RESEARCH ARTICLE | Innovative Methodology

Fluorescence quenching by metal centered porphyrins and porphyrin enzymes

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Olson KR, Gao Y, Arif F, Arora K, Patel S, DeLeon E, Straub KD. Fluorescence quenching by metal centered porphyrins and porphyrin enzymes. Am J Physiol Regul Integr Comp Physiol 313: R340–R346, 2017. First published August 23, 2017; doi:10.1152/ajpregu.00202.2017.—Fluorescence spectroscopy and microscopy have been used extensively to monitor biomolecules, especially reactive oxygen species (ROS) and, more recently, reactive sulfide (RSS) species. Nearly all fluorophores are either excited by or emit light between 450 and 550 nm, which is similar to the absorbance of heme proteins and metal-centered porphyrins. Here we examined the effects of catalase (Cat), reduced and oxidized hemoglobin (Hb and metHb), albumin (alb), manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), iron protoporphyrin IX (hemin), and copper protoporphyrin IX (CuPPIX) on the fluorescence properties of fluorescein. We also examined the effects of catalase and MnTBAP on fluorophores for ROS (dichlorofluorescein, DCF), polysulfides (3’,6’-di-(0-thiosalicylic)fluorescein, SSP4), and H2S (7-azido-4-methylcoumarin, AzMC) previously activated by H2O2, a mixed polysulfide (H2Sn), n = 1–7) and H2S, respectively. All except albumin concentration dependently inhibited fluorescein fluorescence and absorbed light between 450 and 550 nm, suggesting that the inhibitory effect was physical and not catalytic. Catalase inhibition of fluorescein fluorescence was unaffected by sodium azide, dithiothreitol, diamide, tris(2-carboxyethyl)phosphine (TCEP), or iodoacetate, supporting a physical inhibitory mechanism. Catalase and TBAP augmented, then inhibited DCF fluorescence, but only inhibited SSP4 and AzMC fluorescence indicative of a substrate-specific catalytic oxidation of DCF and nonspecific fluorescence inhibition of all three fluorophores. These results suggest caution must be exercised when using any fluorescent tracers in the vicinity of metal-centered porphyrins.

Fluorescein indicators; reactive sulfide species; ROS; antioxidants

Fluorescence quenching by metal centered porphyrins and porphyrin enzymes (ROS; 10, 11, 21, 26) and more recently for detection of important in identifying and tracking reactive oxygen species (ROS). Fluorescein-conjugated macromolecules have been especially used in biomedical research as volume and fluid compartment markers (10, 11, 16, 22), see also Molecular Imaging and Contrast Agent Database (https://www.ncbi.nlm.nih.gov/books/NBK5330/). Fluorescein-conjugated macromolecules have been especially important in identifying and tracking reactive oxygen species (ROS; 10, 11, 21, 26) and more recently for detection of oxidized and reduced thiols in cells (5, 13, 23, 25).

Materials and methods

SSP4 was purchased from Dojindo molecular Technologies (Rockville, MD). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer (PBS) consisted of the following (in mM): 137 NaCl, 2.7 KCl, 8 Na2HPO4, and 2 NaH2PO4. pH was adjusted with 10 mM HCl or NaOH to 7.4 (all but pH experiments) or 6.0, 7.0 or 8.0 (pH experiments). HEPES buffer consisted of the following (in mM): 145 NaCl, 3 KCl, 0.57 MgSO4·7 H2O, 2 CaCl2·2H2O, 5 glucose, 3 HEPES acid, and 7 HEPES sodium salt, pH 7.4.

Hemoglobin as supplied from Sigma-Aldrich is essentially completely oxidized to methemoglobin. Reduced hemoglobin was prepared by slow addition of 1 mM ascorbate until the brown methemoglobin turned red. Initially, we performed this dialysis overnight under nitrogen to remove the ascorbate but this proved unnecessary as ascorbate did not interfere with fluorescence and most experiments were conducted using undialyzed samples.

Compounds of interest were pipetted into black 96-well plates in a darkened room and fluorescence or absorbance was measured on a
Fluorescence was typically measured every 10 min over 90 min, although most reactions were completed within the first 10 min. Excitation/emission (Ex/Em) wavelengths for DCF, SSP4, and 7-azido-4-methylcoumarin (AzMC) were 500/525, 482/515, and 365/450, respectively, per manufacture's recommendations. Excitation or absorbance wavelength for fluorescein was 500 nm and emission wavelength was 525 nm. These wavelengths were selected as a compromise between wavelengths used for DCF, SSP4, and AzMC excitation and the 490/520 Ex/Em wavelengths for fluorescein were reported by Sjoback et al. (24). In some situations dimethyl sulfide (DMSO) was used as the initial solvent and aliquots of corresponding concentrations of DMSO were also analyzed for their autofluorescence and competition with other fluorophores. DMSO did not affect fluorescence at these concentrations.

Results are expressed as means ± SE, and statistical analysis was determined by one-way ANOVA (two-tailed) with Holm-Sidak multiple comparisons. Significance was assumed at $P = 0.05$.

**RESULTS**

In an initial study we added various concentrations of catalase to 10 µM fluorescein and observed that catalase concentration dependently reduced fluorescence (Fig. 1A). As this response could be due to an enzymatic reaction with the catalase or interference with transmission of excitation or emission light, we then examined the effects of other heme proteins and metal-centered porphyrins with these parameters in mind.

As shown in Fig. 1, B–D, catalase, reduced (oxy) hemoglobin, and methemoglobin produced a concentration-dependent inhibition of 1 µM fluorescein fluorescence with apparent IC$_{50}$ values (in µM) of 23, 7, and 15. Catalase, reduced hemoglobin (Fe$^{2+}$), and methemoglobin (Fe$^{3+}$) produced similar inhibition of 3 µM fluorescein (IC$_{50}$ 18, 5, and 18 µM, respectively, not shown). Conversely, albumin did not affect...
fluorescence from 1 µM (or 3 µM, not shown) fluorescein. Metal-centered porphyrins produced a similar concentration-dependent inhibition of 1 µM fluorescein fluorescence (Fig. 2, A–C), although the IC₅₀ values were typically around four- to fivefold greater (hemin, 75 µM; TBAP, 73 µM). This likely reflects the four heme groups per molecule in the proteins. Copper protoporphyrin IX (CuPPIX) was not soluble above 100 µM and the IC₅₀ appeared to be slightly greater than this; at 100 µM CuPPIX fluorescence from 1 µM fluorescein was reduced by 37%. This was also similar to the effects of these compounds on 3 µM fluorescein (IC₅₀; 84, 53, and >100 µM, not shown). To determine if the inhibitory effects were more pronounced at lower fluorescence concentrations, we examined TBAP quenching of 0.1 and 0.01 µM fluorescein. While this reduced fluorescence to 3,000 fluorescence units (0.01 µM fluorescein), the TBAP IC₅₀ values were only slightly lower: 33 and 43 µM for 0.01 and 0.01 µM fluorescein (not shown) compared with 1 or 3 µM fluorescein (73 and 53, respectively). These results suggest that quenching is dependent on the porphyrin concentration but independent of the concentration of fluorophore. Designing experiments with this in mind can ensure minimal loss of the fluorescence signal even with low fluorophore concentrations encountered in cells and tissues.

Figure 2D shows the effect of 100 µM methemoglobin and 1 mM hemin on fluorescence from variable concentrations of fluorescein. In both instances ~30 µM of fluorescein was necessary to overcome the effects of methemoglobin or hemin and produce the same fluorescence as 1 µM fluorescein alone.

Upon recommendation of an anonymous reviewer, we determined if buffer pH or buffer composition affected the ability of TBAP to quench fluorescein. Although reducing the pH of PBS from 7.0 to 6.0 decreased fluorescence from 1 µM fluorescein over 60%, the inhibitory effect of TBAP remained essentially the same (EC₅₀ values 68, 89, and 86 for pH 6, 7, and 8, respectively; Fig. 2E). The inhibitory effect of TBAP on 1 µM fluorescein was also similar in HEPES buffer at pH 7.4 (EC₅₀ ~60 µM TBAP; Fig. 2F).

To determine if quenching was a chemical reaction, we examined catalase quenching in the presence of the catalase.
inhibitor sodium azide, and in the presence of the sulfhydryl reductant dithiothreitol (DTT), the sulfhydryl oxidant, di- amide, the strong reductant tris(2-carboxyethyl)phosphine (TCEP), and the cysteine alkylating agent iodoacetate (IOA). None of these treatments affected catalase inhibition of fluorescein fluorescence (Fig. 3). This supports the hypothesis that the inhibitory effect was physical rather than chemical and most likely it was mediated by quenching light at either, or both, the excitation or emission wavelengths.

Figure 4, A–E, compares light absorption at 500 nm and 525 nm (the excitation and emission wavelengths used for fluorescein) by catalase, methemoglobin, TBAP, hemin, and albumin to the quenching effect these compounds had on fluorescence from 3 µM fluorescein. All compounds except albumin exhibited a concentration-dependent increase in absorption at both wavelengths that correlated with their inhibition of fluorescein fluorescence. Albumin did not absorb an appreciable amount of light at either wavelength nor did it inhibit fluorescein fluorescence. These results suggest that the inhibitory effect is mediated through absorption of both the excitation and emission light. Figure 5 shows that the absorption spectra of these compounds at the IC50 for inhibition of 1 µM fluorescein fluorescence extends well beyond 450 and 550 nm and is considerably greater than the absorption of fluorescein at 490 nm.

To determine whether these compounds could affect fluorescence produced by fluorophores commonly used to measure ROS and RSS, we examined the effects of catalase and TBAP on AzMC activated by 300 µM H2S, SSP4 activated by the mixed polysulfide (H2Sn, n = 1–7), and DCF activated by 300 µM H2O2 (Fig. 6). Similar to their effects on fluorescein fluorescence, both catalase and TBAP inhibited AzMC fluorescence when activated by H2S and SSP4 fluorescence when activated by H2Sn. The effects of catalase and TBAP on DCF fluorescence were different as both initially and concentration dependently increased fluorescence at low concentrations and then inhibited it at high concentrations. We (19) have shown that catalase directly oxidizes DCF and it appears to be more efficacious than catalase (cf. Fig. 6, E and F) in so doing.

**DISCUSSION**

Fluorescent compounds are extensively used in biomedical research. Many of these have been developed to identify and track ROS, especially in and around mitochondria where much of the ROS production is thought to occur (2, 4, 6, 7, 9, 10, 15, 17, 26, 27). Yang et al. (29) identified 19 mitochondrially targeted fluorescent redox sensors ranging from fluorescein-based compounds to green fluorescent proteins and all but two were either excited by or emitted light between 450 and 555 nm. Similarly, interest in reactive thiols has spurred development of fluorescent markers of protein and low-molecular-weight thiols and other reactive disulfides (5, 13, 18, 23, 25, 28).

While interferences with analytes chemically similar to those being measured are frequently (and necessarily) performed (cf. 7), there is less information on other possible and seemingly unrelated interferences, especially light absorption artifacts. Zielonka et al. (30) observed that TBAP absorbs excitation and emitted light from 5 µM of the ROS indicators ethidium cation (E+) and 2-hydroxyethidium cation (2-OH-E+) and concluded that it thereby quenched the fluorescence signal. The IC50 values for TBAP extrapolated...
from their figure appeared to be between 1 and 10 µM. Here we show that a variety of molecules, all with metal porphyrins and chemically unrelated to ROS- or RSS-specific fluorophores, have a similar fluorescence quenching effect. Depending on their concentration these could considerably bias not only ROS and RSS measurements, but compromise the accuracy of any fluorophore with similar excitation/emission wavelengths.

Perhaps the most potentially significant endogenous fluorescence quenching molecules would be hemoglobin and myoglobin and this might considerably impact measurements in red blood cells and skeletal and cardiac muscle. Our results suggest that the hemoglobin concentration in red blood cells, which is approximately 22 mM, would result in complete inhibition of fluorescence (Fig. 2). Similarly, skeletal muscle myoglobin in mammals ranges from 64 mg/g in Northern elephant seals to 8 mg/g in rabbits and 2 mg/g in mice and humans (20). Assuming myoglobin is as effective as hemin in inhibiting fluorescein fluorescence, this would amount to 100% inhibition in the elephant seal, over 90% inhibition in rabbit skeletal muscle, and ~70% inhibition in mice and humans (20). Human cardiac myoglobin is ~120 mM (12), which would also inhibit most fluorescence. Catalase has the potential for local inhibition of fluorescence measurements in red blood cells and peroxisomes as do other endogenous heme proteins, especially those in the respiratory chain. One micromolar of the catalase we used is equivalent to ~4 × 10^5 units. In our experiments this amounted to ~104 units per well. The catalase concentration in red blood cells is ~1 µM (31). This alone might not affect fluorescence from 1 µM fluorophores but could augment interference by hemoglobin.

Fig. 4. Comparison of light absorption at 500 nm and 525 nm (the excitation and emission wavelengths used for fluorescein) of catalase (A), methemoglobin (B), TBAP (C), hemin (D), and albumin (E) (left) to the quenching effect of these compounds had on 3 µM fluorescein fluorescence (right). All compounds except albumin exhibited a concentration-dependent increase in absorption at both wavelengths that correlated with their inhibition of fluorescein fluorescence. All values are mean ± SE, n = 4.
Exogenous application of metal-centered porphyrins could be problematic if coupled with fluorescence measurements. TBAP is often used as a SOD mimetic and peroxynitrite scavenger (although its ability to scavenge ROS is doubtful, 8). When applied to cells, effective concentrations are achieved with ~100 µM (cf. 3). According to our study, this would inhibit fluorescence by more than 40%.

**Perspectives and Significance**

The ability to identify and track biochemical reactions using fluorescence tags has become integral to biological investigations. While there has been considerable attention paid to chemical specificity of the analytes and potentially interfering chemical interactions, comparatively little attention has been focused on potential interferences of unrelated compounds with the light transmitted to or from the fluorophores. Our results show that this photo-quenching interference can be considerable and clearly illustrate that caution must be exercised when using these, and most likely other metal-centered compounds, with any fluorophores that absorb or emit at similar wavelengths.
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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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