A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases

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The enzymatic determination of hydrogen peroxide can be accomplished with high sensitivity and specificity using N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and chemically stable fluorogenic probe for the enzymatic determination of H$_2$O$_2$. Enzyme-catalyzed oxidation of Amplex Red, which is a colorless and nonfluorescent derivative of dihydroresorufin, produces highly fluorescent resorufin, which has an excitation maximum at 563 nm and emission maximum at 587 nm. The reaction stoichiometry of Amplex Red and H$_2$O$_2$ was determined to be 1:1. This probe allows detection of 5 pmol H$_2$O$_2$ in a 96-well fluorescence microplate assay. When applied to the measurement of NADPH oxidase activation, the Amplex Red assay can detect H$_2$O$_2$ release from as few as 2000 phorbol myristate acetate-stimulated neutrophils with a sensitivity 5- to 20-fold greater than that attained in the scopoletin assay under the same experimental conditions. Furthermore, the oxidase-catalyzed assay using Amplex Red results in an increase in fluorescence on oxidation rather than a decrease in fluorescence as in the scopoletin assay. In comparison with other fluorometric and spectrophotometric assays for the detection of monoamine oxidase and glucose oxidase, this probe is also found to be more sensitive. Given its high sensitivity and specificity, Amplex Red should have a broad application for the measurement of H$_2$O$_2$ in a variety of oxidase-mediated reactions and very low levels of H$_2$O$_2$ in food, environmental waters, and consumer products.

Key Words: fluorescence; hydrogen peroxide; horseradish peroxidase; Amplex Red.

Abbreviations used: HRP, horseradish peroxidase; H$_2$DCF, 2',7'-dichlorodihydrofluorescein; H$_2$DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; PMA, phorbol myristate acetate.
in a 96-well fluorescence microplate assay. In addition the reaction mixtures (10 mM resorufin in a spectrofluorometer is reliable only when its concentration is below ~5 μM in a 10-mm cuvette (4); above that concentration the optical density of the solution results in an "inner filter" effect that reduces the apparent fluorescence of the solution and results in nonlinearity of the response. However, the optimal concentration of resorufin required for use as the substrate for HRP in a H₂O₂-mediated reaction usually is higher than 5 μM. Consequently, when an optimally high concentration of resorufin is used, it is not practical to determine a small fraction of oxidation over the high background of the unconsumed substrate nor to accurately measure the fluorescence in the sample due to self-quenching by the substrate.

Preferred for all fluorometric assays is a substrate that is optically transparent and essentially nonfluorescent at the excitation wavelength used to detect the fluorescence production. With such a substrate, a very small fraction of substrate oxidation can result in a significant fluorescence increase from a nearly zero background level. Among substrates that have been used to detect H₂O₂ are p-hydroxyphenylacetic acid and 2',7'-dichlorodihydrofluorescein (H₂DCF) (5-7). Under the action of HRP, p-hydroxyphenylacetic acid can be oxidized by H₂O₂ to form a dimer that is optimally fluorescent above pH 10. The fluorescent dimer exhibits a maximum absorption at ~300 nm and maximum emission at ~400 nm (5). Spectra of the dimer overlap the absorption of certain common biologicals, such as NADPH. While the diacetate form of H₂DCF, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), is widely used in the intracellular assay of oxidants, it requires preactivation by removal of its acetates for the extracellular and solution assay of H₂O₂ (1). H₂DCF itself is used by some researchers to measure H₂O₂ in solution assays (1, 7), but it is chemically unstable, necessitating daily preparation and storage under an inert gas. Furthermore, the H₂DCF assay lacks selectivity since the reagent can be oxidized by either H₂O₂ or superoxide (7), as well as nitric oxide (8). Recently, Amplex Red has been reported as a highly sensitive fluorogenic probe for the measurement of H₂O₂ released from activated human leukocytes (9).

In this paper, we report on the properties and use of N-acetyl-3,7-dihydroxyphenoxazine, Amplex Red, a highly sensitive and stable substrate for HRP with selectivity to hydrogen peroxide. This colorless and nonfluorescent derivative of resorufin allows the detection of as little as 5 pmol H₂O₂ per 100-μL sample (50 mM) in a 96-well fluorescence microplate assay. In addition to its use either to quantitate H₂O₂ in solution or to measure horseradish peroxidase activity, we have successfully applied this substrate in measuring the activity of NADPH oxidase in phagocytes and a variety of other oxidases in solution.

**MATERIALS AND METHODS**

**Materials**

Horseradish peroxidase, monoamine oxidase, glucose oxidase, β-o-glucose, resorufin, resazurin, scopoletin, and benzylamine were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (30%) was obtained from EM Science (Gibbstown, NJ). The following reagents were from Molecular Probes, Inc. (Eugene, OR): Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine, Catalog No. A-6550) and H₂DCFDA.

**Methods**

**Stock solution and stability studies.** The stock solution of Amplex Red was prepared in analytically pure DMSO and stored at ~20°C for up to 6 months. Immediately before use, the thawed stock solution was diluted in 50 mM Tris–HCl buffer, pH 7.4. To test the stability of the reagent in dry form and in stock solution, the background fluorescence of the diluted Amplex Red solution was measured with excitation at 563 nm and emission at 587 nm in comparison with a solution freshly made from the solid reagent. The acetates of a 10 mM stock solution of H₂DCFDA were hydrolyzed for 20 min by 50 mM hydroxylamine, pH 8, followed by 1000-fold dilution in 50 mM Tris, pH 7.4, prior to the stability assay. The stability of Amplex Red and H₂DCF in working solution at room temperature was also tested in 50 mM Tris, pH 7.4, by measuring the change in fluorescence for a period of up to 24 h. The complete oxidation of H₂DCF and Amplex Red was obtained by incubating equal molar concentrations of H₂O₂ and the substrate in the presence of 1 U/ml of HRP. The spontaneous oxidation is taken as the measure of Amplex Red and H₂DCF in solution and expressed as the percentage of complete oxidation.

**Spectral assays.** Amplex Red at 10 μM was incubated with 10 μM H₂O₂ and 1 U/ml HRP in 50 mM Tris, pH 7.4, at room temperature for 5 min and then diluted threefold prior to measurement. The emission spectrum of Amplex Red and its oxidation product were recorded using a spectrofluorometer with excitation at 563 nm (SPF-500 C; SLM/Aminco, Urbana, IL). The emission spectrum of resorufin was obtained from a 3.3 μM resorufin solution in 50 mM Tris, pH 7.4. To compare the absorption spectra of the oxidation products of Amplex Red with those of resorufin and resazurin, solutions of resorufin and resazurin each at 10 μM and the reaction mixtures (10 μM Amplex Red, 1 U/ml HRP
and H₂O₂ at molar ratios to Amplex Red of 1:1, 1:1.5, and 1:2) were recorded with a spectrophotometer (IBM Instruments, Inc., Model 9430). The fluorescence intensities of the above solutions and reaction mixtures were measured using a fluorescence microplate reader with a filter set for excitation and emission at 560 ± 10 and 590 ± 10 nm, respectively (Cytofluor 2350; PerSeptive Biosystems, Framingham, MA).

Thin-layer chromatography. The solutions and reaction mixtures were prepared as described for the measurement of absorption spectra. Each solution and reaction mixture was spotted on a TLC plate. The TLC experiment was performed in a small chamber with a solvent system composed of acetic acid and methanol (3:1, v/v). The results were recorded by marking the individual spots on the TLC plate under a Mineralight lamp (UVP, Inc., San Gabriel, CA).

Kinetics studies. A low concentration of HRP (0.025 U/ml) was chosen to compare the reaction rate of these two compounds because the oxidation of Amplex Red is almost instantaneous at high concentrations of HRP. Solutions of Amplex Red and resoru®n each at 50 μM were incubated with 0.025 U/ml HRP and 20 μM H₂O₂ at room temperature for various periods of time. The fluorescence change of the reaction mixture was recorded versus time using a fluorescence microplate reader.

NADPH oxidase, monoamine oxidase, and glucose oxidase assays. Human neutrophils were prepared as described (10). The activity of NADPH oxidase in human neutrophils was determined by measuring H₂O₂ release from phorbol myristate acetate (PMA)-activated neutrophils (10). The measurement was carried out in a reaction mixture containing 100 ng/ml PMA, 1 U/ml HRP, and either 50 μM Amplex Red or 30 μM scopoletin, using a range in the number of neutrophils in the sample. For the coupled assays of monoamine and glucose oxidases, several concentrations of each enzyme were incubated in the following reaction mixtures: 1 U/ml HRP, 50 μM Amplex Red, and 1 mM benzylamine (for monoamine oxidase) or .10 mg/ml β-D-glucose (for glucose oxidase).

RESULTS

Solubility and Stability of the Substrate

When stored at −20°C as a solid, there is no detectable spontaneous oxidation of Amplex Red over 12 months. The substrate is soluble in DMSO up to 300 mM and in 50 mM Tris, pH 7.4, up to 300 μM when prepared from a DMSO stock solution. The stability of Amplex Red was evaluated both in DMSO and Tris buffer. When kept at −20°C, this substrate in DMSO is stable for more than 6 months, while in Tris buffer there is about 6–8% spontaneous oxidation over 24 h (Fig. 1). In contrast, more than 85% of the H₂DCF is spontaneously oxidized when incubated in Tris buffer for the same period of time, even in the absence of either H₂O₂ or horseradish peroxidase (Fig. 1).

Spectral Properties of the Substrate

Amplex Red is a colorless and nonfluorescent derivative of resoru®n, but the substrate produces a highly fluorescent product in response to H₂O₂ upon the action of HRP (Fig. 2). The oxidation product of Amplex Red exhibits a maximum emission at 587 nm when excited at 563 nm in a reaction buffer at pH 7.4 (Fig. 2). Both the excitation and emission spectra of oxidized Amplex Red are indistinguishable from those of resoru®n; the extinction coefficient is ~54,000 cm⁻¹ M⁻¹, the same as that for resoru®n.

Studies of Stoichiometry

The spectral analysis above suggests that Amplex Red is transformed into resoru®n upon oxidation. To determine the reaction’s stoichiometry, the reaction mixtures were prepared with different molar ratios of H₂O₂ to Amplex Red, and the absorption spectra and the fluorescence intensities were recorded in comparison with resoru®n and resazurin. When Amplex Red is incubated with an equimolar concentration of H₂O₂ in the presence of HRP, the substrate appears to be quantitatively transformed into resoru-
Similarly, when resorufin is incubated with equimolar H$_2$O$_2$ in the presence of HRP, the absorbance and fluorescence of the reaction mixture disappear (data not shown). These data indicate that resorufin becomes further oxidized to a colorless and nonfluorescent compound that does not appear to be resazurin, which is a colored compound (Figs. 3A and 3C). Furthermore, this nonfluorescent compound precipitates when the reaction mixture stands at room temperature for more than 3 h. In a previous report, the oxidation product of resorufin was only described as being a colorless and nonfluorescent compound (4). Our data may shed some light on the identity of this further oxidized compound. As indicated by the product’s absorption spectrum and its behavior in solution and on TLC (Fig. 3C), this further oxidized derivative seems to be a nonfluorescent complex polymer.

To further determine the stoichiometric relationship, the fluorescence of the oxidation product of Amplex Red was also compared with the fluorescence of a known concentration of resorufin. In these experiments, a higher concentration of Amplex Red was incubated with a series of concentrations of H$_2$O$_2$ in the presence of HRP. The fluorescence of the resulting product was determined and compared with that of a known concentration of resorufin. Figure 4 shows that the fluorescence of the oxidation product correlates well with that of resorufin at the concentration equal to the H$_2$O$_2$ concentration used to oxidize Amplex Red. Taken together, we conclude that the reaction stoichiometry of H$_2$O$_2$ and Amplex Red is 1:1; that the fluorescence increase truly indicates the concentration of H$_2$O$_2$ in the reaction mixture (provided that the substrate concentration is higher.

![Fluorogenic Probe for Hydrogen Peroxide](image)

**FIG. 2.** Emission spectra and structures of Amplex Red, resorufin, and the oxidation product of Amplex Red. Solutions of Amplex Red and resorufin each at 3.3 μM and a reaction mixture of 1 U/ml HRP, 3.3 μM Amplex Red, and H$_2$O$_2$ are excited at 563 nm. The emission spectrum of resorufin is almost the same as that of the oxidized Amplex Red product.

![Absorption and Fluorescence Spectra](image)

**FIG. 3.** Characterization of the oxidation product of Amplex Red in comparison with standard resorufin and resazurin. Amplex Red at 10 μM is incubated in a reaction mixture of 1 U/ml HRP with H$_2$O$_2$ at molar ratios to Amplex Red of 1:1 (a), 1:1.5 (c), and 1:2 (d). Solutions with the same concentration of resorufin (b) and resazurin (e) are used as controls. The reaction mixtures and control solutions are recorded with a spectrophotometer and a fluorescence microplate reader, showing their absorption spectra (A) and fluorescence intensities (B), and also characterized with a TLC assay (C).
FIG. 4. Correlation of the fluorescence of the oxidation product of Amplex Red and resorufin. Amplex Red at 150 μM is incubated with H₂O₂ at concentrations from 0 to 20 μM in the presence of 1 U/ml HRP. The fluorescence of the reaction mixtures is measured and compared with the fluorescence of resorufin at the concentration equal to the amount of H₂O₂ used to oxidize the Amplex Red (linear coefficient: r = 0.971).

FIG. 5. Kinetics of the transformation of Amplex Red to resorufin and resorufin to non-fluorescent compounds. The kinetics of Amplex Red and resorufin oxidation are determined by incubating 50 μM Amplex Red or resorufin with 50 μM H₂O₂ and 0.025 U/ml HRP for various periods of time. The fluorescence changes are plotted versus the incubation time (Amplex Red, open circles; resorufin, filled circles).

Kinetics of Oxidation

Since the fluorescent product of the oxidized Amplex Red, resorufin, is itself a known substrate for HRP (4), we compared the kinetics of oxidation of Amplex Red and resorufin by H₂O₂ in a HRP-catalyzed reaction. Under the same experimental conditions, it appears that the transformation rate from resorufin into the non-fluorescent complex polymer is more than 30-fold slower than that from Amplex Red into resorufin (Fig. 5).

Selectivity and Sensitivity

The oxidation of Amplex Red requires the participation of both HRP and H₂O₂ and appears to be selective for H₂O₂ over superoxide (9). When the substrate is incubated with H₂O₂ or HRP alone, no significant fluorescence increase can be detected after 60 min. However, a marked fluorescence increase is detected when the substrate is incubated with H₂O₂ and HRP for only 3 min (Fig. 6). To determine the sensitivity of this substrate for detecting H₂O₂, we performed the experiments using commercial H₂O₂, human neutrophils, and monoamine and glucose oxidases. Using commercial H₂O₂ and HRP, Amplex Red can detect H₂O₂ at a concentration as low as 50 nM, or 5 pmol per 100 μl sample (Fig. 7A), and HRP at a concentration as low as 1 × 10⁻⁵ U/ml, or 1 × 10⁻⁶ U per sample in a 96-well microplate assay (Fig. 7B). When applied to the measurement of NADPH oxidase activity, Amplex Red allows the detection of H₂O₂ from as few as 2000 PMA-activated neutrophils, which is about 5- to 20-fold more sensitive than the detection based on scopoletin (Fig. 8). When this substrate is applied in measuring the activity of monoamine and glucose oxidases in HRP-coupled reactions, we have been able to achieve the sensitivity of 1.2 × 10⁻⁵ U/ml of monoamine oxidase and 6.2 × 10⁻⁵ U/ml of glucose oxidase, which is about...
5- to 20-fold more sensitive than the currently used spectrophotometric method (2, 13).

DISCUSSION

We have characterized Amplex Red, a novel fluorogenic substrate of HRP with specificity for H₂O₂. This substrate has numerous advantages over the existing substrates. Under identical conditions, this substrate is 5- to 20-fold more sensitive than a currently used fluorescent probe, scopoletin, in detecting H₂O₂ from activated human neutrophils or about 5- to 20-fold more sensitive than the conventional spectrophotometric assay for detecting the activity of monoamine and glucose oxidases. In solution assays of H₂O₂ and HRP, this substrate permits detection of 5 pmol H₂O₂ and 1 × 10⁻⁶ U HRP per 100-µl sample in a 96-well microplate assay. The superior sensitivity of Amplex Red results from the following properties: (a) Amplex Red is totally nonfluorescent but upon oxidation becomes highly fluorescent with an extinction coefficient approximately three times higher than that of scopoletin (4); (b) it has an excellent photo- and chemical stability, which allows the reaction to occur with a minimal background over a long period of time; (c) it has a better sensitivity than scopoletin and resorufin to HRP; and (d) it can be used at a high initial concentration, which overcomes the limitation for previously reported substrates in assessing H₂O₂, most of which depend on measurement of a decrease in fluorescence. In addition, the oxidized product of Amplex Red has excitation and emission maxima at 563 and 587 nm, in comparison with those of scopoletin at 360 and 460 nm. These superior spectral properties are important for avoiding interference from autofluorescence in assays involving biological sam-

FIG. 7. Sensitivity of Amplex Red and resorufin to H₂O₂ and HRP. A shows the fluorescence change of Amplex Red and resorufin in response to the indicated concentrations of H₂O₂ in the presence of 1 U/ml HRP (Amplex Red, open circles; resorufin, filled circles); B shows the fluorescence change of Amplex Red and resorufin in response to the indicated concentrations of HRP in the presence of 100 µM H₂O₂. The inset shows an enlargement of the plot to depict the detection limit of H₂O₂ at low concentrations (Amplex Red, left Y axis; resorufin, right Y axis).

FIG. 8. Application of Amplex Red in the measurement of NADPH oxidase. The data show the different sensitivities of Amplex Red (open circles) and scopoletin (filled circles) in detecting NADPH oxidase activity as judged by the measurement of H₂O₂ released from the indicated numbers of neutrophils.
amples, such as body fluids, blood samples, and particularly cell lysates and tissue homogenates.

While resorufin has been previously applied as a substrate to the enzymatic determination of H$_2$O$_2$, it is much less sensitive than its dihydro derivative, Amplex Red (Fig. 7). As discussed above, there are a few factors affecting the sensitivity of resorufin for detecting H$_2$O$_2$. As with scopoletin, resorufin detects H$_2$O$_2$ by measuring a decrease in fluorescence. As indicated in Fig. 7A, the initial oxidation of resorufin does not result in a fluorescence decrease when a high concentration of resorufin is used in the reaction mixture. While use of a very low initial concentration of resorufin in the reaction mixture could avoid this problem (4), lowering the initial concentration also reduces the sensitivity since the optimal enzymatic reaction requires a higher concentration of substrate. More importantly, it is not practical to add the substrate, resorufin, into the reaction mixture sequentially in a kinetic assay of H$_2$O$_2$ at a concentration that is higher than the initial concentration of the substrate. Thus, resorufin is less sensitive for detecting H$_2$O$_2$, and the kinetic rate is slower (Fig. 5).

The published methods, which could provide a comparable sensitivity in detecting H$_2$O$_2$ with our current assay, include the dual-wavelength spectroscopy of the catalase−H$_2$O$_2$ intermediate (11) and the method using the HRP−superoxide dismutase−scopoletin system (12). The dual-wavelength spectroscopy of the catalase−H$_2$O$_2$ intermediate developed by Dr. Chance's group provided a sensitive method for determining the rate of H$_2$O$_2$ generation in the liver in situ, but this method has not been widely used in the solution assay of H$_2$O$_2$ because of the complexity of the procedure and data calculation. In the assay of H$_2$O$_2$ using the HRP−superoxide dismutase−scopoletin system, the fluorescence change resulting from the oxidation of exogenous NADPH, which occurs in the presence of HRP, superoxide dismutase, and scopoletin, is taken as the measure of H$_2$O$_2$. While this novel assay can detect 10 nM H$_2$O$_2$ under the optimal conditions, it may not be appropriate for the measurement of H$_2$O$_2$ from intact cells and cell lysates due to the presence of endogenous NADPH.

As with other fluorogenic probes, Amplex Red may not be suitable for detecting H$_2$O$_2$ at concentrations higher than 20 μM, due to instrument limitation, unless the sample is diluted to reduce its absorbance. Using a Cytoflor 2350, we can quantitate the H$_2$O$_2$ concentration from 50 nM to 20 μM without dilution. For substrates such as scopoletin and resorufin, using the inverse fluorescence assay, the estimated H$_2$O$_2$ concentration may not reflect the actual concentration of H$_2$O$_2$ since the fluorescence decrease at high concentration is not proportional to the amount of oxidized substrate in the reaction mixture. With the advantage of its non-fluorescence, we determined that Amplex Red reacts with H$_2$O$_2$ at a stoichiometry of 1:1. Although the oxidation product of Amplex Red, resorufin, can be further oxidized to a nonfluorescent compound, this further oxidation will not occur significantly unless the H$_2$O$_2$ concentration is higher than the Amplex Red concentration in the reaction mixture.

We have successfully applied the Amplex Red-based assay for the continuous measurement of the activity of NADPH oxidase, monoamine oxidase, and glucose oxidases. These assays are about 5- to 20-fold more sensitive than the current methods (2, 13). Furthermore, we found that Amplex Red is about 10-fold more sensitive to H$_2$O$_2$ than 0-phenylenediamine when the assay is conducted at pH 7.6 (data not shown). The superior sensitivity and stability of Amplex Red may make this new H$_2$O$_2$ probe useful for detecting the very low level of antigens in enzyme-linked immunosorbent assays with longer incubation times. In conclusion, we have characterized a highly sensitive and stable fluorogenic probe for H$_2$O$_2$. This new H$_2$O$_2$ probe should greatly facilitate the measurement of the activity of NADPH oxidase and a variety of other oxidases, as well as H$_2$O$_2$ in food and environmental waters.

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