Loss of Selenium from Selenoproteins: Conversion of Selenocysteine to Dehydroalanine In Vitro

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Characterization of reduced and alkylated rat selenoprotein P by mass spectrometry yielded selenopeptides from which one or more selenium atoms were missing. Predicted selenopeptide mass peaks were accompanied by peaks corresponding to the conversion of one or more selenocysteine residues to dehydroalanine(s). Experiments were carried out to determine whether this loss of selenium occurred in vitro. A selenopeptide was isolated that contained two selenocysteine residues that were both in selenide-sulfide linkages with cysteine residues. After the peptide had been reduced and alkylated, in addition to the predicted mass peak with both selenocysteine residues present, two mass peaks were detected at positions expected for conversion of one and two selenocysteine residues of this selenopeptide to dehydroalanine residues, which was confirmed by tandem mass spectrometry. Similar findings were obtained from a study of another selenoprotein, rat plasma glutathione peroxidase. These results indicate that selenium atoms are lost from selenoproteins during purification and characterization. The loss of selenium from selenoproteins is probably through the mechanism of oxidation of selenocysteine residue to selenoxide followed by *syn*-β-elimination of selenenic acid during sample processing. (J Am Soc Mass Spectrom 2003, 14, 593–600) © 2003 American Society for Mass Spectrometry

Selenoprotein P is a heparin-binding protein [1] that circulates in the plasma as several isoforms [2]. The full-length isoform contains ten selenocysteine residues in its primary structure. During characterization studies of this isoform using mass spectrometry, we observed selenopeptides that had lost some of their selenium atoms. This observation raised the possibilities (1) that tissues can remove selenium atoms from circulating selenoprotein P, possibly as part of a selenium transport mechanism [3, 4], and/or (2) that selenium is lost as a consequence of the isolation procedure.

We carried out several experiments to characterize this removal of selenium from selenoprotein P and to assess its occurrence in plasma glutathione peroxidase. Those experiments indicated that loss of selenium atoms occurs in vitro and is not a result of a selenium transport function of the protein.

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Experimental

Materials

Matrix, α-cyano-4-hydroxycinnamic acid (CHCA), was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ammonium bicarbonate, iodoacetamide, dithiothreitol (DTT), and peptide standards used for mass calibration, des-Arg^1-bradykinin and bovine insulin, were purchased from Sigma Chemical Co. (St. Louis, MO). N-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-treated porcine trypsin was obtained from Promega (Madison, WI). Endoproteinase Glu-C was obtained from Boehringer Mannheim (Indianapolis, IN). The deglycosylation kit, which contained *N*-glycosidase F (PNGase F), endo-*O*-glycosidase, and Sialidase A, was purchased from ProZyme (San Leandro, CA).

Selenoprotein P and Glutathione Peroxidase Purification

Selenoprotein P was purified and its full-length isoform was separated as described previously [2, 5]. Glutathione peroxidase was purified from rat plasma that had been depleted of selenoprotein P by passage over an

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immunoaffinity column [5]. The pellet from a 30 to 50% ammonium sulfate precipitation was dissolved in buffer A (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM GSH) and dialyzed overnight against buffer A. It was then applied to a DEAE cellulose column and eluted with a linear NaCl gradient from 0 to 0.3 M in buffer B (200 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM GSH). Fractions containing glutathione peroxidase activity were combined and concentrated and passed over a Sephacryl 300 column in buffer A. Fractions containing glutathione peroxidase activity were combined, concentrated, and buffer exchanged to buffer C (0.1 M Tris-HCl pH 7.2, 0.2 M ammonium sulfate, 5 mM EDTA, 1 mM GSH). The sample was then applied to a 5 ml HiTrap phenyl sepharose column from Pharmacia (Piscataway, NJ) equilibrated with buffer C. The glutathione peroxidase activity was eluted with a linear gradient composed of equal volumes of buffer C and buffer D (0.08 M Tris-HCl pH 7.2, 0.16 M ammonium sulfate, 5 mM EDTA, 1 mM GSH). Fractions containing glutathione peroxidase activity were combined, concentrated, and applied to a HiTrap Blue column (Pharmacia, Piscataway, NJ) equilibrated with buffer D. Glutathione peroxidase was not retained by this column and appeared in the flow through fractions.

Selenoprotein P Deglycosylation and Enzymatic Digestion of Selenoprotein P and Glutathione Peroxidase

Deglycosylation of the selenoprotein P full-length isoform was accomplished using *N*-glycosidase F, endo-*O*-glycosidase, and sialidase A [2]. Trypsin digestion of the deglycosylated and unmodified selenoprotein P (approximately 10 μg) was carried out as previously described [2]. Purified glutathione peroxidase (approximately 10 μg) was digested using Glu-C according to the protocol used for selenoprotein P [2].

Reverse Phase High Performance Liquid Chromatography (HPLC)

Digestion mixtures were separated on a Vydac microbore C18 column (2.1 × 250 mm) (Vydac, Hesperia, CA) using an HP 1100 HPLC system (Hewlett-Packard Co., Wilmington, DE). The mobile phase consisted of (1) 0.1% trifluoroacetic acid in H_2O and (2) 94.9% acetonitrile, 5% H_2O, and 0.1% trifluoroacetic acid. The combined flow was maintained at 0.2 ml/min. The separation was monitored with a UV detector at 214 nm. The gradient elution conditions were the same as used before [2]. UV monitored peptide peaks were manually collected in microcentrifuge tubes. The HPLC fractions were lyophilized dry and reconstituted in 40 μL of 50% acetonitrile/50% H_2O/0.1% trifluoroacetic acid for mass spectrometry analysis.

Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). The samples were prepared on a stainless steel plate by the dried-droplet method using CHCA as matrix. CHCA was dissolved in 50:49.9:0.1 of water:acetonitrile:trifluoroacetic acid at a concentration of 10 mg/ml. Mass calibration was accomplished using des-Arg^1-bradykinin (MW 903.46) and bovine insulin (MW 5733.58).

Nanoelectrospray MS/MS was performed on a QSTAR quadrupole time-of-flight (Q-TOF) mass spectrometer (PE Sciex, Concord, Ontario, Canada) equipped with a nanoelectrospray ion source (Protana A/S, Odense, Denmark). The peptide sample (approximately 5 pmol/μL) of 2 μL was loaded into the metal-coated glass capillary (Protana A/S, Odense, Denmark). A stable spray was obtained at spray voltage of 800-900 Volts. Positive ion mass spectra were acquired in the profile mode with an ion count accumulation time of 1 sec at a TOF pulse frequency of 6.99 kHz. Peptide sequences were obtained from product ion spectra acquired for ions selected with Q1 and dissociated with collision energy of 30 to 45 eV. The collision gas was set at 6 (arbitrary units).

Results

Selenopeptide Shows Loss of Selenium

During the characterization of full-length selenoprotein P by mass spectrometry, peptides containing one to four selenocysteine residues were generated by proteolytic digestion [2]. Figure 1 shows the MALDI-TOF mass spectrum of such a tryptic peptide (SPPAAUHSQHVSPTEASPNUSUNNK, residues 333–357, U = selenocysteine) that contains three selenocysteine residues. The signal at *m/z* 2880.0 corresponds to the expected selenopeptide with all the three selenocysteine residues alkylated by iodoacetamide. Besides this expected peptide ion signal, additional ion signals at *m/z* 2742.5 and 2604.7 are observed. It is significant to note that all three ions have selenium isotope patterns when studied under reflector MALDI-TOF conditions (data not shown). The ion at *m/z* 2880.0 has an isotope pattern characteristic of three selenium atoms, while the ion at *m/z* 2742.5 has a pattern characteristic of two selenium atoms and the ion at *m/z* 2604.7 has a pattern characteristic of one selenium atom (see on-line supplement of reference 2 for appearance of these patterns). These results imply that the ions at *m/z* 2742.5 and 2604.7 correspond to peptides arising from sequential loss of selenium atoms from the expected selenopeptide. The ion at *m/z* 2742.5 corresponds to the peptide with conversion of one selenocysteine to dehydroalanine, while the ions at *m/z* 2604.7 corresponds to the peptide with conversion of two selenocysteines to dehydroala-

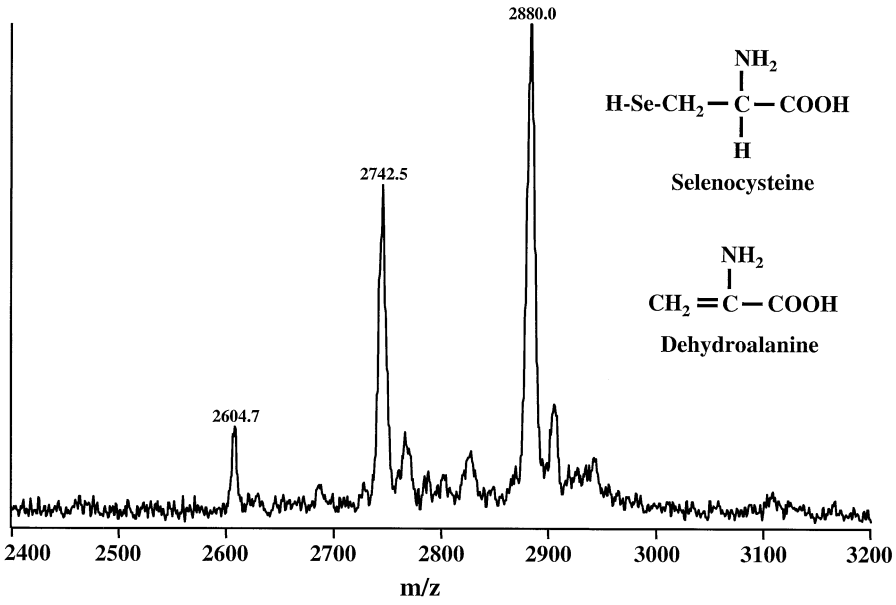

Figure 1. MALDI-TOF mass spectrum of the tryptic peptide from selenoprotein P, ^{333}SPPAAUHSQHVSPTEASPNUSUNNK^{357}. The protein was reduced by DTT, alkylated by iodoacetamide, and deglycosylated with *N*-glycosidase F (PNGase F), endo-*O*-glycosidase, and sialidase A before it was digested with trypsin.

nines. As the number of selenium atoms lost increases, the corresponding ion signal drops sharply. We did not observe the peptide corresponding to the conversion of all three selenocysteine residues to dehydroalanine. Conversion of selenocysteine to dehydroalanine (mass loss of 81) together with the loss of acetamide adduct (mass of 57, dehydroalanine cannot be alkylated by iodoacetamide), would count for the mass loss of 138. This is consistent with the additional ions appearing at intervals of 138 Da below the expected selenopeptide ion.

Selenium Lost in Vitro

The results of Figure 1 are consistent with in vivo or in vitro removal of selenium atoms from selenoprotein P. An experiment was carried out to determine whether removal of selenium atoms occurs during analysis of the protein. To assess removal in vitro, we isolated a tryptic peptide from full-length isoform that had been neither reduced nor alkylated before digestion. This peptide consists of amino acid residues 298–324 (SGSAITUQCAENLPSLCSUQGLFAEEK) and contains two selenocysteine residues. This peptide has been shown to contain two selenide–sulfide (Se–S) bonds from secys304 to cys314 and from cys306 to secys316 (Ma, S.; Hill, K. E.; Burk, R. F.; Caprioli, R. M., manuscript in preparation). As shown in Figure 2a, the ion at *m/z* 2881.4 corresponds to this double Se–S linked peptide. There are no ion signals that correspond to the conversion of selenocysteine residues to dehydroalanine. This suggests that selenocysteine residues involved in selenide-sulfide linkages are not susceptible to loss of the selenium atom.

This peptide was then reduced by DTT and alkylated by iodoacetamide. The mass spectrum in Figure 2b contains an ion at *m/z* 3113.0, corresponding to the peptide with its two selenocysteine residues and two cysteine residues reduced and alkylated by iodoacetamide. The ion at *m/z* 2997.0 most likely resulted from the peptide with one selenide–sulfide bond being reduced and alkylated while the other selenide–sulfide bond remained intact. The ion at *m/z* 3055.6 corresponds to the peptide with both selenide-sulfide bonds reduced, but with one selenocysteine or cysteine residue remaining free. Under these conditions, additional ions at *m/z* 2975.6 and 2837.0 are observed. Those ions correspond to the conversion of one and two selenocysteine residues to dehydroalanine. This result indicates that the loss of selenium can occur in vitro.

The sequences of the peptides arising from conversion of one and two selenocysteine residues to dehydroalanine(s) were confirmed by nanoelectrospray ionization MS/MS. Since peptide 298–324 contains selenocysteine residues at positions 304 and 316, conversion of either of these residues to dehydroalanine would yield a peptide with loss of 1 selenium atom. Therefore, the ion at *m/z* 2975.6 should represent two peptides. The tandem mass spectrum was obtained from the fragmentation of these two isobaric peptides as shown in Figure 3a. The y- and b-ion series were used to label the fragments from the peptide with conversion of the selenocysteine residue at position 304 to dehydroalanine, while Y- and B-ion series were used for the fragments from the peptide with conversion of the selenocysteine residue at position 316 to dehydroalanine. Since the only differences between these two peptides would be for residues 304 and 316 to be

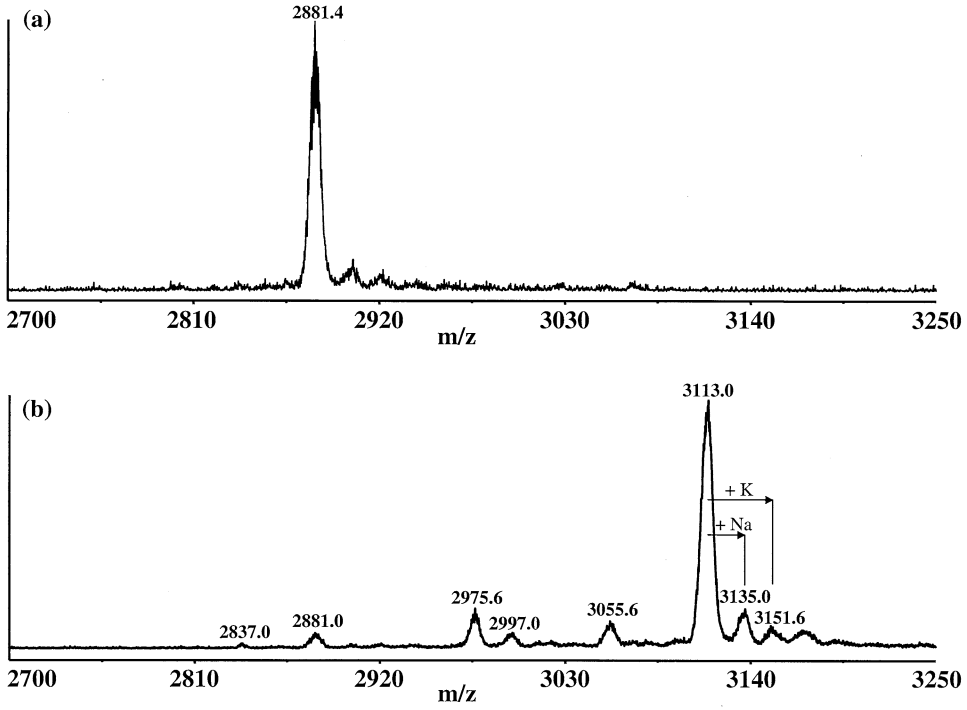

Figure 2. (**a**) MALDI-TOF mass spectrum of the tryptic peptide from unmodified selenoprotein P, ^{298}SGSAITUQCAENLPSLCSUQGLFAEEK^{324}, which contains two selenide-sulfide (Se–S) linkages of secys304 to cys314 and cys306 to secys316. (**b**) MALDI-TOF mass spectrum of the same peptide after reduction with DTT and alkylation with iodoacetamide.

either selenocysteine or dehydroalanine, the fragments of these two peptides should only be different at y_9^+ to y_{20}^+ and at b_7^+ to b_{18}^+ with a difference of 138 Da between the b_n^+ and B_n^+ (n = 7–18) or y_m^+ and Y_m^+ (m = 9–20) ion series. For example, y_{14}^+ arising from the cleavage at the N-terminus of proline 311 is 138 Da greater than Y_{14}^+ ion, while its corresponding b_{13}^+ ion is 138 Da lower than B_{13}^+ (Figure 3a). Figure 3b shows the MS/MS spectrum of the peptide after loss of both selenium atoms. These fragmentation patterns (Figure 3a and b) confirm that the only differences among the peptides at *m/z* 3113.0, 2975.6, and 2837.0 are for residues 304 and 316 to be either selenocysteine or dehydroalanine.

Selenium Lost from Glutathione Peroxidase

Loss of selenium atoms from selenoprotein P in vitro allows the prediction that loss might also occur from other selenoproteins. We studied glutathione peroxidase, which had been purified from rat plasma. This selenoprotein is a homotetramer that contains a selenocysteine at residue 54 of each subunit [6].

The protein was reduced, alkylated, and digested using Glu-C. The peptide mixture was then separated by C18 reverse phase HPLC. Figure 4 shows the MALDI mass spectrum of one of the HPLC fractions that contains major ions at *m/z* 2836.5 and 2505.3. These mass peaks correspond to peptides LGPFGLVILGFPCNQFGKQEPGENSE (residues 70–95), and LGPFGLV ILGFPCNQFGKQEPGE (residues 70–92). The insert spectrum shows the broad up range from *m/z* 3300 to 3580. The ion at *m/z* 3513.2 corresponds to the selenopeptide, YIPFKQYAGKYILFVNVASYUGLTDQYLE (residues 34–62). The ion at *m/z* 3374.9 corresponds to the mass of the same peptide with the selenocysteine at residue 54 converted to dehydroalanine. The MS/MS spectrum shown in Figure 5a confirms the selenopeptide sequence, while the MS/MS spectrum in Figure 5b confirms the peptide sequence after the conversion of selenocysteine to dehydroalanine at residue 54. The results of this experiment clearly indicate that the phenomenon of selenium atom loss is not restricted to selenoprotein P.

Discussion

Several studies have indicated that selenoprotein P serves to transport or distribute selenium to various tissues [3]. Therefore, the mechanism by which the element is removed from the protein for uptake and utilization by cells would be an important part of its transport mechanism. During the course of mass spectrometry characterization studies [2], we observed peptides that had selenium atoms missing (Figure 1). We considered that removal of these selenium atoms might be part of the selenium transport mechanism. To assess this possibility, we carried out detailed experiments using the purified protein.

These mass spectrometry experiments demonstrate that selenium atoms are removed from selenopeptides

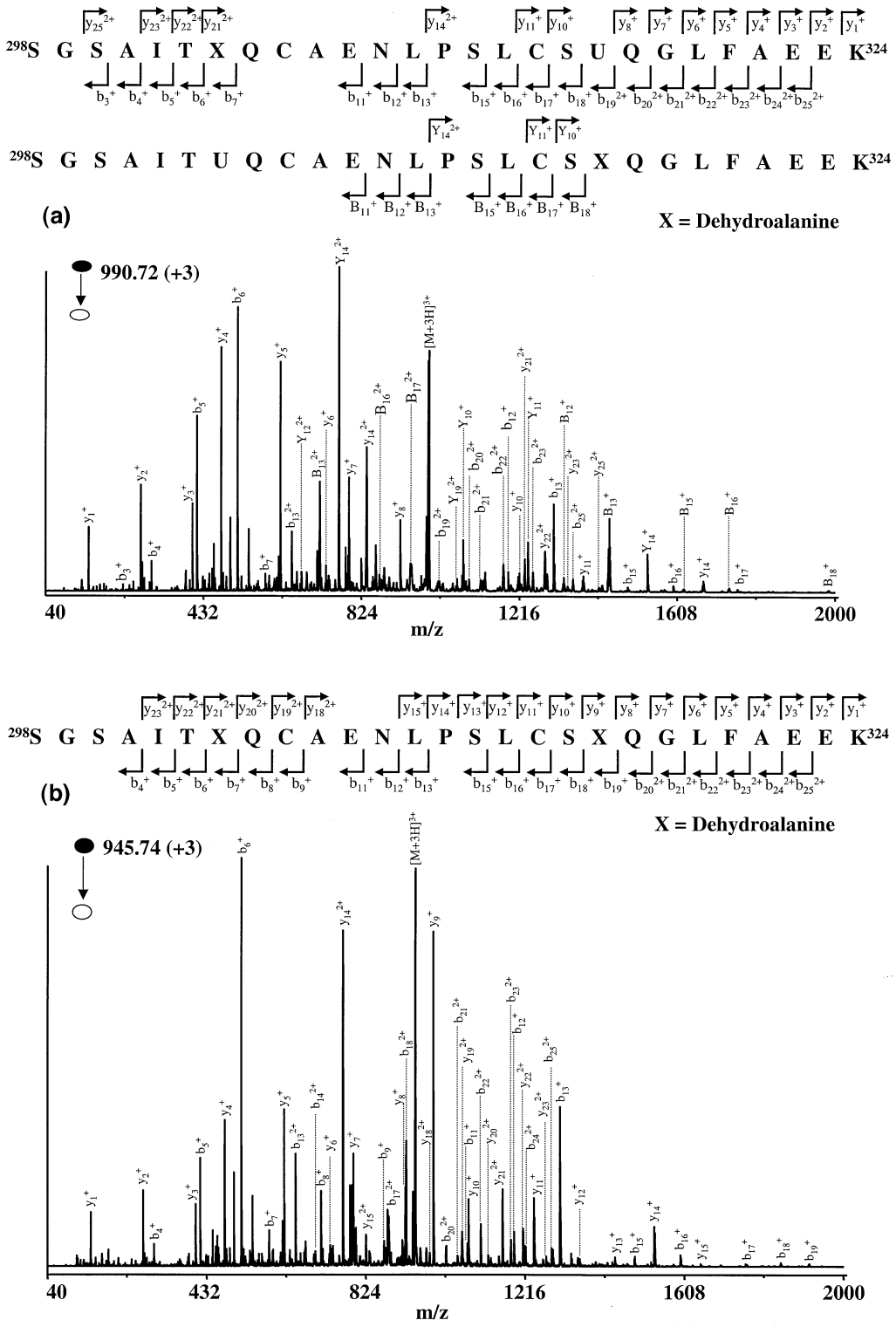

Figure 3. MS/MS spectra of the peptide (residues 298–324) (**a**) after conversion of one selenocysteine (either residue 304 or 316) to dehydroalanine and (**b**) after conversion of both selenocysteine residues at 304 and 316 to dehydroalanine residues.

in vitro. They further demonstrate that this removal is not caused by in-source fragmentation during the MALDI process because the peptides resulting from loss of selenium were also observed when electrospray ionization was employed. Also, MS/MS experiments of the selenopeptide did not show a fragment ion corresponding to loss of selenium. Thus, the loss of selenium appears to occur during the purification and/or modi-

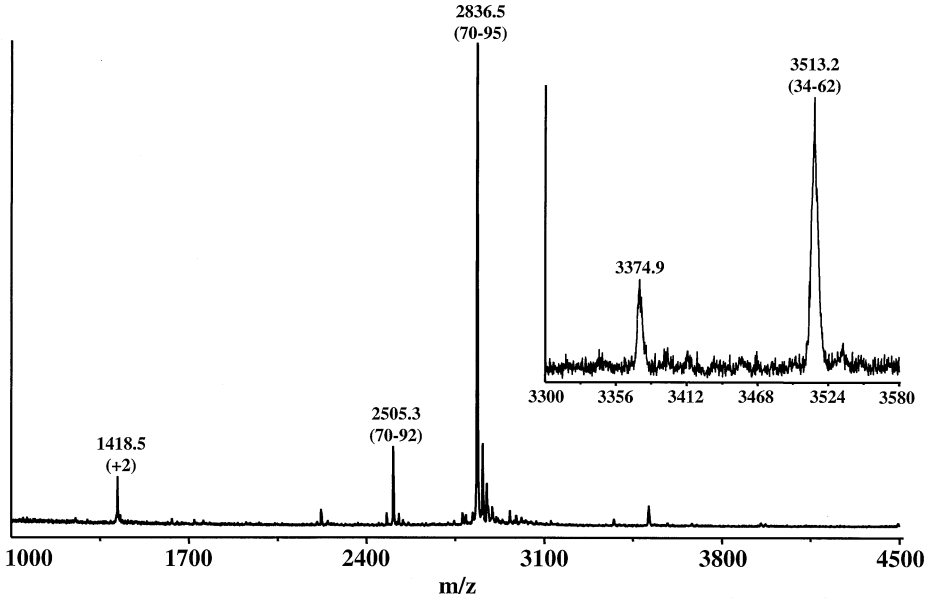

Figure 4. MALDI-TOF mass spectrum of the HPLC separated peptide from Glu-C digestion of glutathione peroxidase, which shows ion signals at 2505.3 (residues 70–92), 2836.5 (residues 70–95), and 3513.2 (residues 34–62). The ion at *m/z* 3374.9 corresponds to the peptide of residues 34–62 after the conversion of selenocysteine 54 to dehydroalanine.

fication of the protein. Analyses of purified selenoproteins for selenium content have sometimes yielded results that were lower than expected [7]. Such results might reflect selenium losses during preparation of the proteins for analysis.

There is precedent for conversion of selenocysteine to alanine. Twenty years ago, an enzyme was described in rat liver that converted free selenocysteine to alanine and elemental selenium [8]. This enzyme, selenocysteine β-lyase, has been studied further and shown to be present in the cytosolic fraction of mouse tissues—principally in liver, kidney, and testis [9]. Those are tissues that synthesize selenoproteins very actively and selenocysteine β-lyase apparently supplies selenium in them for this process.

It seems unlikely that selenocysteine β-lyase could be involved in the removal of selenium from selenoprotein P. The enzyme is intracellular and selenoprotein P is extracellular. The enzyme utilizes free selenocysteine as a substrate and selenoprotein P has the amino acid in its primary structure. Thus, we conclude that this known biological mechanism for catabolism of selenocysteine would not remove selenium atoms from selenoprotein P.

There is renewed interest in β-elimination reactions in seleno-amino acids because of their potential to generate alkyl selenols that might have cancer chemopreventive properties [10, 11]. While studies on this have focused on intracellular processes, it is theoretically possible that extracellular β-elimination of selenium atoms might occur and release selenium from extracellular selenoproteins. This possibility needs to be further investigated.

The findings of this study lead to the conclusion that loss of selenium atoms from selenoprotein P occurs in vitro. Loss of selenium also occurs for glutathione peroxidase from rat plasma, mammalian thioredoxin reductase [12], and glutathione peroxidase from chlamydomonas reinhardtii [13]. Therefore, loss of selenium needs to be considered a possibility in studies of all selenoproteins.

The mechanism of losing selenium from selenoproteins is most likely through oxidation of selenocysteine to selenoxide followed by *syn*-β-elimination of selenenic acid initiated by intramolecular abstraction of the β-proton during sample processing. This mechanism is commonly used to generate unsaturated compounds in synthetic organic chemistry [14], and most recently, has been applied to synthesis of dehydroalanine residues within peptides [15, 16]. A similar mechanism was also proposed as a bio-activation pathway for pyruvate formation from the selenocysteine *Se*-conjugates in rat liver microsomes. Oxidation of selenocysteine *Se*-conjugates by flavin-containing monooxygenases resulted in spontaneous β-elimination of selenenic acid, leading to the formation of pyruvate and ammonia [17].

Amino acid residues containing thioethers are easily oxidized during protein purification, derivatization, and/or digestion. For instance, oxidation of methionine residues in proteins during SDS-PAGE was commonly observed due to residual persulfate in polyacrylamide [18]. Chemical oxidation of methionine was also found to occur during capillary zone electrophoresis with electrospray mass spectrometry analysis as a result of the electrolysis of water during electrophoresis and at the electrospray interface [19, 20]. Alkylated cysteine residues were also found in their oxidized form during or subsequent to protein digestion [21].

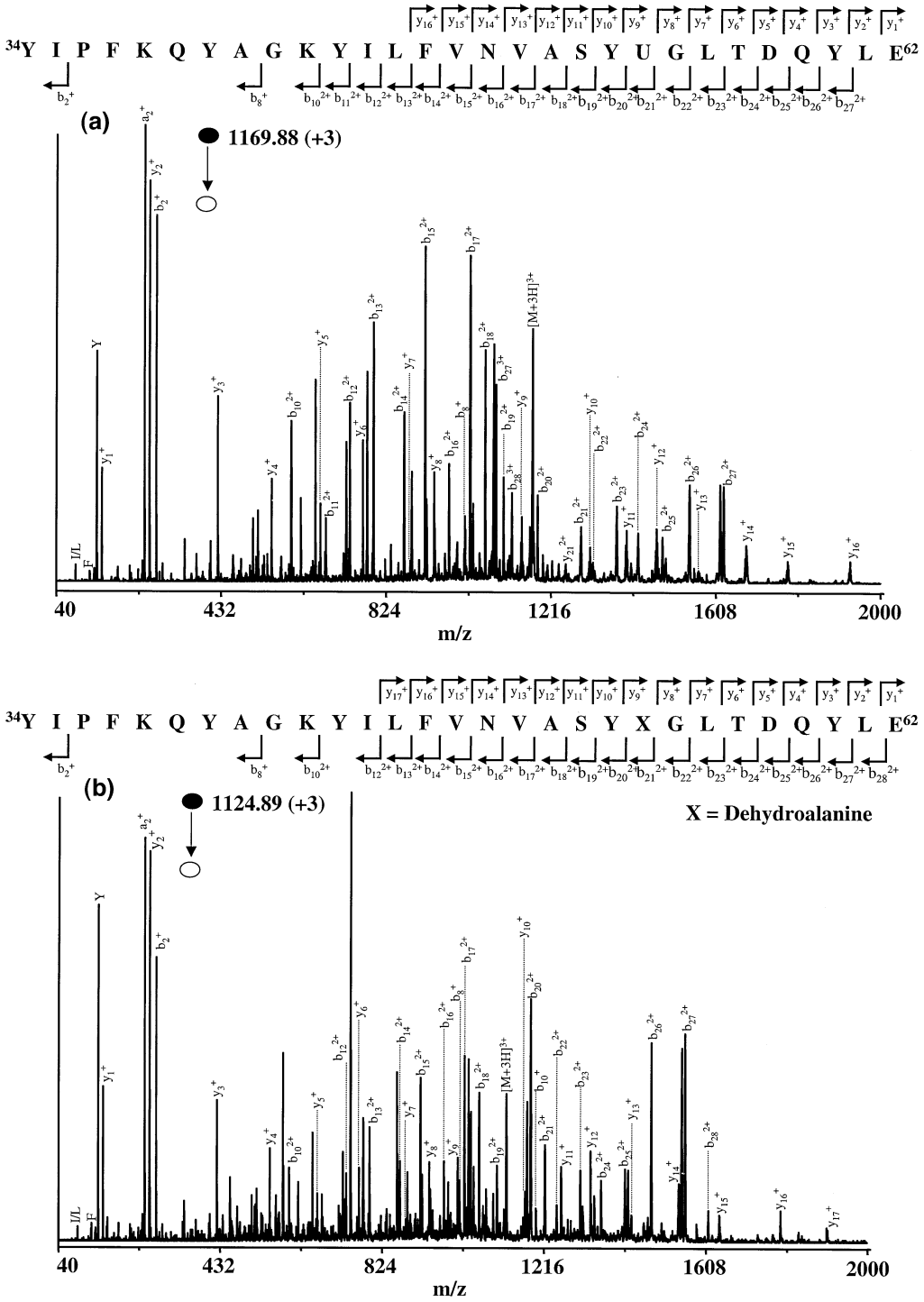

Figure 5. Tandem mass spectra of the selenopeptide (residues 34–62) of glutathione peroxidase shown in Figure 4. (**a**) MS/MS spectrum of the peptide containing selenocysteine obtained from the fragmentation of the triply charged ion at *m/z* 1169.88. (**b**) MS/MS spectrum of the same peptide after conversion of selenocysteine to dehydroalanine obtained from the fragmentation of the triply charged ion at *m/z* 1124.89.

Selenoethers are generally much more prone to oxidation to the corresponding selenoxides than are thioethers to the corresponding sulfoxides. The observation that selenoxides from selenocysteine residues undergo *syn*-β-elimination of selenenic acid more easily than cysteine/methionine-derived sulfoxides can be explained from the weaker bond strength of the C–Se bond (234 kJ/mol) versus the C–S bond (272 kJ/mol) [22] and/or a more facile β-proton abstraction of the selenocysteine moiety [23].

Conclusion

The loss of selenium from rat selenoprotein P and glutathione peroxidase resulted from the conversion of selenocysteine residues to dehydroalanine residues and was found to occur during protein purification, derivatization, and/or digestion. The mechanism of selenium loss from selenoproteins is probably the oxidation of selenocysteine to selenoxide followed by *syn*-β-elimination of selenenic acid, resulting in the formation of a dehydroalanine residue in the protein/peptide sequence. This phenomenon needs to be considered to be a possibility in studies of all selenoproteins.

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References

1. Hondal, R. J.; Ma, S.; Caprioli, R. M.; Hill, K. E.; Burk, R. F. *J. Biol. Chem.* **2001,** *276*, 15823–15831.
2. Ma, S.; Hill, K. E.; Caprioli, R. M.; Burk, R. F. *J. Biol. Chem.* **2002,** *277*, 12749–12754.
3. Burk, R. F.; Hill, K. E.; Read, R.; Bellew, T. *Am. J. Physiol.* **1991,** *261*, E26–E30.
4. Motsenbocker M. A.; Tappel A. L.; Biochim. Biophys. Acta **1982,** *719*, 147–152.
5. Chittum, H. S.; Himeno, S.; Hill, K. E.; Burk, R. F. *Arch. Biochem. Biophys.* **1996,** *325*, 124–128.
6. Yoshimura, S.; Watanabe, K.; Suemizu, H.; Onozawa, T.; Mizoguchi, J.; Tsuda, K.; Hatta, H.; Moriuchi, T. *J. Biochem.* **1991,** *109*, 918–923.
7. Read, R.; Bellew, T.; Yang, J.-G.; Hill, K. E.; Palmer, I. S.; Burk, R. F. *J. Biol. Chem.* **1990,** *265*, 17899–17905.
8. Esaki, N.; Nakamura, T.; Tanaka, H.; Suzuki, T.; Morino, Y.; Soda, K. *Biochemistry* **1981,** *20*, 4492–4496.
9. Mihara, H.; Kurihara, T.; Watanabe, T.; Yoshimura, T.; Esaki, N. *J. Biol. Chem.* **2000,** *275*, 6195–6200.
10. Ip, C.; Zhu, Z.; Thompson, H. J.; Lisk, D.; Ganther, H. E. *Anticancer Res.* **1999,** *19*, 2875–2880.
11. Rooseboom, M.; Vermeulen, N. P. E.; van Hemert, N.; Commandeur, J. N. M. *Chem. Res. Toxicol.* **2001,** *14*, 996–1005.
12. Nordberg, J.; Zhong, L.; Holmgren, A.; Arner, E. S. J. *J. Biol. Chem.* **1998,** *273*, 10835–10842.
13. Fu, L.-H.; Wang, X.-F.; Eyal, Y.; She, Y.-M.; Donald, L. J.; Standing, K. G.; Ben-Hayyim, G. *J. Biol. Chem.* **2002,** *277*, 25983–25991.
14. Reich, H. J.; Renga, J. M.; Reich, I. L. *J. Am. Chem. Soc.* **1975,** *97*, 5434–5447.
15. Okeley, N. M. Zhu Y.; van der Donk, W. A. *Org. Lett.* **2000,** *2*, 3603–3606.
16. Zhu, Y.; van der Donk, W. A. *Org. Lett.* **2001,** *3*, 1189–1192.
17. Rooseboom, M.; Commandeur, J. N. M.; Floor, G. C.; Rettie, A. E.; Vermeulen, N. P. E. *Chem. Res. Toxicol.* **2001,** *14*, 127–134.
18. Patterson, S. D.; Aebersold, R. *Electrophoresis* **1995,** *16*, 1791–1814.
19. Bateman, K. P. *J. Am. Soc. Mass Spectrom.* **1999,** *10*, 309–317.
20. Morand, K.; Talbo, G.; Mann, M. *Rapid Commun. Mass Spectrom.* **1993,** *7*, 738–743.
21. Steen, H.; Mann, M. *J. Am. Soc. Mass Spectrom.* **2000,** *12*, 228–232.
22. Krief, A. In *The Chemistry of Organic Selenium and Tellurium Compounds*, Vol I; Patai, S.; Rappoport, Z., Eds.; John Wiley and Sons: Chichester, 1987; p 675.
23. Miles, E. W. In *Coenzymes and Cofactors. Vitamin B6 Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects, Part B*, Vol I; Dolphin, D.; Poulson R.; Avramovic, O., Eds.; John Wiley and Sons: New York, 1986; p 253.