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ELECTRON ACCEPTOR FUNCTION OF O_2 IN RADICAL N-DEMETHYLATION REACTIONS CATALYZED BY HEMEPROTEINS

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SUMMARY

In aerobic solutions, O_2 consumption correlated well with N-demethylation of N,N-dimethyl-p-toluidine catalyzed by horseradish peroxidase, in the presence or absence of H_2O_2. In the absence of added H_2O_2, superoxide dismutase stimulated, and catalase inhibited, both reactions; in the presence of H_2O_2, argon inhibition of formaldehyde production increased with increasing concentration of horseradish peroxidase. These results provide evidence for competing reactions of the enzymatically-generated substrate radical: oxidation by O_2 increases formaldehyde production, while radical dimerization decreases the yield of this product. Implications of these findings for similar reactions catalyzed by microsomal cytochrome P-450 are suggested.

INTRODUCTION

It has been shown that horseradish peroxidase and other hemeproteins can catalyze the hydroperoxide-dependent N-demethylation of several electron donor substrates of liver microsomal cytochrome P-450 (1,2). EPR, and other, data have supported the following radical mechanism of these reactions (1,2):

$$R_2N\text{-}CH_3 \xrightarrow{-e^-} R_2N\text{-}CH_3^{+\cdot} \xrightarrow{-H\cdot} R_2\overset{+}{N}{=}CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C{=}O \quad (1).$$

More recently, the aminopyrine radical intermediate was detected during the oxidation of this compound by cumene hydroperoxide catalyzed by purified liver microsomal cytochrome P-450 (3). In this report, results of a more detailed study of the oxidation of N,N-dimethyl-p-toluidine (DPT)[1] catalyzed by horse-

[1] Abbreviations: DPT, N,N-dimethyl-p-toluidine; DMA, N,N-dimethylaniline.

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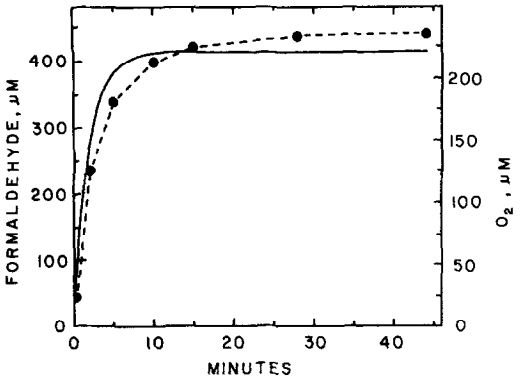

Fig. 1. Kinetics of formaldehyde production and oxygen consumption during the aerobic oxidation of DPT by H_2O_2 catalyzed by horseradish peroxidase. Oxygen (——) and formaldehyde (●) were measured separately in reactions containing air-saturated 0.1 M potassium phosphate buffer pH 6.5, 3 mM DPT, 0.4 mM H_2O_2 and 2 nM peroxidase, at 37°C.

radish peroxidase are described, which demonstrate the involvement of molecular O_2 as an electron acceptor in this reaction.

METHODS

Enzymes which were supplied by Sigma included: horseradish peroxidase (type VI, salt-free powder), catalase (purified powder from bovine blood), and superoxide dismutase (lyophilized powder from bovine blood); their activities were assayed by standard procedures. Metmyoglobin was isolated from beef heart (4). The N-methyl substrates were purchased from Aldrich; DPT and DMA[1] were distilled before use. Formaldehyde was assayed by the Nash procedure (5) after quenching the reaction with 15% trichloroacetic acid. A Clark-type electrode was used to measure O_2 concentrations in reaction mixtures maintained at constant temperature.

RESULTS

The kinetics of formaldehyde production and oxygen utilization correlated closely during the peroxidase-catalyzed oxidation of DPT by H_2O_2, as shown in Fig. 1. The initial rates of change of both parameters were maximal near pH 6.5. At this pH, the dependence of the rates on H_2O_2 concentration could be fit by Michaelis-Menten plots with apparent K_m values of 45 and 86 μM H_2O_2, and turnover numbers of 7.4×10^4 and 5.8×10^4 moles/min/mole enzyme, for formaldehyde production and O_2 uptake, respectively. In the absence of any added H_2O_2, aerobic mixtures of the enzyme and DPT also consumed measurable amounts

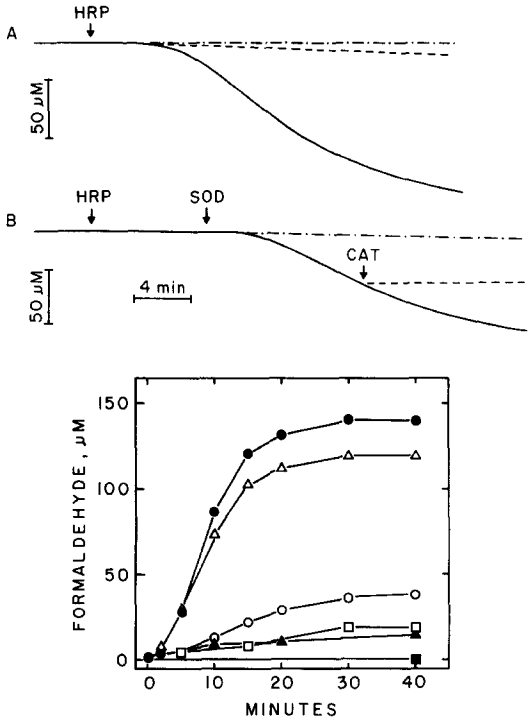

Fig. 2. Oxygen consumption (top) and formaldehyde production in aerobic mixtures of DPT and horseradish peroxidase. Components were 3 mM DPT, 2 nM peroxidase, and 0.1 M potassium phosphate buffer, at the stated pH and temperature, with additions as noted. Panel A and closed symbols, pH 6.0: 25°C (——,●); 37°C (- - -,▲); control for 25°C expt. with DPT or enzyme omitted (—·—,■). Panel B and open symbols, pH 7.0: 25°C (—·—,○); 25°C with 20 μg/ml superoxide dismutase added at indicated point (——) or before initiating the reaction (△); 25°C, with superoxide dismutase, as described, and also 42 nM catalase added at indicated point (- - -) or prior to initiation (□).

of O_2. As shown in Fig. 2, O_2 uptake absolutely required both the enzyme and substrate, and was quite sensitive to temperature and pH. The very low rate of O_2 consumption measured at pH 7.0 and 25°C was greatly stimulated by superoxide dismutase and completely inhibited by the subsequent addition of catalase; moreover, formaldehyde production under identical conditions correlated well with O_2 consumption (Fig. 2).

In the presence of H_2O_2 and argon, the decreasing yield of formaldehyde with increasing enzyme concentration (Table 1) correlated qualitatively with more intense color development: a deep purple with 50 nM enzyme and black with 200 nM enzyme. In preliminary experiments, the conditions required for

TABLE 1

Effect of argon on formaldehyde produced during N-demethylation of DPT by H_2O_2 catalyzed by horseradish peroxidase

Enzyme concentration	2 nM	50 nM	200 nM
Formaldehyde, μM			
Air	390	373	325
Argon	240	150	65

Experimental conditions: 15-min incubations at 37°C in 0.1 M potassium phosphate buffer pH 6.5, with 3 mM DPT and 0.4 mM H_2O_2, under air or argon, as indicated.

oxidizing DPT, with H_2O_2 and horseradish peroxidase (in air-saturated buffers), to a stable purple product absorbing at 545 nm were investigated. The relative amount of this product was decreased, and the amount of formaldehyde increased, by decreasing the concentration of both the enzyme and H_2O_2. The metmyoglobin-catalyzed N-demethylation of DPT resulted in O_2 consumption only in the presence of H_2O_2, and this reaction required considerably larger concentrations of both H_2O_2 and the hemeprotein than in Fig. 1. This result is consistent with the low peroxidatic activities of metmyoglobin toward DPT and other compounds (1,2). The oxidation of DMA by H_2O_2 catalyzed by horseradish peroxidase occurred with concomitant O_2 uptake, which exhibited the same pH dependence, but only 5% the rate of N-demethylation of this compound (2). Measurable O_2 utilization was observed during aminopyrine oxidation by the peroxidase-H_2O_2 system only when the enzyme concentration was less than about 5 nM, and the initial rates of O_2 uptake and product formation displayed different pH behavior (1,6). In the absence of H_2O_2, no measurable O_2 uptake occurred in aerobic solutions of peroxidase and either DMA or aminopyrine, under any conditions examined.

DISCUSSION

These experimental results indicate that the DPT radical, previously detected by EPR during the peroxidase-catalyzed oxidation of DPT by H_2O_2 (2), can reduce O_2 to O_2^-. A similar reaction, between the NAD· radical and O_2, is an

essential component of the NADH oxidase activity of peroxidase, which is, however, inhibited by superoxide dismutase (7). The different effects of superoxide dismutase on the NADH and DPT oxidase activities of this enzyme result from very different rate constants for reduction of Compound II by the respective substrates: 8×10^2 $\underline{M}^{-1}s^{-1}$ for NADH (7) and 1.2×10^5 $\underline{M}^{-1}s^{-1}$ for DPT (8). Enzymatic oxidation of NADH is actually less efficient than a radical chain process, i.e., reduction of O_2 to O_2^- by NAD$^\cdot$, followed by one-electron oxidation of NADH by O_2^-, which sustains the chain (7). Thus, the chain process is inhibited by superoxide dismutase. However, in the case of DPT, catalysis of O_2^- dismutation supplies H_2O_2 for rapid enzymatic generation of the DPT radical, thereby sustaining both O_2 consumption and formaldehyde production, Eq. (1). It is clear that the rates of all reactions involved must fall within critical ranges to propagate the radical chain process. If the rate of any reaction is changed significantly, e.g., decreasing the rate of spontaneous dismutation of O_2^- by increasing the pH (9), then a dramatic effect on the overall rate will occur (cf. Fig. 2). In the absence of added H_2O_2, slow autoxidation of DPT is presumed to form the O_2^- and DPT radicals, which initiate the chain reaction if peroxidase is present and other kinetic requirements are met.

These findings have also demonstrated that, even under the mild conditions of enzymatic generation of radical species, the distribution of stable reaction products will be determined, in a complex way, by many experimental variables. Based on the faster rate of enzymatic N-demethylation of DPT relative to DMA and the greater stability of the DPT radical compared to the DMA radical (2,10), it was suggested (2) that the <u>para</u> methyl substituent of DPT inhibits the radical dimerization previously reported to occur in the peroxidase-catalyzed oxidation of DMA (11). However, we have presented evidence that the DPT radical also undergoes coupling reactions which become relatively more important as the rate of radical generation increases and the concentration of electron acceptors becomes limiting. Previous studies of the

electrochemical oxidation of DPT (12) have suggested that dimerization occurs at the less favorable ortho position. The reactivity of the two substituted aniline radicals contrasts with that of the more stable aminopyrine radical, which, at high radical concentrations, undergoes radical dismutation in preference to dimerization (1,13). In the peroxidase-H_2O_2 system, steady-state levels of the aminopyrine radical intermediate correlate well with formaldehyde produced under many experimental conditions, including high rates of radical generation (1,6). Since an aminopyrine radical can be oxidized by an identical radical, dimerization does not compete with formaldehyde production when other electron acceptors are unavailable.

The facile one-electron oxidation of various N-methyl substituted amines by many chemical and enzymatic systems (1,2) suggests that the microsomal monooxygenation of these compounds may be more complex than has been considered. Two distinctive properties of microsomes are: 1) very low rates of substrate oxidation, and consequent low concentrations of radical species and 2) the relatively high concentration of O_2 dissolved in the lipid phase compared to the aqueous phase. Thus, the oxidation of membrane-bound radicals by O_2 would be much more probable than radical-radical reactions; moreover, EPR detection of radicals under these conditions would be very unlikely. The results of this study do not support the suggestion in a recent review on cytochrome P-450 (14) that radical species of N-methyl compounds would not react with O_2. Studies of the microsomal oxidation of N-methyl compounds are now in progress to test the ideas suggested by these findings.

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