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Reaction-Based Genetically Encoded Fluorescent Hydrogen Sulfide Sensors

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Supporting Information

ABSTRACT: The detection of hydrogen sulfide (H_2S), a toxic gas and an important biological signaling molecule, has been a long-time challenge. Here we report genetically encoded fluorescent protein (FP)-based probes that can selectively detect H_2S. By expanding the genetic codes of *E. coli* and mammalian cells, FP chromophores were modified with the sulfide-reactive azide functional group. These structurally modified chromophores were selectively reduced by H_2S, resulting in sensitive fluorescence enhancement detectable by spectroscopic and microscopic techniques. Exploration of a circularly permuted FP led to an improved sensor with faster responses, and the feasibility of using such a genetically encoded probe to monitor H_2S in living mammalian cells has also been demonstrated.

Hydrogen sulfide (H_2S) is a weak acid in aqueous solutions (pK_{a1} = 7.04, pK_{a2} = 11.96),[1] equilibrating mainly with HS^- at physiological pH. Although it is poisonous at high concentrations, following nitric oxide (NO) and carbon monoxide (CO), H_2S is considered the third most important gasotransmitter for regulating cardiovascular, neuronal, immune, endocrine, and gastrointestinal systems.[2−4] H_2S can be enzymatically generated in the cytosols and mitochondria of mammalian cells from sulfur-containing molecules.[4,5] In addition, in response to physiological signals, intracellularly bound sulfides (e.g., sulfane sulfur) can release H_2S.[6] Furthermore, H_2S-releasing drugs are currently being used to treat cardiovascular and inflammatory diseases.[7,8] Despite the fact that H_2S has been linked to diverse physiological and pathological processes, a large part of the underlying molecular events remain unknown.[4,6]

H_2S is highly diffusible and reactive, so it is difficult to follow transiently generated H_2S. Colorimetric,[9] electrochemical,[10] and chromatographic assays[11] are available to measure H_2S in blood plasma and homogenized tissues, but they often require sample processing and do not provide much spatial and temporal information on H_2S concentrations and distribution in living cells and organisms. The needs for spatiotemporally detecting H_2S have promoted the emergence of several organic molecules, whose fluorescence is responsive to H_2S.[12−15] In particular, the research groups of Chang[12] and Wang[15] showed that the fluorescences of rhodamine and dansyl azide derivatives could be selectively enhanced by H_2S. Inspired by previous results, we undertook the effort to genetically modify chromophores of fluorescent proteins (FPs)[16] to create azide-containing structures through the genetic code expansion of living cells.[17]

FPs are self-sufficient in generating intrinsic chromophores from polypeptide sequences synthesized by cell machineries.[16] In the presence of molecular oxygen, FPs self-catalyze the formation of conjugated chromophores through a series of spontaneous biochemical reactions. Nature, as well as laboratory efforts, have generated FPs containing chromophores derived from tyrosine, phenylalanine, histidine, and tryptophan.[18] Unnatural amino acids (UAAs) have also been used to modify FPs and FP chromophores.[19] We envisioned that if azide-derived chromophores or chromophore precursors could be made by incorporating *p*-azidophenylalanine (*p*AzF) into peptides of FPs, the resulting molecules could then be reduced by H_2S to show selective fluorescence responses (Figure 1).

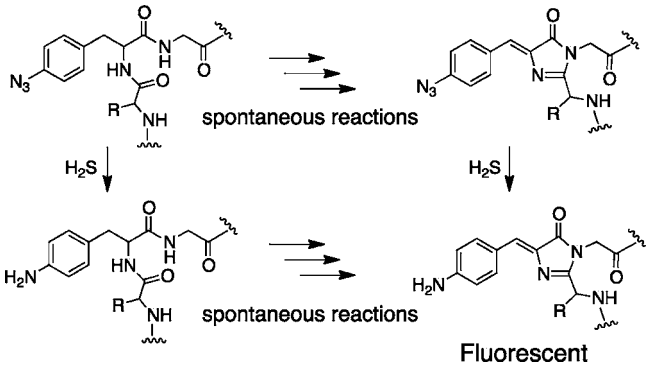

Figure 1. Schematic representation of the mechanism of reaction-based H_2S sensors. When *p*-azidophenylalanine (*p*AzF) is incorporated into the peptide precursor of an FP chromophore, both the precursor and the *p*AzF-derived mature chromophore can react with H_2S. The final fluorescent species harbors a reduced amine-containing mature chromophore.

Previously, Schultz and others developed a genetic code expansion technology to site-specifically introduce UAAs into proteins by coexpressing orthogonal tRNA/synthetase pairs.[17,20] In particular, orthogonal tRNA/synthetase pairs exist in *E. coli* and mammalian cells for the genetic encoding of *p*AzF in response to the amber (TAG) codon.[21,22] We first prepared *p*AzF-containing monomeric teal fluorescent protein (mTFP1)[23] from *E. coli*. mTFP1 was chosen due to its high

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brightness and folding efficiency.[23] The chromophore of mTFP1 is formed from the spontaneous reaction of three residues, Ala66, Tyr67, and Gly68, so we mutated the codon of Tyr67 to TAG. The resulting gene was C-terminally fused to a hexa-His tag and inserted into the plasmid pCDF-1b. The plasmid was then used to co-transform BL21(DE3) *E. coli* cells with pEvol-*p*AzF,[24] a plasmid expressing the *p*AzF-specific tRNA and synthetase. Protein production was induced in the presence of *p*AzF, so that the full-length protein (mTFP1-Tyr67*p*AzF) could be purified using Ni-NTA agarose beads.

The mature chromophore of mTFP1-Tyr67*p*AzF was at least partially formed, according to an absorption band peaked at 377 nm (Supporting Information (SI) Figure S1a). Exciting this band did not give off fluorescence. The protein was, however, slightly fluorescent when excited at 440 nm with a 492-nm emission maximum (Figure S1b). We incubated the protein (5 μM) with excess NaHS (10 mM) in a phosphate-buffered solution (PBS, pH 7.4). No fluorescence change was detectable in the first hour after mixing. Fluorescence enhancement became noticeable only after overnight incubation at room temperature (Figure S1b). We concluded that H_2S could slowly convert mTFP1-Tyr67*p*AzF into more fluorescent species. The initial background fluorescence of the purified protein was probably due to misincorporated tyrosine by the introduced tRNA/synthetase pair[24] to form the highly fluorescent tyrosine-derived chromophore,[23] or the partial conversion of *p*AzF-derived chromophores by intracellular reducing reagents or room light during protein preparation.

Since the reaction between mTFP1-Tyr67*p*AzF and H_2S is too slow for most applications, we decided to explore FPs in the circularly permuted topology. We reasoned that the chromophore of mTFP1 is shielded from the surroundings by its β-barrel structure (Figure S2a), and this might affect the accessibility by H_2S and, thus, the rate of the reaction. Previous research has created circularly permuted FPs (cpFPs) by connecting the original N- and C-termini of FPs through floppy peptides and introducing new N- and C-termini at sites within the folding of FPs.[25] There exist cpFPs that have new N- and C-termini spatially close to their chromophores (Figure S2b). Next, we prepared a *p*AzF-modified cpFP. We cloned the gene encoding a circularly permuted green fluorescent protein (cpGFP) from the Ca^{2+} sensor, inverse pericam.[25] This cpGFP variant has new terminal breaks between the original residues 144 and 145. The codon for its Tyr66 (numbered according to the wild-type GFP; structurally aligned with Tyr67 of mTFP1) was mutated to TAG. A procedure similar to the preparation of mTFP1-Tyr67*p*AzF was performed to prepare the protein cpGFP-Tyr66*p*AzF from *E. coli*. Mixing cpGFP-Tyr66*p*AzF (5 μM) with buffered NaHS (10 mM) resulted in instant and drastic fluorescence enhancement (Figure 2a), and the maximal enhancement was reached in only a few minutes.

Excited at the initial success, we closely followed fluorescence changes induced by NaHS at varying concentrations (Figure S3). The magnitudes and speeds of the changes were highly dependent on the concentrations of NaHS and the cpGFP-Tyr66*p*AzF protein. In general, higher NaHS and lower protein concentrations resulted in stronger enhancement. Parallel incubation of native cpGFP containing a tyrosine-derived chromophore (cpGFP-WT) with NaHS did not change its fluorescence. Therefore, H_2S-induced fluorescence enhancement of cpGFP-Tyr66*p*AzF is expected to be directly associated with the incorporated azide functional group. To further confirm this, we measured the molar masses of proteins before and after H_2S treatment using electrospray ionization time-of-flight mass spectrometry (ESI-TOF) (Figure S4). A major peak, which can be assigned to cpGFP harboring an amine-derived mature chromophore, was observed in the spectrum of the H_2S-treated protein. The freshly purified protein was a mixture containing both the premature and mature azide-containing chromophore. In addition, we compared fluorescence excitation and emission profiles of H_2S-treated cpGFP-Tyr66*p*AzF with an authentic *p*-aminophenylalanine (*p*AmF) containing cpGFP (cpGFP-Tyr66*p*AmF; for preparation see SI). Both showed identical excitation and emission profiles (Figure S5). These results support that the azide functional group was reduced to an amino group by H_2S.

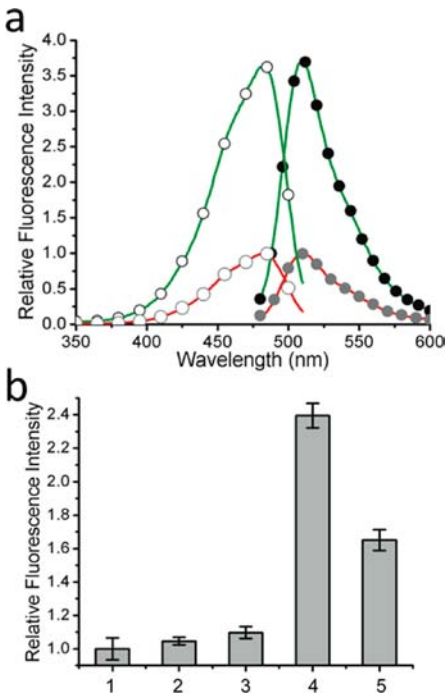

Figure 2. (a) Fluorescence excitation (open circles) and emission spectra (solid circles) of cpGFP-Tyr66*p*AzF before (red lines) and after (green lines) reaction with H_2S. (b) Fluorescence responses of cpGFP-Tyr66*p*AzF (5 μM) when mixed with different reductants (1, blank; 2, 5 mM cysteine; 3, 5 mM glutathione; 4, 1 mM dithiothreitol; 5, 100 μM NaHS).

To further investigate the relationship between the magnitude of fluorescence enhancement and NaHS concentrations, we incubated cpGFP-Tyr66*p*AzF (5 μM) with NaHS from 10 to 200 μM (Figure S6). A linear relationship was observed when <50 μM NaHS was used. We also tested the selectivity of cpGFP-Tyr66*p*AzF in response to H_2S over other cell-relevant reductants (Figure 2b). cpGFP-Tyr66*p*AzF (5 μM) was incubated with 5 mM cysteine or glutathione. For comparison, protein was also mixed with either NaHS (100 μM) or dithiothreitol (1 mM). The commonly used reductant, dithiothreitol, reacted with cpGFP-Tyr66*p*AzF, so caution must be exercised to exclude dithiothreitol when measuring H_2S in *in vitro* studies. Fortunately, cell-relevant reductants cysteine and glutathione at concentration 50× higher than that of NaHS did not affect the fluorescence of cpGFP-Tyr66*p*AzF.

To validate the use of this genetically encoded probe for visualizing H_2S in living mammalian cells, we constructed a mammalian expression plasmid harboring the gene encoding cpGFP-Tyr66TAG (pUCR-H_2S). Another plasmid (pMAH-

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*p*AzF, see SI), which expresses the orthogonal tRNA and synthetase pair for encoding *p*AzF in mammalian cells, was used to co-transfect HeLa cells with pUCR-H_2S. *p*AzF (2 mM) was supplemented in cell culture media. Cells were imaged 72 h after transfection using a confocal fluorescent microscope (Figure 3a,b). Time-lapse imaging was set at one frame per min. Previous work has indicated that H_2S can penetrate cell membranes by simple diffusion,[26] so buffered NaHS (50 μM) was directly added to cells after a few initial frames. NaHS caused fluorescence enhancement of cells expressing cpGFP-Tyr66*p*AzF, notable in the first minute, and the maximal intensity was reached in ~7 min (Figure 3c and movies in SI). In parallel, we also performed control experiments and imaged HeLa cells expressing cpGFP-Tyr66*p*AzF in the absence of NaHS and cells expressing wild-type cpGFP in the presence of NaHS. Neither of these cells showed any noticeable fluorescence change (Figure S7).

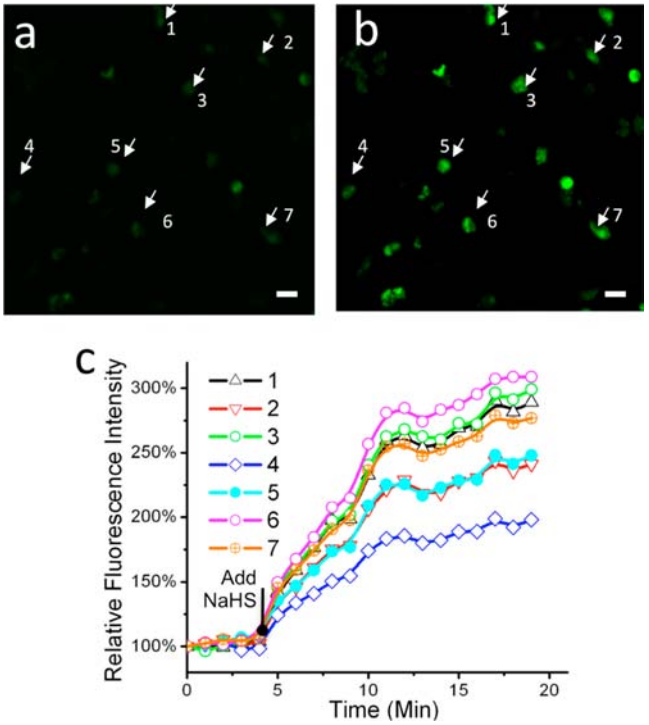

Figure 3. Fluorescence microscopic imaging of living HeLa cells expressing cpGFP-Tyr66*p*AzF before (a) and after (b) the addition of buffered NaHS. Fluorescence intensities of cells indicated in panels a and b are plotted over time and shown in panel c. Scale bar: 100 μm.

It is worth noting that, in the presence of the same amount of NaHS (50 μM), the magnitude of H_2S-induced fluorescence changes of cpGFP-Tyr66*p*AzF-expressing mammalian cells is much higher than that of the purified protein from *E. coli* (Figures 3c and S3). This is beneficial for mammalian applications, and we attribute it to possibly lower concentrations of cpGFP-Tyr66*p*AzF protein in mammalian cells, higher fidelity of mammalian *p*AzF tRNA and synthetase, and less preconversion of azide to amine.

In summary, by exploring FPs in both their natural and circularly permuted topologies, we have developed novel fluorescent probes that are selectively responsive to H_2S in aqueous solutions and in living mammalian cells. Our best cpGFP-based sensor showed fast and selective H_2S-induced fluorescence enhancement. The probe can be genetically encoded, so it offers many advantages, such as the addition of cell localization tags to allocate the probe to specific cell subdomains.[27] This simple and selective strategy for H_2S detection has the potential to facilitate a great field of research on H_2S. In addition, the approach described here represents a substantive departure from most existing FP-based biosensors, which are often relied on recognition of target molecules or enzymatic reactions,[28] while the incorporation of UAAs into FPs enabled the exploration of encoded biosensors based on selective chemical transformations. Currently, we are performing research to improve the dynamic range of the reported H_2S sensor, create H_2S sensors in compatible colors, and extend the strategy to create biosensors for other analytes.

■ ASSOCIATED CONTENT

Supporting Information

General methods for preparing plasmids and proteins, presentations on the topologies of FPs and cpFPs, spectroscopic and microscopic characterizations, mass spectrometry analysis of proteins, and movies showing microscopic imaging of living HeLa cells expressing cpGFP-Tyr66*p*AzF. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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