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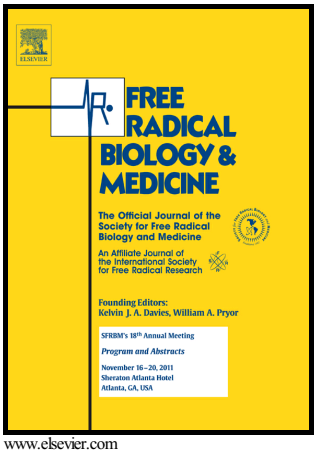

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Systematic *in vitro* assessment of responses of roGFP2-based probes to physiologically relevant oxidant species

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ABSTRACT

The genetically encoded probes roGFP2-Orp1 and Grx1-roGFP2 have been designed to be selectively oxidized by hydrogen peroxide (H_2O_2) and glutathione disulfide (GSSG), respectively. Both probes have demonstrated such selectivity in a broad variety of systems and conditions. In this study, we systematically compared the *in vitro* response of roGFP2, roGFP2-Orp1 and Grx1-roGFP2 to increasing amounts of various oxidant species that may also occur in biological settings. We conclude that the previously established oxidant selectivity is highly robust and likely to be maintained under most physiological conditions. Yet, we also find that hypochlorous acid, known to be produced in the phagocyte respiratory burst, can lead to non-selective oxidation of roGFP2-based probes at concentrations ≥ 2 µM, *in vitro*. Further, we confirm that polysulfides trigger direct roGFP2 responses. A side-by-side comparison of all three probes can be used to reveal micromolar amounts of hypochlorous acid or polysulfides.

LIST OF ABBREVIATIONS

roGFP2, reduction-oxidation sensitive variant of GFP; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; GSSG, oxidized glutathione; Grx1, glutaredoxin 1; Orp1, oxidant receptor peroxidase 1 from yeast; K_2S_x, potassium polysulfide; GSNO, S-nitroso glutathione; $ONOO^-$, peroxynitrite; NO•, nitric oxide; SperNO, spermine Nonoate; DeaNO, Diethylamine Nonoate; HOCl, hypochlorous acid; DTT, dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; AT-2, aldrithiol 2; $O_2^{\bullet-}$, superoxide anion; H_2S, hydrogen sulfide; PBS, phosphate buffered saline; FPLC, fast protein liquid chromatography

Keywords:

genetically encoded redox probes; polysulfides; HOCl; peroxynitrite; glutathione; H_2O_2; nitric oxide

INTRODUCTION

The ability to observe defined intracellular redox changes is of relevance for the understanding of cell and organismal biology. To study both physiological and pathological redox changes, a range of experimental tools, ranging from redox proteomics to chemical and genetically encoded redox probes, has become available [1-3].

One method to optically monitor intracellular redox changes is the use of genetically encoded redox probes based on redox-sensitive green fluorescent protein 2 (roGFP2). In roGFP2, two cysteines sitting on the surface of the GFP beta barrel can form a disulfide bond with each other [4,5]. Disulfide bonding causes a slight conformational change, which influences the location of a proton that moves between the phenoxy group of the fluorophore and an internal glutamate residue [6]. The disulfide bond-induced change in the protonation status of the fluorophore is reflected by a shift in the excitation maximum and thus allows for a ratiometric quantification of the probe's redox state, independent of probe concentration. Importantly, the roGFP2 internal proton relay responsible for the reversible shift in fluorophore protonation is shielded from the protein environment, thus making the probe insensitive to physiologically relevant changes in ambient pH [7].

When expressed inside cells, the thiol-disulfide redox pair of roGFP2 predominantly, if not exclusively, equilibrates with the glutathione (GSH^2/GSSG) redox pair [7,8]. This equilibration, however, is slow and depends on catalysis by endogenous

glutaredoxins [7]. This insight led to the development of Grx1-roGFP2, a composite probe in which human glutaredoxin-1 is translationally fused to roGFP2. The oxidation of Grx1-coupled roGFP2 by glutathione disulfide (GSSG) was found to be at least 100,000 times faster when compared to unfused roGFP2 [9]. Subsequently, the fusion concept was also used to generate a roGFP2-based probe that is highly responsive to hydrogen peroxide. In the roGFP2-Orp1 probe roGFP2 is translationally fused to the yeast thiol peroxidase Orp1, which is highly efficient in reacting with H_2O_2 and in transmitting oxidation to roGFP2 by a thiol-disulfide exchange reaction [10]. The mechanisms, uses and limitations of roGFP2-based probes have been reviewed previously [6,11-13].

Grx1-roGFP2 and roGFP2-Orp1 have been used successfully to determine the glutathione redox potential and changes in H_2O_2 levels, respectively, in various organelles, cell types and model organisms [11]. However, one relevant issue that remains to be fully addressed is the question of whether these probes remain specific for their designated oxidants (GSSG and H_2O_2, respectively) even at very high oxidant concentrations or in the presence of highly reactive oxidants, as they may be formed under special biological circumstances. One such situation is the respiratory burst mounted by activated phagocytes, which is known to produce a wide variety of oxidative and nitrosative species at relatively high concentrations [14]. Since the reactivity of roGFP2-based probes towards some of these highly reactive oxidants, including hypochlorite, has not yet been studied systematically *in vitro*, we aimed to close this gap.

In the present study, we systematically tested and compared the response of recombinant roGFP2, roGFP2-Orp1, and Grx1-roGFP2 to a set of physiologically relevant oxidative and nitrosative agents at defined concentrations of 2 µM and 100 µM *in vitro*. It should be noted that 100 µM is clearly supra-physiological and that even 2 µM is of uncertain physiological relevance for the majority of oxidant species. The highest concentrations actually occurring *in vivo* (under either physiological or pathological conditions) are effectively unknown and remain controversial for almost all oxidant species. Nevertheless, we used these particular concentrations to assess which responses can be imposed upon the probes at high oxidant to probe stoichiometric ratios (10:1 and 500:1). Our results show that roGFP2-Orp1 and Grx1-roGFP2 maintain their discriminatory selectivity for GSSG and H_2O_2, respectively, even at a concentration as high as 100 µM. However, we also show that, in particular, hypochlorous acid and polysulfides can lead to non-selective (i.e. direct) roGFP2 oxidation, at concentrations that may be of physiological relevance under specific physiological or pathological conditions or in specific cell types.

RESULTS

Measurement of the oxidation state of roGFP2-based probes in vitro

To assess and compare the specificity of roGFP2-based sensors *in vitro* we purified recombinant roGFP2, roGFP2-Orp1 and Grx1-roGFP2 from *Escherichia coli*. Freshly purified proteins were reduced with DTT and stored at -80°C until use. In all experiments, we measured the full excitation spectrum (350 - 500 nm) minute-by-minute after addition of the oxidants (Figs. 1-10 in [15]). Subsequently, the ratio of fluorescence intensity at excitation wavelengths 405 and 488 nm, related to the degree of oxidation of the roGFP2 thiol-disulfide redox pair [6], was determined for each individual spectrum. Using this approach, we went on to assess the response of all three probes towards defined oxidants at defined concentrations. The systematic comparison between the three probes allowed deducing which of the probe moieties (roGFP2, Orp1, Grx1) responded to which oxidant at which concentration.

Since oxidants may bleach GFP, especially at high concentrations, we also monitored the excitation spectrum's isosbestic point at 420 nm [4]. A loss in the emission intensity at the excitation wavelength of the isosbestic point indicates a loss of overall fluorescence, i.e. bleaching. In contrast, a shift in the thiol-disulfide state of roGPF2 does not change fluorescence emission at the isosbestic point.

We first measured probe responses under aerobic conditions. To initiate the measurement, we added the recombinant probes under continuous stirring to a final concentration of 0.2 µM to a cuvette filled with buffer. Under these basal conditions roGFP2-Orp1 oxidized within about 20 minutes (**Fig. 1A**, Fig. 1 in [15]), while roGFP2 and Grx1-roGFP2 remained fully reduced during the 60 minute period of

measurement (**Fig. 1B**, **C**; Fig. 1 in [15]). When measured under fully anaerobic conditions, roGFP2-Orp1 remained in a reduced state (**Fig. 1D**; Fig; 1 in [15]). Addition of one volume of aerobic buffer to the anaerobic cuvette, corresponding to a final oxygen concentration of 100 µM, again led to complete probe oxidation after approximately 20 minutes (**Fig. 1E**; Fig. 1 in [15]). This result suggested that under the conditions used in our study, the Orp1 moiety is either oxidized by H_2O_2, potentially present or generated in the buffer system, or by another oxygen-dependent reaction. In contrast, when expressed and measured within *E. coli*, roGFP2-Orp1 remained reduced under aerobic conditions (**Fig. 1F**; Fig. 1 in [15]). We concluded that the pronounced air oxidation of Orp1 observed in our system is an *in vitro* artifact. Generation of H_2O_2 or thiol oxidation itself may be catalyzed by trace amounts of transition metals, which are often found in phosphate buffers. As a consequence, all subsequent measurements of roGFP2-Orp1 were performed under anaerobic conditions, while the other two probes were tested aerobically.

Before testing responses to defined concentrations of oxidants, we established that all three probes showed the expected response to strong thiol-reducing and thiol-oxidizing compounds, which are routinely used to define the fully reduced and fully oxidized reference state of roGPF2-based probes [6]. To this end, DTT was applied to achieve full roGFP2 reduction, and aldrithiol-2 (AT-2, 2,2'-dipyridyldisulfide), a strong thiol-oxidizing agent [16], served to achieve maximal roGFP2 oxidation. All three probes, already fully reduced by DTT treatment following purification, remained reduced after the addition of additional DTT (**Fig. 2A**; Fig. 2 in [15]). All three probes exhibited rapid and complete oxidation after addition of AT-2 at 2 µM. The kinetics of AT-2 induced roGFP2 oxidation was similar for all probes,

confirming that AT-2 directly oxidizes roGFP2 to the disulfide form, independently of fused moieties (**Fig. 2B**; Fig. 2 in [15]). Addition of reduced glutathione (GSH) did not alter the already reduced state of the probes, as expected (**Fig. 2C**; Fig. 2 in [15]).

roGFP2-Orp1 and Grx1-roGFP2 respond to H_2O_2 and GSSG, respectively, with high specificity

First, we tested the response of the three probes to H_2O_2 and GSSG, supplied at a final concentration of 2 µM, which corresponds to a 10-fold molar excess over the probes. As expected, roGFP2-Orp1 was rapidly oxidized by H_2O_2, but not by GSSG. In contrast, Grx1-roGFP2 responded to GSSG, but not to H_2O_2 (**Fig. 3**; Fig. 3 in [15]). Unfused roGFP2 did neither respond to H_2O_2 nor to GSSG within the time frame of the experiment. These results reconfirm that the two fusion probes faithfully discriminate between H_2O_2 and GSSG, at oxidant concentrations of 2 µM, which may be considered rather high in the context of physiological situations. They also reconfirm that the direct reaction between roGFP2 and either H_2O_2 or GSSG is kinetically insignificant at the given concentration and time frame.

roGFP2-Orp1 and Grx1-roGFP2 are easily oxidized by hypochlorous acid

When phagocytes mount a respiratory burst, a set of highly reactive oxygen species is produced, which includes hypochlorous acid (HOCl) [17,18]. Due to the great difficulty of measuring HOCl *in vivo*, the actual concentrations reached in phagocytes during the respiratory burst remain unknown. Estimates suggesting concentrations in the high-µM to low-mM range are based on theoretical calculations [19] and should be taken with caution. In any case, HOCl is a strong oxidant, much less limited by kinetic barriers than for example H_2O_2. Indeed, HOCl is known to cause a variety of

non-specific oxidative protein modifications [20-23]. We thus asked if roGFP2-based probes are non-specifically oxidized by HOCl. Indeed, roGFP2-Orp1 and Grx1-roGFP2 were both oxidized rapidly by the addition of 2 µM HOCl (**Fig. 4A**; Fig. 4 in [15]). Interestingly, however, unfused roGFP2 did not show a response to HOCl at this particular concentration and under the conditions of this experiment. Thus, it seems that the redox-active thiols of the Orp1 and Grx1 moieties are more easily oxidized by HOCl than the thiols of roGFP2.

Probe responses to GSNO and peroxynitrite

S-nitrosoglutathione (GSNO) is an S-nitrosothiol that has been found in a variety of organisms and cell types [24-26]. It is thought to act as an endogenous mediator of protein S-nitrosation and/or S-glutathionylation [27]. We thus tested responsiveness of the three probes to 2 µM GSNO. All three probes showed a slow but significant response to GSNO, and the response of the roGFP2-Orp1 probe was more pronounced (**Fig. 4B**; Fig. 4 in [15]). However, none of the probes reached full oxidation.

Peroxynitrite ($ONOO^-$) possesses oxidizing and nitrosating properties [28]. It forms from the reaction between NO• and superoxide ($O_2^{\bullet-}$) and can be generated during the phagocyte respiratory burst, as well as under various pathological conditions [29-31]. As for most other oxidants, typical or maximal *in vivo* concentrations of $ONOO^-$ remain to be determined. We found that roGFP2-Orp1 responded rapidly to 2 µM $ONOO^-$, while the other two probes were not significantly influenced (**Fig. 4C**; Fig. 4 in [15]). Thus, the thiol peroxidase Orp1 is prone to react with the peroxide $ONOO^-$, in line with the fact that most thiol peroxidases react with $ONOO^-$ [32]. In conclusion,

depending on its *in vivo* concentration, peroxynitrite may also oxidize roGFP2-Orp1 inside cells, thus potentially compromising the specific detection of H_2O_2.

Nitric oxide does not influence the redox state of roGFP2-based probes

Next, we tested the NO• donors spermine NONOate (SperNO) and diethyl amino NONOate (DeaNO). These two NO• donors differ significantly in their half-lives and NO• release kinetics. In contrast to GSNO, NO• should not be able to directly S-nitrosate thiols. Accordingly, none of the probes changed their oxidation state significantly upon addition of 2 µM NO• donors, indicating that NO• does not induce S-nitrosation or thiol oxidation in these probes (**Fig. 4D, E**; Fig. 4 in [15]).

All roGFP2 probes are rapidly oxidized by polysulfides

Hydrogen sulfide (H_2S) is considered a signaling molecule that regulates numerous physiological events [33]. It is increasingly realized that many actions previously attributed to H_2S may actually be mediated by sulfane sulfur species, which include per- and polysulfides [34-36]. Thus, we compared the response of the three probes to polysulfides. As a source for polysulfides we used K_2S_x, which is a mixture of K_2S and polysulfides of varying chain length. Based on our previous measurements, using cyanolysis to determine the concentration of total sulfane sulfur in a K_2S_x solution [34], we used K_2S_x at 0.4 µg mL^{-1}, equivalent to 2 µM polysulfides. All three probes converted rapidly to the oxidized state, confirming our previous observations that polysulfides directly oxidize the redox-sensing thiols of the roGFP2 moiety (**Fig. 4F**; Fig. 4 in [15]).

Response of unfused roGFP2 to very high oxidant concentrations

Using a 10-fold molar excess of oxidant (2 µM) over probe, unfused roGFP2 did not show a significant response to most of the tested oxidants, except for AT-2 and K_2S_x. Considering the theoretical possibility that oxidants may reach ever higher concentrations under special *in vivo* conditions, e.g. in the context of inflammation, we tested the response of roGFP2 to H_2O_2, HOCl, and peroxynitrite at increasingly higher concentrations. For this purpose, the 405/488 nm ratio after 60 minutes was plotted against the oxidant concentration. Unfused roGFP2 reached a maximal 405/488 nm ratio at 20 mM H_2O_2, which corresponds to a 100,000-fold molar excess over the probe (**Fig. 5A**; Fig. 5 in [15]). In stark contrast, 25 µM HOCl (a 125-fold molar excess over the probe) was sufficient to oxidize the probe to the same extent (**Fig. 5B**). At concentrations higher than 37.5 µM, the ratio decreased, due to fluorophore bleaching (Fig. 6 in [15]), also reflected by a decrease in intensity at the isosbestic point. In contrast, H_2O_2 did not cause a loss in overall fluorescence at any concentration (Fig. 5 in [15]). Peroxynitrite added at concentrations above 128 µM (a 640-fold molar excess over the probe) resulted in full oxidation of roGFP2 without notable bleaching (**Fig. 5C**; Fig. 7 in [15]). In conclusion, at extreme non-physiological oxidant concentrations, roGFP2 is directly oxidized.

Response of roGFP2 fusion probes to very high oxidant concentrations

We then asked how the fusion probes roGFP2-Orp1 and Grx1-roGFP2 respond to very high oxidant concentrations. All three probes were treated with 100 µM of oxidizing (**Fig. 6A-C**; Fig 8. in [15]) or nitrosating agents (Fig. **7A-D**; Fig. 9 in [15]), thus applying a 500-fold molar excess. Notably, the roGFP2-Orp1 still allowed the specific detection of H_2O_2 relative to the other two probes (**Fig. 6A**), and the Grx1-roGFP2 probe largely, but not completely, retained specificity (**Fig. 6B**). All three

probes showed a rapid oxidation upon addition of HOCl (**Fig. 6C**), GSNO (**Fig.** 7**A**), and $ONOO^-$ (**Fig. 7B**). Oxidation of roGFP2-Orp1 by HOCl and $ONOO^-$ was slightly more pronounced in comparison to roGFP2 and Grx1-roGFP2. Addition of DeaNO and SperNO led only to minor changes of the oxidation state of the three probes (**Fig. 7C, D**), further supporting the notion that a direct reaction of NO• with thiols is not occurring *in vitro*.

DISCUSSION

The roGFP2-based probes roGFP2-Orp1 and Grx1-roGFP2 have been designed to respond to the oxidants H_2O_2 and GSSG, respectively [9,10]. In these composite probes, the thiol peroxidase (Orp1) and glutaredoxin (Grx1) domains serve as primary sensors and receptors for the oxidant. These transmit the oxidizing equivalent to the attached roGFP2 domain, hence translating a defined oxidation event into a fluorescent response. Thus, in principle, the roGFP2 responses are as sensitive and selective as the oxidant-sensing domains to which they are physically coupled.

While both probes have demonstrated sensitivity and selectivity in a broad variety of model systems and experimental conditions [11], the question arises if there may exist biologically relevant situations under which probe selectivity breaks down. The most obvious candidate for such a situation is the respiratory burst in phagocytes, which is known to generate highly reactive oxidants at relatively high concentrations [17,18]. These in principle may directly oxidize the thiols on roGFP2, thus 'bypassing' the attached oxidant-sensing domain (i.e. Grx or Orp). In such a case the probes would properly report on an oxidation event, yet they would not be able to report on the identity of oxidant.

To this end we compared unfused roGFP2 and the two fusion probes, roGFP2-Orp1 and Grx1-roGFP2, under standardized conditions. We took various measures to exclude non-specific effects or measurement artifacts, i.e. recording of the full excitation spectrum at each time point, monitoring of the isosbestic point and comparison with non-redox-sensitive GFP. We were also careful to perform all

measurements of roGFP2-Orp1 under anaerobic conditions, since we noticed its *in vitro* susceptibility to 'air oxidation'.

First we asked the question if roGFP2-Orp1 and Grx1-roGFP2 retain their specificity at very high H_2O_2 and GSSG concentrations. As expected, roGFP2-Orp1 and Grx1-roGFP2 exhibited high specificity towards H_2O_2 and GSSG when the oxidants were applied at a moderate concentration of 2 µM (**Fig. 3**). But also at a concentration of 100 µM H_2O_2, which is far beyond the expected intracellular concentration range, roGFP2-Orp1 responded in a highly selective manner relative to the other two probes (**Fig. 6**). The lack of roGFP2 and Grx1-roGFP2 responsiveness to even 100 µM H_2O_2 confirms the sluggishness of H_2O_2-dependent thiol oxidation in the absence of a dedicated catalyst. Indeed, our titration experiments revealed that millimolar amounts of H_2O_2 are needed to oxidize unfused roGFP2 to a similar extent as roGFP2-Orp1 and within a similar time frame (**Fig. 5**).

Grx1-roGFP2 maintained much of its selectivity (**Fig. 6**) despite the fact that 100 µM GSSG was applied without the backdrop of a millimolar concentration of reduced glutathione typical of cellular environments. Thus, in conclusion, the kinetic differences between unfused and fused roGFP2 probes are hard to 'override' even by increasing the concentration of the oxidants far beyond physiological levels.

Next we asked how the probes react to other oxidants that may be formed within biological settings (**Fig. 4**). HOCl was of particular interest as it is produced by myeloperoxidase during the phagocyte respiratory burst, potentially reaching relatively high concentrations [37,38]. HOCl is known to react with thiols very

efficiently, with rate constants close to 3 x 10^7 M^{-1} s^{-1} [39-41]. Indeed, we observed that both roGFP2-Orp1 and Grx1-roGFP2 were rapidly oxidized by 2 µM HOCl. Interestingly, however, 2 µM HOCl was not sufficient to oxidize unfused roGFP2, indicating that the cysteines of Orp1 and Grx1 are more sensitive to oxidation under these conditions. Nevertheless, only slightly higher HOCl concentrations also oxidized unfused roGFP2, which was fully oxidized at a concentration of 20 µM HOCl (**Fig. 5**).

Another oxidant of particular interest is peroxynitrite. Its formation is especially relevant in phagocytic immune cells, which are known to produce peroxynitrite in relatively large quantities [42,43]. Peroxynitrite oxidizes cysteine thiols with a rate constant approaching 3 x 10^3 M^{-1} s^{-1} [44], thus it is much less reactive than HOCl, yet more reactive that H_2O_2. At a concentration of 2 µM, peroxynitrite oxidized roGFP2-Orp1, but not roGFP2 or Grx1-roGFP2. This result shows that peroxynitrite is a substrate for the thiol peroxidase domain of roGFP2-Orp1. Thus, like other thiol peroxidases, including peroxiredoxins, Orp1 is not strictly specific to the smallest peroxide H_2O_2, but can be oxidized by other peroxides, including peroxynitrite. At a concentration of 100 µM $ONOO^-$, uncatalyzed thiol reactivity became relevant, as all three probes were oxidized.

Another oxidant of potential biological relevance is GSNO. At 2 µM it induced partial oxidation of all three probes, suggesting that trans-nitrosation of the roGFP2 thiols can induce disulfide bond formation. In contrast, donors of NO• did not have any significant influence on the probes.

Finally, per- and polysulfides are increasingly implicated in H_2S-mediated cytoprotection and redox signaling [34,45].In particular, persulfides are known to be created inside mitochondria by sulfide:quinone oxidoreductase (SQR) [35,46]. In agreement with our previous results [34], all three probes were rapidly and efficiently oxidized by K_2S_x.

The transferability of our *in vitro* results to the *in cellulo* or *in vivo* situation is necessarily limited. On the one hand, typical and maximal *in vivo* concentrations are uncertain for all oxidants. Values reported in the literature cover a wide range. For example, intracellular H_2O_2 concentrations may be restricted to the pico- and nanomolar range [47,48], but are sometimes considered to reach concentrations as high as 2 µM [17,49]. On the other hand, not all probe responses seen *in vitro* may also occur *in vivo*, given kinetic competition between the probe and various antioxidative systems and between different oxidants for reacting with the probe. Oxidation reactions that occur *in vitro* may be outcompeted kinetically by more favorable reactions available *in vivo*. These uncertainties notwithstanding, we determined the reactivity of the roGFP2-based probes towards different oxidants at concentrations of 2 µM and 100 µM *in vitro*. This approach simply shows what is chemically possible when purified probe proteins encounter defined oxidants at defined concentrations in defined molar ratios. Our results may thus serve as a reference, to further investigate potential probe responses in cells and *in vivo*.

CONCLUSION

Having compared the *in vitro* response of three different roGFP2-based probes to

various candidate oxidants at concentrations of 2 µM and 100 µM, we conclude that the previously established oxidant selectivity is very likely to be maintained under most physiological conditions. However, there are at least two possible scenarios that probe users should be aware of. First, probes expressed in phagocytes or -more generally- in inflamed tissues may be exposed to highly reactive oxidants, in particular hypochlorous acid, which, depending on its actual concentration, may lead to indiscriminant roGFP2 oxidation. Within the same context, it must be kept in mind that peroxynitrite has the potential to cause roGFP2-Orp1 responses. Second, the intracellular formation of per- or polysulfides may trigger roGFP2 responses. However, the experimental relevance of these scenarios remains uncertain until confirmed in a biological context. We recommend that users of roGFP2-based probes compare the response of all three probes when investigating a biological context in which the generation of hypochlorous acid or polysulfides can be expected. If all probes respond similarly, then highly reactive species like HOCl or polysulfides are likely to be involved in the phenomenon under study.

MATERIALS AND METHODS

Preparation and quantification of compounds

H_2O_2, NaOCl, DTT, AT-2, GSH, and GSSG were purchased from Sigma-Aldrich (Darmstadt, Germany). The concentration of the HOCl stock solution was determined with a JASCO V-650 spectrophotometer (JASCO, Darmstadt, Germany) ($_{292}$= 350 $M^{-1}\cdot cm^{-1}$). DeaNO, SperNO, GSNO, and $ONOO^-$ were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Stock solutions of approximately 2 mg mL^{-1} of DeaNO and SperNO in 0.01 M NaOH and 2 mg mL^{-1} of GSNO in 0.3 M NaOH were prepared freshly on a daily basis. The exact concentrations of SperNO, DeaNO, GSNO, and $ONOO^-$ were determined with a JASCO V-650 spectrophotometer using the following extinction coefficients as recommended by the manufacturer or elsewhere as referenced: ε_{250}= 6,500 $M^{-1}\cdot cm^{-1}$ (DeaNO); ε_{252}= 8,000 $M^{-1}\cdot cm^{-1}$ (SperNO); ε_{335}= 920 $M^{-1}\cdot cm^{-1}$ (GSNO) [50]; ε_{302}= 1,670 $M^{-1}\cdot cm^{-1}$ ($ONOO^-$). Dilutions of all stressors for probe treatment were done as describe below. Upon dilution of SperNO and DeaNO in aquaeous solutions, decomposition and NO release initiates instantly with half-lives of 2 mins (DeaNO) and 39 mins (SperNO) at pH 7.4 and 37 °C, respectively [51]. The decomposition of DeaNO at 22-25 °C as used in our study has been described as 16 mins, while the half-live of SperNO at room temperature has, to our knowledge, not been reported yet.

Cloning of roGFP2-based probes

Vectors pQE60_roGFP2-orp1-his and pQE60_grx1_roGFP2-his were modified by quick change PCR using primers orp1-QC_fw, orp1-QC_rv, grx1-QC_fw, and grx-QC_rv, in order to remove the internal EcoRI site for further cloning steps (Table 1;

Table 2). Subsequently, the coding regions of roGFP2-orp1 and grx1-roGFP1 were PCR amplified using primer pairs Orp1-His-fw/Grx-Orp1-His-rv and Grx1-His-fw/Grx-Orp1-His-rv. PCR products were subjected to restriction with NdeI and EcoRI and subcloned into equally cut pCC. The coding region of roGFP2 was amplified using template pEQ60_grx1_roGFP2-his-QC and primers roGFP2-fw/roGFP2-rv. The PCR product was subcloned into pCC after restriction with NdeI and EcoRI. Constructs were verified by sequencing.

Overexpression and purification of roGFP2-Orp1-His, Grx1-roGFP2-His, and roGFP2

Plasmids pCC_roGFP2-orp1-His, and pCC_grx1-roGFP2-His were transformed into *Escherichia coli* MG1655. A single colony was used to inoculate 50 mL LB medium containing 100 µg mL^{-1} ampicillin. Cells were grown over night at 37 °C at 125 rpm. The complete culture was used to inoculate 5 Liters LB medium with ampicillin. Cultures were grown until OD_{600} of 0.5 was reached. Overexpression was induced by the addition of 0.5 mM IPTG. Cultures were shifted to 20 °C and incubated over night at 125 rpm. Cells were harvested by centrifugation for 45 min at 4 °C and 6,000 rpm. The pellet was resuspended in 100 mL buffer A (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 10 mM imidazole). Two tablets of cOmplete protease inhibitor cocktail tablets (Roche, Basel, Switzerland) were added prior passaging the cell suspension three times through a TS 0.75 constant cell disruption system at 1.9 kbar and 4 °C (Constant Systems Ltd., Daventry, UK). Lysates were cleared by centrifugation at 6,700 g at 4 °C for 1 h followed by filtration through a 0.2 µm filter. The filtrate was loaded on a Ni-NTA column preequilibrated with buffer A, which was run on a Äkta purifier FPLC system (GE healthcare, Buckinghamshire, UK). The column was

washed with buffer A, followed by 2 % of buffer B (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 500 mM imidazole). Proteins were eluted with a 2-100 % gradient of buffer B at 3 mL fractions with a flow rate of 3.5 mL min^{-1}. Fractions were analyzed by SDS-PAGE. Fractions containing considerable amounts of protein were pooled and dialyzed against buffer C (25 mM HEPES/KOH, 40 mM NaCl, pH 7.3) over night at 4 °C.

Overexpression of roGFP2 and cell harvest was essentially performed as described above for roGFP2-Orp1-His and Grx1-roGFP2-His. The pellet was resuspended in buffer QA (40 mM HEPES/KOH, 40 mM KCl, pH 7.5). After cell lysis, centrifugation, and filtration, the lysate was added onto an 8 x 5 mL HiTrap Q FF column (GE Healthcare) preequilibrated with buffer QA. Protein separation was performed with an 800 mL gradient of 0-100 % buffer QA/buffer QB (40 mM HEPES/KOH, 700 mM KCl, pH 7.5) at a flow rate of 3.0 mL min^{-1} and 3 mL fraction volume. Fractions were analyzed for the presence of roGFP2 by SDS-PAGE and pooled accordingly. DTT was added to the pooled fractions to a final concentration of 2 mM prior to over night dialysis against 5 Liters buffer HA (5 mM potassium phosphate, 2 mM DTT, pH 6.8) at 4 °C. The protein solution was centrifuged at 6,000 rpm for 15 min at 4 °C and loaded onto a self-packed hydroxyapatite column (25 mL column volume) preequilibrated with buffer HA. Fractions containing high amounts of roGFP2 and low amounts of other proteins were pooled, concentrated and rebuffered in buffer GA (40 mM potassium phosphate, 200 mM KCl, 2 mM DTT, pH 7.5). Gelfiltration was performed using a superdex 75 60/600 gelfiltration column (GE Healthcare) at a flow rate of 2.5 mL min^{-1}. Proteins were eluted at a fraction size of 3 mL. Fractions containing highly pure roGFP2 were combined, further

concentrated, and rebuffered into buffer S (50 mM sodium phosphate, 150 mM NaCl, pH 8.0).

Measurement of the redox state of roGFP2, roGFP2-Orp1, and Grx1-roGFP2

The probes were reduced with 20 mM DTT for 30 min at room temperature. DTT was removed using illustra Nap5 columns (GE Healthcare) prior to determination of the protein concentration. All protein solutions were adjusted to 1 mM. Aliquots were stored at -80 °C until usage. Fluorescence measurements were performed in a JASCO FP-8500 fluorescence spectrometer equipped with a Peltier thermo-holder 'EHC-813' at 20 °C for 60 min under continous stirring. Proteins were added to degassed buffer F (PBS, 5 mM EDTA, pH 7.4) at a final concentration of 0.2 µM. Measurement parameters were set to 510 nm (Em), 350-500 nm (Ex), 5 nm slit width (Ex/Em) and medium sensitivity. Spectra were recorded in 1 min steps. Stressors were diluted in buffer F (H_2O_2, HOCl, DTT), DMSO (AT-2), PBS, 5 mM EDTA, pH 5.0 (GSH, GSSG), 10 mM NaOH (DeaNO, SperNO), 100 mM NaOH ($ONOO^-$) and added to a final concentration of 2 µM to 20 mM after the first measurement. Measurements of roGFP2-Orp1 were performed under anaerobic conditions to prevent air oxidation. roGFP2-Orp1 was added to anaerobic buffer F in a 3.5 mL quartz cuvette with a septum-containing, screwable lid to a final concentration of 0.2 µM in an anaerobic chamber. Stressors (2 or 100 µM) in anaerobic buffer were added to the cuvette through a gas-tight syringe connected to the cuvette via a scalp vein set. Calculation of the 405/488 nm ratios and standard deviations (**Figs. 1-7**) was done based on two independent measurements. A representative measurement including the raw data of these spectra can be found in our associated article [15].

Measurement of the redox state of roGFP2-Orp1 expressed in E. coli

Overexpression of roGFP2-Orp1 in MG1655 was performed as described above. The cell density was determined and a volume corresponding to an OD_{600} of 0.5 in 1 mL was harvested and washed twice with PBS. Cells were transfered to a quartz cuvette and fluorescence measurements were performed as described above for the purified probes. A representative measurement of the spectra is shown in Figure 1 in [15].

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Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source of Reference
Strains		
E. coli XL1 blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*	Stratagene
E. coli MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	[52]
Plasmids		
pQE60_roGFP2-orp1-his	pQE60 carrying *roGFP2-orp1*-his_6; pT5-*lac* promoter	[10]
pEQ60_grx1_roGFP2-his	pQE60 carrying grx1-*roGFP2*-$his_{6;}$ pT5-*lac* promoter	[9]
pQE60_roGFP2-orp1-his-QC	pQE60 carrying *roGFP2-orp1*-his_6; removed EcoRI site; pT5-*lac* promoter	This work
pEQ60_grx1_roGFP2-his-QC	pQE60 carrying grx1-*roGFP2*-his_6; removed EcoRI site; pT5-*lac*	This work
pCC	TAC-MAT-Tag-2 derivative; p*tac*	Lab collection
pCC_roGFP2	*roGFP2*; p*tac*	This work
pCC_roGFP2-orp1-His	*roGFP2-orp1* with C-terminal his_6-tag; p*tac*	This work
pCC_grx1-roGFP2-His	*grx1-roGFP2* with C-terminal his_6-tag; p*tac*	This work

Table 2: Primers

Primer name	Sequence
orp1-QC_fw	TCAGGAGGAGAATTTGATATCTCAGAATTTTATAAGCTAGCACC
orp1-QC_rv	GGTGCTAGCTTATAAAATTCTGAGATATCAAATTCTCCTCCTGA
grx1-QC_fw	GATCAGGAGGAGGAGGATCAGGAGGAGAGTTCGT
grx-QC_rv	GAACAGCTCCTCGCCCTTGCTCACGAACTCTCC
Orp1-His-fw	CCCCCCATATGGTGAGCAAGGGCGAGGA
Grx1-His-fw	CCCCCCATATGGCTCAAGAGTTTGTGAAC
Grx1-Orp1-His-rv	GGGGGGAATTCTTAGTGATGGTGATGGTG
roGFP2-fw	AACCCCATATGGTGAGCAAGGGCGAGGA
roGFP2-rv	GGGGGGAATTCTTACTTGTACAGCTCGTC

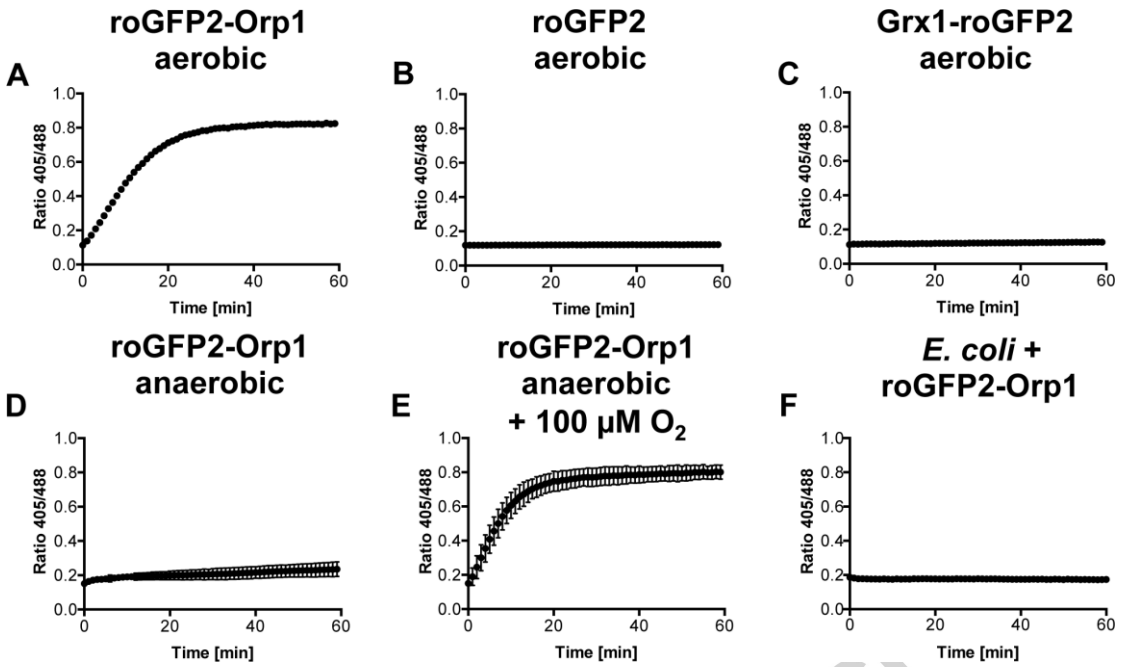

FIG 1. Oxygen sensitivity of roGFP2-based probes. Emission of the probes at 510 nm was measured every minute in the excitation wavelength range of 350 to 500 nm (Fig. 1 in our associated article [15]). The emission values at 405 and 488 nm were used to calculate the 405/488 nm ratios of two independent replicates. Shown are the mean ratios and standard deviations plotted against time. **A** DTT-reduced, purified roGFP2-Orp1 was added to PBS buffer at a final concentration of 0.2 µM under fully aerobic conditions. The probe rapidly oxidized within approximately 20 minutes. **B** roGFP2 was measured under aerobic conditions and did not show any autooxidation. **C** Grx1-roGFP2 was not prone to autooxidation under aerobic conditions. **D** roGFP2-Orp1 measured under strictly anaerobic conditions remained reduced for a total of 60 minutes. **E** Emission of the probe was measured under anaerobic conditions. After one measurement cycle, a buffer volume corresponding to a final oxygen concentration of 100 µM was added. **F** roGFP2-Orp1 was expressed in *E. coli*. Emission spectra were recorded under the same aerobic conditions as in A. *In vivo*, roGFP2-Orp did not show any autooxidation induced by the presence of air.

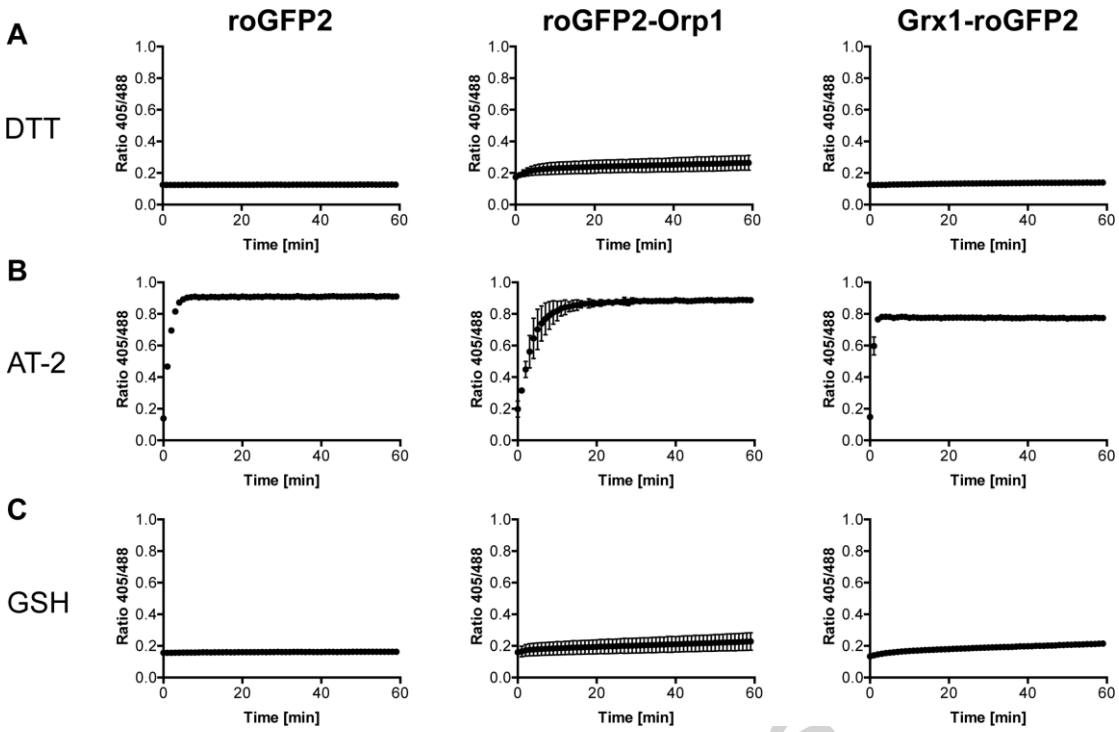

FIG 2. Response of roGFP2-based probes to DTT, AT-2 and GSH. The purified proteins were tested for their responsiveness to a 10-fold molar excess of DTT (**A**), AT-2 (**B**), and GSH (**C**). Emission at 510 nm was measured in the excitation wavelength range of 350 to 500 nm. All compounds were added to a final concentration of 2 µM after recording the first spectrum. Mean ratios and standard deviations (error bars) were calculated from the fluorescence emission at 405 and 488 nm of two independent replicates for a total of 60 minutes (Fig. 2 in our associated article [15]). The probes remained reduced after the addition of DTT and GSH. AT-2, in contrast, induced rapid oxidation of all three probes.

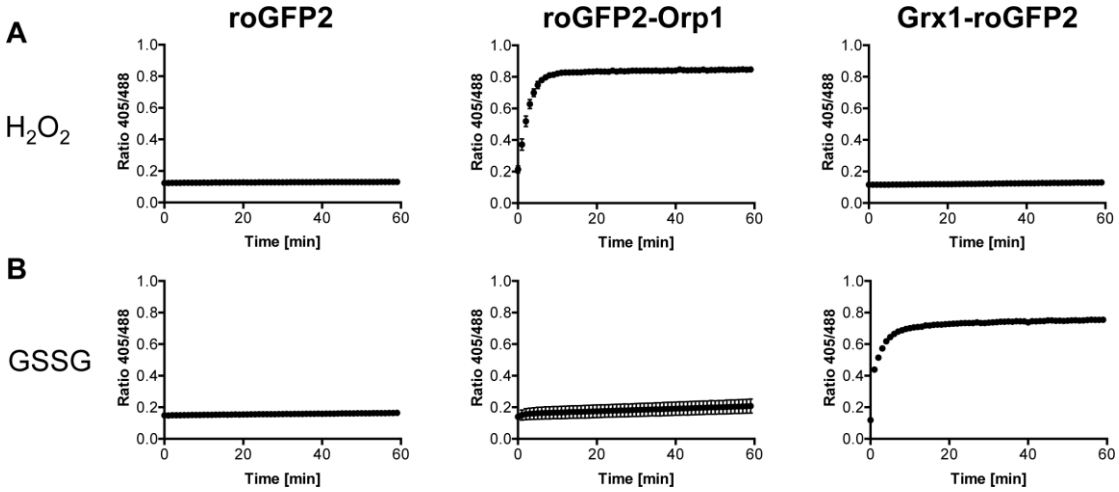

FIG 3. Response of roGFP2-based probes to H_2O_2 and GSSG. For each probe and condition, a total of 60 fluorescence excitation spectra in the wavelength range of 350 to 500 nm were recorded for 60 minutes. Oxidants were added to a final concentration of 2 µM after the first spectrum measurement. A representative spectrum for each measurement is shown in Fig. 3 in our associated article [15]. Mean ratios at 405/488 nm and standard deviations (error bars) were calculated from two independent measurements. roGFP2-Orp1 specifically and rapidly reacted to the addition of a 10-fold molar excess of H_2O_2 (**A**), while roGFP2 and Grx1-roGFP2 remained reduced. In contrast, only Grx1-roGFP2 reported the presence of GSSG (**B**), indicating high specificity and sensitivity of the two hybrid probes for these two compounds.

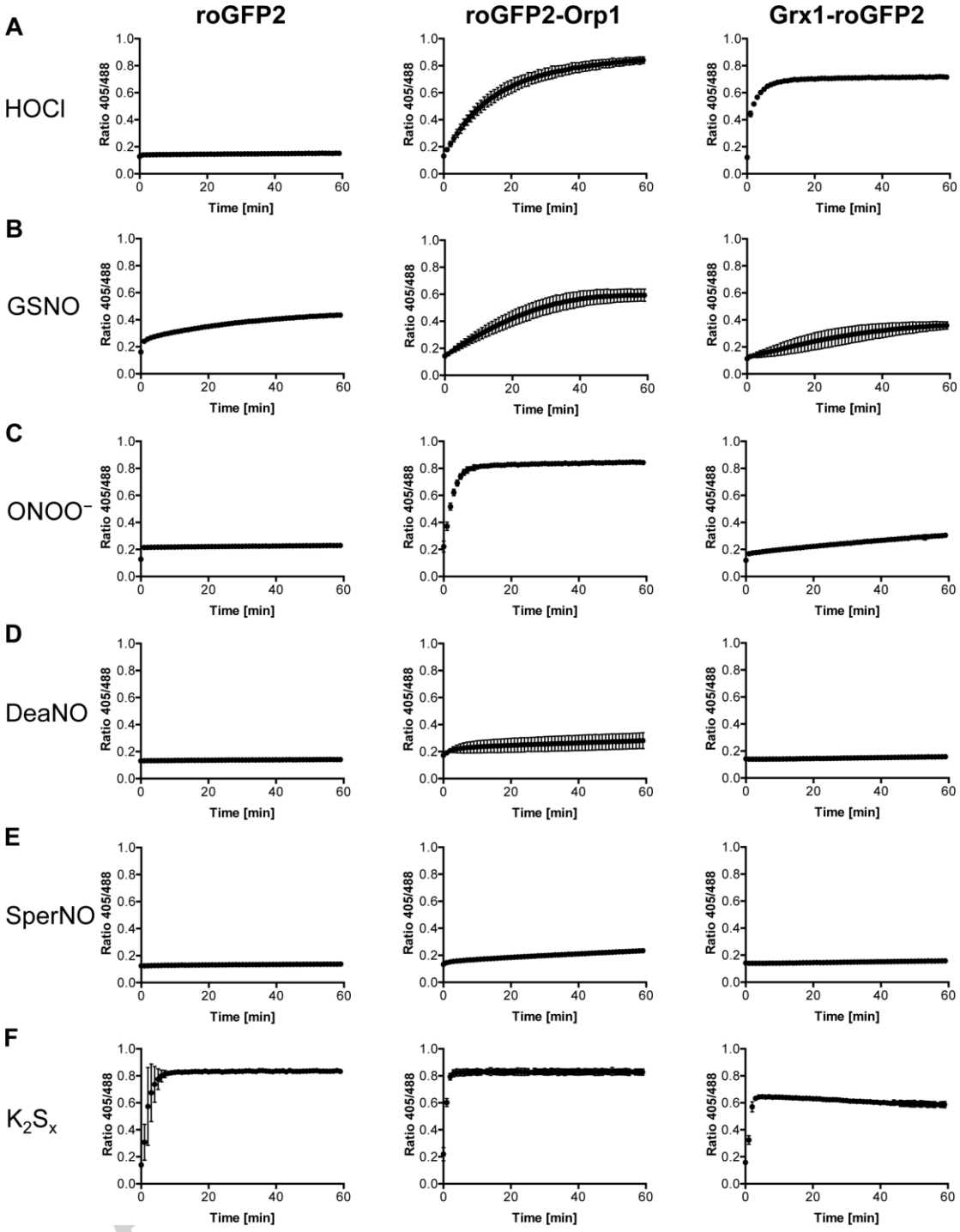

FIG 4. Responses of roGFP2-based probes to various oxidative and nitrosative species. All three probes were tested for their response to a 10-fold molar excess of the indicated stressors. For each oxidant and probe, a series of 60 fluorescence excitation spectra in the range of 350 to 500 nm with one spectrum each minute was recorded (a representative measurement is shown in Fig. 4 of our associated article

[15]). Oxidants were added to a final concentration of 2 µM after the first measurement. The mean ratios at 405/488 nm and standard deviations (error bars) shown here were calculated from two independent measurements. While the probes differed in their response to HOCl (**A**), GSNO (**B**), $ONOO^-$ (**C**), and K_2S_x (**F**), none of them showed a response to the presence of DeaNO (**D**) and SperNO (**E**).

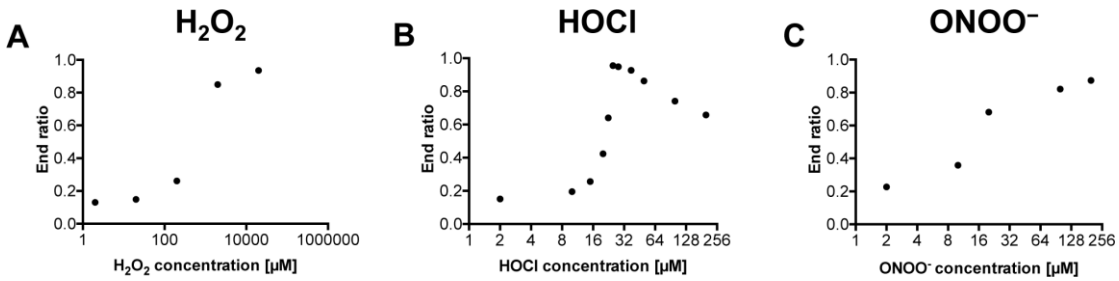

FIG 5. Response of unfused roGFP2 to increasing concentrations of HOCl, H_2O_2, and $ONOO^-$. Unfused roGFP2 was treated with the indicated oxidants at the indicated concentrations. For each oxidant concentration (each data point), a 60-minute measurement with one spectrum each minute in the range of 350-500 nm was recorded (data are shown in Figs. 5-7 in our associated article [15]). Oxidants were added after the first measurement. End ratios were calculated by dividing the emission values at 405 nm and 488 nm after 60 minutes of measurement. At concentrations high enough, roGFP2 clearly becomes oxidized in the presence of H_2O_2 (**A**), HOCl (**B**), and $ONOO^-$ (**C**).

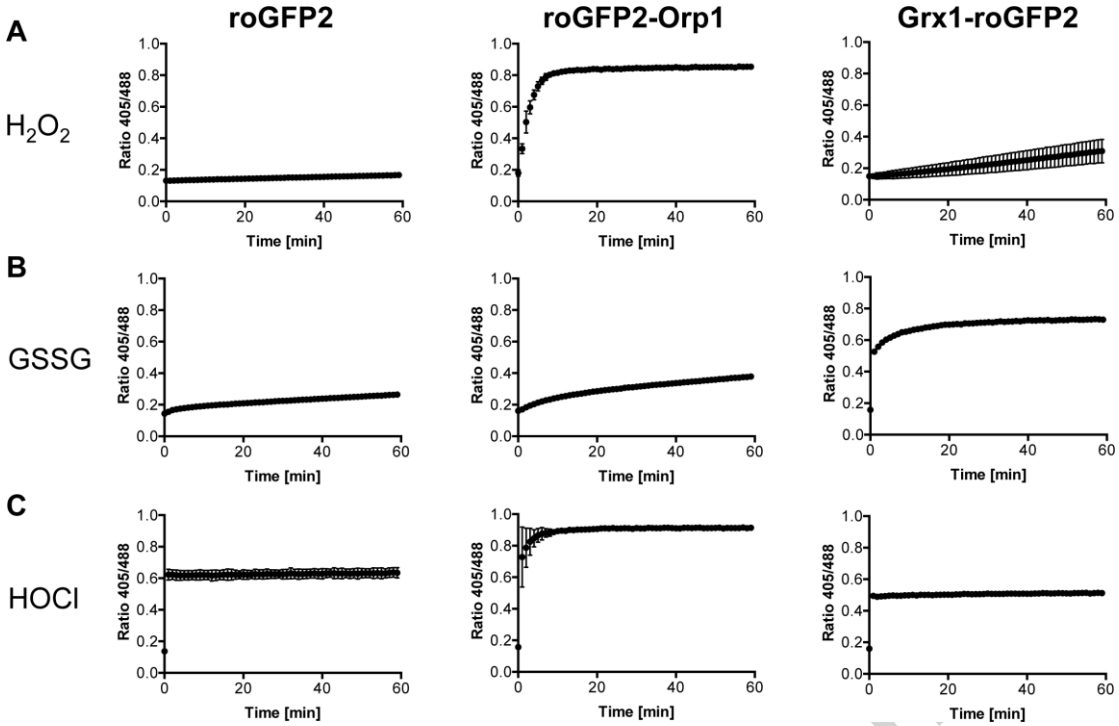

FIG 6. Response of roGFP2-based probes to 100 µM of H_2O_2, GSSG and HOCl. Response of the three probes to 100 µM of the indicated oxidants was analyzed. For each experiment, 60 spectra in a time frame of 60 minutes were recorded (data are shown in Fig. 8 in our associated article [15]). Ratios and standard deviations (error bars) were calculated based on the emission at 405 and 488 nm at every single time point from two independent measurements. **A** Even at 100 µM, Grx1-roGFP2 did not show a considerable response to H_2O_2. **B** Addition of GSSG led to only a slight increase in the 405/488 nm ratios for roGFP2-Orp1 and roGFP2. **C** All three probes were oxidized in the presence of 100 µM HOCl. However, the final 405/488 nm ratios differed significantly.

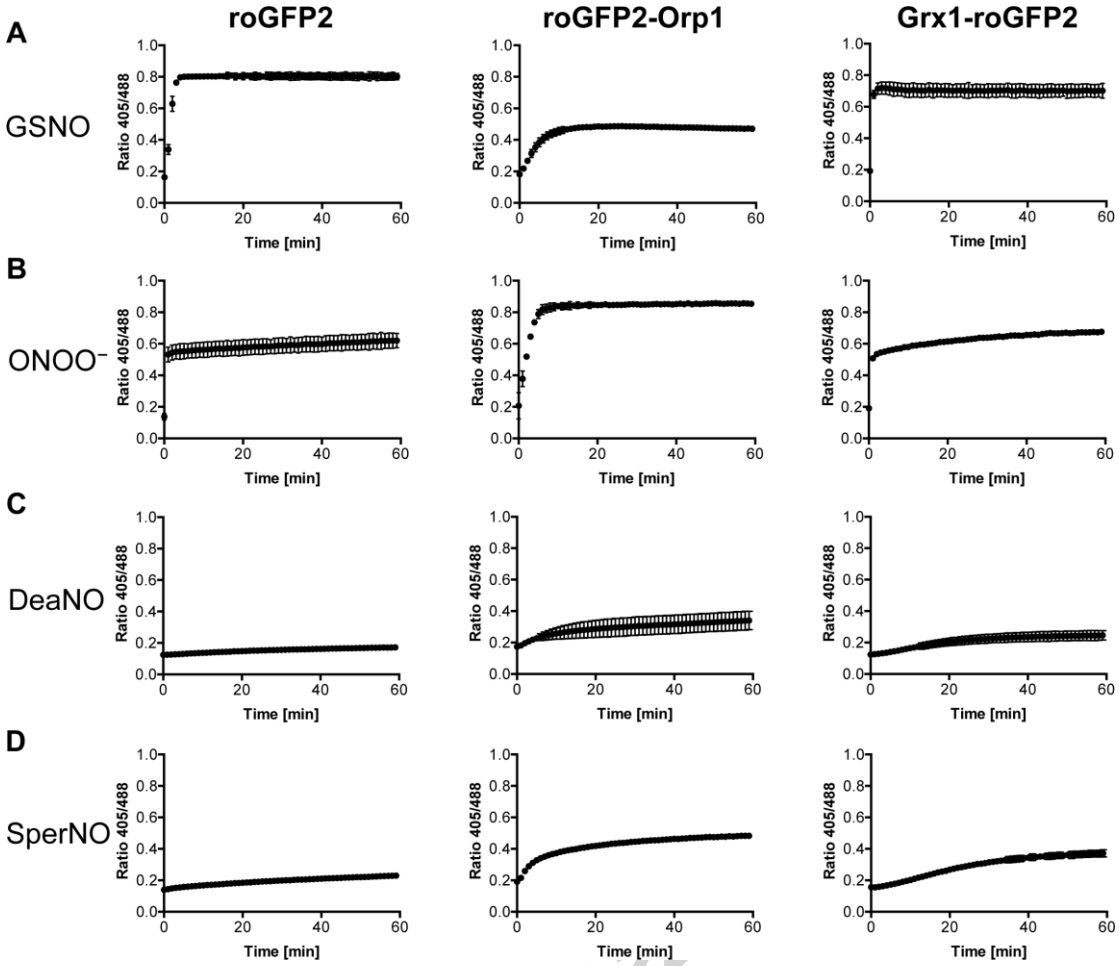

FIG 7. All roGFP2-based probes are oxidized by 100 µM of GSNO or $ONOO^-$. Behavior of the probes in the presence of 100 µM of GSNO (**A**), $ONOO^-$ (**B**), DeaNO (**C**), and SperNO (**D**) was tested. Each graph is based on a total of 60 spectra in the excitation wavelength range of 350 to 500 nm recorded in a time series of 60 minutes (data are shown in Fig. 9 in our associated article [15]). Oxidants were added after the first spectrum. Each spectrum was used to calculate the ratio of 405/488 nm at the given time point. Data points represent the mean values and standard deviations (error bars) of two independent measurements. While there were only minor changes of the oxidation states in the presence of DeaNO and SperNO, the three probes were rapidly oxidized in the presence of GSNO and $ONOO^-$. The maximal 405/488 nm ratio however, differed between roGFP2-Orp1 and the other two probes roGFP2 and Grx1-roGFP2.

Highlights:

- Comparison of the *in vitro* response of 3 roGFP2-based probes to a panel of oxidants
- The probes' selectivity is maintained in the absence of highly reactive species
- HOCl and polysulfides lead to unspecific probe oxidation
- Peroxynitrite oxidizes roGFP2-Orp1 with kinetics comparable to H_2O_2
- Similar responses of probes indicate the presence of highly reactive species