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 recently been used 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), and manganese- and iron-centered porphyrins (MnTBAP, iron protoporphyrin IX (hemin), and copper protoporphyrin IX (CuPPIX)) on the fluorescence properties of fluorescein. We also examined the effects of catalase on DCF fluorescence for ROS (dichlorofluorescein, DCF), polysulfides, and H2S (7-azido-4-methylcoumarin, Am J Physiol Regul Integr Comp Physiol 313: R340–R346, 2017).—Fluorescence quenching by metal centered porphyrins and porphyrin enzymes.

We recently examined the relationship between ROS and reactive sulfide species (RSS) using two of these compounds, dichlorofluorescein (DCF) to identify ROS and 3′,6′-di(0-thiosalicyl) fluorescein (SSP4) to identify RSS (19). When activated by ROS or RSS both DCF and SSP4 fluoresce due to the formation of fluorescein. We noticed in these studies that while polysulfides oxidized SSP4 and increased fluorescence, high catalase concentrations (40 μM) appeared to interfere with polysulfide-induced SSP4 fluorescence. We attributed this to catalase-mediated consumption of polysulfides or our previous experiences with inherent variability of different lot numbers of SSP4 (19). However, in retrospect it was not clear if the inhibitory effect of catalase was due to reaction with polysulfides or SSP4 or reaction with the resultant fluorescein. In the present work we examined the effects of catalase directly on fluorescein, and finding evidence for optical quenching we then examined the effects of hemoglobin and other metal-centered redox-active porphyrins, hemin, manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), protoporphyrin IX, CuPPIX, and H2S (7-azido-4-methylcoumarin, AzMC) previously activated by H2O2, a mixed polysulfide (H2Sx, 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 dithiothreitol, diamide, or 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 nonselective 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.

FLUORESCINE AND FLUORESCINE-TAGGED COMPOUNDS are widely used in biomedical research as volume and fluid compartment tracers and covalently attached to macromolecules for cytometry or to track and/or identify chemical reactions (1, 14, 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
SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). 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

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 IC50 values (in µM) of 23, 7, and 15. Catalase, reduced hemoglobin (Fe2+), and methemoglobin (Fe3+) produced similar inhibition of 3 µM fluorescein (IC50 18, 5, and 18 µM, respectively, not shown). Conversely, albumin did not affect fluorescence.
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 fluorescein 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.

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**Fig. 2.** Concentration-dependent inhibitory effects of metal porphyrins on 1 µM fluorescein fluorescence (A–C), effects of 100 µM methemoglobin (meHb) and 1 mM hemin (D) on increasing concentrations of fluorescein, and effects of buffer pH and HEPES buffer (E and F), tetrakis (4-benzoic acid) porphyrin chloride (TBAP) (A), hemin (B), and copper protoporphyrin IX (CuPPIX) (C) concentration dependently decreased fluorescein fluorescence. D: both meHb and hemin suppressed fluorescein fluorescence up to ~30 µM fluorescein. E: effects of TBAP on 1 µM fluorescein fluorescence in phosphate-buffered saline at pH 6.0, 7.0 and 8.0. Although absolute fluorescence was decreased at pH 6.0, percent inhibition of fluorescence occurred at approximately the same TBAP concentration at all three pH. F: inhibitory effect of TBAP on 1 µM fluorescein is similar in HEPES buffer at pH 7.4. Fluorescence values (left ordinate, solid symbols) are means ± SE, n = 4; percent decrease (right ordinate, open symbols) was calculated from mean fluorescence values.
inhibited AzMC fluorescence when activated by H$_2$S and effects on fluorescein fluorescence, both catalase and TBAP on AzMC activated by 300 nM H$_2$O$_2$. 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 independent of H$_2$O$_2$ or other oxidants. Although not examined, it is likely that TBAP also 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 absorbed excitation and emitted light from 518 nm to 525 nm and these compounds at the IC$_{50}$ for inhibition of 1 µM fluorescent extended 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 H$_2$S, SSP4 activated by the mixed polysulfide (H$_2$S$_n$, $n = 1–7$), and DCF activated by 300 µM H$_2$O$_2$ (Fig. 6). Similar to their effects on fluorescein fluorescence, both catalase and TBAP inhibited AzMC fluorescence when activated by H$_2$S and SSP4 fluorescence when activated by H$_2$S$_n$. 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 independent of H$_2$O$_2$ or other oxidants. Although not examined, it is likely that TBAP also directly oxidizes DCF and it appears to be more efficacious than catalase (cf. Fig. 6, E and F) in so doing.
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 is 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.
Exogenous application of metal-centered porphyrians could be problematic if coupled with fluorescence measurements. TBAP is often is 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.
E.R.D. performed experiments. K.R.O. interpreted results of experiments; K.R.O. prepared figures; K.R.O. drafted
AUTHOR CONTRIBUTIONS

No conflicts of interest, financial or otherwise, are declared by the author(s).

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