Domain structure of bi-functional selenoprotein P

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Human selenoprotein P (SeP), a selenium-rich plasma glycoprotein, is presumed to contain ten selenocysteine residues; one of which is located at the 40th residue in the N-terminal region and the remaining nine localized in the C-terminal third part. We have shown that SeP not only catalyses the reduction of phosphatidylcholine hydroperoxide by glutathione [Saito, Hayashi, Tanaka, Watanabe, Suzuki, Saito and Takahashi (1999) *J. Biol. Chem.* **274**, 2866–2871], but also supplies its selenium to proliferating cells [Saito and Takahashi (2002) *Eur. J. Biochem.* **269**, 5746–5751]. Treatment of SeP with plasma kallikrein resulted in a sequential

limited proteolysis (Arg-235–Gln-236 and Arg-242–Asp-243). The N-terminal (residues 1–235) and C-terminal (residues 243–361) fragments exhibited enzyme activity and selenium-supply activity respectively. These results confirm that SeP is a bi-functional protein and suggest that the first selenocysteine residue is the active site of the enzyme and the remaining nine residues function as a selenium supplier.

Key words: bi-functional protein, domain structure, glutathione peroxidase (GPx), plasma kallikrein, selenium, selenoprotein.

INTRODUCTION

Selenium is an essential micronutrient, and is incorporated into proteins in the form of selenocysteine (Sec) and selenomethionine. The term ‘selenoprotein’ is restricted to Sec-containing proteins [1], and is to be distinguished from proteins that non-specifically incorporate selenomethionine. Sec is encoded by a UGA codon, formerly known only as a stop codon, in the open reading frame of selenoprotein mRNA that is accompanied by a Sec insertion sequence (SECIS) element in the 3′-untranslated region in eukaryotes [2]. In animals 25 selenoproteins have been found [3], and some of them have been shown to exert biological functions [4].

Selenoprotein P (SeP) is a selenium-rich extracellular glycoprotein [5], and human SeP has been purified and studied by several groups [6–8]. The sequence of the cDNA predicts that human SeP contains 10 Sec residues (in contrast with other species, which contain 12 or 17 Sec residues) encoded by UGA stop codons in the open reading frame and two SECIS elements in the 3′-untranslated region of its mRNA [9]. The function of SeP *in vivo* is currently unknown, although several pieces of indirect evidence suggest that SeP serves in antioxidative defence [10] and in selenium transport [11]. SeP has been reported recently to catalyse the reduction of phospholipid hydroperoxide by GSH [8] or thioredoxin [12], to function as a peroxynitrite scavenger [13], and to be a cell survival-promoting factor [14,15]. Recently, we demonstrated that SeP functions as a selenium-supply protein, delivering selenium to the cells [16]. More recently, data on SeP-knockout mice reveal that SeP plays a pivotal role in delivering hepatic selenium to target tissues [17,18] and support our hypothesis described above. They also reported that SeP-knockout mice display a complex phenotype characterized by significant growth retardation, neurologically by seizures and a movement disorder, and by reduced fertility in males [17–20].

It is currently unknown which Sec residue in SeP is the active site of the enzyme and which function as a selenium supplier. In a previous paper [8], we reported that the recovery of SeP was very low if di-isopropyl fluorophosphate [*i*Pr₂P-F (or DFP)]; one of the most potent serine-protease inhibitors] was not added to each fraction during purification. This suggests that the degradation of SeP by an *i*Pr₂P-F-sensitive protease occurs in the course of purification without *i*Pr₂P-F. In this study we first searched for this *i*Pr₂P-F-sensitive serine protease, and identified plasma kallikrein, which plays a key role in the activation of blood coagulation, as a SeP-cleaving enzyme. Next, we determined the cleavage sites of SeP by plasma kallikrein, and showed that the N-terminal and C-terminal fragment exhibited enzyme activity and selenium-supply activity respectively. We finally propose the domain structure of bi-functional SeP.

EXPERIMENTAL

Chemicals

*i*Pr₂P-F was obtained from Kishida Chemical Co. (Osaka, Japan); t-butyl hydroperoxide from Nacalai (Kyoto, Japan); 1-palmitoyl-2-linoleoyl-3-phosphatidylcholine, GSH, GSH reductase, and RPMI-1640 medium from Sigma-Aldrich (St. Louis, MO, U.S.A.); soybean lipoxygenase from Biozyme Laboratories Ltd. (Blaenavon, Gwent, Wales, U.K.); Ni²⁺-nitrilotriacetate-agarose from Qiagen Inc. (Chatsworth, CA, U.S.A.); aprotinin from Wako Pure Chemical Co. (Osaka, Japan); human plasma kallikrein and human Factor XIIa from Enzyme Research Laboratories Inc. (South Bend, IN, U.S.A.); and human Factors IXa, Xa and XIa from Haematologic Technologies Inc. (Essex Junction, VT, U.S.A.). Human Factor VIIa [21], human activated protein C [22] and human α -thrombin [23] were donated by the

Abbreviations used: *i*Pr₂P-F, di-isopropyl fluorophosphate; GPx, glutathione peroxidase; cGPx, cellular GPx; PC-OOH, phosphatidylcholine hydroperoxide; Sec, selenocysteine; SECIS, Sec insertion sequence; SeP, selenoprotein P; SeP-CF, C-terminal 20 kDa proteolysis fragment of SeP; SeP-NF, N-terminal 56 kDa proteolysis fragment of SeP.

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Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Human frozen plasma was kindly donated by the Hokkaido Red Cross Blood Center (Hokkaido, Japan). Phosphatidylcholine hydroperoxide (PC-OOH) was prepared from phosphatidylcholine by oxidation with soybean lipoxygenase, as described previously [8]. SeP was purified from human plasma as described previously [8]. SeP-depleted human serum was prepared with six kinds of immobilized anti-human SeP monoclonal antibodies as described previously [16,24]. Other chemicals were of the highest quality commercially available.

Electrophoresis

SDS/PAGE was performed according to the method of Laemmli [25] in slab gels (12.5 % gel). After electrophoresis, proteins in the gel were detected with Coomassie Brilliant Blue and silver staining methods. Coomassie Brilliant Blue G-25 (0.01 %) in 10 % acetic acid and 30 % methanol was used for staining for 30 min, and then the gel was destained in 10 % acetic acid and 30 % methanol. Silver staining was conducted according to the protocol outlined by the manufacture in the silver staining kit (Daiichi Pure Chemicals Co., Tokyo, Japan).

Western-blot analysis

Six monoclonal anti-SeP antibodies (BD1, BD3, BF2, AE2, AH5 and AA3) were used for this study, as described previously [24]. After electrophoresis, proteins in the gel were electroblotted on to a PVDF membrane (Millipore Co., Bedford, MA, U.S.A.). Proteins in the membrane were detected with monoclonal antibodies, peroxidase-conjugated secondary antibody and an enhanced chemiluminescence (ECL[®]) Kit (Amersham International, Uppsala, Sweden). To detect naturally occurring SeP fragment in human plasma, rabbit polyclonal antibody to SeP C-terminal fragment and ECL[®] Advance Western Blotting Detection Kit (Amersham) were used.

Amino-acid-sequence analysis

After electrophoresis, proteins in the gel were electrically transferred to a Pro Blot membrane (Applied Biosystems) in 20 mM Tris containing 150 mM glycine and 20 % methanol. The proteins on the membrane were stained with 0.1 % Coomassie Brilliant Blue G-250 in 1 % acetic acid and 40 % methanol for 1 min, and then the membrane was destained with 50 % methanol. The protein bands were cut out and their N-terminal amino acid sequences were analysed with a 473A Protein Sequencer (Applied Biosystems).

Quantitative amino acid analysis for determination of protein molar concentration

The protein was dialysed against distilled water and hydrolysed in 6 M HCl for 24 h, 48 h or 72 h, or in 3 M mercaptoethanesulphonic acid for 24 h. After removal of the solvent *in vacuo*, quantitative amino acid analysis was performed on a Pico-Tag[™] system (Millipore Waters Chromatography, Milford, MA, U.S.A.) [8].

Selenium and enzyme assay

Selenium concentration was determined according to the fluorimetric method of Bayfield and Romalis [26]. Enzyme activities were examined by following the oxidation of NADPH in the presence of GSH reductase, which catalyses the reduction of the oxidized GSH, as described previously [12].

Figure 1 Fragmentation of partially purified SeP

Partially purified SeP was incubated for 48 h at 37 °C with *iPr*₂P-F (2 mM) or aprotinin (10 µg/ml) and subjected to Western-blot analysis using a mixture of six monoclonal antibodies against human SeP, as described in the Experimental section. Lane 1, none; lane 2, *iPr*₂P-F; lane 3, aprotinin.

Figure 2 Cleavage of SeP by plasma serine proteases

Purified SeP was incubated with plasma serine proteases [protease/SeP = 1:20 (w/w)] for 1 h at 37 °C and subjected to SDS/PAGE. After electrophoresis, proteins in the gel were stained with 0.1 % Coomassie Brilliant Blue G-250. Lane 1, none; lane 2, plasma kallikrein; lane 3, active protein C; lane 4, α -thrombin; lane 5, Factor XIIa; lane 6, Factor XIa; lane 7, Factor Xa; lane 8, Factor IXa; lane 9, Factor VIIa.

Selenium-supply assay

Selenium-supply activities were examined by the measurement of cellular glutathione peroxidase (cGPx) activity, which is an index of selenium status, as described previously [16].

RESULTS

Identification of plasma kallikrein as a SeP-cleaving enzyme

Incubation of partially purified SeP fractions (post heparin-Sepharose chromatography as in [8]), without any addition of protease, resulted in the fragmentation of SeP, as detected by Western blotting (Figure 1). When a potent serine protease inhibitor, *iPr*₂P-F, was added to the incubation mixture, no cleavage of human SeP was observed. These results indicate that an *iPr*₂P-F-sensitive serine protease is contaminated with partially purified fractions, and cleaves SeP when activated in the purification process. As many serine proteases of the blood coagulation system are known to bind heparin-Sepharose, we next examined the reactivity of eight blood coagulation system proteases (plasma kallikrein, active protein C, α -thrombin, and Factors XIIa, XIa, Xa, IXa, VIIa) against purified SeP. Purified SeP was incubated with the proteases at an enzyme/substrate concentration of 1:20 (w/w) at 37 °C for 1 h and resolved by SDS/PAGE. As shown in Figure 2, only plasma kallikrein

Figure 3 Time course of cleavage of SeP by plasma kallikrein

Purified SeP was incubated with plasma kallikrein [protease/SeP = 1:20 (w/w)] for the indicated times at 37 °C and subjected to SDS/PAGE. After electrophoresis, proteins in the gel were stained with 0.1 % Coomassie Brilliant Blue G-250.

Figure 4 Limited proteolysis of SeP by plasma kallikrein

N-terminal amino-acid-sequence analysis of intact SeP and three SeP fragments was conducted, as described in the Experimental section. **(A)** N-terminal amino acid sequence of SeP and three SeP fragments. **(B)** the sequential limited proteolysis of SeP by plasma kallikrein. Individual amino acid residues are indicated by their position and single letter code.

cleaved purified SeP into two fragments. To confirm that the fragmentation of partially purified SeP described above is caused by plasma kallikrein, the effect of aprotinin (a specific inhibitor of plasma kallikrein) was examined. As shown in Figure 1 (lane 3), aprotinin inhibited the fragmentation of partially purified SeP.

Limited proteolysis of SeP by plasma kallikrein

SeP was incubated with plasma kallikrein and resolved by SDS/PAGE. As shown in Figure 3, SeP was first cleaved by plasma kallikrein to yield two fragments of 56 and 21 kDa. The 21 kDa fragment was subsequently cleaved to yield a 20 kDa fragment. Figure 4(A) shows the N-terminal amino acid sequences of intact SeP and the three SeP fragments. N-terminal amino-acid-sequence analysis revealed that the 56 kDa fragment was derived from the N-terminal side of SeP. The 21 kDa band on SDS/PAGE showed an amino acid sequence corresponding to the sequence starting with the Gln-236 of SeP [9]. The 20 kDa band showed another sequence starting at the Asp-243 of SeP [9]. Thus, the sequential limited proteolysis of SeP by plasma kallikrein was demonstrated as shown in Figure 4(B). The fragments of 56 and 20 kDa were termed SeP-NF and SeP-CF respectively.

Isolation and characterization of SeP fragments

Immobilized metal chelate affinity chromatography is generally used for the purification of recombinant proteins containing six

Figure 5 SDS/PAGE and Western-blot analysis of purified SeP fragments

(A) Isolated SeP-NF (lane 1) and SeP-CF (lane 2) were subjected to SDS/PAGE. After electrophoresis, proteins in the gel were detected with the silver staining method. Isolated SeP-NF **(B)** and SeP-CF **(C)** were subjected to SDS/PAGE and Western-blot analysis. Proteins in the membrane were detected with rat anti-human SeP monoclonal antibody BD1 (lane 1), BD3 (lane 2), BF2 (lane 3), AE2 (lane 4), AH5 (lane 5) and AA3 (lane 6) respectively.

consecutive histidine residues at their N-terminus. As SeP has two histidine-rich regions within SeP-NF and was reported to bind to this resin [8], the SeP preparation treated with plasma kallikrein for 24 h was applied to the immobilized metal chelate column. As expected, SeP-NF bound strongly to the resin and was eluted by competition with imidazole, a histidine analogue, although SeP-CF passed through the resin (results not shown). The isolated SeP-NF and SeP-CF preparations gave single stained bands on SDS/PAGE with mobility corresponding to 56 kDa and 20 kDa respectively (Figure 5A). Western-blot analysis revealed that five monoclonal antibodies (BD1, BD3, BF2, AE2 and AH5) are specifically reactive to SeP-NF, though AA3 monoclonal antibody is reactive to SeP-CF only (Figures 5B and 5C).

We next measured the abilities of full-length SeP and its fragments to catalyse the GSH-dependent reduction of phospholipid hydroperoxide, PC-OOH (Figure 6). A dose-dependent reduction of PC-OOH by full-length SeP and SeP-NF was observed. The specific activity of SeP-NF to PC-OOH (21 min⁻¹) was almost one-half lower than that of full-length SeP (49 min⁻¹). On the other hand, SeP-CF did not show any enzymatic activity.

When Jurkat cells were cultured solely in the presence of SeP-depleted serum, the activity of cGPx, a major Se-dependent enzyme, decreased to 17 % that of the control. To compare full-length SeP with its fragments as a selenium supplier, we studied the effect on the cellular cGPx activity (Figure 7). Full-length SeP was the most effective, with a 50 % effective dose (ED₅₀) of 5 nM (selenium equivalent), followed by SeP-CF (ED₅₀ of 42 nM). SeP-NF had no effect up to 50 nM.

Detection of naturally occurring SeP fragment in human plasma

We reported previously that immunoblot analysis of the immunoprecipitate of human plasma by anti-SeP antibodies gave only one major 69 kDa band [24]. This suggests that SeP is present in one major form. To confirm whether a small amount of naturally occurring SeP fragment exists in plasma, we examined immunoblot analysis of human plasma using a very sensitive detection kit (ECL[®] Advance Western Blotting Detection Kit).

Figure 6 Reduction of PC-OOH by SeP and its fragments

The activities were measured by monitoring the oxidation of NADPH, as described in the Experimental section. Protein concentration was determined by quantitative amino acid analysis. Open circles, intact SeP; closed circles, SeP-NF; open squares, SeP-CF. The values represent means $\pm$ S.D. of three separate experiments.

Figure 7 Selenium-supply activity of SeP and its fragments

The activities were determined by the measurement of cellular GPx activity [index of selenium (Se) status], as described in the Experimental section. Open circles, intact SeP; closed circles, SeP-NF; open squares, SeP-CF. The values represent means $\pm$ S.D. of three separate experiments.

A small amount of SeP fragment with the same molecular mass of SeP-CF was detected, as shown in Figure 8. The quantitative estimation of SeP fragment using a calibration curve of purified SeP-CF indicates that 1.2% (1.3 nM) of total SeP (110 nM) exists as a cleaved form.

DISCUSSION

Plasma kallikrein is a serine proteinase that releases the hypotensive peptide, bradykinin, from the plasma substrate high-molecular-weight kininogen [27]. Plasma kallikrein can cleave plasma zymogens, such as Factor XII [28] and plasminogen [29]. In addition, complement fragments, such as C1s [30], C2b [31] and iC3b [32], are cleaved by plasma kallikrein. We have reported that a 120 kDa plasma protein, termed PK-120 [33–35], is rapidly cleaved by plasma kallikrein. As demonstrated here, SeP is also cleaved by plasma kallikrein. A time-course study indicates sequential limited proteolysis. The first proteolysis at Arg-235–Gln-236, results in the production of the N-terminal (residues 1–235, SeP-NF) and C-terminal (residues 236–361) fragments, and the second proteolysis at Arg-242–Asp-243 results in the

Figure 8 Detection of a cleaved SeP fragment in human plasma by Western-blot analysis

The immunoprecipitate of human plasma (freshly prepared) by rabbit anti SeP-CF antibodies (lane 1) and a various amount of purified SeP-CF (lane 2, 16 fmol; lane 3, 32 fmol; lane 4, 64 fmol) were subjected to Western-blot analysis. Proteins in the membrane were detected with rat anti-human SeP monoclonal antibody AA3.

production of a small peptide (residues 236–242) and the C-terminal (residues 243–361; SeP-CF) fragment. In the rat for example, multiple forms of SeP are reportedly present in plasma [36,37]. It is known that one form (47 kDa) terminates at the second UGA, while another form (57 kDa) terminates at a UAA stop codon after all 10 UGAs have been translated as Sec [37]. The second UGA codon in rat SeP mRNA corresponds to the Gln-236-encoded CAG codon in human SeP mRNA. Surprisingly, these facts show that an analogue of human SeP-NF exists in rat plasma. Kryukov and Gladyshev [38] found a zebrafish SeP gene (designated zSelPa) that contained two SECIS elements and encoded a protein containing 17 Sec residues, the largest number of Sec residues found in any known protein. In contrast, a second SeP gene (designated zSelPb) was also identified that contained one SECIS element and encoded a protein with a single Sec. The stop codon in zSelPb corresponds to the Gln-236-encoded CAG codon in human SeP mRNA. More surprisingly, these facts suggest that an analogue of human SeP-NF (zSelPb) exists in zebrafish plasma.

The N-terminal fragment, which contains only the first isolated Sec, is capable of reducing PC-OOH in the presence of GSH. All selenoproteins with enzymic activity are reported to contain Sec in their active sites. The substitution of Sec for Cys in selenoproteins is reported to decrease the enzyme activity by about three orders of magnitudes [39]. These suggest that the first isolated Sec might be the active site of this enzyme. Comparison of the amino acid sequence of SeP-NF with the whole sequences of four types of GPx indicates that these proteins are structurally unrelated. As thioredoxin protein and the N-terminal half of GPx protein share the same tertiary structure, but the different amino acid sequences, SeP-NF and GPx are suggested to share the same tertiary structure. Further studies to clarify the tertiary structure of SeP are necessary in the future. The specific activity of the SeP-NF enzyme was 43% lower than that of full-length SeP. It is supposed that the cleavage of SeP by plasma kallikrein causes SeP-NF to change its tertiary structure.

Selenium is well known to be essential for cell culture when using a serum-free medium, but not when a medium containing serum is used [40]. We recently reported that SeP functions as a selenium-supply protein, delivering selenium to the cells [16]. More recently, SeP-knockout mice studies reveal that SeP transports selenium to peripheral tissues [17,18]. To clarify which

Figure 9 Domain structure of human SeP

Se, selenium.

Sec residue(s) in SeP delivers selenium to the cells, we measured the selenium-supply activities of SeP fragments. Only SeP-CF supplies selenium to the cells, suggesting that the remaining nine Sec residues function as a selenium supplier. In spite of the calculation of specific activity taking into consideration different Sec content of fragment versus full-length SeP, the specific activity of SeP-CF selenium supply was much lower than that of the full-length SeP. It is supposed that SeP-NF is not essential for delivering selenium to the cells, but increases the binding of SeP-CF to the cells and the selenium-supply activity of SeP-CF. Though the existence of SeP receptors has been inferred from binding studies [41], the mechanism of selenium supply by SeP is currently unknown. As selenium is covalently bound in SeP, the supply mechanism is supposed to require a receptor-mediated uptake, digestion of SeP by proteases and peptidases, the breakdown of Sec for the release of its selenium by Sec lyase [42], and the provision of selenium for biosynthesis of selenophosphate [43]. Further studies on confirming these points, as well as the identification and characterization of the SeP receptor, will provide important information on the precise roles of SeP fragments.

The structure–function relationship of SeP is shown in Figure 9. We propose that SeP is composed of two domains: the N-terminal domain, which contains one Sec residue, and the C-terminal domain, which contains multiple Sec residues, and is connected with a bridge containing two His-rich regions. The former displays enzymic activity, reducing phospholipid hydroperoxide in the presence of GSH, while the latter may demonstrate selenium-supplier activity, delivering selenium to cells. Furthermore, SeP is reported to bind to endothelial cells, presumably via the His-rich regions and a second different N-terminal domain [44,45]. Thus, SeP has a unique protein structure, and is proposed to be a bi-functional protein. Further studies are required to prove the above hypothesis and to establish the physiological roles of this interesting protein.

It was observed that 1.2% of SeP exists as a cleaved smaller form in human plasma (Figure 8). Although it is currently unknown whether this smaller form is derived from cleavage by plasma kallikrein, it is likely that human SeP may be cleaved by plasma kallikrein under some pathological conditions, and these smaller forms may function in an unknown fashion. Binding of SeP to endothelial cells suggests that the cleavage of SeP by plasma kallikrein results in the release of SeP-CF from endothelial cells. It has been reported that proteases are activated at inflammatory sites [46], and that selenium modulates the inflammatory and immune responses [47]. Therefore, it is possible that plasma-kallikrein-processed SeP fragments regulate inflammation by controlling selenium action in the cells. Through an examination of this point, we intend to clarify the *in vivo* functions of SeP and its fragments. Furthermore, the possibility

remains that the hexapeptide released from SeP by the plasma kallikrein digestion is a bioactive peptide like bradykinin.

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