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ABSTRACT: Methionine oxidation is involved in regulating protein activity and often leads to protein malfunction. However, tools for quantitative analyses of protein-specific methionine oxidation are currently unavailable. In this work, we developed a biological sensor that quantifies oxidized methionine in the form of methionine-R-sulfoxide in target proteins. The biosensor "tpMetROG" consists of methionine sulfoxide reductase B (MsrB), circularly permuted yellow fluorescent protein (cpYFP), thioredoxin, and protein G. Protein G binds to the constant region of antibodies against target proteins, specifically capturing them. Then, MsrB reduces the oxidized methionine in these proteins, leading to cpYFP fluorescence changes. We assessed this biosensor for quantitative analysis of methionine-R-sulfoxide in various proteins, such as calmodulin, IDLO, LegP, Sacde, and actin. We further developed an immunosorbent assay using the biosensor to quantify methionine oxidations in specific proteins such as calmodulin in animal tissues. The biosensor-linked immunosorbent assay could prove to be an indispensable tool for detecting methionine oxidation in a protein-specific manner. This is a versatile tool for studying the redox biology of methionine oxidation in proteins.

Peroxide, superoxide, and hydroxyl radical are highly reactive oxygen species (ROS).[1] In aerobic organisms, ROS are generated as by-products of the mitochondrial respiratory chain reaction, but they can also be produced during specific immune responses.[2] Accumulated ROS in cells can exert both beneficial or detrimental influence on numerous cellular events by altering functions of various macromolecules, including proteins, lipids, and nucleic acids.[3-5] In particular, several amino acid residues in proteins can be readily oxidized by ROS. Sulfur-containing amino acids, including methionine (Met), are especially easily oxidized by ROS. Oxidation of sulfur in methionine produces either methionine-*S*-sulfoxide (MetSO) or methionine-*R*-sulfoxide (MetRO).[6] MetSO and MetRO are reduced by MsrA and MsrB, respectively, which are conserved in animals, including mammals.

Emerging evidence suggests that reversible oxidation/reduction of methionine residues is an adaptation for functional regulation of proteins.[7-10] Oxidation of methionine residues 281 and 282 within the regulatory region of calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) increases its enzymatic activity even in the absence of to Ca^{2+}/Calmodulin.[11] Oxidation of methionine residue in hypochlorite-responsive transcription factor (HypT), a protein expressed in *Escherichia. coli*, induces the expression of various genes required for escaping cell death due to the oxidative stress mediated by neutrophils during an innate immune response.[12] In F-actin, the stereoselective oxidation of methionine 46 and 49 catalyzed by molecule interacting with CasL (MICAL) family of proteins results in actin depolymerization and the generation of G-actin monomers.[13, 14] These oxidized methionine residues involved in altering protein functions were shown to be reduced by either MsrA or MsrB, which brings the proteins back to the original reduced state. For example, G-actin monomers disassembled by oxidation of the two conserved methionine residues are reassembled to F-actin polymer upon treatment with MsrB.[15, 16] Additionally, the oxidation of specific methionine residues in proteins may alter protein structure, thereby diminishing activity.[17-19]

Therefore, it can be surmised that global methionine oxidation may confer differential effects on individual protein function. It is critical to identify proteins where methionine oxidation levels regulate protein function. However, detecting methionine oxidation in a specific protein has been challenged due to the spontaneous oxidation that occurs during sample preparation. To eliminate this oxidation source, Sun and his colleagues replaced the ammonium persulfate (APS) with a Flavin mononucleotide/diphenyliodonium chloride/sodium toluenesulfinic acid system and UV light.[20] Also artificial oxidation could be distinguished by using the use of isotopes through LC-MS/MS.[21, 22] But high cost and incomplete specificity still remains a challenge to be overcome.[23, 24] In recent, an advanced approach was developed by Tarrago and his colleagues. It is a ratiometric fluorescent sensor that detects a level of global methionine oxidation *in vitro* and *in vivo*.[25]

However, there is still a need for measuring the degree of methionine oxidation of specific protein target. Here, we introduce a new method for measuring methionine oxidation in a target-specific manner based on the strategy developed from the sensor devised by Tarrago and his colleagues. Using the ability of protein G to bind immunoglobulins, we made a recombinant protein composed of yeast MsrB, cpYFP, and yeast Trx3, followed by protein G with a short amino acid linker. The biosensor was then applied in immunosorbent assays to measure methionine oxidation of specific purified proteins from cell lysates and organ extracts.

MATERIALS AND METHODS

Construction of the target protein MetRO protein G (tpMetROG) biosensor. All polymerase chain reactions (PCR) and site-directed mutagenesis were performed using the KOD Plus polymerase (TOYOBO, Osaka, Japan). *MsrB* and *Trx3* were amplified from the *S. cerevisiae* genomic DNA using PCR with a specific pairs of primers (listed in Supplementary Table S1) containing overlapping extensions for *cpYFP*. *cpYFP* was amplified from the H_2O_2 sensor, HyPer[26] using PCR with a specific pairs of primers (listed in Supplementary Table S1). *Flexible linker* and *protein G* were de novo synthesized (listed in Supplementary Table S2) from Cosmogenetech (South Korea). *MsrB* and *Trx3* amplicon were subsequently fused to *cpYFP* by PCR. Sequentially *flexible linker* and *protein G* were fused to C terminal of Trx3 by PCR with a specific pairs of primers (listed in Supplementary Table S1). The fused MsrB/cpYFP/Trx3/flexible linker/protein G was purified using a PCR/Gel purification kit (Bioneer, Daejeon, Republic of Korea). For the construction of the MsrB/cpYFP/Trx3/flexible linker/protein G expression plasmid, purified fragments were proportionally mixed with the pET 21a cloning vector (Addgene, MA, USA) plasmid that was previously digested by BamHI and XhoI restriction enzyme (Thermo fisher scientific, MA, USA) and further ligated using T4 DNA ligase (Invitrogen, MA, USA). Then the ligation mixture was transformed into Escherichia coli (E. coli) DH5α (Invitro-gen, MA, USA) for selection and amplification for the correct recombinant plasmid.

Expression and purification of tpMetROG and methionine-rich proteins (MRPs). All constructed plasmids were introduced into *E. coli* Rosetta2 (DE3) (Novagen, WI, USA) grown in Luria-Bertani medium (Formedium, Norfolk, England) containing ampicillin (50 μg/ml) at 37 °C. When absorption at 600 nm (A_{600}) reached ~0.6, synthesis of the recombinant protein was induced by adding 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold biotechnology, MO, USA) at 18 ℃. After 18-24 h incubation, the cells were collected by centrifugation at 3000 g for 30 min. Pellets were resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, 5 mM 2-mercaptoethanol, pH 8.0) and subsequently sonicated. Proteins were purified using the HisTrap HP column (GE Healthcare, IL, USA) and eluted with buffer B (buffer A supplement 500 mM imidazole). Protein solutions were concentrated using Amicon Ultra-15 30 kDa centrifugal filters (Millipore, MA, USA). Initial protein concentrations were measured using the Pierce bicinchoninic acid (BCA) protein kit (Thermo Scientific, MA, USA). The recombinant proteins were aliquoted and stored at -80 °C.

For the analysis of MRPs, we selected four proteins, including IDLO from *Idiomarina loihiensis* L2TR, Sacde from *Saccharophagus degradans* 2-40, LegP from *Legionella pneumophila* (LegP), calmodulin from *Mus musculus*.[27] All proteins were prepared as His-tag forms using a pET-21a (+) cloning vector (Novagen, WI, USA). All constructed plasmids were introduced into *E. coli* Rosetta2 (DE3) (Novagen, WI, USA) grown in Luria-Bertani medium containing ampicillin (50 μg/ml) at 37 °C. The purification was carried out similarly as described above.

Spectroscopic characterization of tpMetROG biosensors with oxidized MRPs. For assessing the tpMetROG biosensor, purified proteins (10-20 μM) were reduced using 10 mM dithiothreitol (DTT) at room temperature for 30 min. After reduction, the proteins were desalted using HiTrap Desalting columns (GE Healthcare, IL, USA) in 50 mM Tris-HCl, pH 7.5, and were diluted to 1-5 μM in the same buffer for recording fluorescence. Purified MRPs (calmodulin, IDLO, LegP, Sacde) were oxidized using 40 mM hydrogen peroxide (H_2O_2) at room temperature for 2 h. Residual H_2O_2 was removed by centrifugation (14,000 g, 10 minutes, 4 °C) using Amicon Ultra-0.5 centrifugal filter with 10 K cutoffs. The centrifugation step was repeated five times. The protein concentrations were measured using the Pierce bicinchoninic acid (BCA) protein kit (Thermo Scientific, MA, USA). Fully reduced tpMetROG (1 μM) in working buffer (50mM Tris-HCl, 150mM NaCl, pH 7.5) were incubated with MRP (1-2 μM), partially oxidized MRP (1-2 μM) at room temperature for 30 min. Fully oxidized tpMetROG (1 μM) were prepared by incubating fully reduced tpMetROG with N-acetyl methionine sulfoxide (100 μM) at room temperature for 30 min. Methionine (Met) and methionine sulfoxide (MetO) (Sigma-Aldrich, MO, USA) were prepared in 1 mM in 50 mM Tris-HCl (pH 8.0). Fully reduced tpMetROG were incubated with Met (100 μM) or MetO (100 μM) at room temperature for 30 min.

The fluorescence spectrum is recorded in Costar black clear-bottom 96 well microplates using SpectraMax I3 (Molecular Devices, CA, USA). Absorbance spectra were recorded from 360 to 800 nm in a 1 nm step increment. Excitation spectra were recorded from 380 nm to 510 nm with 535 nm emission and a 530-nm cutoff in a 1 nm step increment. The device was typically set to 10 reads per well with the photomultiplier tube (PMT) set on 'auto.' Emission spectrum were recorded from 500 nm to 750 nm with an excitation wavelength of 410 nm, in 1 nm step increment. The normalized fluorescence intensity (NFI) was obtained by dividing all spectrum values by the maximum value recorded at 535 nm emission wavelength. The percentage of oxidized tpMetROG was represented as relative fluorescence intensity (RFI), and RFI was determined using the following equation (equation A).

$$\text{RFI of tpMetROG} = (R_{Reduced} - R) / (R_{Reduced} - R_{Oxidized}) \quad \text{(equation A)}$$

Where R is measured $F_{500\ nm}/F_{415\ nm}$ ratio of the excitation spectrum. $R_{Reduced}$ is the $F_{500\ nm}/F_{415\ nm}$ ratio of fully reduced tpMetROG, and ROxidized is the $F_{500\ nm}/F_{415\ nm}$ ratio of fully oxidized tpMetROG.[28]

pH calibration and kinetic measurement. For the analyzing the pH influence, following buffers were used: 50 mM Phosphate, 150 mM NaCl (pH 5.8, 6.4 and 7.0); 50 mM Tris-HCl, 150 mM NaCl (pH 7.5, 8.0, 8.3 and 9.0). Fully reduced tpMetROG (1 μM) or inactive tpMetROG (1 μM) was incubated with IDLO (2 μM) or oxidized IDLO (2 μM) in a Costar black clear-bottom 96 well microplate for 30 min at 37 ℃. The Fluorescence spectrum is recorded using SpectraMax I3 (Mo-

lecular Devices, CA, USA). The Fluorescence ratio (FR ($F_{500\ nm}/F_{415\ nm}$)) was calculated as the ratio of fluorescence

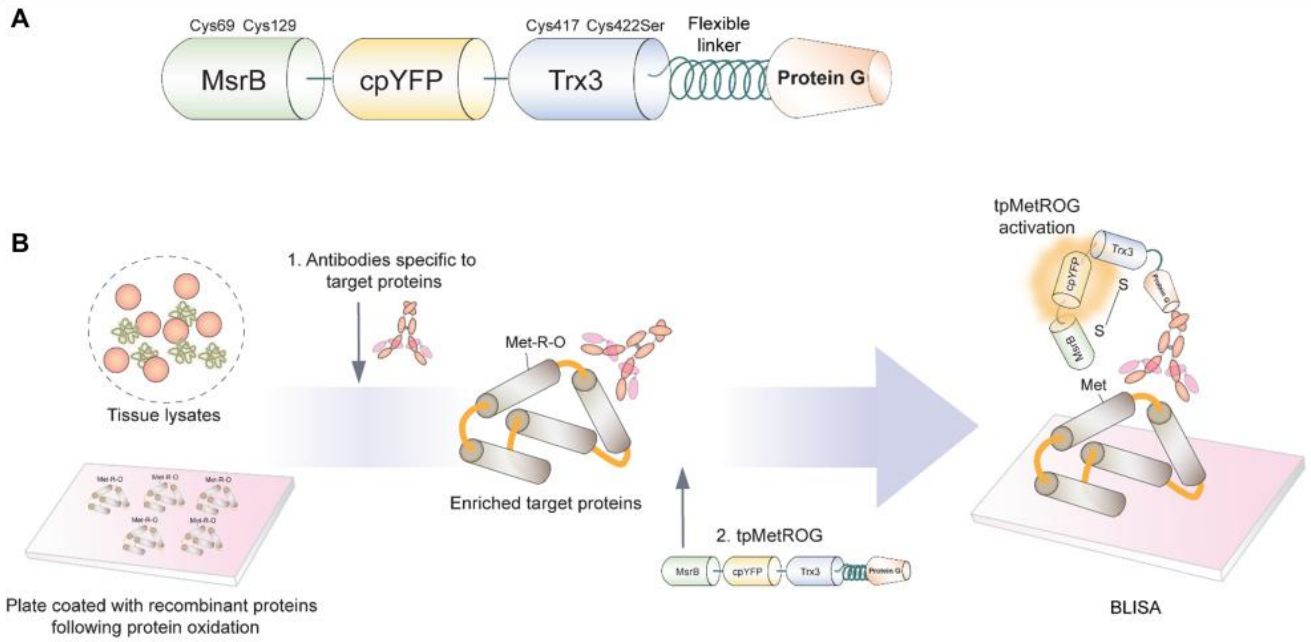

Figure 1. Schematic representation of tpMetROG biosensor and its application in an immunosorbent assay. (A) Schematic representation of the tpMetROG biosensor construct encoding *cpYFP* flanked by *MsrB1* and *Trx3* at the N- and C- terminus, and *protein G* linked to the C-terminus of *Trx3* by a peptide linker. (B) Schematic representation of the biosensor-linked immunosorbent assay using tpMetROG. First, the plate is coated with the protein or a sample containing a protein to measure the oxidation state of methionine. After adding the primary antibody for a target protein, the unbound antibody is washed away, and tpMetROG is added at low temperature. The unbound tpMetROG is washed using the working buffer. After incubation at 37 ℃, the tpMetROG fluorescence spectrum was measured.

at 500 nm and 415 nm for the excitation spectrum with an emission wavelength of 535 nm.

For measuring kinetic reactivity of tpMetROG with various substrates, the reduced biosensor (1 μM) was incubated for 60 min with buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), DTT (1 mM), oxidized calmodulin (2 μM) or reduced calmodulin (2 μM) as control. The examination of the excitation spectrum and calculating $F_{500\ nm}/F_{415\ nm}$ was performed as described above. The $F_{500\ nm}/F_{415\ nm}$ (R) were normalized to the value at t = 0 (R_0)

Application of tpMetROG using purified MRPs in antibody-based immunoassays. tpMetROG was applied in antibody-based immunoassays. Purified MRPs were oxidized with 10-40 mM hydrogen peroxide (H_2O_2) at room temperature for 2 h. Residual H_2O_2 was removed by repeating five cycles of washing-centrifugation (14,000 g, 10 minutes, 4 °C) using Amicon Ultra-0.5 centrifugal filter with 10 K cutoffs. Protein concentrations were measured using the Pierce bicinchoninic acid (BCA) protein kit (Thermo Scientific, MA, USA). MRPs were prepared at 2 μM in 200 μl buffer A. Black immunoplates (SPL Life sciences, Korea) were coated with MRPs for 2 hours at room temperature and then blocked with SuperBlock (Thermo Scientific, MA, USA). Dilutions of anti-idlo (1:1000; Convance), anti-sacde (1:1000; Convance), anti-legp (1:1000; Convance), or anti-calmodulin (1:1000; Convance) antibodies were added to the plates for 2 h at room temperature. After washing with a wash buffer (100 mM phosphate, 150 mM sodium chloride, 0.05% tween 20, pH 7.2), the plates were incubated with the fully reduced tpMetROG (1 μM) in 200 μl working buffer (50mM Tris, 150mM NaCl, pH 7.5) for 2 h at 18 ℃. After the plate were washed again with working buffer, 200 μl working buffer was added and incubated at 37 ℃ for 30 min. Then, fluorescence was recorded using SpectraMax I3. Excitation spectrum was recorded from 380 nm to 510 nm with 535 nm emission and a 530-nm cutoff in 1 nm increments. The percentage of oxidized tpMetROG is represented as RFI.

Application of tpMetROG using whole cell lysates. tpMetROG was used to analyze the oxidation of calmodulin obtained from the lysates of Raw264.7 and U87MG cells. Raw264.7 cells were cultured in HyClone RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% FBS, penicillin 100 U/ml, and 100 μg/ml streptomycin. U87MG cells were cultured in HyClone DMEM (Thermo Fisher Scientific, MA, USA) supplemented with 10% FBS, penicillin 100 U/ml, and 100 μg/ml streptomycin. Cells were oxidized with 15, 30, and 45 mM H_2O_2 for 40 minutes at 37 ℃ or 5, 10, and 15 mM NaOCl for 15 min at 37 ℃ or with 100 ng/ml LPS for 6 h -16 h. After the treatment, cells were washed three times with cold PBS, and the harvested cells were recovered by centrifugation. Cells were lysed in RIPA buffer (30 mM HEPES, pH 7.3, containing 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100 and 1% (w/v) Na-deoxycholate), and the lysates were centrifuged at 16000 g for 10 min at 4 ℃. The protein concentrations of the supernatant were measured using the Pierce BCA protein kit. The same amount of total protein (1.6 mg/ml, 200 μl) was coated on Black immunoplates, incubated for 2 h at room temperature, and blocked with SuperBlock. A dilution of anti-calmodulin antibodies (1:1000; Convance) was applied for 2 h at room temperature. After washing with a wash buffer (100 mM phosphate, 150 mM sodium chloride, 0.05% tween 20, pH 7.2), the plates were incubated with the fully reduced tpMetROG (1 μM) in 200 μl working buffer (50mM Tris, 150mM NaCl, pH 7.5) for 2 h at 18 ℃. After the plate were washed again with

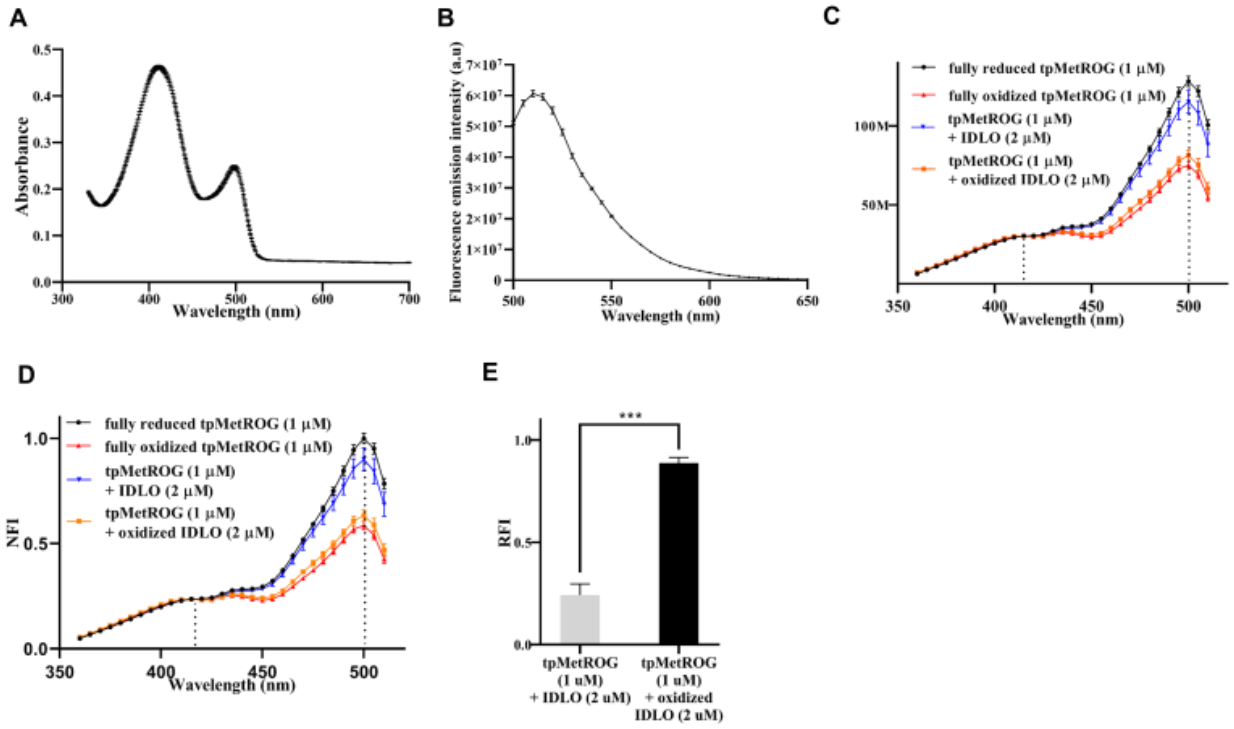

Figure 2. Optical spectral analysis of the tpMetROG sensor. (A) Absorption spectrum of reduced tpMetROG. (B) The emission spectrum of reduced tpMetROG (1 μM) when the maximum absorbance wavelength (410 nm) was used as the excitation wavelength. (C) Excitation spectrum and (D) normalized fluorescence intensity (NFI) of fully reduced tpMetROG (1 μM) incubated with or without IDLO (2 μM), partially oxidized or N-acetyl methionine sulfoxide (100 μM) when emission wavelength was fixed at 535 nm. The dotted vertical lines indicate the 415 and 500 nm. (E) A relative fluorescence intensity (RFI) of tpMetROG (1 μM) incubated with IDLO (2 μM) or partially oxidized IDLO (2 μM). Data are presented as means ± SD and are representative of 3 replicates. *** $p < 0.001$, as determined by two-tailed paired *t*-test.

working buffer, 200 μl working buffer was added and incubated at 37 ℃ for 30 min. Then, fluorescence was recorded in same manner as described above.

Quantification of oxidized methionine from mouse tissues using tpMetROG. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University (KUIACUC-2019-0107). 10-12 weeks old MsrB1 knock-out,[29] and age- and sex-matched C57BL/6J (Orientbio) mice were used in this study. Mice were anesthetized by intraperitoneal injection of sterile avertin (tribromoethanol: 200 mg/10 ml/kg) and euthanized by cervical dislocation. The small intestine, large intestine, brain, heart, liver, kidneys, and lungs were dissected under sterile conditions. Each organ was immediately rinsed with PBS and weighed. Each organ was placed in a 15 ml polystyrene tube containing 2 ml of 0.2 % Tween 20/TBS and homogenized on ice with a Polytron PT1200E homogenizer (Kinematica AG, Luzern, Switzerland). The homogenates were centrifuged at 16000 g for 10 min at 4 ℃. The protein concentration of the supernatants was measured using the Pierce BCA protein kit. Same amount of total protein (3.2 mg/ml, 200 μl) was coated into Black immunoplates and incubated for 2 h at room temperature and then blocked with SuperBlock. Anti-calmodulin (1:1000; Convance) antibodies were applied for 2 h at room temperature. After washing with a wash buffer (100 mM phosphate, 150 mM sodium chloride, 0.05% tween 20, pH 7.2), the plates were incubated with the fully reduced tpMetROG (1 μM) in 200 μl working buffer (50mM Tris, 150mM NaCl, pH 7.5) for 2 h at 18 ℃. After the plate were washed again with working buffer, 200 μl working buffer was added and incubated at 37℃ for 30 min. Then, fluorescence was recorded in same manner as described above.

Analysis of stereospecific oxidation in F-actin using tpMetROG. Recombinant mouse Mical1 and oxidized form of F-actin were prepared as described previously.[30] Mical1-oxidized F-actin and actin were coated on Black immunoplates and incubated for 2 h at room temperature and then blockedwith SuperBlock. A proper dilution of mouse monoclonal anti-β actin antibody (1:1000) (Santa Cruz Biotechnology, TX, USA) was applied for 2 h at room temperature. After washing with a wash buffer (100 mM phosphate, 150 mM sodium chloride, 0.05% tween 20, pH 7.2), the plates were incubated with the fully reduced tpMetROG (1 μM) in 200 μl working buffer (50mM Tris, 150mM NaCl, pH 7.5) for 2 h at 18 ℃. After the plate were washed again with working buffer, 200 μl working buffer was added and incubated at 37 ℃ for 30 min. Then, fluorescence was recorded in same manner as described above.

Statistical analysis. Data are presented as mean ± standard error. To compare two groups, the two-tailed unpaired *t*-test, Mann-Whitney, or Wilcoxon test was used with or without the assumption of normality. The number of asterisks indicates the degree of statistical significance according to the p-value. All statistical analyses were calculated using Prism 8 software (GraphPad Software, CA, USA).

RESULT

Construction of a biosensor detecting the oxidation state

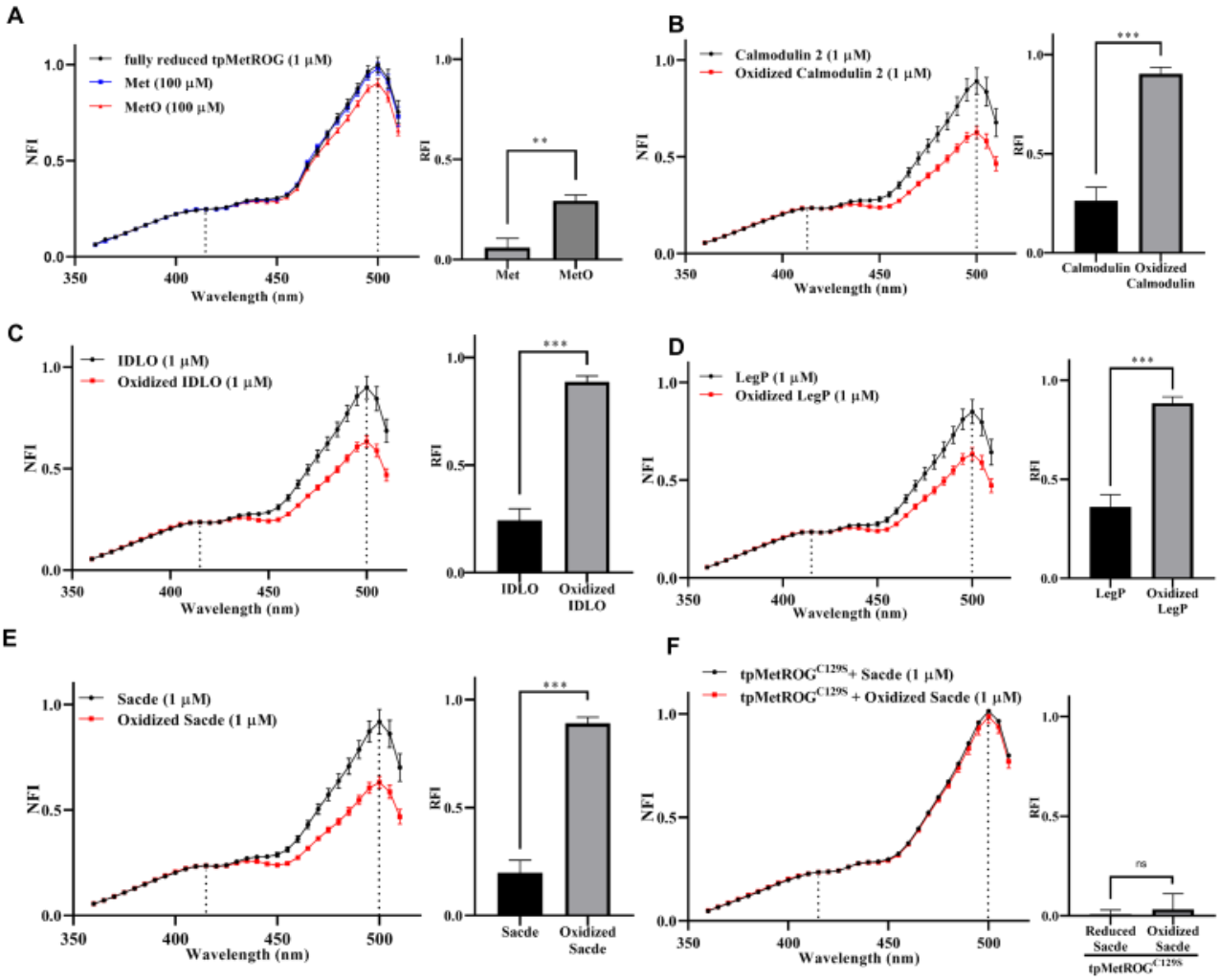

Figure 3. Analysis of various MRPs using tpMetROG. (A) Normalized fluorescence intensity (NFI) and relative fluorescence intensity (RFI) of reduced tpMetROG (1 μM) incubated with methionine (Met, 10 μM) or methionine sulfoxide (MetO, 10 μM) when emission wavelength was fixed at 535 nm. (B) NFI and RFI of reduced tpMetROG (1 μM) incubated with (B) calmodulin (1 μM) or partially oxidized calmodulin (1 μM), (C) IDLO (1 μM) or partially oxidized IDLO (1 μM), (D) LegP (1 μM) or partially oxidized LegP (1 μM), (E) Sacde (1 μM) or partially oxidized Sacde (1 μM) when emission wavelength was fixed at 535 nm. (F) NFI and RFI of inactive mutant tpMetROG^{C129S} incubated with Sacde (1 μM) or partially oxidized Sacde (1 μM) when emission wavelength was fixed at 535 nm. The dotted vertical lines indicate the 415 and 500 nm. Data are presented as means ± SD and are representative of 3 replicates. ** $p < 0.01$, *** $p < 0.001$; ns, not significant, as determined by two-tailed paired *t*-test.

of methionine residues in target proteins. We prepared a fusion protein named tpMetROG, consisting of MsrB, circularly permuted yellow fluorescent protein (cpYFP), and thioredoxin3 (Trx3), peptide linker, and protein G in succession (Fig 1A). Protein G is a cell surface protein from *Streptococci*, and its B1 domain binds to the Fc region of immunoglobulin G (IgG).[25, 31] Once the biosensor reacts with MetRO, a stable disulfide bond is formed between MsrB and Trx3, causing changes in the spectral properties of cpYFP; (1) Reduction of the methionine R-sulfoxide residue of a target protein leads to the formation of sulfenic acid on catalytic cysteine (Cys129) of MsrB, (2) the resolving cysteine (Cys69) in proximity attacks the sulfenic acid intermediate to form an intramolecular disulfide, reducing the sulfenic acid, (3) the disulfide bond between two cysteines is reduced by Trx3 by a similar mechanism as in (2), resulting in the formation of a transient intermolecular bond between Cys69 of MsrB and Cys417 of Trx3. In our recombinant Trx3, the resolving cysteine (Cys422) has been substituted to serine, which makes the transient disulfide bond between MsrB and Trx3 stable, thereby affecting the structure of cpYFP permanently.[25, 28, 32] Importantly, this tpMetROG biosensor is designed to bind to the Fc region via the Protein G, allowing it to detect methionine sulfoxide in a protein-specific manner using antibodies (Fig. 1B). Using the sensing ability of tpMetROG, we designed a method named biosensor-linked immunosorbent assay (BLISA), similar to the indirect ELISA (Fig 1B); as follows: (1) Coat proteins from the tissue lysates on the plate. (2) Add primary antibody for a target protein. (3) Wash away the unbound antibody and then add tpMetROG at low temperature. (4) Wash away the unbound tpMetROG and then add the working buffer with increasing temperature. (5) Measure the change of fluorescence intensity in response to the reduction of methionine sulfoxides of a target protein.

Optical properties of tpMetROG. When purified tpMetROG was analyzed for absorbance spectra, two peaks

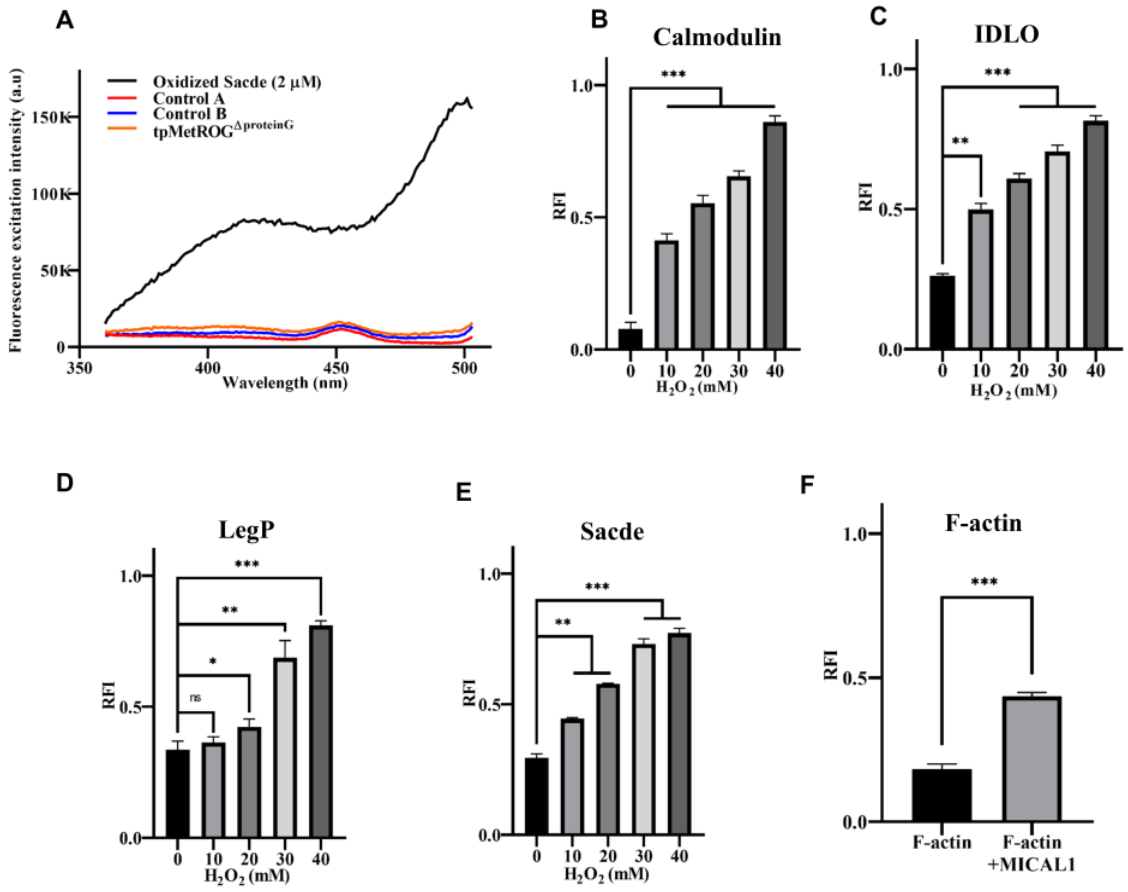

Figure 4. Characterization of tpMetROG used in immunosorbent assays. (A) Fluorescence excitation intensity of tpMetROG was measured after tpMetROG was incubated with antibodies in the Sacde coated plate. Control A: tpMetROG incubated with anti-sacde antibody in a non-coated plate. Control B: tpMetROG incubated in sacde coated plate without antibody. tpMetROG $^{\Delta protein\ G}$; tpMetROG lacking protein G incubated with antibodies in the Sacde coated plate. (B-F) Relative fluorescence intensity (RFI) of tpMetROG after tpMetROG was incubated with antibodies in a plate coated with purified protein. RFIs were obtained from the excitation spectrum recorded with a 535 nm emission wavelength, according to the oxidation state of calmodulin, IDLO, LegP, or Sacde. (F) Detection of stereospecific oxidation states of MICAL1-oxidized actin using tpMetROG. Static measurements of fluorescence in MICAL1-oxidized actin using tpMetROG. Actin oxidized by MICAL1 (0.2 μM) for 2 h was coated on a plate, incubated with mouse monoclonal anti-β actin antibody, and the RFI of tpMetROG in the immunoassay after oxidation was measured. Data are presented as means ± SD and are representative of 3 replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant, as determined by two-tailed paired t-test.

appeared at 410 nm and 500 nm (Fig. 2A). The purified tpMetROG showed a single peak at 515 nm in the emission spectrum (Fig. 2B) and two peaks at 415 nm and 500 nm in the excitation spectrum (Fig. 2C). To characterize the optical properties of tpMetROG and the changes upon Met oxidation, the excitation spectrum was analyzed by incubating tpMetROG with or without partially oxidized, fully oxidized, or untreated IDLO. For readability, we used the normalized fluorescence intensity (NFI) of the excitation spectrum (Fig. 2D), calculated by dividing all excitation spectrum values by the maximum value of the excitation spectrum. Indeed, the NFIs of tpMetROG at 500 nm decreased after reacting with the fully or partially oxidized IDLOs, but the NFIs at 415 nm were not changed. The NFIs at 500 nm showed the greatest difference for all tested samples, and the NFIs at 415 nm were constant (Fig. 2D). Based on this observation, the NFI at both wavelengths were selected to calculate a ratio of fluorescence intensity (RFI) by dividing the 500 nm value by the 415 nm value to quantify methionine oxidation (See the materials and methods). The RFI increased quantitatively after incubation with the oxidized IDLO, reflecting the degree of protein methionine oxidation (Fig. 2E).

Characterization of tpMetROG with various oxidized MRPs. To examine whether tpMetROG can be used as a biosensor for various proteins, the purified tpMetROG was incubated with free MetRO and oxidized MRPs. The spectrum of the NFI obtained by incubating tpMetROG with Met was similar to that obtained after incubating tpMetROG without any substrate. However, a slight reduction was observed at 500 nm in the NFI when the biosensor was incubated with free MetRO (Fig. 3A). Accordingly, the RFI also showed a slight increase, suggesting that tpMetROG senses free MetRO with low efficiency (Fig. 3A). Next, a significant reduction in theNFIs at 500 nm and increase in the RFIs were observed when tpMetROG was incubated with several oxidized MRPs such as mouse calmodulin, hypothetical protein (accession no. YP_155605) from Idiomarina loihiensis L2TR (IDLO), hypothetical protein (accession no. YP_095088) from Legionella

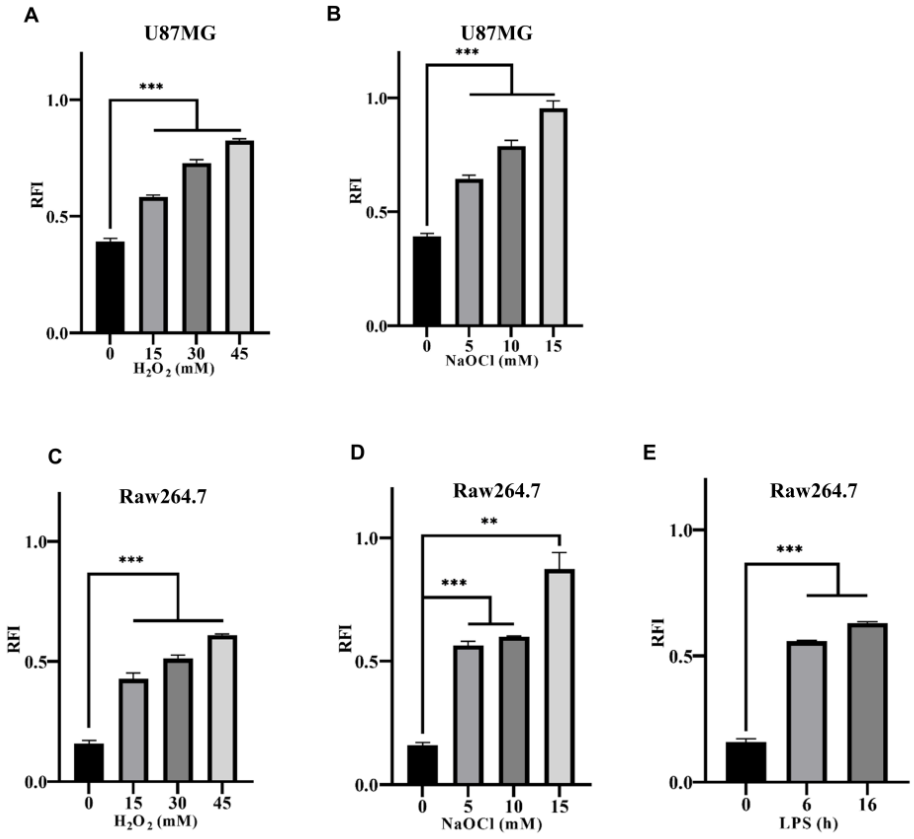

Figure 5. Quantitative analysis of calmodulin methionine oxidation in cell lysates. Relative fluorescence intensity (RFI) of tpMetROG after tpMetROG was incubated with antibodies in the plate coated with U87MG and Raw264.7 cell lysates. (A-D) RFI of tpMetROG in the immunoassay with oxidized cell lysates. U87MG or Raw264.7 cells were treated with H_2O_2 (0 mM, 15 mM, 30 mM, 45 mM, A, B) or NaOCl (0 mM, 5 mM, 10 mM, 15 mM, C, D) for 45 min (H_2O_2) or 15 min (NaOCl). Cell lysates were coated in a plate, incubated with an anti-calmodulin (1:1000; Covance) antibody, followed by sensing using tpMetROG. (E) RFI of tpMetROG in the immunoassay with LPS treated Raw264.7 cell lysates. Lysates from Raw264.7 cells treated with 100 ng/ml LPS for 0, 6 h, or 16 h were used to quantify methionine oxidation of calmodulin. Data are presented as means ± SD and are representative of 3 replicates. ** $p < 0.01$, *** $p < 0.001$; ns, not significant, as determined by two-tailed paired t-test.

(Fig. 3B-E). Finally, there were no changes in the NFI at 500 nm and the RFI when mutated tpMetROGC129S (cysteine in the catalytic part of MsrB replaced by serine) was incubated with the oxidized Sacde (Fig. 3F). Collectively, these results indicate that tpMetROG can quantitatively measure the methionine oxidation in proteins. Next, we measured the kinetic change in the biosensor to determine the optimal incubation time with the substrate. tpMetROG was incubated with calmodulin (2 μM), oxidized calmodulin (2 μM), or dithiothreitol (DTT) (1 mM) for 1500 s. Then, the RFIs after every 30 s (R) were normalized to the RFI at t=0 (R_0) and are presented as R/R_0, (Figure S1A). A rapid decrease and increase of R/R_0 were observed up to 150 s in the reaction with oxidized calmodulin and DTT. After 150 seconds, R/R_0 reaches a stable plateau until the end of the reaction, indicating that incubation for 150 seconds could be sufficient to obtain a stable RFI value (Figure S1A). We examined the effect of pH on tpMetROG activity. The fluorescence ratio was recorded while tpMetROG or tpMetROG^{C129S} was incubated with reduced or oxidized IDLO in the buffers with different pH values (5.8 to 9.0)(Figure S1B). The fluorescence intensity (FI, $F_{500\ nm}/F_{415\ nm}$) of all used sensors increases to the same level with increase of pH. In particular, tpMetROG^{C129S} with reduced IDLO, tpMetROG^{C129S} with oxidized IDLO, and tpMetROG with reduced IDLO show the same value and increase rate with increase of pH. Accordingly, this data supports the idea that tpMetROG^{C129S} can be used as a control dead mutant to determine pH dependency of tpMetROG under variable pH conditions. However, in vitro BLISA assay are performed at controlled pH by using the working buffer (50mM Tris, 150mM NaCl, pH 7.5)(see the materials and methods). Thus, the control dead mutant does not need to be applied for BLISA to determine pH dependency. Nevertheless, in vivo application of tpMetROG needs to consider pH variation and so this control dead mutant will be a useful tool to determine pH dependency of fluorescence intensity.

Application of tpMetROG in the immunosorbent assay for purified proteins. Indirect ELISA intends to quantify specific protein antigen, which requires the primary and enzyme-labeled secondary antibody. The tpMetROG biosensor was applied to develop a novel method, BLISA, a modified version of indirect ELISA. The biosensor (instead of specific protein antigen) that has an affinity for the constant region of the primary antibody replaces the enzyme-labeled secondary antibody to quantify the methionine oxidation of specific protein. Before applying it to various protein substrates, we performed control experiments to examine non-specific interactions (Fig. 4A). For the oxidized Sacde, the biosensor was

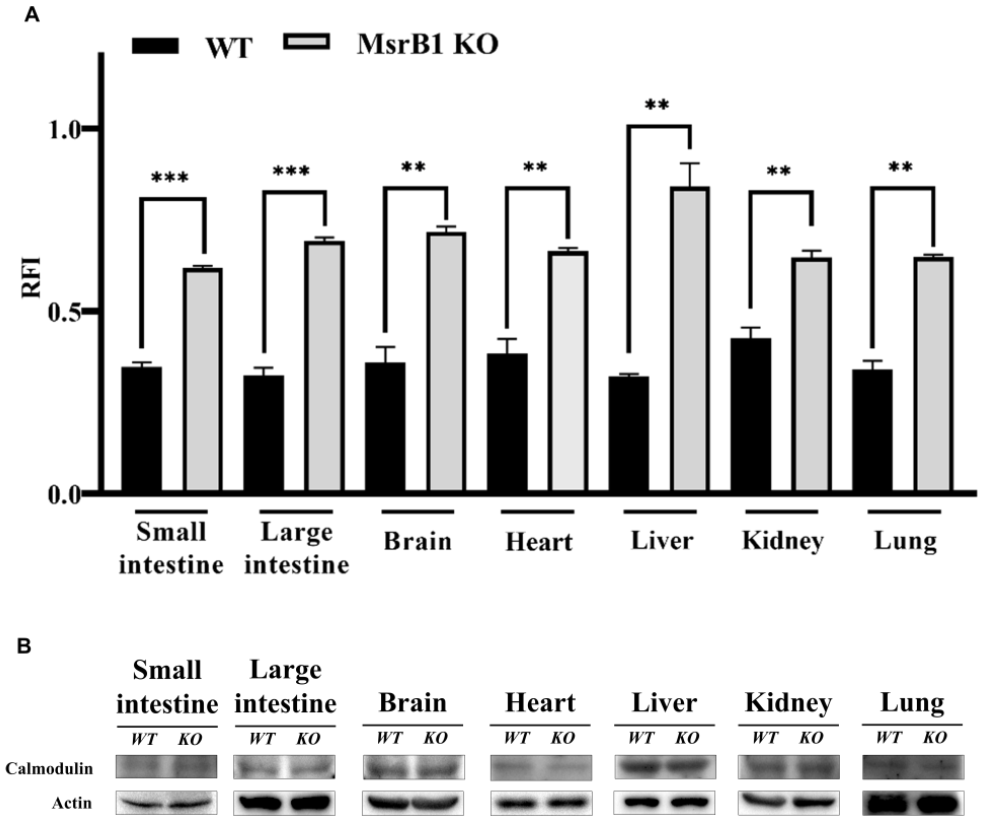

Figure 6. Quantitative analysis of calmodulin methionine oxidation in tissue extracts. Lysates of the small intestine, large intestine, large intestine, brain, heart, liver, kidney and lung isolated from WT mice and MsrB1 knockout mice were used to compare the state of methionine oxidation of calmodulin. Organ extracts were coated in a plate, incubated with an anti-calmodulin (1:1000; Covance) antibody, following by sensing using tpMetROG. (A) RFI of tpMetROG in immunoassay according to MsrB1 knock-out and WT mice organ extracts. (B) Expression level of calmodulin in each organ was analyzed by western blot in MsrB1 knock-out and WT mice. Data are presented as means ± SD and are representative of 3 replicates (A). ** $p < 0.01$, *** $p < 0.001$; ns, not significant, as determined by two-tailed paired t-test.

pneumophila (LegP), and hypothetical protein (accession no.YP_527410) from Saccharophagus degradans (Sacde) incubated with anti-Sacde antibody in the oxidized Sacde-coated plate. In the control A experiment, the biosensor was incubated with an anti-Sacde antibody in a non-coated plate, and in the control B experiment, the biosensor was incubated with oxidized Sacde without anti-Sacde antibody. The experiments with tpMetROG$^{\Delta protein\ G}$ indicate that the sensor lacking protein G was incubated with an anti-Sacde antibody in the oxidized Sacde-coated plate (Fig. 4A). The NFI, including the value at 500 nm, of control A, control B, and tpMetROG$^{\Delta protein\ G}$ were almost similar as a basal level, whereas the NFI of the oxidized Sacde showed a dramatic increase, indicating that the tpMetROG biosensor could successfully detect the methionine oxidation in a specific protein during BLISA. Therefore, it shows a successful development of functional BLISA to analyze methionine oxidation of a specific protein without any experimental noise.

Next, we applied BLISA to various protein substrates with various oxidation levels; calmodulin, IDLO, LegP, and Sacde were oxidized by short-term exposure to H_2O_2 (10 mM, 20 mM, 30 mM, and 40 mM) and used to coat plates. The RFI for all proteins (calmodulin (Fig. 4B), IDLO (Fig. 4C), LegP (Fig. 4D), and Sacde (Fig. 4E)) gradually increased as the concentration of H_2O_2 used to oxidize proteins increased. Finally, we applied BLISA to the active MICAL1-treated F-actin. Active MICAL1 contains flavin-containing monooxygenase (FMO) and calponin-homology domain (CH), which stereoselectively oxidize the two conserved methionine residues of F-actin to MetRO.[16, 30] As a result, the active MICAL1-treated F-actin shows increased RFI compared to the untreated F-actin (Fig. 4F). Taken together, these results prove that BLISA using tpMetROG can be applied to detect methionine oxidation in a protein-specific manner.

Analysis of Methionine oxidation of calmodulin in cells using BLISA. We examined methionine oxidation of calmodulin in cells using BLISA after the induction of oxidative stress. Human glioblastoma U87MG cells (Fig 5A-B) and mouse macrophage Raw264.7 cells (Fig 5C-D) were incubated with various concentrations of H_2O_2 (15 mM, 30 mM, and 45 mM) or NaOCl (5 mM, 10 mM, and 15 mM) for 30 min, and the cell lysates were used to coat plates. After treating with anti-calmodulin antibody and tpMetROG, the RFIs were analyzed (Fig 5A-D). As expected, the RFIs for calmodulin gradually increased as the concentration of H_2O_2 or NaOCl used increased in all tested cell lines.

Lipopolysaccharides (LPS) trigger the production of ROS in the macrophages by binding with toll-like receptor 4.[33] BLISA was applied to quantify the methionine oxidation of calmodulin in Raw264.7 cells stimulated with LPS for 6 and 16 h. The RFIs of calmodulin dramatically increased in LPS-

stimulated cells for 6 and 16 hours (Fig. 5E). Therefore, these results demonstrate that the tpMetROG biosensor is able to quantify protein-specific methionine oxidation occurring in the cells.

Analysis of methionine oxidation in calmodulin obtained from various tissues of MsrB1 knock-out mice using BLISA. Methionine oxidation of calmodulin in animal tissues was analyzed using BLISA and tpMetROG. For this study, tissue samples of the small intestine, large intestine, brain, heart, liver, kidney, and lung were collected from MsrB1 knock-out and wild-type (WT) mice. Interestingly, the RFIs for calmodulin were much higher in all organ tissues from MsrB1 knock-out mice than those from WT mice (Fig. 6). Consequently, this result demonstrates that the ablation of MsrB1 leads to severe methionine oxidation of calmodulin, thereby supporting the idea that MsrB1 is essential for maintaining the reduced state of methionine in proteins.

DISCUSSION

Oxidation of methionine residues often leads to structural abnormalities in protein and their malfunction. Therefore, it is critical to quantitatively measure the methionine oxidation status of proteins under various physiological conditions. Recently, two strategies were used to quantify the amount of methionine sulfoxide residues in proteins *in vivo*; the LC-MS using an isotope labeling with $^{18}O^{34}$ and the fluorescence-based biosensor using cpYFP.[25] However, these methods have limitations in terms of complexity and the lack of specificity. In particular, considering that the oxidation of specific methionine residues in proteins, and not all methionines, is associated with the changes in protein functions, it is essential to quantify oxidation of a specific methionine residue within a protein. In this regard, the tpMetROG biosensor is an advanced tool that employs a fluorescent biosensor and antibodies against specific proteins, allowing for quantifying methionine oxidation of target proteins; it could be applied for any proteins if specific antibodies are available. Accurate analysis of methionine sulfoxide residues in specific proteins using LC-MS/MS has been challenging due to a high degree of spontaneous methionine oxidation during sample preparation. However, the immunosorbent assay using the tpMetROG adopted the sampling process that does not require incubation with buffers containing methanol or heating; thereby, preventing the spontaneous methionine oxidation.

Calmodulin is a multifunctional calcium-binding protein expressed in all eukaryotic cells.[35] Upon calcium binding, calmodulin undergoes conformational changes, which facilitate its interaction with various target proteins such as the kinases, phosphatases, phosphodiesterases, and ion channels.[35, 36] However, oxidation of the methionine residues of calmodulin also leads to conformational changes that allow interaction with various target proteins without binding calcium.[37-40] The tpMetROG biosensor was able to quantify the oxidation of methionine residues in calmodulin under oxidative stress. Therefore, this biosensor can potentially be used to predict the changes in calmodulin functions associated with methionine oxidation. Many proteins, such as nitrogen assimilation transcription factor nirA (NirA), nuclear factor of kappa B inhibitor alpha (IkBα), HypT, CaMKII, actin, and, calcineurin are functionally altered by reversible oxidation and reduction of methionine residues causing structural changes or altered interactions.[41] In particular, the two conserved methionine residues of actin are stereoselectively oxidized to methionine-*R*-sulfoxide by MICALs, which results in actin depolymerization. The tpMetROG biosensor allows understanding the functional role of oxidation and reduction of methionine residues and conducting research on methionine residues in protein as the potential regulators of cellular function under various physiological conditions.

However, this novel biosensor still has some limitations. Currently, its application is limited to in vitro analysis. In vivo analysis such as live-cell imaging and immunohistochemistry should be developed to expand its use and contribution to various biological studies for better understanding association of methionine oxidation and functional regulation within a specific protein. Also, this sensor is devised for quantifying relative change of methionine oxidation in a specific protein, whereas the absolute value of methionine oxidation in a single protein cannot be analyzed in the present way. To make this possible, analysis of the number of methionine oxidation in a specific protein by using LC-MS/MS can be applied to examine correlation of the number of methionine oxidation and fluorescence intensity. Future study will be conducted to overcome this hurdle of tpMetROG sensors in order to expand its application.

CONCLUSION

We developed the recombinant fluorescence-based sensor that includes MsrB, cpYFP, Trx3, and protein G. This sensor could successfully replace the secondary antibody to measure the oxidation of methionine residues of specific proteins in an immunosorbent assay, BLISA. In particular, methionine oxidation of calmodulin upon oxidative stress could be quantified in cells and tissues. For future research, this advanced tool would be useful for studying mechanisms linking methionine oxidation to changes in the function of various proteins. We need to consider the extended application of this biosensor, beyond the immunosorbent assay, as a versatile tool.

ASSOCIATED CONTENT

Supporting Information.
Supporting Information Available: The following files are available free of charge.
pH dependence of tpMetROG; This information contains kinetic reactivity and pH dependence of tpMetROG, oligonucleotides used in this study, *de novo* synthesized DNA sequences

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J.H.K. and B.C.L. conceived and supervised the research. H.M.L. designed the experiment and carried out the experiments with support from S.K., A.L, M.K., Y.J.R, Y.H.J., H.W.J. and H.-J.L. The manuscript was written by H.M.L., D.W.C., L.T., V.N.G., J.H.K and B.C.L. All authors reviewed and edited the manuscript.

Notes

The authors have filed a patent application relating to the technology.

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ABBREVIATIONS

MsrB, methionine sulfoxide reductase B; cpYFP, circularly permuted yellow fluorescent protein; ROS, Reactive oxygen species; Met, methionine; MetSO, methionine-*S*-sulfoxide; MetRO, methionine-*R*-sulfoxide; CaMKII, calcium/calmodulin-dependent protein kinase II; HypT, hypochlorite-responsive transcription factor; MICAL, molecule interacting with CasL; NFI, normailized fluorescence intensity; RFI, relative fluorescence intensity; MRP, methionine rich protein; IgG, immunoglobulin G; BLISA, biosensor-linked immunosorbent assay; NirA, nitrogen assimilation transcription factor nirA; IkBα, nuclear factor of kappa B inhibitor alpha.

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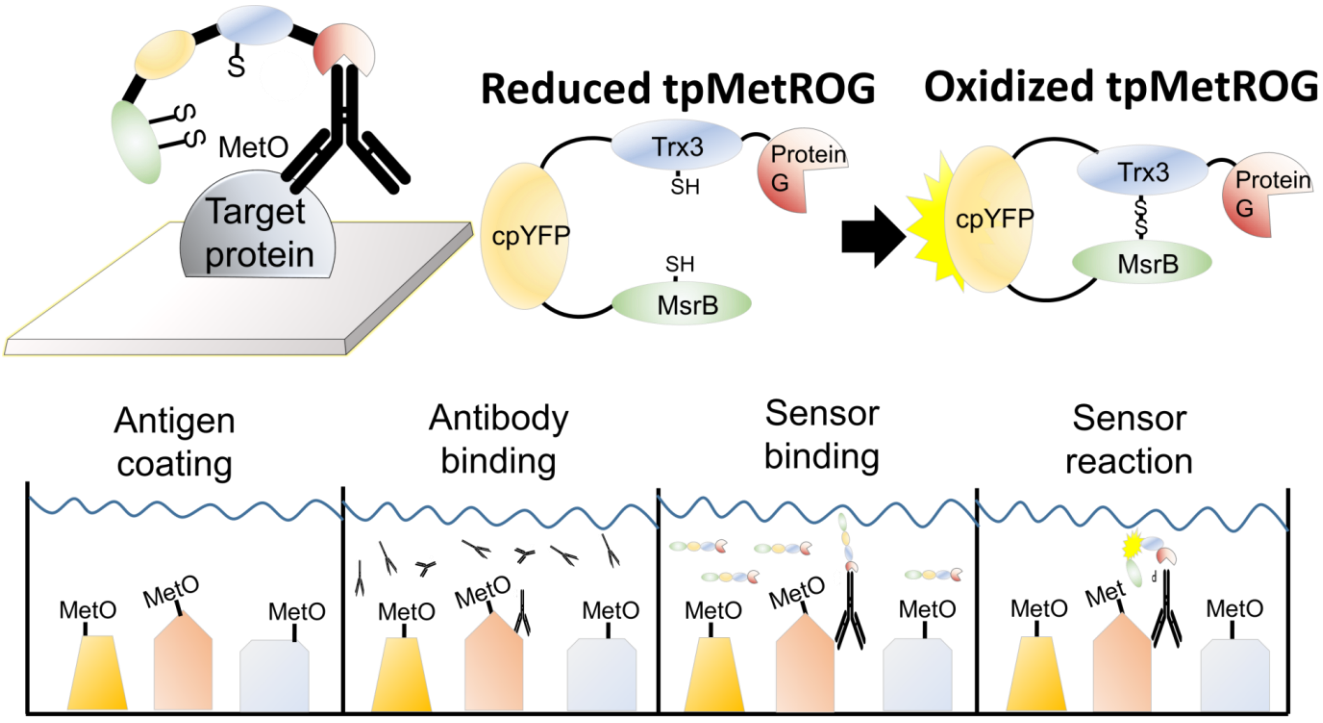
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Protein G
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Antigen coating
Antibody binding
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