A Fluorimetric Assay for Hydrogen Peroxide, Suitable for NAD(P)H-Dependent Superoxide Generating Redox Systems

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We report a simple and sensitive fluorimetric method for quantitative assay of the production rate of hydrogen peroxide, and indirectly of superoxide, during electron transfer reactions. The assay requires the inclusion of superoxide dismutase, catalase, and 6% methanol in the tested reaction system, to stochiometrically produce formaldehyde per molecule of H_2O_2 generated. The reaction is terminated by adding 2 vol of Nash reagent and heating at 60°C for 10 min, to convert accumulated formaldehyde to diacetyldihydrolutidine (DDL). The standard curve for formaldehyde, based on the fluorescence of DDL, is highly reproducible and allows measurement of 1 μM amounts in the reaction sample (coefficient of variation <15%). The excitation and emission wavelengths of DDL at 412 and 505 nm are distant from those of NAD(P)H. Thus, the method can be used in NAD(P)H-dependent enzymatic systems to measure both NAD(P)H oxidation and superoxide production in the same sample. We validated the assay in a mitochondrial P450 system determining the fraction of total electron flow that is channeled to oxy-radical formation. The assay should be useful in the study of this and other superoxide/H_2O_2 generating systems. © 1994 Academic Press, Inc.

Oxygen radicals are generated during a variety of cellular processes, such as electron transfer reactions, and cellular injury (1,2). The assay of these radicals is difficult because of their high reactivity and short half lives. One group of commonly used assays is based on the spectrophotometric measurement of molecules that react with superoxide radical, e.g., nitroblue tetrazolium (3), epinephrine (4), and cytochrome c (5). These methods are relatively simple but because of frequent nonspecific color reactions most of these cannot be used in quantitative assays (6). Some of these molecules, such as nitroblue tetrazolium and cytochrome c, are not suitable for study of enzymatic electron transfer systems that can reduce the molecule directly rather than through oxy-radicals (3,6).

Another approach to assay oxy-radicals is the use of spin-traps with ESR[2] spectroscopic equipment (7). This method is difficult to use for quantitative kinetic assays because the rates of reaction of the spin-trap molecule with oxygen radicals often do not reflect the rate of radical formation.

A third approach, employed here, is based on the dismutation of superoxide to hydrogen peroxide. This approach can be used for quantitative assays since superoxide can be converted to H_2O_2 by superoxide dismutase (SOD) at a very high rate ($k = 2 \times 10^9$ M $\times$ s^{-1}), limited by diffusion rate (8,9). In contrast to superoxide, H_2O_2 is relatively stable. It can be assayed by H_2O_2-dependent peroxidation or hydroxylation of compounds that can be measured by absorbance (10–12) or fluorescence (13–17). Previously we observed that the most convenient spectrophotometric method (12) does not provide an accurate stoichiometric result (18), and that its sensitivity is limited to 5–10 μM of superoxide. Fluorimetric methods are generally more sensitive and permit estimation of <1 μM quantities. However, these methods cannot be used in reactions with pyridine nucleotides such as NAD(P)H, because of their strong overlapping fluorescence (15,19).

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[2] Abbreviations used: ESR, electron spin resonance; SOD, superoxide dismutase; DDL, diacetyldihydrolutidine.
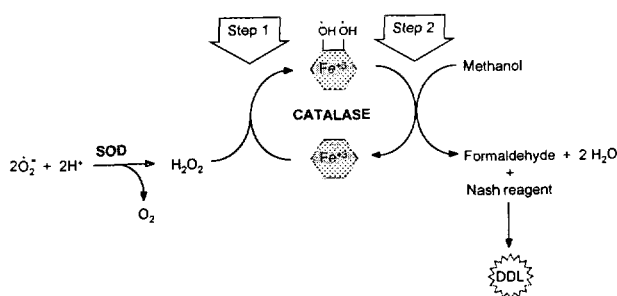
FIG. 1. *Reaction scheme for the fluorimetric assay* of superoxide and H_2O_2. Superoxide is converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is then used in the conversion of methanol into formaldehyde by catalase in two steps. Finally, formaldehyde is reacted with the Nash reagent to form the fluorescent product diacetyldihydrolutidine (DDL).

We report a fluorimetric assay for H_2O_2, based on peroxidation of methanol to formaldehyde (12). Formaldehyde is then quantitated by the fluorescence of its reaction product with the Nash reagent (20). Our assay is simple and highly sensitive, and can be used in enzymatic systems including NAD(P)H as an electron donor.

MATERIALS AND METHODS

All reagents and enzymes were purchased from Sigma, Aldrich, or BDH. Concentrations of NADPH and H_2O_2 were determined using extinction coefficients ($mM^{-1} \times cm^{-1}$) of 6.2 at 340 nm and 0.043 at 240 nm, respectively. The Nash reagent was freshly prepared by adding 0.3 ml of glacial acetic acid and 0.2 ml of acetyl acetone to 100 ml of 2 M ammonium acetate (20).

As a model system for superoxide production we used a *mitochondrial P450 system* that transfers electrons from NADPH to P450 via two electron transfer proteins, adrenodoxin reductase and adrenodoxin (21). In the absence of substrate, P450 reduces O_2 directly, producing superoxide radicals (18). The rate of NADPH oxidation of this system was determined at 340 nm during the linear phase of the reaction (18).

Enzymatic reactions generating superoxide were carried out at 37°C, in 0.1 or 0.5 ml of 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer, pH 7.2, and 100 mM KCl. In addition to the protein components of the P450 system (18), the reaction included 6% methanol, 10 U/ml bovine erythrocyte superoxide dismutase, and 200 U/ml bovine liver catalase that are necessary for the conversion of superoxide to H_2O_2 and subsequent oxidation of methanol to formaldehyde.

The reactions were stopped by adding 2 reaction vol of Nash reagent, and immersing immediately in a 60°C bath for 10 min. The reaction mixture was then transferred to a 2-ml glass test tube, or a 3-ml cuvette, and

fluorescence ($\lambda_{ex} = 412$ nm, $\lambda_{em} = 505$ nm) was measured using a Perkin–Elmer LS-5B spectrometer. If necessary, the samples and the standards were adjusted to the minimum volume necessary for measurement, with reaction buffer. The slope of the standard curve may rise slightly over 24 h without the curve losing its linearity. Thus, samples can be read within 24 h as long as the standard solutions are processed and read together with the samples. Blank readings show no significant change in 24 h. For later measurement the samples were stored in the dark at 4°C.

In all experiments, control tubes included all reaction components, except the catalytic amount of reductase used to start the superoxide production, and their fluorescence was subtracted from the sample readings.

RESULTS AND DISCUSSION

H_2O_2-Dependent Formation of Formaldehyde

The present assay is based on the reaction scheme shown in Fig. 1. Superoxide is first dismutated to H_2O_2. Catalase then utilizes H_2O_2 to oxidize methanol by a two-step process: (i) destruction of H_2O_2 by two electron reduction, forming two hydroxyl radicals complexed with the heme of the enzyme (termed compound I in the literature); (ii) oxidation of the alcohol by this complex (22). In the absence of an alcohol, compound I is reduced by a second molecule of H_2O_2 to form O_2 and H_2O, completing the usual catalase reaction.
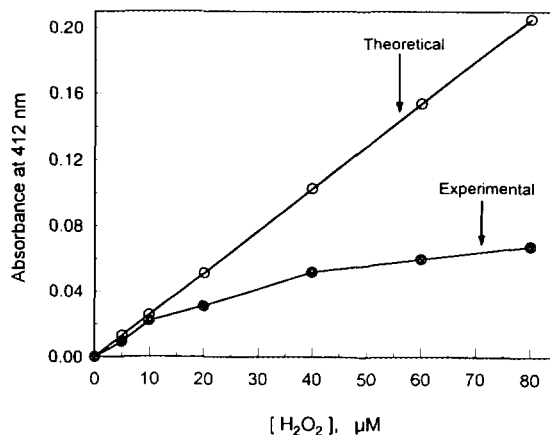

FIG. 2. The effect of H_2O_2 concentration on alcohol oxidase activity of catalase assayed by methanol peroxidation to formaldehyde. H_2O_2 was added from a stock solution of 100 μM to a mixture of 10 mM Hepes buffer, 200 U/ml catalase, 10 U/ml SOD, and 6% methanol, to complete a final volume of 0.5 ml. The solutions were then reacted with 1 ml of Nash reagent for 10 min at 60°C, and absorbance was read at 412 nm. Therefore, while the abscissa values give concentration in 0.5 ml of reaction mixture, the ordinate absorbance values correspond to the concentration in 1.5 ml after addition of the Nash reagent. The theoretical curve was calculated by multiplying the final H_2O_2 concentration (in 1.5 ml) by 7.7 based on the extinction coefficient of final product, diacetyldihydrolutidine (DDL) (20).

Since H_2O_2 can reduce compound I at a much higher rate than alcohol (2.6×10^7 vs 0.2–1×10^3 $M^{-1} \times s^{-1}$) (22), alcohol oxidase activity of catalase predominates only at low concentrations of H_2O_2. As shown in Fig. 2, beyond 10 μM of H_2O_2, the alcohol oxidase activity of catalase loses its linear dependence on H_2O_2. The high concentrations of methanol (1.5 M) (12) and catalase in the reaction mixture prevent accumulation of H_2O_2 as each molecule of H_2O_2 is immediately consumed in conversion of methanol into formaldehyde. Thus, the reaction conditions favor reduction of compound I by methanol and not by H_2O_2. The alternative approach of adding methanol and catalase at the end of the reaction is not optimal, because the accumulated H_2O_2 would drive the reaction toward the usual catalase reaction rather than alcohol oxidation.

In the presence of reduced forms of transition metals, such as Fe, Cu, and Vd, H_2O_2 is decomposed by the Fenton reaction (1). Trace amounts of these metals are generally in oxidized form. Moreover, our assay conditions do not favor Fenton reaction because SOD prevents the reduction of metals by superoxide, and catalase reacts much faster with the H_2O_2 than the transition metals (1). Therefore, the addition of heavy metal chelators, such as desferrioxamine, is not necessary to protect against decomposition of H_2O_2.

Assay of Formaldehyde

The present assay uses the Nash method to quantitate formaldehyde (20). The reaction is terminated by adding

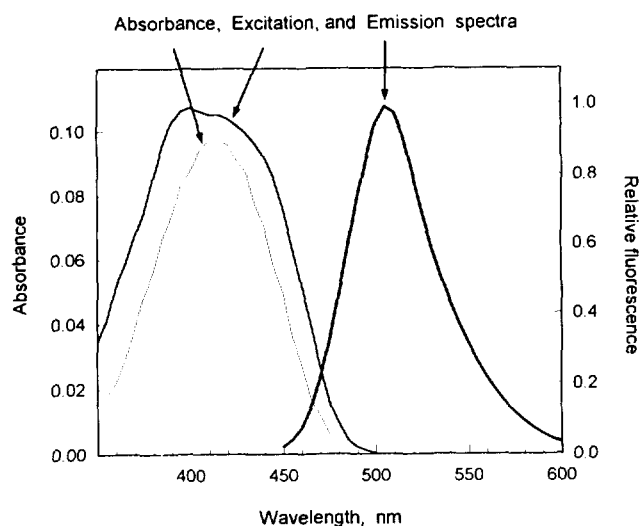Absorbance, Excitation, and Emission spectra

FIG. 3. Absorption, excitation, and emission spectra of diacetyldihydrolutidine (DDL), product of the reaction of formaldehyde with the Nash reagent. Formaldehyde (37 μM) in Hepes buffer was reacted with the Nash reagent as described in text. The absorbance corresponds to 12.3 μM of DDL after 1:3 dilution with the Nash reagent. Excitation and emission spectra were recorded with $\lambda_{em} = 505$ nm and $\lambda_{ex} = 412$ nm, respectively.

TABLE 1

Effect of Some Buffers and Reagents on the Fluorimetric Assay

Tested reactant	Concentration	% Interference
Blank (Hepes)	10 mM	0
K phosphate	10 mM	0
Tris	10 mM	0
NADPH	1 mM	5
Dimethyl sulfoxide	1.5%	<2
Dimethyl formamide	1.5%	14
Tween 20	0.3%	6
Bovine serum albumin	0.4 mg/ml	3
Adrenodoxin	9 μM	26

The fluorescence of 10 mM Hepes buffer was taken as 100%, hence its interference expressed as 0%. The percentage interference of tested reagents is based on their fluorescence intensities relative to the blank.

the Nash reagent and heating to convert accumulated formaldehyde to diacetyldihydrolutidine (DDL) (23).

Noting that DDL is fluorescent we established a method for formaldehyde assay based on the fluorescence rather than absorbance of DDL (20). DDL fluoresces with a broad excitation spectrum and a sharp emission spectrum peaking at 505 nm (Fig. 3). The excitation wavelength of 412 nm was chosen to avoid uv region of NAD(P)H absorbance, and to permit the use of glass tubes for fluorescence measurement. At this λ_{ex} up to 1 mM NADPH did not show significant interference with the assay (Table 1). In contrast, in other fluorimetric methods, even low levels of NAD(P)H (<50 μM) emit strong overlapping fluorescence prohibiting their use in systems with NAD(P)H (19).

The standard curve based on the fluorescence of DDL is highly reproducible and permits the measurement of 1 μM of H_2O_2 in the reaction sample (0.33 μM after 1:3 dilution of the sample with Nash reagent) (Fig. 4). The coefficient of variation in the range of 1–10 μM of formaldehyde was <15%. In contrast, the spectrophotometric assay of DDL is limited in its sensitivity to about 10 μM H_2O_2 in the sample (3.3 μM after 1:3 dilution of the sample with Nash reagent) because of its relatively low extinction (7.7 $mM^{-1} \times cm^{-1}$) (20). For the spectrophotometric measurement of 10 μM DDL the coefficient of variation was unacceptably high (31%); the method was not usable below this concentration. Thus, the fluorimetric method improves the limit of detection of the assay nearly 10-fold, relative to the spectrophotometric method (12).

The fluorimetric assays for H_2O_2 that use fluorescent probes as substrates for horseradish peroxidase are more sensitive than the present method (19). However, these assays cannot be used in the presence of NADPH for several reasons: The fluorescence spectrum of NADPH overlaps with that of most fluorogenic sub-
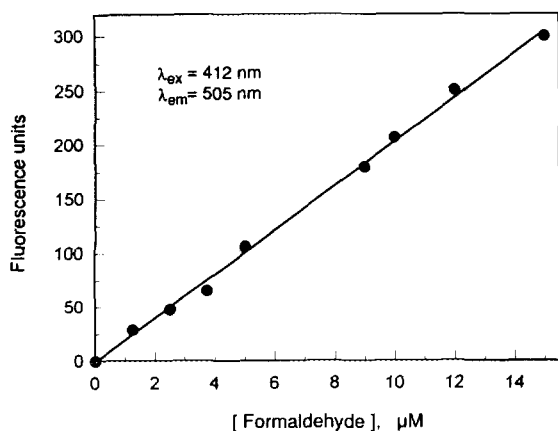
FIG. 4. Standard curve for DDL fluorescence based on formaldehyde reaction with the Nash reagent. The standards were prepared by adding aliquots of a stock solution of 125 μM formaldehyde to complete a final volume of 0.5 ml of 10 mM Hepes buffer, and then reacted with the Nash reagent as described in the text. The values on the abscissa represent the formaldehyde concentration before dilution with the Nash reagent. Since the formation of one molecule of formaldehyde depends on one molecule of H_2O_2, the fluorescence values can be used to estimate directly the H_2O_2 concentration in the reaction samples. Fluorescence was measured at $\lambda_{ex} = 412$ nm, and $\lambda_{em} = 505$ nm. The correlation coefficient (r) was >0.99 in all assays. Each point represents the mean of three independent measurements. The standard deviation at each point was <15% of the mean in the range of 1–15 μM formaldehyde.

strates of horseradish peroxidase, causing major interference with the assay (Fig. 5 and Refs. 15, 19). The fluorescence of fluorescin product does not interefere with that of NADPH, but fluorescin is not specific for H_2O_2 and may react with hydroperoxides generated as intermediates by redox systems (24). In the presence of peroxidase and H_2O_2, NADPH is oxidized, producing superoxide (1). The magnitude of this effect depends on the concentration of peroxidase and H_2O_2 and could contribute significant interference with the assay.

In terminating the reaction, the high concentration of salt in the Nash reagent inhibits the activity of our enzyme system (25) and the sample can be taken directly for fluorescence measurement without previously used trichloroacetic acid precipitation step (12). Although our assays were carried out in Hepes, other common buffers can also be used without interference (Table 1). High concentration of bovine serum albumin also shows little interference (Table 1), yet proteins such as adrenodoxin with an absorbance peak (414 nm) close to the excitation wavelength of 412 nm may interfere with the assay. Fluorescence contribution of such proteins may be eliminated by including them in the blank.

Validation of the Assay in an Enzymatic Electron Transfer System

We developed this assay to quantitate the electrons that leak from mitochondrial P450 systems, and react

with O_2 to form oxy-radicals. In these systems electrons donated from NADPH are utilized in P450-catalyzed steroid hydroxylation:

NADPH $\rightarrow$ adrenodoxin reductase $\rightarrow$

adrenodoxin $\rightarrow$ cytochrome P450.

Thus, the total electron flow can be quantitated simply by monitoring NADPH oxidation at 340 nm. In the absence of a steroid substrate, all electrons from NADPH are consumed in superoxide formation (18). Thus, theoretically, the stoichiometry of superoxide formation and NADPH oxidation would be expected to be equal. Indeed, when the reaction was carried out under these conditions, and superoxide was converted to H_2O_2 by SOD, the rate of H_2O_2 formation correlated highly ($r > 0.95$) with the rate of NADPH oxidation. This indicates that the present method converted superoxide to DDL quantitatively. The presence of 6% methanol in the reaction solution did not affect the activity of the enzymes. Catalase had a small effect (<20%) on electron leakage and cholesterol side chain cleavage reaction catalyzed by the P450. Nevertheless, the effect of these reactants should be checked in each system.

The findings above validate the fluorimetric assay and demonstrate that it allows estimation of both NADPH oxidation and superoxide/H_2O_2 formation in the same sample. Therefore, the assay should be useful in the quantitation of superoxides or H_2O_2 in the presence of pyridine nucleotides, in reconstituted P450 and other oxy-radical generating enzymatic systems as in neutrophils, liver and other organs (1,2,6,26). The method may also be useful for crude preparations after sample clean-up by a previously described method (12).
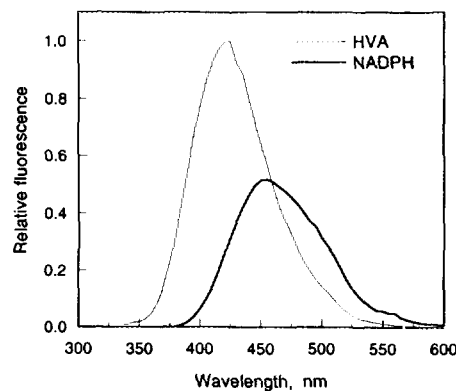

FIG. 5. Emission spectra of NADPH and homovanillic acid-H_2O_2 adduct (2,2-dihydroxy-3,3 dimethoxybiphenyl-5,5-diacetic acid). H_2O_2 (2 μM) in Hepes buffer was reacted with 450 μM homovanillic acid, in the presence of 0.5 U/ml horseradish peroxidase. NADPH concentration in the same buffer was 20 μM. Excitation wavelength was 315 nm.

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