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Biochemistry

Metalloselenonein, the selenium analogue of metallothionein: Synthesis and characterization of its complex with copper ions

(synthetic peptide/selenocysteine)

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ABSTRACT **We used an automated peptide synthesizer to produce a peptide, metalloselenonein, that contains selenocysteine residues substituted for all cysteine residues in *Neurospora crassa* copper metallothionein. Metalloselenonein binds 3 mol of Cu(I) per mol. This adduct shows a broad absorption band between 230 and 400 nm and a fluorescence band at 395 nm, which can be attributed to copper–selenolate coordination. The circular dichroism spectrum of the copper–metalloselenonein complex shows a positive band around 245 nm attributable to asymmetry in metal coordination.**

A selenocysteine residue occurs as an integral moiety in the active center of the selenium-containing enzymes such as glycine reductase, formate dehydrogenase, and glutathione peroxidase (1). The catalytic role of the selenocysteine residue is attributed to the high reactivity of the selenol group: selenols have much lower redox potentials than their sulfur counterparts (2). Thus, attention has been paid to synthesis of selenocysteine-containing polypeptides and proteins, which may have properties that the sulfur counterparts do not have (3).

Metallothioneins are low molecular weight, cysteine-rich proteins that bind various kinds of metal ions (4). The biological functions of metallothioneins have been proposed to be involved in provision of physiological metals for metalloenzymes and in storage and detoxification of heavy metals. These functions depend on high affinity for metal ions and the reactivity of cysteine residues in a metal-thiolate cluster. Here we describe the synthesis of metalloselenonein, in which all the cysteine residues in the *Neurospora crassa* copper metallothionein are replaced by selenocysteines, and its interaction with copper ions.

MATERIALS AND METHODS

β-Chloro-L-alanine was synthesized from L-serine (5); disodium diselenide was prepared by the method of Klayman and Griffin (6); L-selenocysteine was prepared from disodium diselenide and β-chloro-L-alanine (7); *Se*-(*p*-methylbenzyl)-L-selenocysteine was obtained from L-selenocysteine and α-bromo-*p*-xylene by a modification of the procedure of Erickson and Merrifield (8); *N*-(*tert*-butyloxycarbonyl)-*Se*-(*p*-methylbenzyl)-L-selenocysteine was prepared from *Se*-(*p*-methylbenzyl)-L-selenocysteine and *S*-(*tert*-butyloxycarbonyl)-4,6-dimethyl-2-thiopyrimidine by a modification of the procedure of Nagasawa *et al.* (9). Other reagents, the best grade commercially available, were used without further purification.

Metalloselenonein was synthesized in an Applied Biosystems 430A peptide synthesizer programmed as shown in Table 1. The synthesis was started on 1 g of Boc-Lys(ClZ)-OCH_2-Pam resin (0.5 mmol/g of resin; ClZ, *p*-chlorobenzyloxycarbonyl) (10). The *tert*-butyloxycarbonyl (Boc) group was used for the N^{α} terminus, and the side-chain protecting groups were as follows: Asp(OBzl), Sec(MeBzl), and Ser(OBzl) (Bzl, benzyl; MeBzl, methylbenzyl; Sec, selenocysteine). The coupling reactions were carried out with 2.0 mmol of protected amino acid. The yield in the coupling reaction was determined with ninhydrin (11).

Table 1. Solid-phase synthesis protocol

Step	Reagents and operation	Mixing time, min
1. Deprotection	60% CF_3COOH in DMF	1
2. Wash	CH_2Cl_2 (three times)	1
3. Neutralization	10% DIEA in DMF (twice)	1
4. Wash	DMF (six times)	1
5. Coupling	Symmetric anhydride method in DMF	20
6. Wash	DMF (twice),	1
	CH_2Cl_2 (four times)	1
7. Ninhydrin test		

DMF, dimethylformamide; DIEA, *N*,*N*-diisopropylethylamine.

The peptidyl resin produced (about 1.0 g) was treated with anhydrous hydrogen fluoride containing 15% anisole and 2.5% ethyl methyl sulfide at 0°C for 1 hr. After evaporation of hydrogen fluoride, the residue was washed with diethyl ether and extracted with 2 M acetic acid, and the extract was lyophilized.

The crude peptide was purified by reverse-phase high-performance liquid chromatography (HPLC). Special care was taken to avoid air oxidation during the purification procedure. All buffers were kept under a constant stream of nitrogen. Preparative separations were done with an Ultron C_{18} column (25 × 1 cm; Shinwakako, Kyoto) at a flow rate of 2.4 ml/min, whereas analytical separations were carried out on an Ultron N-C_{18} column (15 × 0.46 cm) at a flow rate of 0.8 ml/min. Both columns were programmed with a 30-min linear gradient from 5% to 50% acetonitrile in water. Trifluoroacetic acid was added at a concentration of 0.1% (vol/vol) to water and acetonitrile. Peptides were detected at 210 nm.

Amino acid analysis was performed with a Beckman 7300 amino acid analyzer. Peptides were hydrolyzed in 6 M HCl (Pierce) at 108°C in a sealed vessel under reduced pressure for 9 hr with a Waters Pico-Tag automatic acid hydrolysis system. Selenocysteine was determined after conversion into *Se*-carboxymethylselenocysteine with iodoacetic acid. The numbers of amino acid residues were calculated on the basis of a molecular weight of 2548 for metalloselenonein.

All procedures were performed in a nitrogen glove box in which oxygen concentration was kept below 10 ppm. All solutions were degassed prior to use. Metalloselenonein (390

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nmol) was incubated in 20 mM Tris·HCl buffer (pH 8.6) containing 5.85 μmol of $CuCl_2$ and 1.2 mmol of 2-mercaptoethanol at 30°C for 30 min. The solution was applied to an Asahipak GS-220 HPLC column (500 × 7.6 mm; Asahi-Kasei, Tokyo) equilibrated with 20 mM Tris·HCl buffer (pH 8.6). Absorption spectra were recorded using a Shimadzu MPS-2000 spectrophotometer in a sealed cuvette under reduced pressure. Circular dichroism spectra were recorded in a Jasco J-600 spectropolarimeter using a 1-mm cuvette at room temperature (about 20°C). Fluorescence spectra were measured with a Hitachi MPF-4 fluorescence spectrophotometer at 25°C. The copper content was determined by the method of standard additions with a Shimadzu AA-670G atomic absorption spectrophotometer equipped with a graphite furnace atomizer. Absorption was monitored at 324.8 nm with deuterium-arc background correction. We took an average of three independent determinations. All solutions used for copper analysis were passed through a Chelex (Bio-Rad) column (1 × 10 cm) to remove free copper ions and stored in polyethylene bottles.

RESULTS

We synthesized metalloselenonein by the solid-phase method (12) with an automated peptide synthesizer. Its sequence is H-Gly-Asp-Sec-Gly-Sec-Ser-Gly-Ala-Ser-Ser-Sec-Asn-Sec-Gly-Ser-Gly-Sec-Ser-Sec-Ser-Asn-Sec-Gly-Ser-Lys-OH, where Sec means selenocysteine. The amino terminus and the side chains of amino acids were protected with acid-labile groups: amino terminus, *tert*-butyloxycarbonyl group; aspartic acid and serine, benzyl group. However, no masking group for the selenol of selenocysteine has been developed. We used a *p*-methylbenzyl group to protect the thiol of cysteine. Several techniques have been developed to reduce side reactions during the peptide synthesis and were used in the present synthesis. For example, Pam resin, which has acid-stable linkage to the resin support, was used to reduce loss of premature peptides during acidolytic deprotection cycles (13, 14). We used symmetrical anhydride coupling in dimethylformamide for 20 min to obtain satisfactory coupling yields of >99% for all coupling steps except for the step of Ala^8-Ser^9 synthesis: only 96.7% coupling yields were achieved.

Metalloselenonein synthesized as described above was purified by preparative HPLC after reduction with $NaBH_4$. The final preparation of metalloselenonein was eluted as a single symmetrical peak upon analytical HPLC (Fig. 1), and was found to be homogeneous by amino acid analysis of the acid hydrolysates (Table 2). The overall recovery in the preparative HPLC was about 2%. The low yield is probably attributable to the irreversible adsorption of metalloselenonein to the HPLC column. Selenocysteine is oxidized to give selenocysteic acid by performic acid oxidation in the same manner as cysteine. However, selenocysteic acid decomposes during acid hydrolysis of proteins. Therefore, we analyzed selenocysteine in the form of *Se*-carboxymethylselenocysteine after alkylation with iodoacetic acid (15).

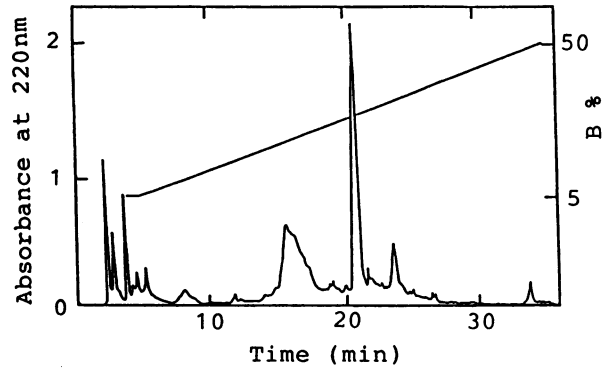

FIG. 1. Reverse-phase HPLC profile of metalloselenonein. The crude peptide was applied to a column equilibrated with 0.1% trifluoroacetic acid in 5% acetonitrile. The column was washed with the same buffer at a flow rate of 0.8 ml/min followed by elution with a linear gradient between 0.1% trifluoroacetic acid in water and 50% acetonitrile containing 0.1% trifluoroacetic acid (solvent B).

Table 2. Amino acid analysis of metalloselenonein

Amino acid	Number of residues	
	Calculated	Found
Ala	1	1.0
Asx	3	2.4
Gly	6	6.0
Lys	1	0.7
Sec	7	7.1*
Ser	7	6.6

*Selenocysteine (Sec) was determined as *Se*-carboxymethylselenocysteine after alkylation with iodoacetic acid.

Selenium compounds are generally highly susceptible to oxidative degradation. However, we have found that the selenol of metalloselenonein is oxidized much more slowly than that of selenocysteine. When metalloselenonein (0.4 mM) was incubated in 0.1 M Tris·HCl buffer (pH 8.0) at 37°C for 2 hr, all of the initial selenocysteine residues remained intact, whereas 52% of free selenocysteine was oxidized to selenocysteine under the same conditions.

The metalloselenonein complex with Cu(I) was isolated by HPLC with an Asahipak GS-220 gel filtration column. The first major fraction ($M_r \approx 2700$) contained 3 mol of copper per mol. The absorption spectrum of the copper complex was characterized by a broad absorption band between 230 and 400 nm with a shoulder around 260 nm (Fig. 2). However, metalloselenonein itself showed no absorption above 260 nm. Therefore, the broad absorption band around 260 nm observed for the copper complex is most probably attributable to a copper–selenolate complex. The circular dichroism of the copper–metalloselenonein complex revealed a negative band at 220 nm and a positive band at 245 nm (Fig. 2). Metalloselenonein itself showed only a negative band at 220

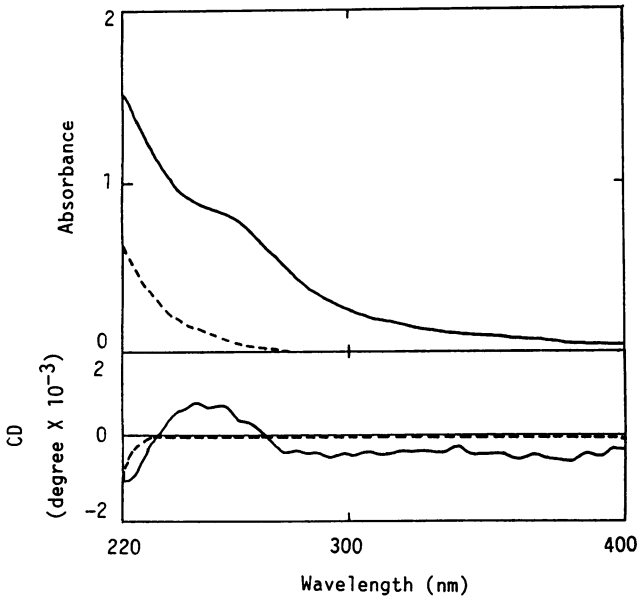

FIG. 2. Absorption spectra and circular dichroism spectra of metalloselenonein. The solid lines show the spectra of the copper complex of metalloselenonein in 20 mM Tris·HCl buffer (pH 8.6), and the dotted lines show the spectra of metalloselenonein in 50 mM HCl.

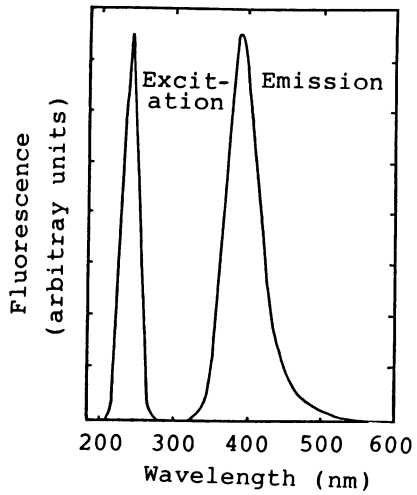

FIG. 3. Fluorescence spectrum of copper–metalloselenonein complex.

nm, which is attributable to an amide transition. Therefore, the positive band at 245 nm shows asymmetry in the copper–selenolate coordination. The copper–metalloselenonein complex showed an emission spectrum with a maximum at 395 nm when it was excited at 245 nm (Fig. 3). Addition of 1 M HCl to the solution containing the complex led to complete disappearance of not only the absorption band around 260 nm but also the fluorescence band at 395 nm. This indicates displacement of copper with protons in the complex upon addition of HCl. Furthermore, both electron-spectral bands were lost when the complex was oxidized in air.

DISCUSSION

The selenium analogues of various sulfur-containing peptides and proteins have been prepared previously. The acid-labile sulfur in several iron-sulfur proteins was replaced by selenium: Fe_2S_2 putidaredoxin (16) and parsley ferredoxin (17); Fe_4S_4 ferredoxin from *Clostridium pasteurianum* (18). A derivative of β-galactosidase whose methionine residues were extensively replaced by selenomethionine residues was obtained from a selenium-resistant mutant of *Escherichia coli* grown on sodium selenate (27). Selenium analogues of glutathione (19), oxytocin (20), and somatostatin (21) were synthesized by the manual liquid-phase method (22), which is much less efficient than the automatic solid-phase method based on symmetric anhydride coupling chemistry. By using an automated peptide synthesizer according to the standard protocol, we have achieved the synthesis of a peptide (metalloselenonein) that contains selenocysteine residues in place of all cysteine residues in copper metallothionein of *N. crassa*.

The copper–metalloselenonein complex showed a broad absorption band between 230 and 400 nm with a shoulder around 260 nm. This is probably attributable to a Se*-Cu(I) transition in the copper–selenolate complex. The circular dichroism spectrum of the Cu–metalloselenonein complex showed a positive band around 245 nm. This indicates asymmetry in the copper–selenolate complex because the band is absent from the spectrum of free metalloselenonein. The fluorescence spectrum of the copper–metalloselenonein complex is also attributable to transitions of a charge-transfer type of the Cu(I)–selenolate complex. Metalloselenonein binds three copper ions per molecule, in contrast to the native and reconstituted metallothionein of *N. crassa*, which binds six copper ions per molecule (23, 24). In the copper complex of metallothionein, copper atoms are contained in a compact polynuclear cluster with the thiolate of the cysteine residues (25). The difference in ionic radius between sulfur and selenium (ref. 26 and references therein) most likely accounts for the observed difference in the coordination mode between the two copper complexes.

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1. Stadtman, T. C. (1980) *Annu. Rev. Biochem.* **49,** 93–110.
2. Williams, R. J. P. (1978) in *New Trends in Bioinorganic Chemistry* (Academic, New York), pp. 253–260.
3. Wu, Z. P. & Hilvert, D. (1989) *J. Am. Chem. Soc.* **111,** 4513–4514.
4. Nordberg, M. & Kojima, Y. (1979) in *Metallothionein*, eds. Kagi, J. H. R. & Nordberg, M. (Birkhaeuser, Basel), pp. 41–117.
5. Walsh, C., Schonbrumm, A. & Abels, R. P. (1971) *J. Biol. Chem.* **246,** 6855–6866.
6. Klayman, D. J. & Griffin, S. T. (1973) *J. Am. Chem. Soc.* **95,** 197–199.
7. Chocat, P., Esaki, N., Tanaka, H. & Soda, K. (1985) *Anal. Biochem.* **148,** 485–489.
8. Erickson, B. W. & Merrifield, R. B. (1973) *J. Am. Chem. Soc.* **95,** 3757–3763.
9. Nagasawa, T., Kuroiwa, K., Narita, K. & Isowa, Y. (1973) *Bull. Chem. Soc. Jpn.* **46,** 1269–1272.
10. Mitchell, A. R., Kent, S. B. H., Engelhard, M. & Merrifield, R. B. (1978) *J. Org. Chem.* **43,** 284–285.
11. Sarin, V. K., Kent, S. B. H., Tam, J. P. & Merrifield, R. B. (1981) *Anal. Biochem.* **117,** 147–157.
12. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85,** 2149–2154.
13. Merrifield, R. B. (1986) *Science* **232,** 341–347.
14. Mitchell, A. R., Erickson, B. W., Ryabtsev, M. N., Hodges, R. S. & Merrifield, R. B. (1976) *J. Am. Chem. Soc.* **98,** 7357–7362.
15. Cone, J. E., Martin del Rio, R., Davis, J. N. & Stadtman, T. C. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2659–2663.
16. Fee, J. A. & Palmer, G. (1971) *Biochim. Biophys. Acta* **245,** 175–195.
17. Wilson, G. S., Tsibris, J. C. M. & Gunsalus, I. C. (1973) *J. Biol. Chem.* **248,** 6059–6061.
18. Meyer, J. & Moulis, M. M. (1981) *Biochem. Biophys. Res. Commun.* **103,** 667–673.
19. Theodoropoulos, D., Schwartz, I. L. & Waltoder, R. (1967) *Biochemistry* **6,** 3927–3932.
20. Walter, R. & Chan, W. Y. (1967) *J. Am. Chem. Soc.* **89,** 3892–3898.
21. Hartrodt, B., Neubert, K., Bierwolf, B., Blech, W. & Jakubke, H.-P. (1980) *Tetrahedron Lett.* **21,** 2393–2396.
22. Popenoe, E. A. & du Vigneaud, V. (1953) *J. Am. Chem. Soc.* **75,** 4879–4880.
23. Lerch, K. (1980) *Nature (London)* **284,** 368–370.
24. Kull, F. J., Reed, M. F., Elgren, T. E., Ciardelli, T. L. & Wilcox, D. E. (1990) *J. Am. Chem. Soc.* **112,** 2291–2298.
25. Smith, T. A., Lerch, K. & Hodgson, K. O. (1986) *Inorg. Chem.* **25,** 4677–4680.
26. Klayman, D. L. & Günther, W. H. H. (1973) *Organic Selenium Compounds: Their Chemistry and Biology* (Wiley, New York).
27. Coch, E. H. & Green, R. C. (1971) *Biochim. Biophys. Acta* **230,** 223–236.