Supporting Information

1. General information

Fmoc-Gly-(Et)Cys(Trt)-OH and Fmoc-Lys(*i*Noc)-OH were prepared by previously described methods.[1,2] The ESI mass spectra were recorded using a LCQ DECA XP Plus (Thermo Fisher, MA). Circular dichroism spectrum was measured using a J-820 spectropolarimeter (Jasco, Tokyo). The native Fd was expressed according to the reference.[3] The electron transfer activity was measured by the previously established method[4] using U-3900H spectrophotometer (Hitachi, Tokyo). The amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo) after hydrolysis with 6 M HCl at 180 °C for 25 min in an evacuated sealed tube. The content of the peptides in the powders was estimated based on the amino acid analysis.

2. Synthesis of peptide segments of the Se-Fd

2-1. *i*Noc-[Lys(*i*Noc)[4]]-Se-Fd (1-33)-SC_6H_4-*m*-OH **1**

Fmoc-Rink amide MBHA resin (Fmoc-NH-resin, 1.3 g, 0.40 mmol) was treated with 20% piperidine/1-methyl-2-pyrrolidinone (NMP) for 1 min with vortex mixing. The reaction was repeated with fresh reagent for 5 min. Fmoc-Lys(Boc)-OH (0.94 g, 2.0 mmol), dissolved in 0.45 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetra- methyluronium hexafluorophosphate (HBTU) in DMF (4.2 mL, 1.9 mmol) and *N,N'*-diisopropylethylamine (DIEA) (0.7 mL, 4 mmol), was added to the resin and vortexed for 12 min at 50 °C. Following the same procedure, another Lys residue was introduced and the Fmoc-$[Lys(Boc)]_2$-NH-resin was obtained. After removal of the Fmoc group, a solution of Fmoc-Gly-(Et)Cys(Trt)-OH (0.54 g, 0.80 mmol), *N,N'*-diisopropylcarbodiimide (DIC) (0.25 mL, 1.6 mmol) and 1-hydroxybenzo- triazole (HOBt) (0.22 g, 1.6 mmol) in 1,2-dichloroethane (DCE) (4.0 mL) was added to the resin, and the mixture was vortexed overnight at room temperature. The obtained Fmoc-Gly-(Et)Cys(Trt)-$[Lys(Boc)]_2$-NH-resin (0.40 mmol) was divided in two, and the synthesis was continued on the half of the resin using microwave- assisted peptide synthesizer except for Asp^{11}-Gly^{12} and Lys^4. For introduction of Asp^{11}-Gly^{12}, Fmoc-Asp(OBu^t)-(Dmb)Gly-OBt, which was prepared by mixing Fmoc-Asp(OBu^t)- (Dmb)Gly-OH (250 mg, 0.40 mmol), DIC (120 μL, 0.80 mmol), and HOBt (110 mg, 0.80 mmol) in DCE (2.0 mL) for 30 min at room temperature, was reacted overnight at room temperature. For coupling of Lys^4, Fmoc-Lys(*i*Noc)-OH (210 mg, 0.40 mmol), dissolved in 0.45 M HBTU in DMF (0.84 mL, 0.38 mmol) and DIEA (140 μL, 0.80 mmol), was added to the resin and vortexed for 12 min at 50 °C. After the completion of peptide chain assembly, *i*Noc-ONp (110 mg, 0.40 mmol) and DIEA (140 μL, 0.80 mmol) in DCE (2.0 mL) were added to the resin and the mixture was vortexed overnight at room temperature. The resulting resin was washed with MeOH (x3) and dried in vacuo to yield *i*Noc-Ala-Thr(Bu^t)-

Tyr(Bu^t)-Lys(*i*Noc)- Val-Thr(Bu^t)-Leu-Val-Arg(Pbf)-Pro-Asp(OBu^t)-(Dmb)Gly-Ser(Bu^t)-Glu(OBu^t)- Thr(Bu^t)-Thr(Bu^t)-Ile-Asp(OBu^t)-Val-Pro-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Tyr(Bu^t)-Ile-Leu-Asp(OBu^t)-Val-Ala-Glu(OBu^t)-Glu(OBu^t)-Gln(Trt)-Gly-(Et)Cys(Trt)-[Lys(Boc)]$_2$-NH-resin (1.6 g). A part of the resin (310 mg, 37 μmol) was treated with the TFA cocktail (TFA-triisopropylsilane-H_2O-1,2- ethanedithiol, 92.5 : 2.5 : 2.5 : 2.5, 6.0 mL) for 2 h at room temperature. The reaction mixture was filtered, and the filtrate was concentrated by a N_2 stream and precipitated with ether. The precipitate was washed with ether (x3) and dried in vacuo. The residue was dissolved in 50% MeCN aq. containing 6 M urea and 5% AcOH (12 mL). 3-Hydroxybenzenethiol (0.6 mL) was added to the reaction mixture and the solution was vortexed for 15 h at 37 °C. 3-Hydroxybenzenethiol was extracted with ether (x3), and the mixture was purified by RP-HPLC to give the peptide **1** (16 mg, 3.9 μmol, 11%). ESI mass, found: m/z 1345.1, calcd for $[M+3H]^{3+}$: 1345.1. Amino acid analysis: $Asp_{3.96(4)}Thr_{3.10(4)}Ser_{0.75(1)}$ $Glu_{6.25(6)}Pro_{2.25(2)}Gly_{2.00(2)}Ala_{1.96(2)}Val_{3.88(4)}Ile_{1.86(2)}$ $Leu_{1.98(2)}Tyr_{1.94(2)}Lys_{1.00(1)}Arg_{0.96(1)}$.

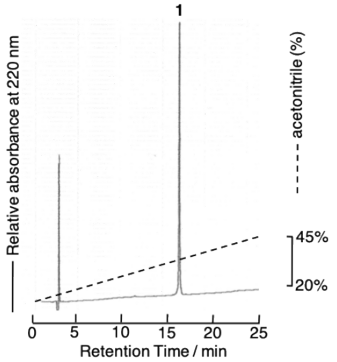

Figure S1. HPLC profile of purified peptide **1**. Elution conditions: column, Mightysil RP-18 GP Ⅱ (4.6 × 150 mm, Kanto Chemical, Japan) at the flow rate of 1 ml/ min; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA.

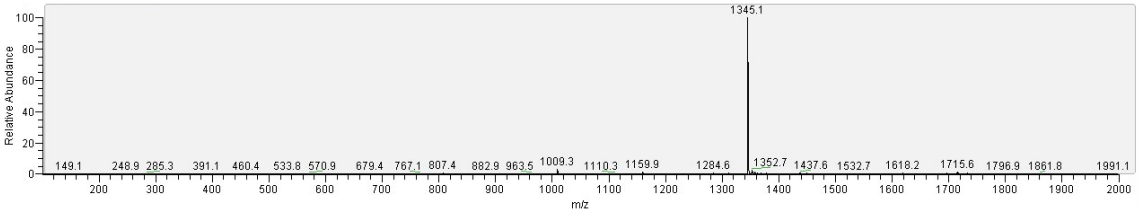

Figure S2. ESI mass spectrum of peptide **1**.

2-2. H-[Lys(*i*Noc)^{51,72}, Sec(MPM)^{40,45,48}]-Se-Fd (34-73)-SCH_2CH_2COOH **2**

Starting from Fmoc-Gly-(Et)Cys(Trt)-[Lys(Boc)]$_2$-NH-resin (0.10 mmol), the peptide chain was elongated by microwave-assisted peptide synthesizer except for Lys^{51,72} and Sec^{40,45,48}. Lys residues were introduced as described for the preparation of the peptide **1**. For introduction of Sec, Fmoc-Sec(MPM)-OBt, which was prepared by mixing Fmoc-Sec(MPM)-

OH (100 mg, 0.20 mmol), DIC (46 μL, 0.30 mmol) and HOBt (40 mg, 0.30 mmol) in DMF (2.0 mL) for 30 min at room temperature, was added to the resin and vortexed for 1 h at room temperature. The resulting resin was washed with MeOH (x3) and dried in vacuo to yield H-Leu-Asp(OBu^t)-Leu-Pro-Phe-Ser(Bu^t)-Sec(MPM)-Arg(Pbf)-Ala-Gly-Ala-Sec(MPM)-Ser(Bu^t)-Thr(Bu^t)-Sec(MPM)-Ala-Gly-Lys(*i*Noc)-Leu-Leu-Glu(OBu^t)-Gly-Glu(OBu^t)-Val-Asp(OBu^t)-Gln(Trt)-Ser(Bu^t)-Asp(OBu^t)-Gln(Trt)-Ser(Bu^t)-Phe-Leu-Asp(OBu^t)-Asp(OBu^t)-Asp(OBu^t)-Gln(Trt)-Ile-Glu(OBu^t)-Lys(*i*Noc)-Gly-(Et)Cys(Trt)-[Lys(Boc)]$_2$-NH-resin (1.4 g). A part of the resin (1.0 g, 72 μmol) was treated with the TFA cocktail (TFA-triisopropylsilane-H_2O-dimethyl sulfide, 92.5 : 2.5 : 2.5 : 2.5, 20 mL) for 2 h at room temperature. The reaction mixture was filtered, and the filtrate was concentrated by a N_2 stream and precipitated with ether. The precipitate was washed with ether (x3) and dried in vacuo. The residue was dissolved in a buffer containing 6 M GdnHCl, 0.1 M sodium acetate and 5% 3-mercaptopropionic acid (1.0 mL), which was adjusted pH 4.5 by 1 M NaOH. The reaction mixture was vortexed for 12 h at room temperature and the resultant mixture was purified by RP-HPLC to give the peptide **2** (9.2 mg, 1.8 μmol, 2.5%). ESI mass, found: m/z 1708.5, 1282.1, 1025.5, calcd for $[M+3H]^{3+}$: 1708.4, $[M+4H]^{4+}$: 1281.5, $[M+5H]^{5+}$: 1025.4. Amino acid analysis: $Asp_{5.72(6)}Thr_{0.50(1)}Ser_{2.39(4)}Glu_{5.50(6)}Pro_{0.85(1)}Gly_{4.00(4)}$ $Ala_{2.93(3)}Val_{1.14(1)}Ile_{0.88(1)}Leu_{4.80(5)}Phe_{1.94(2)}Lys_{2.07(2)}Arg_{0.95(1)}$.

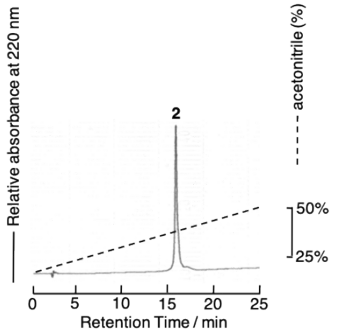

Figure S3. HPLC profile of purified peptide **2**. Elution conditions are the same as those of Figure S1.

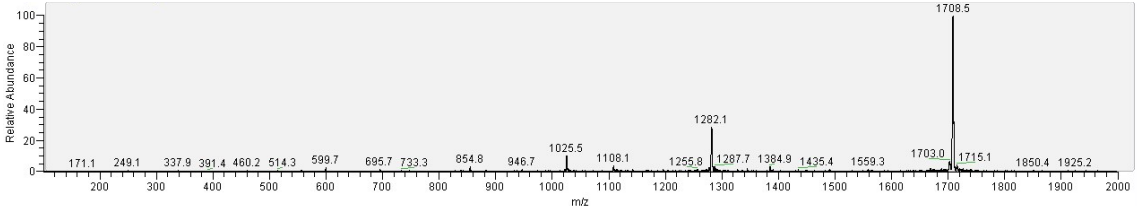

Figure S4. ESI mass spectrum of peptide **2**.

2-3. H-[Cys(MPM)[86], Lys(*i*Noc)[87], Sec(MPM)[78]]-Se-Fd (74-97)-OH **3**

Starting from Fmoc-Tyr(Bu^t)-Wang resin LL (360 mg, 0.10 mmol), peptide chain was elongated by microwave-assisted peptide synthesizer, except for the Sec^{78} and Lys^{87}, which were introduced using Fmoc-Sec(MPM)-OH and Fmoc-Lys(*i*Noc)- OH as described for the preparation of peptide **2**, respectively. After the peptide chain elongation, the resulting resin was washed with MeOH (x3) and dried in vacuo to yield H-Phe-Val-Leu-Thr(Bu^t)-Sec(MPM)-Val-Ala-Tyr(Bu^t)-Pro-Arg(Pbf)- Ser(Bu^t)-Asp(OBu^t)-Cys(MPM)-Lys(*i*Noc)-Ile-Leu-Thr(Bu^t)-Asn(Trt)-Gln(Trt)-Glu (OBu^t)-Glu(OBu^t)-Glu(OBu^t)-Leu-Tyr(Bu^t)-Wang resin (730 mg). A part of the resin (200 mg, 29 μmol) was treated with the TFA cocktail (TFA- triisopropylsilane-H_2O- dimethyl sulfide, 92.5 : 2.5 : 2.5 : 2.5, 4.0 mL) for 2 h at room temperature. The reaction mixture was filtered, and the filtrate was concentrated by a N_2 stream and precipitated with ether. The precipitate was washed with ether (x3) and dried in vacuo. The residue was purified by RP-HPLC to give the peptide **3** (11 mg, 3.5 μmol, 12%). ESI mass, found: m/z 1629.9, 1087.1, calcd for $[M+2H]^{2+}$: 1629.8, $[M+3H]^{3+}$: 1086.9. Amino acid analysis: $Asp_{1.99(2)}Thr_{1.56(2)}Ser_{0.71(1)}Glu_{3.73(4)}Pro_{0.86(1)}Ala_{1.00(1)}$ $Val_{2.02(2)}Ile_{0.94(1)}Leu_{2.99(3)}Tyr_{1.99(2)}Phe_{0.97(1)}Lys_{0.99(1)}Arg_{0.69(1)}$.

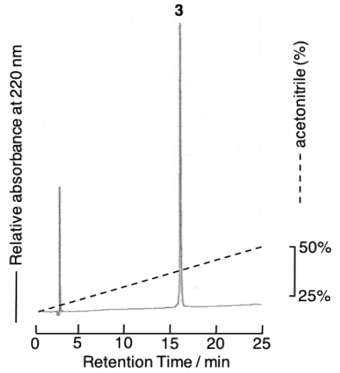

Figure S5. HPLC profile of purified peptide **3**. Elution conditions are the same as those of Figure S1.

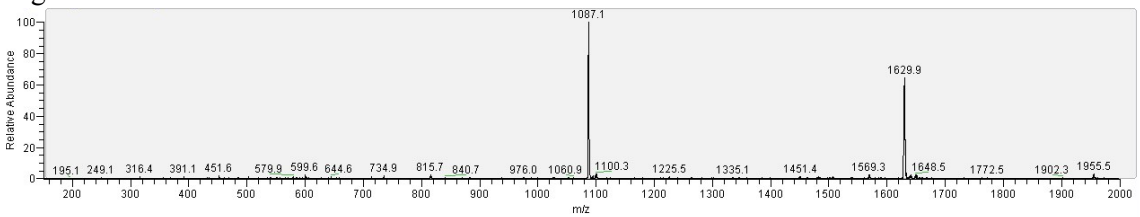

Figure S6. ESI mass spectrum of peptide **3**.

3. Synthesis of the Se-Fd

3-1. *i*Noc-[Cys(MPM)^{86}, Lys(*i*Noc)^{4,51,72,87}, Sec(MPM)^{40,45,48,78}]-Se-Fd (1-97)-OH **5**

Peptide **1** (6.0 mg, 1.5 μmol) and **2** (5.1 mg, 1.0 μmol) were dissolved in DMSO (100 μL) containing 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) (7.3 mg, 45 μmol) and DIEA (5.2 μL, 30 μmol), and the mixture was vortexed for 4 h at room temperature to give peptide **4** (ESI mass, found: m/z 1807.1, 1505.9, 1291.1, 1130.1, 1004.5, 903.9, calcd for $[M+5H]^{5+}$: 1806.7, $[M+6H]^{6+}$: 1505.7, $[M+7H]^{7+}$: 1290.8, $[M+8H]^{8+}$: 1129.5, $[M+9H]^{9+}$: 1004.2, $[M+10H]^{10+}$: 903.8). Without isolation of peptide **4**, peptide **3** (3.9 mg, 1.2 μmol) in DMSO (100 μL) containing HOOBt (2.5 mg, 15 μmol), DIEA (1.3 μL, 7.5 μmol) and a tiny portion of AgCl were added, and the mixture was vortexed for 3 h at room temperature. The mixture was precipitated with ether, and then the precipitate was washed twice with ether. The crude product was purified by gel filtration chromatography using G3000PW$_{XL}$ (7.5 x 300 mm, Tosoh, Tokyo) in 50% CH_3CN aq. containing 0.1% TFA at a flow rate of 0.7 mL / min to yield polypeptide **5** (6.8 mg, 560 nmol, 56%). ESI mass, found: m/z 1741.1, 1523.7, 1354.5, 1219.2, 1108.6, 1016.5, calcd for $[M+7H]^{7+}$: 1741.0, $[M+8H]^{8+}$: 1523.5, $[M+9H]^{9+}$: 1354.3, $[M+10H]^{10+}$: 1219.0, $[M+11H]^{11+}$: 1108.3, $[M+12H]^{12+}$: 1016.0. Amino acid analysis: $Asp_{11.2(12)}Thr_{5.09(7)}Ser_{3.97(6)}Glu_{16.0(16)}Pro_{2.60(4)}$ $Gly_{6.00(6)}Ala_{5.73(6)}Val_{6.43(7)}Ile_{3.52(4)}Leu_{9.35(10)}Tyr_{3.25(4)}Phe_{2.76(3)}Lys_{4.01(4)}$ $Arg_{2.70(3)}$.

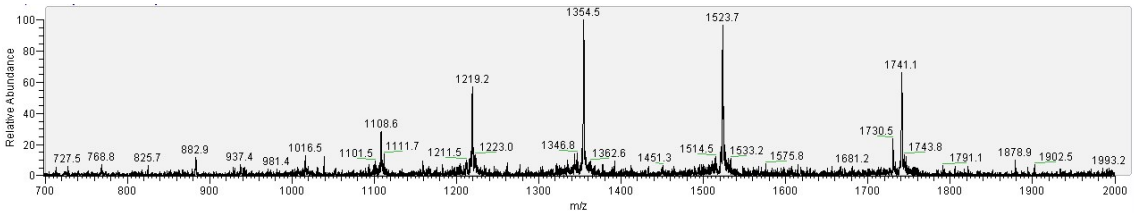

Figure S7. ESI mass spectrum of polypeptide **5**.

3-2. Apo Se-Fd (1-97) **7**

Polypeptide **5** (3.0 mg, 250 nmol) was dissolved in TFA cocktail (TFA-thioanisole-2,2'-dipyridyldisulfide, 90 : 5 : 5, 1.0 mL) and the mixture was vortexed for 30 min at room temperature. The reaction mixture was concentrated by a N_2 stream and precipitated with ether. The precipitate was washed with ether (x3) and dried in vacuo to yield the 2-pyridylsulfanylated polypeptide **6** (ESI mass, found: m/z 1985.3, 1701.7, 1489.4, 1323.8, 1191.9, 1083.4, 993.4, calcd. for $[M+6H]^{6+}$: 1985.1, $[M+7H]^{7+}$: 1701.6, $[M+8H]^{8+}$: 1489.1, $[M+9H]^{9+}$: 1323.7, $[M+10H]^{10+}$: 1191.4, $[M+11H]^{11+}$: 1083.2, $[M+12H]^{12+}$: 993.0.) The residue was dissolved in 50% AcOH aq. containing 3 M GdnHCl (2.5 mL), and to the mixture was added powdered Zn (ca. 150 mg), which was activated by washing three times with 1 M HCl aq. followed by washing three times with H_2O. The reaction mixture was vigorously vortexed for 1 h at room temperature. After filtration, the mixture was purified by RP-HPLC using YMC-Pack Protein-

RP (4.6 x 150 mm, YMC, Kyoto) at a flow rate of 1 mL / min to yield the apo Se-Fd **7** (1.6 mg, 150 nmol, 60% over two steps). ESI mass, found: m/z 1817.9, 1558.5, 1363.8, calcd for $[M+6H]^{6+}$: 1817.6, $[M+7H]^{7+}$: 1558.8, $[M+8H]^{8+}$: 1363.6. Amino acid analysis: $Asp_{11.7(12)}Thr_{5.57(7)}Ser_{4.08(6)}Glu_{13.3(16)}Pro_{2.79(4)}Gly_{6.00(6)}$ $Ala_{5.91(6)}Val_{7.30(7)}Ile_{3.75(4)}Leu_{9.65(10)}Tyr_{3.91(4)}Phe_{2.81(3)}Lys_{4.00(4)}Arg_{2.89(3)}$.

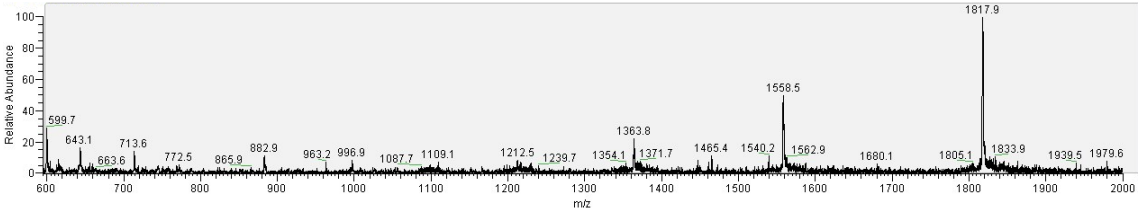

Figure S8. ESI mass spectrum of apo Se-Fd **7**.

3-3. Holo Se-Fd (1-97) **8**

For the reconstitution of Se-Fd, all handling was carried out in the N_2 chamber except for purification and buffers were also degassed by the N_2 bubbling. To the mixture of dithiothreitol (DTT) (640 mg, 4.1 mmol) and $Na_2S•9H_2O$ (1.3 mg, 5.4 μmol) in 50 mM Tris buffer containing 50 mM NaCl (pH 8.0), $FeCl_3•6H_2O$ (3.5 mg, 13 μmol) in the same buffer was added dropwisely. The resultant solution (12.7 mL) was added to Apo Se-Fd **7** (1.4 mg, 130 nmol) dissolved in 50 mM tris(hydroxymethyl)aminomethane (Tris) containing 8 M urea and 50 mM NaCl (0.33 mL, pH 8.0). the apo Se-Fd solution and the mixture was vortexed for 5 h at room temperature. 0.5 M EDTA solution (26 μL, 13 μmol) was added to the mixture for chelating the excess amount of Fe^{3+}. The solution was then filtered and the filtrate was purified by ion-exchange chromatography to yield the holo Se-Fd **8** (0.38 mg, 34 nmol, 26%). ESI mass, deconvoluted: m/z 11078.9, calcd for 11075.3. $Asp_{11.5(12)}Thr_{5.31(7)}Ser_{3.75(6)}Glu_{13.9(16)}Pro_{4.89(4)}Gly_{6.00(6)}Ala_{5.91(6)}Val_{7.53(7)}Ile_{3.87(4)}Leu_{11.1(10)}$ $Tyr_{3.93(4)}Phe_{2.97(3)}Lys_{4.10(4)}$ $Arg_{3.00(3)}$.

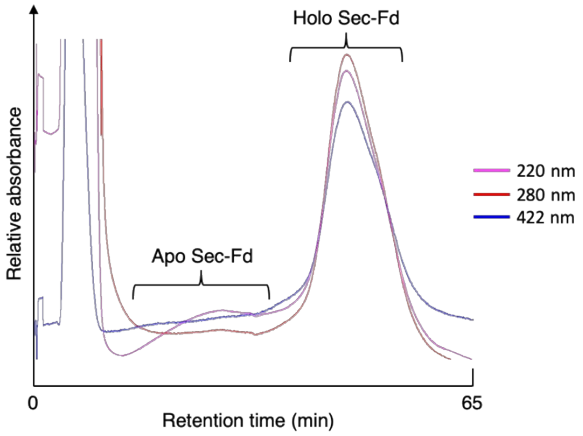

Figure S9. Elution profile of ion-exchange chromatography of holo Se-Fd. Elution conditions: Column, HiTrap Q HP (5 mL) at the flow rate of 1 mL/min; eluent A, 50 mM Tris (pH 7.5), eluent B, 50 mM Tris, 1 M NaCl (pH 7.5); gradient (B%), 28% to 45% in 0-90 min.

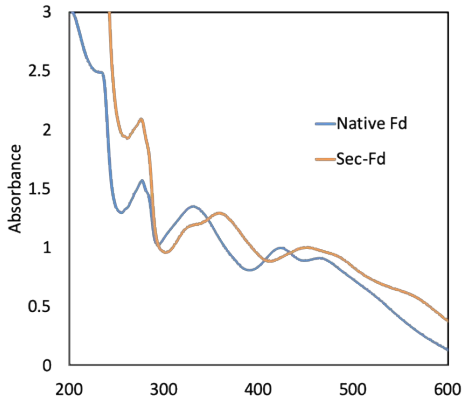

Figure S10. UV-Vis spectra of native- and Se-Fd. The spectra were normalized at 422 nm and 450 nm for native- and Se-Fd, respectively.

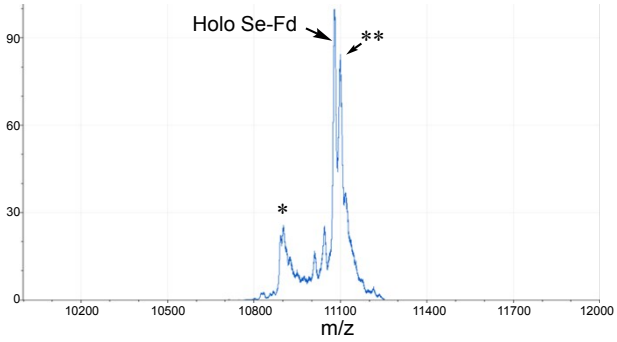

Figure S11. Deconvoluted ESI mass spectrum of holo Se-Fd. The ESI spectrum was recorded by JMS-T100LC (JEOL, Tokyo). The asterisked peak derives from the decomposition of [2Fe-2S] cluster during mass analysis. Figure S8. Deconvoluted ESI mass spectrum of holo Se-Fd. The ESI spectrum was recorded by JMS-T100LC (JEOL, Tokyo). The asterisked peak (*) derives from the decomposition of [2Fe-2S] cluster during mass analysis. The double asterisked peak (**) indicates the ammonium and the sodium ion adducts caused by the measurement solvent.

4. CD spectrum measurement

CD spectra of holo Fds in 50 mM Tris buffer containing 400 mM NaCl (pH 7.5) were measured between 197 nm and 250 nm at 4 °C using a 0.1 cm light path length cell. The spectra is shown in Fig. 3.

5. Electron transfer activity measurement

Electron transfer activity of holo Se-Fd was measured by previously established method by monitoring the absorbance of reduced cytochrome C (Cyt C) with ferredoxin-$NADP^+$ reductase (FNR).[4] Briefly, to the mixture of oxidized Cyt C (200 μM) and FNR (40 nM) in 50 mM Tris buffer containing 100 mM NaCl, holo Se-Fd was added at the final concentration of 1, 3, 5, 10, 15, 20 and 40 μM, respectively. A NADPH solution was then added to the solution at the final concentration of 100 μM and the mixture was immediately mixed by pipetting. The increasing absorption of reduced Cyt C was monitored at 550 nm. Electron transfer activity of native Fd was also measured by same procedure. Using the obtained reduction rate at each concentration of Se-Fd and native Fd, fitting curve was made by Origin® and Km, kcat and kcat/Km values were calculated. These values are shown in Table 1.

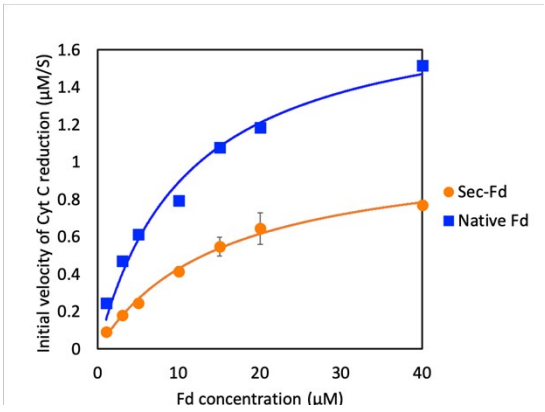

Figure S12. Initial ve

6. References

1. H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara, Y. Nakahara, *Tetrahedron Lett.*, **2007**, *48*, 25-28.

2. Y. Asahina, S. Kamitori, T. Takao, N. Nishi, H. Hojo, *Angew. Chem. Int. Ed.,* **2013**, *52*, 9733-9737.

3. M. Risa, N. Muraki, K. Shinmura, H. Kubota-Kawai, Y.-H. Lee, M. M. Nowaczyk, M. Rogner, T. Hase, T. Ikegami, G. Kurisu, Biochem., **2015**, *54*, 6052-6061.

4. Y. Onda, T. Matsumura, Y. Kimata-Ariga, H. Sakakibara, T. Sugiyama, T. Hase, *Plant Physiol.*, **2000**, *123*, 1037-1045.