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Novel peroxiredoxin-based sensor for sensitive detection of hydrogen peroxide

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ABSTRACT

A series of genetically encoded sensors have been developed to detect the important signaling molecule H_2O_2 in living cells. However, more responsive and sensitive biosensors need to be developed. To address these demands, we used *E. coli* as a platform to develop a novel fluorescent H_2O_2 sensor, which we refer to as TScGP. This sensor employs a circularly permuted YFP (cpYFP) and is based on a redox relay between peroxiredoxin (Prx) and thioredoxin (Trx). Structurally, cpYFP is sandwiched between a fungal PrxA and a C-terminal cysteine mutated TrxA that can form a stabilized disulfide bond between PrxA and TrxA in response to H_2O_2. We confirmed that TScGP can be used for detecting exogenous H_2O_2 in the range of 0.5–5 μM with high selectivity and rapidly detecting H_2O_2 within 30 s in *E. coli*. To demonstrate an application, cellular H_2O_2 production by menadione was detected directly by TScGP. Our results demonstrated that using Prx-Trx combination as a sensing moiety is another strategy in designing H_2O_2 sensor with high performance.

1. Introduction

Hydrogen peroxide (H_2O_2) has important functions in cellular physiology [1–3]. Understanding the spatial and temporal aspects of H_2O_2 in living cells is now considered essential in physiological and pathological research. Currently, the most effective method for measuring H_2O_2 uses genetically encoded fluorescent sensors. These sensors will necessarily consist of (at least) two components. The first is a fluorescent reporter protein, and the second is a sensitive protein domain that can undergo a conformation change in response to H_2O_2. Two typical genetically encoded H_2O_2 sensors are Hyper and roGFP2-Orp1 [4,5]. These sensors employ circularly permuted fluorescent protein (cpFP) and redox-sensitive green fluorescent protein 2 (roGFP2) and are based on OxyR and Orp1, respectively. The cpFPs are highly responsive to conformational changes in sensitive domains to which they are fused, whereas roGFP responds to oxidation of its cysteine pair to a disulfide bond by its fusion protein [6–8]. OxyR is a bacterial transcription factor that senses H_2O_2 by way of a conformation change. Orp1 is an oxidant receptor that can oxidize the yeast transcription activator Yap1 in the presence of H_2O_2, and thiol-disulfide exchange between Orp1 and roGFP also occurs under the same conditions. Another practical strategy is the use of a Förster resonance energy transfer (FRET) pair from two fluorescent proteins to sense structural changes imposed by the H_2O_2-driven oxidation of the sensing proteins being inserted between the fluorescent donor and the acceptor [9]. Therefore, based on existing sensors, at least three different designs can be envisaged.

Peroxiredoxins (Prxs) are thiol-specific peroxidases that catalyze the reduction of H_2O_2. During the reduction process, H_2O_2 first oxidizes the peroxidatic cysteine residue (C_P) of Prx to cysteine sulfenic acid (C_P–SOH), which can be resolved by the "resolving" Cys (C_R) of Prx to form a protein disulfide bond (C_P-C_R). H_2O_2-oxidized Prxs are subsequently reduced by thioredoxin (Trx). Trx directly reduces the C_P-C_R in Prx by disulfide exchange. Subsequently, TrxR catalyzes the reduction of oxidized Trx (Trx-S2) using NADPH as the electron donor. Prxs have a significantly higher reactivity and sensitivity towards H_2O_2 than those of both OxyR and Orp1 [10,11], which potentially enables Prxs to provide superior H_2O_2 sensors. Recently, Morgan et al. designed a fluorescent sensor based upon the redox relay between the yeast peroxiredoxin Tsa2 and the fluorophore roGFP2 and applied it in yeast [12]. More

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recently, a novel FRET sensor based on human peroxiredoxin 2 sandwiched between two fluorescent proteins has been applied in human cells [13]. As expected, both sensors achieved much higher sensitivities than other sensors based on less reactive peroxidase domains. Until now, no Prx-based sensors have been constructed to be available for bacteria. Moreover, it is noteworthy that whether the natural redox relay between Prx and Trx capable of designing for H_2O_2 sensor as a new tool.

In this study, we developed a novel bacterial H_2O_2 sensor by inserting a circularly permuted YFP (cpYFP) into a fungal PrxA domain and a TrxA mutant (TrxA-C39S) domain. TrxA forms a transient disulfide bond with the C_P of PrxA which is stabilized by mutating the C-terminal cysteine of TrxA [14]. The intermolecular bond between PrxA and TrxA-C39S causes a significant conformation change of the sensor that can be observed by ratiometric fluorescent signal changes. Trx is the natural redox-partner of Prx, which allows the reaction to proceed smoothly. Taking advantage of the reaction mechanism of Trx-Prx, the new sensor was validated to respond more sensitive and rapidly to H_2O_2 than Hyper.

2. Materials and methods

2.1. Construction of fluorescent sensors

All PCRs were performed using PrimeSTAR HS DNA Polymerase (Takara, Japan). To generate the sensitive domain of the sensor, the sequences encoding *A. nidulans* PrxA and PrxA (C61S) were amplified from plasmids that were previously constructed [15] using primers Pr1 and Pr2 (the sequences are available in Supplementary Table 1), containing overlapping extensions to pET28a and cpYFP, respectively. TrxA mutants (C39S) were generated by site-directed mutagenesis using the recombinant plasmid (pET28a-TrxA) constructed previously [16] as the template. The sequence encoding TrxA (C39S) was then amplified from the resulting plasmid using primers Pr7 and Pr8 that contained overlapping extensions to the cpYFP and the pET28a, respectively.

The sequence of cpYFP was the same as that used in the MetO sensor [17]. The sequence was synthesized and then amplified using primers Pr3 and Pr4. All of the PCR products were purified using a Gel Extraction Kit (Omega Bio-tek, USA). For the construction of the sensor expression plasmid, the three purified fragments were proportionally mixed with the pET28a plasmid that was previously digested by *Eco*RI and *Hind*III restriction enzymes (Takara, Japan) and kept at 37 °C for 30 min according to the protocol outlined in the ClonExpressMultiS One Step Cloning Kit (Vazyme, China). Then, half of the mixture was transformed into DH5α for amplification and screening for the correct recombinant plasmid. For constructing several chimeras with various linkers, a set of primers with specific linkers and homologous arms were designed and are listed in Supplementary Table 1, and recombinant plasmids with various chimeras were generated as above mentioned.

Hyper was constructed following the method described previously [4], except that the Hyper-encoding DNA fragment was inserted into the *Eco*RI/*Hind*III sites of pET28a instead of *BamH*I/*Hind*III sites of pQE30.

2.2. Expression and purification of fluorescent sensors

All constructed plasmids were introduced into *E. coli* BL21 (DE3). Transformed cells were grown in LB and induced by 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) under 20 °C. The cells were collected and then disrupted by sonication in buffer A (20 mM NaH_2PO_4, 0.5 mM NaCl, pH 7.4) containing 5 mM 2-mercaptoethanol. Proteins were purified using HiTrap Chelating HP (GE Healthcare, USA) and eluted with buffer B (buffer A supplemented with 0.5 M imidazole). Protein solutions were concentrated using Amicon Ultra concentrators (Millipore, USA) in 50 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) containing 1 mM dithiothreitol (DTT).

2.3. Fluorometric characterization of TScGP in vitro

For the characterization of TScGP *in vitro*, aliquots of purified proteins were added to phosphate buffer (pH 7.0) and then transferred to a spectrophotometer cuvette. After recording the initial fluorescence spectrum using an F-4600 fluorescence spectrophotometer (HITACHI, Japan), various amounts of H_2O_2 or oxidants were added to the reaction mixture and the spectra were recorded immediately. Excitation spectra were recorded typically from 380 to 510 nm under the following condition: the emission wavelength was 530 nm, the excitation and emission slits were both 5 nm, the wavelength scan speed was 2400 nm/min, the PMT voltage was 700 V, and the response was 0.5 s. Emission spectra were recorded from 505 to 600 nm with an excitation wavelength of 485 nm. The other conditions were the same as those used to obtain excitation spectra.

For selectivity test, *tert*-butyl hydroperoxide (t-BOOH), oxidized glutathione (GSSG), S-Nitrosoglutathione (GSNO), 3-morpholinosydnonimine (SIN-1), xanthine, xanthine oxidase and catalase (from bovine liver) were all purchased from Sigma-Aldrish. Xanthine-xanthine oxidase system was used to generate superoxide radicals. Catalase was used to deplete the H_2O_2 generated during spontaneous dismutation of superoxide. 1-(hydroxy–NNO–azoxy)-L-prolin disodium salt (PROLI NONOate) (Cayman Chemical, USA) was used as NO donor. All of the above reagents were prepared freshly before being used.

For pH calibration, purified proteins were added to phosphate buffer with different pH values ranging from 5.8 to 8.2. Reproducibility was determined at pH 7.0 and 7.5.

2.4. Characterization of TScGP in vivo

E. coli BL21 (DE3) expressing the sensor TScGP in LB media were centrifuged at 10,000×*g* prior to each assay, rinsed twice and then resuspended in phosphate buffer (pH 7.0). Cells expressing TScGP were diluted in 1 ml quartz cuvettes. Kinetic assays of the response to H_2O_2 with TScGP-expressing cells were conducted in 1 ml cuvettes.

For the detection of H_2O_2 in *E. coli* that were treated with menadione sodium bisulfite (MSB), solid MSB was dissolved in sterile distilled water to prepare a 100 mM stock solution prior to testing. Before adding MSB to the cuvette, the fluorescence intensities were measured and then the MSB stock solution was added to the diluted *E. coli* cell suspensions to provide a final concentration of 0.5 mM. Sterile distilled water was used as a control. Fluorescence intensities were acquired every 3 min for a period of 50 min.

2.5. Statistical analysis

In all figures, the results are presented either as a representative example of a single experiment that was repeated at least in triplicate or as at least three experiments. The results obtained are presented as the means ($n = 3$) ± SD.

3. Results

3.1. Generation of a fluorescence indicator for H_2O_2

We genetically fused *A. nidulans* PrxA and TrxA-C39S (the C-

terminal cysteine of the TrxA active site was mutated to serine [14]) to the N- and C-termini of cpYFP, respectively, to obtain a three-protein chimera (chimera 1). Considering the mechanism of reduction of PrxA by TrxA, a stable disulfide bond is expected to be formed between PrxA and TrxA-C39S following a reaction with H_2O_2, thereby resulting in conformational changes within the fusion protein. We investigated the responsivity of chimera 1-expressed *E. coli* cells as well as the purified recombinant protein to H_2O_2, but did not observe the expected change in the fluorescence ratio (Fig. 1b and c). There are two possible reasons for the absence of a reaction. One reason is the loss of the peroxidase activity of PrxA in the fusion protein, and the other reason is the difficulty of forming an intermolecular disulfide bond between PrxA and TrxA due to steric hindrance. We confirmed the presence of peroxidase activity by the fused PrxA in chimera 1 and determined that the fusion protein remained capable of catalyzing the oxidation of H_2O_2 but required additional natural TrxA (Supplementary Fig. 1). This result indicated that the fused PrxA was still active and that a nonfunctional H_2O_2 sensor could be due to steric hindrance.

Amino acid linkers can be introduced into fusion proteins to improve their structural flexibility and to reduce steric hindrance. Linkers with various lengths and structures [18,19] were tested and three chimeras (chimera 2, 3, 4) were obtained (Supplementary Fig. 2). All three chimeras produced typical cpYFP-based fluorescent spectra, but none produced a satisfactory signal response to H_2O_2 in both *in vitro* and *in vivo* experiments (Fig. 1b and c). Next, we tentatively exchanged the locations of PrxA and TrxA in chimera 2 and acquired a new sensor, chimera 5, named TScGP (Fig. 1a). Surprisingly, TScGP exhibited reactivity to H_2O_2 with an expanded dynamic range both *in vitro* and *in vivo* (Fig. 1b and c). Purified TScGP produced two excitation peaks at 415 and 500 nm and one emission peak at 520 nm (Fig. 1d). The addition of H_2O_2 to TScGP caused an increase in the excitation peak at 500 nm and a decrease in the excitation peak at 415 nm, thereby producing a ratiometric behavior response to H_2O_2. As expected, the emission fluorescence upon excitation at 485 nm was substantially increased by the addition of H_2O_2 (Fig. 1d). To confirm that a disulfide bond was indeed formed between C31 of PrxA moiety and C36 of TrxA-C39S moiety during the oxidation process, TScGP$^{PrxA\ C31S}$ and TScGP$^{TrxA\ C36S}$ mutants were generated and subsequently evaluated. As expected, both of the purified mutated sensors abolished the H_2O_2-induced fluorescence changes (Fig. 1e and f). Additionally, DTT treatment induced a dramatic decrease in the fluorescence ratios of the H_2O_2-oxidized wild-type sensor, suggesting that the disulfide bond between PrxA and TrxA-C39S was disrupted (Fig. 1d). These results indicate that TScGP functions on the basis of an interaction between the reactive cysteines of PrxA and TrxA.

3.2. In vitro characterization of TScGP

To evaluate the sensitivity of TScGP, we investigated the necessary minimal H_2O_2 concentrations to induce fluorescence changes for the purified sensor. We placed three concentrations of TScGP (45 nM, 90 nM, 180 nM) into the reaction buffer and titrated the sensors against increasing concentration of H_2O_2 until the fluorescence fold change achieved nearby 1.2. We found that the response minimal concentrations of H_2O_2 were 16, 40, and 80 nM corresponding to the above three concentrations of TScGP. It seemed high concentration of sensor caused detriment to its sensitivity. Comparing the performances of the 45 nM of TScGP with 25 nM of Hyper who has a response minimum at 25 nM [4], we demonstrated that TScGP exhibited slightly more sensitive than Hyper *in vitro*.

To evaluate the selectivity of TScGP, we tested several oxidants (Fig. 2**)**. *T*-butyl hydroperoxide (t-BOOH), another natural substrate for most peroxiredoxins, unavoidably induced changes in fluorescence of TScGP, as with other peroxiredoxin-based fluorescent H_2O_2 sensors [12,13]. Oxidized glutathione (GSSG), an important endogenous oxidant, was unable to drive TScGP oxidation, even at a high concentration (1 mM). Although nitric oxide (NO) is classified as a reactive oxygen species, NO released from two popular NO donors, 40 μM PROLI NONOate and 10 μM GSNO, also appeared to be unable to induce changes in TScGP fluorescence. Some

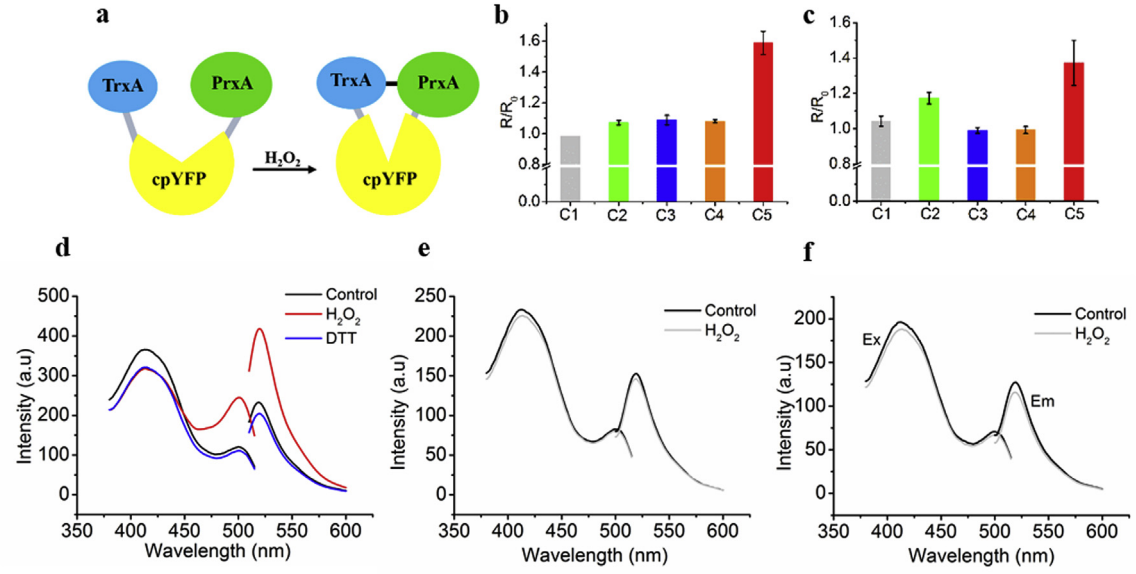

Fig. 1. Fluorescence responses of various PrxA-cpYFP-TrxA chimeras to H_2O_2. (**a**) Model for H_2O_2 sensing of the designed H_2O_2 sensor. After reaction with H_2O_2, a disulfide is formed between C_P and C_R of PrxA. Reduction of this bond by the catalytic cysteine of the Trx moiety induces a conformational change in the sensor, leading to spectral changes for cpYFP. (**b**) Fluorescence ratio changes of various chimeras in *E. coli* cells upon reaction with 100 μM H_2O_2. (**c**) Fluorescence ratio changes of various purified chimeras to 1 μM H_2O_2. $F_{500\ nm}/F_{415\ nm}$ of the oxidized chimeras (R) were normalized to the value of its reduced form (R_0). (**d**, **e** and **f**) Fluorescence spectra of purified TScGP (d) and its inactive counterparts TScGP$^{RrxA\ C31S}$ (e) and TScGP$^{TrxA\ C36S}$ (f) in response to 1 μM H_2O_2 or 1 mM DTT.

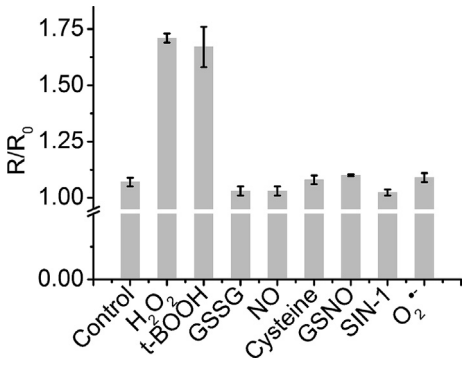

Fig. 2. Effect of various oxidants on fluorescence ratio changes of TScGP. 0.2 μM TScGP was treated with the following oxidants: H_2O_2 (1 μM), *tert*-butyl hydroperoxide (t-BOOH, 1 μM), glutathione disulfide (1 mM), PROLI NONOate (NO donor, 40 μM), cysteine (10 μM), *S*-nitrosoglutathione (10 μM) and 3-morpholinosydnonimine (SIN-1). (0.5 mM). $O_2^{\bullet-}$ (Xanthine (100 μM)-xanthine oxidase (20 mU/ml) plus catalase (500 U/ml)).

peroxiredoxins are also reported to be sensitive to oxidation by peroxynitrite [20,21]. However, the signal for TScGP did not change after treatment of peroxynitrite released from 0.5 mM 3-morpholinosydnonimine (SIN-1). We also examined the response of TScGP towards superoxide radicals, an intracellular precursor of hydrogen peroxide, using xanthine-xanthine oxidase system plus catalase [4]. Addition of enough catalase could completely prevent the oxidation of TScGP (Fig. 2). Therefore, TScGP exhibits specificity for H_2O_2 and its derivative organic hydroperoxide and does not react with other oxidants.

As with most cpYFP-based sensors, TScGP fluorescence is dependent on pH (Supplementary Fig. 3). The effect of pH on H_2O_2 reactivity can be corrected when TScGP fluorescence is normalized by its inactive counterpart that is measured in parallel experiments.

3.3. Performance of TscGP in cultured E. coli cells

To determine the fluorescence changes of TScGP in response to H_2O_2, *E. coli* cells expressing the TScGP sensor were monitored by fluorescence microscopy. A significant enhancement in the fluorescence intensity excited by a 488 nm laser after addition of H_2O_2 was observed (Supplementary Fig. 4), which is consistent with the increase in fluorescence intensities that were obtained using a spectrophotometer with an excitation wavelength of 485 nm after the addition of H_2O_2 (Fig. 1d).

Because of the instability of H_2O_2, a high-quality H_2O_2-sensor requires a rapid reaction capability. We measured the timing of H_2O_2-induced fluorescence changes in cells that express TScGP (Fig. 3a). We found that by exogenously adding either high or low concentrations of H_2O_2, the normalized $F_{500\ nm}/F_{415\ nm}$ excitation ratio (R/R_0) reached a peak value within 30 s. To make a direct experimental comparison of TScGP with Hyper, we produced Hyper and investigated its response rate. As shown in Fig. 3b, reaching the end-point oxidation state, Hyper cost about 1 min, which is consistent with that in previous report [5]. Our results indicated that TScGP is a more rapid response sensor.

Next, we assessed and compared the sensitivity of TScGP with Hyper expressed in *E. coli* cells under the same conditions. We suspended bacterial cells in phosphate buffer and titrated the sensors against increasing concentrations of H_2O_2 from 0.5 to 25 μM (Fig. 3a and b). 0.5 μM of H_2O_2 significantly changed the signal of TScGP, and 5 μM of H_2O_2 saturated the sensor (Fig. 3a). By comparison, the minimal concentration of H_2O_2 needed to activate Hyper was 5 μM (Fig. 3b), which is consistent with the previous report [4]. Thus, the minimal detection limit of TScGP should be more than 10-fold lower than that of Hyper under our experimental conditions. These results demonstrate that TScGP is a very sensitive H_2O_2 sensor and may be vailable for imaging or measuring trace amounts of H_2O_2 during cell signaling.

To dynamically test cellular substrates, reversibility of the sensor is required. Removal of H_2O_2 from H_2O_2-stimulated TScGP expressed cells led to a rapid decrease of the fluorescence ratio (Fig. 3c). This result indicates TScGP is a rapid and reversible H_2O_2-sensor, and the endogenous reduction system of *E. coli* may be responsible for the sensor reduction.

3.4. Menadione-generated H_2O_2 in E. coli

Menadione is a cytotoxic compound that undergoes a redox-cycling reaction resulting in the production of ROS, including superoxide and H_2O_2. Although H_2O_2-resistance genes have been reported to respond to menadione, menadione-catalyzed H_2O_2 production has rarely been determined in cells [22,23]. To investigate whether menadione generates H_2O_2 in *E. coli*, we exposed the cell-expressed TScGP to menadione for 50 min and monitored the fluorescence changes over time (Fig. 4). The R/R_0 for the active sensor began to increase after approximately 6 min, reached a plateau at 20 min, and then remained stable. These results demonstrate that menadione generated H_2O_2 in *E. coli*, but the process requires several minutes. The lag time between menadione addition and H_2O_2 production may be due to the generation of the intermediate product, superoxide.

4. Discussion

H_2O_2 is increasingly recognized to have important roles in cellular and organismal physiology. To characterize H_2O_2 biology, development of genetically-encoded fluorescent H_2O_2 sensors is necessary. Two classical H_2O_2 sensors, Hyper and roGFP-Orp1, have greatly increased the ability to detect H_2O_2 in living cells. However, these sensors' sensing moieties (OxyR and Orp1, respectively) limit their performance. In combination with the Trx system, peroxiredoxin (Prx) reacts with H_2O_2 approximately 100-fold faster than Orp1 and OxyR, which indicates that Prx can be a more efficient sensing moiety for the sensor. As part of the natural reducing pattern of Prx, Trx will covalently trap the H_2O_2-oxidized Prx, thus provoking a conformational change that can be sensed by cpYFP. Therefore, we constructed a novel H_2O_2 sensor (TScGP) in which cpYFP is sandwiched between Prx and Trx domains. Genetically-encoded TScGP was characterized as a rapid and sensitive H_2O_2 sensor.

The recently developed roGFP2-Tsa2ΔC_R is another Prx-type H_2O_2 sensor and was developed based on a redox relay between a yeast peroxiredoxin (Tsa2) and roGFP2. As with roGFP2-Orp1, the fluorescence ratio for roGFP2-Tsa2ΔC_R treated with H_2O_2 reached its end-point oxidation state value in several minutes [5,12]. Thus, even though Tsa2 is an efficient peroxidase, roGFP2-Tsa2ΔC_R is not a rapid sensor for H_2O_2. The non-natural redox relay between Tsa2 and roGFP2, and the relatively slow reaction rate of roGFP may reduce the reaction rate of the sensor. Whereas, the efficient oxidant transfer route between Trx and Prx may ensure TScGP a rapid response to H_2O_2.

At an early stage in our work, we fused PrxA and TrxA to the N- and C- termini of cpYFP, but the products were unable to respond to H_2O_2. However, exchanging the locations of PrxA and TrxA in chimera 2 created a satisfactory sensor. These results suggest that N- or C-terminal fusions of fluorescent proteins might influence Prx behavior. In fact, even the presence of a short tag, such as an N-terminal His-tag, on human PrxIII was reported to markedly

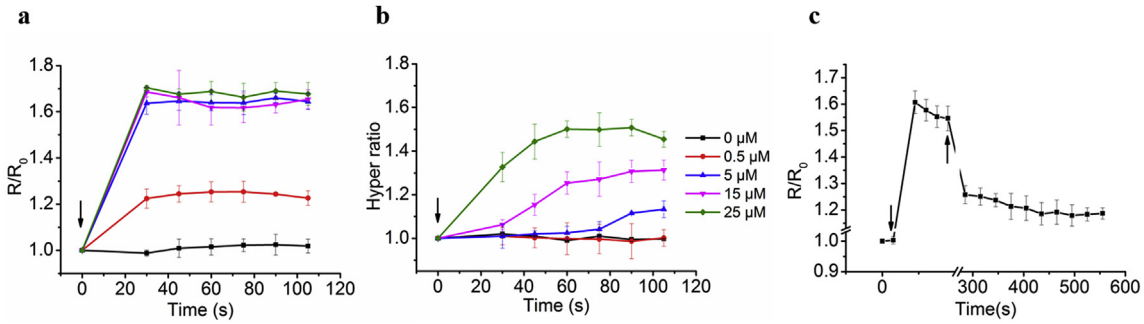

Fig. 3. Performance of TscGP and Hyper in *E. coli* cells. (**a, b**) Time course for the fluorescence ratio in TScGP (a) and Hyper (b)-expressing *E. coli* cells after stimulation with indicated concentrations of H_2O_2. The arrow indicates the time point of the H_2O_2 addition. (**c**) Reversibility of fluorescence changes in TScGP-expressing *E. coli* cells treated with 10 μM H_2O_2 (downward arrow), followed by washing (upward arrow).

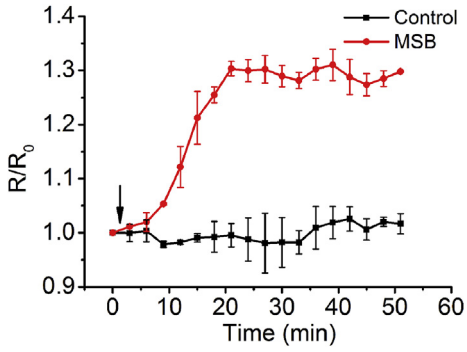

Fig. 4. Time course for the R/R_0 ratio in TScGP-expressing *E. coli* cells after stimulation with 0.5 mM menadione. The arrow indicates the time point of addition of menadione.

enhance its dodecamer stability and lead to a 3.0–3.5-fold decrease in activity [24]. Additionally, the N-terminal myc-tag was found to destabilize the Tsa1 high molecular weight structure and alter its function [25]. Alternatively, other research analyzed the essential factors during the interaction of Prx with Trx and concluded that the C-terminal tail of Prx is especially important for Prx-Trx binding [26]. Therefore, it appears that there is no preferred arrangement for the locations of elements in the design of Prx-based sensors.

TScGP is a dynamic sensor that offers the possibility of monitoring intracellular H_2O_2 in real time. Although no natural reducing partner for the fungal PrxA exist, there must be a functional substitutable reducing system in *E. coli* cells. The amino acid sequence of *A. nidulans* PrxA exhibits a high degree of homology (38.5%) with that of Ahp1 (a yeast Prx), whose peroxidase activity can be supported by the Trx/TrxR system of *E. coli* [27]. Therefore, oxidized TScGP may be reduced in the same manner. One possible mechanism is that the intramolecular disulfides between Cys31 of PrxA and Cys36 of TrxA in TScGP were reduced by the redox-active cysteines of *E. coli* Trx in a typical thiol-disulfide exchange manner. However, glutathione/glutaredoxin (GSH/Grx) is also an alternative reducing system for TScGP recycling in a manner similar to the reduction of roGFP-based sensors. These issues remain to be addressed.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.07.062.

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