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A FRET-Based Approach to Ratiometric Fluorescence Detection of Hydrogen Peroxide

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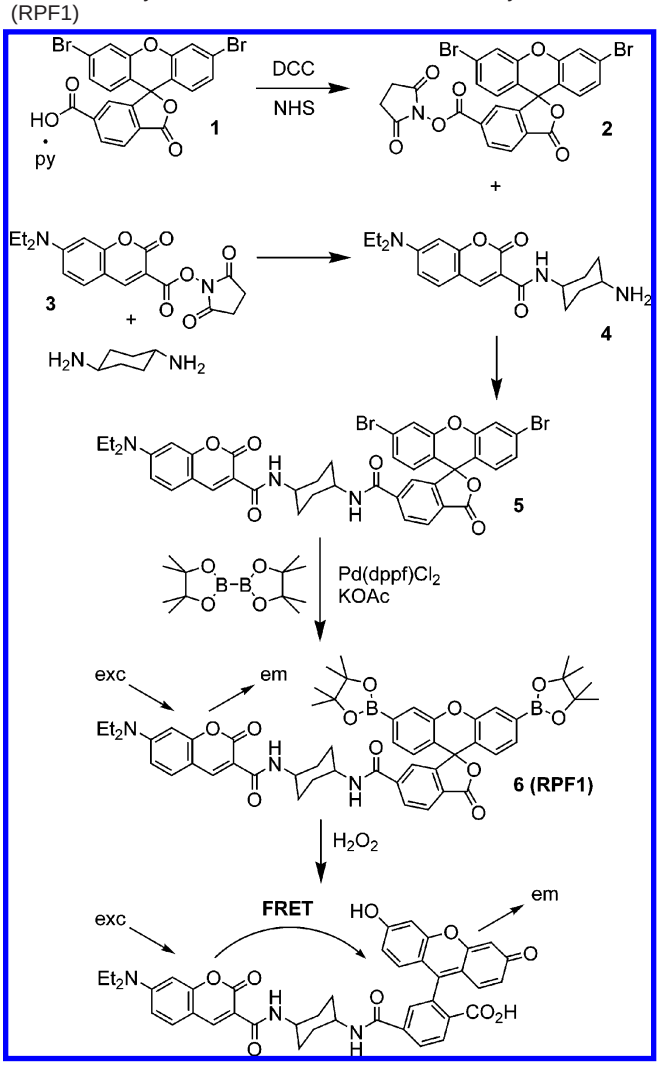
Hydrogen peroxide is an essential oxygen metabolite in living systems, and mounting evidence supports its role as a messenger in cellular signal transduction.[1,2] This byproduct of aerobic respiration is far from benign, however, as overproduction of H_2O_2 and other reactive oxygen species (ROS) from the mitochondrial electron transport chain leads to oxidative stress and the subsequent functional decline of organ systems.[3] Accumulation of oxidative damage over time is connected to debilitating human diseases where age is a risk factor, including Alzheimer's and related neurodegenerative diseases,[4] as well as cardiovascular disorders[5] and cancer.[6]

The far-ranging impacts of H_2O_2 homeostasis on human health and disease provide motivation to devise new diagnostic methods for detecting and quantifying its production from endogenous sources. Synthetic fluorescent probes offer one approach to this goal, and reagents that respond to H_2O_2 by an emission increase have been reported recently.[7−14] These intensity-based probes are of practical value, but their application toward quantitative measurements of changes in H_2O_2 concentrations in heterogeneous biological samples can be complicated by variations in excitation intensity, emission collection efficiency, sample thickness, and/or probe concentration and environment. Ratiometric probes that afford simultaneous recording of two measurable signals in the presence and absence of analyte minimize these factors and can, in principle, allow for accurate and quantitative readouts.[15] To this end, we now present the synthesis and properties of Ratio-Peroxyfluor-1 (RPF1), a new ratiometric fluorescent reporter for H_2O_2. RPF1 features good selectivity for H_2O_2 over other ROS in water, visible wavelength excitation and emission profiles, and is capable of detecting endogenous H_2O_2 production from viable mitochondria.

Our strategy for ratiometric detection of H_2O_2 is based on modulating fluorescence resonance energy transfer (FRET) in a two-fluorophore cassette comprised of a coumarin donor and a boronate-protected fluorescein acceptor linked by a rigid spacer. This approach to controlling FRET by electronic spectral overlap as opposed to modulating the physical separation of donor and acceptor dyes is inspired by optical reporters of phosphatase activity.[16] In the absence of H_2O_2, the boronate protecting groups force the acceptor to adopt a closed, colorless, and nonfluorescent lactone form. Spectral overlap between coumarin emission and fluorescein absorption is minimized, FRET is suppressed, and only blue donor emission is observed upon excitation of the coumarin chromophore. Upon selective reaction with H_2O_2 to generate the open, colored, and fluorescent fluorescein moiety, the acceptor shows a strong absorption in the coumarin emission region. Spectral overlap is enhanced, and excitation of the donor coumarin chromophore results in increased green fluorescein acceptor emission by FRET. Changes in $[H_2O_2]$ can be detected by measuring the ratio of blue and green fluorescence intensities. Scheme 1 outlines the synthesis and proposed activation of RPF1 based on this design.

Scheme 1. Synthesis and Activation of Ratio-Peroxyfluor-1 (RPF1)



RPF1 was evaluated under simulated physiological conditions (DPBS buffer, 1% FBS, pH 7.4). As expected, the optical properties of the parent cassette are dominated by the coumarin chromophore. RPF1 displays a single absorption band in the visible region centered at 420 nm, with blue-colored fluorescence from a corresponding emission band centered at 464 nm (Figure 1a). The spectral data are consistent with minimal FRET from the coumarin donor to the closed, colorless fluoran acceptor. Upon treatment with H_2O_2, excitation at 420 nm produces a bright green-colored fluorescence. The resulting emission spectrum possesses one major band centered at 517 nm with a minor band at 461 nm, consistent with increased FRET from the coumarin donor to the open, colored fluorescein acceptor (Figure 1a). The fluorescence response is accompanied by concomitant growth of a visible wavelength absorption band

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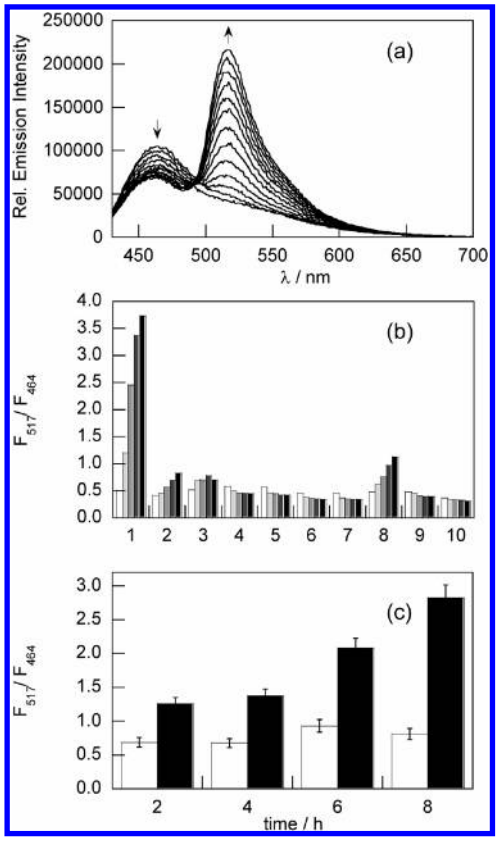

Figure 1. (a) Ratiometric fluorescence response of 1 μM RPF1 to 200 μM H_2O_2. Spectra shown were acquired before H_2O_2 addition and 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min after H_2O_2 was added. (b) Relative reactivities of 1 μM RPF1 to various ROS. (1) H_2O_2; (2) *tert*-butyl hydroperoxide (TBHP); (3) O_2^-; (4) NO; (5) NO^+; (6) •OH; (7) •O^{t}Bu; (8) OCl^-; (9) O_3; (10) $^1O_2$. Data shown are for 10 mM O_2^-, 2 mM $^1O_2$, and 200 μM for all other ROS. Bars represent emission intensity ratios at 5 (white), 15 (light gray), 30 (gray), 45 (dark gray), and 60 min (black) after addition of the appropriate ROS. (c) Fluorometric analysis of H_2O_2 produced by viable yeast mitochondria using 1 μM RPF1. Bars show emission intensity ratios for untreated control mitochondria (white) and mitochondria stimulated with the cytochrome bc_1 inhibitor antimycin A (0.54 mg/mL) to disrupt the electron transport chain. All measurements were acquired in DPBS with 1% FBS, pH 7.4, with excitation at 420 nm.

characteristic of fluorescein, and high-resolution mass spectrometry confirms that a pendant fluorescein is generated from the reaction between RPF1 and H_2O_2. The ratio of fluorescein- to coumarin-type emission intensities ($\lambda_{517}/\lambda_{464}$) upon excitation at 420 nm varies from 0.45 in the absence of H_2O_2 to 3.7 after H_2O_2 treatment after 1 h, a ca. 8-fold emission ratio increase due to FRET modulation. The observed rate constant for H_2O_2-mediated deprotection of RPF1 under pseudo-first-order conditions (1 μM dye, 1 mM H_2O_2) is $k_{obs} = 2.7(1) \times 10^{-4}$ s^{-1}. Although the reaction of RPF1 with peroxide is not rapid, its relatively large dynamic range allows this probe to detect the low micromolar levels of H_2O_2 required for cellular signaling.[17]

The ratiometric emission response of RPF1 is highly H_2O_2 selective. Figure 1b shows the fluorescence responses of RPF1 to various ROS over time. The FRET-based probe shows a >5-fold higher emission ratio response to H_2O_2 over similar ROS, such as superoxide (O_2^-) or *tert*-butyl hydroperoxide (TBHP). RPF1 is also >8-fold more responsive to H_2O_2 than *S*-nitrosocysteine (SNOC), an NO^+ donor in vitro, as well as nitric oxide gas (NO). The coumarin−fluorescein cassette is also >8-fold more selective for H_2O_2 over highly oxidizing ROS, such as hydroxyl radical (•OH), *tert*-butoxy radical (•O^{t}Bu), singlet oxygen ($^1O_2$), and ozone (O_3). Finally, RPF1 is >3-fold more reactive toward H_2O_2 than to hypochlorite ion (−OCl).

Our next goal was to evaluate the ability of RPF1 to detect endogenous production of H_2O_2 from living biological samples. Assays employed purified mitochondria from *Saccharomyces cerevisiae*. The yeast mitochondria were stimulated with antimycin A (0.54 mg/mL), a cytochrome bc_1 inhibitor,[18] to trigger generation of H_2O_2 and other ROS by uncoupling of the respiratory electron transport chain. Samples were treated with antimycin A for various times and analyzed directly with RPF1. The ratiometric emission data collected in Figure 1c show clear increases in H_2O_2 production from antimycin A-inhibited mitochondria over untreated control samples; H_2O_2 levels detected by RPF1 (0.2 μM/min) are within ranges reported using other analytical techniques.[19,20] In addition, control experiments show that RPF1 does not react with antimycin A, and stimulated mitochondria without dye give no fluorescence. The results demonstrate that RPF1 is capable of monitoring and quantifying changes in endogenous [H_2O_2] through a ratiometric fluorescence response.

To summarize, we have presented the synthesis and properties of RPF1, a new type of ratiometric fluorescence reporter for hydrogen peroxide. This FRET-based reagent features good selectivity for H_2O_2 over competing ROS as well as visible wavelength excitation and emission profiles to minimize damage and autofluorescence from biological samples. Experiments with viable mitochondria show that RPF1 can detect and quantify endogenous H_2O_2 production, establishing the potential utility of this approach for probing peroxide biology in living systems.

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Supporting Information Available: Synthetic and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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