-glycosidic linkage to serine or threonine residues. However, in agreement with previous reports (3, 4), purified selenoprotein A samples reacted positively in a glycoprotein stain when using the periodic acid–Schiff reagent procedure. Moreover, as shown in lane 4 of Fig. 1, selenoprotein A, after being electroblotted from the SDS gel to a polyvinylidene difluoride membrane, gave a positive reaction with a commercial glycan detection kit. In the case of glycoproteins, carbonyl groups generated by periodate oxidation of carbohydrate react with the basic fuchsin–sulfite complex to give pink-colored protein bands. It was noticed, however, that the positive reaction with selenoprotein A developed much more slowly than that with a reference glycoprotein. When a sample of the protein that had been hydrolyzed with CF₃COOH was analyzed by high-pH anion exchange chromatography as described in *Materials and Methods*, only trace amounts of ubiquitous saccharides were detected (data not shown). Also analysis by GC/MS of protein after methanolysis and treatment with trimethylsilyl reagent failed to show the presence of saccharide except for trace amounts of contaminating glucose or mannose (<0.1 mol/mol of protein). The results of these quantitative analyses further indicated that selenoprotein A is not a glycoprotein.

Amino Acid Sequence and Molecular Mass of Selenoprotein A. Definitive evidence that there are no glycosyl groups

![Fig. 1. SDS/PAGE analysis and glycan detection kit analysis of purified selenoprotein A. Proteins were stained with Coomassie blue. Lanes: 1, molecular weight markers (yeast phosphorylase, 97,000; bovine serum albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; soybean trypsin inhibitor, 20,000; and α-lactalbumin, 14,000); 2, selenoprotein A; 3, alkali-treated selenoprotein A; 4, selenoprotein A (after electrophoretic separation from an SDS/polyacrylamide gel to a polyvinylidene difluoride membrane) treated with a glycan detection kit (Oxford GlycoSystem).](image1)

![Fig. 2. Reversed-phase HPLC of the *S. aureus* V8 protease digest of carboxymethylated selenoprotein A. The V8 digest was chromatographed on a C₁₈ column, and peptides were eluted with a gradient system consisting of 0.1% CF₃COOH (solvent A) and 80% acetonitrile in solvent A (solvent B).](image2)
attached to selenoprotein A came from direct Edman degradation of the N-terminal region of the intact carboxymethylated protein and a series of peptides comprising the remainder of the protein molecule. The N-terminal 15 amino acids that could be determined directly were Ser-Arg-Phe-Thr-Gly-Lys-Lys-Ile-Val-Ile-Gly-Asp-Arg-Asp, and these confirm the deduced amino acid sequence (8). The protein used in this study clearly was not N-blocked, and the N-terminal residue, serine, corresponds to residue 2 of the deduced amino acid sequence. In earlier preparations of the protein that lacked a free N terminus (3), an N-formyl group on the predicted N-terminal methionine residue may have been retained. Peptides generated by treatment of carboxymethylated selenoprotein A with S. aureus V8 protease were separated by reversed-phase HPLC and are designated SP1 to SP15 (Fig. 2). These 15 peptides could be completely sequenced by automated Edman degradation. The N-terminal amino acid of SP-12, corresponding to the N-terminal region of the protein, also was identified as serine rather than methionine. The molecular mass of SP-12 was determined by matrix-assisted laser desorption MS to be 2570.0 (MH+), a value in good agreement with the calculated molecular mass, 2569 (data not shown). The C-terminal amino acid of SP-11 was identified as aspartic acid, which corresponds to the C-terminal amino acid of the protein (Asp-157) originally determined by carboxypeptidase digestion (3) and later deduced from the gene sequence (8). Radioactivity from the 75Se-labeled selenocysteine residue was recovered in SP-15, which spans Cys-40 (carboxymethylated) and Glu-52. In the amino acid sequence analysis system used, phenylthiohydantoin (PTH)-conjugated carboxymethylcysteine was eluted at the same position (4.1 min) as that of PTH-asparagine. PTH-carboxymethylselenocysteine gave two signals presumably due to oxidation; one was at the same position as that of PTH-glutamine and the other was located 6.1 min between PTH-glycine (5.6 min) and PTH-alanine (7.0 min). By direct amino acid analysis, carboxymethylcysteine was detected as a peak at 3.5 min, and carboxymethylselenocysteine was detected as a peak at 3.7 min.

The peptide containing Asn-68 through Glu-79 could not be recovered from the V8 protease digestion mixture fractionated on the C18 column. To isolate this peptide region, carboxymethylated selenoprotein A was digested with trypsin, and the resulting peptides were fractionated by using a C18 reversed-phase HPLC column (Fig. 3). Edman degradation of the N-terminal region of a trypptic peptide, TP-2, identified the sequence to be -Phe-Gly-Ala-Glu-Asn-Leu-Val-Val-Leu-Ile-Gly-Ala-Ala-Glu-Ala-Gly-Leu- Ala-. The preponderance of hydrophobic amino acids in this peptide region could account for the failure to recover the V8 peptide (Asn-68 to Glu-79) from the C18 column because of its strong binding.

The complete amino acid sequence of the C. sticklandii selenoprotein A is shown in Fig. 4. Since all of the serine, threonine, and asparagine residues predicted by the gene sequence were identified, it is clear that none of these were modified by glycosylation. It has been reported (6) that the crystalline surface layer glycoprotein of Clostridium thermohydrodsulfuricum contains oligosaccharide O-linkage to the phenolic hydroxyl group of tyrosine. However, the single

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\begin{align*}
\text{Ser} & - \text{Phe} - \text{Thr} - \text{Gly} - \text{Lys} - \text{Lys} - \text{Ile} - \text{Val} - \text{Ile} - \text{Gly} - \text{Asp} - \text{Arg} - \text{Asp} - \text{Cys} - \text{Phe} - \text{Ser} - \text{Ser} - \text{Thr} - \text{Glu} - \text{Lys} - \text{Pro} - \text{Ile} - \text{Asp} - \text{Cys} - \text{Val} - \text{Sec} - \text{Thr} - \text{Ala} - \\
\text{Gly} & - \text{Val} - \text{Ile} - \text{Gly} - \text{Asp} - \text{Arg} - \text{Asn} - \text{Glu} - \text{Lys} - \text{Ile} - \text{Lys} - \text{Glu} - \text{Ala} - \\
\text{Thr} & - \text{Lys} - \text{Gly} - \text{Asp} - \text{Arg} - \text{Asn} - \text{Leu} - \text{Val} - \text{Leu} - \text{Val} - \text{Ile} - \text{Gly} - \text{Ala} - \\
\text{Ala} & - \text{Glu} - \text{Ala} - \text{Glu} - \text{Thr} - \text{Val} - \text{Glu} - \text{Leu} - \text{Ala} - \text{Thr} - \text{Val} - \text{Glu} - \text{Leu} - \text{Gly} - \\
\text{Gly} & - \text{Asp} - \text{Pro} - \text{Thr} - \text{Phe} - \text{Ala} - \text{Glu} - \text{Asp} - \text{Glu} - \text{Val} - \text{Arg} - \text{Asp} - \text{Val} - \text{Ala} - \\
\text{Leu} & - \text{Arg} - \text{Val} - \text{His} - \text{Val} - \text{Ala} - \text{Glu} - \text{Ly} - \text{Phe} - \text{Lys} - \text{Asp} - \text{Val} - \text{Glu} - \text{Leu} - \text{Gly} - \\
\text{Asp} & - \text{Ala} - \text{Glu} - \text{Ile} - \text{Phe} - \text{Asp} - \text{Glu} - \text{Val} - \text{Gly} - \text{Met} - \text{Met} - \text{Glu} - \text{Met} - \text{Val} - \\
\text{Leu} & - \text{Asn} - \text{Val} - \text{Asp} - \text{Glu} - \text{Ile} - \text{Glu} - \text{Val} - \text{Met} - \text{Glu} - \text{Ser} - \text{Ile} - \text{Arg} - \text{Ser} - \\
\text{Gln} & - \text{Phe} - \text{Cys} - \text{Lys} - \text{Glu} - \text{Asp} - \text{Asp} -
\end{align*}
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FIG. 3. Reversed-phase HPLC of the trypptic digest of carboxymethylated selenoprotein A. The trypptic digest was chromatographed on a C4 column, and peptides were eluted with a gradient system consisting of 0.1% CF3COOH (solvent A) and a 5:3:2 (vol/vol) mixture of 2-propanol/acetonitrile/solvent A (solvent B).

FIG. 4. Complete amino acid sequence of C. sticklandii selenoprotein A. Arrowheads indicate residues determined by Edman degradation. Sec, selenocysteine; CMS, carboxymethylated selenoprotein A; SP, S. aureus V8 peptides of CMS; TP, trypptic peptides of CMS.
tyrosine residue in selenoprotein A also was identified, thus eliminating this as an additional possible glycosylation site.

Further proof of the absence of group(s) resulting from posttranslational modification was furnished by electrospray ionization mass spectrometric analysis of intact selenoprotein A. The molecular mass (17,011) calculated from the amino acid sequence determined in this study is consistent with the observed mass (17,022.7) found by electrospray ionization mass spectrometric analysis (Fig. 5). A significantly higher molecular mass of 17,142 should have been observed if the N-terminal methionine residue (+131) had been retained on the protein. These results together with the amino acid se-
quence data clearly show the absence of glycosyl groups on the selenoprotein A preparation examined in the present study. The N-terminal sequence data clearly show that the N-blocked selenoprotein A preparation studied earlier retained the usual N-formylmethionine initiation residue, since cleavage of the formyl group is incomplete under some conditions of growth. The larger apparent molecular mass of selenoprotein A reported here and observed in other studies (3, 8, 11), based on migration in SDS gels, could be due entirely to its SDS-binding capacity.

The results of these studies also refute the earlier view that the presence of glycosyl groups on selenoprotein A might facilitate interaction with proteins B and C for assembly of the glycin reductase complex. In view of the significant hydrophobic character of certain regions of the protein molecule, particularly in the segment encompassed by Val-70 to Ala-90 as evidenced by hydropathy analysis (ref. 18; data not shown), this property may be the important determinant for interaction of the selenoprotein with the membrane-associated hydrophobic proteins B and C.

**Periodic Acid Oxidation of Selenoprotein A.** Since glycosyl groups on selenoprotein A are excluded as the basis of the positive periodic acid–Schiff reagent test, the possibility was considered that carbonyl groups were generated by oxidation of the protein itself. Accordingly, the protein dissolved in 10% (vol/vol) acetic acid was treated with 0.5% periodic acid in the dark at room temperature for 2 hr. After separation of the protein from the reaction mixture by gel filtration on Bio-Gel P-4, the oxidized protein was subjected to matrix-assisted laser desorption MS. As shown in Fig. 6, two fragments of molecular mass ca. 4 kDa (peak I) and ca. 13 kDa (peak II) were found. After reaction of the oxidized protein with 2,4-dinitrophenylhydrazine (19) followed by separation of the fragments by gel filtration, the 4-kDa fragment exhibited an electronic absorption spectrum typical of a 2,4-dinitrophenylhydrazone with an absorption maximum at 375 nm (data not shown). The 375-nm peak was not present in the spectrum of the 13-kDa fragment. These preliminary data suggest that periodate oxidation of selenoprotein A causes peptide bond cleavage and carbonyl group generation, thus providing an explanation of the positive tests observed with the glycan test kit and the periodic acid–Schiff reagent. Cleavage in the region of the -Asp-Asp-Gln- sequence (residues 126–128) would be predicted if the smaller fragment containing the carbonyl group function originates from the C-terminal end of the protein. Periodate oxidation of 75-Se-labeled protein together with end-group analysis of the two peptide fragments generated should furnish additional information needed to fully explain the false-positive glycoprotein test given by selenoprotein A.

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