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Thiol-Dependent Membrane Transport of Selenium through an Integral Protein of the Red Blood Cell Membrane

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The molecular details of the selenium metabolism and transport in living systems are still not completely understood, despite their physiological importance. Specifically, little is known about the membrane transport of selenium from most of the selenium containing compounds. In the present study, we investigated the mechanism for the membrane transport of selenium from red blood cells (RBCs) to the blood plasma. When the selenium distribution in the RBC ghost membrane after treatment with selenious acid was analyzed, nearly 70% of the selenium in the membrane was found to bind to the anion exchanger 1 (AE1) protein, which suggested that the integral protein AE1 is responsible for the membrane transport of selenium. The thiol dependency of the selenium export from the RBC to the blood plasma was examined using membrane permeable thiol reagents, i.e., *N*-ethylmaleimide (NEM) and tetrathionate (TTN). Treatment of the RBC with NEM, a thiol-alkylating reagent, resulted in modification of the thiol groups in the amino-terminal cytoplasmic domain (N-CPD) of the AE1, but not those in the membrane domain. Such an NEM treatment provided a marked inhibition of the selenium export from the RBC to the blood plasma. In addition, the treatment with TTN, a thiol-oxidizing reagent that forms intermolecular disulfide bonds, appeared to oxidize thiol groups in both the N-CPD and the membrane domain of AE1, which resulted in complete inhibition of the selenium export even during the initial period in which the export had a maximum velocity when using the thiol reagent-free treatment. Such complete inhibition of the selenium export from the TTN-treated RBC appeared to be due to the oligomerized AE1 proteins resulting from the intermolecularly formed disulfide bonds. These inhibitory effects using NEM and TTN suggested that thiol groups in the integral protein AE1 play essential roles in the membrane transport of the selenium from the RBCs to the blood plasma.

Introduction

Essential microelement selenium in all higher forms of animal life is the component of the 21st naturally occurring amino acid (selenocysteine, Sec).[1] Since the discovery of its biological importance in 1957,[2] interest in studying the biochemical nature of this trace element has increased. Defects such as liver necrosis, white muscle disease, and certain cardiac and skeletal muscle degenerations are associated with a selenium deficiency.[3] The insertion of Sec into proteins is genetically directed by the UGA codon that normally serves as the stop codon, and the resulting Sec-containing proteins broadly occur in the entire body.[4,5] In humans, it has catalytic and/or structural roles in the 25 Sec-containing proteins that were deduced from a selenoproteome analysis.[6] The best-understood Sec-containing proteins are glutathione peroxidases that regulate the redox state in living systems by catalyzing the reduction of hydrogen peroxide and phospholipid hydroperoxides.[7]

Selenium in living systems comes from various selenium compounds including both inorganic and organic species. Despite its physiological importance, the molecular details of the selenium metabolism and transport in living systems are still not completely understood. Because of the highly reactive and unstable properties of selenium, its transport mechanism has to preserve the physicochemical state of the selenium during transport. Thus, biogenic substances, probably macromolecules, such as proteins that allow low-mass selenium species to bind, are thought to be responsible for the transport of selenium.[8−10] Although selenite ($Se^{IV}O_3^{2-}$) is not

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a major source of selenium, selenium-enriched yeast (a common form of selenium supplement) is found to contain significant amounts of selenite,[11,12] and selenite is also used to supplement infant formulas and other products.[13,14] Highly bioavailable selenite is most frequently used as a source compound for the treatment of a selenium deficiency.[15] Intravenously administered selenite is immediately taken up into red blood cells (RBCs) through the anion exchanger 1 (AE1) protein in the plasma membranes,[16] and then reappeared in the blood plasma after nonenzymatic reduction in RBCs. We already reported that selenium from selenious acid ($H_2Se^{IV}O_3$, SA) is mostly bound to the reactive thiol groups of hemoglobin (Hb) on its β chain through selenotrisulfide,[17] and then transferred to thiol groups of the amino-terminal cytoplasmic domain (N-CPD) of AE1 based on the intrinsic interactions between Hb and the extreme amino-terminal sequence of AE1.[18] The selenium export from RBCs to the blood plasma occurs in an oxygen-linked fashion; it is enhanced by increasing the proportion of the deoxygenated Hb with a higher binding affinity for the N-CPD than the oxygenated Hb. Subsequently, the selenium transport from the blood plasma to the peripherals involves a relay mechanism of thiol exchange that occurs between the selenotrisulfide and thiol compounds (selenotrisulfide relay mechanism: R-SSeS-R + R′-SH → R′-SSeS-R + R″-SH → R-SSeS-R″).[19] On the basis of previous studies, we investigated the mechanism underlying the missing membrane transport process of selenium from the N-CPD of the AE1 protein to the blood plasma. So far, little is known about the membrane transport of selenium from most of the selenium containing compounds, including selenite. In our previous study using RBC inside-out vesicles, we found that selenium bound to Hb is not transported to the N-CPD deleted RBC membrane at all.[18] Therefore, the selenium transport from Hb to the N-CPD of AE1 was thought to be the prerequisite event for the subsequent membrane transport of selenium to the blood plasma.

Human AE1, the most abundant protein in the RBC plasma membrane, plays a critical role in the carbon dioxide transport system in which carbon dioxide is carried as bicarbonate in the blood plasma. Since the solubility of carbon dioxide in the blood plasma is rather low, the carbon dioxide molecule that diffuses into the RBC is converted into the bicarbonate anion by cytosolic carbonic anhydrase. The bicarbonate anion is then transported out of the RBC by AE1 during the exchange for a chloride anion (Cl^-/HCO_3^- anion exchange). AE1, a 911-amino acid long glycoprotein, involves two structurally distinct domains, that is, the amino-terminal domain (Met1-Pro403, N-CPD) is cytosolic with a molecular weight of 43 kDa, and the membrane embedded domain with the carboxyl-terminal tail (Gln404-Val911, molecular weight of 52 kDa) traverses the plasma membrane multiple times (Figure 1).[20] The membrane domain also contains a single *N*-linked glycosylation site, Asn642, on its extracellular surface.[21] The extreme sequence of the N-CPD binds multiple glycolytic enzymes[22] and hemoglobin.[23] Other parts of this domain also provide binding sites for the cytoskeletal proteins ankryin,[24] protein 4.2,[25] and protein 4.1.[26] The membrane domain mediates anion transport, while its cytosolic carboxyl-terminal tail contains acidic motifs that constitute a core binding site for cytoplasmic carbonic anhydrase II.[27] It has been reported that AE1 has many diverse important biological functions in RBCs. In the present study, we investigated the participation of free thiol groups of the cysteine residues in the AE1 protein during the membrane transport of selenium from RBCs to the blood plasma.

Experimental Section

Materials. Fresh human venous blood was collected in a heparinized VENOJECT II tube (TERUMO, Tokyo, Japan). Each sample was centrifuged at 1,400 *g* for 10 min at room temperature, and the plasma, buffy coat and upper 10% of the RBC layers were removed by aspiration. The precipitated RBCs were washed three times with isotonic phosphate buffer (pH 7.4). Selenious acid ($H_2Se^{IV}O_3$, SA) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). 2,3-Diaminonaphthalene (DAN) and 2,2′-bicinchoninic acid (BCA) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). α-Chymotrypsin (α-Chy, from bovine pancreas, 35−65 units/mg) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) for determination of the thiol content was from Sigma Co. (St. Louis, MO), *N*-ethylmaleimide (NEM) and potassium tetrathionate (TTN) for chemical modifications of the thiol groups were from Nacalai Tesque, Inc. and Sigma Co., respectively. All other chemicals were of commercial reagent or special grades and used as received.

Determination of Protein Concentration. The protein concentrations were colorimetrically determined by the BCA protein assay using bovine serum albumin as the reference.[28] BCA and Cu(II) sulfate were added to the RBC membrane samples dissolved in a 5 mM sodium phosphate solution (pH 8) containing 1% sodium dodecyl sulfate (SDS). The mixtures were then allowed to react at 60 °C for 30 min. The produced color was monitored at 562 nm.

Determination of Thiol Contents. The RBC membrane samples were suspended in a 5 mM sodium phosphate solution (pH 8) containing 1% SDS, and added to an equal volume of a 1 mM DTNB solution. After incubation for 60 min, the absorbance at 420 nm was monitored, and then determined by the DTNB method.

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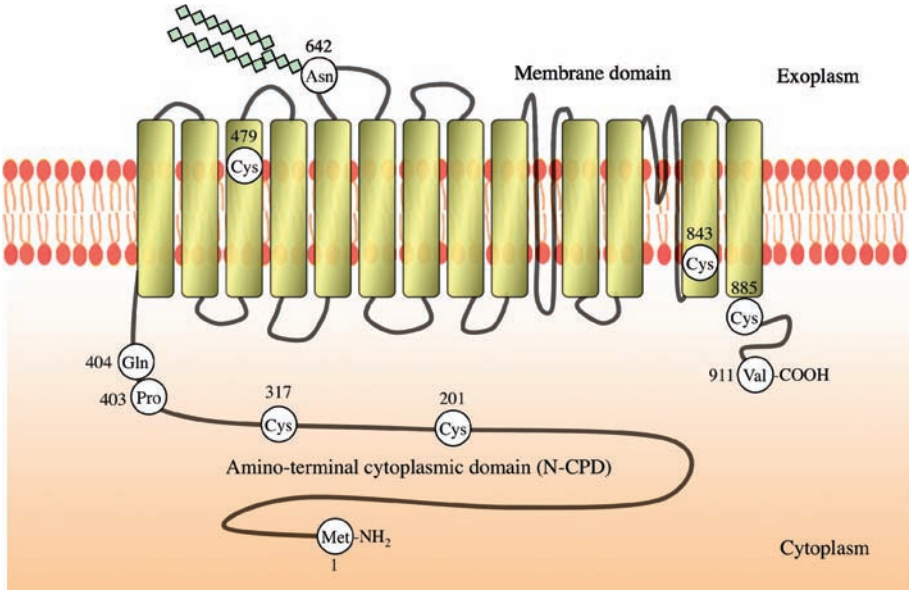

Figure 1. Locations of cysteine residues in a topographical model of the anion exchanger 1 (AE1).[24]

Determination of Selenium Concentration. The selenium concentrations in the specimens were fluorometrically determined using DAN after acid digestion with a 1:5 mixture by volume of perchloric acid and nitric acid.[29] The selenium standard solution [1000 ppm as selenium(IV) dioxide in 0.5 M nitric acid] for the fluorometry was obtained from Kanto Chemical Co., Inc.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting Analysis. After measurement of the protein concentration by the BCA method, the crude membrane protein was dissolved in a mixture of 1% SDS, 0.01 M tris(hydroxymethyl)aminomethane-HCI (pH 8), and 10% sucrose. The membrane protein samples (20−40 μg) were electrophoresed on a 7.5 or 15% polyacrylamide disk gel with an electrophoresis buffer [0.1% SDS, 25 mM tris(hydroxymethyl)aminomethane and 192 mM glycine] using a pageRun AE-6531 M (ATTO Corporation, Tokyo, Japan). Unless otherwise noted, the gel electrophoresis was carried out under a non-reducing condition. The molecular mass calibration was carried out using Precision Plus Protein standards that contain ten bands of 10−250 kDa (Bio-Rad Laboratories, CA, U.S.A.). The locations of the proteins in the gels were determined by Coomassie blue (CB) staining (0.1% CB in 30% methanol-acetic acid mixed solution). For the immunoblotting analysis, the membrane proteins on the gels were transferred onto a P membrane (polyvinyl difluoride membrane, ATTO Corporation). The membrane samples were blocked with 1% nonfat milk in 0.1% Tween-20 supplemented 25 mM tris(hydroxymethyl)aminomethane-HCl-buffered saline. The mouse monoclonal antihuman anion exchanger 1 (AE1) antibody, that is directed against within 20 kDa from the amino-terminal domain, (Alpha Diagnostic Int'l Inc., San Antonio, TX) at a 1:3,000 dilution was then applied, followed by goat antimouse antibody-horseradish peroxidase conjugate (Alpha Diagnostic Int'l, Inc.) at a 1:17,500 dilution. An LAS-1000 luminescent image analyzer (Fujifilm Co., Tokyo, Japan) was used to detect the AE1 immunoblot.

Isolation of RBC Ghost Membranes and Their α-Chy Digestion and NaOH Extraction. RBC ghost membranes were isolated according to the method of Steck et al.[30] Packed RBCs were osmotically hemolyzed by the addition of 40 volumes of a 5 mM cold sodium phosphate solution (pH 8). The hemolysate was centrifuged at 22,000 *g* and 4 °C for 20 min and then the supernatant was removed by aspiration. To remove hemoglobin bound to the membranes, the red pellets were washed three times with a 5 mM sodium phosphate solution (pH 8) and once with a 0.5 mM sodium phosphate solution (pH 8), and then white unsealed RBC ghost membranes were obtained. The Hb-free white RBC ghost membranes were digested with α-Chy (final concentration, 0.2 mg/mL) in 0.01 M phosphate buffer (pH 7.4) at 37 °C for 10 min, and centrifuged again at 22,000 *g* and 4 °C for 15 min. The white RBC ghost membranes or α-Chy-treated membranes were combined with a 0.1 M sodium hydroxide solution, and the mixture was immediately centrifuged again at 35,000 *g* and 4 °C for 60 min. The obtained pellets were washed three times with a 5 mM sodium phosphate solution (pH 8).

Treatments of Red Blood Cells with Selenious Acid, *N*-ethylmaleimide, and Tetrathionate. The purified RBCs were treated with SA (final concentration 8 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min [hematocrit 20% (v/v)]. After washing three times with isotonic phosphate buffer (pH 7.4), RBCs were treated with NEM (final concentration 1 mM) for 20 min at 35 °C or TTN (final concentration 20 mM) for 1 h at 37 °C in isotonic phosphate buffer (pH 7.4).

Selenium Export Experiments. The SA-treated RBC precipitate was combined with an equal volume of human blood plasma, and the mixture was incubated in a water bath with shaking at 60 min^{-1}. Aliquots were withdrawn from the mixture at appropriate time intervals. After centrifugation at 1400 *g* for 10 min at 4 °C to separate the RBCs and the blood plasma, the selenium contents in the RBCs and the blood plasma were determined by the DAN method subsequent to acid digestion. The selenium content in the RBC precipitates before mixing with the blood plasma (3.1 ± 0.1 μg/mL-RBC) was defined as 100% in Figures 5 and 7.

Statistical Analysis. All data were presented as the mean and standard error (S. E.) ($n \geq 3$). Statistical analyses were performed using PRISM 4 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). The multiple mean values were compared by a one or two-way analysis of variance with a Bonferroni posthoc test. Comparisons were considered statistically significant at $P < 0.05$.

Results and Discussion

Thiol-containing biomolecules in the bloodstream, such as glutathione and hemoglobin in RBCs and albumin in the blood plasma, participate in the metabolism and transport of

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selenium species inside the RBCs and the subsequent transport to the peripherals.[17−19] Therefore, the thiol groups of the cysteine residues in the AE1 are also thought to play an important role in the membrane transport of selenium. As schematically seen in a current topographical model of the AE1 monomer (Figure 1),[31] the AE1 contains five cysteine residues at amino acid positions 201, 317, 479, 843, and 885; the first two of these are found in the N-CPD, and the other three are in the membrane domain.[32] Glycophorin A is the second abundant integral protein in the RBC plasma membrane, and includes the interaction sites with the membrane domain of AE1. However, glycophorin A is unlikely to directly participate in the membrane transport of selenium because it is a single polypeptide of 131 amino acids free of the cysteine residue that is reactive with selenium species.[33]

To analyze the selenium distribution in the RBC plasma membrane after treatment with SA, the RBC ghost membrane proteins were separated by SDS-PAGE [Figure 2 a, lanes (1) left], and then an immunoblotting analysis using the anti-AE1 antibody that was directed against an amino-terminal moiety identified AE1 protein [Figure 2 a, lanes (1) right]. RBC membranes can be enriched for AE1 by a high pH extraction of peripheral proteins such as spectrin and ankyrin; after such an extraction, AE1 makes up 70% of the membrane protein.[34] Such a high pH extraction of the RBC ghost membrane practically resulted in the preferable separation of AE1 from the peripheral proteins [Figure 2 a, lane (2)]. While α-chymotrypsin (α-Chy) digestion can produce separation of the N-CPD and the membrane domain by cleaving the peptide bond between Tyr359 and Lys360 of the AE1.[35] Thus, the RBC ghost membranes were digested with α-Chy, and then extracted with NaOH to remove the peripheral proteins. In the electrophoregram of the RBC membrane sample after treatments with α-Chy and NaOH, the band of the whole AE1 protein disappeared, while cleaved fragments appeared in a molecular weight range below 50 kDa [Figure 2 a, lane (4)]. Immunoblotting analysis using the anti-AE1 antibody confirmed that the proteins remaining in this membrane were free from the N-CPD (data not shown). Furthermore, during the course of the membrane separation, changes in the protein and thiol contents in the RBC membranes were also followed. AE1 is known to be one-fourth of the total membrane protein.[36] The protein contents in the RBC membranes decreased because of the separation of the membranes, as compared to that in the RBC ghost membrane; nearly 60% of the protein was removed from the RBC ghost membrane by the NaOH extraction, and over 70% was removed by treatment with α-Chy and NaOH (Figure 2 b). The thiol contents in the RBC membranes also decreased in a similar fashion because of these treatments (Figure 2 c). The observed changes in the protein and thiol contents verified the removal of the peripheral proteins, such as the thiol-containing spectrin.[37] Consequently, these analytical data could verify that the high pH extraction subsequent to α-Chy digestion can successfully separate the N-CPD and the membrane domain of AE1 from the RBC ghost membrane.

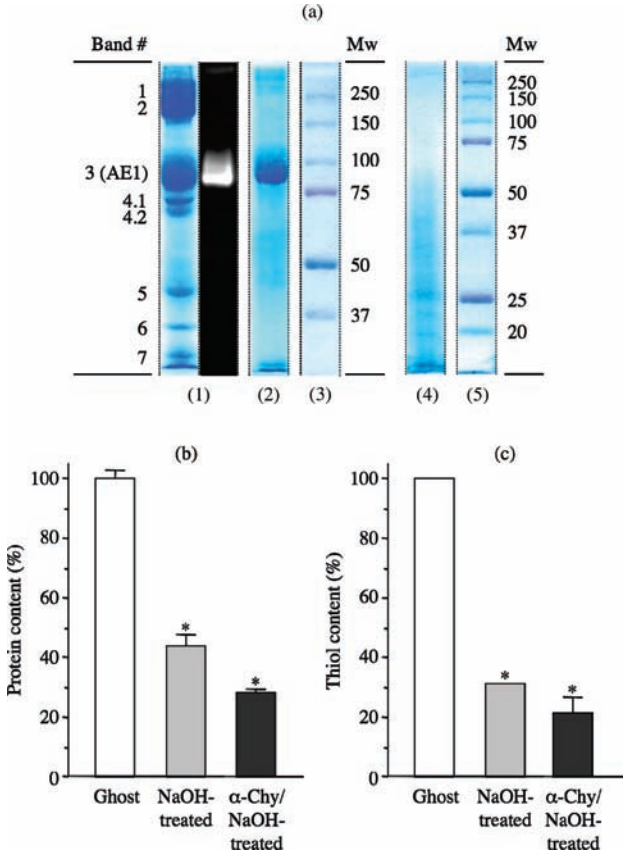

Figure 2. Electrophoretic and immunoblotting analyses of RBC membrane proteins (a) and protein (b) and thiol (c) contents in RBC membranes. (a) Lanes (1), SDS-PAGE (left) and its immunoblotting with anti-AE1 antibody (right) of RBC ghost membrane, 7.5% polyacrylamide disk gel; lane (2), NaOH-extracted RBC membrane, 7.5% polyacrylamide disk gel; lanes (3), M_w marker in kDa for (1) and (2); lane (4), SDS-PAGE of NaOH-extracted RBC membrane subsequent to α-chymotrypsin digestion, 15% polyacrylamide disk gel; lane (5), M_w marker in kDa for (4). Actual protein content in RBC ghost membranes [panel (b) white column] was 1.7 ± 0.1 mg/mL-RBC. Actual thiol content in RBC ghost membranes [panel (c) white column] was 132.2 ± 9.6 nmol-thiol/mL-RBC. Data express mean and S. E. (n=3−8) *, Significantly different from the corresponding values for the RBC ghost membrane with $P < 0.05$.

The selenium distribution in the RBC ghost membrane after treatment with SA was analyzed according to the above separation technique. Sequential treatment of the RBC ghost membrane with α-Chy and NaOH gave 47.5 ± 3.4 [Figure 3, bar (a), mean ± S. E.] and 31.5 ± 3.8 [Figure 3, bar (b)] % selenium releases from the membrane, respectively. The remaining selenium (21.0 ± 0.5%) [Figure 3, bar (c)] was found in the stripped membrane involving the membrane domain of AE1. As a result, nearly 70% of the selenium in the RBC ghost membrane treated with SA appeared to bind to the AE1 protein, which suggests that the N-CPD and the membrane domain are responsible for the membrane transport of selenium.

The thiol-dependency of the selenium export from the RBCs to the blood plasma was examined using the membrane permeable thiol reagents, *N*-ethylmaleimide (NEM)

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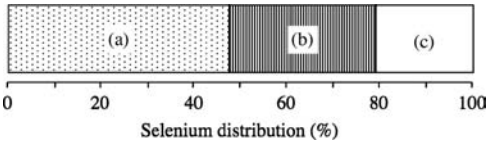


Figure 3. Selenium distribution in RBC ghost membrane after treatment of selenious acid (SA). (a), % released from the α-chymotrypsin (α-Chy) digestion; (b), % released from the NaOH extraction; (c), % remaining in the membrane after the NaOH extraction. RBC ghost membranes from SA (8 μM) treated RBC were incubated with 0.2 mg/mL α-Chy for 10 min in isotonic phosphate buffer (pH 7.4). After washing, the membranes were treated with 0.1 M NaOH. The selenium content in the RBC ghost membrane after the treatment of SA was defined as 100%. Actual selenium amounts for bars (a), (b), and (c) were 56.2 ± 12.1, 35.5 ± 3.2, and 24.5 ± 4.6 ng, respectively (mean ± S. E., $n = 3$).

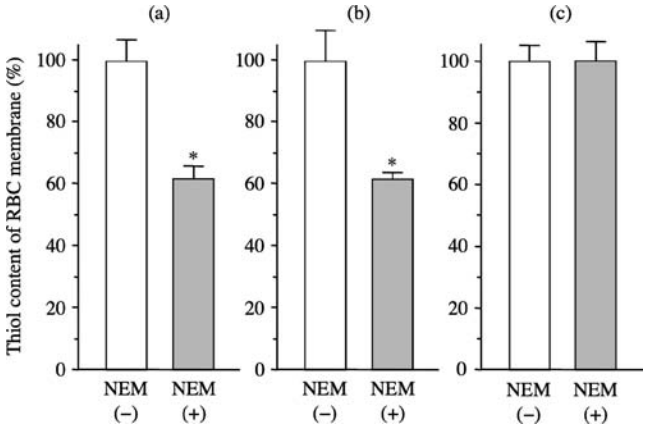


Figure 4. Changes in thiol content of RBC membranes because of *N*-ethylmaleimide (NEM) treatment. RBCs were incubated with selenious acid (final concentration 8 μM) for 10 min, and then treated with NEM (final concentration 1 mM) for 20 min at 35 °C in isotonic phosphate buffer (pH 7.4). (a), % thiol content in RBC ghost membrane; (b), % thiol content in NaOH-extracted RBC membrane; (c), % thiol content in NaOH-extracted RBC membrane subsequent to α-chymotripsin digestion. Actual thiol contents for NEM (−) in panels (a), (b), and (c) were 78.3 ± 5.7, 64.9 ± 6.5 and 57.5 ± 3.3 nmol-thiol/mg-protein, respectively. (mean ± S. E., $n = 3$). *, Significantly different from the corresponding values for the NEM-free treatment with $P < 0.05$.

and tetrathionate (TTN).[38−40] NEM treatment can result in alkylation of thiol groups of proteins [Scheme 1 a]. The thiol contents of the RBC membranes treated with NEM were compared to those without NEM. The RBC ghost membrane showed the reduced thiol levels, as compared to the respective control membrane samples (Figure 4 a). A similar decrease in the thiol content was also found in the NaOH-extracted membrane remaining the N-CPD of the AE1 Figure 4 b], whereas no such change was observed for the RBC membrane treated with α-Chy and NaOH (Figure 4 c). Therefore, the NEM treatment used in this study resulted in the alkylation of thiol groups in the N-CPD of AE1, but not those in the membrane domain. Such an NEM treatment significantly inhibited the selenium export from the RBC to the blood plasma (Figure 5), which suggests that the membrane transport of selenium from the RBC to the blood plasma is dependent on the thiol groups in AE1 protein.

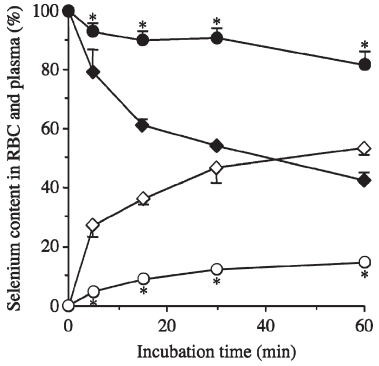
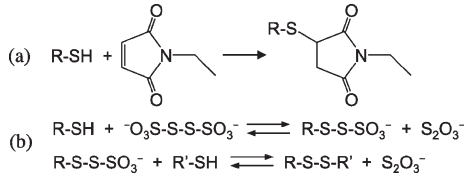


Figure 5. Inhibitory effect of *N*-ethylmaleimide (NEM) treatment on the selenium export from RBC to the blood plasma at 37 °C. Before mixing with the blood plasma, RBCs were incubated with selenious acid (final concentration 8 μM) for 10 min, and then treated with or without NEM for 20 min at 35 °C in isotonic phosphate buffer (pH 7.4). Circle symbol, treated with NEM (final concentration 1 mM); diamond symbol, treated without NEM. Solid symbol, in RBC; open symbol, in the blood plasma. Data express mean and S. E. ($n = 3$). *, Significantly different from the corresponding time points of the NEM-free treatment with $P < 0.05$.

Scheme 1. Thiol Modification by the Reaction of *N*-ethylmaleimide (NEM) (a) and Tetrathionate (TTN) (b) with Membrane Protein Thiol Groups



TTN can oxidize thiol groups to form the disulfide bond between the thiol-containing substances (Scheme 1 b).[41−43] TTN treatment caused decreases in the thiol content not only in the RBC ghost membrane (Figure 6 a) and the NaOH-extracted membrane (Figure 6 b) but also in the RBC membrane treated with α-Chy and NaOH [Figure 6 c]. Thus, the TTN treatment appeared to oxidize the thiol groups in both the N-CPD and the membrane domain of the AE1. TTN treatment of the RBCs resulted in complete inhibition of the selenium export to the blood plasma, even during the initial period when the export has a maximum velocity in the case of the TTN-free treatment (Figure 7). Such an inhibitory effect demonstrates that thiol groups in both the N-CPD and the membrane domain are critically responsible for the membrane transport of selenium through the AE1 protein.

To investigate the inhibitory effect of the TTN treatment from the structural viewpoint of the AE1, the TTN-treated RBC membranes before and after the selenium export experiment were analyzed by SDS-PAGE. Although AE1 exists as a homodimer and/or tetramer in the native membrane, AE1 is detected in the monomeric state under a non-reductive SDS-PAGE condition, as shown in Figure 2. Reinhart et al. indicated that AE1 could be covalently cross-linked to a dimer in the membrane by intermolecular

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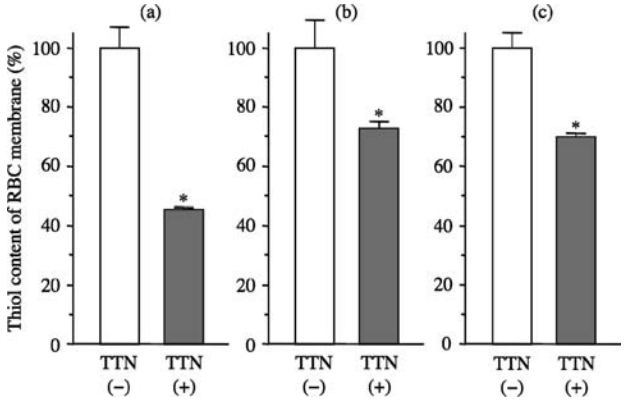

Figure 6. Changes in thiol content of RBC membranes because of tetrathionate (TTN) treatment. RBCs were incubated with selenious acid (final concentration 8 μM) for 10 min, and then treated with TTN (final concentration 20 mM) for 1 h at 37 °C in isotonic phosphate buffer (pH 7.4). (a), % thiol content in RBC ghost membrane; (b), % thiol content in NaOH-extracted RBC membrane; (c), % thiol content in NaOH-extracted RBC membrane subsequent to α-chymotripsin digestion. Actual thiol contents for TTN (−) in panels (a), (b), and (c) were the same as those in Figure 4 (n = 3). *, Significantly different from the corresponding values for the TTN-free treatment with $P < 0.05$.

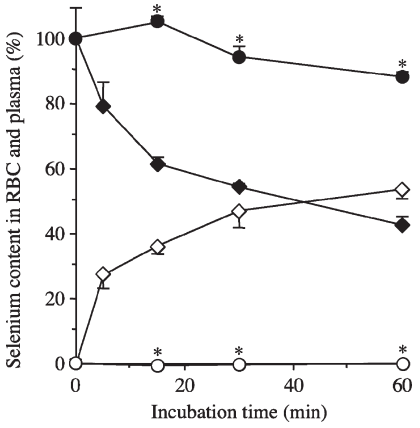

Figure 7. Inhibitory effect of tetrathionate (TTN) treatment on the selenium export from RBC to the blood plasma at 37 °C. Before mixing with the blood plasma, RBCs were incubated with selenious acid (final concentration 8 μM) for 10 min, and then treated with or without TTN for 1 h at 37 °C in isotonic phosphate buffer (pH 7.4). Circle symbol, treated with TTN (final concentration 20 mM); diamond symbol, treated without TTN. Solid symbol, in RBC; open symbol, in the blood plasma. Data express mean and S. E. (n = 3). *, Significantly different from the corresponding time points of the TTN-free treatment with $P < 0.05$.

disulfide bond formation between the cytoplasmic cysteine residues.[44] An SDS-PAGE analysis of the NaOH-extracted RBC membrane sample from the TTN-treated RBC before the selenium export experiment showed AE1 in oligomeric states [Figure 8 a, lanes (1)]. The formed AE1 oligomers were converted to the monomeric and/or lower order oligomers (dimer and trimer) by treatment with dithiothreitol [Figure 8 a, lane (2)]. Because of the reversible thiol oxidation manner of TTN, the intermolecularly formed disulfide bonds of AE1 proteins could be cleaved by endogenous reducing materials such as glutathione in the RBC during the incubation with the blood plasma. When the RBC membrane sample after the selenium export experiment was analyzed by SDS-PAGE, the oligomerized AE1 proteins still remained

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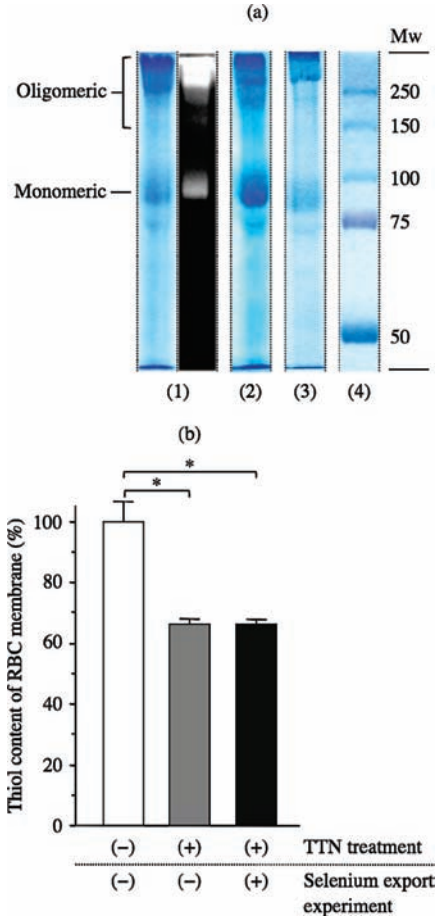

Figure 8. Electrophoretic and immunoblotting analyses of proteins (a) and thiol contents (b) of TTN-treated RBC membranes before and after the selenium export experiment. (a) Lanes (1), SDS-PAGE (left, 7.5% polyacrylamide disk gel) and its immunoblotting with anti-AE1 antibody (right) of the NaOH-extracted RBC membranes after treatment with TTN (final concentration 20 mM) for 1 h at 37 °C in isotonic phosphate buffer (pH 7.4); lane (2), SDS-PAGE of the membrane sample (1) after treatment with dithiothreitol (final concentration 4 mM) for 45 min at 37 °C in isotonic phosphate buffer (pH 7.4), 7.5% polyacrylamide disk gel; lane (3), SDS-PAGE of the NaOH-extracted RBC membranes at 60 min in the selenium export experiment, 7.5% polyacrylamide disk gel; lane (4), M_w marker in kDa. Data express mean and S. E. (n = 3−5) *, Significantly different with $P < 0.05$.

[Figure 8 a, lane (3)]. In addition, no change in the thiol content was also observed between the TTN-treated RBC membrane samples before and after the selenium export experiment (Figure 8 b). Consequently, the complete inhibition of selenium export from the TTN-treated RBC appeared to be ascribed to the intermolecularly formed disulfide bonds resulting in the oligomerized AE1 proteins.

In aqueous media, thiol reagents can react with ionized thiol groups (R−S$^-$) about 5×10^9 times faster than with unionized ones (R−SH).[45] If a thiol group is on the membrane lipid-accessible surface and in the protein interior, it will not be reactive, because ionization of the thiol group is suppressed because of the low dielectric constant of the environment.[46] Thus, thiol groups in the water-accessible surface will be much more reactive with thiol reagents than those in the membrane lipid-accessible surface or in the protein interior,

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and will be covalently modified by the reagents. In the case of the AE1 protein, thiol groups of Cys201 and Cys317 in the N-CPD and Cys885 of the carboxyl-terminal tail should be water-accessible. In addition, Cys843 in the membrane domain would also be a water-accessible residue, since the AE1 protein is reported to undergo palmitoylation through this thiol group in the RBC.[47] As the Cys479 residue is on a membrane lipid-accessible surface, it is quite unlikely that this thiol group would be modified under the reaction conditions used in this study. In fact, the thiol groups in both the NEM- and the TTN-treated membranes remain to be modified. The TTN treatment used in this study could possibly modify the thiol groups of four cysteine residues (Cys201, Cys317, Cys843, and Cys885), except for that of Cys479. Taking into consideration that selenium bound to Hb is not transported to the N-CPD deleted RBC membrane at all,[18] selenium bound to the N-CPD of AE1 may be intra- and/or intermolecularly relayed to the thiol groups in the membrane domain. The thiol group of Cys479 on a lipid-accessible surface could possibly be involved as the next relay site of selenium to be exported from the other thiol groups in the AE1 to the blood plasma, since the AE1 protein seems to show a more dynamic movement because of conformational changes of its two domains than originally proposed.[48] Casey et al. mutated each of these cysteines to serine to examine their role in the function of the AE1, and stated that the cysteine residues of the human AE1 are not required for the anion exchange and cytoskeletal binding roles of the protein,[49] while the membrane domain can mediate anion transport even after the removal of the N-CPD.[50,51] Consequently, the mechanism for the thiol-dependent membrane transport of selenium should be different from that for the Cl^-/HCO_3^- anion exchange. In addition, the selenium export from RBC to the blood plasma was blocked at 4 °C (data not shown), which means that this event is also an energy-dependent pathway. The AE1 protein is known to undergo extensive covalent modification in RBC, including *N*-glycosylation and phosphorylation, but the functions of these modifications are still unknown in the mammalian AE1.[52,53] Besides the thiol-dependent mechanism, the membrane transport of selenium seems to involve other mechanisms that result from such modifications of the cytosolic moiety of the AE1.

In summary, our data suggested that the selenium export from the RBC to the blood plasma is primarily dependent on the thiol groups in the integral protein AE1 of the RBC plasma membrane; the treatment of NEM, a thiol-alkylating agent, provided a marked inhibition of the selenium export from the RBC to the blood plasma. In addition, the treatment of TTN, a thiol-oxidizing agent that forms an intermolecular disulfide bond, resulted in complete inhibition of the selenium export. Thiol groups in the integral protein AE1 are thought to play essential roles in the membrane transport of selenium, as thiol groups in hemoglobin (Cysβ93) and human serum albumin (Cys34) aided in the transport of selenium.[18,19] Anion exchange proteins are widely distributed over cells and tissues in mammals that require selenium as a micronutrient. These proteins include AE1 expressed in the RBC and the kidney; AE2 found in the kidney, lymphocytes, and stomach; and AE3 is in brain, heart, and retina,[32] and contain the membrane domain that are structurally quite similar to each other, and amino-terminal cytosolic domain. Such anion exchange proteins other than erythroid AE1 may also participate in the membrane transport of selenium.

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