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The Effect of Alkaline Denaturation on Organic Hydroperoxide-supported N-Demethylase Activities of Catalase

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The enzymic activities of various preparations of catalase were determined. Catalase C-100, which is a twice-crystallized preparation, showed the highest catalatic activity, whereas C-40, which is a lyophilized preparation, showed the highest N-demethylase activities towards aminopyrine. The ethyl hydroperoxide (EHP)-supported peroxidatic activity towards methanol increased in parallel with the increase in the catalatic activity. On the other hand, the order of increasing N-demethylase activities towards aminopyrine was not the same as that of increasing catalatic activity. Treatment with alkali followed by neutralization increased the N-demethylase activities of C-40 and C-100 and decreased both the peroxidatic activity towards methanol and the catalatic activity. When the alkali-treated catalase solution was neutralized and allowed to reconstitute, the increased levels of N-demethylase activities only slightly decreased with time up to 2 hr. The decreased peroxidatic activity towards methanol was restored nearly completely, whereas only 7.6% of the original catalatic activity of C-40 was restored after reconstitution for 24 hr. Gel chromatographic experiments showed that treatment with alkali results in the dissociation of catalase into subunits, and that the recombination of the subunits takes place fairly rapidly on neutralization of the alkali-treated catalase. The present results suggest that catalase molecules with a loosely combined quaternary structure, which are formed by lyophilization or by treatment with alkali followed by neutralization, are capable of catalyzing the organic hydroperoxide-supported N-demethylation of aminopyrine, whereas they cannot decompose hydrogen peroxide efficiently.

Keywords——catalase; alkaline denaturation; N-demethylation of aminopyrine; organic hydroperoxide-supported peroxidation; cumene hydroperoxide; ethyl hydroperoxide; gel filtration chromatography; peroxidation of methanol; peroxidatic activity of catalase; lyophilized catalase

The decrease in the catalatic activity of catalase upon mild denaturation and the appearance of and increase in the peroxidatic activity towards phenolic compounds have been studied by many workers.[2)] However, hydrogen peroxide has been employed as the peroxide substrate in these studies. Since the rate of decomposition of hydrogen peroxide is much faster than that of the peroxidatic reaction, interpretation of the results is not straightforward. Marklund employed methyl and ethyl hydroperoxides as peroxide substrates, since alkyl-hydroperoxides are not rapidly decomposed by catalase, and hence changes in the peroxidatic activity can be observed clearly.[3)] He also studied the tryptic digestion of catalase and suggested that the active sites for phenolic compounds are sterically shielded.

Sies and Summer claimed that catalase does not react with organic hydroperoxides such as *tert*-butyl hydroperoxide (BHP) and cumene hydroperoxide (CHP).[4)] Oshino and Chance 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.[5)] On the other hand, Azizov *et al.* used BHP as a peroxide substrate to determine the peroxidatic activity of catalase towards guaiacol.[6)] He reported that the development of peroxidatic activity is observed after alkaline denaturation or prolonged drying of catalase and suggested that the catalatic and the peroxidatic centers differ in nature. Kadlubar *et al.* reported that aminopyrine and dimethylaniline are rapidly oxidized by organic hydroperoxides, such as CHP and BHP, in the presence of catalase.[7)] However, this activity was not well characterized.

In previous papers, we reported that aminopyrine is oxidized to the aminopyrine cation radical by CHP or ethyl hydroperoxide (EHP) in the presence of catalase.[8] On the basis of studies on the inhibitory effects of CHP, EHP, and sodium azide, it was suggested that the active sites of catalase for the CHP- and EHP-supported N-demethylations of aminopyrine are different from those for the catalatic reaction and the EHP-supported oxidation of methanol respectively. A lyophilized preparation of catalase (Sigma C-40, from beef liver) was used throughout the above studies.

Since it seems likely that our results[8] are closely related to those by Marklund[3] and Azizov *et al.*,[6] we have now investigated the relationship between the catalatic and the CHP- or EHP-supported peroxidatic activities of various preparations of catalase and we have found that the EHP- and CHP-supported N-demethylase activities of catalase towards aminopyrine are largely due to catalase molecules with a loosely combined quaternary structure, which are formed by lyophilization or by treatment with alkali followed by neutralization.

Experimental

Materials——Catalase preparations used for this study were C-10, C-40, and C-100 from Sigma Chemicals, and purified powder and twice-crystallized suspension (PL-2X) from P–L Biochemicals. All preparations were from beef liver. The concentration of protein was determined by the biuret method. Aminopyrine, EHP, and CHP were obtained and purified as described previously.[8] The concentrations of hydroperoxides were determined by iodometric titration.[9] Water was purified by the use of a Millipore MILLI–R/Q system. Sephacryl S-200 Superfine was obtained from Pharmacia Fine Chemicals. All other chemicals used were of reagent grade.

Methods——The CHP- and EHP-supported peroxidatic activities of catalase were determined at 25° and expressed conveniently in terms of the concentration of formaldehyde formed 5 min after initiating the reaction. Formaldehyde was assayed by the Nash procedure,[10] after the reaction mixture had been mixed with an equal volume of 10% trichloroacetic acid and the solution had been contrifuged to remove precipitated protein. Ultraviolet and visible absorption spectra were recorded on a Hitachi 340 spectrophotometer at 25°. Catalatic activity was determined from the decrease in absorbance at 240 nm of an H_2O_2 solution (12.5 mM) in 0.05 M sodium phosphate, pH 7.0, upon the addition of catalase at 25°.[11]

Alkaline denaturation of catalase: 0.2 ml of catalase solution (6 mg/ml in 2% NaCl) was made alkaline (final pH, 11.9) by the addition of 0.3 ml of NaOH solution containing 0.02 M NaCl. After various time intervals the solution was neutralized (final pH, 7.45) by the addition of 2.05 ml of 0.1 M sodium phosphate (pH 7.4). The catalase was then allowed to stand for 1—120 min, and 0.3 ml of aminopyrine (60 mM) or methanol (60 mM) and 0.15 ml of EHP or CHP (30 mM) were added to the catalase solution in that order.

Ascending gel chromatography was carried out on a K26/70 column of Sephacryl S-200 Superfine (column height 45 cm) at 5°. The rate of flow of solution through the column was controlled by means of a pump. As an eluent, 0.08 M sodium phosphate (pH 7.45) containing 0.025 M NaCl was used. The elution profiles in the column effluent were analyzed automatically at 280 nm by the use of an Atto MINI UV MONITOR I. The column was calibrated with a gel filtration calibration kit from Pharmacia Fine Chemicals.

Results

Enzymic Activities of Various Preparations of Catalase

The purity index, catalatic activity, and CHP- and EHP- supported peroxidatic activities of various preparations of catalase were determined, and the results are shown in Table I. The purity index was a good indication of the catalatic activity except in the case of PL-2X. The EHP-supported peroxidatic activity towards methanol increased in parallel with the increase in the catalatic activity. On the other hand, the orders of increasing EHP- and CHP-supported N-demethylase activities were not the same as the order of increase of the purity index or the catalatic activity. Catalase C-100, which is a twice-crystallized preparation, showed the highest catalatic activity, whereas C-40, which is a lyophilized preparation, showed the highest N-demethylase activity.

Alkaline Denaturation

When catalase C-40 was exposed to pH 11.9 at 25° for 5 min and then the enymic activities were measured at pH 11.9, the EHP- and CHP-supported N-demethylase activities towards

Table I. Purity Index, Catalatic Activity, and Organic Hydroperoxide-supported Peroxidatic Activities[a] of Various Preparations of Catalase

Catalase preparation	C-10	C-40	C-100	PL-powder	PL-2X
Purity index (A_{405}/A_{275})[b]	0.43	0.67	0.80	0.34	0.65
Catalatic activity[11] Kat. f.	3800	16100	32900	3100	27600
EHP-supported peroxidatic activity towards methanol	123	350	430	112	400
CHP-supported N-demethylase activity	27	251	95	30	25
EHP-supported N-demethylase activity	47	193	40	52	33

a) The CHP- and EHP-supported activities were expressed conveniently in terms of the concentration of formaldehyde (μM) formed 5 min after initiating the reaction. The reaction mixture contained one preparation of catalase (0.4 mg/ml), 6 mM aminopyrine or methanol, and 1.5 mM CHP or EHP. All experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.45) at 25°.

b) P. Jones and D.N. Middlemiss, *Biochem. J.*, **130**, 411 (1972).

aminopyrine were only 9 and 33% of those of the native C-40 measured at pH 7.45. Thus, the alkali-denatured catalase was neutralized and allowed to reconstitute for 1—120 min at pH 7.45 before determination of enzymic activities.

Figure 1 shows the dependence of the catalatic and peroxidatic activities of catalase C-40 on the duration of incubation at pH 11.9 and 25° (the catalase was kept at pH 7.45 for 1 min before activity determination). The catalatic activity and the EHP-supported peroxidatic activity towards methanol decreased remarkably, whereas the EHP- and CHP-supported N-demethylase activities towards aminopyrine increased considerably. Exposure for 2.5—5 min to pH 11.9 gave a maximal increase in the N-demethylase activities. A longer

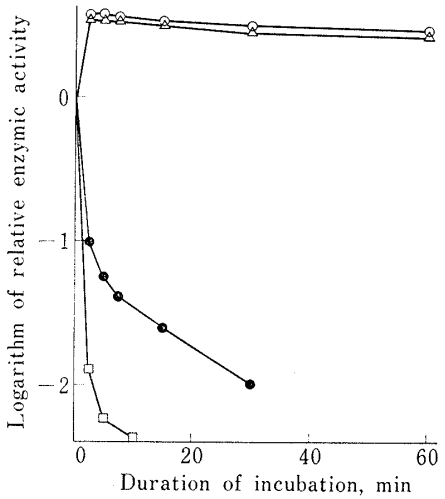

Fig. 1. Dependence of the Catalatic and Peroxidatic Activities of Catalase C-40 on the Duration of Incubation at pH 11.9 and 25°

The alkali-denatured catalase was allowed to reconstitute for 1 min at pH 7.45 before determination of enzyme activities. The reaction mixture for the determination of the peroxidatic activities contained 0.4 mg/ml catalase, 6 mM aminopyrine or methanol, and 1.5 mM CHP or EHP. △, CHP-supported N-demethylase activity; ○, EHP-supported N-demethylase activity; ●, EHP-supported peroxidatic activity towards methanol; □, catalatic activity.

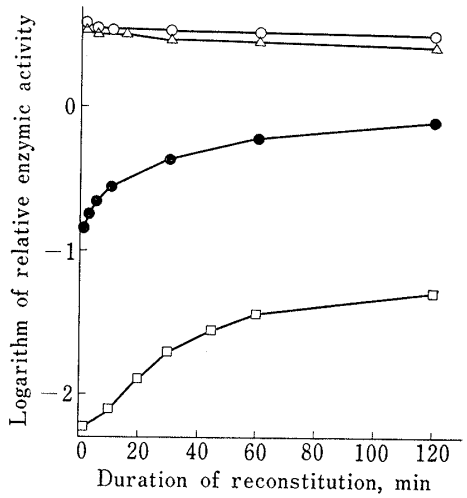

Fig. 2. Dependence of the catalatic and Peroxidatic Activities of Catalase C-40 on the Duration of Reconstitution at pH 7.45 and 25°

Catalase was exposed to pH 11.9 for 5 min at 25°, and then the solution was neutralized to pH 7.45 and kept for an appropriate time. The final concentrations of various agents and the symbols are the same as in Fig. 1.

duration of incubation slightly diminished the increase in the N-demethylase activities.

Figure 2 shows the dependence of enzymic activities of catalase C-40 that had been alkali-denatured for 5 min at pH 11.9 and 25° on the duration of reconstitution at pH 7.45 and 25°. As the duration of reconstitution became longer, the increase in the N-demethylase activities were slightly diminished, whereas the EHP-supported peroxidatic activity towards methanol was restored nearly completely. On the other hand, only 7.6% of the original catalatic activity was recovered even after reconstitution for 24 hr.

Figure 3 shows the time dependence of the N-demethylation of aminopyrine catalyzed by native and denatured C-40. The concentration of formaldehyde formed during the CHP-supported N-demethylation catalyzed by the alkali-denatured C-40 increased rapidly with time and reached a limiting value after 8 min. The limiting concentration of formaldehyde (0.75 mM) was equal to half the concentration of CHP added and was only slightly larger than that for the CHP-supported N-demethylation catalyzed by the native C-40 (0.70 mM). On the other hand, the limiting concentration of formaldehyde for the EHP-supported N-demethylation was increased significantly by denaturation with alkali, and approached that for the CHP-supported N-demethylation.

When C-100 was exposed to pH 11.9 for 5 min at 25° and the solution was kept at pH 7.45 for 1 min, the EHP- and CHP-supported N-demethylase activities increased to 16.7 and 7.9 times those of the native enzyme, respectively. However, since the EHP- and CHP-supported N-demethylase activities of the native C-100 were 21 and 38% of those of the native C-40, the increased activities of the denatured C-100 were 94 and 93% of those of the denatured C-40, respectively. The EHP-supported peroxidatic activity towards methanol and the catalatic activity of the denatured C-100 were 38 and 5.4% of those of the native C-100, respectively. The dependence of the enzymic activities of the denatured C-100 on the duration of reconstitution at pH 7.45 and 25° was similar to that of the denatured C-40.

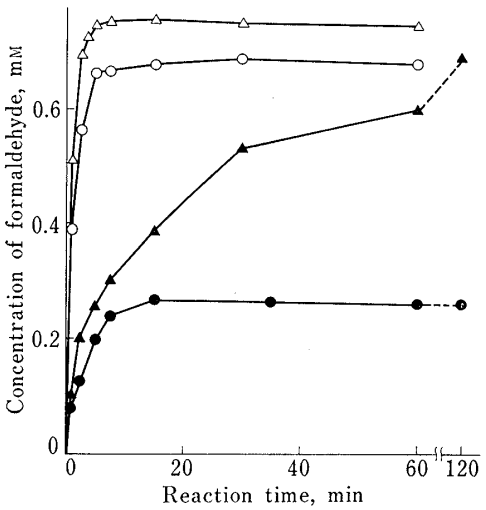

Fig. 3. Time Dependence of the N-Demethylation of Aminopyrine catalyzed by Native and Alkali-denatured Catalase C-40

The reaction mixture contained 0.4 mg/ml catalase, 6 mM aminopyrine, and 1.5 mM CHP or EHP. ▲, CHP-supported N-demethylation catalyzed by native C-40; ●, EHP-supp supported N-demethylation catalyzed by native C-40; △, CHP-supported N-demethylation catalyzed by alkali-denatured C-40; ○, EHP-supported N-demethylation catlalyzed by alkali-denatured C-40.

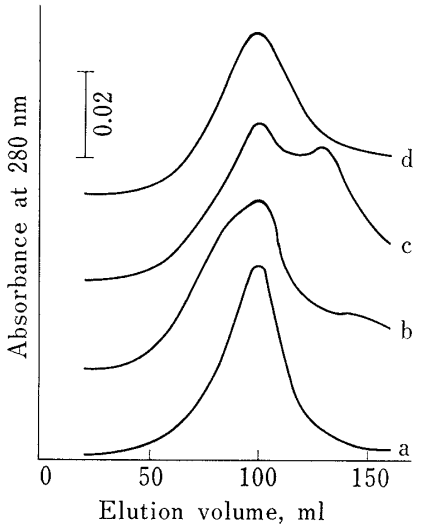

Fig. 4. Elution Profiles of 1 ml of Catalase Preparations (16.7 mg/ml) from a Sephacryl S-200 Superfine Column

Column, K 26/70 with flow adaptor; bed dimensions, 2.6×45 cm; void volume, 86 ml; eluent, 0.08 M sodium phosphate (pH 7.45) containing 0.025 M NaCl; ascending flow rate, 2.2 ml/min. a, native C-100; b, native C-40; c, C-40 exposed to pH 11.9 for 2 hr at 0° then kept at pH 7.45 for 1 min; d, C-40 exposed to pH 11.9 for 2 hr at 0° then kept at pH 7.45 for 30 min.

The alkaline denaturation of C-40 was also carried out at pH 11.9 and 0°. The dependences of the EHP- and CHP-supported N-demethylase activities on the duration of denaturation were similar to those for the C-40 denatured at 25° within 10 min. However, the increases remained constant up to 2 hr. When C-40 was exposed to pH 11.9 for 2 hr at 0° and then the solution was neutralized to pH 7.45, the N-demethylase activities decreased very slowly and were about 2.8 times those of the native C-40 even after reconstitution for 2 hr, while only 2.5% of the original catalatic activity had been recovered by that time.

Gel Filtration Chromatography of Catalase Preparations

In order to suppress recombination of subunits of catalase during chromatography, reverse flow gel filtration chromatography was carried out with a Sephacryl S-200 Superfine column at 5°. The results are shown in Fig. 4. The elution profile of the native C-40 showed a much broader peak than that of the native C-100, and there was a shoulder at an elution volume corresponding to a molecular weight of *ca.* 60000. When C-40 was exposed to pH 11.9 at 0° for 2 hr followed by reconstitution at pH 7.45 and 0° for 1 min, then 1 ml of the solution (catalase 16.7 mg) was chromatographed, two peaks were obtained in the elution profile (Fig. 4c). The peak at smaller elution volume coincided with that of C-100, and the other one corresponded to that with a molecular weight of *ca.* 60000. When the duration of reconstitution was increased to 30 min, the latter peak decreased in height considerably and became obscure (Fig. 4d). Since the concentration of catalase in the fractions was too low, the N-demethylase activities of the fractions could not be determined.

Discussion

All carefully characterized catalases are oligomers containing four tetrahedrally arranged 60000-dalton subunits. Each subunit consists of a single polypeptide chain that associates with a single prosthetic group, ferric protoporphyrin IX.[12] Tanford and Lovrien reported that commercial lyophilized beef liver catalase is partially dissociated to half-size and quarter-size molecules.[13] Deisseroth and Dounce separated the lyophilized catalase by Sephadex column chromatography and found that a conformationally changed whole-molecular catalase, which has the same spectral and hydrodynamic characteristics as the native enzyme, shows low catalatic activity.[14] Azizov *et al.*[5] reported that development of the peroxidatic activity (substrate: BHP, donor: guaiacol) is observed after prolonged drying of catalase. They also detected peroxidatic activity of catalase molecules which do not differ in hydrodynamic properties from enzyme molecules with unimpaired quaternary structure.

The present experimental findings that the elution profile of C-40 showed a much broader peak than that of C-100, together with a shoulder at an elution volume corresponding to quarter-size molecules, and that the N-demethylase activities of C-100 (which has the highest catalatic activity among the catalase preparations studied) are lower than those of C-40, suggested that the N-demethylase activity of catalase is due to subunits and/or conformationally changed whole molecules of catalase.

Samejima *et al.* reported that beef liver catalase at pH 12 dissociates completely into four subunits within a few minutes and loses its catalatic activity, and that the enzyme recombines when the solution is neutralized.[15] Marklund found that the methyl hydroperoxide-supported peroxidatic activity towards phenolic compounds is strongly increased upon alkaline denaturation of crystalline beef liver catalase, and that longer exposure to high pH leads to a decrease in all activities.[3]

The experimental finding that catalase C-40 showed N-demethylase activity to some extent even at pH 12 indicates that the subunits of catalase do have N-demethylase activity. The gel chromatographic experiment showed that the recombination of the subunits takes place fairly rapidly. On the other hand, the increases in the N-demethylase activities of the alkali-

denatured C-40 decreased only slightly after reconstitution for 2 hr, while the catalatic activity did not increase to an appreciable value. Therefore, in contrast to the previous conclusion,[5] the present results suggest that the bulk of the N-demethylase activities is due to recombined whole molecules which have low catalatic activity.

Although C-40 showed the highest activity in both the EHP- and CHP-supported N-demethylations, the orders of increasing N-demethylase activities were not the same. The alkaline denaturation of C-40 considerably increased the limiting concentration of formaldehyde formed in the EHP-supported N-demethylation of aminopyrine, while it did not appreciably increase that formed in the CHP-supported reaction. The following explanations may be offered for these findings: (a) since EHP reacts with catalase fairly rapidly without addition of reductants,[12] EHP is consumed for both the EHP-supported N-demethylation and the decomposition of EHP itself; (b) the active sites for both reactions are considered to be different,[8] and denaturation with alkali increases the N-demethylase activity and decreases the activity for the decomposition of EHP itself, hence causing a large increase in the proportion of EHP consumed for the former reaction; (c) since the latter reaction does not produce formaldehyde, a net increase in the limiting concentration of formaldehyde is observed; (d) since CHP does not react with catalase without addition of reductants,[4,8] the total amount of CHP is always consumed for the N-demethylation, and hence the limiting concentration of formaldehyde formed in the CHP-supported N-demethylation remains nearly constant in spite of the increase in the N-demethylase activity upon denaturation; (e) the rate of the EHP-supported N-demethylation depends on both active sites, whereas that of the CHP-supported one depends on only one site, and therefore, the order of increasing CHP-supported N-demethylase activity is not necessarily the same as that of the EHP-supported activity.

The order of increasing EHP-supported peroxidatic activity towards methanol was the same as that of the catalatic activity, and alkaline denaturation decreased both activities considerably. However, the peroxidatic activity was almost completely restored on reconstitution, whereas only 7.6% of the catalatic activity was restored even after reconstitution for 24 hr at 25°. This suggests that the active sites for both activities are similar but not identical.

The present results together with the previous conclusions[3,5,8] lead to the following suggestions: (a) hydrogen peroxide is decomposed most efficiently by catalase with a tightly combined quaternary structure, (b) a pair of hydroperoxides and reductants, both of which have fairly small molecular sizes, such as EHP and methanol, can approach the active centers in the catalase molecules described above and undergo the peroxidatic reaction, whereas if one or both have large molecular sizes, such as EHP or CHP and aminopyrine, close approach is not possible; (c) although structural requirements for catalase molecules in the peroxidatic activity towards methanol are less strict than in the catalatic activity, as shown by the recovery of the former activity on reconstitution, cooperation of two or more hematins[16] is also considered to be essential for the appearance of the former activity, because catalase seems to be the only hemoprotein capable of catalyzing the peroxidation of alcohols;[17] (d) catalse molecules with a loosely combined quaternary structure, formed by lyophilization or by alkaline denaturation followed by reconstitution, permit the access of a pair of hydroperoxides and reductants with large molecular sizes, such as CHP and aminopyrine, and catalyze the N-demethylation, whereas they cannot decompose hydrogen peroxide efficiently; (e) the N-demethylase activities cannot be due to impurities in catalase, since C-100, which is a twice-crystallized preparation and has high catalatic activity, shows N-demethylase activity comparable to that of C-40 on denaturation with alkali.

It is clear that the work reported here is only suggestive in nature as far as the mechanism of catalase action is concerned, and it is hoped that the present work will stimulate further interest in the mechanism of the peroxidatic reactions catalyzed by catalase.

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