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TRYPTIC DIGESTION AND ALKALINE DENATURATION OF CATALASE

THE INFLUENCE ON CATALATIC ACTIVITY, PEROXIDATIC ACTIVITY TOWARDS PHENOLIC COMPOUNDS, AND THE REACTIVITY WITH METHYL- AND ETHYL-HYDROPEROXIDE

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SUMMARY

Tryptic digestion of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) leads to a gradually increased peroxidatic activity towards phenolic compounds. The reactivity with ethylhydroperoxide increases 2–3 times after brief digestion but decreases thereafter. The catalatic activity and the reactivity with methylhydroperoxide disappear rapidly. The results indicate a loosening of the structure around the hematins, increasing the reactivity with peroxide and hydrogen donor substrates formerly sterically hindered. This change may occur to some extent without loss of the catalase-specific ability to react rapidly with hydroperoxides. The ability to peroxidize ethanol is apparently not lost before the loss of high reactivity with hydroperoxides.

After brief exposure of catalase to pH $>$ 12 leading to its dissociation into subunits, the enzyme recombines when the pH is lowered (Samejima, T., McCabe, W. J. and Tsi Yang, J. (1968) *Arch. Biochem. Biophys.* 127, 354–360). After this procedure most ($\sim$ 70%) of the catalatic activity is recovered. The reactivity with ethylhydroperoxide is doubled and the peroxidizing activity towards phenolic compounds is strongly increased. The results indicate an increased accessibility of the hematins of recombined catalase. However, they do not become reducible by dithionite. After longer exposure to high pH, all enzymic activities are decreased and the hematins become reducible.

INTRODUCTION

Many papers describe the decrease in the catalatic activity of catalase upon denaturation and the appearance of and increase in the peroxidatic activity towards phenolic compounds[1–7]. However, H_2O_2, which is very rapidly disproportionated by

native catalase, has been employed as peroxide substrate in these studies of the comparatively slow peroxidatic reactions. Interpretation of the results is, therefore, complicated by the fact that a disappearance of the catalatic activity may lead to the appearance of and an apparent increase in the peroxidatic activity even if there is no real increase.

This difficulty was avoided in the present study, in which the changes in peroxidatic activity of catalase upon denaturation were studied using alkylhydroperoxides as peroxide substrates. Alkylhydroperoxides are not rapidly decomposed by catalase[8], and hence true changes in peroxidatic activity could be recorded. Furthermore, the changes in the reactivity of catalase with alkylhydroperoxides, not previously investigated, and the changes in catalatic activity were studied.

The denaturation methods employed, tryptic digestion and exposure to alkaline pH, were chosen so that the agents used would not interfere with reagents for the enzyme assay (*e.g.* H_2O_2-urea[9]) or interact with the hematin groups of the enzyme (as do guanidine and formamide[5]).

The catalatic activity (a)[10] and two peroxidatic reactions, with alcohols (b)[11–13] and phenolic compounds (c)[14,15] as hydrogen donors, were studied.

$$\text{(a)}\quad H_2O_2 + \text{catalase} \xrightarrow{k_1} \text{Compound I} \tag{1}$$

$$H_2O_2 + \text{Compound I} \xrightarrow{k_4'} \text{catalase} + 2\,H_2O + O_2 \tag{2}$$

$$\text{(b)}\quad \text{ROOH} + \text{catalase} \xrightarrow{k_1} \text{Compound I} (+ \text{ROH}) \tag{3}$$

$$RCH_2OH + \text{compound I} \xrightarrow{k_4} \text{catalase} + RCHO + H_2O \tag{4}$$

$$\text{(c)}\quad \text{ROOH} + \text{catalase} \xrightarrow{k_1} \text{Compound I} (+ \text{ROH}) \tag{5}$$

$$\text{Compound I} + AH_2 \xrightarrow{k_7} \text{Compound II} + AH\cdot \tag{6}$$

$$\text{Compound II} + AH_2 \xrightarrow{k_4} \text{catalase} + AH\cdot \tag{7}$$

The most common designations for the rate constants have been used. Both alkylhydroperoxides and H_2O_2 can function in the first step in all three reactions whereas the second step in (a) is specific for H_2O_2 (ref. 8). Reaction 7 (k_4) is the rate-limiting step for the peroxidative reaction against phenolic compounds when the peroxide is added in excess[16].

The rate of formation of Compound I from catalase and hydroperoxides ("k_1") decreases rapidly with increasing size of the hydroperoxide[10,17–20]. This has been interpreted as being due to steric hindrance to the access to the hematins of catalase and not to a decreasing reactivity of the peroxides per se, as no such dependence on size is seen for Compound I formation with *e.g.* horseradish peroxidase.

The non-reducibility of native catalase by dithionite and a low peroxidative activity towards phenolic compounds[15,16], which are comparatively large molecules, has also been explained as being due to a low accessibility of the catalase hematins.

The peroxidatic activities towards phenolic compounds of many hemo-

proteins[2,4,5,21–23] and hematin complexes with nitrogen bases[24,25] are high and may sometimes approach those of true peroxidases when the reaction with hydrogen donor substrate ("k_4" Formula 7) is made rate limiting. The activity is often increased by denaturation[4,5,21,22], probably due to increased exposure of the hematins. Hence this type of reaction appears to be an inherent property of a complexed hematin group which is only partially dependent on the protein parts.

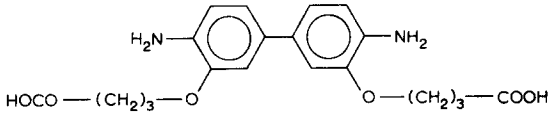
On the other hand, if the reaction with H_2O_2 is made rate limiting, large differences are seen, peroxidases (and catalases) giving rate constants of the order of 10^7 $M^{-1} \cdot s^{-1}$ whereas other hemoproteins and hematin complexes react much more slowly[4,5,22,24,26] ($k \leq 10^4$ $M^{-1} \cdot s^{-1}$). Hence, a rapid reaction with H_2O_2 seems to be a distinctive feature of peroxidases and catalases which must depend on a very special configuration of the protein parts near the hematins.

Catalases seem to be the only hemoproteins capable of catalyzing the peroxidation of alcohols[27], and their catalatic activities are $\approx$ 10 000 times higher than that of a free hematin or that of horseradish peroxidase (here Reaction 2 is crucial) which seems to be the most active of other hemoproteins[27].

In the following discussion both the "k_1" and "k_4" reactions of catalase are tentatively assumed to depend on (1) a "steric factor" determining accessibility and (2) the reactivity of the peroxide or hydrogen donor substrate with the hematins. The "active sites" for the different classes of substrates are not necessarily identical, though they are probably associated with the hematins. The sterical hindrance afforded by the protein may, therefore, differ.

MATERIALS AND METHODS

H_2O_2, Perhydrol, Merck. Pyrogallol, Baker and Adamson. Guaiacol was double distilled *in vacuo*. Dicarboxidine, γ,γ'-(4,4'-diamino-3,3'-diphenyldioxi dibutyric acid)



was a generous gift from AB Kabi, Stockholm. Ethylhydroperoxide, AB Ferrosan, Malmö. Methylhydroperoxide was synthesized according to Criegee[28]. The H_2O_2 content of the two hydroperoxides was 1.6% and 0.75%, respectively, as determined by Ti(IV)[29] which does not react with alkylhydroperoxides. Water was double distilled.

Crystalline bovine liver catalase was obtained from Boehringer. It was dissolved in 30 mM sodium phosphate, pH 7.4, and passed through a Sephadex G-200 column (110 cm). $A_{405}/A_{280} = 0.95$. ε_{405} was taken as $3.24 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ (30) and it was assumed to contain 3 hematins/molecule. All rate constants were calculated per molar of hematin. Horseradish peroxidase, fraction III b (31), $\varepsilon_{403} = 1.00 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$. Trypsin-TPCK, 200 units $\cdot$ mg^{-1}, Worthington Biochemical Corp.

Unless otherwise indicated the experiments were performed at 25 °C. For spectrophotometry a Beckman Acta III was used.

Tryptic digestion of catalase. Catalase (13.5 μM) was incubated with trypsin

(5 mg/ml) in 30 mM sodium phosphate, pH 7.40, at 25 °C. After measured time intervals the reaction was quenched by the transfer of aliquots of the solution to 5 volumes of 100 mM sodium acetate, pH 4.25, 0 °C.

Alkaline denaturation of catalase. Catalase (2.24 μM) was incubated in 95 mM sodium phosphate, pH 12.2, at 0 °C. After measured time intervals aliquots of the solution were neutralized (final pH, 7.75) by the addition to 1.7 vol. of 100 mM sodium phosphate, pH 6.0, 0 °C. The catalase was then allowed to reconstitute (*c.f.* ref. 33) for at least 15 h before determination of enzymic activities.

Catalatic activity was determined from the decrease in absorbance at 230 nm of a H_2O_2 solution (8.3 mM) in deaerated 50 mM sodium phosphate, pH 6.9, upon the addition of catalase.

"k_1, *alkylhydroperoxide, ethanol*". The rate constant for the reaction between catalase and alkylhydroperoxides as determined using ethanol as hydrogen donor substrate. Catalase was added to solutions containing 50 μM methylhydroperoxide or 80 μM ethylhydroperoxide and 500 mM ethanol in 50 mM sodium phosphate, pH 6.9. After measured time intervals aliquots were analyzed for hydroperoxide content from the increase in A_{440} upon the addition of peroxidase (0.3 μM) and dicarboxidine (0.5 mM).

Under these conditions k_1 (Formula 3) will be rate limiting and may be obtained from the pseudo first-order decrease in alkylhydroperoxide. With native catalase ethyl- and methylhydroperoxide gave $k_1 = 2.3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ and $6.1 \cdot 10^5$ $M^{-1} \cdot s^{-1}$, respectively, in good agreement with results obtained by stopped-flow technique[17].

"k_4, *pyrogallol, dicarboxidine, guaiacol*". The peroxidatic reactions with phenolic compounds were studied with the reaction between "phenol" and catalase rate limiting. Methylhydroperoxide (1–3 mM) was added to solutions containing catalase and pyrogallol (8 mM), or dicarboxidine (0.5 mM), or guaiacol (10 mM) in 100 mM sodium phosphate, pH 6.0. The rate constants were calculated from the initial rate of increase in absorbance at 400 nm, 440 nm and 470 nm, respectively, and the molar absorptivities (in terms of reduced alkylhydroperoxide) $1.1 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, $12.2 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ and $6.0 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, respectively. With native catalase, the rate constants were 290, 200 and 10 $M^{-1} \cdot s^{-1}$, respectively.

"k_1, *ethylhydroperoxide, pyrogallol*". The rate constant for the reaction between ethylhydroperoxide and catalase, using pyrogallol as hydrogen donor substrate, was obtained from the first-order approach of a level in A_{400} upon addition of ethylhydroperoxide (20 μM) to catalase and 8.3 mM pyrogallol in deaerated 50 mM sodium phosphate, pH 6.0. Native catalase gave $k_1 = 1.6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

RESULTS AND DISCUSSION

Tryptic digestion

Fig. 1 shows changes in various enzymic activities of catalase upon tryptic digestion. The peroxidatic activities towards phenolic compounds increase. The activity towards the initially most slowly reacting substrate, guaiacol, is increased most, $>$ 60 times, reaching $k_4 \approx 640$ $M^{-1} \cdot s^{-1}$. Pyrogallol attains a maximal k_4 of 1100 $M^{-1} \cdot s^{-1}$.

The catalatic activity decreases rapidly, whereas the rate constants for the

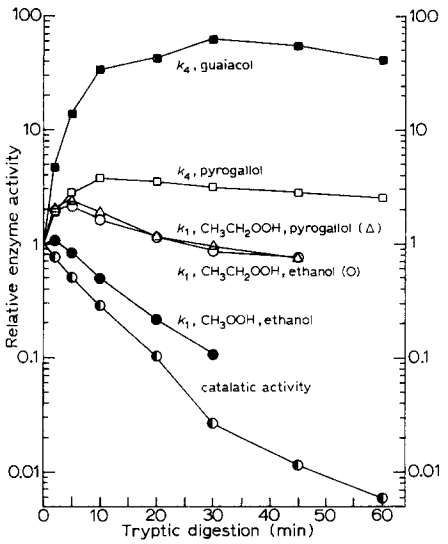


Fig. 1. Changes in enzymic activities of catalase upon tryptic digestion. The tryptic digestion and enzymic analyses were performed as described in Materials and Methods.

reactions between the alkylhydroperoxides and those catalase molecules still capable of peroxidizing ethanol increase initially. This effect is most pronounced with ethylhydroperoxide which reacts most slowly with native catalase.

The results indicate that tryptic digestion leads to exposure of the hematins, increasing their inherent "phenolic compound" peroxidizing ability. Furthermore, exposure of the hematins may occur to some extent without loss of the specific enhancement by the protein of the reactivity with hydroperoxides as indicated by the increase in "k_1, ethylhydroperoxide" and "k_1, methylhydroperoxide" especially the former, and also by the decrease in the difference between them.

The changes in "k_1, ethylhydroperoxide" in Fig. 1 as measured with the non-specific substrate pyrogallol, is essentially equal to the changes in "k_1, ethylhydroperoxide" as measured with ethanol. In spite of the denaturation "k_1, ethylhydroperoxide" remains fairly high and the catalase molecules contributing the essential part of the reactions must retain a high reactivity with hydroperoxides. These molecules do apparently in the same time retain the catalase-specific ability to peroxidize ethanol, otherwise higher values for "k_1, ethylhydroperoxide" would have been obtained with pyrogallol.

With native catalase the rate of the catalatic reaction is primarily determined by the first step, "k_1" (ref. 10). The fairly large difference in the decrease in "k_1, ethylhydroperoxide" and "k_1, methylhydroperoxide" in Fig. 1 indicates a decrease in importance of the steric factor in "k_1" which suggests that k_1 for H_2O_2, a smaller molecule, should decrease more rapidly than "k_1, methylhydroperoxide". This could account for the more rapid decrease in the catalatic activity as compared with 'k_1, methylhydroperoxide". However, it is difficult to judge whether a loss of ability to perform the first reaction or the second reaction is primarily responsible for the decrease in catalatic activity. It is probable that both catalase specific abilities are generally lost in parallel, as are possibly also the reactions involved in the ethanol peroxidation.

Alkaline denaturation

If catalase is exposed to pH $\geq$ 12 it is dissociated within a few minutes into four subunits and loses its catalatic activity[32,33]. The activity may be partially recovered if the solution is neutralized. The longer the exposure to high pH the less activity could be recovered. A reconstituted catalase was passed through a DEAE-cellulose column and $\approx$ 50% of the protein could be recovered. It could not be distinguished from native catalase with respect to catalatic activity, sedimentation coefficient and ORD spectrum[33].

Fig. 2 demonstrates the changes in several enzymic activities of catalase after exposure to high pH and subsequent recombination in neutral solution. The reactions which are rapid with native catalase, catalatic activity and "k_1, methylhydroperoxide" are decreased, whereas the reactivity with ethylhydroperoxide and the peroxidizing activity towards "phenols" are increased, more the lower they are with native catalase. The results indicate an increased accessibility of the hematins. The changes are related to the dissociation into subunits and the subsequent recombination upon lowering of the pH. Longer exposure to high pH leads to a decrease in all activities.

In contrast to the previous conclusions[33] the present results indicate that the recombined catalatically active enzyme is different from native catalase.

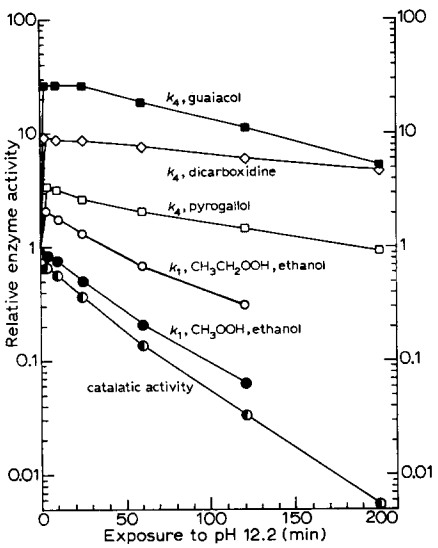


Fig. 2. Changes in enzymic activities of catalase as a result of exposure to pH 12.2 and subsequent recombination at neutral pH. The alkaline denaturation and enzymic analyses are described in Materials and Methods.

4-min exposure to high pH gives a maximal increase in peroxidatic activities, but does not make the hematins reducible by dithionite. This seems to indicate that the nonreducibility of catalase is not merely related to steric hindrance around the hematins. After longer exposure reducibility appears as evidenced by a band at 559 nm, but not as rapidly as catalatic activity is lost. Most hematins appear to be reducible after 120 min and 200 min.

CONCLUSIONS

Pyrogallol, dicarboxidine and guaiacol were very slowly peroxidized by native catalase. Upon denaturation of the enzyme, large real increases in the peroxidatic activity were found, more the lower the activity was initially. The result supports the notion that the "active sites" for phenolic compounds are sterically shielded. It seems unlikely that this type of reaction is of physiological relevance for catalase.

The reactivity of catalase with hydroperoxides is roughly inversely dependent on their size. The idea, that this is due to steric hindrance around the "active sites" for hydroperoxides is supported by the present findings. Upon limited denaturation, the reactivity with ethyl and methylhydroperoxides was increased, more with ethylhydroperoxide, which reacted most slowly initially.

The cytochemical staining of peroxisomes by the diaminobenzidine-H_2O_2 method[34], is dependent on an increased peroxidatic activity and an extensive loss of catalatic activity of the peroxisomal catalase, due to denaturation brought about by fixation and incubation at high pH[35–37].

In the present experiments, the major increase in peroxidase activity was obtained while 30–60% of the catalatic activity still remained. An extensive loss of catalatic activity was accompanied by a decrease in peroxidatic activity. The use of methylhydroperoxide instead of H_2O_2 might allow the development of improved staining procedures for peroxisomes.

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