[Chem. Pharm. Bull. 33(10)4471—4477(1985)]

Cumene Hydroperoxide-Supported *N*-Demethylation of *N*,*N*-Dimethylanilines Catalyzed by Catalase

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(Received January 21, 1985)

The stoichiometry and kinetic mechanism of the cumene hydroperoxide (CHP)-supported *N*-demethylation of *N*,*N*-dimethylaniline (DMA) catalyzed by catalase were studied. The formations of formaldehyde, 2-phenyl-2-propanol and *N*-methylamiline, and the consumption of DMA exhibited a 1 : 1 : 1 : 1 stoichiometry up to 5 min after initiation of the reaction. Plots of reciprocal initial velocity *versus* the reciprocal concentration of either substrate at several different fixed concentrations of the other substrate resulted in a family of straight lines which converged to a common intersection point on the left side of the ordinate and above the abscissa, suggesting a sequential mechanism involving the formation of a ternary complex of catalase, DMA and CHP followed by one or more reactions and the subsequent release of the products. *N*,*N*,*N*′,*N*′-Tetramethylbenzidine radical cation was detected by electron spin resonance spectroscopy during the CHP-supported oxidation of DMA, whereas the corresponding amine radical cations were detected during the oxidations of *N*,*N*-dimethyl-*p*-toluidine and *N*,*N*-dimethyl-*p*-anisidine. These results suggest that the mechanism of the CHP-supported oxidation of DMA catalyzed by catalase is the same as that of aminopyrine.

Keywords——aminopyrine; *N*,*N*-dimethylaniline; catalase; cumene hydroperoxide; *N*,*N*-dimethyl-*p*-toluidine; *N*,*N*-dimethyl-*p*-anisidine; ESR; peroxidatic reaction; *N*-demethylation

Catalase ($H_2O_2 : H_2O_2$ oxidoreductase; EC 1.11.1.6) is a very efficient catalyst for the decomposition of H_2O_2 (catalatic reaction), but it also catalyzes the oxidation of primary alcohols, phenols, sodium azide, and hydroxylamine by H_2O_2 or ethyl hydroperoxide (EHP) (peroxidatic reaction).[1] A ping-pong mechanism involving the initial formation of catalase compound I has been proposed for the catalase-mediated reactions.[1b] Some workers claimed that *tert*-butyl hydroperoxide (BHP) and cumene hydroperoxide (CHP) do not support the peroxidatic reaction,[2] whereas other workers claimed that BHP and CHP do support the peroxidatic activity of catalase towards guaiacol[3] and aminopyrine.[4] In the previous papers,[5] we reported that aminopyrine (AMP) is oxidized to the AMP radical cation by CHP or EHP in the presence of catalase. Treatment with alkali followed by neutralization increased the *N*-demethylase activity of catalase and decreased both the peroxidatic activity towards methanol and the catalatic activity, and we suggested that the active site of catalase for the CHP-supported *N*-demethylation of AMP is different from that for the catalatic reaction.[5d] The results of steady-state kinetic analysis indicated that the CHP- and EHP-supported oxidations of AMP catalyzed by catalase proceed by a sequential mechanism involving the formation of a ternary complex,[5e] which is different from that generally proposed for catalase-mediated reactions.

In order to clarify the active species of the reaction, peroxyphenylacetic acid (PPAA) was used as a peroxide instead of CHP. Electron spin resonance (ESR) studies on the reaction of AMP with PPAA catalyzed by catalase indicated that homolytic oxygen–oxygen bond cleavage and oxy radicals generated from PPAA play no major role in the reaction, and hence

the PPAA-supported oxidation was considered to proceed in a similar manner to the CHP-supported oxidation of AMP.[5f]

However, the stoichiometry of the reaction was not established, since the oxidation of AMP generally gives very complex products.[6] The present study was undertaken to establish the stoichiometry and kinetic mechanism of the CHP-supported *N*-demethylation of *N*,*N*-dimethylanilines. *N*,*N*-Dimethylaniline (DMA) was used as a representative of the anilines, because DMA is most frequently employed as an *N*-methyl substrate for the monooxygenation and peroxidation reactions of hemeproteins,[7] and the results were compared with those for AMP. The oxidations of *N*,*N*-dimethyl-*p*-toluidine and *N*,*N*-dimethyl-*p*-anisidine were also studied briefly for comparison.

Experimental

Materials——Catalase (from bovine liver, C-40) was used as supplied by Sigma. AMP and CHP were obtained and purified as described previously.[5b] *N*,*N*-Dimethylaniline, *N*,*N*-dimethyl-*p*-toluidine, and *N*-methylaniline were obtained from commercial sources, and were distilled before use. *N*,*N*-Dimethyl-*p*-anisidine was prepared by the method of Thomas *et al.*[8] and purified by column chromatography on alumina with benzene as an eluent. 4-Methylaminoantipyrine was prepared by the method of Morita.[9] The buffer solution used for the kinetic study was 0.1 M NaH_2PO_4–Na_2HPO_4 (pH 7.4). Water was purified by the use of a Millipore MILLI-R/Q sytem. All other chemicals used were of reagent grade.

Methods——ESR spectra were recorded on JEOL JES-FE 1X spectrometer, equipped with a Union-Giken MX-7 mixing device and an ES-LC 11 flat cell. Free radicals were generated by the continuous flow method. One reservoir was filled with aqueous buffer solution containing CHP, and the other one was filled with a mixture of catalase and an aniline containing acetone (4%). Each solution was allowed to warm to 37 °C, then deoxygenated by passing nitrogen, and fed to the mixer through a micro tube pump at a flow rate of 2 ml/min. Computer simulation of the spectrum was carried out using a JEOL EC-100 computer system.

Formaldehyde was assayed by the Nash procedure,[10] after the reaction had been quenched with 10% trichloroacetic acid and the solution centrifuged to remove precipitated protein. For determination of 2-phenyl-2-propanol (cumenol), the reaction mixture (2.7 ml) was made acidic by the addition of 0.2 ml of 10% HCl, and 0.1 ml of 1 mM benzyl alcohol was added to the mixture as an internal standard. Two ml of the resulting mixture was extracted with 12 ml of ether by the use of Extrelut,® and 400 μl of 0.1 M citric acid–sodium citrate buffer (pH 4.5) was added to the ether extract. The ether was evaporated off and 100 μl of the residue was analyzed by high performance liquid chromatography (HPLC). HPLC was carried out with a Waters Associates model 510 solvent delivery system, U6k universal LC injector, Z-module radial compression separation system with 8NV C-18 (5 μ) cartridge, and Uvidec-100 IV UV detector (Japan Spectroscopic Co.). The mobile phase was a mixture of the citrate buffer and methanol (1 : 1), and the flow rate was 1.5 ml/min. The detector was operated at 254 nm.

For the determination of DMA and *N*-methylaniline, the reaction was quenched by the addition of an equal volume of chilled methanol (−40 °C), the mixture was filtered with a Millipore SJHV004NS (0.45 μm) and aliquots of 50 μl of the filtrate were injected into the HPLC apparatus and eluted with a mobile phase of 0.1 M citrate buffer (pH 3.5)–methanol (1 : 1) at a flow rate of 1.5 ml/min. The detector was operated at 254 nm.

Results

The rates of the CHP-supported oxidation of some aromatic amines catalyzed by catalase are given in Table I. As to the oxidations of AMP and 4-methylaminoantipyrine (MAP), the determination of cumenol by HPLC was unsuccessful because of interference from the oxidation products. Although 4-aminoantipyrine (AAP) and aniline did not give formaldehyde as a product, they were also oxidized in this system as evidenced by the formation of cumenol. However, attempts to identify the oxidation products of AAP and aniline were unsuccessful.

CHP-Supported Oxidation of AMP

Time courses of the formation of formaldehyde produced during the CHP-supported oxidations of AMP and MAP in the presence and absence of AAP are shown in Fig. 1. Demethylation of AMP was greatly depressed by addition of AAP, whereas that of MAP was

Table I. Catalytic Activities of Catalase for the CHP-Supported Oxidation of Aromatic Amines

Substrate	Rate (nmol product/min) HCHO formation	Cumenol formation
Aminopyrine	24	N.D.
4-Methylaminoantipyrine	45	N.D.
4-Aminoantipyrine	None	31
N, *N*-Dimethylaniline	44	46
N-Methylaniline	32	43
Aniline	None	10
N, *N*-Dimethyl-*p*-toluidine	38	36
N, *N*-Dimethyl-*p*-anisidine	48	47

The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), substrate (0.5 mM), catalase (0.125 mg/ml), and CHP (0.125 mM). The reaction was initiated by addition of CHP, and the mixture was incubated at 25 °C for 5 min, then assayed for formaldehyde and cumenol as described under Experimental. N.D.: not determined.

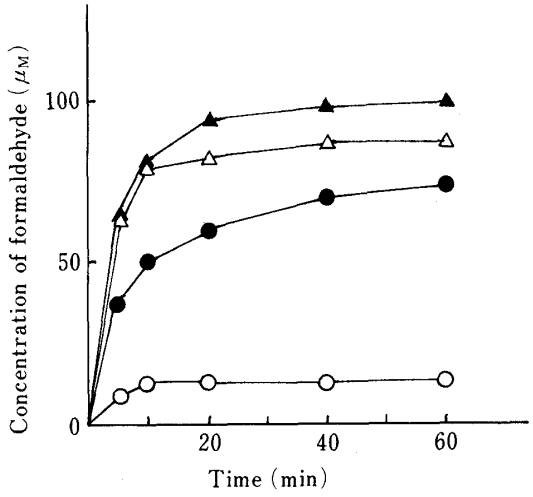

Fig. 1. Time Dependence of the CHP-Supported Oxidation of AMP and MAP

The reaction mixture contained 0.1 M phosphate buffer (pH 7.4), catalase (0.125 mg/ml), substrate (0.5 mM), and CHP (0.125 mM) at 25 °C.

AMP with (○) and without (●) addition of AAP (0.5 mM); MAP with (△) and without (▲) addition of AAP (0.5 mM).

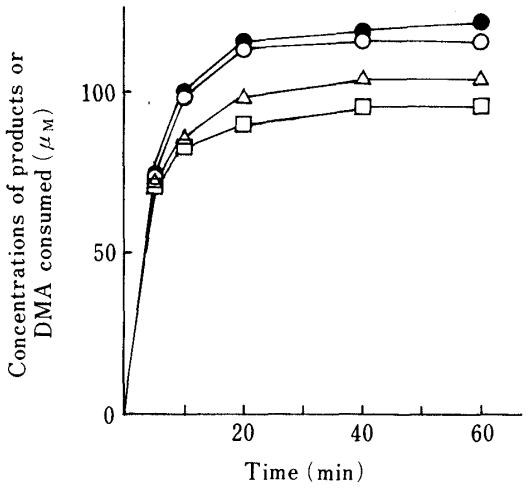

Fig. 2. Time Dependence of the CHP-Supported Oxidation of DMA

The reaction mixture contained 0.1 M phosphate buffer (pH 7.4), catalase (0.125 mg/ml), DMA (0.5 mM), and CHP (0.125 mM) at 25 °C

Formaldehyde formation (○); *N*-methylaniline formation (□); cumenol formation (●); DMA consumption (△).

only slightly depressed. Since AAP was also oxidized in this system without giving formaldehyde and the rate of oxidation is considered to be in the order MAP > AAP > AMP (Table I), these results indicate that MAP formed by the demethylation of AMP is oxidized immediately to AAP, and AAP formed is also oxidized rapidly without giving formaldehyde. Thus the products and stoichiometry of the demethylation of AMP could not be determined.[5b]

CHP-Supported Oxidation of DMA

Time courses of the formation of formaldehyde, cumenol and *N*-methylaniline, and the consumption of DMA are shown in Fig. 2. Acetophenone was not detected in the reaction mixture. The molar ratio of the four compounds was 1 : 1 : 1 : 1 up to 5 min. After 10 min, the concentrations of *N*-methylaniline formed and DMA consumed became less than those of

formaldehyde and cumenol formed, whereas the ratio of the latter two compounds remained 1 : 1. Since *N*-methylaniline is also oxidized in this system, as shown in Table I, the decrease in the relative concentrations of *N*-methylaniline formed and DMA consumed can be ascribed to the oxidation of *N*-methylaniline formed from DMA. However, the rates of oxidation of *N*-methylaniline and aniline (Table I) are sufficiently small that the reaction does not distort the stoichiometry of the reaction up to 5 min. Therefore, the amount of formaldehyde formed during the initial 5 min was used to calculate the initial rate of the reaction. The initial rate of formaldehyde formation was linear with respect to catalase concentration in the reaction mixture up to a final concentration of 0.15 mg/ml.

When the concentration of DMA was varied at several different fixed concentrations of CHP, double reciprocal plots of the initial rate of the demethylation reaction against the concentration of DMA resulted in a family of straight lines which converged to a common intersection point on the left side of the ordinate and above the abscissa as shwon in Fig. 3. The double reciporocal plots of velocity against the concentration of CHP also gave a family of straight lines having a common point of intersection on the left side of the ordinate and above the abscissa (not shown). These results are indicative of a sequential mechanism,[11] either ordered or rapid equilibrium random, involving the formation of a ternary complex between enzyme, DMA, and CHP, followed by one or more reactions and the subsequent release of the two products. The secondary plot obtained when the slopes and ordinate intercepts from the primary plot were replotted against the reciprocals of the corresponding concentrations of DMA (inset, Fig. 3) was linear. The K_m for DMA calculated from this plot

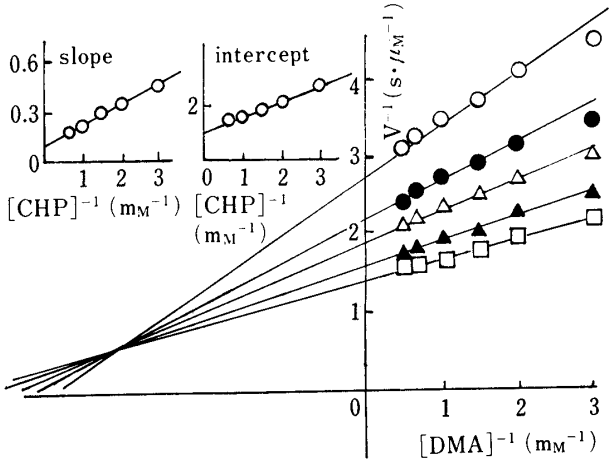

Fig. 3. Initial Velocity Patterns for DMA Oxidation with DMA as the Variable Substrate

The reaction mixtures contained catalase (0.125 mg/ml) and the indicated concentration of DMA in 0.1 M phosphate buffer (pH 7.4).

The concentration of CHP was: □, 1.5; ▲, 1.0; △, 0.68; ●, 0.5; ○, 0.33 mM. The insets are replots of the slopes and intercepts from the reciprocal plots *versus* the reciprocals of the corresponding CHP concentration.

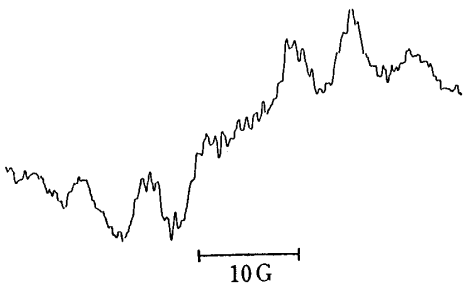

Fig. 4. The ESR Spectrum Produced during the CHP-Supported Oxidation of DMA Catalyzed by Catalase

The reaction mixture contained catalase (0.5 mg/ml), DMA (5 mM), and CHP (0.5 mM) in 0.1 M citrate buffer (pH 5.8). Spectrometer settings: power, 20 mW; modulation amplitude, 3.2 G; scan rate, 25 G/min; time constant, 1 s; gain, 6.3 × 1000.

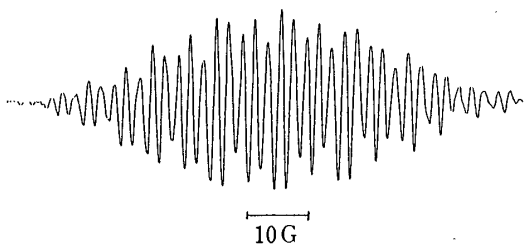

Fig. 5. The ESR Spectrum Produced during the CHP-Supported Oxidation of *N*,*N*-Dimethyl-*p*-anisidine Catalyzed by Catalase

The reaction mixture contained catalase (0.125 mg/ml), *N*,*N*-dimethyl-*p*-anisidine (0.5 mM), and CHP (0.125 mM) in 0.1 M phosphate buffer (pH 7.4). Spectrometer settings: power, 20 mW; modulation amplitude, 0.63 G; scan rate, 25 G/min; time constant, 0.3 s; gain, 2 × 1000.

was 0.13 mM while the K_m for CHP was 0.2 mM.

Detection and Identification of Enzymatically Produced Radicals

With catalase as the catalyst, the oxidation of *N*,*N*-dimethylanilines by CHP yielded ESR signals centered at $g = 2.003$, as expected for radicals derived from this class of compounds. In order to generate ESR signals sufficiently intense for analysis, it was necessary to use higher concentrations of both the reactants and the enzyme than those used for the kinetic studies. However, the ESR spectrum produced from DMA was still very weak, and hence much larger modulation amplitude was needed to obtain a fairly distinct spectrum, as shown in Fig. 4.

The spectrum was identical to that previously obtained by the use of horseradish peroxidase (HRP) and H_2O_2, and assigned to the radical cation of *N*,*N*,*N'*,*N'*-tetramethylbenzidine (TMB).[7a] The radical cation of DMA was not detected. This is because the radical cation is very unstable and undergoes a rapid dimerization to form TMB.[12] Therefore, detection of the TMB radical cation suggests that the initial step of the CHP-supported oxidation of DMA catalyzed by catalase is a one-electron transfer to form the radical cation of DMA.

Although the ESR spectrum obtained from the oxidation of *N*,*N*-dimethyl-*p*-toluidine was poorly resolved, it was reasonably well simulated by the use of the following ESR parameters ($A_1 = 10.9$ (1N), $A_2 = 11.8$ (6H), $A_3 = 5.2$ (2H), $A_4 = 1.2$ (2H), $A_5 = 9.8$ G (3H), line width 2.5 G), which agree well with those previously reported for the *N*,*N*-dimethyl-*p*-toluidine radical cation generated by the use of HRP and H_2O_2.[7b]

The CHP-supported oxidation of *N*,*N*-dimethyl-*p*-anisidine gave a well resolved ESR spectrum, as shown in Fig. 5. The hyperfine splitting constants of this spectrum ($A_1 = 10.15$ (1N), $A_2 = 10.45$ (6H), $A_3 = 4.20$ (2H), $A_4 = 1.75$ (2H), $A_5 = 1.80$ G (3H)) agree well with those previously reported for the *N*,*N*-dimethyl-*p*-anisidine radical cation generated electrochemically.[13]

Discussion

Hollenberg and Kedderis reported that the H_2O_2- and EHP-supported *N*-demethylation of DMA by HRP resulted in the formation of equimolar amounts of *N*-methylaniline and formaldehyde with no other products, and that the initial rate kinetics were consistent with a ping-pong kinetic mechanism.[7c] They also reported that benzoyl peroxide, which also supported th HRP-catalyzed demethylation of DMA at a rate somewhat greater than that of the H_2O_2- or EHP-supported demethylation, did not form compound I, and they suggested that either compound I of HRP is not involved in the demethylation or that the mechanism of the benzoyl peroxide-supported demethylation is different from that of the H_2O_2- or EHP-supported demethylation. However, they gave no kinetic evidence for this view. Based on an inhibition study with spin-trapping reagents they proposed that substrate free radicals are not free intermediates in the demethylation reaction, and that hydroxyl radicals, superoxide anions, and singlet oxygen are not free intermediates in the reaction. On the other hand, Griffin *et al.* [7a,b] suggested that organic free radicals were formed as free intermediates in the HRP-catalyzed demethylation of *N*-methyl-substituted anilines by H_2O_2. Hollenberg and Kedderis[7c] objected to Griffin's suggestion on the grounds that high concentrations of HRP and the anilines were used for the ESR study.

The present study clearly indicates that the CHP-supported demethylation of DMA catalyzed by catalase proceeds not by a ping-pong mechanisms but by a sequential mechanism involving the formation of a ternary complex, that is, the mechanism of the CHP-supported demethylation of DMA is the same as that in the case of AMP and different from that of the H_2O_2- or EHP-supported demethylation catalyzed by HRP. Although the radical cation of DMA was not detected by ESR, this was anticipated in view of the instability of radical

cations, since the ESR spectrum of the radical cation has not been reported hitherto. However, the detection of the radical cation of TMB strongly suggests that the radical cation of DMA is formed during the CHP-supported demethylation of DMA, since the TMB radical cation is generally considered to be formed from the DMA radical cation.[12] Furthermore, the radical cations of *N*,*N*-dimethyl-*p*-toluidine and *N*,*N*-dimethyl-*p*-anisidine were detected during the CHP-supported demethylations of the parent anilines. Although rather high concentrations of the aniline and catalase were used to obtain a well-resolved ESR spectrum of the former radical, the presence of the latter radical was confirmed at the same concentrations of the reactants and catalase as those used for the kinetic study of DMA. Therefore, the radical cations of the anilines are the true intermediates in the demethylations and not the products of side reactions.

The following schemes are proposed for the CHP-supported demethylation of DMA. At

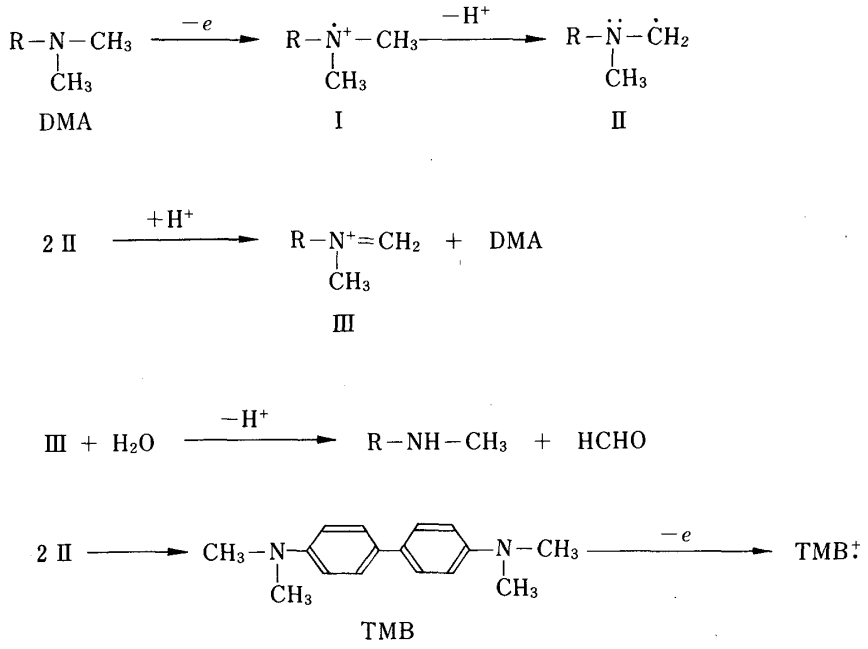

Chart 1

low concentrations of DMA the radical II undergoes disproportionation to form DMA and the iminium cation III, and the latter is hydrolyzed to *N*-methylaniline and formaldhyde, whereas at higher concentrations of DMA II undergoes dimerization to form TMB, which is further oxidized to form the TMB radical cation. The facts that the demethylation obeyed a sequential mechanism and that acetophenone, which is a product of the decomposition of cumyloxy radical,[14] was not detected in the reaction mixture indicate that the active species in the CHP-supported demethylation is neither compound I of catalase nor cumyloxy radical. Catalase may catalyze the CHP-supported demethylation of DMA by binding both substrates in close proximity to each other, thus lowering the activation energy barrier by increasing the probability of productive collisions, rather than through the intermediacy of an oxidized enzyme species or oxyradicals.

Acknowledgement The authors wish to thank Mr. Takayoshi Akioka for his help with some of the experiments.

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