A Novel Peptide SeCys-Gly-Pro-Cys, An Active Site Model of Thioredoxin: Synthesis and Glutathione Peroxidase-like Activity

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ABSTRACT

A tetrapeptide, Secys-Gly-Pro-Cys, is a selenium analog of the peptide moiety occuring at the active site of thioredoxin, which forms an intramolecular 14-membered disulfide loop structure in the oxidized state. We have synthesized the selenium-containing tetrapeptide, and studied its glutathione peroxidase-like activity. The tetrapeptide showed glutathione peroxidase-like activity, which was three times higher than those of glutaselenone and diphenyldiselenide. The high catalytic activity is considered to be due to the intramolecular selenosulfide bond formation in the catalysis.

INTRODUCTION

Selenium belongs to the VIb group of the periodic table and possesses both matalic and non-metalic characteristics. Organoselenium compounds, in general, are less stable and more reactive than the corresponding sulfur analogs. In 1984, a biologically active organoselenium compound named ebselene was synthesized (1, 2). Many of the biological properties of ebselene were found to be related with its glutathione peroxidase-like activity. A number of attemps were made to modify the structure of ebselene, and structure-activity relationships of a series of anti-inflammatory benzisoselenazolones have studied by Parnham *et al.* (3) and Tarino (4). Wilson*et al.* (5) and Cotgreave*et al.* (6) also reported pharmacological effects of the related compounds. The pharmacological actions of synthetic organic selenium compounds were reviwed by Parnham and Graf (7). We have synthesized a selenocysteine analog of glutathione disulfide (γ-glutamyl-selenocysteinylglycine), named it glutaseleone, and showed its glutathione peroxidase-like activity.

Thioredoxin is a ubiquitous protein which is involved in a variety of biochemical redox reactions, such as reduction of inorganic sulfate and methionine sulfoxide (8, 9) in microorganisms and biosynthesis of deoxyribonucleotides (10) in all living cells. It also plays an important regulatory role in photo-

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synthesis (11). All prokaryotic and eukaryotic thioredoxins contain a conserved sequence, -Cys-Gly-Pro-Cys-, at the active site (12-15). This moiety of forms an intramolecular 14-membered disfulfide loop structure in the oxidized state, and is reduced to a dithiol by an FAD- containing enzyme, thioredoxin reductase. The peptide medels for the thioredoxin active site, Boc-Trp-Cys-Gly-Pro-Cys-NHMe and Boc-Cys-Gly-Pro-Cys-NHMe, were synthesized chemically (16, 17). The models were oxidized to form an intramolecular disufide bond, and the resulting β-turn conformation was characterized. We have shown that glutaseleone selenosulfide (GSeSG) is the key intermadiate in glutathione peroxidase-like reaction catalyzed by glutaseleone. The active site model of thioredoxin is expected to serve as a template for intramolecular selenosulfide bond formation. We here describe synthesis and characterization of a tetrapeptide, SeCys-Gly-Pro-Cys, and its glutathione peroxidase-like activity.

MATERIALS AND METHODS

N- (*p*-Methoxybenzyloxycarbonyl) -*Se*- (*p*-methoxybenzyl) -L-seleno-cysteine (PMZ-SeCys (Se-MBz) -OH) was synthesized from *Se*- (*p*-methoxybenzyl) -L-selenocysteine and *p*-methoxybenzyl azidoformate (11). *S*-Benzylcysteine was purchased from Wako Pure Chemicals, and derivatized to *S*-benzyl-L-cysteine benzyl ester with benzyl bromide (18). *N*-*t*-Butyloxycarbony-L-proline (Boc-Pro) and *N*-*t*-butyloxycarbonyl-glycine (Boc-Gly) were synthesized from Boc-SDP and L-proline or glycine (19). Reduced and oxidized forms of glutathione, hydrogen peroxide, *o*-phthalaldehyde, *N*-ethylmaleimide, sodium hydroxide, ethylenediamine-tetraacetic acid (EDTA) and sodium azide were purchased from Nacalai Tesque (Kyoto, Japan). Diphenyldiselenide was from Aldrich (98%). Other reagents were of analythical grade.

Melting point was measured with a Micro Melting Point Determinational Apparatus MM-2 (Shimadzu, Kyoto, Japan) ; optical rotations with Perkin-Elmer 241 (Perkin-Elmer and Co, Ueberkingen) ; absorption spectra with a Shimadzu UV-260 spectrophotometer; ^{1}H-nmr spectra with a VXR-200 (Varian) ; mass spectra with JMS-DX 300 (Jeol) .Elemental analysis was done with a Yanaco CHN corder MT-3 (Yanagimoto).

SeCys-Gly-Pro-Cys (16mg ; 0.035mmol) was dissolved in 10mM potassium phosphate buffer (pH 7.0) in 99.8% D_2O (0.6ml), and mixed with sodium borohydride (2mg ; 0.05mmol) .The solution was transferred to a NMR tube, and nitrogen gas was flashed. ^{1}H-^{1}H-COSY spectrum was recorded on a Varian VXR-200 spectrophotometer at an ambient temperture.

Glutathione peroxidase-like activity was determined with *o*-phthalaldehyde. The assay mixture (2ml) contained 50mM potassium phosphate buffer (pH7.0), 1mM GSH, 5mM EDTA, 1mM NaN_3, and 50 μM organoselenium compounds. The reaction was initiated by addition of hydrogen peroxide (final concentration, 0.2mM), and the reaction mixture was incubated at 38℃. A 100 μl-aliquot of the reaction

mixture was taken every 10min and mixed with 20 μl of 0.1M *N*-ethylmaleimide. The mixture was kept at room temperature for 30min, and mixed with 280 μl of 0.1M NaOH. An aliquot of 100 μl of the resulting mixture was added to the mixture with 100 μl of *o*-phthalaldehyde solution (1mg/ml in methanol) and 1 ml of 0.1M NaOH, and the mixture was incubated at room for 15min. Fluorescence of the mixture was measured with a fluorescence spectrophotometer by excitation on 350nm and emission at 410nm. The rate of production of GSSG was plotted against incubation time. H_2O_2 was replaced by H_2O in a control experiment. Glutaselenone was omitted in a blank experiment.

RESULTS

S-Benzyl-L-cysteine benzyl ester (4.6g ; 14mmol) and triethylamine (2ml) dissolved in dimethylformamide (28ml) were added to a solution of *N*-*t*-butyloxycarbonyl-L-proline (2.06g ; 9.6mmol), *N*, *N*'-dicyclohexylcarbodiimide (2.3g), and 1-hydroxybenzotriazole (1.5g) in dimethylformamide (40ml) at 0℃. After 20h, the mixture was filtered and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (100ml) , and washed successively with 5% critic acid (10ml, twice), 5% sodium bicarbonate (10ml, twice), and saturated sodium chloride solution (10ml, twice). The ethyl acetate layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The product was crystallized from ethyl acetate and ether (yield, 3.5g ; 7.0mmol) ; mp, 84℃ $[\alpha]^{25}_{D}$, −86° (c = 0.5, acetone) ; EI-MS (m/z) , 498[M^+] ; Found : C, 65.04% ; H, 7.06% ; N, 5.49% ; $C_{27}H_{34}N_2O_5S$ requires C, 65.03% ; H, 6.87% ;N, 5.62% ; ^{1}H-nmr (200MHz, $CDCl_3$) ; δ 1.4 (9H, s, Boc), 1.6 (2H, br, Pro-γ-CH_2), 1.9-2.2 (2H, b, Pro-β-CH_2), 2.8 (2H, m, Cys-β-CH_2), 3.4 (2H, b, Pro-γ-CH_2), 3.6 (2H, s, S-CH_2-Ph), 4.3 (1H, m, Pro-α-CH), 4.7 (1H, m, Cys-α-CH), 5.1 (2H, s, Cys-OCH_2-Ph), 7.2 (5H, s, Benzyl), and 7.3 (5H, s, Benzyl).

N-*t*-Butyloxycarbonyl-L-prolyl-*S*-benzyl-L-cysteine benzyl ester (3.5g, 7mmol) was treated with 4M HCl in ethyl acetate (20ml) for 30 min at room temperature, and solvent was removed by evaporation. The residue was dissolved in a mixture of dimethylformamide (14ml) and triethylamine (1ml) to give a solution of the amine component.

A solution containing *N*-*t*-butyloxycarbonyl glycine (2.5g, 14mmol), 1-hydroxybenzotriazole (2.1g) and dicyclohexylcarbodiimide (3.22g) dissolved in dimethylformamide (56ml) was mixed with the solution of amine component, and stirred at 4℃ for four days. The mixture was filtered and evaporated under reduced pressure to give a white precipitate. The precipitate was dissolved in ethyl acetate (100ml), and washed as described above. Ethyl acetate layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation to give a pale yellow residue. The product, Boc-Gly-Pro-Cys (S-Bz) -OBz, was crystallized from ethyl acetate at -20℃ (yield, 2g ; 3.5mmol) ; mp, 65℃ ; $[\alpha]^{25}_{D}$, -89℃ (c=1.0, acetone) ;FAB-MS (m z) , 556[M+1] ; ^{1}H-nmr (200MHZ, $CDCl_3$) : δ 1.4 (9H, s, Boc) , 1.6-2.2 (4H, br, Pro-β, γ-C_2H_4), 2.8 (2H, dq, Cys-CH_2) , 3.4 (2H, m, Pro-δ-CH_2),

3.6 (2H, s, S-CH_2-Ph), 3.9 (2H, dd, Gly-CH_2), 4.6 (1H, d, Pro-α-CH), 4.7 (1H, m, Cys-α-CH), 5.1 (2H, s, Cys-OCH_2-pH), 7.2 (5H, s, Benzyl), and 7.3 (5H, s, Benzyl).

N-t-Butyloxycarbonyl-glycyl-L-prolyl-*S*-benzyl-cysteine benzyl ester (2g, 3.5mmol) was treated with 4M HCl in ethyl acetate (20ml) for 30 min at room temperature, and the solvent was evaporated. The residue was dissolved in a mixture of dimethylformamide (7ml) and triethylamine (0.5ml) to give a solution of the amine component. The amine component solution was added to a mixture of *N*-(*p*-methoxybenzyloxycarbonyl) - *Se*- (*p*-methoxybenzyl) -selenocysteine (2.3g, 5.2mmol), 1-hydroxybenzo-triazole (0.8g), and dicyclohexylcarbodiimide (1.2g) dissolved in dimethylformamide (10ml), and stirred at 4℃ for four days. The reaction mixture was filtered and the solvent was removed by evaporation. White residue was dissolved in ethyl acetate (100ml), and washed as described above. The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated to give white solid (yeild, 3.0g ; 3.3mmol), mp, 120℃ ; $[\alpha]^{25}_D$, -70.8° (c=0.4 ; chloroform) ; Found : C, 58.78% ; H, 5.63% ; N, 6.24% ; $C_{44}H_{50}N_4O_9SeS$ requires C, 59.39% ; H, 5.66% ; N, 6.24% ; ^{1}H-nmr (200MHz, $CDCl_3$) : δ 2.0 (3H, b, Pro-β-CH, γ-CH_2), 2.3 (1H, b, Pro-β-CH), 2.9 (4H, m, Cys-β-CH2, SeCys-β-CH_2), 3.5 (2H, m, Pro-δ-CH_2), 3.6 (2H, s, S-CH_2-Ph), 3.71 (2H, s, Se-CH_2-Ph), 3.75 (3H, s, $BzOCH_3$), 3.77 (3H, s, $BzOCH_3$), 4.0 (2H, b, Gyl-CH_2), 4.4 (1H, m, SeCys-α-CH), 4.6 (1H, d, Pro-α-CH), 4.8 (1H, m, Cys-α-CH), 5.0 (2H, dd, Cys-OCH_2-Ph), 5.1 (2H, s, PMZ), 7.2 (4H, dd, Benzyl), and 7.3 (14H, m, Benzyl).

Liquid ammonia (30ml) distilled over sodium metal under nitrogen gas was introduced to the fully protected tetrapeptide (1g, 1.1mmol). Sodium metal was added in a very small piece to the magnetically stirred mixture at 33℃ until the blue color persists for 10 min. The mixture was decolorized with a little solid ammonium sulfate, then the ammonia was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in distilled water (30ml) and desalted by Micro Acilyzer S1 (Asahi Kasei, Kawasaki, Japan), then lyophilized to give yellow hygroscopic powder (yield, 0.3g, ; 0.65mmol) ; mp, 170-180℃ (decomposition) ; $[\alpha]^{25}_D$, -40.0℃ (c=0.2, H_2O) ; C, 34.46% ;H, 4.87% ;N, 12.14% ; $C_{13}H_{23}H_4O_5SeSCl$ requires C, 33.81% ; H, 5.02% ; N, 12.13% ; UV, λ max 200mn (ε =12000), λ max 310nm (ε =620) ; fluorescence at 390nm and 620nm by excitation at 310nm ; ^{1}H-nmr (200MHz, D_2O : δ 1.9-2.0 (3H, b, Pro-β-CH, λ-CH_2), 2.1 (1H, b, Pro-β-CH), 2.6-2.8 (4H, m, Cys-β-CH_2, SeCys-β-CH_2), 3.2 (1H, m, Pro-δ-CH), 3.5 (2H, m, Pro-δ-CH, SeCys-α-CH), 3.6 (1H, b, Gly-α-CH), 4.0 (1H, b, Gly-α-CH), and 4.2 (2H, b, Cys-α-CH, Pro-α-CH).

The spin systems of the constitutive amino asids were assigned by ^{1}H-^{1}H-COSY. Since the sample was dissolved in D_2O, amide protons were not observed. Although many signals were overlapped with each other, characteristic spin systems were assigned to the constitutive amino acids : δ 4.1- (δ 1.9, 2.1) -δ -1.9- (δ 3.2, 3.5) was assigned as the signals of the proline residue, because the ^{1}H-^{1}H-COSY spectrum of L-proline gave δ 4.3 (α) -δ 1.9, 2.3 (β) -δ 1.9 (λ) -δ 3.3 (δ). The two protons at

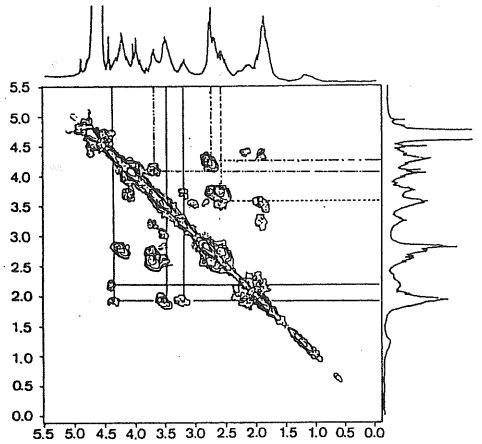

Fig. 1. ^{1}H-^{1}H-COSY spectrum of SeCys-Gly-Pro-Cys.

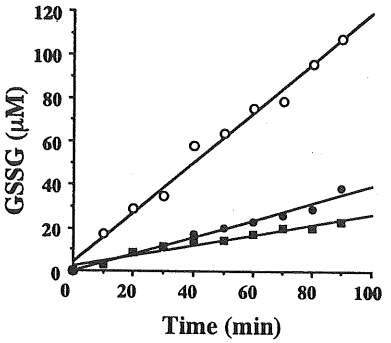

Fig. 2. Glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys (○), glutaselenone (●) and diphenyldiselenide (■). Organoselenium compounds used were at the concentration of 50mM.

δ 3.7 and 4.1 showed correlation, and were assigned to glycine residue. Glycine showed a sharp singlet at δ 4.0, and the signal splitting may derive from conformational constraint on glycine. The complicated signal at δ 2.6-2.8 was assigned to be derived from Cys-β-CH_2 and SeCys-β-CH_2. Sharp signal was assigned to that of Cys-β-CH_2, and broad signal was that of SeCys-β-CH_2. The α-proton signals of SeCys and Cys were assigned to those at δ 3.5 and 4.2, respectively.

The tetrapeptide, SeCys-Gly-Pro-Cys, showed glutathione peroxidase-like activity, which was almost three times higher than those of LL-glutaseleone and diphenyldiselenide. Three organoselenium compounds were added to the reaction mixture at a final concentration of 50 μM. The reaction catalyzed by the tetrapeptide was turned over every 40min. However, those catalyzed by LL-glutaselenone and diphenyldiselenide was not turned over even after 90min.

Glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys, and LL-glutaselenone was affected by

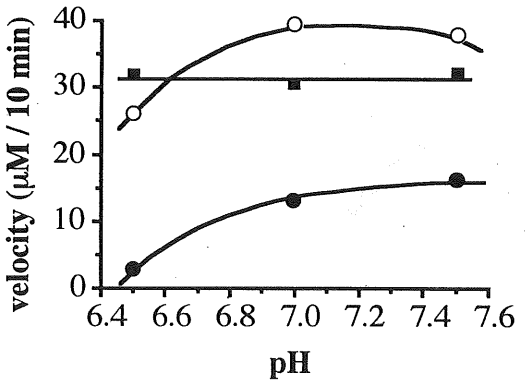

Fig. 3. Effect of pH on glutathione peroxidase-like activity of SeCys-Gly-Pro-Cys (○), glutaselenone (●) and diphenyldiselenide (■). Formation of glutathione disulfide was determined by the o-phthalaldehyde method.

pH of the assay mixture. However, disphenyldiselenide showed almost the same activity in the pH range tested (Fig. 3). Rate of decomposition of hydrogen peroxide may be influenced by dissociation: selenolate anion decomposes peroxides more efficiently than selenol. Selenol group of selenocysteine-containing peptides are alkyl selenols, whereas that of diphenyldiselenide is an aryl selenol. Effect of pH on the deprotonation is more significant for alkyl selenols than for aryl selenols.

DISCUSSION

A selenium analog of the active site sequence of thioredoxin was synthesized by a liquid phase method. The peptide bonds of the tetrapeptide were formed from cysteine to selenocysteine for the smallest loss of selenocysteine. The selenol group of L-selenocysteine was protected with *p*-methoxybenzyl group. It was also used for chemical synthesis of glutaselenone, and proved to be a good protecting group.

α -Amino group of *S*-benzyl-L-cysteine benzyl ester, L-proline, and glycine were protected with *t*-butyloxycarbonyl (Boc) group, which was selectively removed for peptide elongation. The amino group of L-selenocysteine was protected with *p*-methoxybenzyloxycarbonyl (PMZ) group. All the protecting group of fully protected tetrapeptide, including the PMZ group were removed in a final deprotection step with sodium in liquid ammnoia. This step may result in cleavage of X-proline bonds (X=amino acids), in particular in the presence of methanol or moisture (20). Such cleavage was also observed for lysyl-proline bond when *p*-toluenesulfonyl group was removed with Na/NH_3 (21). However, in the previous reports on synthesis of peptide models of thioredoxin activce site (16, 17), glycine-proline bond was not cleaved by the deprotection. In the present synthesis, the fully protected tetrapeptide was crystallized from ethylacetate.

Formation of disulfide bond between cysteine residues in polypeptide generates compact structures. Intramolecular disulfide bonds occur in peptide hormons, e. g. oxytocin and vasopressin (22), insulin (23), and snake toxins such as siamensis (24). Loop structures formed by disulfide bond may have con-

siderable flexibility when many amino acid residues occur between the two cysteine residues. However, when only a few amino acid residues occur, constrains are introduced to the loop structure.

Conformation analysis of synthetic tetrapeptides, Cys-X-Y-Cys (X, Y=Proline, Glycine, Valine, and α-aminoisobutyric acid) has been carried out (16, 17, 25-27). Pro-Y segment has a structure of reverse or β-turn (26). X-Pro also has similar reverse or β-turn conformations (27). Boc-Cys-Gly-Pro-Cys-NHMe has an all-*trans* peptide backbone (17) with two consecutive β-turns at Gly-2-Pro-3 and Pro-3-Cys-4.

Conformation of model peptides is also stabilized by two intramolecular hydrogen bonds (Cys-1-CO and Cys-4-NH, Gly-2-CO, and NHMe) (17). Intramolecular hydrogen bond was also identified in Boc-Cys-Pro-Val-Cys-NHMe (25) ; between Cys-1-CO and Cys-4-NH. Thus, the selenium containing peptide, SeCys-Gly-Pro-Cys, is also expected to have a similar consecutive β-turns in the Gly-Pro-Cys segment. A hydrogen bond between SeCys-1-CO and Cys-4-NH may also be formed and stabilize the cyclic conformation. Such a conformational constraint promotes the interaction between the selenol group and the thiol group at ends of the molecule in the reaction with peroxidase and glutathione.

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