# Denatured Hemoproteins as Catalysts in Lipid Oxidation[1]

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## ABSTRACT

Purified catalase and peroxidase were denatured by heat, acid and urea. Denaturation resulted in up to 22-fold increase in nonenzymatic lipid oxidation activity concomitant with loss of enzymatic activity. It is proposed that the increased nonenzymatic activity is due to increased exposure of the heme group. Acid-splitting of the hemoproteins into apoprotein and hemin had the greatest influence on both of the catalytic activities and recombination reversed the effect. Urea-denatured hemoprotein possessed increased nonenzymatic activity due to increased exposure of the protein-bound heme, however, peroxidase increased less than catalase which is consistent with the fact that peroxidase is the more heat stable enzyme. Nonenzymatic activity of the heat denatured hemoproteins was maximum when catalase was treated at 90 C for 2 min and peroxidase at 100 to 125 C for 5 to 30 min.

## INTRODUCTION

Catalase and peroxidase can function as catalysts in two different ways. One is to act as an enzyme in the decomposition of a substrate, such as hydrogen peroxide, the other is to catalyse nonenzymatically the oxidation, by molecular oxygen, of unsaturated compounds, e.g., unsaturated fatty acids. Both the enzymatic and nonenzymatic activities are performed at the iron porphyrin group present in these proteins. In addition, the enzymatic activity is dependent on the secondary and tertiary structure which form the active site.

Nonenzymatic activity on unsaturated fatty acids is well known for most native hemoproteins such as hemoglobin, myoglobin, catalase, peroxidase and cytochrome c (1). In a preliminary report from our laboratory we showed that the nonenzymatic activity on linoleic acid of bovine liver catalase and horseradish peroxidase increased significantly when they were treated with heat or acid (2). Purified lactoperoxidase responded to heat treatment in the same way (3). One explanation of this behavior might be that denaturation led to conformation changes in the protein whereby the catalytically active heme group became more favourably exposed to the surrounding lipid molecules during the reaction.

The work presented in this paper concerns the increased nonenzymatic activity of catalase and peroxidase due to increased exposure of the heme group. The investigations involved, except heat treatment, chemical denaturation of the protein. Manipulation of pH was used for the splitting and recombination of the apoprotein and hemin. Heat treatment and 8 M urea were used to alter the conformation of the proteins.

## EXPERIMENTAL PROCEDURES

Purified bovine liver catalase (E.C.1.11.1.6) containing 39,000 units/mg (Boehringer, Germany), horseradish peroxidase Reinheitszahl (RZ), the absorbance ratio $A_{403nm}/A_{275nm}$, 1.73 and 1.08 (Worthington, USA) and hemin chloride, iron content 8.7% (NBC, USA) were used as catalysts. The catalase was delivered as a crystal suspension. The crystals were dissolved during dialysis against 10 mM $NaHCO_3$ at 4 C overnight and insoluable material was removed by centrifugation. Peroxidase, RZ 1.08, delivered as a lyophilized powder, was further purified by ion exchange chromatography and gel filtration prior to use. One hundred milligrams of the impure peroxidase was dialyzed against 1 mM tris (hydroxymethyl) aminomethane hydrochloric acid (THAM-HCl) pH 7.2 at 4 C. After low speed centrifugation to remove insoluable material, the peroxidase was passed through a 2.5x38 cm column of O-(diethylaminomethyl)-cellulose (DEAE-cellulose) (DE-52 preswollen, Whatman, England). The cellulose was first treated with a 0.1 M pyridiniumchloride solution containing 20% (w/v) NaCl and then equilibrated with 1 mM THAM-HCl pH 7.2. Five milliliter fractions were taken every 15 min and the absorbancy at 403 nm and 275 nm was measured in each colored fraction. The tubes containing peroxidase were pooled so that an average RZ value of 2.0 or more was obtained. The peroxidase solution was concentrated to approximately 3 ml by vacuum dialysis. The concentrated peroxidase was then passed through a 2.5x95 cm column of Sephadex G-150 (Pharmacia, Sweden) by upward flow elution in 1 mM THAM-HCl pH 7.2. Fractions were collected and absorbancy measured as above. Again, the tubes containing peroxidase were pooled until the final peroxidase solution had an RZ value of about 3. The purified peroxidase was concentrated by vacuum dialysis.

Hydrogen peroxide, 30% (Merck, Germany) was used in the enzymatic assay of catalase and peroxidase and *cis-cis* linoleic acid, 98% (Fluka, Switzerland) for the nonenzymatic assay. Analytical grade HCl and urea were used as chemical denaturants. The urea was recrystallized three times from 80% ethanol prior to use.

Spectrophotometric measurements were made in a Perkin-Elmer Model 124 instrument. The enzymatic activity was measured spectrophotometrically for catalase (4) and peroxidase, the latter with O-dianisidine as the hydrogen donor (Worthington, enzyme manual, 4-69). The enzymatic activity of catalase and peroxidase is expressed as the absorbance change per min·mg enzyme. In Figure 3, for convenience, the enzymatic activities after heat treatment are expressed as per cent of the activity of the untreated enzymes. The nonenzymatic activity was measured at pH 6.5 with 10 mM linoleic acid as the substrate according to a previously described polarographic method (5) and is expressed as nmole $O_2$ consumed per minute. The catalyst concentration used in each case is specified in the legends to tables and figures.

Except for the pH experiments, the hemoproteins were kept as stock solutions containing 1-10 mg protein per milliliter in 10 mM sodium phosphate buffer pH 6.5. The hemoprotein concentration was determined spectrophotometrically by use of the millimolar absorbance coefficient $\epsilon_{mM} = 324$ at 405 nm for catalase (6) and $\epsilon_{mM} = 91$ at 403 nm for peroxidase (7). Hemin chloride was converted into hematin by dissolution in alkali, and was stored as a 1 mM stock solution in 0.1 M NaOH from which dilutions were made immediately before assay. The influence of pH on the nonenzymatic activity was measured by adding the hemoproteins from stock solutions in distilled water to the linoleic acid emulsions in universal citrate-phosphate-borate buffer covering the pH range of 2.90-9.20. The ionic strength of the emulsion was adjusted to I=0.2 at each pH by the addition of KCl.

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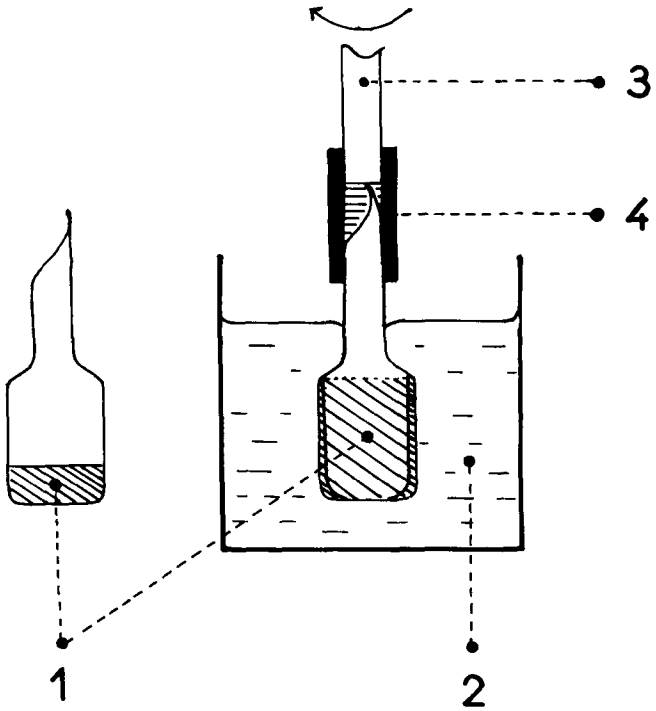


FIG. 1. Heat treatment of hemoproteins by the spinning ampoule technique. 1, hemoprotein solution in a sealed ampoule; 2, temperature controlled glycerol bath; 3, motor shaft; 4, latex tubing.

Heat treatment was performed over the temperature range of 25-140 C for periods of 2-30 min by a spinning technique illustrated in Figure 1. One-milliliter samples of hemoprotein solution were transferred to thin-walled (0.45 mm) 3 ml glass ampoules. The ampoules were sealed and then attached to the shaft of a variable speed electric motor. During these procedures the ampoule was kept at 0 C by immersing in a small ice bath. Rotation of the ampoule at a suitable speed distributed the solution over the inner glass wall. The spinning ampoule was then transferred from the ice bath to a temperature controlled glycerol bath, kept there for the predetermined time and then, still spinning, returned to the ice bath. Enzymatic and nonenzymatic assays were made immediately after the treatment.

Denaturation by chemical means was performed by dialysing the hemoprotein solution against a large excess of 8 M urea in 0.1 M sodium phosphate at pH 6.5. Splitting

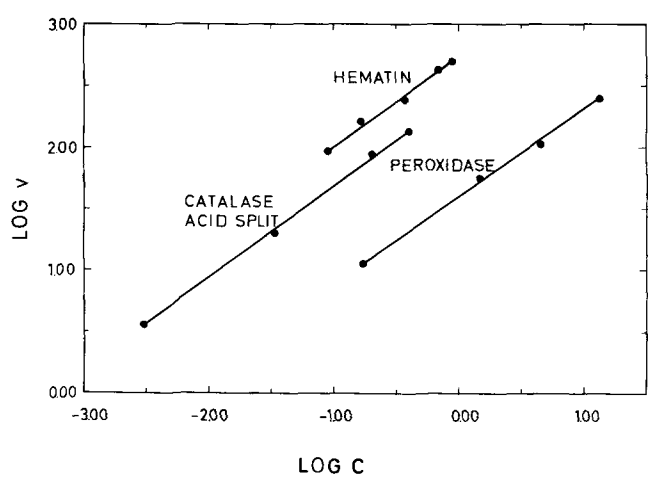


FIG. 2. Nonenzymatic activity of hemoproteins and hematin vs. concentration; v in nmole $O_2$/min, C in $\mu$M.

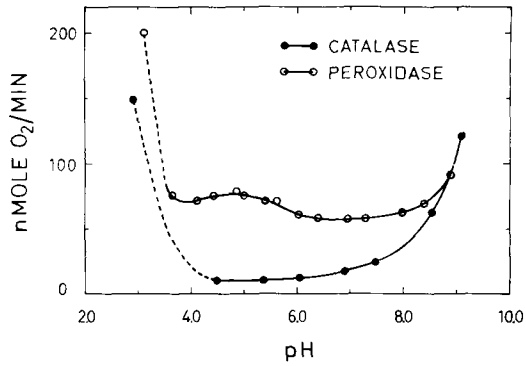


FIG. 3. Nonenzymatic activity of catalase and peroxidase (RZ=1.73) vs. pH, I=0.2. Values obtained in the lower pH range (---) are time dependent. Assay: 1.6 nmole catalase or 5.7 nmole peroxidase, 37.1 $\mu$mole linoleic acid in 91 mM phosphate buffer pH 6.5, 25 C, in a total volume of 4.11 ml.

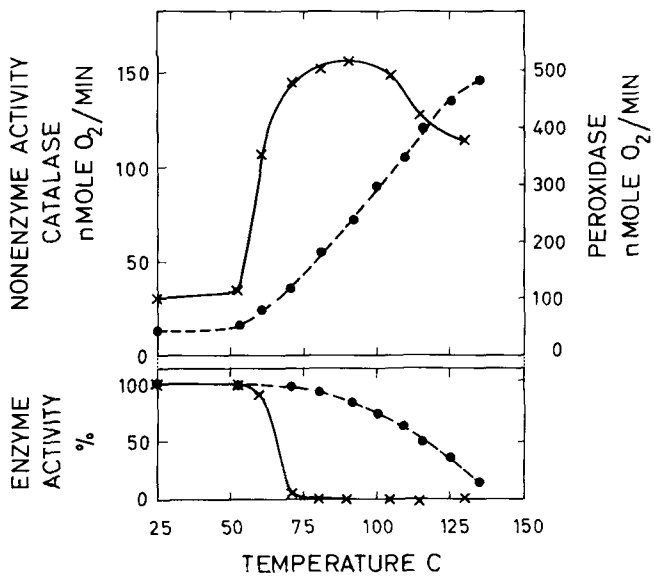


FIG. 4. Nonenzymatic and enzymatic activity of catalase x—x, and peroxidase (RZ 1.73) •----• after heat treatment for 2 min at the temperatures indicated. Nonenzymatic assay: 1.6 nmole catalase, 5.7 nmole peroxidase, 37.1 $\mu$mole linoleic acid in 91 mM phosphate buffer pH 6.5, 25 C, in a total volume of 4.11 ml.

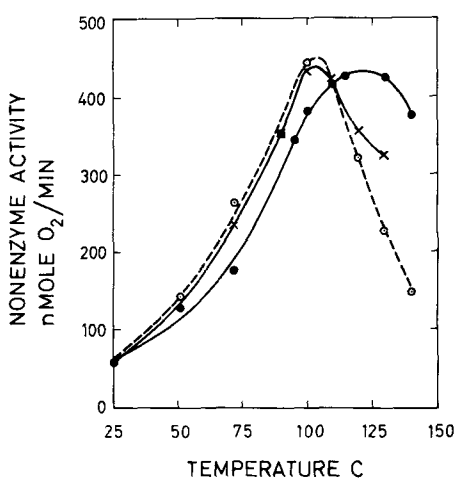


FIG. 5. Nonenzymatic activity of peroxidase (RZ=1.73) after heat treatment for 5 min •——•, 15 min x——x and 30 min o-----o at the temperatures indicated. Assay: 5.7 nmole peroxidase 37.1 $\mu$mole linoleic acid in 91 mM phosphate buffer pH 6.5, 25 C, in a total volume of 4.11 ml.

TABLE I

Enzymatic and Nonenzymatic Activity of Native and Denatured Peroxidase (RZ[a]=3.17)[b]

| Treatment of peroxidase | Enzymatic activity, $A_{460}$/min·mg | Nonenzymatic activity, nmole $O_2$/min |
|---|---|---|
| Native pH 6.5 | 13900 | 22 |
| Acid split pH 2.1 | 110 | 326 |
| Apoprotein pH 7.5 | 63 | 0 |
| Hematin pH 7.5 | 0 | 221 |
| Split and recombined pH 6.5 | 10500 | 66 |
| 8 M urea pH 6.5 | 8300 | 90 |
| Heated 100 C, 15 min, pH 6.5 | 3280 | 232 |

[a]RZ, Reinheitszahl, the absorbance ratio $A_{403nm}/A_{275nm}$.

[b]Nonenzymatic assay: 3.6 nmole peroxidase, 37.1 μmole linoleic acid in 4.11 ml 91 mM phosphate buffer, pH 6.5, 25 C.

into apoprotein and hemin and subsequent recombination was made according to a previously described method (8). In the present experiment enzymatic and nonenzymatic assay was run both after the splitting reaction (HCl addition, pH 2.1) and after recombination (NaOH addition, pH 6.5) without intermediate separation of the cleavage products. In separate experiments such fractionation was made twice in acid acetone (9). The precipitated apoprotein and the hemin obtained after evaporation of the combined organic solvents were dissolved in 1% $NaHCO_3$ and 10 mM NaOH respectively. After dilution to the original concentrations the apoprotein and hematin were assayed separately.

## RESULTS

The reaction rate, v, of the nonenzymatic oxidation of linoleic acid was related to the hemoprotein or hematin concentration, C, according to $v = k \cdot C^{0.7}$ (Fig. 2) over the entire concentration range used in this investigation. These data ascertained that no critical hemoprotein concentration was exceeded where antioxidative effects appears according to previous work (10,11). It also showed that free heme, heme free but in the presence of protein and protein bound heme, had the same nonenzymatic kinetics.

The nonenzymatic activity of both catalase and peroxidase was not influenced by the variation of pH in, or slightly below, the neutral range (Fig. 3) whereas both more acid and alkaline conditions increased their activities. The influence of pH on the nonenzymatic activity of catalase is almost a mirror image of the curve previously obtained for the pH dependent enzyme activity of catalase (12).

Heat treatment for 2 min in the temperature range of 25-140 C increased the nonenzymatic activity of both catalase and peroxidase (Fig. 4), and decreased the enzymatic activity. The nonenzymatic activity of catalase was maximum around 90 C whereas the enzymatic activity was completely lost above 75 C. In contrast to catalase, peroxidase continuously increased in nonenzymatic and decreased in enzymatic activity over the entire experimental range. Increased exposure times, 5, 15 and 30 min, revealed a maximum in the nonenzymatic activity of peroxidase being reached in the range of 100 to 125 C (Fig. 5). In the catalase samples representing the curve after the maximum in Figure 4, aggregation could be followed visually whereas no observations of that kind was made in the corresponding samples of peroxidase in Figure 5.

The enzymatic and nonenzymatic activity of acid-split and urea-treated peroxidase and catalase were compared with those of the untreated proteins and with those treated by a time-temperature combination sufficient to raise the nonenzymatic activity to the peak values as shown in Figures 4 and 5. The results are given in Tables I and II. Spectra of the differently treated hemoproteins were recorded and are shown in Figures 6 and 7.

Table I summarizes the results obtained with peroxidase. Splitting into apoprotein and hemin resulted in a large increase in the nonenzymatic activity, at the same time enzymatic activity was nearly completely lost. A rapid but unavoidable partial recombination of the apoprotein and hemin might take place under the assay conditions and therefore the value obtained is probably too low. Recombination under controlled conditions restored 76% of the original enzymatic activity and again decreased the nonenzymatic activity. During the splitting reaction, the apoprotein and the released hemin occurred together in the solution but separation of the two parts in acid acetone showed that the nonenzymatic activity was associated with the separated hematin alone. The treatment of peroxidase with 8 M urea also increased the nonenzymatic activity, but to a lesser extent than splitting. Also the enzymatic activity was less affected, 60% of it remained, indicative of no severe damage to the protein which was further supported by the fact that the spectrum of the urea treated peroxidase closely resembled that of the native one (Fig. 6). There was a 10-fold increase in the nonenzymatic activity after treatment at 100 C for 15 min and at the same time the enzymatic activity was reduced by approximately 75%. The spectrum (Fig. 6) showed a reduced absorbance in the Soret region and a weak band around 350 nm which indicated alterations in the heme-protein binding.

Catalase gave similar results to peroxidase (Table II). All denaturating treatments of catalase led to a considerable

TABLE II

Enzymatic and Nonenzymatic Activity of Native and Denatured Catalase[a]

| Treatment of catalase | Enzyme activity, $A_{240}$/min·mg | Nonenzymatic activity, nmole $O_2$/min |
|---|---|---|
| Native pH 6.5 | 2410 | 15 |
| Splitting pH 2.1 | 0 | 333 |
| Apoprotein pH 7.5 | 0 | 125 |
| Hematin pH 7.5 | 0 | 203 |
| 8 M urea pH 6.5 | 0 | 305 |
| Heated 100 C, 2 min, pH 6.5 | 0 | 206 |

[a]Nonenzymatic assay: 0.9 nmole catalase, 37.1 μmole linoleic acid in 4.11 ml 91 mM phosphate buffer, pH 6.5, 25 C.

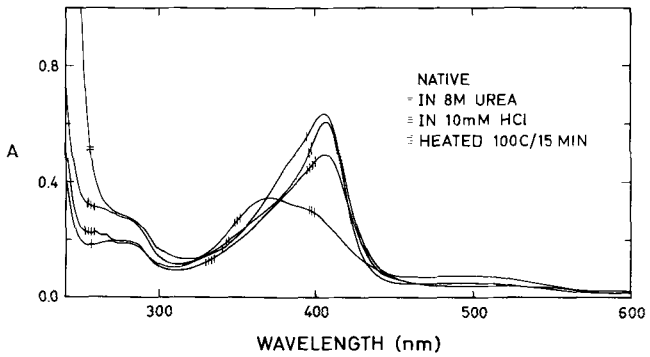


FIG. 6. Spectra of native and denatured peroxidase (RZ=3.17) 7.0 μM. Native, urea- and heat-treated peroxidase in 0.1 M sodium phosphate pH 6.5.

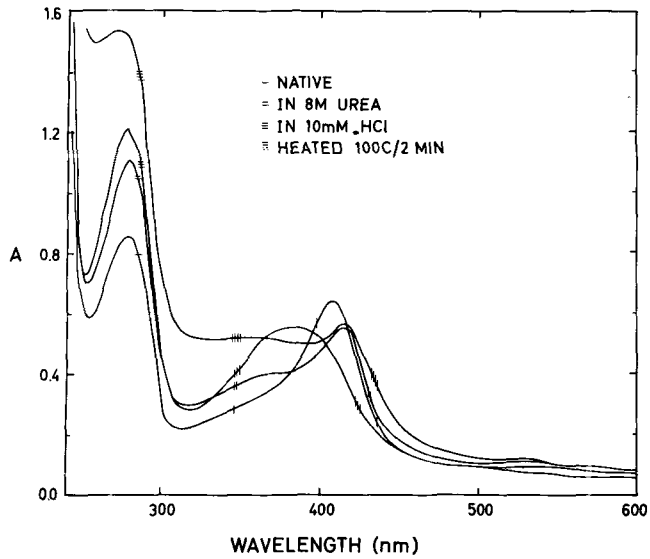


FIG. 7. Spectra of native and denatured catalase, 2.0 μM. Native, urea- and heat-treated catalase in 0.1 M sodium phosphate pH 6.5.

increase in nonenzymatic activity and a complete loss of enzyme activity. As with peroxidase, acid-splitting of catalase had the largest effects but it was found that the protein obtained after fractionation in acid acetone still had a considerable nonenzymatic activity but no enzymatic activity. It is important to note that the incompletely cleaved protein fraction had a nonenzymatic activity eight times as large as that of the native protein. Efforts to recombine apoprotein and hemin led to precipitation of the protein and were therefore omitted. Treatment by urea or heat did not increase the nonenzymatic activity as much as splitting. However, the spectra indicated that catalase was more affected by the treatments than peroxidase (Fig. 7).

Heat treatment of catalase at temperatures higher than 100 C for at least 2 min led to less increased nonenzymatic activity than treatment up to 90 C (Fig. 4) possibly due to a growing tendency for the denatured protein to aggregate at the higher temperatures, which also was followed visually. Heat treatment of catalase in the presence of 8 M urea gave however, the same results as the urea treatment alone fairly independent of the additional heat treatment (Table III). The nonenzymatic activity of catalase treated by the combination of heat and urea was much higher than the heat treatments alone which led to coagulation of the protein.

## DISCUSSION

Results from the introductory experiments clearly show the contrasting effects by pH (Fig. 3) and heat (Fig. 4) on the nonenzymatic and enzymatic activity both of peroxidase and catalase. The marked effect on the nonenzymatic activity by low pH was consistent both with the facts that heme cleavage increases considerably below pH 4.5, especially at high ionic strength (13), and that hematin had the highest nonenzymatic catalytic activity (Fig. 2).

Loss of enzyme activity is a sensitive criterion for induced conformation changes in the protein molecule. Such changes involve random coil formation when intramolecular bonds like hydrogen and disulfide bonds are broken in strong urea and guanidine solutions in the presence of reducing agents and by mild heat treatment (14). Strong heat treatment on the other hand causes intermolecular bonding resulting in the formation of aggregates and finally coagulation. It seems reasonable to assume that peroxidase and catalase, like myoglobin and hemoglobin, carry their heme groups in hydrophobic pockets within the globin molecule (15). A major conformation change in these proteins must result in the unmasking of the heme groups which are then more exposed to the surrounding solution, thus increasing the probability of collision with the relatively large linoleic acid molecules. Aggregation caused by strong heat treatment will, on the other hand, counterbalance the effect of unmasking, which might be the explanation to the shape of the nonenzymatic activity curves in Figure 4 and 5. The above hypothesis was supported by the following results.

Both with catalase and peroxidase, acid cleavage of the heme from the apoprotein produced the largest effect both on the enzymatic and nonenzymatic activity. The enzymatic activity was almost completely lost and the nonenzymatic activity increased 15- to 22-fold (Tables I and II). The heme released by acid cleavage might be expected to represent the maximal possible increase in the nonenzymatic activity. However, 3.6 nmole hematin had 496 nmole $O_2$ per minute activity (calculated from Fig. 2) as compared with 325 for acid-split peroxidase (Table I and 333 for 0.9 nmole (3.6 nmole heme) acid-split catalase (Table II). Apart

TABLE III

Nonenzymatic Activity of Catalase Heat-Treated in the Presence of 8 M Urea[a]

| Treatment of catalase | | Nonenzymatic activity, nmole $O_2$/min | Aggregation[b] |
|---|---|---|---|
| Control 25 C, | no addition | 13 | - |
| | in 8 M urea | 165 | - |
| 75 C, 5 min, | no addition | 60 | + |
| | in 8 M urea | 165 | - |
| 125 C, 5 min, | no addition | 45 | + |
| | in 8 M urea | 148 | - |

[a]Nonenzymatic assay: 0.4 nmole catalase, 37.1 μmole linoleic acid in 4.11 ml 91 mM phosphate buffer pH 6.5, 25 C.

[b]+, Visible; -, Not visible.

from interference of the apoprotein present in the reaction mixture and chemical differences between the commercial and the separated hemins, the lower figures obtained can be explained by partial recombination of the hemin and apoprotein under the assay conditions (pH 6.5) as mentioned under Results. Controlled recombination of acid split peroxidase was finished within 30 min but during the first 10, 20 and 30 sec of the reaction as much as 22%, 29% and 37% of the total recombination took place. During assay, the maximal slope of the reaction curve, used as the rate measure, was usually not reached until 20 to 30 sec after addition of the catalyst. During this time considerable recombination occurred resulting in decreased nonenzymatic activity. In the spectra of acid split peroxidase and catalase (Fig. 6 and 7) absorption maxima are located at 370-380 nm, which indicates that the free hemin had dimerized or polymerized (16). It is, however, not known how polymerization of hemin influences the nonenzymatic activity.

After separation of the apoprotein from hemin in acid acetone, no nonenzymatic activity remained in the protein fraction obtained from peroxidase in contrast to that from catalase which contained considerable activity (Tables I and II). Previous work has revealed that acid denatured bovine liver catalase still contained 20-25% helical structure (17). The same authors, in another investigation, showed by enzyme activity measurements, that the denaturation of catalase at pH 3 consisted of an initial fast reaction followed by a slower one. No differences were, however, detected in the spectra obtained at different times during the experiment (6). These findings open the possibility that incompletely disordered polypeptide chains in our experiment were still able to bind hemin at unspecific sites of the protein. Bovine serum albumin is capable of unspecific and strong binding of hematin (18) and, in a separate experiment to be reported later, we found that a bovine serum albumin - hemin complex had a nonenzymatic activity and a visible spectrum comparable to those of acid denatured catalase. After both neutral and acid acetone fractionation, the albumin protein retained nonenzymatic activity. The fraction of nonenzymatic activity found in protein obtained after acid acetone precipitation of catalase was more than eight times that of the native catalase (Table II) and the result indicates that heme bound to denatured protein was sufficiently exposed to enable increased nonenzymatic activity. Additional evidence for this conclusion is the increased nonenzymatic activity following urea treatment of peroxidase and catalase (Tables I and II). The corresponding spectra (Fig. 6 and 7) did not show any heme-splitting. The spectrum of peroxidase in 8 M urea had changed very little from that of the native peroxidase, even after prolonged storage, an expected result in view of the fact that 60% of the enzyme activity remained (Table I). Urea treatment of catalase for 16 hr caused a small red shift in the Soret band. In 8 M urea, in the absence of a reducing agent, bovine liver catalase dissociates into two subunits, each having half the molecular weight of the catalase (19). Dissociation of bacterial catalase into subunits at pH 12.8 was accompanied by a blue shift in the Soret band (20). Similar results were obtained when beef erythrocyte catalase was dissociated into subunits through lyophilization and when the same enzyme was kept in 8 M urea for 1 hr (21). The same authors also observed that prolonged storage of bovine liver catalase caused a similar red shift in the Soret band, as that obtained in the present investigation. The reasons for the red shift are not known. They suggested that it was due to the presence of bilirubin in the liver enzyme.

Treatment of peroxidase and catalase with 8 M urea thus led to increased exposure of the heme which was still bound to the protein or protein subunit essentially at the original site, the structure of which is stabilized by the heme as long as the latter is not removed. In contrast to our results with peroxidase and catalase, spectra of metmyoglobin, after prolonged storage at high concentrations of urea or guanidine hydrochloric acid, were similar to those obtained from hematin alone in urea or guanidine solutions (22). Even in this case the heme is believed to be bound unspecifically to the protein because separation of the heme is known to occur primarily at low pH preferably in the presence of chloride ions (13) when competing ligands are absent.

Heat treatment increased the nonenzymatic activity of peroxidase and catalase by factors of 10 and 14 respectively. The spectra (Fig. 6 and 7) differed a little from those obtained on urea treatment but they did not show appreciable hemesplit, since no blue shifts in the Soret bands were induced. For peroxidase, the reduced absorbance at 403 (Fig. 6) and the 75% loss of enzyme activity clearly showed, however, that the globin-heme bonds had been altered. The spectrum of heat-treated catalase (Fig. 7) resembled that of the urea-treated catalase in the red shift of the Soret band. The increased absorbance at the lower wavelengths most probably depends on increased light scattering due to heat induced protein aggregation.

For catalase the effect of heat treatment was less than that obtained on urea treatment due to aggregate formation. The diminishing effect of aggregate formation on the nonenzymatic activity is evident from the results in Table III. Strong heat treatment in the presence of urea gave the same result as urea treatment alone but much higher values than heat treatment alone where aggregation was observed. Urea prevented aggregation of the heat denatured protein. Previous workers have shown that urea could be used to dissolve heat precipitated $\beta$-galactosidase (23) and $\alpha$-amylase (24).

This investigation has shown that nonenzymatic lipid oxidation catalyzed by the heme in peroxidase and catalase increased while enzymatic activity decreased when the protein was denatured by acid, urea and heat. Denaturation increased heme exposure thus nonenzymatic activity. The largest increase of nonenzymatic activity was obtained by acid cleavage of the hemoprotein into hemin and apoprotein, but there was evidence that heme which still remained bound to the denatured protein was almost as active as free hemin. Heat denaturation led to more complex situations where the contribution of partially randomized hemoprotein, free hematin and protein aggregates is still unknown. These questions are presently being studied.

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