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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_2O_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_2O_2 was determined to be 1:1. This probe allows detection of 5 pmol H_2O_2 in a 96-well fluorescence microplate assay. When applied to the measurement of NADPH oxidase activation, the Amplex Red assay can detect H_2O_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_2O_2 in a variety of oxidase-mediated reactions and very low levels of H_2O_2 in food, environmental waters, and consumer products.

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

The ability to detect hydrogen peroxide (H_2O_2) resulting from NADPH oxidase activation is an important tool for studying phagocyte function. The initial product of NADPH oxidase-mediated oxygen reduction is primarily superoxide anion. Subsequently, superoxide is converted to H_2O_2 either spontaneously or by superoxide dismutase. Because of its stability and membrane permeability, H_2O_2 is usually chosen as an analyte among other oxidants to quantitate the extracellular release of reactive oxygen reduction products in phagocytes. H_2O_2 is also a coproduct of a variety of oxidases, and its detection is the basis for measuring the activity of these oxidases (1, 2). Both chromogenic and fluorogenic substrates are currently used to measure the activity of NADPH oxidase, HRP,[1] and other oxidases. While assays using chromogenic substrates require only a regular spectrophotometer, they are usually less sensitive than assays that use fluorogenic substrates. Absorption- and fluorescence-detecting microplate readers, which are particularly useful instruments for enzyme assays, are now available in many research and clinical laboratories.

Several substrates have been used for the fluorometric quantitation of H_2O_2. Under the action of HRP, H_2O_2 is detected using these substrates by measuring either an increase or a decrease in the substrate's fluorescence upon oxidation. H_2O_2 oxidizes scopoletin, a naturally occurring fluorescent compound, to a nonfluorescent product (an inverse fluorescence measurement). Although extensively used for the assay of H_2O_2 production (3), scopoletin has a low extinction coefficient and short wavelength spectra (4). Because of its high extinction coefficient and good chemical and photostability, resorufin has been used in an inverse fluo-

[1] Abbreviations used: HRP, horseradish peroxidase; H_2DCF, 2′,7′-dichlorodihydrofluorescein; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; PMA, phorbol myristate acetate.

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rescence assay to detect H_2O_2 by measuring the disappearance of its fluorescence upon horseradish peroxidase-mediated oxidation to a nonfluorescent derivative (4). However, the determination of H_2O_2 using this inverse fluorescence measurement has intrinsic limitations. For example, detection of the fluorescence of 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 H_2O_2-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 oxidization can result in a significant fluorescence increase from a nearly zero background level. Among substrates that have been used to detect H_2O_2 are *p*-hydroxyphenylacetic acid and 2′,7′-dichlorodihydrofluorescein (H_2DCF) (5–7). Under the action of HRP, *p*-hydroxyphenylacetic acid can be oxidized by H_2O_2 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 H_2DCF, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), is widely used in the intracellular assay of oxidants, it requires preactivation by removal of its acetates for the extracellular and solution assay of H_2O_2 (1). H_2DCF itself is used by some researchers to measure H_2O_2 in solution assays (1, 7), but it is chemically unstable, necessitating daily preparation and storage under an inert gas. Furthermore, the H_2DCF assay lacks selectivity since the reagent can be oxidized by either H_2O_2 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 H_2O_2 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 H_2O_2 per 100-μL sample (50 nM) in a 96-well fluorescence microplate assay. In addition to its use either to quantitate H_2O_2 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-D-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 H_2DCFDA.

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 H_2DCFDA 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 H_2DCF 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 H_2DCF and Amplex Red was obtained by incubating equal molar concentrations of H_2O_2 and the substrate in the presence of 1 U/ml of HRP. The spontaneous oxidation is taken as the measure of Amplex Red and H_2DCF in solution and expressed as the percentage of complete oxidation.

Spectral assays. Amplex Red at 10 μM was incubated with 10 μM H_2O_2 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 H_2O_2 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 resorufin each at 50 μM were incubated with 0.025 U/ml HRP and 20 μM H_2O_2 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 H_2O_2 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

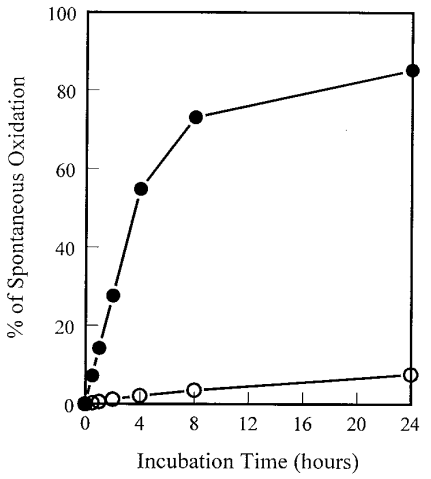

FIG. 1. Spontaneous oxidation of Amplex Red and dichlorodihydrofluorescein (H_2DCF) in solution. Solutions of Amplex Red and H_2DCF are prepared at concentrations of 10 μM in 50 mM Tris, pH 7.4, and incubated in the dark for the indicated times. The maximal fluorescence (complete oxidation) of Amplex Red and H_2DCF is obtained by incubation with 10 μM H_2O_2 and 1 U/ml HRP. The percentage of spontaneous oxidation is calculated by using the fluorescence of complete oxidation as 100%. The data are plotted versus the incubation time (Amplex Red, open circles; H_2DCF, filled circles). Each point is the average of three determinations.

(Fig. 1). In contrast, more than 85% of the H_2DCF is spontaneously oxidized when incubated in Tris buffer for the same period of time, even in the absence of either H_2O_2 or horseradish peroxidase (Fig. 1).

Spectral Properties of the Substrate

Amplex Red is a colorless and nonfluorescent derivative of resorufin, but the substrate produces a highly fluorescent product in response to H_2O_2 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 resorufin; the extinction coefficient is ~54,000 cm^{-1} M^{-1}, the same as that for resorufin.

Studies of Stoichiometry

The spectral analysis above suggests that Amplex Red is transformed into resorufin upon oxidation. To determine the reaction's stoichiometry, the reaction mixtures were prepared with different molar ratios of H_2O_2 to Amplex Red, and the absorption spectra and the fluorescence intensities were recorded in comparison with resorufin and resazurin. When Amplex Red is incubated with an equimolar concentration of H_2O_2 in the presence of HRP, the substrate appears to be quantitatively transformed into resoru-

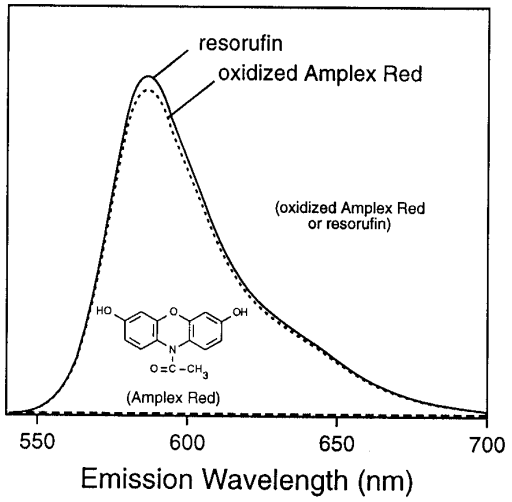

FIG. 2. Emission spectra and structures of Amplex Red, resorufin, and the oxidation product of Amplex Red. Solutions of Amplex Red and resorufin each at 3.3 μM and a reaction mixture of 1 U/ml HRP, 3.3 μM Amplex Red, and H_2O_2 are excited at 563 nm. The emission spectrum of resorufin is almost the same as that of the oxidized Amplex Red product.

fin. The wavelength and intensity of the absorption spectrum of the oxidized Amplex Red product are identical to those of resorufin at the same concentration (Fig. 3A). Both the fluorescence emission wavelength and intensity of resorufin and the oxidized product of Amplex Red are almost the same (Fig. 2 and Fig. 3B). However, if the concentration of H_2O_2 is higher than that of Amplex Red in the reaction mixture, both the absorption and fluorescence intensities decrease and nearly disappear when the ratio of H_2O_2 to Amplex Red reaches 2 (Figs. 3A and 3B). Similarly, when resorufin is incubated with equimolar H_2O_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_2O_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_2O_2 concentration used to oxidize Amplex Red. Taken together, we conclude that the reaction stoichiometry of H_2O_2 and Amplex Red is 1:1; that the fluorescence increase truly indicates the concentration of H_2O_2 in the reaction mixture (provided that the substrate concentration is higher

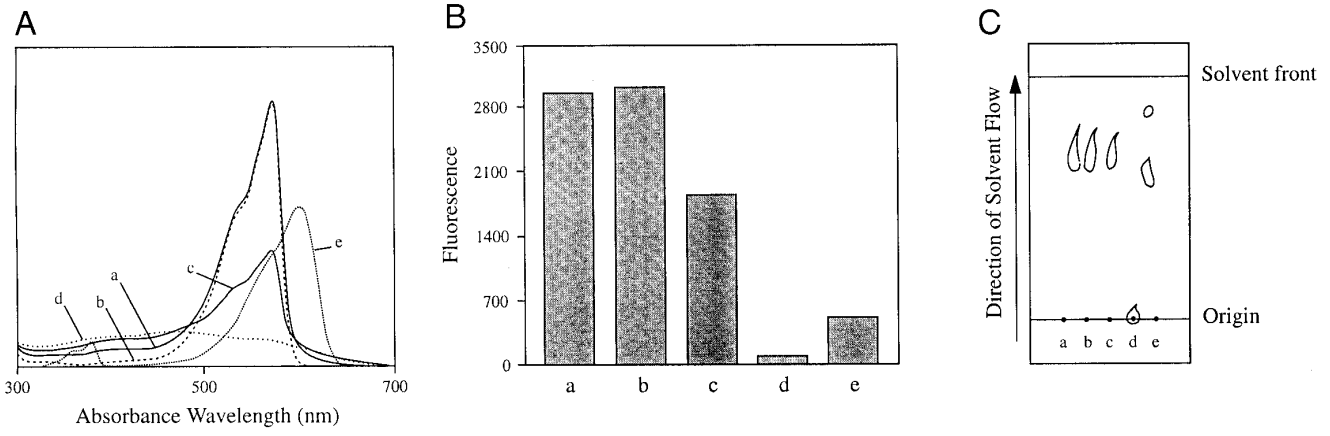

FIG. 3. Characterization of the oxidation product of Amplex Red in comparison with standard resorufin and resazurin. Amplex Red at 10 μM is incubated in a reaction mixture of 1 U/ml HRP with H_2O_2 at molar ratios to Amplex Red of 1:1 (a), 1:1.5 (c), and 1:2 (d). Solutions with the same concentration 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).

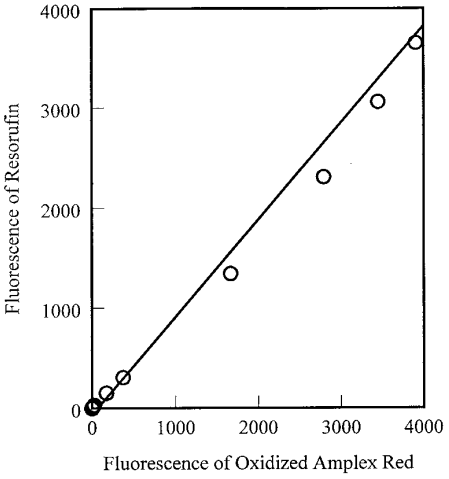

FIG. 4. Correlation of the fluorescence of the oxidation product of Amplex Red and resorufin. Amplex Red at 150 μM is incubated with H_2O_2 at concentrations from 0 to 20 μM in the presence of 1 U/ml HRP. The fluorescence of the reaction mixtures is measured and compared with the fluorescence of resorufin at the concentration equal to the amount of H_2O_2 used to oxidize the Amplex Red (linear coefficient: $r = 0.971$).

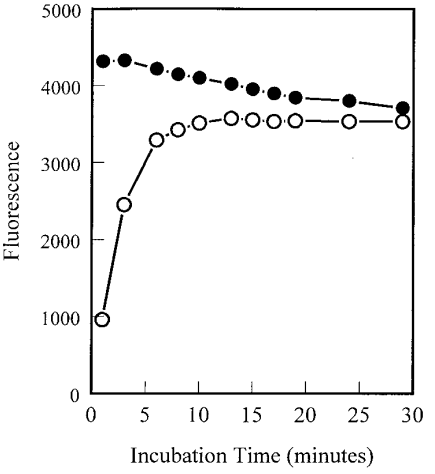

FIG. 5. Kinetics of the transformation of Amplex Red to resorufin and resorufin to nonfluorescent compounds. The kinetics of Amplex Red and resorufin oxidation are determined by incubating 50 μM Amplex Red or resorufin with 50 μM H_2O_2 and 0.025 U/ml HRP for various periods of time. The fluorescence changes are plotted versus the incubation time (Amplex Red, open circles; resorufin, filled circles).

than the H_2O_2 concentration); and that the oxidation product, resorufin, can be further oxidized into a nonfluorescent complex polymer when the ratio of H_2O_2 to substrate is greater than 1.

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 H_2O_2 in a HRP-catalyzed reaction. Under the same experimental conditions, it appears that the transformation rate from resorufin into the nonfluorescent 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 H_2O_2 and appears to be selective for H_2O_2 over superoxide (9). When the substrate is incubated with H_2O_2 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 H_2O_2 and HRP for only 3 min (Fig. 6). To determine the sensitivity of this substrate for detecting H_2O_2, we performed the experiments using commercial H_2O_2, human neutrophils, and monoamine and glucose oxidases. Using commercial H_2O_2 and HRP, Amplex Red can detect H_2O_2 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 H_2O_2 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

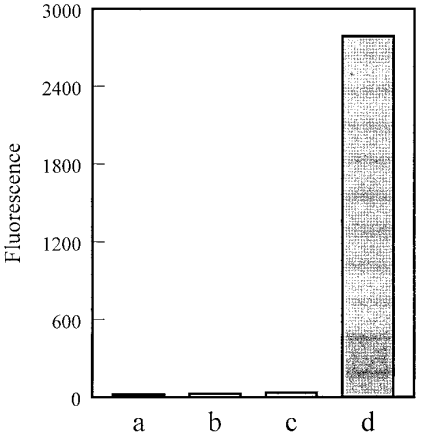

FIG. 6. The requirement for HRP of Amplex Red in detecting H_2O_2. The experiments are carried out by incubating Amplex Red in buffer (a), with H_2O_2 (b) or HRP alone (c), and with both H_2O_2 and HRP (d).

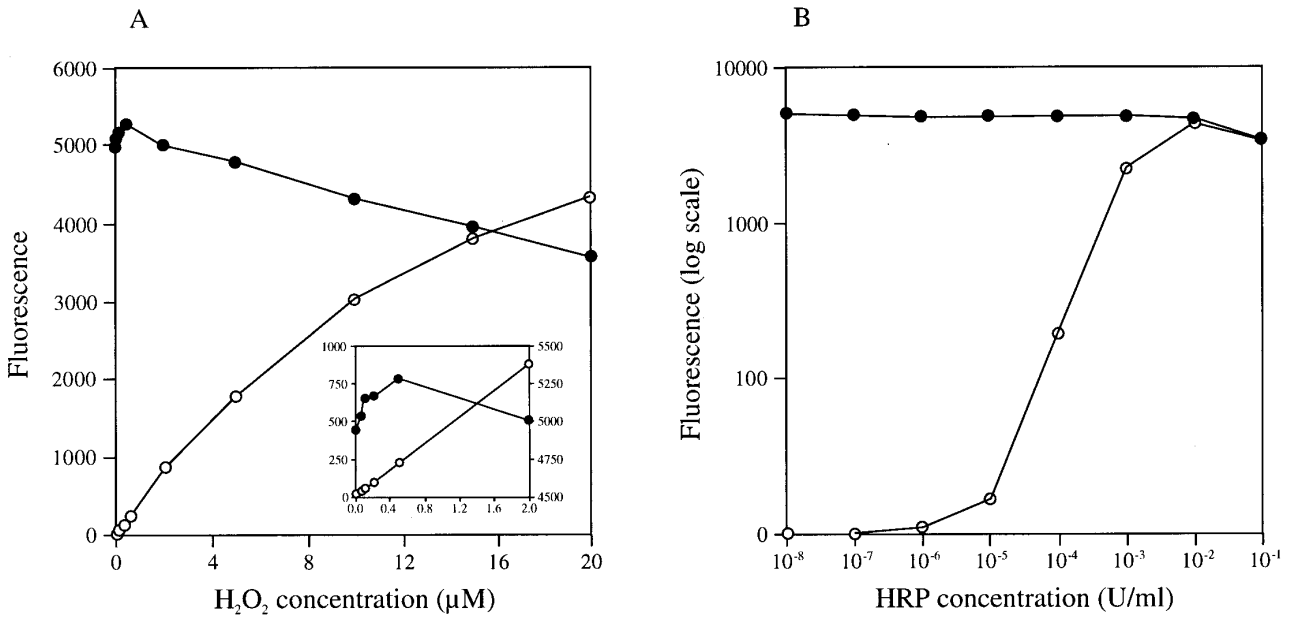

FIG. 7. Sensitivity of Amplex Red and resorufin to H_2O_2 and HRP. A shows the fluorescence change of Amplex Red and resorufin in response to the indicated concentrations of H_2O_2 in the presence of 1 U/ml HRP (Amplex Red, open circles; resorufin, filled circles); B shows the fluorescence change of Amplex Red and resorufin in response to the indicated concentrations of HRP in the presence of 100 μM H_2O_2. The inset shows an enlargement of the plot to depict the detection limit of H_2O_2 at low concentrations (Amplex Red, left *Y* axis; resorufin, right *Y* axis).

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 H_2O_2. 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 H_2O_2 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 H_2O_2 and HRP, this substrate permits detection of 5 pmol H_2O_2 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 H_2O_2, 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-

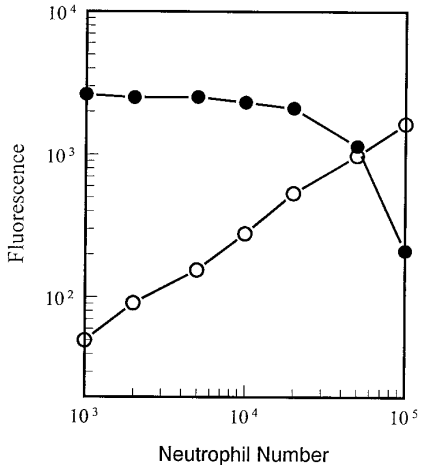

FIG. 8. Application of Amplex Red in the measurement of NADPH oxidase. The data show the different sensitivities of Amplex Red (open circles) and scopoletin (filled circles) in detecting NADPH oxidase activity as judged by the measurement of H_2O_2 released from the indicated numbers of neutrophils.

ples, 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_2O_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_2O_2. As with scopoletin, resorufin detects H_2O_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_2O_2 at a concentration that is higher than the initial concentration of the substrate. Thus, resorufin is less sensitive for detecting H_2O_2, and the kinetic rate is slower (Fig. 5).

The published methods, which could provide a comparable sensitivity in detecting H_2O_2 with our current assay, include the dual-wavelength spectroscopy of the catalase–H_2O_2 intermediate (11) and the method using the HRP–superoxide dismutase–scopoletin system (12). The dual-wavelength spectroscopy of the catalase–H_2O_2 intermediate developed by Dr. Chance's group provided a sensitive method for determining the rate of H_2O_2 generation in the liver *in situ*, but this method has not been widely used in the solution assay of H_2O_2 because of the complexity of the procedure and data calculation. In the assay of H_2O_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_2O_2. While this novel assay can detect 10 nM H_2O_2 under the optimal conditions, it may not be appropriate for the measurement of H_2O_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_2O_2 at concentrations higher than 20 μM, due to instrument limitation, unless the sample is diluted to reduce its absorbance. Using a Cytofluor 2350, we can quantitate the H_2O_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_2O_2 concentration may not reflect the actual concentration of H_2O_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 nonfluorescence, we determined that Amplex Red reacts with H_2O_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_2O_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 HRP than *o*-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_2O_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_2O_2. This new H_2O_2 probe should greatly facilitate the measurement of the activity of NADPH oxidase and a variety of other oxidases, as well as H_2O_2 in food and environmental waters.

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