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Selenoprotein A of the clostridial glycine reductase complex: Purification and amino acid sequence of the selenocysteine-containing peptide

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ABSTRACT **A selenium-containing protein, selenoprotein A, is an essential component of the clostridial glycine reductase complex. This enzyme complex catalyzes the reductive deamination of glycine, which is coupled to the esterification of orthophosphate resulting in the formation of ATP. Sequence information was obtained by automated Edman degradation of peptides generated by digesting carboxamidomethylated selenoprotein A with chymotrypsin or trypsin or with endoproteinase Arg-C followed by *Staphylococcus aureus* V8 protease. The sequence near the selenocysteine (Sec) residue is -Cys-Phe-Val-Sec-Thr-Ala-Ala-Gly-Ala-Met-Asp-Leu-Glu-Asn-Glu-Lys-. Selenium-containing peptides isolated from digests of carboxamidomethylated selenoprotein A with trypsin or endoproteinase Arg-C were found to be blocked at the amino terminus. The sequence of the selenocysteine-containing peptide from selenoprotein A shows no homology with those of two other selenoproteins, glutathione peroxidase and formate dehydrogenase.**

The catalytic activity of the clostridial glycine reductase complex depends on the presence of a selenium-containing protein component, selenoprotein A (1). This enzyme complex catalyzes the reductive deamination of glycine to ammonia and acetate, which is coupled to the esterification of orthophosphate resulting in the formation of ATP (2). Selenoprotein A contains selenium at a single selenocysteine residue (3) within the polypeptide chain. Previous studies have shown that the amino terminus of selenoprotein A is blocked (4). The presence of this blocked amino terminus and its proximity to the selenocysteine residue have made sequence analysis of this protein difficult. In this paper, a partial sequence of the selenium-containing peptide is reported. This sequence should permit the synthesis of oligonucleotide probes that might be used in the molecular cloning of the selenoprotein A gene.

MATERIALS AND METHODS

Purification of Selenoprotein A. Selenoprotein A was purified from *Clostridium sticklandii* grown in the presence of [^{75}Se]selenite as described (5). The peak fractions from the DEAE HPLC step were carboxamidomethylated and subjected to proteolytic digestion.

Carboxamidomethylation of Selenoprotein A. Selenoprotein A was alkylated with iodo[1-^{14}C]acetamide (NEN) by a modification of the procedures of Cone *et al.* (3) and Günzler *et al.* (6). To 3 mg of [^{75}Se]selenoprotein A (≈0.3 μCi/mg; 1 μCi = 37 kBq) in 3.0 ml of H_2O was added 2.1 g of solid guanidine hydrochloride and 0.5 ml of 1 M Tris·HCl buffer (pH 8.0). The resulting solution (5.0 ml) was maintained under nitrogen for ≈30 min at room temperature. Several grains of KBH_4 were then slowly added, and the solution was maintained in the dark under nitrogen. Additional borohydride was added at 10 and 20 min. After 30 min of reduction, 170 μl of acetone was added to the solution, followed by 50 μCi of iodo[1-^{14}C]acetamide in 50 μl of 0.1 M NaOH. The iodo[1-^{14}C]acetamide vial was rinsed with 280 μl of 0.1 M NaOH containing 9.25 mg of iodoacetamide and the rinse was added to the protein mixture. The alkylation reaction was allowed to proceed for 20 min in the dark under a gentle stream of nitrogen and was quenched with 0.5 ml of 2-mercaptoethanol. The solution was then dialyzed versus H_2O.

Amino Acid Analysis. Amino acid analysis using precolumn derivatization with either *o*-phthaldialdehyde (7) or phenylisothiocyanate was found to be superior to cation-exchange chromatography for the simultaneous resolution of carboxymethylcysteine, carboxymethylselenocysteine, and the 16 other amino acids resulting from acid hydrolysis of proteins. In both cases, the derivatives of carboxymethylcysteine and carboxymethylselenocysteine are eluted early in the chromatogram between the derivatives of glutamate and serine, in a flat baseline area that permits accurate identification and quantitation. The procedures for hydrolysis and derivatization were essentially as described by Bidlingmeyer *et al.* (8) with the following modifications. For hydrolysis, 20 μl of 5.7 M HCl was added to each tube as well as to the bottom of the reaction vial. Hydrolyses were carried out at 155°C for 40 min.

Chromatography of the phenylthiocarbamoyl derivatives was performed by a modification of the general procedure described by Heinrickson and Meredith (9). A Hewlett–Packard 1090 liquid chromatograph with a diode-array detector and a 10-cm IBM C_{18} column (catalogue no. 8635789), 3-μm particle diameter, was used for analysis. Solvent A was 200 mM ammonium acetate adjusted to pH 6.0 with H_3PO_4. Solvent B was H_2O and solvent C was acetonitrile. Signal detection was at 247 nm (10-nm bandwidth), with the signal at 350 nm (100-nm bandwidth) as reference. The column was equilibrated with 25% A/74.5% B/0.5% C at 52°C. The following biphasic linear gradient system was used: from 0 to 9 min, linear gradient to 27.5% A/67.5% B/5% C; from 9 to 20 min, linear gradient to 37.5% A/37.5% B/25% C; regeneration was accomplished by a linear gradient from 20 to 25.5 min to 50% B, 50% C followed by a gradient to 100% C over the next 3 min, followed by 2 min at 100% B; reequilibration occurs in 8 min under initial solvent conditions. For optimal recovery of carboxymethylselenocysteine, the reconstituted phenylthiocarbamoyl amino

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Abbreviations: TLCK, 7-amino-1-chloro-3-tosylamido-2-heptanone ("tosyllysine chloromethyl ketone"); Sec, selenocysteine.

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acid derivatives were treated with a few grains of KBH_4 prior to analysis.

Chymotryptic Digestion of Selenoprotein A and Purification of Selenocysteine-Containing Peptide. Carboxamidomethylated selenoprotein A was digested at 3 mg/ml in 0.2 M Tris·HCl buffer (pH 8.0) with 60 μg of 7-amino-1-chloro-3-tosylamido-2-heptanone-treated chymotrypsin (TLCK-chymotrypsin, Sigma). The digestion was carried out at 32°C for 12 hr, after which an additional 15 μg of chymotrypsin was added and the incubation continued for another hour. The reaction was stopped with 20 μl of 6 M HCl, and then 0.5 ml of 7 M guanidine hydrochloride adjusted to pH 2.2 with trifluoroacetic acid was added to maintain solubility.

Peptides were resolved by reverse-phase HPLC using a Vydac 218TP54 C_{18} column (The Separations Group, Hesperia, CA). The column was equilibrated with 0.05% trifluoroacetic acid and elution was achieved with an acetonitrile/0.05% trifluoroacetic acid gradient. The major ^{75}Se peak, eluted at 23.3 min, was dried in a Speed-Vac (Savant) vacuum concentrator and redissolved in 0.02 M sodium phosphate buffer (pH 3.0) containing 30% acetonitrile. This fraction was then applied to a sulfopropyl HPLC column (SP-5PW, Bio-Rad) that had been equilibrated in 0.02 M sodium phosphate, pH 3.0/30% acetonitrile. The ^{75}Se-containing peak was again dried and then desalted on a C_{18} column.

Endoproteinase Arg-C Digestion and Peptide Purification. The conditions for the digestion of carboxamidomethylated selenoprotein A with endoproteinase Arg-C (Boehringer Mannheim) were essentially the same as those used with chymotrypsin, except that the reaction was not quenched with HCl nor was guanidine hydrochloride added. Instead, the resulting peptides were diluted with 5 volumes of H_2O and chromatographed on a DEAE HPLC column (DEAE-5PW, Bio-Rad) equilibrated in 0.02 M sodium phosphate buffer (pH 7.0) containing 30% acetonitrile.

***Staphylococcus aureus* V8 Protease Digestion and Peptide Purification.** The selenocysteine-containing peptide from the endoproteinase Arg-C digestion of selenoprotein A was subsequently digested with *S. aureus* V8 protease (Miles). The dried selenocysteine-containing peptide (300 nmol) was dissolved in 0.5 ml of 0.5 M Tris·HCl (pH 8.0). The digestion was initiated by the addition of 15 μl of *S. aureus* V8 protease (1 mg/ml). The reaction was allowed to proceed at 32°C for 4 hr and was stopped by the addition of 0.5 ml of 6 M guanidine hydrochloride containing 1% trifluoroacetic acid. The pH of the solution was adjusted to pH 2.0 with HCl and the peptides were separated on a Vydac C_{18} column (10 cm), using an acetonitrile/0.05% trifluoroacetic acid gradient system. The first ^{75}Se-containing peptide (23 min) was rechromatographed by loading in 10% acetonitrile/0.05% trifluoroacetic acid and elution with an acetonitrile gradient developed at 0.5%/min over 40 min.

Sequencing. N-terminal amino acid sequencing was performed by A. Smith at the protein-sequencing facility of the University of California at Davis. In the initial experiments, peptides were sequenced using a Beckman 890M spinning-cup sequencer. In later work, an Applied Biosystems (Foster City, CA) vapor-phase sequencer was used.

Radioactivity Measurements. Enzyme preparations and fractions collected from HPLC columns were monitored for ^{75}Se with a Beckman 5000 gamma counter. The ^{14}C content of samples was measured in Aquasol (New England Nuclear) by liquid scintillation spectrometry with an LKB Rack Beta scintillation counter. Radioactivity due to ^{14}C was calculated from the difference between liquid scintillation counts and gamma counts after relative efficiency corrections were made. A 50-μl aliquot of each 1-ml (1-min) fraction was used for radioactivity measurement.

RESULTS

A single selenium-containing peptide was obtained from a digest of carboxamidomethylated selenoprotein A with TLCK-chymotrypsin. The initial purification of this peptide was achieved by reverse-phase HPLC (Fig. 1*A*). As seen from the A_{215} signal, the major ^{75}Se-containing peptide, eluted at 23.5 min, was a mixture of at least two unresolved peptides. Efforts to purify this peptide to homogeneity by other reverse-phase methods (including the use of shallower gradient conditions, different organic solvents, alternative ion-pair reagents, and alternative stationary phases) failed. The peptide was, however, successfully purified by cation-exchange chromatography on a sulfopropyl HPLC matrix (Fig. 1*B*). In this chromatographic system, 30% acetonitrile was kept in the mobile phase to ensure solubility of the peptide. The purified peptide, eluted at 4 min, was diluted and applied to a C_{18} column for desalting prior to compositional and sequence analysis. The composition of this octapeptide is shown in Table 1. Upon N-terminal sequence analysis, the peptide was found to have the sequence Val-Sec-Thr-Ala-Ala-Gly-Ala-Met (where Sec is selenocys-

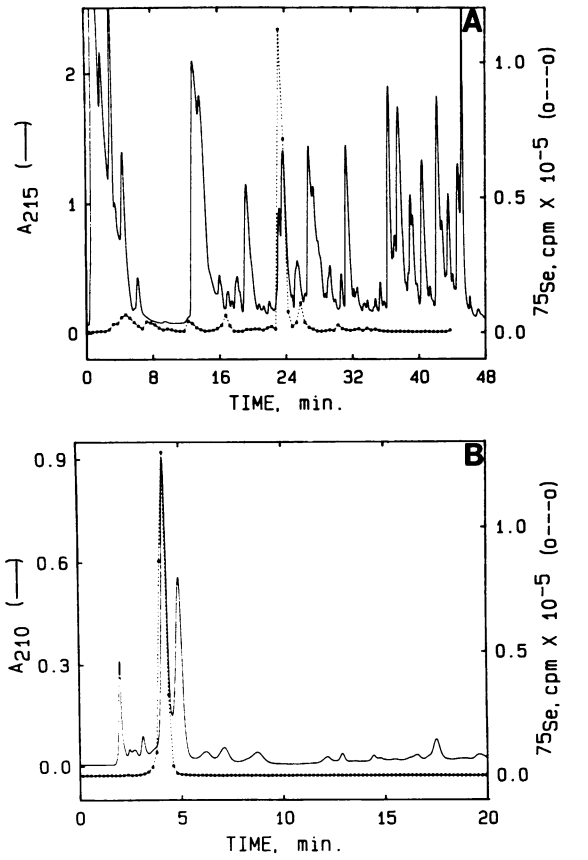

FIG. 1. (*A*) Reverse-phase HPLC of a chymotryptic digest of carboxamidomethylated [^{75}Se]selenoprotein A. The digest was chromatographed on a C_{18} column (Vydac 218TP54) equilibrated with 0.05% trifluoroacetic acid (solvent A). Peptides were eluted with an acetonitrile/0.05% trifluoroacetic acid (solvent B) gradient as follows: 0–5 min, 100% A; 5–25 min, gradient to 15% B; 25–40 min, gradient to 30% B; 40–45 min, gradient to 100% B. (*B*) Sulfopropyl HPLC of ^{75}Se-labeled chymotryptic peptide from carboxamidomethylated selenoprotein A. The peak ^{75}Se fraction from the reverse-phase C_{18} column (eluted at 23.5 minutes) was applied to an SP-5PW column (Bio-Rad) equilibrated in 0.02 M sodium phosphate, pH 3.0/30% acetonitrile. The column was washed for 3 min at 1 ml/min with the equilibration buffer, and retained material was eluted with a linear gradient over 25 min to 50% B (where B is 0.5 M sodium phosphate, pH 3.0/30% acetonitrile).

Table 1. Amino acid composition of chymotryptic peptide from carboxamidomethylated selenoprotein A

Amino acid	Observed, nmol	Normalized*
Gly	1.8	1.4
Thr	1.3	1.0
Ala	5.0	3.8
Val	1.3	1.0
Met	1.4	1.1
Sec	—	(1)†

*Normalization was made relative to valine.
†Carboxymethylselenocysteine was estimated by ^{75}Se radioactivity.

teine). As expected, >80% of the ^{75}Se and ^{14}C radioactivity was recovered in the second degradation cycle.

When carboxamidomethylated selenoprotein A was digested with endoproteinase Arg-C, a single major selenium-containing peptide was obtained. This peptide was purified by chromatography on a DEAE HPLC column (Fig. 2*A*) by salt elution in the presence of acetonitrile. Amino acid analysis of this peptide indicated that the peptide was rather large, containing 36–41 residues. Sequence analysis of ≈25 nmol of this peptide yielded an unexpectedly low signal at

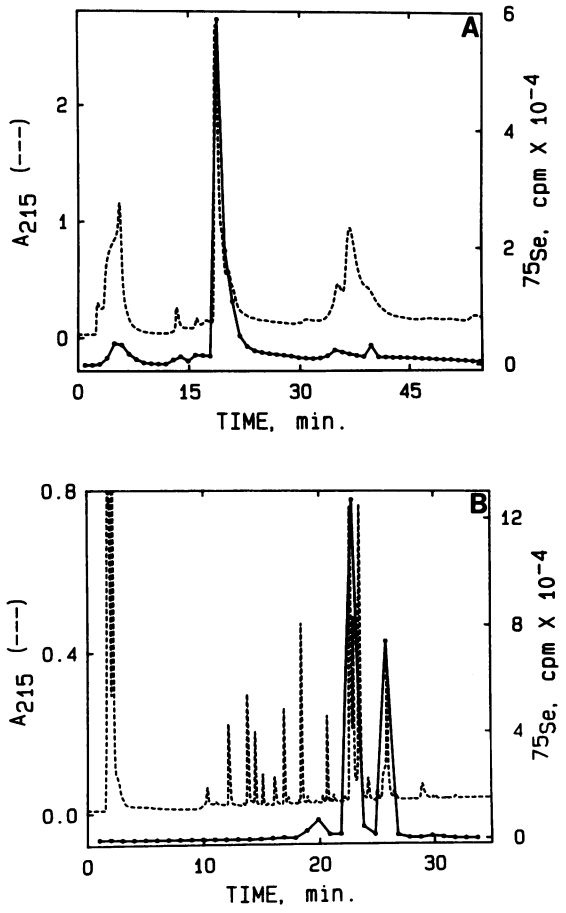

FIG. 2. (*A*) DEAE HPLC of ^{75}Se-labeled endoproteinase Arg-C peptide from carboxamidomethylated selenoprotein A. The column (DEAE-5PW) was equilibrated in 0.02 M sodium phosphate buffer, pH 7.0/30% acetonitrile. The column was washed for 10 min at 1 ml/min with the equilibration buffer and then retained material was eluted with a linear gradient to 100% B (where B is 0.4 M sodium phosphate, pH 7.0/30% acetonitrile). (*B*) Reverse-phase HPLC of ^{75}Se-labeled *S. aureus* V8 digest of endoproteinase Arg-C peptide (eluted at 19 minutes) described in *A*. Peptides were separated on a 10-cm C_{18} column (Vydac) equilibrated with 0.05% trifluoroacetic acid. After a 5-min wash, peptides were eluted with a linear gradient to 70% acetonitrile/0.05% trifluoroacetic acid in 45 min.

the 2-nmol level, which probably resulted from a contaminating peptide. This endoproteinase Arg-C peptide most likely contains the amino terminus of selenoprotein A, which previously was shown to be blocked (4).

When the endoproteinase Arg-C peptide was digested with *S. aureus* V8 protease, two ^{75}Se-containing peptides were obtained that could be resolved by reverse-phase chromatography on a C_{18} column (Fig. 2*B*). Amino acid analysis (Table 2) revealed similarity between the two peptides, with peptide II containing five or six more amino acids than peptide I. Upon sequence analysis peptide I was found to have the sequence Cys-Phe-Val-Sec-Thr-Ala-Ala-Gly-Ala-Met-Asp-Leu-Glu. Cycle 4 contained 79% of the ^{75}Se counts, whereas cycle 1 and cycle 4 contained 12% and 74%, respectively, of the ^{14}C counts. This difference in ^{14}C labeling is due to the greater reactivity of selenocysteine as compared to cysteine with the alkylating agent under the conditions employed. Peptide II was found to have a blocked amino terminus. It was concluded that peptide II was an extension of peptide I with the following extra amino acids: 2 Ala, Leu, Asx, Glx, and possibly Met. Since V8 protease will occasionally cleave at aspartic residues (10, 11), it is likely that aspartate precedes cysteine in the sequence and that the Glx residue is a glutamine.

Further efforts to obtain additional sequence on the amino-terminal side of the selenocysteine residue were not successful. When CNBr cleavage of carboxamidomethylated selenoprotein A was attempted, ^{75}Se radioactivity did not appear to correspond to any particular peptide in the chromatogram. It appears that the CNBr may cause oxidation and/or elimination of selenium within the protein. Interestingly, when carboxamidomethylated selenoprotein A was digested with trypsin, which was not L-1-tosylamido 2-phenylethyl chloromethyl ketone (TPCK)-treated, two selenium-containing peptides were obtained (data not shown). On reverse-phase chromatography, the earlier eluted peptide, containing ≈20% of the total ^{75}Se radioactivity, was found to have the sequence Val-Sec-Thr-Ala-Ala-Gly-Ala-Met-Asp-Leu-Glu-Asn-Gln-Lys. This peptide has the same amino terminus as the chymotryptic peptide described above and probably resulted from cleavage of the Phe-Val bond by trace amounts of chymotrypsin in this particular trypsin preparation.

Table 2. Amino acid composition of V8 peptides from carboxamidomethylated selenoprotein A

	Peptide I		Peptide II	
Amino acid	Observed, nmol	Normalized*	Observed, nmol	Normalized†
Asx	1.00	1.1	1.19	1.9
Glx	0.84	0.9	1.30	2.1
Cys‡	0.39	0.4	0.30	0.5
Sec‡	0.36	0.4	0.25	0.4
Gly	0.95	1.0	0.84	1.3
Thr	0.73	0.8	0.52	0.8
Ala	2.97	3.2	3.31	5.3
Val	0.86	0.9	0.75	1.2
Met	1.30	1.4	1.08	1.7
Leu	0.96	1.0	0.95	1.5
Phe	0.45	0.5	0.36	0.6

Peptides I and II correspond to peaks at 23.3 and 26.3 min, respectively, as shown in Fig. 2*B*.
*Normalization was made relative to the average values of Asx and Glx.
†Normalization was made relative to twice the average values of Asx and Glx.
‡Cysteine and selenocysteine were quantitated as the carboxymethyl derivatives.

DISCUSSION

The precise function of selenoprotein A in the reaction catalyzed by the glycine reductase complex has not been elucidated. It has been suggested that the selenium in this protein serves as a redox center, since a selenol group is generated upon reaction with dithiothreitol, the electron donor for the *in vitro* catalytic reaction (1, 12). As shown in the present work, the proximity of a cysteine residue to the selenocysteine site suggests that together these residues could serve as a redox center. The previously demonstrated susceptibility to oxidation in air of both the selenol and the two sulfhydryl groups in protein A (4) is in accord with this view. In the selenoprotein subunit of an *Escherichia coli* formate dehydrogenase (13), a pair of cysteines is located near the selenocysteine residue. Thus, the formation and subsequent reduction of a unique seleno-sulfide bond may be a common feature in the reactions catalyzed by these proteins.

In addition to selenoprotein A, sequence information also exists for two other selenocysteine-containing proteins, glutathione peroxidase (6, 14, 15) and formate dehydrogenase (13). In both of these proteins, the UGA termination codon corresponds to the selenocysteine residue. Specific selenium incorporation into proteins appears to occur by either a pretranslational or cotranslational mechanism. A pretranslational mechanism would be similar to the manner in which certain Gram-positive bacteria synthesize glutamine (16). In these organisms, the $tRNA^{Gln}$ is initially charged with glutamate. The glutamyl-$tRNA^{Gln}$ then undergoes amidation, forming glutaminyl-$tRNA^{Gln}$, which is used for protein synthesis. An analogous series of reactions occurs in the formation of formylmethionine (17, 18). A similar mechanism has been considered for the selenylation of *E. coli* formate dehydrogenase (13). A suppressor tRNA that recognizes the UGA termination codon could be directly charged with selenocysteine or charged with a precursor amino acid, which is then converted to selenocysteine.

In studies on the expression of β-galactosidase fused to truncated forms of formate dehydrogenase, selenium is required for synthesis of β-galactosidase only when the UGA codon is present upstream from the fusion site (13). Immunological studies with selenoprotein A (5) and glutathione peroxidase (19) also have linked the lack of biologically available selenium to the cessation of protein synthesis. It appears, then, that the level of a selenium-containing metabolite functions in the regulation of the expression of these proteins. The ability to clone and express high levels of these proteins may facilitate the elucidation of the molecular mechanism(s) by which selenium is incorporated as selenocysteine residues. Additionally, through the use of site-directed mutagenesis, it should be possible to examine the function of the selenocysteine residue in the reactions catalyzed by these enzymes.

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