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COUPLING OF DIHYDRORIBOFLAVIN OXIDATION TO THE FORMATION OF THE HIGHER VALENCE STATES OF HEMEPROTEINS

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SUMMARY: The reactions between hydrogen peroxide and hemeproteins have been coupled to the oxidation of dihydroriboflavin so as to provide a simple method for measuring the rate constant of hemeprotein peroxidation. Dihydroriboflavin rapidly reduces the higher oxidation states of iron and the hydroxy radicals which are the products of the hemeprotein / hydrogen peroxide reaction. The rapid reduction of these highly reactive compounds prevents the hemeproteins from undergoing irreversible chemical modifications and thus allows the kinetics of peroxidation to be studied. The rate constants at pH 7.2 and 23°C for the peroxidation of horseradish peroxidase, myoglobin, and ferrocytochrome *c* are found to be 6.2 x 10^6, 7.5 x 10^4, and 8 x 10^3 $M^{-1}s^{-1}$, respectively. These studies suggest that reduced riboflavin might efficiently protect cells from oxidative damage such as that occurring in inflammation and reperfusion injury. © 1991 Academic Press, Inc.

The higher oxidation states of peroxidases are participants in the catalytic mechanisms of these enzymes. In order to better understand the structure-activity relationships, the higher oxidation states generated by mixing peroxidases and peroxides have been detected, quantitated, and studied extensively by direct uv-visible spectroscopy (1-3).

Such experiments are difficult or impossible to conduct with many hemeproteins other than peroxidases because of the high reactivities of their higher oxidation states. With such hemeproteins, the higher oxidation state derivatives undergo reactions which lead to irreversible modification and inactivation of the proteins and degradation of cellular components (1-12). In the presence of peroxides, hemeproteins catalyze the peroxidation of cellular lipids (5, 6, 13-17). The ferryl, Fe(IV)O, form of myoglobin has been postulated to be critical in the damage associated with reperfusion injury of cardiac and other muscles (18, 19).

In this paper we report a simple method for measuring rates of hemeprotein peroxidation based on the rapid reduction of the higher oxidation states by dihydroriboflavin. The method is not limited to those hemeproteins with stable higher oxidation states and is thus a useful technique for studying the mechanism of peroxide-dependent, hemeprotein-catalyzed cellular damage. The results of these studies suggest that reduced flavins might play a role in protecting cells from oxidative damage induced by hemeproteins in the presence of peroxide.

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MATERIALS AND METHODS

Materials. Horse heart metmyoglobin was obtained from ICN, horse heart ferricytochrome *c* (type III) and horseradish peroxidase were from Sigma, and riboflavin from Eastman. Hydrogen peroxide (30%) from EM Science was quantitated by photometry at 240 nm ($\varepsilon = 43.6\ M^{-1}cm^{-1}$).

General methods. Buffers for kinetic studies were prepared with sodium acetate (pH 3-5), potassium phosphate (pH 6-8), and sodium carbonate (pH 9-11). Spectral studies were conducted with a Uvikon Kontron 810 spectrophotometer using 1-cm light-path quartz cuvettes.

Photochemical reductions were carried out using EDTA as the source of electrons (20-22). The sample containing riboflavin and hemeprotein was placed in a quartz cuvette immersed in a Pyrex ice-water bath and flushed with N_2 in the dark for 20 min. In the dark, no reduction occurred on the experimental time-scale. Irradiation was carried out with a 650-watt tungsten-halogen lamp and the extent of reduction of riboflavin and ferric hemeprotein was spectrally monitored. During the irradiation, residual O_2 was eliminated thereby avoiding complicating oxidation reactions. A 5-min exposure was more than sufficient to completely reduce flavin and the ferric forms of myoglobin and cytochrome *c*. The ferric form of horseradish peroxidase was not reduced under these conditions.

The rate of formation of ferryl myoglobin from myoglobin and H_2O_2 was measured directly by spectrophotometry (10, 18). Metmyoglobin (0.2 to 2 μM) was first subjected to photochemical reduction with 0.03 μM riboflavin and 0.4 mM EDTA in 17 mM potassium phosphate, pH 7.2, at 23°C. The formation of the ferryl state of myoglobin was initiated by adding H_2O_2 in 1 to 10-fold excess over myoglobin under anaerobic conditions. Absorbance in the Soret region was followed with time and the spectrum of the final product was recorded. The riboflavin concentration was sufficiently low to avoid reducing ferryl myoglobin with dihydroflavin to a significant extent, but high enough to accomplish the photochemical reduction of metmyoglobin.

A method for measuring the rate of hemeprotein peroxidation by coupling to dihydroriboflavin oxidation. The reactions of myoglobin, ferrocytochrome *c*, and horseradish peroxidase with H_2O_2 were studied at 23°C by coupling to the oxidation of dihydroriboflavin. Rates were determined using 0.003 to 5 μM hemeprotein, 10 to 100 μM riboflavin, 0.5 to 10 μM H_2O_2, ~1 mM EDTA, and 10 to 35 mM buffer. The sample containing riboflavin, hemeprotein, EDTA, and buffer was made anaerobic, and a few μl of an anaerobic solution of H_2O_2 were then placed on the wall of the cuvette such that there was no mixing with the sample. After five min of flushing with N_2, the sample was subjected to photoreduction as described above. The peroxidation was then initiated by mixing the H_2O_2 and sample. The final concentration of dihydroriboflavin was greater than the final concentration of H_2O_2, so that the hemeprotein was protected during the reaction cycles. The oxidation of dihydroriboflavin was monitored by absorbance at 445 nm ($\varepsilon = 12.2\ mM^{-1}cm^{-1}$). Second-order rate constants were derived from either the dependence of the observed pseudo-first order rates on hemeprotein concentration or the dependence of the initial rates on H_2O_2 and hemeprotein concentrations. Corrections were made for the direct reaction of dihydroriboflavin with H_2O_2.

RESULTS

Addition of H_2O_2 to a photochemically-reduced mixture of riboflavin and hemeprotein initiated coupled oxidation of dihydroriboflavin to riboflavin, as exemplified in Fig. 1. In the pH range 3 to 8, the oxidation in the absence of hemeprotein was negligible in comparison with the hemeprotein-stimulated reaction. The observed initial reaction rates with horseradish peroxidase, myoglobin, and ferrocytochrome *c* increase linearly with hemeprotein concentration and H_2O_2 concentration, but are independent of dihydroriboflavin concentration. The observed half-life times of reaction show linear dependence on hemeprotein concentration. Thus the reactions are first-order with respect to hemeprotein and H_2O_2 and zero-order with respect to dihydroriboflavin. In each of the

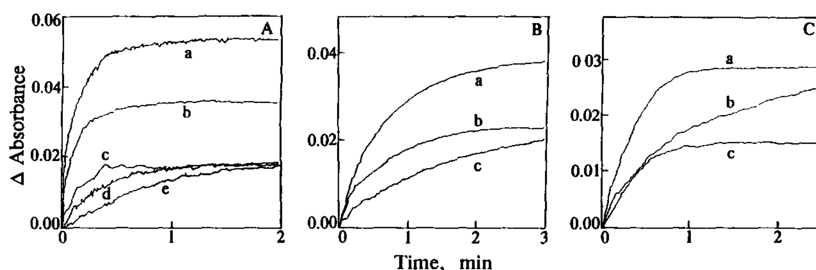

FIG. 1. Hemeprotein peroxidations as followed by coupling the reaction to dihydroriboflavin oxidation. Change of absorbance at 445 nm was monitored with time. All solutions contained 0.93 mM EDTA and 33 mM potassium phosphate buffer, pH 7.2. (A) Horseradish peroxidase in the presence of 42 μM dihydroriboflavin. a, 13 nM peroxidase and 5.2 μM H_2O_2; b, 13 nM peroxidase and 3.7 μM H_2O_2; c, 13 nM peroxidase and 1.4 μM H_2O_2; d, 6.6 nM peroxidase and 1.4 μM H_2O_2; e, 3 nM peroxidase and 1.4 μM H_2O_2. (B) Myoglobin in the presence of 22 μM dihydroriboflavin. a, 0.43 μM myoglobin and 3.7 μM H_2O_2; b, 0.43 μM myoglobin and 2.2 μM H_2O_2; c, 0.22 μM myoglobin and 2.2 μM H_2O_2. (C) Ferrocytochrome *c* in the presence of 21 μM dihydroriboflavin. a, 4.5 μM cytochrome *c* and 2.4 μM H_2O_2; b, 2.3 μM cytochrome *c* and 2.4 μM H_2O_2; c, 4.5 μM cytochrome *c* and 1.2 μM H_2O_2.

reactions, the stoichiometry is 1.0 ± 0.2 mole of dihydroriboflavin oxidized per mole of H_2O_2 consumed and the final spectrum of the oxidized dihydroriboflavin is identical to that of riboflavin.

As measured with this coupling system, the second-order rate constants, *k*, of hemeprotein peroxidations at neutral pH and 23°C are 6.2 x 10^6, 7.5 x 10^4, and 8 x $10^3 M^{-1}s^{-1}$ for horseradish peroxidase, myoglobin, and ferrocytochrome *c*, respectively. The rate constants are unchanged over the pH range from 3 to 8 (Fig. 2). In alkaline solution (pH 8 - 10) the plots of log *k* *vs* pH show a slope of -1, indicating the involvement of a single proton-dissociating group. pK_a values of 9.2, 8.2, and 7.8 were obtained for horseradish peroxidase, myoglobin, and ferrocytochrome *c*, respectively. Spectral analysis shows that during the course of the reaction, both myoglobin and cytochrome *c* remain in the ferrous state generated by the photochemical reduction procedure, but horseradish peroxidase remains in the ferric state both after the photochemical reduction procedure and during the peroxidation reaction (Fig. 3).

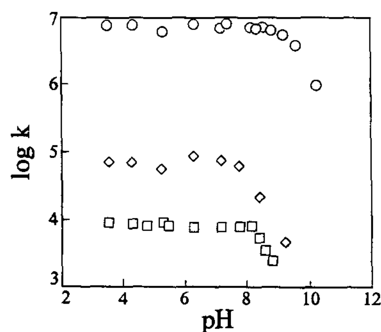

FIG. 2. pH profiles of the secondary rate constants for peroxidation of hemeproteins. See text for details.

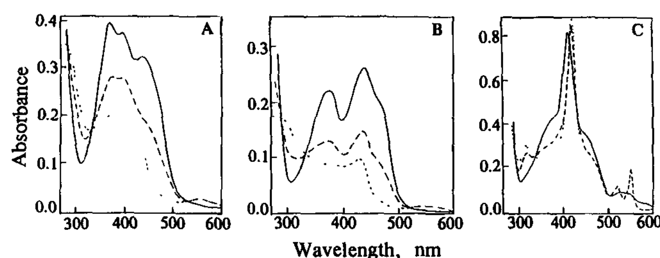

FIG. 3. Spectral changes occurring upon hemeprotein peroxidations. (A) Horseradish peroxidase. The solution contained 1.9 μM horseradish peroxidase, 14 μM riboflavin, 0.9 mM EDTA, and 16 mM potassium phosphate buffer, pH 7.2. (—), before irradiation; (···), after irradiation; (– – –), 5 min after the solution was made 8 μM in H_2O_2. (B) Myoglobin. The solution contained 0.5 μM metmyoglobin, 20 μM riboflavin, 1 mM EDTA, and 35 mM sodium acetate buffer, pH 5.3. (—), before irradiation; (···), after irradiation; (– – –), 10 min after the solution was made 7 μM in H_2O_2. (C) Cytochrome *c*. The solution contained 4.5 μM cytochrome *c*, 20 μM riboflavin, 0.9 mM EDTA, and 33 mM potassium phosphate buffer, pH 7.2. (—), before irradiation; (···), after irradiation; (– – –), 15 min after the solution was made 10 μM in H_2O_2.

As measured by direct spectrophotometry (Fig. 4), the formation of ferryl myoglobin from myoglobin and H_2O_2 followed simple second-order kinetics. A rate constant of 8.0×10^4 $M^{-1}s^{-1}$ was calculated. During the peroxidation, the appearance of a spectrum with absorbance maxima at 424, 550, and 584 nm confirmed that ferryl myoglobin is the primary product of this reaction (10). After a few minutes of reaction, the ferryl myoglobin began to gradually revert to the ferric form. Under the same conditions, it was not possible to directly measure the formation of peroxidation products of cytochrome *c* because a rapid bleaching reaction occurred immediately after mixing ferrocytochrome *c* with H_2O_2.

DISCUSSION

The coupling of dihydroriboflavin oxidation to the reactions between hemeproteins and H_2O_2 provides the basis for a convenient and rapid method for measuring the rates of peroxidation of

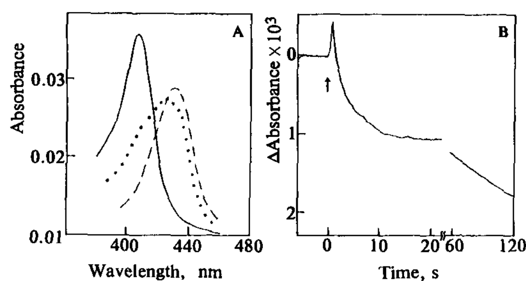

FIG. 4. Reaction of myoglobin and H_2O_2 as followed by direct photometric measurement of the formation of ferryl myoglobin. (A) Formation of ferryl myoglobin. The solution contained 0.22 μM metmyoglobin. See text for details. (—), before irradiation; (– – –), after irradiation; (···), 1 min after the solution was made 1.2 μM in H_2O_2. (B) Absorbance change at 432 nm with time. H_2O_2 was added as indicated by the arrow. The slower step corresponds to ferryl myoglobin conversion to metmyoglobin.

hemeproteins. The overall reaction is: Flavin-H_2 + H_2O_2 → Flavin + 2 H_2O. In contrast to the use of direct spectrophotometry for measuring the rate of formation of the higher valence states of hemeproteins, the coupling method described in this paper is not limited to those hemeproteins which yield stable oxidation products in the presence of peroxide. Horseradish peroxidase, myoglobin, and cytochrome *c* all remain intact under the conditions of the assay (Fig. 3). The peroxidation reactions are zero-order with respect to dihydroriboflavin, indicating that this compound reduces the products of the peroxidation reaction rapidly, thereby protecting the hemeprotein from being destroyed and making the peroxidation reaction the rate-limiting step. The reduced flavin plays two additional roles in the assay—it serves in the photochemical reduction reaction to shuttle reducing equivalents from EDTA to hemeproteins and it serves as the reagent the disappearance of which is used as a measure of the overall coupled reaction.

Whereas the procedure is effective in measuring the rate of peroxidation for each of the three hemeproteins, each peroxidation proceeds by a different scheme. Horseradish peroxidase, which remains predominantly in its ferric form throughout the procedure, catalyzes the peroxidation of dihydroriboflavin as follows:

$$\text{Horseradish peroxidase-Fe(III)} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + \text{Flavin}\cdot H_2 \rightarrow \text{Horseradish peroxidase-Fe(III)} + \text{Flavin} + H_2O \quad (2)$$

The rate constant of $6.2 \times 10^6\ M^{-1}s^{-1}$ obtained in this procedure may be assigned to the rate-limiting step, the formation of Compound I. This value is similar to the value of $10^7\ M^{-1}s^{-1}$ reported for rate constant of the direct photometric measurement of the formation of Compound I (3) and the value of $2 \times 10^6\ M^{-1}s^{-1}$ measured at pH 6 in a coupling system using the redox dye 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) as the coupler (23).

In contrast, myoglobin catalyzes the reaction between H_2O_2 and dihydroriboflavin as follows:

$$\text{Myoglobin-Fe(II)} + H_2O_2 \rightarrow \text{Myoglobin-Fe(IV)O} + H_2O \quad (3)$$

$$\text{Myoglobin-Fe(IV)O} + \text{Flavin-}H_2 \rightarrow \text{Myoglobin-Fe(II)} + \text{Flavin} + H_2O \quad (4)$$

Prior to adding H_2O_2, irradiation in the presence of EDTA yields dihydroriboflavin which in turn reduces ferric myoglobin. Adding H_2O_2 generates ferryl myoglobin in the rate-limiting step (24, 25), and the ferryl myoglobin then rapidly oxidizes dihydroriboflavin. This scheme is confirmed by the results of the direct spectrophotometric measurement of ferryl myoglobin formation (Fig. 4). The observed rate constant of $8.0 \times 10^4\ M^{-1}s^{-1}$ for the direct measurement agrees well with the value of $7.5 \times 10^4\ M^{-1}s^{-1}$ obtained with the coupled system. Previously, the value for k had been inferred from a variety of experiments to be much greater than $10^3\ M^{-1}s^{-1}$ (18, 24, 25).

Unlike peroxidase and myoglobin, cytochrome *c* does not readily bind and activate H_2O_2 to form a ferryl state, but nonetheless is bleached by peroxides (13) and induces lipid peroxidation (13-16, 26). The catalysis of dihydroriboflavin peroxidation by cytochrome *c* observed in the present study is proposed to proceed by the following scheme involving a Fenton-type reaction:

$$\text{Cytochrome } c\text{-Fe(II)} + H_2O_2 \rightarrow \text{Cytochrome } c\text{-Fe(III)} + OH\cdot + OH^- \quad (5)$$

$$\text{Cytochrome } c\text{-Fe(III)} + \text{Flavin}\cdot H_2 + OH^- \rightarrow \text{Cytochrome } c\text{-Fe(II)} + \text{Flavin-H}\cdot + H_2O \quad (6)$$

$$OH\cdot + \text{Flavin}\cdot H_2 \rightarrow H_2O + \text{Flavin-H}\cdot \quad (7)$$

$$2\ \text{Flavin-H}\cdot \rightarrow \text{Flavin}\cdot H_2 + \text{Flavin} \quad (8)$$

Reaction 5 would be rate-limiting, and oxidation of dihydroriboflavin would proceed rapidly by its reactions with ferricytochrome *c* and hydroxy radical. Reaction 5 has been proposed as the initial reaction between ferrocytochrome *c* and H_2O_2 (27) and reactions 6, 7, and 8 have been reported to proceed rapidly (28-30). We propose that the bleaching of cytochrome *c* which we observe in the absence of dihydroriboflavin results from reaction of cytochrome *c* with hydroxy radical, and that dihydroriboflavin protects cytochrome *c* by reducing the hydroxy radical. The reported cytochrome *c -dependent* oxidation of membranes might likewise be attributed to hydroxy radicals generated by such a redox cycle, rather than to radicals generated by iron released from cytochrome *c* (13).

Our results show that coupling to dihydroriboflavin is a useful tool with which to investigate peroxidation reactions of peroxidases and non-peroxidase hemeproteins. Its usefulness arises from its rapid reaction with the higher oxidation states of hemeproteins and hydroxy radical. Oxidations of hemeproteins by either superoxide or peroxides and related radical generations are currently under intense study because of their relevance to cellular damage. Our results suggest that flavins might be good candidates as therapeutic agents for decreasing the oxidative damage resulting from processes like ischemia and reperfusion. Arguments have been made that reducing compounds could diminish the extent of ferryl myoglobin production and / or increase the rate of ferryl state myoglobin reduction during reperfusion (31-34). Reduced riboflavin is an appealing candidate for use as such a therapeutic agent because this naturally occurring compound can enter cells, is readily reduced intracellularly, and is non-toxic at effective concentrations.

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