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ATTACHMENT OF SELENOCYSTEINE IN THE CATALYTIC SITE OF GLUTATHIONE PEROXIDASE

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Summary

Selenocysteine in the catalytic site of glutathione peroxidase was stabilized by conversion to the carboxymethyl derivative. A selenium-containing tryptic fragment was partially purified by column chromatography through cellulose phosphate, Sephadex G-25 superfine, DEAE-Agarose, and again through Sephadex G-25 superfine. Automated sequential Edman degradation yielded a residue of the phenylthiohydantoin of carboxymethyl-selenocysteine, indicating that the selenocysteine in the native enzyme is located within the polypeptide chain.

Introduction

Glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) catalyzes the reaction ROOH + 2 GSH → ROH + GSSG + H_2O (1), where ROOH is any of a wide variety of hydroperoxides (2,3), ROH is the corresponding alcohol, GSH is reduced glutathione, and GSSG is oxidized glutathione. This enzyme apparently functions in protection of the cell from oxidant damage (4-6) and is the only enzyme in animal tissues known specifically to contain the element Se[1] (7-10). Recently, the Se of rat liver glutathione peroxidase was shown to be in the form of Se-Cys, the Se-containing analogue of Cys, and this Se-Cys was shown to function as the catalytic site (11).

The presence of this unusual amino acid in the catalytic site raises the question of the manner in which the Se-Cys is attached in the glutathione peroxidase molecule. In this report the partial purification of a Se-containing tryptic peptide of glutathione peroxidase is described, and it is shown that residue 11 of this Se-peptide contains Se-Cys within the polypeptide chain

[1]Abbreviations used: Se, selenium; Se-Cys, selenocysteine; CM-Se-Cys, carboxymethyl-selenocysteine; CM-Cys, carboxymethyl-cysteine; PTH, phenylthiohydantoin; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; and N,N-diPhe, N,N-diphenylthiourea.

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rather than attached as a side-chain group or as the N- or C-terminal amino acid.

Materials and Methods

Materials. Rats were purchased from Simonsen Laboratories, Inc.; ^{75}Se (9 Ci/mmol) and Aquasol scintillation fluid from New England Nuclear; iodoacetic acid, cellulose phosphate, and Sephadex from Sigma Chemical Co.; DEAE-Agarose from Bio-Rad Laboratories; ethylenimine and fluorescamine from Pierce Chemical Co.; trypsin from Worthington Biochemical Corp.; TPCK from Cyclo Chemical Co.; polyamide sheets for thin-layer chromatography from Schleicher and Schuell; and the miniPump was from Milton Roy, Inc.

Enzyme preparation and hydrolysis. Glutathione peroxidase that contained ^{75}Se as a radioactive tracer was purified from cytoplasm of livers from male Sprague-Dawley rats to approximately 50% purity as described previously (11). The enzyme was reacted with iodoacetic acid on the Se-Cys residue to produce CM-Se-Cys (11) and then with ethylenimine at pH 8.0 to derivatize all Cys residues present. Trypsin hydrolysis of the derivatized enzyme was performed at 37°C after addition of 0.5 mg trypsin previously treated with TPCK (12) to remove possible chymotrypsin contamination. After 3 hr and again after 12 hr, further additions of 0.25 mg trypsin were made. The total incubation time was 15 hr.

Peptide purification. The tryptic hydrolyzate was acidified to pH 2 by addition of HCl and applied to a 1.5 X 30 cm column of cellulose phosphate equilibrated in 0.05 M H_3PO_4, pH 2.5 (13). The flow rate was maintained by pumping at 2 ml/min. The column was first eluted with 50 ml of 0.025 M KCl, 0.05 M H_3PO_4, pH 2.5, and then with a 500-ml linear gradient of 0.025 to 0.05 M KCl in 0.05 M H_3PO_4, pH 2.5. The major peak of ^{75}Se radioactivity eluted in the gradient and was collected, pooled, and concentrated. The peptide obtained was applied to a 1.5 X 100 cm column of Sephadex G-25 superfine equilibrated in 10% acetic acid, 10% ethanol and was eluted at a flow rate of 0.2 ml/min. The major peak of ^{75}Se radioactivity was pooled, concentrated, and applied to a 1.5 X 30 cm column of DEAE-Agarose equilibrated in 0.01 M ammonium bicarbonate at a flow rate of 1 ml/min. A 400-ml linear gradient of 0.01 M to 0.10 M ammonium bicarbonate was used to elute the ^{75}Se-containing peak, which was then pooled and concentrated. This partially purified peptide was then freed of residual ammonia by chromatography through a 1.5 X 100 cm column of Sephadex G-25 superfine equilibrated in 10% acetic acid, 10% ethanol at a flow rate of 0.2 ml/min.

Peptide analysis. Amino acid analysis of the purified peptide was performed on a Durrum D-500 amino acid analyzer after hydrolysis in 6 N HCl *in vacuo* at 110°C for 24 hr.

The peptide sample was sequenced using a Beckman 890C Sequencer and the DMAA peptide program 102974. The peptide sample was applied in the spinning cup along with 2.8 mg of N-acylated cytochrome *c* as carrier. Each anilinothiazolinone-amino acid residue produced by the sequencer was manually converted to the PTH derivative by incubation in 0.2 ml 1 N HCl at 80°C for 10 min. Extraction with ethyl acetate yielded most PTH-amino acids in the organic phase. Each PTH-amino acid residue was assayed for ^{75}Se radioactivity and identified by gas chromatography, trimethylsilylation gas chromatography (14), and by two-dimensional thin-layer chromatography on 5 X 5 cm polyamide sheets (15).

Standards of [^{3}H]CM-Se-Cys were prepared according to the method of Fletcher (16) and of PTH-[^{3}H]CM-Se-Cys according to the technique of Edman and Henschen (17).

Assays. ^{75}Se was assayed using a Packard model 5110 auto-gamma spectrometer with 30% efficiency. Protein and peptide amine groups were assayed by a

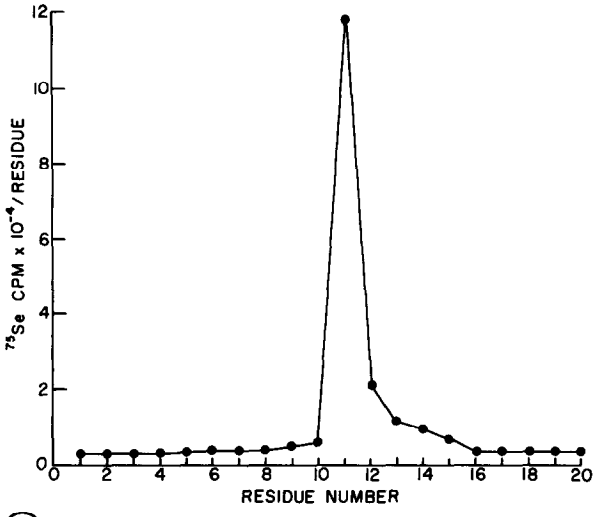
micro-fluorescamine procedure (18). ^{3}H was assayed in 10 ml of Aquasol scintillation fluid and counted in a Beckman CPM-100 liquid scintillation counter. Se was assayed according to the method of Watkinson (19).

Results

A glutathione peroxidase sample of 50% purity had the labile Se-Cys converted to the stable CM-Se-Cys and the Cys converted to aminoethyl-Cys. The Se-containing peptide from a tryptic digest of derivatized glutathione peroxidase was isolated by column chromatography through cellulose phosphate, Sephadex G-25 superfine, DEAE-Agarose, and again through Sephadex G-25 superfine. Twenty-five per cent of the ^{75}Se in the native enzyme was recovered in this partially purified tryptic peptide (45 nmoles of Se). The Se-containing peptide had a molecular weight of 2000 as compared with calibration standards on Sephadex chromatography. This peptide was approximately 60% pure as determined by the ratio of N-terminal amino acids on the first cycle of the sequencer. Thirty percent of the rest of the sample consisted of one contaminating peptide. Amino acid analysis of the acid hydrolyzate showed lack of purity of the isolated peptide sample since integral ratios of amino acids could not be obtained.

Several residues, principally the hydrophobic amino acids, were tentatively identified early in the sequence for each of the two major peptides in the sample. The residue of greatest interest, however, was the one that contained Se, and this residue was rigorously identified.

Residue 11 was the only residue with a significant amount of ^{75}Se radioactivity (14 nmoles of Se) indicative of the Se-Cys-containing fragment (Figure 1). Gas chromatography of residue 11 and of its trimethylsilyl derivative identified it as PTH-CM-Cys, PTH-CM-Se-Cys, or PTH-Ser. However, only the Se-Cys in the native glutathione peroxidase sample had been carboxymethylated, and absence of CM-Cys was confirmed on amino acid analysis of the acid hydrolyzate. Standard PTH-CM-Se-Cys eluted from the gas chromatograph at a position similar to that of PTH-CM-Cys and PTH-Ser. Two-dimensional poly-



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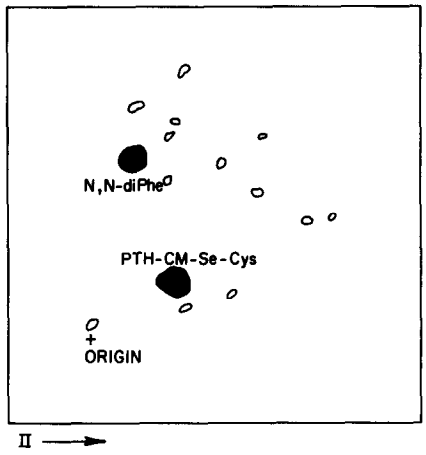

(2)

Figure 1. Amount of ^{75}Se radioactivity released per residue of peptide sequence.

Figure 2. Two-dimensional polyamide thin-layer chromatography on 5 X 5 cm plates (15) of residue 11 co-spotted with PTH-[^{3}H]CM-Se-Cys. The other major spot is the artifact N,N-diPhe. Dimension I: toluene/n-pentane/acetic acid (60:30:16, v/v); Dimension II: 25% aqueous acetic acid. Closed spots are major; open spots are minor.

amide thin-layer chromatography of residue 11 spotted together with standard PTH-[^{3}H]CM-Se-Cys showed that, aside from a large spot of the artifact N,N-diphenylthiourea, there was only one large spot similar to where PTH-CM-Cys would have migrated if it had been present (Figure 2). This spot was cut out and counted, and ^{75}Se and ^{3}H were found to be coincident.

Thus, residue 11 behaved chromatographically similar to PTH-CM-Cys and PTH-Ser, co-chromatographed consistently with standard PTH-[^{3}H]CM-Se-Cys, and was the only residue with a significant amount of ^{75}Se radioactivity. Since the two major peptides released amino acids at the 10-20 nmol level, and since residue 12 contained two major amino acids, we concluded that residue 11 was not C-terminal.

Discussion

Of the three known Se-containing enzymes (7,20,21), clostridial glycine reductase and mammalian glutathione peroxidase have been shown to contain

their selenium as Se-Cys (11,21). The Se moiety of bacterial formate dehydrogenase remains unknown. The Se-Cys in glutathione peroxidase was also shown to function as the catalytic site (11). How this unusual amino acid is attached to the polypeptide chain at the catalytic site and how it becomes incorporated into or onto the chain are important questions. The Se-containing peptide of glutathione peroxidase was isolated for the purpose of elucidating the structure of this attachment and discriminating between Se-Cys that is located within the polypeptide as a normal amino acid would be, and Se-Cys that is attached to a preformed polypeptide backbone via a side-chain group or the N- or C-terminus. Sequential Edman degradation would release free CM-Se-Cys as the PTH derivative only if it were internally located in the chain or if it had a free N-terminus.

Although we have been unable at this point to elucidate the entire sequence of the tryptic-peptide that contains the Se-Cys catalytic site of glutathione peroxidase, we have identified the type of attachment of the critical amino acid. The iodoacetic acid-derivatized Se-Cys in rat liver glutathione peroxidase is located within the polypeptide backbone and is neither attached to a side-chain residue nor on the N- or C-terminus. If the Se-Cys had been attached to a side-chain group, then residue 11 of the tryptic peptide would have been a two or a three amino acid-containing structure. If the Se-Cys had been N- or C-terminal, it would have been released as the first or last residue, respectively.

This leaves open the question of the mechanism of incorporation of the Se-Cys into glutathione peroxidase. Apparently, Se-Cys is not preformed and then attached to an otherwise completed polypeptide chain. It is possible that the Se-Cys is formed *in situ* from some previously incorporated amino acid, such as Cys or Ser, that would be susceptible to post-translational modification. This *in situ* reaction might occur similarly to the cysteine synthase reaction, which can produce Cys from serine and Na_2S, if H_2Se or its equivalent were substituted for the Na_2S (22). Alternatively, the Se-Cys

could be incorporated during translation via the action of a Se-Cys tRNA and its charging enzyme. We are experimentally searching for a Se-Cys tRNA. Specificity for Se incorporation might be obtained from an appropriate sequence surrounding the insertion site (23), and we are attempting to elucidate the sequence around the Se-Cys.

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