Hemoglobin-mediated selenium export from red blood cells

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Abstract   On the basis of the fact that selenium (Se) from selenite binds to hemoglobin (Hb), we investigated the missing process in the selenium export from red blood cells (RBCs), i.e., the transfer of selenium bound to Hb to RBC membrane proteins.   To elucidate the molecular events of the Hb-associated selenium export from RBC, an Hb-Se complex was synthesized from thiol-exchange of Cys-β93 in Hb with penicillamine-substituted glutathione selenotrisulfide, as a model of major metabolic intermediates, and then interactions between the Hb-Se complex and RBC inside-out vesicles (IOVs) were examined.   Selenium bound to Hb was transferred to the IOV membrane on the basis of the intrinsic interactions between Hb and the cytoplasmic domains of Band 3 protein (CDB3).   The observed selenium transfer was inhibited by the pretreatments of IOVs with iodoacetamide and the α-chymotrypsin digestion, indicating that the Hb mediates the selenium transfer to the thiol groups of CDB3.   In addition, it was found that deoxygenated Hb with a high binding affinity for CDB3 more favorably transferred selenium to the IOV membranes than oxygenated Hb with a low affinity.   When selenium export from RBC to the plasma was examined by continuously introducing nitrogen gas, the selenium export rate was promoted with an increase in the rate of deoxygenated Hb.   Overall, these data suggested that Hb could possibly play a role in the selenium export from RBC treated with selenite in an oxygen-linked fashion.

Keywords Band 3 protein · Hemoglobin · Inside-out vesicle · Red blood cell · Selenium

Abbreviations *N*-CPD: Amino-terminal cytoplasmic domain・AE1: Anion exchanger 1・*C*-CPD: Carboxy-terminal cytoplasmic domain・α-Chy: α-Chymotrypsin・CDB3: Cytoplasmic domains of Band 3 protein · DAN: 2,3-Diaminonaphtalene · DIDS: 4,4'-Diisothiocyanato-2,2'-stilbene disulfonate · DTNB: 5,5'-Dithiobis (2-nitrobenzoic acid)・GSH: Glutathione・GPx: Glutathione peroxidase ・

GSSeSG: Glutathione selenotrisulfide・GPA: Glycophorin A・Hb: Human hemoglobin・IOV: Inside-out vesicle・MALDI-TOF: Matrix-assisted laser desorption ionization-time of flight・Pen: L-Penicillamine・PenGSH: L-Penicillamine-substituted glutathione (γ-L-Glu-L-Pen-Gly)・RBC: Red blood cell

Introduction

Oxygen is essential for all higher forms of animal life. Vertebrates have evolved with two principal mechanisms for supplying their peripheral tissues/cells with a continuous and adequate flow of oxygen to acquire much energy from glucose. The first mechanism is a circulatory system that actively delivers oxygen to the peripherals. The second is the use of oxygen-carrying molecules to overcome the limitation imposed by the low solubility of oxygen in water. Hemoglobin (Hb) molecules in red blood cells (RBCs) serve as the oxygen carrier from the respiratory to peripheral tissues and also play a vital role in the transport of cellular waste carbon dioxide in the opposite direction [1]. On the other hand, selenium is also an essential trace element in mammals and is broadly distributed over the entire body and incorporated into selenoproteins in the form of selenocysteine that is known the 21st amino acid. The family of selenium-dependent glutathione peroxidases (GPx-1, GPx-2, GPx-3, GPx-4 & GPx-6) is the best-known example of the selenoproteins [2]. These enzymes play a critical role in the antioxidant defense against the deleterious actions of free radicals and lipid peroxides that unavoidably occur due to the oxygen utilization [3]. The selenium element as the central building block of GPxs is thought to be delivered to the entire body via the bloodstream.

In humans, the chemical form of selenium from food sources is mainly organic selenomethionine and selenocysteine, while inorganic selenite (SeO_3^{2-}) is rare but an effective source compound most frequently used in the selenium supplementation for medical treatments. Selenite is immediately taken up from the plasma into RBC through the Band 3 protein (anion exchanger 1) and then returned to the plasma after the reductive metabolism in RBC [4,5]. However, little is known about the definitive delivery mechanisms of selenium from selenite after the RBC uptake. It has been speculated further that reductive metabolites such as selenide excreted into the bloodstream are bound to albumin and transferred to tissues/cells. If the oxygen-carrying Hb molecule participates in the selenium delivery to the peripheral blood and/or tissues in order to extinct the harmful substances that are generated by the accompanying oxygen utilization, it is likely to be a compensatory function of Hb. In human Hb, the β chain contains a thiol group (Cys-β93) that probably reacts with reductive metabolites of selenite. This thiol group is highly conserved among species while its actual physiological function has remained unknown. In this study, we synthesized a metabolic intermediate of selenium in RBCs as a chemical

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tool to reveal the missing process, i.e., the transfer of selenium bound to hemoglobin to RBC membrane proteins.

Materials and methods

Materials

L-Pen and iodoacetamide were obtained from Tokyo Chemical Industry Co., Ltd. The human hemoglobin was from Sigma Co. {oxy-Hb : deoxy-Hb : ferri-Hb = 6 : 9 : 86 (UV-VIS photometry), Hb purity; 98.9%, the number of reactive thiol groups determined by the DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] method; 1.45 per Hb tetramer} method. α-Chymotrypsin (from bovine pancreas, 35–65 units/mg) was purchased from Nacalai Tesque, Inc. Sinapinic acid used as a matrix substance for MALDI-TOF mass spectrometry was obtained from Fluka. All other chemicals were of commercial reagent or special grades and used as received.

Determination of selenium and protein concentrations

The selenium concentrations were fluorometrically determined using 2,3-diaminonaphtalene (DAN) after the digestion with a one to five mixture by volume of perchloric acid and nitric acid [6]. The selenium standard solution [1,000 ppm as selenium (IV) dioxide in 0.5 M nitric acid] for the fluorometry was obtained from Kanto Chemical Co., Inc. The protein concentrations were measured by BCA protein assay [7].

Preparation of RBCs and oxy-Hb

Fresh human venous blood was collected in a heparinized vacutainer tube. 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). The isolated RBCs were hemolyzed with 40 volumes of 5 mM phosphate solution (pH 8), and centrifuged at 22,000 g for 10 min. The supernatant was dialyzed against 0.5 mM phosphate solution (pH 8) using a Spentra®/Por Membrane (molecular weight cutoff; 6–8 kDa) at 4 °C. The transition state of the purified oxy-Hb was measured from visible absorption spectra (from 560 to 630 nm) according to the method of Zijistra et al. [8] by UV-VIS photometry (oxy-Hb : deoxy-Hb :

ferri-Hb = 87 : 10 : 3, the number of reactive thiol groups determined by the DTNB method; 1.69 per Hb tetramer).

Analysis of selenium distribution in selenite-treated RBCs

The isolated RBCs were treated with varying concentrations of selenite (0.8–80 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min [hematocrit 20% (v/v)]. The RBCs were hemolyzed by the addition of two volumes of deionized water, and centrifuged at 28,000 g for 1 h. The supernatant and the red pellet were then separated. Furthermore, the supernatant was diluted and ultrafiltered using an Ultrafree®-MC (30 kDa nominal molecular weight limit). The selenium contents of the supernatant (cytosol, whole), the filtrate (cytosol, molecular weight less than 30 kDa), and the red pellets (the plasma membrane) were determined. To investigate the membrane-bound selenium in detail, the selenite-treated RBCs were hemolyzed by the addition of 40 volumes of 5 mM phosphate solution (pH 8). The hemolysate was centrifuged at 22,000 g for 15 min and the supernatant was aspirated. The pellets were washed three times with 5 mM phosphate solution (pH 8) and once with 0.5 mM phosphate solution (pH 8). White unsealed ghosts were obtained and their selenium and protein concentrations were determined.

Synthesis of PenGSSeSGPen

Penicillamine-substituted glutathione (γ-L-Glu-L-Pen-Gly, PenGSH) was synthesized by the conventional liquid-phase synthesis reaction. Purified PenGSH had a 1.08 thiol group/molecule when determined by the DTNB method. ^{1}H NMR (D$_2$O): 1.43 (s, 3H), 1.49 (s, 3H), 2.14 (q, 2H, J = 7.2 Hz), 2.57 (t, 2H, J = 7.8 Hz), 3.77 (t, 1H), 3.87 (s, 2H), 4.47 (s, 1H). FAB MS: calcd for C$_{12}$H$_{21}$N$_3$O$_6$S m/z 336.1, found: 336.0. Anal. calcd for C$_{12}$H$_{21}$N$_3$O$_6$S · CF$_3$COOH · 2H$_2$O C, 34.64; H, 5.40; N, 8.66, found: C, 35.89; H, 5.47; N, 9.01. PenGSH (10 mM) was allowed to react with selenious acid (2.5 mM) in deionized water with stirring for 12 h at room temperature. The resultant was chromatographed on a COSMOSIL 5C18-AR-II at the flow rate of 7 mL/min with a detection wavelength of 210 nm. The column was programmed with a 70-min linear gradient from 100 to 50% of eluent A [10% (v/v) acetonitrile in water containing 0.005% (v/v) trifluoroacetic acid] in eluent B (acetonitrile). The peak assigned to PenSSeSPen was fractionated, and then followed by lyophilization. ^{1}H NMR (D$_2$O): δ 1.44 (s, 3H), 1.49 (s, 3H), 2.10 (m, 2H), 2.51 (t, 2H, J = 7.5 Hz), 3.72–3.78 (m, 3H), 4.55 (s, 1H), MALDI-TOF MS: calcd for C$_{24}$H$_{40}$N$_6$O$_{12}$S$_2$^{80}Se m/z 748.1, found: 749.8. Anal. calcd for

$C_{24}H_{40}N_6O_{12}S_2Se \cdot 2CF_3COOH \cdot 4H_2O$ C, 32.10; H, 4.81; N, 8.02; Se, 7.54, found C, 32.08; H, 4.56; N, 7.93, Se, 7.33. λ_{max} in deionized water: 268 nm (ε_{mM} = 1.60) (Fig. S1).

Preparation of Hb-Se complex

PenGSSeSGPen (0.5 mM) was combined with Hb (50 mM as tetramer) in 0.5 mM phosphate solution (pH 7.4). The mixture was incubated for 10 min at 37 °C, and then the unreacted PenGSSeSGPen was removed by passing through a Sephadex G-50 (fine) column equilibrated with 0.5 mM phosphate solution (pH 7.4). The ferri-Hb and ferri-Hb-Se concentrations were measured using a molar extinction coefficient of Hb at 407 nm (ε = 352.3 mM^{-1} cm^{-1}). The oxy-Hb concentrations and the transition states of the prepared Hb-Se complex were measured by UV-VIS photometry (ferri-Hb-Se; oxy-Hb : deoxy-Hb : ferri-Hb = 10 : 3 : 87, oxy-Hb-Se; oxy-Hb : deoxy-Hb : ferri-Hb = 72 : 0 : 28). The purified Hb-Se complex contained 0.78 selenium/Hb tetramer.

Preparation of IOV and selenium transfer experiment

The IOVs were prepared from the white ghosts according to the method of Steck and Kant with slight modifications [9]. The apparent purity of the used IOVs was estimated to be 87.9 ± 0.6% (mean ± s. e. m.) from the determination of the acetylcholinesterase activity. The IOVs were mixed with the Hb-Se complex in 10 mM phosphate buffer (pH 6) at ambient temperature, and the mixture was centrifuged at 22,000 g for 15 min. The binding affinity of the Hb-Se complex with Hb to IOV was compared using the Langmuir type binding equation. In a study of the selenium transfer, the mixture of the Hb-Se complex and IOV was centrifuged at 22,000 g for 15 min, and then the precipitated IOVs were washed with 20 mM phosphate buffer (pH 8) containing 0.5 M sodium chloride to remove the IOV-bound Hb and Hb-Se complex. The released Hb amounts in the washing buffer and a SDS-PAGE analysis of the IOV (Fig. S2) indicated that Hb and the Hb-Se complex were completely washed out from the IOV membranes. The Hb-Se complex solutions of various transition states (oxy-Hb-Se content; 10 to 70%, ferri-Hb-Se content; 30 to 90 mol%) were prepared by mixing of the oxy-Hb-Se and ferri-Hb-Se.

Selenium export experiment from selenite-treated RBCs to the plasma

The isolated RBCs were treated with selenite (8 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min. The plasma was separately placed in a tightly sealed glass vessel, and was exposed to gentle

N$_2$ gas bubbling at 37 °C for 30 min. The RBC suspension was combined with the plasma in the sealed glass vessel [hematocrit 50% (v/v)], and then incubated with N$_2$ gas bubbling at 37 °C. Aliquots of the RBC suspension were pipetted out at appropriate time intervals and centrifuged at 1,400 g and 4 °C for 10 min to separate it from the plasma. The selenium contents in the RBCs and the plasma were separately determined.

Statistical analysis

All data were presented as the mean ± s. e. m. (n = 5 or more). Statistical analyses were performed using a program PRISM 4 (GraphPad Software Inc.). Multiple mean values were compared by a one- or two-way ANOVA with a Bonferroni post-hoc test. Comparisons were considered statistically significant at $P < 0.05$.

Results and discussion

The hemolysate of RBC was separated into three fractions [low (< 30 kDa) and high (> 30 kDa) mass fractions, and the plasma membrane] and the selenium contents of each fraction were determined. Represented in Fig. 1 (A) is selenium distributions in RBC treated with varying selenite concentrations. Most of the selenium (> 95%) in the RBC hemolysate was found in the high mass fraction, namely Hb, and the rest was in the low mass fraction and the plasma membrane. This selenium distribution was similar to each other for the three different selenite concentrations used in the RBC treatments. The selenium distributions remained the same up to 3 h after the uptake of selenite into the RBC, which demonstrates that most of selenium is stably bound to Hb. The selenium contents in the plasma membrane increased with an increase in the selenite concentration used for the treatment of RBC, although the distribution rate of selenium in the RBC was very low [Fig. 1 (B)].

Painter reported that the reaction of selenite with low mass thiol-containing compounds (RSH) yielded selenotrisulfide (RSSeSR) in vitro (SeO$_3$$^{2-}$ + 4 R–SH → R–SSeS–R + R–SS–R + 3 H$_2$O) [10]. Recently, glutathione selenotrisulfide (GSSeSG) was actually identified in a yeast extract by mass spectrometric techniques [11]. Since Hb did not allow the forming of Hb selenotrisulfide (HbSSeSHb) in the reaction with selenite probably due to a steric hindrance, the Painter reaction with glutathione (GSH) could also be involved in the metabolism of selenite in the RBC. We have demonstrated that selenotrisulfide preferentially reacts with the Cys-β93 by the thiol exchange mechanism to form HbSSeSR, and selenium is not eliminated from Hb in the presence of GSH [12]. Consequently,

selenite in the RBC is thought to transform into GSSeSG, and subsequently, GSSeSG could react with Cys-β93 to form the HbSSeSG. These results are also consistent with previous observations that selenite is bound to Hb after undergoing the GSH-mediated reduction in RBC [4,5].

After the RBC was treated with selenite, selenium-bound Hb was not able to be directly characterized because the fraction of the selenium–bound Hb is fairly lower than that of the unbound one, in addition, the separation of the two species is also quite difficult. While the naturally occurring GSSeSG easily decomposes in vitro at physiological pH to generate the red elemental selenium (Se_0) that is not practically present in vivo [13,14]. An in vitro reaction of Hb with GSSeSG inevitably results in the generation of not only HbSSeSG, but also unfavorable species such as the Hb-Se_0 complex. To avoid this chemical diversity, we synthesized a new penicillamine-substituted glutathione selenotrisulfide [PenGSSeSGPen, Fig. 2 (A)] as a model of the metabolic intermediate GSSeSG. PenGSSeSGPen was isolatable and chemically stable in isotonic phosphate buffer (pH 7.4) at 37 °C for 4 h or longer without any degradation [Fig. 2 (B)]. PenGSSeSGPen and Hb were co-incubated in isotonic phosphate buffer (pH 7.4) at 37 °C, and the unreacted PenGSSeSGPen was monitored by reversed-phase liquid chromatography. PenGSSeSGPen completely disappeared within 10 min after the incubation with Hb [Fig. 2 (C)]. The resulting material was subjected to a MALDI-TOF mass spectrometric analysis. In addition to the peaks assigned to the α- and β-chains (mass number 15191.9 and 15935.8 respectively), a separate peak appeared at mass number 16350.0 and its peak intensity increased with an increase in the PenGSSeSGPen/Hb molar ratio (Fig. 3). The mass number of 414.2, which is greater than the free β-chain, corresponded to that of the PenGSSe- moiety (calculated mass number for $C_{12}H_{20}N_3O_6S_2{}^{80}Se$ 412.3), suggesting the formation of Hb-SeSGPen (Hb-Se complex). These mass spectral data were identical to our previous work, in which penicillamine selenotrisulfide (PenSSeSPen) reacted only with the reactive Cys-β93 via the thiol exchange mechanism [12]. In addition, no remarkable difference in the structural organization between the Hb-Se complex and Hb was observed in the circular dichroism spectral data over the range from 190 to 350 nm (Fig. S3).

Since Hb molecules never pass through the RBC plasma membrane, selenium bound to Hb molecules must dissociate from Hb and then cross the plasma membrane for the selenium to be exported to the plasma. In addition, selenium release from the Hb-Se complex is not observed by glutathione treatment. It is known, however, that the amino-terminal cytoplasmic domain (*N*-CPD, Met1–Pro403) of Band 3 offers the binding sites for Hb and the cytoskeletal proteins [15–21]. Band 3, a 911 amino acid integral membrane protein, catalyzes the electro–neutral exchange of Cl^-/HCO_3^- across the plasma membrane. This membrane protein also anchors a subpopulation of Hb molecules to RBC membranes, however, the functional significance of this behavior is not clear.

We first examined whether the Hb-Se complex would bind to the inner surface of the RBC membrane, as the non-treated Hb does using the RBC inside-out vesicles (IOVs). When the binding constants of the Hb-Se complex and Hb for IOV were estimated using the Langmuir type binding equation [22], the values obtained for the Hb-Se complex and Hb were 2.10 ± 0.43 and 1.86 ± 0.26 (μM^{-1}), respectively (mean ± s. e. m., P = 0.64, Fig. S4). In similar experiments, we confirmed that PenGSSeSGPen and selenite were not interactive with the IOVs (Fig. S5). When the IOVs were treated with the Hb-Se complex and thoroughly washed out with the washing buffer to remove the Hb bound to them, 2.58 pmol selenium per mg of IOV protein remained on the IOVs (Fig. 4). Taking these results into account, the Hb–Se complex is evidently capable of binding to the inner surface of the RBC membrane in a similar fashion to Hb, and that Hb can deliver Se to the RBC membrane components.

Attempts were then made to elucidate the binding sites of selenium to be delivered by Hb. The free cysteine residues (Cys201 and Cys317) of N-CPD are known to form a disulfide linkage with Cys-β93 under catalytic oxidative conditions [23]. X-ray crystal structure analysis indicates that the cytosolic part of one end of the Band 3 docks between the β chains of the Hb molecules. Cys-β93 of Hb is positioned near the potential target thiol groups in the Band 3 [16]. Recently, Stamler et al. demonstrated the concerted nitric oxide/oxygen systemic delivery by which nitric oxide is bound to Cys-β93, and then delivered to Cys201 and Cys317 of N-CPD based on the intrinsic interactions of Hb with N-CPD [24,25].

The pretreatment of IOV with iodoacetamide, a thiol-alkylating agent, resulted in the inhibition of the selenium delivery from the Hb-Se complex to the IOVs in a concentration-dependent manner [Fig. 4 (A)]. The treatments with iodoacetamide gave no remarkable changes in the binding affinity of the Hb-Se complex for IOV (the binding constant: 1.64). The selenium delivery to IOVs was also inhibited by the pretreatment with α-chymotrypsin (α-Chy) which selectively cleaves the Tyr359–Lys360 bond of N-CPD [Fig. 4 (A)] [26]. The reverse treatment with α-Chy (when the IOVs were first treated with the Hb-Se complex and then digested with α-Chy) gave ~ 50% selenium elimination [Fig. 4 (B)], which was similar to the result shown in Fig. 4 (A) (SDS-PAGE analyses of IOVs before and after the α-Chy treatment were shown in Fig. S2). These results support the concept that Cys201 and Cys317 of N-CPD are implicated in the selenium delivery mechanism from the Hb-Se complex to the IOVs.

In addition, the selenium delivery was tested using IOVs that were prepared from the DIDS (4,4'-diisothiocyanato-2,2'-stilbene disulfonate)-pretreated RBC. DIDS, an inhibitor of the anion exchange function of Band 3, binds to the membrane domain residues, Lys539 and Lys851, of the Band 3 protein

and induces its conformational changes [27,28]. The amount of selenium delivered to the DIDS-pretreated IOVs was less compared to the non-treated IOVs' [Fig. 4 (C)], indicating the participation of Band 3 in the selenium delivery.

To verify the results from our model experiments using PenGSSeSGPen and IOV, the unsealed membrane ghosts of the RBCs were treated with selenite and characterized. The treatment with α-Chy afforded ~ 50% release of the selenium bound to the membrane ghosts (Fig. 5), which matched the results from the model experiments (Fig. 4). While ~ 50% of the membrane-bound selenium was located on N-CPD, the rest seems to be on the cytoplasmic domain of Band 3 other than N-CPD or other membrane components. To further explore the binding sites other than N-CPD, the membrane ghosts were treated with dithiothreitol which reductively cleaves di- and trisulfide bonds. The selenium bound was almost quantitatively eliminated from the membrane ghosts (Fig. 5), thus, indicating that the thiol groups on the inner surface of the RBC membrane participated in the selenium delivery by Hb.

Band 3 contains two cytoplasmic domains; one is N-CPD and the other is the carboxy-terminal cytoplasmic domain (C-CPD, Asn880–Val911) responsible for the anion exchange function [28]. It was recently reported that an assembly of C-CPD with the cytoplasmic moiety of glycophorin A (GPA) is another possible Hb binding site [29]. C-CPD also contains one free cysteine residue (Cys885) that is possible to react with the selenotrisulfide moiety ($GSSeS^-$) of the Hb-Se complex, whereas GPA contains no cysteine residues in the cytoplasmic moiety. The mechanism underlying the selenium delivery to the RBC membrane by Hb may also involve the reactive cysteine residues of C-CPD of Band 3.

Hb molecules are present in three physiologically pertinent forms in the RBC (oxy-, deoxy- and ferri-Hb), and bind to N-CPD at physiological pH and ionic strength in an oxygen-linked fashion, with deoxy- and ferri-Hb having higher affinities for the RBC inner membrane [16,19]. Therefore, the ability of selenium delivery by Hb molecules to the IOVs was examined by varying the molar ratio of the oxy- to ferri-Hb-Se complexes. There were no differences in the amount of selenium bound to when the oxy- and ferri-Hb-Se complexes were used in this experiment, while the binding constant of oxy-Hb for the IOVs (0.51 ± 0.09 μM^{-1}) was significantly lower than that of the ferri-Hb (2.10 ± 0.43 μM^{-1}) (mean ± s. e. m., $P < 0.05$, Fig. S6). The amount of selenium transferred to the IOVs increased with a decrease in the oxy-Hb-Se (or an increase in ferri-Hb-Se) fraction (Fig. 6). These data indicate that the selenium delivery by Hb to the inner surface of the RBC membrane is responsible for the Hb transition states.

When Hb in the RBC comes in contact with the capillaries in oxygen-metabolizing tissues, a large fraction of the O_2 saturation is lost to the venous exchange. Practically, all Hb molecules are saturated

by O_2 in the alveoli of the lung while ~ 70% of the Hb is deoxygenated in the active muscle capillaries due to the release of O_2 [1]. To further verify the dependence of the selenium delivery on the Hb transition states, we examined the selenium export from the RBC to the plasma by continuously introducing N_2 gas after the selenite treatment. The selenium content in the RBC significantly decreased due to N_2 gas introduction, while the selenium amount exported to the plasma increased correspondingly (Fig. 7). Under the conditions used in this experiment, the oxy-Hb content in the RBC decreased from 91.4 ± 1.5% before mixing with the plasma to 24.9 ± 4.0% at 60 min after the incubation, whereas the fraction of the deoxy-Hb increased from 0.8 ± 1.2% to 67.6 ± 4.4%. Thus, the selenium release from the RBCs to the plasma was facilitated by the increase in the deoxy-Hb fraction.

The data in this study support the concept that Hb involves the selenium delivery to the peripherals where the rate of the deoxy-Hb in the RBC becomes much higher than that of the oxy-Hb due to the oxygen release. Consequently, selenium supplied by selenite seems to be exported from the RBC to the plasma as follows: selenite is taken up into RBC and transformed into the Hb-Se complex via GSSeSG. Thereafter, the selenium bound to Hb is transferred to the RBC inner membrane surface based on the intrinsic interactions between Hb and the cytoplasmic domain of the Band 3 protein, and then exported to the peripheral blood and/or tissues. Accordingly, Hb molecules may respond to a reduction in peripheral O_2 pressure with the release of selenium from the RBCs.

In the present study, we demonstrated that the interactions between the Hb molecule and the cytoplasmic domain of the Band 3 protein mediate the selenium delivery to the RBC plasma membrane and the subsequent export from the RBC. These observations indicate that selenium could possibly, in part, be exported to the peripheral blood and/or tissues by the Hb molecule in an oxygen–linked fashion. Selenium metabolites such as GSSeSG can be bound to a conserved thiol group (provided by Cys-β93 in human Hb), producing the selenium-bound Hb. This could be followed by the selenium transfer to the Band 3 protein, with selenium departing from the RBC. This would preferentially occur in oxygen-utilizing tissues, where deoxygenation triggers Hb to change its conformation from the oxygen-bound Hb structure to the deoxygenated one. As mentioned above, selenium is a critical element for the antioxidant defense against the oxidative damages to be generated by the respiration. Therefore, if the peripherals receive selenium from Hb together with O_2 in defensive compensation for the oxidative damages, the concerted delivery of selenium and O_2 to the peripheral blood and/or tissues may be a purposive function of Hb. A better understanding of the systemic delivery mechanisms selenium from selenite is also of significance from the viewpoints of human medical treatments and toxicology [3]. Further experiments should focus on the elucidation of the in vivo selenium delivery by Hb to the peripheral blood and/or tissues in an oxygen-linked fashion.

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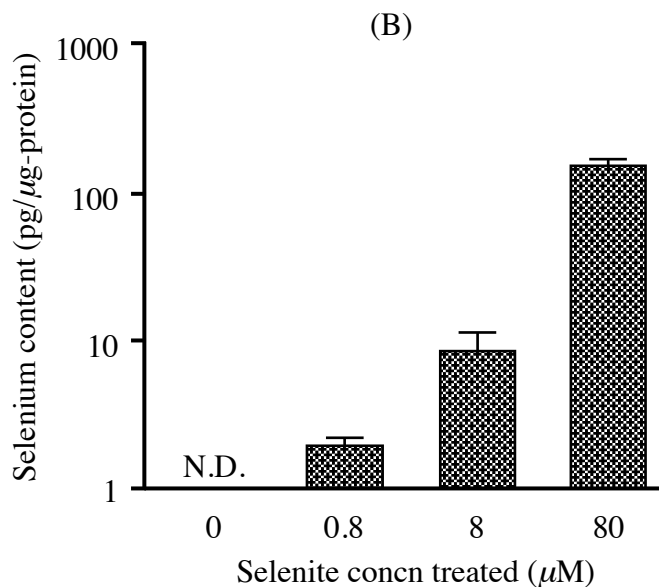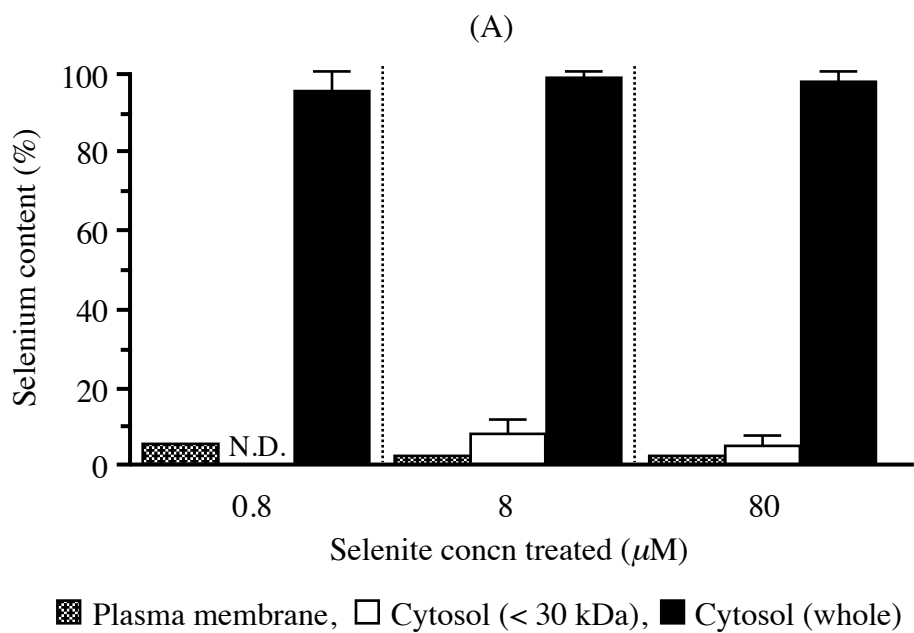
Fig. 1 (A) Selenium distribution in RBC after the treatment with selenite. The RBCs were incubated with selenite (0.8–80 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min to a hematocrit of 20% (v/v). The selenite-treated RBCs were hemolyzed and centrifuged at 22,000 g for 15 min. The obtained supernatant was further ultrafiltered using an Ultrafree®-MC. The selenium contents of the supernatant (whole cytosol), the red pellets (the plasma membrane) and the filtrate (molecular weight less than 30 kDa in the cytosol) were measured. (B) Selenium contents in the plasma membrane after the treatment of selenite. The RBCs [hematocrit 20% (v/v)] were incubated with selenite (0.8–80 μM) in isotonic phosphate buffer (pH 7.4) for 10 min. The selenite-treated RBCs were hemolyzed and centrifuged at 22,000 g for 15 min. The precipitated red pellets were thoroughly washed with 5 and 0.5

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mM phosphate solution (pH 8) to remove the membrane-bound Hb. Data are mean ± s. e. m., n = 5. n. d.; not detected.
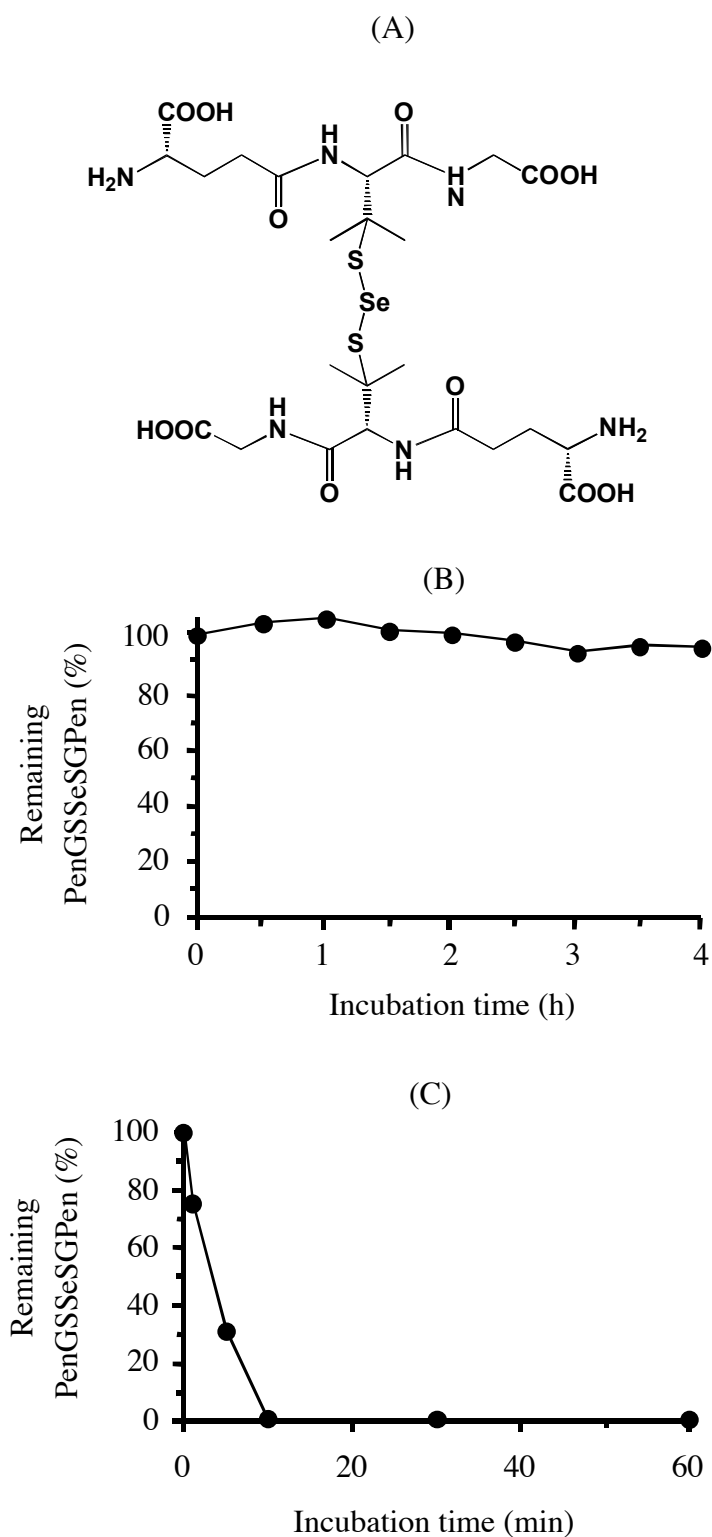
(A)

(B)

(C)

Fig. 2 (A) Chemical structure of PenGSSeSGPen. (B) Chemical stability of PenGSSeSGPen (100 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C. (C) Reaction of PenGSSeSGPen (20 μM) with Hb (200 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C. Remaining PenGSSeSGPen was determined by RPLC.
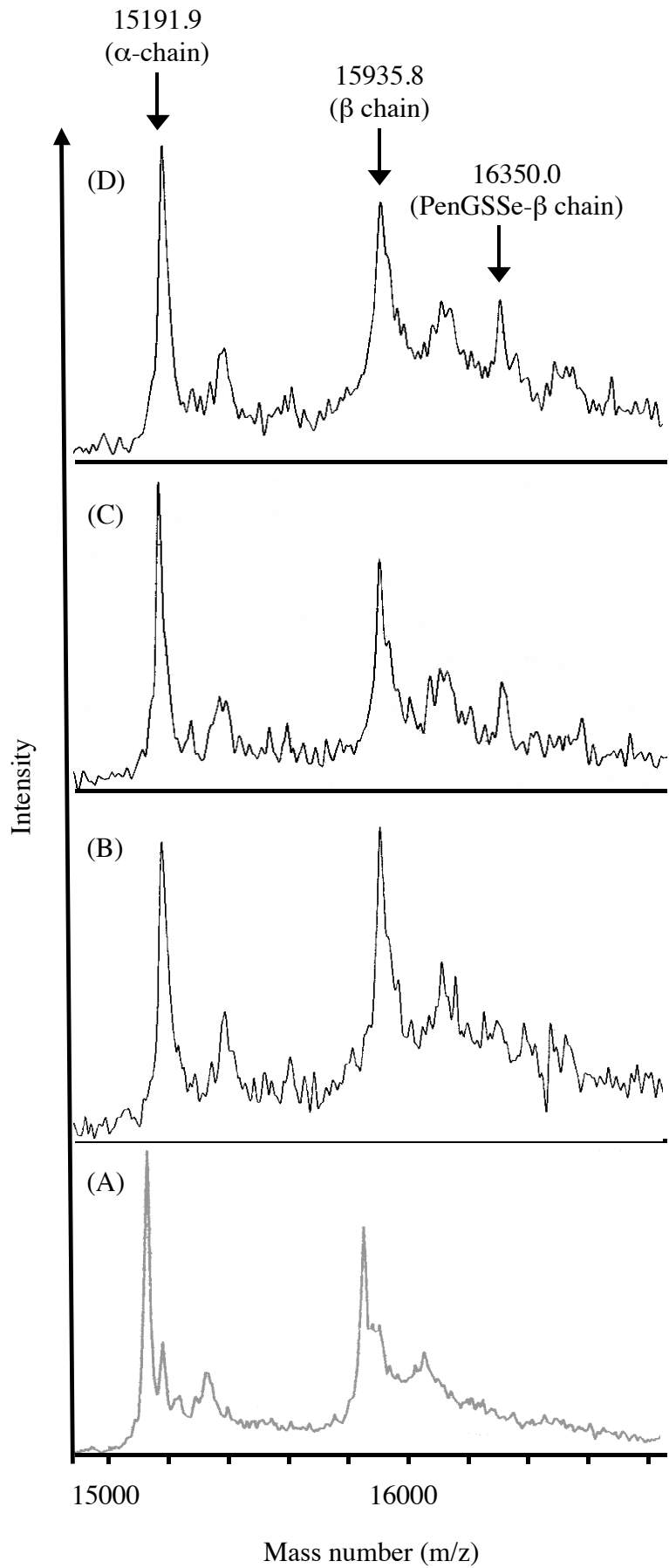
15

15191.9
(α-chain)

15935.8
(β chain)

16350.0
(PenGSSe-β chain)

(D)

(C)

(B)

(A)

Intensity

15000   16000

Mass number (m/z)

Fig. 3 MALDI-TOF mass spectra of Hb treated with increasing PenGSSeSGPen concentrations. Hb to PenGSSeSGPen concentration ratio (A) 1 : 0, (B) 1 : 5, (C) 1 : 10, (D) 1 : 20. Hb (5 μM) was treated with varying concentration ratio of PenGSSeSGPen in deionized water at 37 °C for 10 min. The sample solutions were combined with the matrix solution [sinapinic acid in 34% (v/v) acetonitrile and 20% (v/v) trifluoroacetic acid] by one to ten volume ratios, and an aliquot was applied on an AnchorChip® target (Bruker Daltonics Inc, USA). The mass spectra were obtained using an Ultraflex (Bruker Daltonics Inc, USA), operated in the linear positive ion mode. The molecular mass calibration was accomplished using a #206355 Protein Calibration Standard (Bruker Daltnics, Inc, USA).
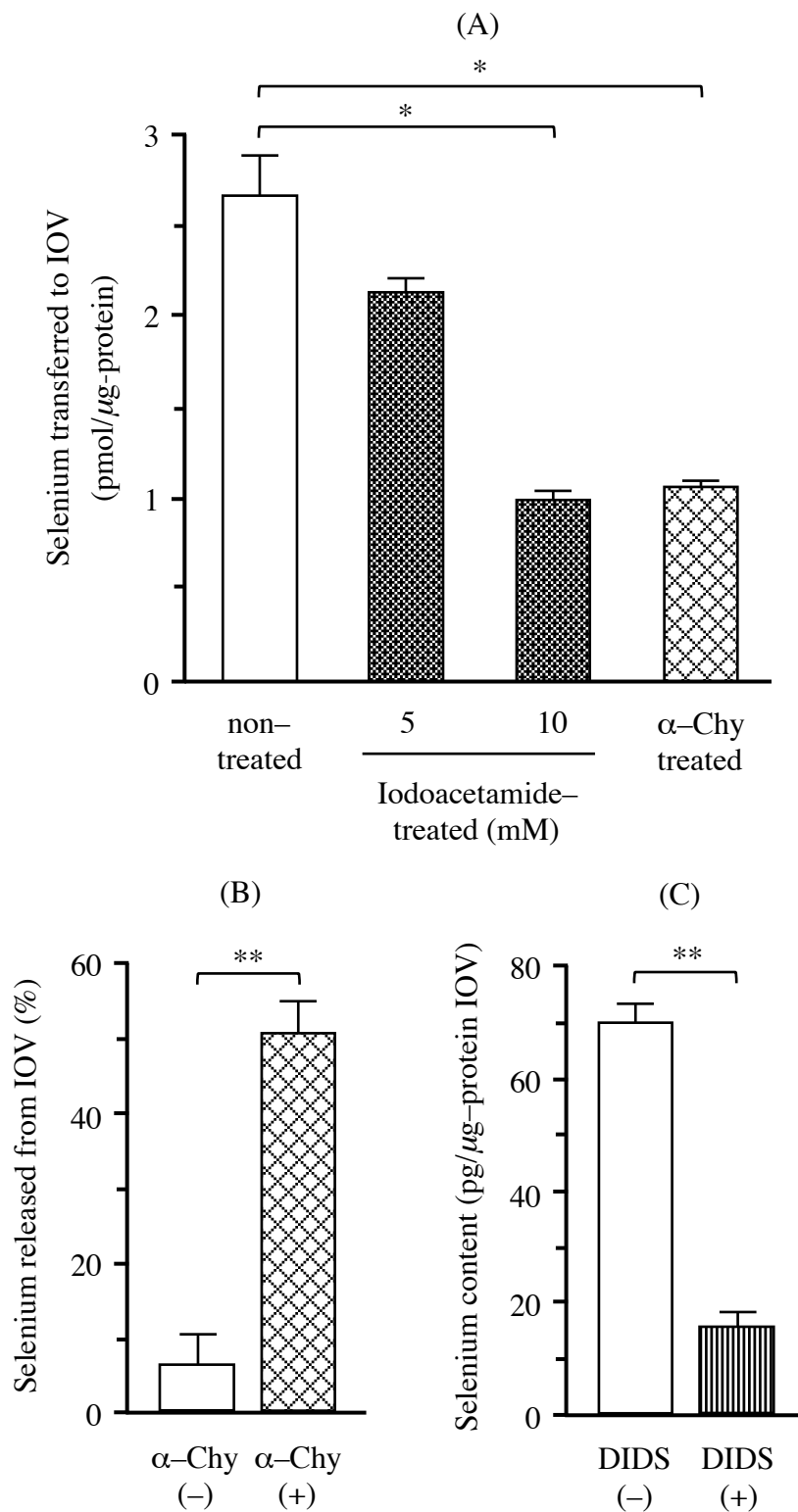
(A)

(B) (C)

Fig. 4 (A) Inhibitory effects of iodoacetamide and α-Chy on the selenium delivery from Hb-Se complex to IOVs. Non-treated and iodoacetamide (5 and 10 mM) or α-Chy (200 μg/mL)-pretreated IOVs (170 μg-protein/mL) were combined with the Hb-Se complex (1 μmol-Hb/L) in 0.01 M phosphate buffer (pH 6). After centrifugation at 22,000 g for 15 min, the precipitated IOVs were

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washed with 0.02 M phosphate buffer (pH 8) containing 0.5 M NaCl and their selenium contents were determined by the DAN method. (B) Selenium delivery from the Hb-Se complex to α-Chy-pretreated IOVs. IOV suspension (170 μg-protein/mL) was pretreated with the Hb-Se complex (1 μmol-Hb/L) in 0.01 M phosphate buffer (pH 6) at room temperature. After centrifugation at 22,000 g for 15 min, the precipitated IOVs were washed three times with 0.02 M phosphate buffer (pH 8) containing 0.5 M NaCl to remove the membrane-bound Hb and Hb-Se complex. The Hb-removed IOVs were treated with α-Chy (200 μg/mL) in 0.01 M phosphate buffer (pH 7.4) at 37 °C, and centrifuged at 22,000 g for 15 min. The selenium contents in the washing buffer and IOV were determined by the DAN method. The selenium content of the IOV before the removal of Hb was defined as 100%. (C) Effect of DIDS on selenium delivery from Hb-Se complex to IOVs. RBCs were pretreated with 100 μM selenite, and the obtained IOVs were treated with 50 μM DIDS. Data are mean ± s. e. m., n = 5. *; $P < 0.05$, **; $P < 0.01$.

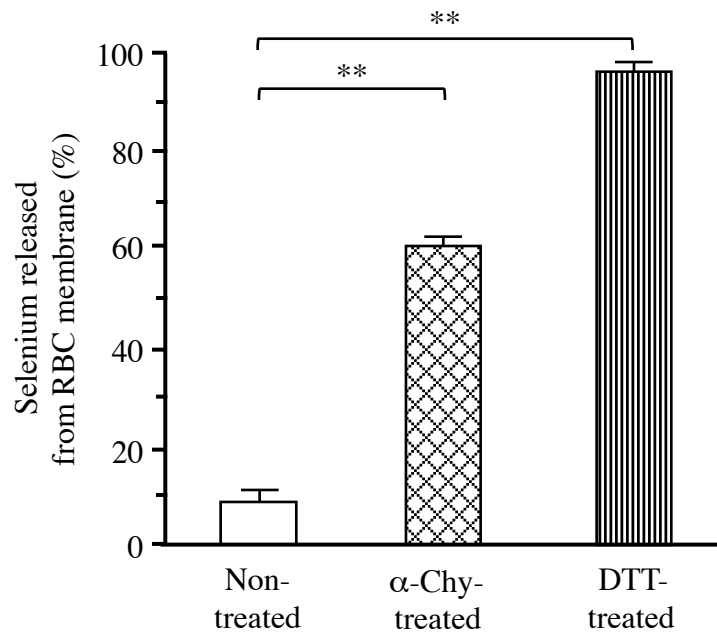Fig. 5 Selenium release from selenite-treated RBC membrane ghosts by α-Chy and dithiothreitol treatments. The isolated RBCs [hematocrit 20% (v/v)] were incubated with selenite (80 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min. The selenite-treated RBCs were hemolyzed and centrifuged at 22,000 g for 15 min. The precipitated red pellets were thoroughly washed with 5 and 0.5 mM phosphate solution (pH 8) to remove the membrane-bound Hb. The Hb-free white RBC membrane ghosts were treated with α-Chy (200 μg/mL) or dithiothreitol (2 mM) in 0.01 M phosphate buffer (pH 7.4) at 37 °C for 10 min, and centrifuged again at 22,000 g for 15 min. The selenium contents in the washing buffer and RBC membrane were determined by the DAN method. The selenium content of the RBC membrane before treatment was defined as 100%. Data are mean ± s. e. m., n = 5. **; $P < 0.01$.
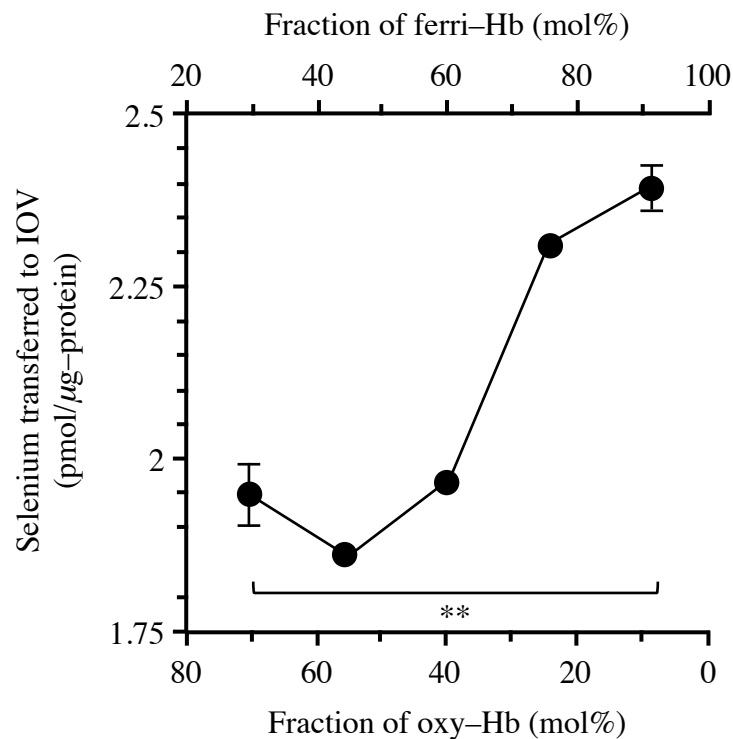
Fig. 6 Effect of the transition form of Hb on the selenium delivery from the Hb-Se complexes to the IOVs. An IOV suspension (170 μg-protein/mL) was co-incubated with mixtures of the oxy-Hb- and ferri-Hb-Se complexes (1 mmol-Hb/L) at various ratios (oxy-Hb; 10–70 mol%, ferri-Hb; 30–90 mol%) in 0.01 M phosphate buffer (pH 6) at 37 °C for 10 min. After centrifugation at 22,000 g for 15 min, the precipitated IOVs were washed with 20 mM phosphate buffer (pH 8) containing 0.5 M NaCl and their selenium contents were determined by the DAN method. Data are mean ± s. e. m., n = 4. **; $P < 0.01$.
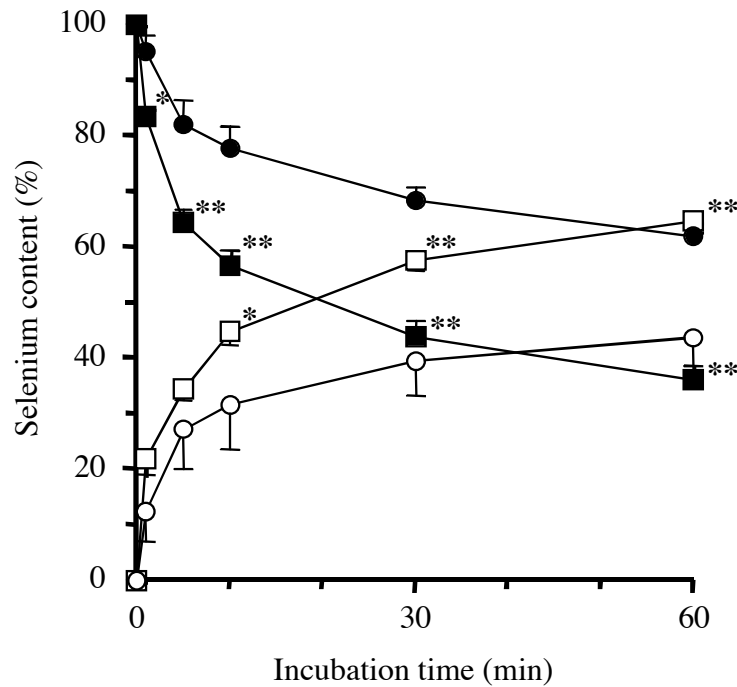
Fig. 7 Effects of nitrogen gas introduction on the selenium release from the selenite-treated RBCs to the plasma. ■, RBC with N_2 gas; □, plasma with N_2 gas; ●, RBC without N_2 gas; ○, plasma without N_2 gas. The RBCs [hematocrit 20% (v/v)] were incubated with selenite (8 μM) in isotonic phosphate buffer (pH 7.4) at 37 °C for 10 min. The selenite-treated RBCs and the plasma that was pretreated with or without N_2 gas for 30 min were combined [hematocrit 20% (v/v)] and then incubated at 37 °C for the indicated time. The selenium contents in RBCs before incubation with the plasma were defined as 100%. Data are mean ± s. e. m., n = 17. Significantly different from the corresponding time points of the N_2 gas-free experiment, *; $P < 0.05$, **; $P < 0.01$.

Supplementary material

Fig. S1 Reversed–phase liquid chromatographic analysis of reaction mixture of selenite and PenGSH and absorption spectrum of isolated PenGSSeSGPen

Fig. S2 SDS–PAGE of IOVs

Fig. S3 Circular dichroism spectra of ferri–Hb–Se complex and ferri–Hb

Fig. S4 Langmuir plots for binding of Hb (A) and ferri–Hb–Se complex (B) to IOV

Fig. S5 Hb mediated–selenium binding to IOV

Fig. S6 Langmuir plots for binding of oxy–Hb prepared from RBC and ferri–Hb to IOV.