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Kinetics of the Organic Hydroperoxide-supported Oxidation of Aminopyrine catalyzed by Catalase

Hiroteru Sayo* and Mikio Hosokawa

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Ikawadani-cho, Tarumi-ku, Kobe 673, Japan

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The kinetic mechanism of the ethyl hydroperoxide (EHP)- and cumene hydroperoxide (CHP)-supported oxidations of aminopyrine catalyzed by catalase was investigated by the use of a stopped-flow spectrophotometer. Plots of reciprocal velocity *versus* the reciprocal concentration of either substrate at several different fixed concentrations of the other substrate converged to common points of intersection on the left side of the ordinate and above or below the abscissa, suggesting a sequential mechanism involving the formation of a ternary complex between catalase, aminopyrine, and EHP or CHP followed by one or more reactions and the subsequent release of the products. Potassium cyanide was a competitive inhibitor with respect to EHP and non-competitive with respect to CHP and aminopyrine. These results, which indicate that neither CHP nor aminopyrine binds directly to the heme iron, support our previous suggestion that the binding group involved in the CHP-supported oxidation of aminopyrine is different from that for the catalatic reaction.

Keywords——catalase; cumene hydroperoxide; ethyl hydroperoxide; aminopyrine; oxidation of aminopyrine; aminopyrine cation radical; kinetics of peroxidation; inhibition by potassium cyanide

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 nitrite, sodium azide, and hydroxylamine by H_2O_2 (peroxidatic reaction).[1] Some aliphatic hydroperoxides can also support the peroxidatic reactions of catalase.[2] Sies and Summer[3] claimed that catalase does not react with organic hydroperoxides such as *tert*-butyl hydroperoxide (BHP) and cumene hydroperoxide (CHP). Oshino and Chance[4] also reported that catalase does not react with BHP in the presence of ethanol, and that the addition of BHP to catalase does not form catalase compound I. Since catalase compound I, which retains two oxidizing equivalents above the native enzyme, is generally considered to be the primary intermediate in the catalatic and peroxidatic reactions of catalase, lack of formation of a spectroscopically distinct intermediate, compound I, in the reaction of catalase with BHP or CHP was considered to indicate that BHP or CHP does not support the peroxidatic reaction of catalase.

In the previous papers,[5] we reported that aminopyrine is oxidized to the aminopyrine cation radical by CHP or EHP in the presence of catalase. CHP which had been added to the catalase solution prior to addition of aminopyrine impaired the *N*-demethylase activity of catalase, whereas the catalase thus impaired showed normal catalatic activity. Methanol was not oxidized appreciably in the catalase–CHP system. 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. These experimental results strongly suggested that the active site of catalase for the CHP-supported *N*-demethylation of aminopyrine is different from that for the catalatic reaction.

Since the organic hydroperoxide-supported oxidations involve a single enzyme, catalase, and two substrate molecules, the hydroperoxide and aminopyrine, this system is amenable to a steady-state kinetic analysis using the techniques established for two substrate enzymes.[6] Potassium cyanide, which has already been demonstrated to occupy the sixth coordination

position of catalase hematin,[7] was used as an inhibitor.

In the present paper, we have characterized the EHP- and CHP-supported oxidations of aminopyrine catalyzed by catalase. The results of the steady-state kinetic analysis indicate that the reactions proceed by a sequential mechanism involving the formation of a ternary complex.

Experimental

Materials——Catalase (from bovine liver, C-40) was used as supplied by Sigma. The concentration of protein was determined by the biuret method, with bovine serum albumin as the standard.[8] Aminopyrine, EHP, and CHP were obtained and purified as described previously.[5b] The buffer solution used in this 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 system. All other chemicals were of reagent grade.

Methods——The initial rate of formation of the aminopyrine cation radical was determined at 25°C by the use of a Union-Giken RA 601 stopped-flow spectrophotometer, equipped with a system 71 data processor. One reservoir was filled with the catalase solution, and the other one was filled with a mixture of aminopyrine and CHP or EHP solutions. Potassium cyanide was added to the mixture when necessary. The concentration of the aminopyrine cation radical was determined from the absorbance at 565 nm. An extinction coefficient of $\varepsilon = 2.23$ mM^{-1} cm^{-1} was used.[9]

Results

Determination of Initial Velocity

The first step of the EHP- and CHP-supported oxidations of aminopyrine is a one-electron transfer to form the blue-violet cation radical of aminopyrine.[5] The concentration of the radical can be determined very sensitively by spectrophotometry.[9] The increase in the absorbance of the reaction mixture at 565 nm as a function of time for the EHP- and CHP-supported oxidations of aminopyrine by catalase is shown in Fig. 1. Although the aminopyrine radical decomposes fairly rapidly in aqueous buffer solutions, the initial increase in the absorbance can be used to obtain the initial rate of oxidation of aminopyrine. For the studies reported here, the initial velocity was obtained from the difference in the absorbances between 1 s and 2 s after initiating the reaction. Since the initial velocity of aminopyrine radical

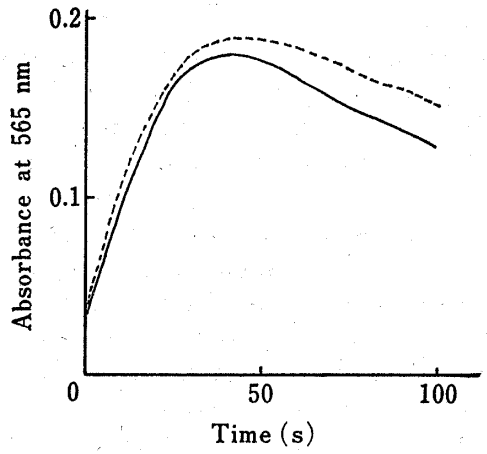

Fig. 1. Oxidation of Aminopyrine supported by EHP or CHP at 25°C

The reaction mixture contained 0.3 mg/ml catalase, 2 mM aminopyrine, and 2 mM EHP or CHP in 0.1 M sodium phosphate buffer (pH 7.4).
——, EHP-supported oxidation; ·····, CHP-supported oxidation.

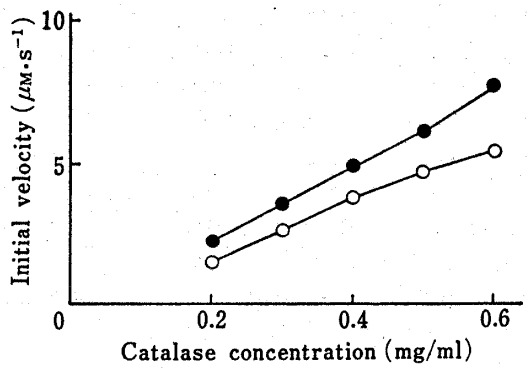

Fig. 2. Plots of the Initial Velocity against the Concentration of Catalase at 25°C

The reaction mixtures contained 2 mM aminopyrine, 2 mM EHP or CHP, and the indicated concentration of catalase in 0.1 M sodium phosphate buffer (pH 7.4).
○, EHP-supported oxidation; ●, CHP-supported oxidation.

formation was linear with the catalase concentration between 0.2 and 0.4 mg/ml, the concentr ation of catalase was set at 0.3 mg/ml throughout the study (Fig. 2). The kinetic mechanism of the EHP- and CHP-supported oxidations of aminopyrine was investigated by means of initial velocity studies at 25°C in which the concentrations of the two substrates were systematically varied, and the results were analyzed on the basis of the steady state assumption.

EHP-supported Oxidation of Aminopyrine

When the concentration of EHP was varied at several different fixed concentrations of aminopyrine, double reciprocal plots of velocity against the concentration of EHP resulted in a family of straight lines which converged to a common intersection point on the left side of the ordinate and below the abscissa (Fig. 3). The double reciprocal plots of velocity against the

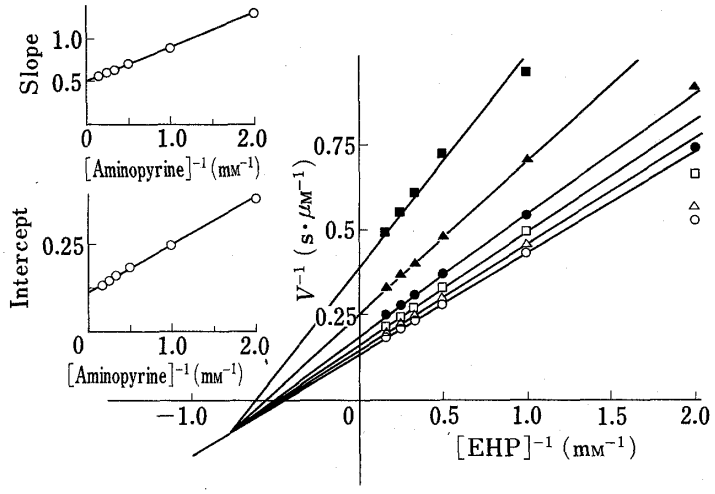

Fig. 3. Initial Velocity Patterns for Aminopyrine Oxidation with EHP as the Variable Substrate

The reaction mixtures contained 0.3 mg/ml catalase and the indicated concentration of EHP in 0.1 M sodium phosphate buffer (pH 7.4).
The concentrations of aminopyrine were: ■, 0.5 mM; ▲, 1 mM; ●, 2 mM; □, 3 mM; △, 4 mM; ○, 6 mM. The insets are replots of the slopes and intercepts from the reciprocal plots *versus* the reciprocals of the corresponding aminopyrine concentrations.

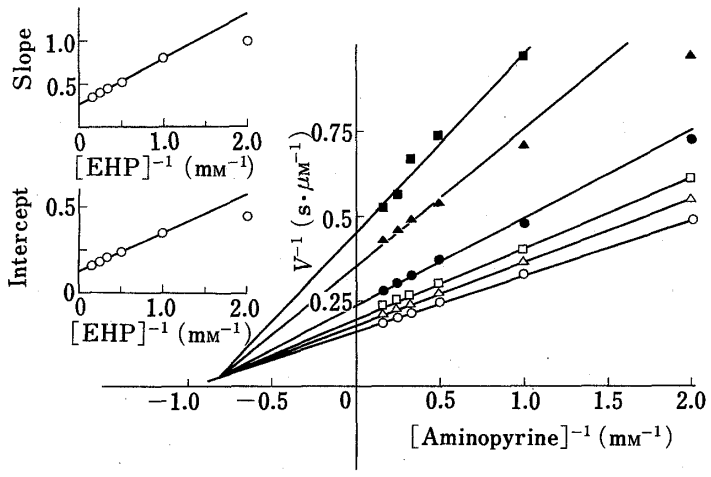

Fig. 4. Initial Velocity Patterns for Aminopyrine Oxidation with Aminopyrine as the Variable Substrate

Assay conditions were as described in Fig. 3. The concentrations of EHP were: ■, 0.5 mM; ▲, 1 mM; ●, 2 mM; □, 3 mM; △, 4 mM; ○, 6 mM. The insets are replots of the slopes and intercepts from the reciprocal plots *versus* the reciprocals of the corresponding EHP concentrations.

concentration of aminopyrine at several fixed concentrations of EHP gave a family of straight lines having a common point of intersection on the left side of the ordinate and above the abscissa (Fig. 4).

These results are indicative of a sequential mechanism,[6] either ordered or rapid equilibrium random, involving the formation of a ternary complex between enzyme, aminopyrine, and EHP, followed by one or more reactions and the subsequent release of the two products. The secondary plots obtained when the slopes and ordinate intercepts from the primary plots were replotted against the reciprocals of the corresponding concentrations of aminopyrine (inset, Fig. 3) or EHP (inset, Fig. 4) were linear in all cases. The true kinetic constants for both substrates were calculated from the slopes and intercepts of these secondary plots as described by Engel.[6c] The K_m for EHP calculated in this manner was 2.1 mM while the K_m for aminopyrine was 1.3 mM.

The double reciprocal plots obtained when the initial velocity was determined as a function

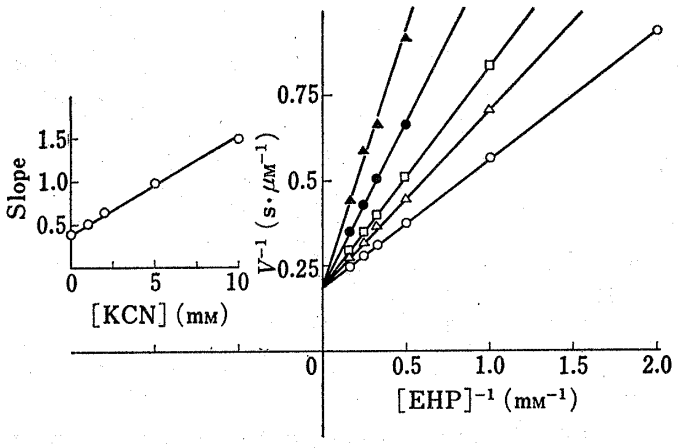

Fig. 5. Double Reciprocal Plots of Potassium Cyanide Inhibition Data with EHP as the Variable Substrate

The reaction mixtures contained 0.3 mg/ml catalase, 2 mM aminopyrine, various concentrations of KCN, and the indicated concentrations of EHP in 0.1 M sodium phosphate buffer (pH 7.4). The concentrations of KCN were: ○, 0 mM; △, 1 mM; □, 2 mM; ●, 5 mM, and ▲, 10 mM. The inset is a replot of the slopes *versus* the corresponding concentrations of KCN.

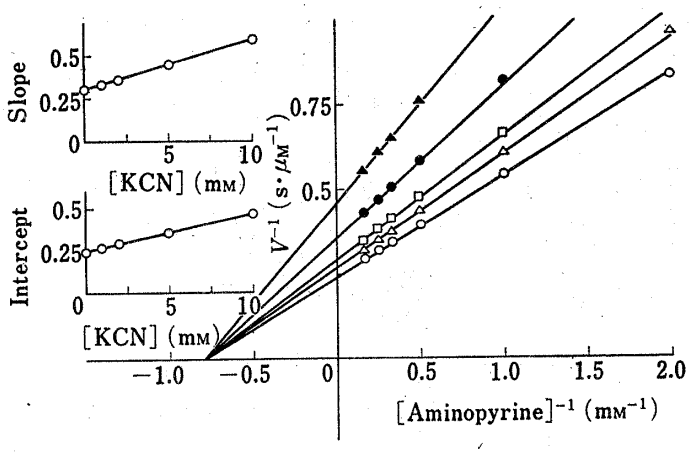

Fig. 6. Double Reciprocal Plots of Potassium Cyanide Inhibition Data with Aminopyrine as the Variable Substrate

The reactions were the same as described in Fig. 5 except the EHP concentration was 2 mM in all reactions and the concentration of aminopyrine was varied as indicated. The insets are replots of the slopes and intercepts *versus* the corresponding concentration of KCN.

of EHP in the presence of 0, 1, 2, 5, and 10 mM potassium cyanide and a fixed concentration of aminopyrine are shown in Fig. 5. The common intersection point on the ordinate indicates that cyanide was a competitive inhibitor with respect to EHP. The replot of the slopes *versus* the inhibitor concentration (inset, Fig. 5) gave a straight line, demonstrating the purely competitive nature of the inhibition. The K_i for cyanide was 3.3 mM.

When the concentration of EHP was held constant at 2 mM and the concentration of aminopyrine was varied in the presence of various fixed concentrations of potassium cyanide (0, 1, 2, 5, and 10 mM), the double reciprocal plots of the initial rate data (Fig. 6) gave a family of straight lines having a common point of intersection on the abscissa. The replots of the slopes or intercepts *versus* the inhibitor concentration (inset, Fig. 6) gave straight lines, which indicated that cyanide was a non-competitive inhibitor with respect to aminopyrine. The K_i for cyanide was 10 mM.

CHP-supported Oxidation of Aminopyrine

The double reciprocal plots of velocity against the concentration of CHP at several fixed

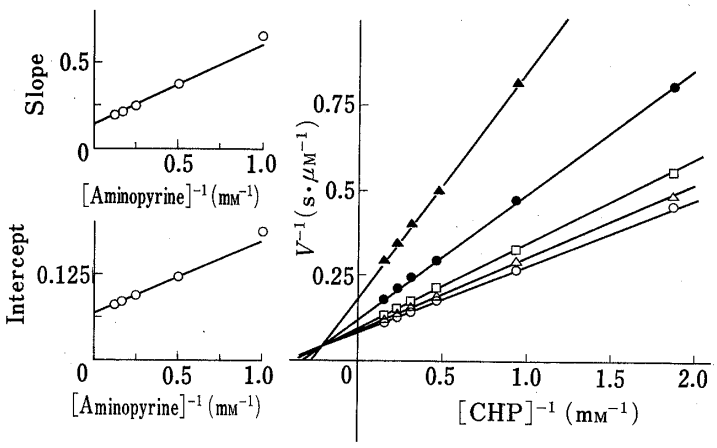

Fig. 7. Initial Velocity Patterns for Aminopyrine Oxidation with CHP as the Variable Substrate

Assay conditions were as described in Fig. 3, except that CHP was used instead of EHP. The concentrations of aminopyrine were: ▲, 1 mM; ●, 2 mM; □, 4 mM; △, 6 mM; ○, 8 mM.

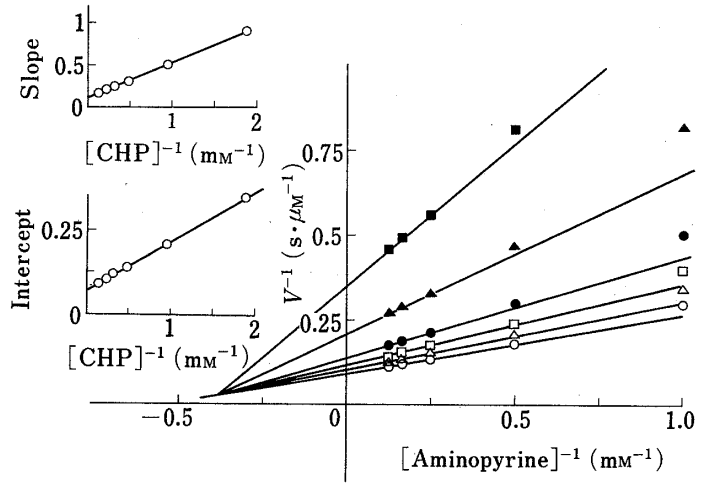

Fig. 8. Initial Velocity Patterns for Aminopyrine Oxidation with Aminopyrine as the Variable Substrate

Assay conditions were as described in Fig. 4, except that CHP was used instead of EHP. The concentrations of CHP were: ■, 0.5 mM; ▲, 1 mM; ●, 2 mM; □, 3 mM; △, 4 mM; ○, 6 mM.

concentrations of aminopyrine gave a family of straight lines having a common point of intersection in the fourth quadrant (Fig. 7). The double reciprocal plots of velocity against the concentration of aminopyrine at fixed concentrations of CHP also gave a family of straight lines having a common point of intersection in the fourth quadrant (Fig. 8). The secondary plots of the slopes and ordinate intercepts from the primary plots against the reciprocals of corresponding concentrations of aminopyrine (inset, Fig. 7) or EHP (inset, Fig. 8) were linear in all cases. These results are indicative of a sequential mechanism involving the formation of a ternary complex between catalase, aminopyrine, and CHP. The K_m for CHP was 2.3 mM while the K_m for aminopyrine was 1.8 mM.

When the concentration of aminopyrine was held constant and the concentration of CHP was varied in the presence of various levels of potassium cyanide, the double reciprocal plots of the initial velocity gave a family of straight lines having a common point of intersection on

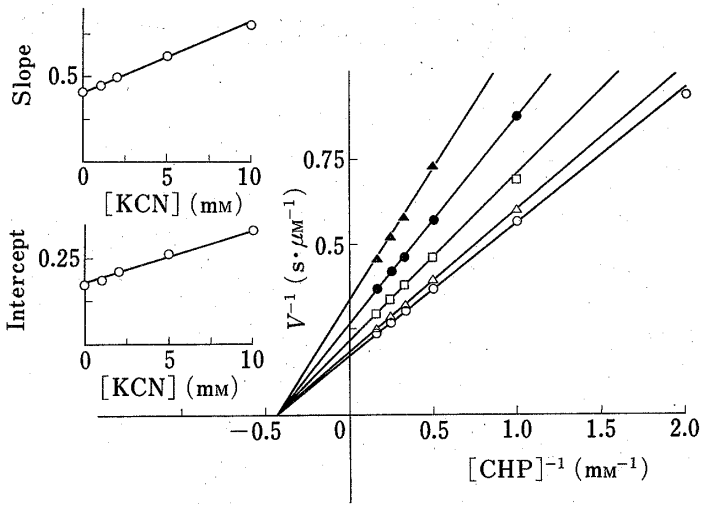

Fig. 9. Double Reciprocal Plots of Potassium Cyanide Inhibition Data with CHP as the Variable Substrate

The reactions were the same as described in Fig. 5 except that CHP was used instead of EHP. The concentrations of KCN were the same as described in Fig. 5.

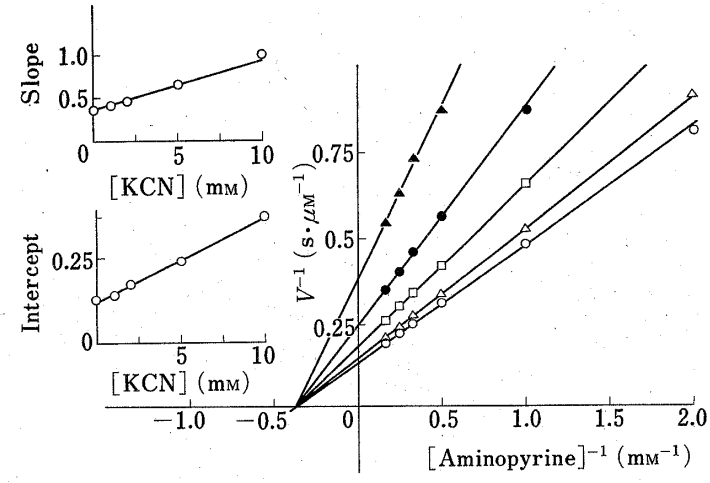

Fig. 10. Double Reciprocal Plots of Potassium Cyanide Inhibition Data with Aminopyrine as the Variable Substrate

The reactions were the same as described in Fig. 6 except that CHP was used instead of EHP. The concentrations of KCN were the same as described in Fig. 6.

the abscissa (Fig. 9). The replots of the slopes or intercepts *versus* the inhibitor concentration (inset, Fig. 9) gave straight lines, which indicated that cyanide was a non-competitive inhibitor with respect to CHP. The K_i for cyanide was 9.5 mM.

The double reciprocal plots of the initial rate data obtained when the aminopyrine concentration was varied in the presence of various levels of potassium cyanide and a fixed concentration of CHP are shown in Fig. 10. The intersection of the lines on the abscissa indicates that cyanide was also a non-competitive inhibitor with respect to aminopyrine. The K_i for cyanide was 6.0 mM.

Discussion

Chance and his coworkers[2] have proposed a ping-pong mechanism involving the initial formation of catalase compound I, which is formed by the reaction of catalase with a hydroperoxide and then reacts with electron donors, for the catalase-mediated reactions. They also claimed that catalase does not react with BHP or CHP.[3,4]

Our previous results on the EHP- and CHP-supported oxidations of aminopyrine by catalase led to the conclusions that the binding site of catalase for the EHP-supported oxidation of aminopyrine is different from that for the oxidation of methanol, and that the binding site of catalase for the CHP-supported oxidation is different from that for the catalatic reaction. These conclusions suggested that the EHP- and CHP-supported oxidations of aminopyrine do not involve catalase compound I as an intermediate.

The intersecting Lineweaver–Burk patterns obtained when the concentration of one substrate was varied systematically in the presence of different fixed concentrations of the second substrate (Figs. 3, 4, 7, and 8) indicate that the overall mechanism for both the EHP- and CHP-supported oxidations is sequential, either ordered or rapid equilibrium random, involving the formation of a ternary complex of catalase, hydroperoxide, and aminopyrine. These results conclusively ruled out a ping-pong mechanism in which EHP or CHP reacts with catalase to form catalase compound I, followed by the binding of aminopyrine to the compound I, followed by a second reaction and subsequent release of the aminopyrine free radical, and which would be characterized by a series of parallel lines in the Lineweaver–Burk plots.

The inhibition patterns observed with cyanide indicate that the cyanide binding is competitive with EHP and non-competitive with CHP and aminopyrine. Since cyanide is known to bind to catalase as an axial ligand, the competitive nature of the inhibition with respect to EHP suggests that EHP binds directly to the hematin iron. In contrast, the non-competitive nature of the inhibition with respect to CHP and aminopyrine suggests that neither CHP nor aminopyrine binds directly to the hematin iron. These results support our previous suggestion that the binding group involved in the CHP-supported oxidation of aminopyrine is different from that for the catalatic reaction. Since cyanide was not a dead-end inhibitor for the reaction, the present results could not differentiate between ordered and rapid equilibrium random mechanisms. However, the results presented in this report indicate that the EHP- and CHP-supported *N*-demethylations of aminopyrine proceed by mechanisms distinct from that generally proposed for catalase-mediated reactions.[2] It is not yet clear whether there is a difference in reaction mechanism between the EHP- and CHP-supported *N*-demethylations of aminopyrine.

Liver microsomal cytochrome P-450 can use organic hydroperoxides in place of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen to support a variety of reactions including *N*- and *O*-dealkylations and the hydroxylation of aromatic substrates. Several laboratories have suggested that the peroxide-supported reactions proceed *via* a "peroxidase-type" mechanism, implicating a compound I-type intermediate in the hydroxylation reactions.[10] However, Koop and Hollenberg recently suggested a sequential mechanism involving the formation of a ternary complex between cytochrome P-450, *p*-nitroaniline, and

BHP.[11]

It remains to be determined whether the EHP- and CHP-supported oxidations of aminopyrine by catalase have any relation to the physiological role of catalase.

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