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Biochemistry

Chemical characterization of the selenoprotein component of clostridial glycine reductase: Identification of selenocysteine as the organoselenium moiety

(selenoprotein chemistry/carboxymethylselenocysteine/aminoethyl selenocysteine)

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ABSTRACT **A small, heat-stable selenoprotein, one of the components of the glycine reductase complex, was labeled with ^{75}Se by growth of *Clostridium sticklandii* in the presence of $Na_2{}^{75}SeO_3$. The selenium-containing moiety, which is essential for the biological activity of the protein, was shown to be a selenocysteine residue. It was isolated as its Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives from digests of the pure ^{75}Se-labeled protein that had been reduced and treated with the various alkylating agents prior to hydrolysis. In each instance the ^{75}Se-labeled moiety obtained from an alkylated protein sample and the corresponding alkyl derivative of authentic selenocysteine were indistinguishable.**

Several studies of the native selenoprotein detected a chromophore (UV_{max} 238 nm) that appeared upon reduction of the protein with KBH_4 and rapidly disappeared upon exposure to oxygen. This oxygen-labile chromophore is thought to be the ionized -SeH group of the selenocysteine residue.

An acidic, heat-stable protein of molecular weight about 12,000 is one of the components of the enzyme system that catalyzes the reductive deamination of glycine (1, 2). The biological activity of this protein depends on the presence of 1 g-atom of covalently bound selenium per mol of protein (2, 3). Selenium also is known to be essential for the biological activity of mammalian (4) and avian (5) glutathione peroxidase and formate dehydrogenase of *Escherichia coli* (6–8) and various anaerobic bacteria (3, 9, 10). The selenium-containing moieties of these two enzymes have not been identified. In this communication evidence is presented that the organoselenium moiety of the clostridial glycine reductase selenoprotein is a selenocysteine residue. We have thus identified an essential selenium-containing residue in a protein.

MATERIALS AND METHODS

The selenoprotein of the glycine reductase system was isolated from sonic extracts of *Clostridium sticklandii* (1, 2). Chromatography on Affi-Gel 501, a mercuribenzoate-agarose preparation supplied by Bio-Rad Laboratories, was used as a final isolation step for some preparations that were otherwise difficult to free of the last traces of impurities.* Cells grown in the presence of 1 μM $Na_2{}^{75}SeO_3$ and 2 mM Na_2S were the source of the ^{75}Se-labeled protein (2). The yeast extract and tryptone in the culture medium supplied additional sulfur, and the resultant high ratio of sulfur to selenium suppressed nonspecific substitution of the latter for sulfur in the proteins and other constituents of the cell.

Other reagents were purchased as follows: carboxypeptidase A-diisopropylfluorophosphate and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-trypsin, Worthington Biochemical Corp.; selenocystine, selenocystamine, and selenomethionine, Sigma Chemical Co.; *Se*-methylselenocysteine and *S*-aminoethylcysteine, Cyclo Chemical Co.; ethyleneimine, Pierce Chemical Co.; iodo[*1*-^{14}C]acetamide, New England Nuclear Corp.; and $Na_2{}^{75}SeO_3$, Amersham-Searle.

A Beckman model 121 amino acid analyzer was used as an analytical instrument and, also, for preparative scale procedures. For the latter, samples were applied directly to the top of the appropriate column; the eluate was diverted and collected in 1-min samples on a fraction collector. In both instances standard sodium citrate buffers supplied by Beckman Instruments, Inc. were used. The buffers at pH 3.25 and pH 4.25 contained thiodiglycol.

Selenocystine was reduced to selenocysteine with KBH_4, and the Se-carboxamidomethyl, Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives of the selenol were prepared by procedures similar to those used for synthesis of the corresponding S-alkyl derivatives of cysteine (11). On cellulose thin-layer sheets these derivatives migrated with the following R_F values: in 2-propanol/HCOOH/H_2O (60:3:15) *Se*-carboxamidomethylselenocysteine, 0.18 and *Se*-carboxymethylselenocysteine, 0.35; in tertiary butyl alcohol/methylethylketone/88% HCOOH/H_2O (40:30:15:15) *Se*-carboxymethylselenocysteine, 0.47, *Se*-carboxyethylselenocysteine, 0.50, and selenocystine, 0.1 in 1-propanol/5% NH_4OH (70:30) *Se*-carboxymethylselenocysteine, 0.44; in $CHCl_3$/CH_3OH/15% NH_4OH (40:40:10) *Se*-aminoethylselenocysteine, 0.46; and in 2-propanol/28% NH_4OH/H_2O (60:1.5:30) *Se*-aminoethylselenocysteine, 0.79, and selenocystine, 0.72. In each instance the R_F of the corresponding sulfur compound was similar.

RESULTS

The unusual absorption spectrum of the selenoprotein of glycine reductase (Fig. 1) is due to its high content of phenylalanine relative to tyrosine and to the absence of tryptophan. Almost identical spectra are exhibited by the parvalbumin of dogfish (12) and the calcium-binding protein component of troponin from rabbit muscle (13). The spectrum of the latter, like that of the selenoprotein, exhibits a shoulder in the 295- to 320-nm region. In neither instance is the nature of the responsible chromophoric group known.

The selenoprotein routinely is isolated under conditions that are not strictly anaerobic and is obtained in an oxidized form. This, however, is readily converted to its fully reduced form by treatment with borohydride. The ultraviolet absorption spectrum of the reduced protein (Fig. 1) shows a marked increase in absorbance below 270 nm, and the difference spectrum (reduced minus oxidized) has a maximum at about 238 nm. Upon exposure to air there is an immediate return to the

* This procedure, to be described elsewhere, makes use of the greater affinity of -SeH over -SH groups for Hg.

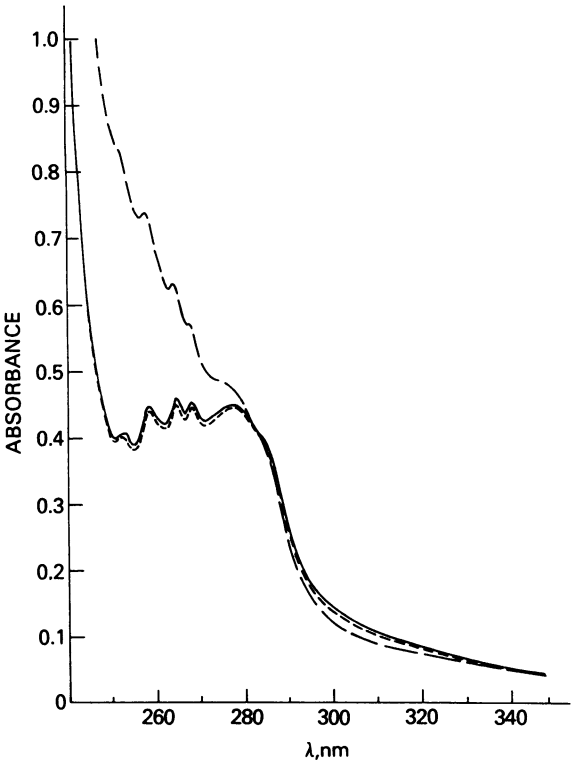

FIG. 1. Ultraviolet absorption spectra of the glycine reductase selenoprotein. The absorption spectrum of the protein (about 4 mg in 1 ml of 0.2 M Tris·HCl, pH 8) first was recorded directly (——), then within 3–5 min after the addition of a few grains of KBH_4 (— —), and, finally, after the cuvette had stood open to the air for an hour (- - - - - - -).

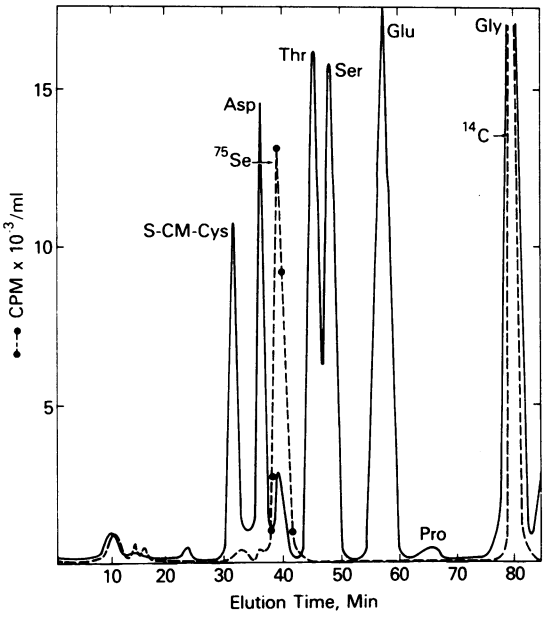

FIG. 2. Amino acid analyzer chromatograms of a carboxypeptidase A digest of carboxymethylated [^{75}Se]selenoprotein. [^{75}Se]Protein (3.77 mg) in 0.5 M Tris·HCl, pH 8.1, containing 6 M guanidine·HCl, 2 mM EDTA, and 12 mM dithiothreitol, was reduced with KBH_4 and alkylated with sodium iodoacetate. After separation from reagents on Sephadex G-25, the alkylated protein was incubated with carboxypeptidase A-diisopropylfluorophosphate for 72 hr. Passage of the digest over a column of Sephadex G-25 separated a salt fraction that contained 68% of the ^{75}Se. This was lyophilized; one half was analyzed on the long column of the amino acid analyzer for the amino acid profile. The other half, after addition of [*1*-^{14}C]glycine (140 × 10^3 cpm), was applied directly to the analyzer column, and 1-min fractions were collected and examined for radioactivity. The solid line shows the damped ninhydrin profile (about 30% of the total absorbance at 570 nm). S-CM-Cys, *S*-carboxymethylcysteine.

absorption spectrum of the original protein solution (Fig. 1). Comparable spectral changes observed when diselenides, e.g., selenocystine and selenocystamine, are reduced to selenols with KBH_4 under the same conditions (data not shown) are attributable to the selenide anion (14). The sulfide anion exhibits similar absorbance, and there are two half-cystine residues in the selenoprotein; but, at pH 7–8, where these experiments were conducted, the -SH group usually exists mostly in the protonated form because of its higher pK_a value. It is likely, therefore, that the extremely oxygen-labile chromophoric group of the reduced selenoprotein is the ionized form of the single -SeH group.

Attempts to isolate the radioactive selenium-containing moiety from native ^{75}Se-labeled protein in amounts sufficient for its identification were unsuccessful. Even after it was recognized that the protein must be reduced with an effective reducing agent such as borohydride and immediately alkylated under strictly anaerobic conditions prior to the preparation of digests, the greater susceptibility of selenoethers to oxidation (as compared to thioethers) made the isolation procedure difficult and prevented the use of many techniques commonly used by protein chemists for characterization of organosulfur compounds. Identification by mass analysis of the relatively small amounts of derivatives isolated from the selenoprotein proved to be impractical because of extensive decomposition and elimination of red selenium caused by esterification procedures or by heating. In our hands, the carboxymethyl-, carboxyethyl-, and aminoethyl-derivatives of the selenoprotein were the most satisfactory, both from the standpoint of ease of preparation and stability to hydrolysis and fractionation procedures. These derivatives of the ^{75}Se-labeled protein were prepared, in general, by standard procedures (15) used for alkylation of protein sulfhydryl groups, with the exception that the protein was reduced with a slight excess of borohydride, usually in the presence of dithiothreitol, and the alkylating agent was added under argon without prior destruction of residual KBH_4.

Characterization of [^{75}Se]Carboxymethylselenocysteine Isolated from the Alkylated [^{75}Se]Protein. On the amino acid analyzer, synthetic *Se*-carboxymethylselenocysteine is eluted immediately after aspartic acid in the position normally identified as methionine sulfone, and 8–10 min after *S*-carboxymethylcysteine. Fig. 2 shows the elution profiles of ninhydrin-reactive material and of radioactivity obtained when a carboxypeptidase A digest of carboxymethylated [^{75}Se]protein was chromatographed on the amino acid analyzer. The ^{75}Se peak of the radioactive profile coincides exactly with a ninhydrin-reactive peak that eluted at 39 min in the position of authentic *Se*-carboxymethylselenocysteine. In this experiment marker [^{14}C]glycine, added to the sample to be counted prior to chromatography, served as an internal standard for precise alignment of peaks. The ^{75}Se-labeled substance recovered from the fraction that eluted at 39 min migrated as a single radioactive, ninhydrin-positive spot with the same R_F (0.47) as *Se*-carboxymethylselenocysteine on cellulose thin-layer sheets developed in tertiary butyl alcohol/methylethylketone/88% HCOOH/H_2O (40:30:15:15) and separated from methionine sulfone, R_F 0.36. The material that eluted at 32 min in the peak labeled S-CM-Cys (Fig. 2) was derived from the half-cystine residues of the selenoprotein. Data (to be reported elsewhere)

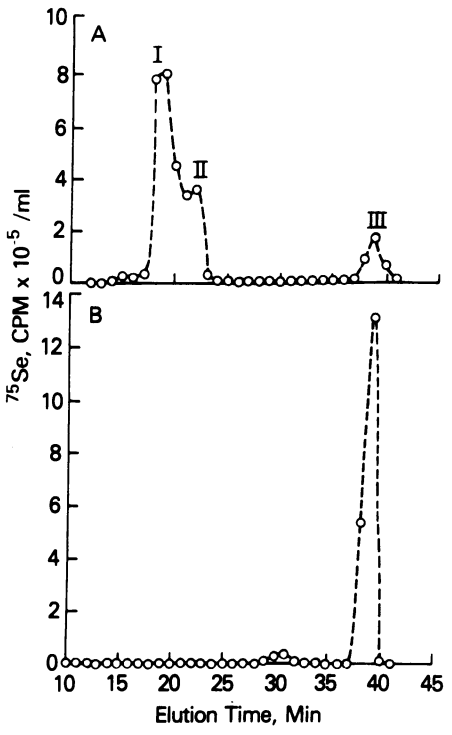

FIG. 3. Amino acid analyzer chromatograms of [^{75}Se]compounds from an acid hydrolysate of carboxamidomethylated selenoprotein. [^{75}Se]protein, reduced and alkylated with iodoacetamide, was hydrolyzed in 6 M HCl, under reduced pressure for 21 hr at 110°. Immediately after removal of HCl at 50° under reduced pressure, followed by adsorption to Dowex-50-H$^+$ and elution with 2 M NH_4OH, 92% of the radioactivity of the protein was found in the cationic fraction. Storage of this cationic amino acid fraction as a dry sample for 2 weeks at 5° resulted in the conversion of 20% of the ^{75}Se to anionic material. Chromatography of the remaining radioactive cationic material on the amino acid analyzer column separated it into three ^{75}Se-labeled fractions (Fig. 3A). The compounds that eluted near the breakthrough volume in peaks I and II were separated from the citrate buffer by treatment with Dowex-50-H$^+$. The radioactivity profile of this material, after reduction with KBH_4, acidification, and reapplication to the analyzer column, is shown in Fig. 3B. The ^{75}Se in peaks I and II of Fig. 3A (2.5 × 10^6 cpm) appeared in the position of peak III in Fig. 3B (1.87 × 10^6 cpm).

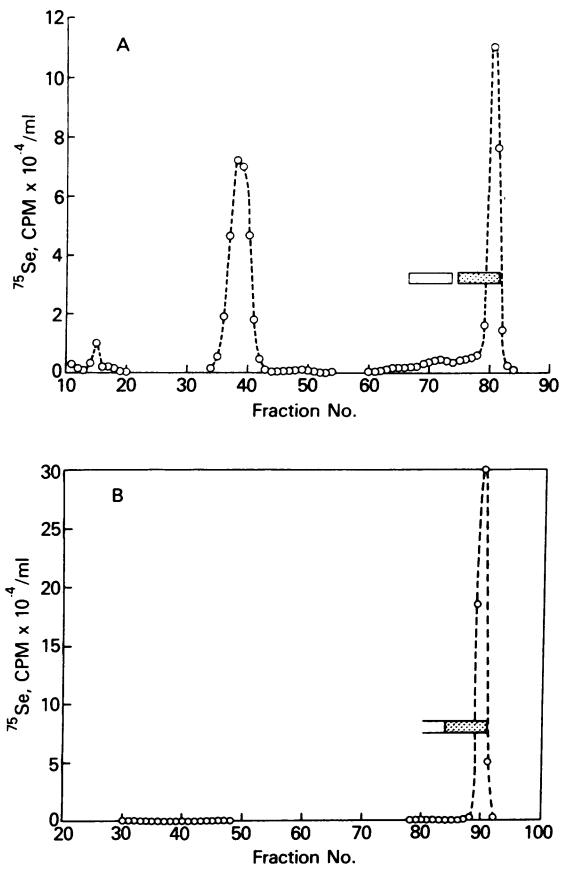

FIG. 4. Chromatography of ^{75}Se-labeled amino acid fraction from an acid hydrolysate of carboxyethylated selenoprotein. [^{75}Se]Protein (11 × 10^6 cpm) was reduced with KBH_4 and alkylated with $BrCH_2CH_2COOK$. The protein was separated from reagents and hydrolyzed in 6 M HCl in an autoclave at 121° for 6 hr. After removal of HCl under reduced pressure, the amino acids were adsorbed to Dowex-50-H$^+$ and eluted with 2 M NH_4OH. This cationic fraction, after removal of ammonia under reduced pressure contained 8.3 × 10^6 cpm (75% of the ^{75}Se). (A) The ^{75}Se profile of an aliquot of the cationic fraction applied to the amino acid analyzer column without prior reduction. The peak that eluted in fractions 36–41 contained 284,900 cpm, and the peak that eluted just after proline contained 227,900 cpm. (B) The ^{75}Se profile of an equal aliquot that was reduced with KBH_4 immediately before application to the column. The single peak that eluted after proline contained 538,300 cpm. Aliquots of individual fractions, after removal of citrate buffer by adsorption to Dowex-50-H$^+$ columns, were analyzed by thin-layer chromatography to determine the elution positions of glutamic acid (open bars) and proline (cross-hatched bars) relative to the radioactive peaks.

show there are two cysteine residues and one selenocysteine moiety per 12,000 daltons.

The carboxymethyl derivative of selenocysteine is prone to spontaneous oxidation and, in some instances, samples were found to contain varying amounts of oxidized forms that were eluted from the amino acid analyzer column near the breakthrough volume. However, subsequent treatment of these compounds with KBH_4 converted them to a form that was indistinguishable from the original selenoether. This behavior is illustrated by the data of Fig. 3. Here the radioactive profile of the amino acid fraction from a 6 M HCl hydrolysate of carboxamidomethylated [^{75}Se]protein shows two major peaks that eluted much earlier (peaks I and II of Fig. 3A) than the normal position of the selenoether (peak III of Fig. 3A). However, when the labeled material from peaks I and II was treated with KBH_4 and rechromatographed, all of the ^{75}Se was eluted at 39 min in the selenoether position (Fig. 3B). In this case the ^{75}Se-labeled derivatives that eluted from the analyzer column in peaks I and II were formed subsequent to acid hydrolysis. An aliquot of the hydrolysate described in Fig. 3 that was chromatographed immediately after removal of HCl and before treatment with Dowex-50 exhibited a single major radioactive peak that eluted in the position of *Se*-carboxymethylselenocysteine (peak III of Fig. 3A). At this time no ^{75}Se was found in the positions corresponding to peaks I and II of Fig. 3A.

Pure samples of [^{75}Se]carboxymethylselenocysteine isolated from the labeled selenoprotein as shown in Fig. 3A and B were indistinguishable from the corresponding derivative of the authentic amino acid in several other respects. Both showed only end absorption in the ultraviolet range and were transparent above 250 nm. Upon reaction with ninhydrin they gave a typical purple color and they cochromatographed on cellulose thin-layer sheets in a number of solvent systems. The *N*-dinitrophenyl derivative of the ^{75}Se-labeled compound was prepared and compared with the [*1*-^{14}C]carboxymethyl-*N*-dinitrophenyl derivative of the authentic compound. These compounds cochromatographed on silica sheets in $CHCl_3$/CH_3OH/glacial CH_3COOH (95:5:1), R_F 0.23, and in 1-pro-

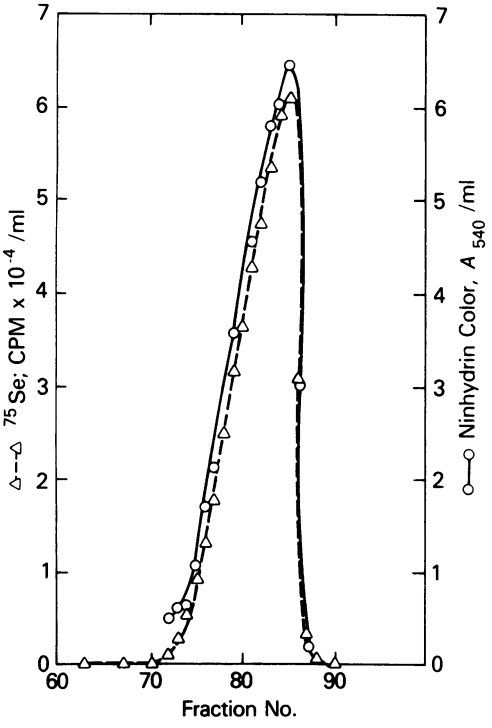

FIG. 5. Elution profiles of ^{75}Se (△- - -△) and carrier *Se*-carboxyethylselenocysteine (○——○). Aliquots (50 μl) of fractions collected directly from the amino acid analyzer column were analyzed for amino acid content by the Rosen modification of the Cocking-Yem quantitative ninhydrin method (16). ^{75}Se was measured in 100-μl aliquots by scintillation spectrometry. No radioactive or ninhydrin-positive oxidation products of the selenoethers were detected in the earlier portion of the elution profile.

panol/28% NH_4OH (70:30), R_F 0.52, as judged by coincidence of yellow color and of radioactivity and failure to react with ninhydrin.

Characterization of the Carboxyethylated ^{75}Se-Labeled Moiety of the [^{75}Se]Protein. To further characterize the selenium-containing residue of the selenoprotein, we prepared the carboxyethylated derivative as described in the legend of Fig. 4. Chromatography of the labeled amino acid fraction from the hydrolyzed [^{75}Se]protein on the amino acid analyzer column (Fig. 4A) separated one radioactive peak that emerged just after proline in the position expected for *Se*-carboxyethylselenocysteine and another that eluted much earlier from the column. The latter was not detected in a parallel sample (Fig. 4B) that had been reduced with KBH_4 prior to chromatography. Instead, all of the ^{75}Se was found in the single peak that followed proline. Another sample of the hydrolysate, analyzed at a different time, contained 80% of the total ^{75}Se in a compound that eluted as a single radioactive peak corresponding to fraction 15 of Fig. 4A, but this also, upon subsequent reduction with KBH_4, was converted quantitatively to the form that migrated to the same position as authentic *Se*-carboxyethylselenocysteine. These data indicate that the carboxyethyl, like the carboxymethyl, derivative is prone to spontaneous oxidation under some conditions. With this derivative, also, it was possible to isolate an appreciable amount of the pure oxidized form from a large sample of the unfractionated protein hydrolysate applied to the analyzer column. When an aliquot of this isolate was mixed with carrier *Se*-carboxyethylselenocysteine, reduced with KBH_4, and rechromatographed on the analyzer column, the profile shown in Fig. 5 was obtained. The exact coincidence of the radioactive peak and the peak of ninhydrin-reactive material

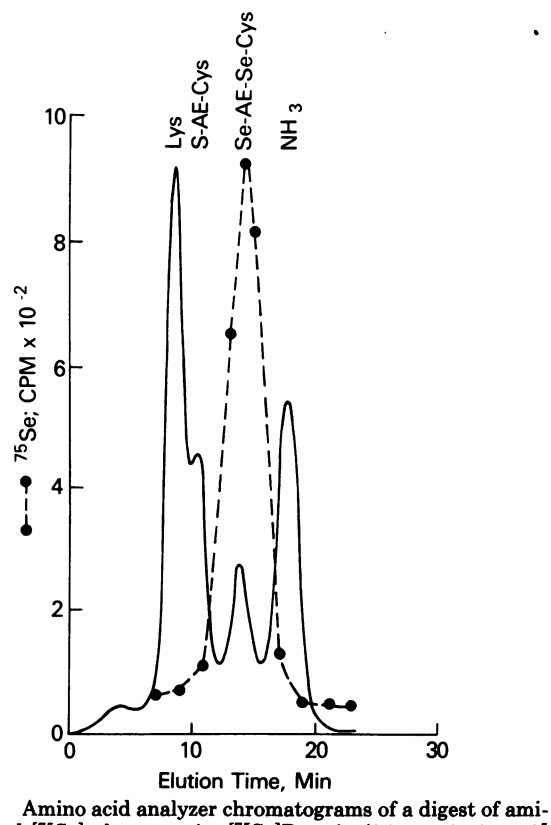

FIG. 6. Amino acid analyzer chromatograms of a digest of aminoethylated [^{75}Se]selenoprotein. [^{75}Se]Protein (2.5 mg; 2.16 × 10^5 cpm) in 100 mM potassium phosphate, pH 7.2, containing 0.3 M NaCl, 1 mM dithiothreitol, and 1 mM MgK_2EDTA was reduced with KBH_4 and reacted with ethyleneimine. The alkylated protein, after transfer to 0.2 M NH_4HCO_3, pH 8.6, on a polyacrylamide P-2 column, was digested with TPCK-trypsin for 17 hr. A small peptide fraction that contained most of the ^{75}Se was separated on a P-2 column and digested further with carboxypeptidase A. The ninhydrin profile (solid line) is of an aliquot of this digest analyzed directly on the "short" column of the amino acid analyzer. Fractionation of another aliquot of the digest on a P-2 column in 44% HCOOH separated a major peak of ^{75}Se with the salt fraction. This was lyophilized and chromatographed on the "short" column of the amino acid analyzer in the presence of carrier *Se*-aminoethylselenocysteine (Se-AE-Se-Cys). The ^{75}Se profile (broken line) was determined in 1-min fractions collected directly from the column.

indicates that the two amino acid derivatives coeluted in the expected position and is further evidence of their identity.

Characterization of ^{75}Se-Labeled Moiety of the Selenoprotein as *Se*-Aminoethylselenocysteine. The aminoethyl derivatives of authentic selenocysteine and cysteine are eluted from the "short" column of the amino acid analyzer as discrete peaks ahead of ammonia. Enzyme digests of a sample of [^{75}Se]selenoprotein that had been reduced and alkylated with ethyleneimine contained two components that eluted from the analyzer column between lysine and ammonia in these same positions (Fig. 6); the fraction corresponding to *Se*-aminoethylselenocysteine contained the ^{75}Se. The peak showing the elution position of the radioactive derivative, determined in a sample to which unlabeled carrier had been added, is superimposed on the ninhydrin profile of Fig. 6. The broad, symmetrical radioactive peak indicates that the labeled amino acid coeluted throughout with the carrier compound. This ^{75}Se-labeled fraction, when reapplied to the analyzer column, exhibited a single, ninhydrin-positive, basic component that was eluted ahead of ammonia precisely at the position of *Se*-aminoethylselenocysteine.

DISCUSSION

From the evidence presented in this paper, it is reasonably certain that the selenium moiety of the glycine reductase selenoprotein is a selenocysteine residue.

Initial studies with the selenium moiety liberated from the native selenoprotein by acid or enzymic hydrolysis indicated it to be considerably less stable than the methyl derivatives of either selenocysteine or selenohomocysteine, e.g., *Se*-methylselenocysteine or selenomethionine. However, not until considerable experience had been gained in preparing and studying various types of derivatives of the available selenoamino acids was it possible to conclude that the selenoamino acid in the protein probably was a selenocysteine residue rather than a known *Se*-methylselenoether or an unknown compound. Eventually, the various ^{75}Se-labeled alkyl derivatives prepared from the reduced [^{75}Se]protein could be shown to be identical, in all of the properties that we studied, to those of the corresponding derivatives of authentic selenocysteine.

The forms of the carboxymethyl and carboxyethyl selenoethers that were detected in early peaks of some of the amino acid analyzer profiles undoubtedly were oxidation products, because treatment with borohydride converted them to compounds indistinguishable from the original selenoethers. The early emergence of these derivatives from the analyzer column and the ease of their reduction to the corresponding selenoether facilitated isolation of the latter in appreciable amounts from hydrolysates of the selenoprotein. However, formation of these compounds was fortuitous, and conditions for controlled oxidation of the selenoethers have not been defined. In the sulfur series, the sulfone and sulfoxides of *S*-carboxymethylcysteine can be formed by limited oxidation of the thioether with performic acid. By analogy with the chromatographic behavior of these products, which are eluted from the amino acid analyzer column near the breakthrough volume and just after cysteic acid (17), it is likely that the oxidized forms of the selenoethers were the corresponding selenoxides and selenones.

In view of the fact that the fully reduced selenoprotein contains one selenocysteine -SeH group and two cysteine -SH groups, it is possible that the native oxidized form of the protein is a dimer with the extra group forming an intermolecular bridge which could be Se-Se, Se-S, or S-S. However, if the unexplained absorbance exhibited by the protein in the ultraviolet region between 295 and 320 nm is a contribution of a constituent that is not yet identified, then this might afford an intramolecular site of interaction for the extra -SeH or -SH group.

Although the biological role of the selenocysteine moiety of the selenoprotein is not known, it is likely that it participates in the oxidation-reduction step of the catalytic reaction. Alternatively, it could serve as acceptor of the postulated carboxymethyl intermediate generated from glycine prior to its reduction to acetate. The highly selective incorporation of a single selenocysteine residue in the selenoprotein also poses an interesting biochemical problem as to the mechanism whereby this is introduced. Continuing investigations on the clostridial glycine reductase selenoprotein and on the formate dehydrogenase of *C. sticklandii* have as their aim the elucidation of some of these problems.

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