Institute of Medical Physics and Biophysics
Medical Department, Leipzig University
Author Manuscript

Published in final edited form as:
Free Radical Research, 2015 Jun; 49:768-76.
Available at:
http://dx.doi.org/10.3109/10715762.2014.999676.

DETECTION OF THE HALOGENATING ACTIVITY OF HEME PEROXIDASES IN LEUKOCYTES BY AMINOPHENYL FLUORESCEIN

Jörg Flemmig, Johannes Remmler, Josefin Zschaler & Jürgen Arnhold

Institute for Medical Physics and Biophysics, Medical Faculty, Leipzig University, Leipzig, Germany

Abstract

The formation of hypochlorous and hypobromous acids by heme peroxidases is a key property of certain immune cells. These products are not only involved in defense against pathogenic microorganisms and in regulation of inflammatory processes, but contribute also to tissue damage in certain pathologies. After a short introduction about experimental approaches for the assessment of the halogenating activity in vitro and in cell suspensions, we are focusing on novel applications of fluorescent dye systems to detect the formation of hypochlorous acid (HOCl) in leukocytes. Special attention is directed to properties and applications of the non-fluorescent dye aminophenyl fluorescein that is converted by HOCl, HOBr, and other strong oxidants to fluorescein. This dye allows the detection of the halogenating activity in samples containing free myeloperoxidase and eosinophil peroxidase as well as in intact granulocytes using fluorescence spectroscopy and flow cytometry, respectively.

Abbreviations:

APF, aminophenyl fluorescein; DCF, 2,7-dichlorofluorescein diacetate; DTNB, 5,5'-dithiobis(2-nitrobenzoicacid); EGCG, (–)-epigallocatechin-3-gallate; EPO, eosinophil peroxidase; FRET, fluorescence resonance energy transfer; HE, hydroethidine; HPF, hydroxyphenyl fluorescein; LPO, lactoperoxidase; MDM, monochlorodimedon; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; Por, porphyrin; ROS, reactive oxygen species; SNAPF, sulfonaphthoaminophenyl fluorescein; TNB, 5-thio-2-nitrobenzoic acid

Correspondence to: Jürgen Arnhold, Institute for Medical Physics and Biophysics, Medical Faculty, Leipzig University, Härtelstraße 16-18, 04107 Leipzig, Germany,
E-mail: juergen.arnhold@medizin.uni-leipzig.de

Keywords:

myeloperoxidase, hypochlorous acid, neutrophils, aminophenyl fluorescein, eosinophil peroxidase, hypobromous acid, eosinophils

Introduction

Human immune cells express several heme peroxidases that are able to oxidize (pseudo)halides to their corresponding (pseudo)hypohalous acids. These products are involved in specific functions such as killing of pathogenic microorganisms and regulation of immune responses, but can also contribute to tissue damage in certain pathologies. Thus, the specific quantification of the halogenating activity of these enzymes is of high scientific and medical interest. There are several approaches for measuring the halogenating activity of isolated heme peroxidases under *in vitro* conditions. However, it is still a challenge to deter- mine this enzymatic activity inside their hosting cells under *ex vivo* conditions in cell suspensions.

Here, we give a short overview about halide oxidation by heme peroxidases and methods applied for the detection of the corresponding products in cells. In the second part of this review, we will focus on the properties and application of aminophenyl fluorescein (APF) that considerably improved the detection of hypochlorous (HOCl) and hypobromous (HOBr) acids in stimulated leukocytes.

Halide oxidation by heme peroxidases

Human heme peroxidases

In mammals, the heme proteins myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are the only enzymes known to be able to oxidize Cl^- to HOCl and Br^- to HOBr [1–3]. The ability of EPO to oxidize Cl^- is restricted to acidic pH values [3,4]. Both enzymes are also known to oxidize I^- and SCN^- [2,3]. Taking into account physiologic (pseudo)halide concentrations in blood, their known reaction rates with activated peroxidases, and assuming neutral pH values, MPO oxidizes mainly Cl^- and SCN^-, whereas EPO uses presumably Br^- and SCN^- as substrates [5,6]. Oxidation of iodide by MPO and EPO is of little importance due to the low iodide concentration (< 1 µM [7]) in blood and tissues with the exception of thyroid gland.

MPO is mostly found in neutrophils and to a lesser extent in monocytes. Both cell types play an important role in early activation of host response to pathogenic microorganisms, trauma, and other disturbances of normal homeostasis. MPO contributes to pathogen inactivation, regulation of the inflammatory process, and also to tissue injury [8]. Eosinophil granulocytes contain EPO that has been implicated not only in protecting against helminthic parasites but also in promoting tissue damage in asthma and other diseases [9,10].

A related heme peroxidase is lactoperoxidase (LPO) found in external secretions such as saliva, tears, milk, and bronchial secretions [11]. This enzyme has protective functions at mucous surfaces and in secretions by oxidizing SCN^- to hypothiocyanite ^-OSCN. LPO is also able to use I^- as substrate, but not Cl^- or Br^- [12].

Halogenation and peroxidase cycles

To induce the (pseudo)halogenating activity, all the mentioned heme peroxidases need to be activated by hydrogen peroxide [13]. During this interconversion (Figure 1, Reaction 1), the ferric heme (Por-Fe^{3+}; Por denotes the porphyrin ring) group is oxidized to an oxo-ferryl species ($^{\bullet+}$Por-Fe^{4+}=O) additionally bearing a cation radical function at the Por ring. This intermediate is known as compound I in peroxidase chemistry [14]. During (pseudo)halide oxidation, compound I is reduced in a two-electron step to the ferric enzyme form (Figure 1, Reaction 2) [15,16]. Reactions 1 and 2 comprise the so-called "halogenation cycle". Alternatively, compound I can be reduced to the ferric enzyme form by two consecutive one-electron processes via compound II (Por-Fe^{4+}–OH), having the heme iron in the oxo-ferryl state but lacking an additional radical function (Figure 1, Reactions 3 and 4). In these reactions different small substrates including tyrosine, tryptophan, nitrite, hydrogen peroxide, xenobiotics, and others will be oxidized by abstracting one electron under formation of substrate radicals [17–21]. Reactions 1, 3, and 4 comprise the peroxidase cycle.

Redox properties of hypohalous acids and hypothiocyanite

The hypohalous acids HOCl and HOBr are powerful two-electron oxidants. Their standard reduction potentials at pH 7 (E'°) and other key properties are listed in Table I. The indicated standard reduction potentials at pH 7 for HOCl and HOBr increase with decreasing pH by 0.0295 V per decreasing pH unit as two electrons and one proton will be transferred during their reduction to the halide ion [4]. The corresponding half reactions for these reductions are

$$HOCl + 2\,e^- + H^+ \longrightarrow Cl^- + H_2O \text{ and}$$

$$HOBr + 2\,e^- + H^+ \longrightarrow Br^- + H_2O$$

In case of hypothiocyanite, the corresponding half reaction is at slightly acidic and neutral pH values:

$$^-OSCN + 2\,e^- + 2\,H^+ \longrightarrow SCN^- + H_2O$$

The standard reduction potential for the couple $^-OSCN/SCN^-$, H_2O increases with decreasing pH by 0.059 V per decreasing pH unit.

Both HOCl and HOBr are powerful agents that oxidize numerous amino acids and other small molecules [26–28]. Hypothiocyanite is a much weaker oxidant as reflected by its lower standard reduction potential (Table I). It oxidizes presumably thiol and selenol residues [29,30].

Substrates suitable for detecting the formation of HOCl/HOBr *in vitro* and in leukocytes

Detection of hypohalous acids in biological samples

Several approaches have been developed to measure and quantify the formation of HOCl under *in vitro* conditions in peroxidase samples or crude enzyme preparations. An overview about common substrates used for the detection of HOCl and also HOBr is given in Table II.

Taurine reacts with HOCl to taurine chloramine that can be assayed either by 3,3',5,5'-tetramethylbenzidine, dihydrorhodamine, or 5-thio-2-nitrobenzoic acid (TNB) [48]. In the

presence of MPO, taurine interacts with the reversible high-spin complex between MPO compound I and chloride under formation of taurine chloramine and ferric MPO [49,50]. This enables taurine to detect the chlorinating activity of MPO at pH 7.4. Other methods for the detection of HOCl production by MPO, like the application of MDM or unsaturated phosphatidylcholines, measured a lower chlorinating activity at pH 7.4 [31] or worked only at more acidic pH values [4,45,51]. Moreover, HOBr is better detectable than HOCl at neutral pH values [32,52]. A differentiation between the formation of HOCl and HOBr has been described applying taurine in combination with 3,3',5,5'-tetramethylbenzidine and iodide [32]. Otherwise, applying mass spectrometric approaches, the formation of these hypohalous acids can also be distinguished using tyrosine [36] or unsaturated phosphatidylcholines [46,47] as target.

Among the methods listed in Table II, only applications of monochlorodimedon (MDM) and TNB enable the continuous monitoring of hypohalous acid formation. However, the TNB assay is highly unspecific [40,41] and should only be applied when interferences with other reactive species are ruled out.

Unspecific approaches for detection of halogenating activity in leukocytes

The activation of neutrophils is highly associated with the production of reactive oxygen species (ROS) due to reactions mainly driven by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and MPO. In suspensions of these cells, the involvement of MPO metabolites in generation of time-dependent chemiluminescence signals (using luminol [53–55] or pholasin [56]) can be assessed by MPO inhibitors and also in case of MPO release from cells by HOCl scavengers. Unfortunately, these approaches allow only the detection of summary effects concerning the whole cell suspension.

Data about oxidant generation based on the analysis of numerous single cells can be obtained via flow cytometry by applying appropriate cell-permeable dyes that change their fluorescent properties upon interaction with ROS. Among them are the non-fluorescent 2,7-dichlorofluorescein diacetate (DCF) and dihydrorhodamine 123 that are converted upon interaction with HOCl or other ROS into a fluorescent species [57,58]. In addition to HOCl, DCF responds also to numerous other oxidants such as hydroxyl radicals, peroxynitrite, nitric oxide, as well as hydrogen peroxide in the presence of peroxidases [59]. Applying DCF, an increased ROS production was detected in mice microglial cells after inhibition of MPO as well as in microglial cells from MPO-deficient mice [60]. Dihydrorhodamine 123 is oxidized by HOCl, peroxynitrite, iron-mediated processes, and hydrogen peroxide in the presence of peroxidases [59]. Signals of this dye were strongly reduced in neutrophils from patients with complete MPO deficiency [61].

Development of more specific methods for chlorinating activity in leukocytes

During the last years, several cell-permeable substrates have been introduced that interact more specifically with HOCl without any interfering activation by superoxide anion radicals or hydrogen peroxide. These approaches enable the specific study of MPO-driven reactions in single neutrophils by means of flow cytometry. An overview about these novel fluorescence-based HOCl-specific detection systems is given in Table III.

In most cases, non-fluorescent dyes are converted upon interaction with HOCl or applying the MPO–hydrogen peroxide–chloride system into a fluorescent species [62–70]. This has been achieved by introduction of specific reactive groups into fluorophores whereby the parent molecule lost its ability to fluoresce. Selected structures for these molecules with

emphasis on the reactive site are given in Figure 2. In these dyes, fluorescence quenching is overcome either by cleavage of a *p*-aminophenyl moiety [62,63] or by oxidation of a functional residue [64,65]. Two examples for non-fluorescent spirocyclic rhodamine derivatives are also shown in Figure 2. Hypochlorous acid induces, via ring opening and oxidative conversions, the formation of highly fluorescent rhodamines [66–70].

Another principle is realized in HOCl-sensitive fluorescent quantum dots, where HOCl induces a fluorescence quenching [72]. A further HOCl detection mechanism is used in rhodamine-thiosemicarbazide-derived probes bearing an additional coumarin subunit. Upon excitation of the coumarin moiety, a fluorescence resonance energy transfer (FRET) takes place only at the rhodamine subunit when the thiosemicarbazide part has been modified by HOCl [71].

Concerning HOCl-induced conversions of these detection systems, only the structures of the target and the resulting final molecule are reported. However, there is a lack of detailed reaction mechanisms of these dyes with HOCl. In most cases, authors claimed that their detection system is highly specific for HOCl by comparing the fluorescence response to HOCl with other oxidants. However, HOBr was not included in these test systems. Interferences with other oxidants are reported for APF [62] (see also below). In case of the HKOCl-1 system, a small interference with peroxynitrite was found [64].

Although the applicability of HOCl-sensitive probes has been demonstrated on selected examples for determination of chlorinating activity in leukocytes and biological imaging, further systematic investigations of their properties are mandatory for future applications.

Properties of the APF/HPF system

Activation of APF and HPF by strong oxidants

The dye 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl)benzoic acid or, as shortly named “APF”, is among the substrates applied for measuring the chlorinating activity in leukocytes [62]. In this non-fluorescent molecule, an aminophenyl moiety is covalently attached to fluorescein (Figure 3). The properties of APF and examples for application are reviewed in the next paragraphs.

Together with APF a related dye, namely 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl)benzoic acid (shortly called “hydroxyphenyl fluorescein” or “HPF”), was introduced [62]. In contrast to APF, HPF bears a hydroxyl group instead of an amino group on the phenyl residue attached to the 6′-position of fluorescein. Both non-fluorescent fluorescein derivatives are converted into fluorescein upon reaction with strong oxidants such as hydroxyl radicals (generated by a Fenton system) or peroxynitrite. It has been shown that their fluorescence is not activated by singlet oxygen, superoxide anion radicals, hydrogen peroxide, nitric oxide, peroxyl radicals, or light-induced autoxidation [62]. In another study, however, an increasing fluorescence signal derived from APF was reported in the presence of singlet oxygen, while HPF was less sensitive against this oxidant [73].

Interaction of HOCl with APF, but not with HPF, also yielded a fluorescent species. Thus, by comparing the fluorescence answers in neutrophils in the presence of APF or HPF, it is possible to evaluate the specific response to HOCl [62]. Hypobromous acid also converts APF (but not HPF) into a fluorescent species [74,75]. This approach allows the detection of the brominating activity in eosinophils [75]. In order to relate APF-derived signals in

biological samples to the formation of hypohalous acids and to exclude effects of other strong oxidants, the application of peroxidase inhibitors is mandatory.

Preliminary data suggest that APF is also oxidized by MPO compounds I and II with rates of 6.5×10^5 $M^{-1}s^{-1}$ and 2.5×10^4 $M^{-1}s^{-1}$, respectively, at 25 °C and pH 7 [unpublished data]. Considering the marked difference between concentrations of chloride (around 0.1 M) and, if applied, APF (only few μM) and the rate of MPO compound I with chloride being 2.5×10^4 $M^{-1}s^{-1}$ at 15 °C and pH 7 [2], conditions are expected in neutrophil samples favoring a priority oxidation of chloride instead of APF by compound I of MPO.

Mechanistic details of fluorescence dequenching in APF and HPF

The activation of APF and HPF includes the cleavage of the aryloxyphenol group from the xanthene moiety of these molecules by highly reactive oxidants, resulting in the release of fluorescein that is well known for its intense fluorescence [62]. In APF and HPF, a benzene unit in the form of a *p*-benzoquinone monoamine (APF) or a *p*-benzoquinone hydroxyl moiety (HPF) is covalently attached to the core xanthene moiety at the 6'-position of fluorescein. In fluorescein derivatives, fluorescence is controlled by photo-induced intramolecular electron transfer processes. A fluorescence quenching might result by electron transfer between different molecule parts in dependence on redox properties of these substructures [76–79]. In APF and HPF, strong oxidants overcome this quenching by release of the electron-rich benzoquinone moiety from the 6'-position of fluorescein. This initial reaction is assumed to start by abstraction of a hydrogen atom from the phenolic hydroxyl group in HPF or from the aminogroup in APF [62]. This mechanism is apparently valid for the action of strong oxidants like hydroxyl radicals and peroxynitrite but unlikely for hypohalous acids. Apparently, HOCl acts on APF via the formation of an intermediate chloramine on the *p*-benzoquinone monoamine group and subsequent abstraction of the benzene moiety. A similar mechanism should be valid for HOBr. Hypochlorous acid is well known for its ability to form *N*-chloramines with substrates bearing amino groups [80–82].

The formation of fluorescein upon interaction of hypohalous acids with APF was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mono- and dichlorinated products as well as mono- and dibrominated products of fluorescein were found after incubation of APF with increasing amounts of hypohalous acids [75]. Comparable experiments with HPF primarily resulted in chlorinated and brominated products of the dye without the formation of fluorescein [75]. To overcome additional halogenations of resulting fluorescein, APF should be used in excess over HOCl/HOBr.

Selected examples for the application of APF

Detection of the halogenating activity of MPO and EPO

Using APF, the *in vitro* halogenating activity of both MPO and EPO can be easily followed after enzyme activation by hydrogen peroxide by monitoring the fluorescence increase around 525 nm due to the formation of fluorescein [75]. At pH 7.4, the increase of fluorescence in the MPO–hydrogen peroxide system depended highly on the presence of chloride ions. However, the sole application of Br^- to MPO caused a much slower and less pronounced increase in the fluorescence [75]. The MPO inhibitor 4-aminobenzoic acid hydrazide totally abolished any halogenating activity of MPO.

The concentration-dependent inactivation of MPO by chlorite was associated with a decrease in APF fluorescence [83].

Halide oxidation by the EPO–hydrogen peroxide system can also be followed by APF. Here, bromide induced a much stronger and faster response than chloride. Interestingly, the application of both bromide (at 100 µM) and chloride (at 100 mM) showed a cooperative effect in this investigation leading to a faster increase in APF destruction than in the sole presence of bromide [75]. The sole addition of SCN^- to the MPO/EPO–hydrogen peroxide system did not induce a fluorescence increase in the presence of APF indicating the inability of ^-OSCN to react with APF [75].

The APF/HPF system was also applied for measuring the halogenating activity of MPO in murine tissue and organ samples, whereby the MPO activity in extra- and intracellular protein fractions were separately measured [84]. All investigated tissue homogenates contained substances that inhibited the APF/HPF-derived fluorescence. An increased APF-derived fluorescence versus control was only found in the extracellular protein fraction of spleen and lung tissue. These authors raised considerable doubt on the specificity of the APF/HPF method and other applied MPO assays for correct measurements of MPO activity in biological samples as interfering substances like other peroxidases, hemoglobin, myoglobin, and tissue inhibitors may disturb the results. Moreover, poor specificity of the APF/HPF system was also demonstrated comparing MPO activity in wild-type and MPO -knockout mice. To improve the measurement of MPO activity in biological samples, they proposed the previous capture of MPO by a specific antibody [84].

Detection of the formation of HOCl by other enzymes

APF was successfully applied to demonstrate the transient formation of HOCl during irreversible inactivation of prokaryotic chlorite dismutase [85]. This heme *b*-dependent oxidoreductase converts chlorite into chloride and dioxygen [86].

Applications of APF to isolated neutrophils and eosinophils

Phorbol ester-stimulated porcine neutrophils increase their fluorescence in the presence of APF, but not HPF, as shown by bioimaging of the cells [62]. We applied flow cytometry analysis for measuring APF-mediated fluorescence in human phorbol ester-stimulated neutrophil and eosinophil samples. In these cell types, the peroxidase inhibitor 4-aminobenzoic acid hydrazide and the NADPH-oxidase inhibitor diphenyleneiodonium chloride abolished the APF-induced fluorescence, while the inducible nitric oxide synthase inhibitor N^G-monomethyl *L*-arginine was ineffective [75]. In human neutrophils treated with hydrogen peroxide, the modulating effect of the dietary flavonoid (–)-epicatechin on the APF response was also investigated. (–)-Epicatechin enhanced the chlorinating activity by overcoming the hydrogen peroxide-mediated accumulation of inactive compound II of MPO [87].

The APF approach can also be applied for detecting peroxidase-containing cell populations in diluted human and rodent blood samples by flow cytometry after depletion of red blood cells by hypotonic lysis [88]. In this study, the mixed leukocyte-enriched cell fraction was incubated with APF and H_2O_2. Peroxidase-containing cell fractions, such as neutrophils, eosinophils, and monocytes, were identified by antibodies against CD16, CCR3, and CD14, respectively, and related to scattering parameters of these cells [88]. In future, this approach might allow the screening of peroxidase-deficiency in blood samples by measuring the halogenating activity. Until now, only the peroxidase activity could be addressed during blood analysis [89,90]. Using this approach, factors affecting the halogenating activity of leukocytes in blood samples can be also systematically investigated.

Applications of APF/HPF to leukemic cells

MPO-transfected leukemia K562 cells markedly increased their ROS formation in the presence of (–)-epigallocatechin-3-gallate (EGCG) as visualized by increased values for APF- and HPF-induced fluorescence [91]. The As_2O_3-mediated apoptosis of HL-60 cells, which are known to contain MPO, was also enhanced by EGCG, whereby the degree of apoptosis correlated with ROS formation [91]. Authors concluded that MPO activity and the enhanced formation of hydroxyl radicals are responsible for these effects. As (–)-epicatechin and their derivatives are able to significantly enhance the chlorinating activity of MPO [34,87], these data underline the cytotoxic role of MPO-derived oxidants.

Conclusions

APF is a suitable tool for assessment of the formation of hypohalous acids and other strong oxidants in biological samples. Especially, the halogenating activities of MPO in neutrophils and EPO in eosinophils can be measured by APF using flow cytometry. The application of peroxidase inhibitors is mandatory in control measurements to exclude any potential interference from other strong oxidants. The discrimination between the formation of HOCl and HOBr as well as between the contribution of neutrophils and eosinophils by means of APF fluorescence remains challenging.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

The authors gratefully acknowledge the financial support provided by the Sächsische Aufbaubank from a funding of the European Regional Development Fund (ERDF) (SAB Project Nr: 100116526) and the German Federal Ministry of Education and Research (BMBF 1315883).

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*Table **I**. Redox properties of hypohalous acids and hypothiocyanite*

Oxidized form	pK_a value	$E'°$(HOX/X^-, H_2O) at pH 7 and 25 °C[a]	Formation at pH 7.4 by
HOCl	7.53[b]	1.28 V	MPO
HOBr	8.8[c]	1.13 V	MPO, EPO
$^-$OSCN	5.3[d]	0.56 V	MPO, EPO, LPO

[a]X denotes the corresponding (pseudo)halide form, for details see [22];
[b]at 25 °C [23];
[c]at 25 °C [24];
[d]at 25 °C [25].

*Table **II**. Common substrates for the detection of HOCl and HOBr*

Substrate	HOCl-induced conversions	Conversions by HOBr	Remarks
Taurine	Formation of taurine chloramine [31]	Formation of taurine bromamine [32]	Both haloamines can be differentiated by their reaction with 3,3',5,5'-tetramethylbenzidine in the presence of I^- [32]
MDM	Formation of dichlorodimedon [33]	Bromination of MDM [33]	MDM is also oxidized by MPO compound I [34]
Tyrosine	Formation of 3-chlorotyrosine and 3,5-dichlorotyrosine [35,36]	Formation of 3-bromotyrosine [37]	
TNB	Oxidation to the colorless 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [38]	HOBr oxidizes TNB to DTNB [39]	Unspecific oxidation by other oxidants [40,41]
Hydroethidine (HE)	Formation of 2-chloroethidium [42]		HE is also oxidized by other strong oxidants [43]
Unsaturated phosphatidylcholines	Formation of chlorohydrins [44,45]	Formation of bromohydrins [46]	No reaction with $^-$OSCN [47]

*Table **III**. Fluorogenic substrates for assessment of the chlorinating activity in leukocytes*

Substrate	Measuring principle	References
APF	Formation of fluorescein from non-fluorescent APF after cleavage of the 4-aminophenyl moiety	[62]
sulphonaphtho-APF (SNAPF)	Formation of a fluorescent product from non-fluorescent SNAPF after cleavage of the 4-aminophenyl moiety	[63]
HKOC-1	Formation of a fluorescent product upon oxidation of the *p*-methoxyphenol moiety of the BODIPY-based dye HKOC-1	[64]
HCSe	Formation of a fluorescent product upon oxidation of the selenium-containing BODIPY-based dye HCSe	[65]
Rhodamine 19	Formation of rhodamine 19 from a non-fluorescent spyrocyclic rhodamine derivative	[66]
Rhodamine 19-S (R19-S)	Formation of rhodamine 19 from spyrocyclic sulfur-containing non-fluorescent R19-S	[67]
HySOx	Conversion of the non-fluorescent spyrocyclic sulfur-containing tetramethylrhodamine derivative HySOx into a fluorescent product	[68,69]
MMSIR derivatives	Formation of a fluorescent product from non-fluorescent spyrocyclic silicon rhodamine	[69,70]
Rhodamine-thiosemicarbazides	Cyclization to rhodamine-oxadizoles, FRET-based mechanism	[71]
Carboxylate coating CdSe-ZnS quantum dots	Fluorescence quenching of quantum dots by HOCl	[72]

Figures 1-3

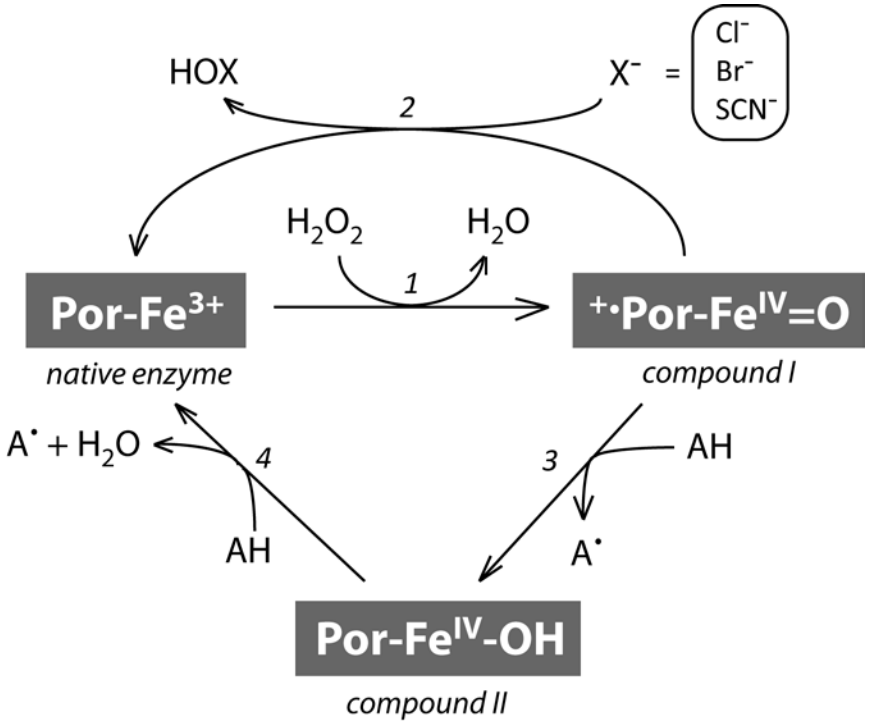

Figure 1. Halogenation (reactions 1 and 2) and peroxidase cycle (reactions 1, 3, and 4) of heme peroxidases. Por-Fe denotes the porphyrin-iron complex. Further explanations are given in the text.

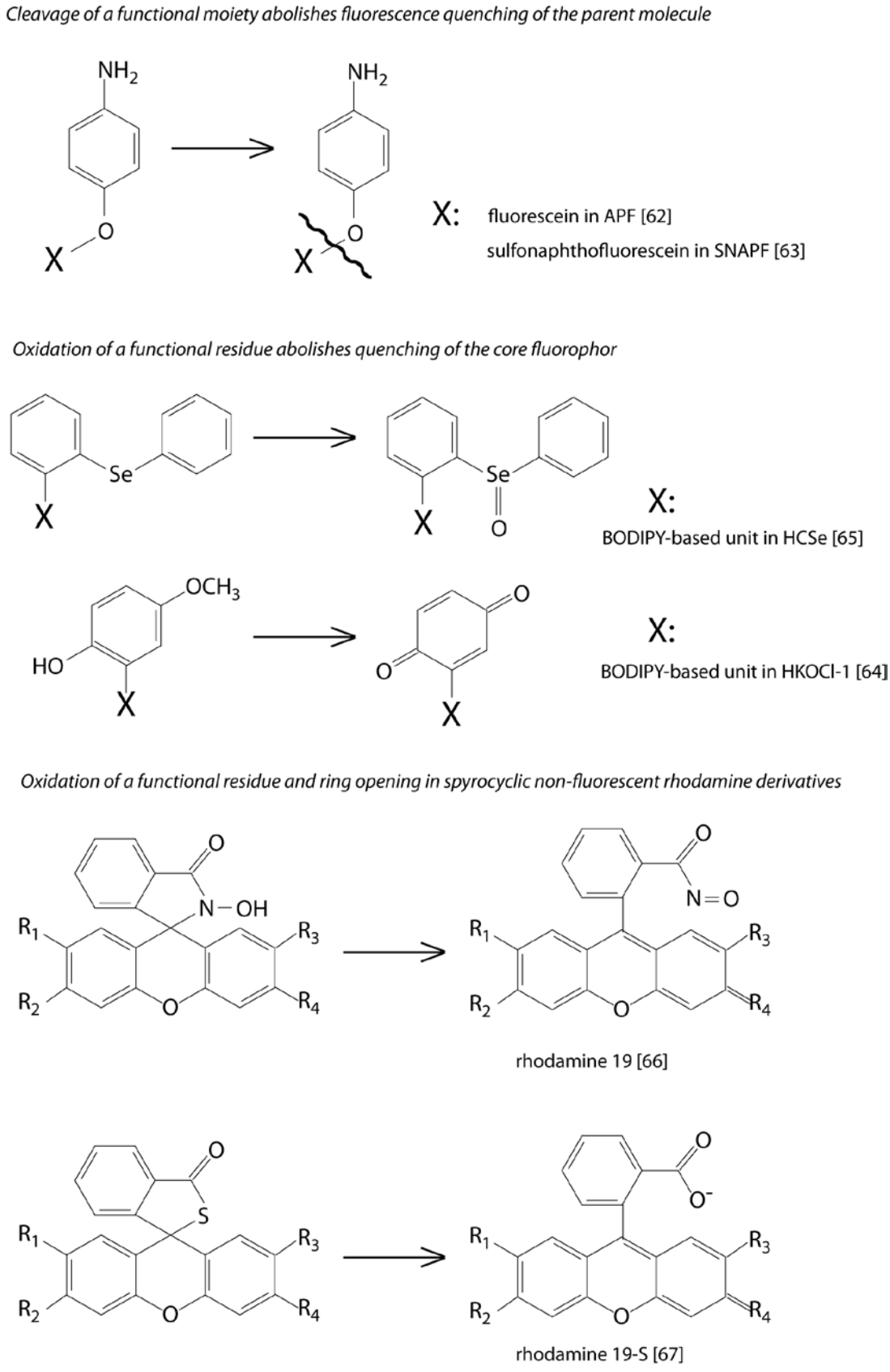

Figure 2. Main principles and examples for HOCl-mediated conversion of non-fluorescent dyes into fluorophores

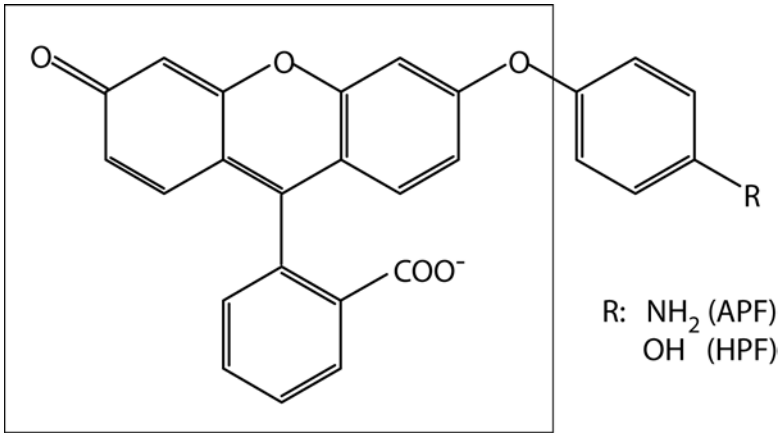

Figure 3. Chemical structures of the fluorescein derivatives APF and HPF. The core fluorescein molecule is highlighted by the rectangle.