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Non-oxygen-forming pathways of hydrogen peroxide degradation by bovine liver catalase at low hydrogen peroxide fluxes

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Abstract

Heme catalases are considered to degrade two molecules of H_2O_2 to two molecules of H_2O and one molecule of O_2 employing the catalatic cycle. We here studied the catalytic behaviour of bovine liver catalase at low fluxes of H_2O_2 (relative to catalase concentration), adjusted by H_2O_2-generating systems. At a ratio of a H_2O_2 flux (given in $\mu M/min^{-1}$) to catalase concentration (given in μM) of $10\,min^{-1}$ and above, H_2O_2 degradation occurred via the catalatic cycle. At lower ratios, however, H_2O_2 degradation proceeded with increasingly diminished production of O_2. At a ratio of $1\,min^{-1}$, O_2 formation could no longer be observed, although the enzyme still degraded H_2O_2. These results strongly suggest that at low physiological H_2O_2 fluxes H_2O_2 is preferentially metabolised reductively to H_2O, without release of O_2. The pathways involved in the reductive metabolism of H_2O_2 are presumably those previously reported as inactivation and reactivation pathways. They start from compound I and are operative at low and high H_2O_2 fluxes but kinetically outcompete the reaction of compound I with H_2O_2 at low H_2O_2 production rates. In the absence of NADPH, the reducing equivalents for the reductive metabolism of H_2O_2 are most likely provided by the protein moiety of the enzyme. In the presence of NADPH, they are at least in part provided by the coenzyme.

Keywords: *Catalase, hydrogen peroxide, NADPH, compound I, compound II, reductive, metabolism*

Introduction

Bovine liver catalase is a homotetramer with one prosthetic heme b group and one NADPH tightly bound as additional cofactor to each subunit [1–3]. It belongs to the monofunctional (typical) catalases and within this class to the small subunits enzymes. Catalases degrade two molecules of hydrogen peroxide (H_2O_2) to one molecule of O_2 and two of H_2O. In their native, resting state the heme group of the typical catalases exists as porphyrin-iron(III) complex (ferricatalase) [1,2,4]. The degradation of hydrogen peroxide proceeds in a cyclic process, the catalatic cycle, by two consecutive reactions. In the first step, upon reaction of ferricatalase with one equivalent of H_2O_2, one equivalent of H_2O is released, and compound I is formed as an intermediate by a two-electron oxidation (Reaction 1, Figure 1). Compound I, which contains an oxyferryl (Fe(IV)=O) group associated with a porphyrin π-cation radical, is

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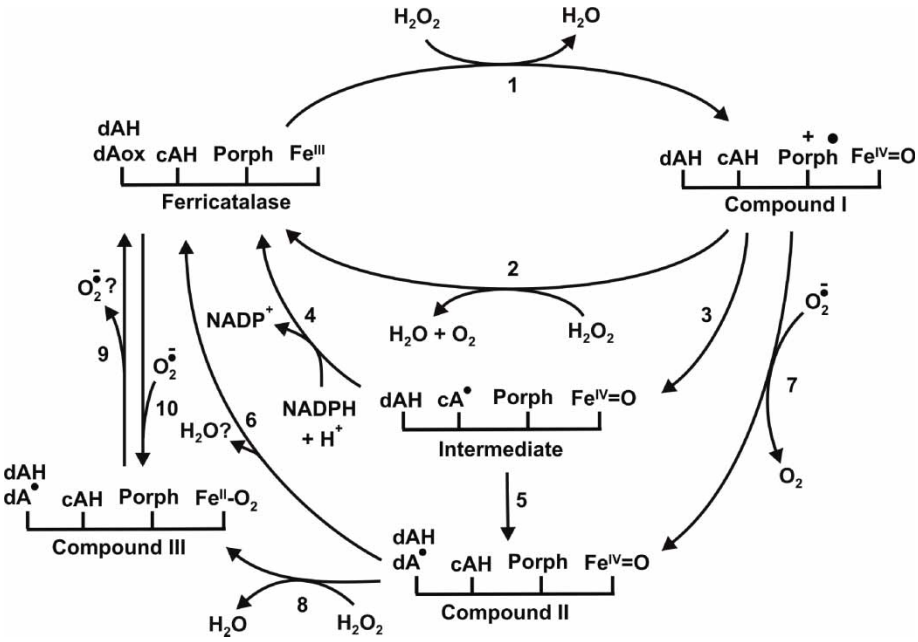

Figure 1. Catalytic pathways of bovine liver catalase. cAH, amino acid (reduced form) of the protein moiety close to the heme; cA•, amino acid (one-electron oxidized form) of the protein moiety close to the heme; dAH, amino acid (reduced form) of the protein moiety distant to heme; dA•, amino acid (one-electron oxidized form) of the protein moiety distant to heme; dAox, amino acid (two-electron oxidized form) of the protein moiety distant to heme.

subsequently re-reduced to ferricatalase in another two-electron transfer reaction by a second molecule of H_2O_2, thereby releasing a second molecule of H_2O and one molecule of O_2 (Reaction 2).

Beside the two active forms, ferricatalase and compound I, bovine liver catalase and related catalases can exist in two other forms named compounds II and III. These forms are inactive with regard to catalatic H_2O_2 degradation [5,6]. Compound II results from one-electron reduction of compound I and is a catalase species with an oxyferryl-ligated porphyrin. Compound II may be formed by reductants of relatively small size, such as $O_2^{\bullet-}$ (Reaction 7). As an alternative, it is discussed that a poorly understood "endogenous donor" provides the reducing equivalents (Reactions 3 and 5) [7–9]. Compound III (oxycatalase) is regarded as having similarities to the oxycompound of hemoglobin. It can arise from action of H_2O_2 on compound II (Reaction 8) or from action of $O_2^{\bullet-}$ on ferricatalase (Reaction 10). Compounds II and III revert spontaneously to ferricatalase, presumably by substracting a second one-electron reducing equivalent, e.g. from another "endogenous donor" (Reaction 6), and by liberating $O_2^{\bullet-}$ (Reaction 9), respectively. The tightly bound NADPH in conjunction with free or transiently bound NADPH or NADH protects the enzyme from inactivation as compound II (Reaction 4) [7–13].

The reactions involving compounds II and III or NADPH are usually considered as inactivation and reactivation reactions but are generally not discussed in terms of a viable catalytic cycle. However, if they really exist, and there is good evidence for that, then kinetics requires that these reactions act as true catalytic pathways. This especially applies to low H_2O_2 concentrations where the re-reduction of compound I by H_2O_2 (Reaction 2) can no longer outcompete these routes. In the present study, experiments are performed with bovine liver catalase to verify this hypothesis.

Materials and methods

Materials

Catalase from beef liver (suspension, 64,000 units mg^{-1} protein, EC 1.11.1.6), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), superoxide dismutase from bovine erythrocytes (EC 1.15.1.1), xanthine oxidase prepared from cow milk (EC 1.1.3.22), and NADPH were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Chelex 100 (chelating resin; iminodiacetic acid), H_2O_2, and xanthine were obtained from Sigma (Deisenhofen, Germany). Amplex Red and resorufin were products of Molecular Probes (Eugene, OR, USA).

Solutions were prepared by using purified water received from a TKA-LAB (Niederelbert, Germany, type HP 6 UV/UF). Buffers were treated with Chelex 100 (1.5 g per 50 ml) to minimize contamination by transition metal ions.

Catalase was diluted with K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 8.0; 1:5, v/v). After dissolution by preincubation for 4 min at 37°C, the enzyme was dialyzed against K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) at 4°C for about 24 h before use.

Experimental systems

Experiments were performed in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) at 37°C unless otherwise stated. H_2O_2 was generated enzymatically by using either the system glucose oxidase and glucose (10 mM) or xanthine oxidase and xanthine (0.1 mM). In the xanthine oxidase–xanthine system superoxide dismutase (160 U ml^{-1}) was additionally included. Different ratios of the rate of H_2O_2 generation to catalase concentration (νH_2O_2/[catalase]) were adjusted by varying the glucose oxidase and xanthine oxidase activities, respectively, between 1 and 6 μM min^{-1} and the catalase concentration between 0.004 and 2 μM (concentrations of catalase and of its intermediates given refer to the tetramer). Experiments where O_2 consumption and O_2 generation were monitored were performed in a thermostated incubation vessel equipped with a Clark-type O_2 sensor (Saur, Reutlingen, Germany). Experiments without continuous O_2 monitoring were performed in Erlenmeyer flasks in a shaking water bath. In these experiments, samples were taken at predetermined time points for measurements in separate assays. For the generation of serial absorption spectra, experiments were performed directly in micro quartz cuvettes.

Determination of enzymatic activities

The catalytic activity of catalase was assessed by either measuring H_2O_2 degradation or the formation of O_2 from H_2O_2; both measurements were performed in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) in the presence of 10 mM H_2O_2. Hydrogen peroxide degradation was followed at 25°C by monitoring its UV–visible absorption at 240 nm as originally described by Chance and Herbert [14] and later improved by Aebi [15]. Degradation of H_2O_2 to O_2 was determined with a Clark-type oxygen sensor at 37°C using the experimental set-up described above.

The glucose oxidase and xanthine oxidase activities were determined by measuring the rate of O_2 consumption in the presence of glucose (10 mM) and xanthine (0.1 mM), respectively, at 37°C in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) as given above.

Assessment of H_2O_2 concentration

Hydrogen peroxide was quantified by a peroxidase assay using Amplex Red as the peroxidase substrate [16]. Resorufin formed from Amplex Red was measured fluorimetrically (RF-1501 spectrofluorometer, Shimadzu, Kyoto, Japan) at pH 7.0 ($\lambda_{exc} = 532$ nm, $\lambda_{em} = 583$ nm). Stock solutions of Amplex Red (2 mM) and resorufin (1 mM) were prepared in purified dimethyl sulfoxide and stored at − 20°C. The assay was performed at room temperature as follows: 1.9 ml KH_2PO_4–K_2HPO_4 buffer (50 mM, pH 7.0) was added to a fluorescence cuvette and baseline fluorescence was read. Subsequently, 4 μl resorufin (first diluted with the KH_2PO_4– K_2HPO_4 buffer to 5 μM, final concentration 10 nM) was added and the fluorescence determined again. Afterwards, 100 μl of the sample, 10 μl peroxidase (from a stock solution, 3800 U ml^{-1}), and 2 μl Amplex Red (2 μM final concentration) were added. After a reaction time of 1 min fluorescence was read again. H_2O_2 concentration was calculated by comparing the increase in fluorescence induced by the sample with the one induced by resorufin (internal standard) assuming a 1:1 stoichiometry between H_2O_2 consumed and resorufin formed from Amplex Red.

Determination of compounds I, II and III

Absorption spectra of catalase were obtained on a Specord S100 diode array spectrometer (Analytic Jena, Jena, Germany) using micro quartz cuvettes. The spectra were run in the absence of ethanol and, following addition of ethanol to a final concentration of 2 mM, 2 and 90 min after addition of the alcohol. In the presence of millimolar concentrations of ethanol, compounds I, II and III revert to ferricatalase [6,7,13]. While the reversion occurs within minutes for compound I, it takes several minutes for compounds II and III (about 20 min for a 50% reversion of compound II into ferricatalase [6]). From the differences in the absorbance at 405 nm, 2 min after addition of ethanol (A_{a2}) and prior to addition of ethanol (A_b), the concentration of compound I was calculated according to Lardinois [6] using the equation $[\text{compound I}] = A_{a2} - A_b/(\varepsilon_{ferri} - \varepsilon_{\text{compound I}})$ 1 cm with $\varepsilon_{ferri} = 3.24 \times 10^5\ M^{-1}\,cm^{-1}$ and $\varepsilon_{\text{compound I}} = 2.19 \times 10^5\ M^{-1}\,cm^{-1}$ per tetramer.

The absorbance at 405 nm determined 90 min after the addition of ethanol was used to calculate the amount of ferricatalase that could be recovered following incubation with H_2O_2 using the extinction coefficient (ε_{ferri}) as given above. Compound II was estimated from the increase in absorbance of the isosbestic point between compound I and ferricatalase at 435 nm, and compound III from the characteristic twin absorption peaks at 545 and 585 nm [17].

In the absence of H_2O_2, the concentration of ferricatalase was estimated by measuring its absorption at 405 nm, applying again the extinction coefficient (ε_{ferri}) given above.

Statistics

All experiments were repeated at least twice. Spectra and traces shown in the figures are representative of all the corresponding experiments performed. The data are expressed as means ± SD.

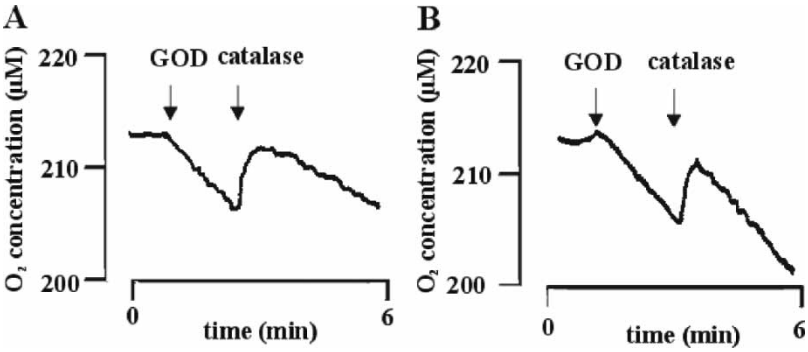

Figure 2. Effect of catalase on O_2 consumption by the glucose oxidase–glucose system. Experiments were performed at 37°C in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) containing 10 mM glucose. Glucose oxidase (GOD) at a concentration producing 4 μM H_2O_2 min^{-1} and bovine liver catalase (A, 0.05 μM; B, 2 μM) were added where indicated. O_2 consumption was monitored polarographically by using a Clark-type oxygen sensor. Typical experiments are shown.

Results

Concentration dependence of H_2O_2 degradation to O_2

Hydrogen peroxide was produced by the H_2O_2-generating system glucose oxidase–glucose. Glucose oxidase converts one molecule of glucose by consuming one molecule O_2 to yield one molecule of gluconolactone and one molecule H_2O_2. In the absence of catalase, O_2 was consumed at a constant rate of about 4 μM min^{-1} after addition of glucose oxidase due to the formation of H_2O_2 (Figure 2). Upon addition of 0.05 μM catalase, the O_2 consumption decreased by approximately 50%, as can be deduced from the lower slope (Figure 2A). Hence, half of the H_2O_2 produced by glucose oxidase was converted back to O_2 according to the catalatic cycle (Reactions 1 and 2). Upon addition of 2 μM catalase, under otherwise unchanged conditions, however, we did not find any evidence for H_2O_2 degradation by the catalatic cycle (Figure 2B). O_2 consumption continued to proceed at almost the same rate. The immediate increase of the O_2 level upon addition of catalase under both conditions resulted from the degradation of H_2O_2 which had already been produced.

To study this unexpected effect on O_2 consumption upon the addition of the higher catalase concentration in more detail, we performed experiments at varying H_2O_2 production rates and catalase concentrations. At $\nu(H_2O_2)$/[catalase] ratios above around 10 min^{-1}, catalase behaved as expected, i.e. decreased O_2 consumption by about 50% (Figure 3). Here, $\nu(H_2O_2)$ is the H_2O_2 flux (production rate) in μM min^{-1} taken as the activity of the added glucose oxidase, and [catalase] denotes the catalase concentration in μM. At $\nu(H_2O_2)$/[catalase] ratios below 10 min^{-1}, the reconstitution of 50% O_2 consumption was no longer observed. Instead, with decreasing ratios, the effect of catalase on O_2 consumption became increasingly less pronounced until, at $\nu(H_2O_2)$/[catalase] ratios below *ca.* 1 min^{-1}, an effect of catalase on the O_2 consumption was no longer visible (Figure 3). Moreover, at ratios below this limit, the O_2 consumption was even somewhat, but reproducibly, increased to values above 100% of the O_2 consumption before addition of catalase. Addition of NADPH was almost without any effect on this behaviour. Again, in spite of the presence of NADPH, the effect of catalase on O_2 consumption became increasingly less at $\nu(H_2O_2)$/[catalase] ratios below 10 min^{-1}. Comparable results were obtained in experiments where H_2O_2 was generated by the system xanthine oxidase-xanthine (data not shown). Control

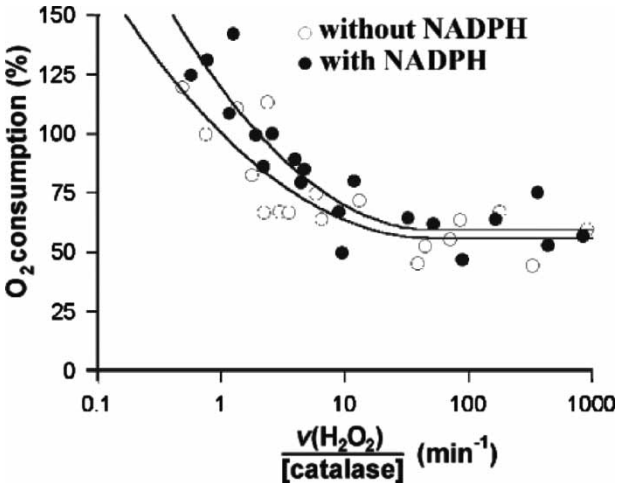

Figure 3. Effect of catalase on the rate of O_2 consumption by the glucose oxidase–glucose system in dependence of the $\nu(H_2O_2)$/[catalase] ratio. Experiments were performed as given in the legend to Figure 2. In the experiments performed with NADPH, the nucleotide was applied at a concentration of 1 mM. The rates of O_2 consumption were calculated from O_2 consumption following addition of catalase and given as percentage of the rate of O_2 consumption by the glucose oxidase–glucose system prior to addition of catalase. The lines are empirical, non-linear fits of the data points in the absence and presence of NADPH to build-in function #8115 of Table Curve 2D (SPSS Inc.) and are only intended to guide the eye.

experiments proved that catalase itself does not consume O_2 in the absence of H_2O_2 under otherwise identical conditions. Obviously, at $\nu(H_2O_2)$/[catalase] ratios below $10\,min^{-1}$ the stoichiometry of H_2O_2 decomposition does no longer follow that of the usual catalatic reaction cycle, both in the presence and absence of NADPH.

Formation of compounds I, II and III

The formation of compounds I, II and III was studied by their UV–visible absorptions at a $\nu(H_2O_2)$/[catalase] ratio of 10 and $1\,min^{-1}$, respectively (Figure 4). The well-known behaviour of catalase [6,7,13,18] was observed, and there were not any

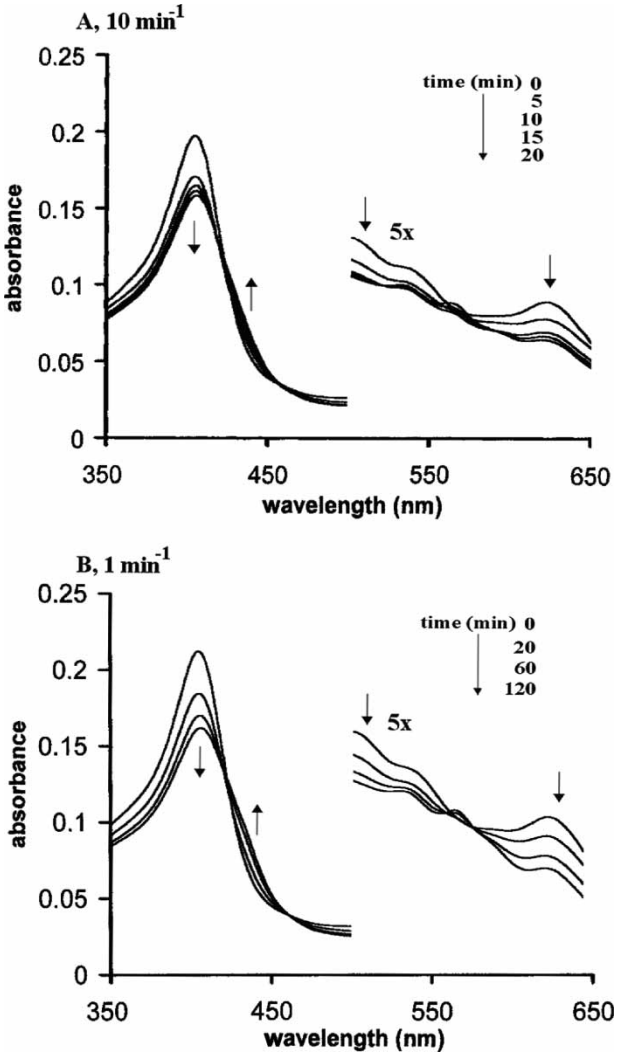

Figure 4. Absorption spectra of catalase at a high and a low $\nu(H_2O_2)$/[catalase] ratio. Bovine liver catalase (0.6 μM) was incubated at 37°C in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) containing 10 mM glucose. Immediately after recording the zero-time spectra, formation of H_2O_2 was started by the addition of glucose oxidase at a concentration to achieve a H_2O_2 generation rate of either $6\,\mu M\,min^{-1}$ (A, $\nu(H_2O_2)$/[catalase] = $10\,min^{-1}$) or $0.6\,\mu M\,min^{-1}$ (B, $\nu(H_2O_2)$/[catalase] = $1\,min^{-1}$). At the times indicated further spectra were recorded. In A, the experiments were ceased after 20 min to prevent limitation by O_2 deficiency.

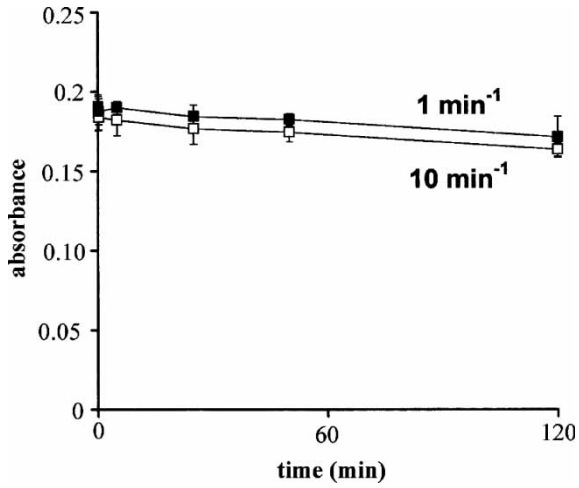

Figure 5. Effect of a high and a low $\nu(H_2O_2)$/[catalase] ratio on the absorbance of catalase at 405 nm determined after addition of ethanol. Bovine liver catalase (0.6 μM) was incubated at 37°C in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) containing 10 mM glucose. Immediately after taking the zero-time aliquot, formation of H_2O_2 was started by the addition of glucose oxidase at a concentration to achieve a H_2O_2 generation rate of either $6\,\mu M\,min^{-1}$ ($\nu(H_2O_2)$/[catalase] = $10\,min^{-1}$) or $0.6\,\mu M\,min^{-1}$ ($\nu(H_2O_2)$/[catalase] = $1\,min^{-1}$). At the time points indicated aliquots were taken and spectra were recorded 90 min after addition of ethanol (2 mM). Each value represents the mean ± SD of three experiments performed in duplicate.

significant differences in the spectral alterations at both ratios. In the absence of NADPH, both compound I and compound II became detectable within minutes after starting H_2O_2 formation by addition of glucose oxidase. No evidence for compound III formation was found. Notably, under all conditions studied, more than 80% of the initial enzyme concentration could be recovered as ferricatalase by the addition of ethanol, even after 120 min of incubation in the presence of H_2O_2 (Figure 5). Comparable results were obtained in the presence of NADPH except for the fact that NADPH largely suppressed the formation of compound II both at the low and the high $\nu(H_2O_2)$/[catalase] ratio (not shown). Thus, at all conditions studied, compounds I and II are formed as to be expected. A contribution of compound III to the absorption spectra appears to be negligible, and there is no spectroscopic evidence for an irreversible alteration at the enzyme's heme site.

Maintenance of the catalytic activity

In the absence of NADPH, an inactivation of catalase, e.g. by compound II formation, may also account for the deviation from the usual catalatic reaction cycle at low $\nu(H_2O_2)$/[catalase] ratios. However, we did not find any evidence for an accumulation of H_2O_2 both at low and high $\nu(H_2O_2)$/[catalase] ratios: upon addition of a second batch of catalase at time points 5–20 min after the first addition of catalase no additional O_2

was released, except for a short increase in the level of O_2 due to the atmospheric oxygen dissolved in the catalase solution (data not shown). Unfortunately, at $\nu(H_2O_2)$/[catalase] ratios where the steady-state H_2O_2 concentration was estimated to remain below 1 μM direct H_2O_2 determination proved impossible, although the peroxidase–Amplex Red method, applied here is already one of the most sensitive assays. Its lower limit for direct H_2O_2 determination is around 50 nM but was found to be around 1 μM in the presence of glucose oxidase–glucose and/or catalase. In the absence of catalase, H_2O_2 production by glucose oxidase could be clearly monitored as soon as a concentration of 1 μM had been exceeded, even at low H_2O_2 production rates.

We additionally determined the activity of catalase in separate assays following incubation at a $\nu(H_2O_2)$/[catalase] ratio of 10 and 1 min^{-1}, respectively. In these assays, H_2O_2 decomposition proceeded by the catalatic cycle due to the high H_2O_2 concentration (10 mM) applied. Determining the catalytic activity of catalase by measuring H_2O_2 degradation spectroscopically revealed that the enzyme lost between 20 and 30% of its initial catalytic activity within the first minute of incubation, independent of whether the incubation was performed at low or high $\nu(H_2O_2)$/[catalase] ratios (Figure 6). Thereafter, the catalytic activity remained almost constant for additional 60 min and then somewhat decreased under both conditions. Similar results were obtained when the catalytic activity of catalase was followed by measuring

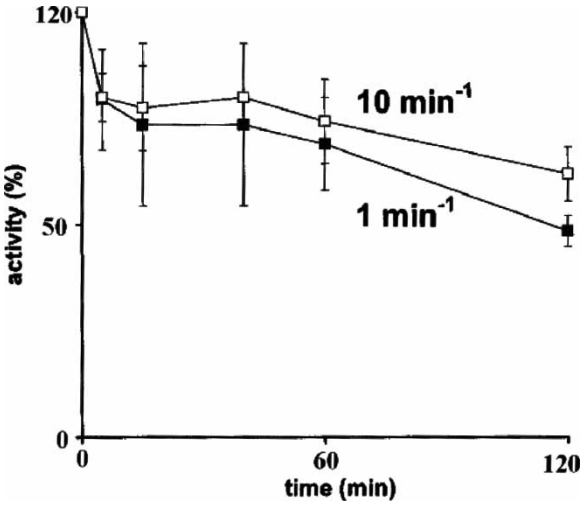

Figure 6. Effect of hydrogen peroxide at a high and a low $\nu(H_2O_2)$/[catalase] ratio on the catalytic activity of catalase. Bovine liver catalase (0.6 μM) was incubated at 37°C in K_2HPO_4–KH_2PO_4 buffer (50 mM, pH 7.0) containing 10 mM glucose. Formation of H_2O_2 was started by the addition of glucose oxidase at a concentration to achieve a H_2O_2 generation rate of either 6 μM min^{-1} ($\nu(H_2O_2)$/[catalase] = 10 min^{-1}) or 0.6 μM min^{-1} ($\nu(H_2O_2)$/[catalase] = 1 min^{-1}). At the time points indicated aliquots were taken and the catalytic activity of catalase was determined spectroscopically. Each value represents the mean ± SD of three experiments performed in duplicate.

O_2 formation polarographically (data not shown). Thus, despite of some loss in activity, a major fraction of the enzyme's catalytic activity was retained over the time periods studied both at low and high $\nu(H_2O_2)$/[catalase] ratios, in accordance with the results on H_2O_2 accumulation and the spectroscopic data described above. The loss in activity may be explained by compound II formation, which, therefore, would be of relevance only at high H_2O_2 concentrations.

Discussion

As can be read in each textbook of biochemistry, catalase degrades two molecules of H_2O_2 to one molecule of O_2 and two molecules of H_2O according to Reactions 1 and 2 of the catalatic cycle. As demonstrated by the present results with bovine liver catalase, however, this stoichiometry is only followed at high H_2O_2 fluxes, i.e. at $\nu(H_2O_2)$/[catalase] ratios of 10 min^{-1} and above. Below this ratio, H_2O_2 is increasingly degraded by pathways not leading to the liberation of O_2. This behaviour can conclusively be explained by considering other well-known reactions of catalase (Figure 1). In this case, however, the protein moiety of the enzyme needs to be assumed to be involved in the catalytic reactions as electron transferring component. Compound I, formed by Reaction 1, is the decisive intermediate under all circumstances. At low H_2O_2 fluxes, however, Reaction 2 proceeds only very slowly and may no longer compete with the "non-O_2-forming" routes starting from compound I. In the absence of NADPH, compound II may play a central role on this pathway. Formation of compound II is well-known to occur in the absence of NADPH [6–9,13], as was also observed here. It can be formed by one-electron reduction of compound I by small reductants such as $O_2^{\cdot-}$ (Reaction 7). In the absence of such exogenous reductants, as was the case in the present study, a reductant within the protein structure of catalase, the so-called "endogenous donor", is considered to cause the conversion of compound I to II (Reactions 3 and 5) [7–9]. That the endogenous donor is identical with the protein moiety of the enzyme is strongly supported by the fact that protein-based radicals of catalase have repeatedly been reported, among others also for bovine liver catalase [11–19]. Furthermore, compound I is a very strong oxidant, reactive enough to oxidize various amino acid residues of its protein [20,21]. Based on the observation that compound II formation is prevented by NADPH but that NADPH does not reduce compound I directly, it has been proposed [7,8] that the formation of compound II from compound I proceeds via a specific intermediate characterized by an oxidized amino acid residue in close vicinity to the heme group (Reactions 3 and 5). Compound II is known to spontaneously revert to ferricatalase [5–7,13,17]. Again, it is assumed that the electron required for this one-electron reduction step derives from an endogenous donor (Reaction 6),

viz. the protein of the enzyme. Hence, in addition to Reaction 1, a path following Reactions 3 + 5 + 6 is presumably responsible for the degradation of H_2O_2 at low H_2O_2 concentrations in the absence of NADPH (Note: ferricatalase formed via compound II would be two-electron oxidized in its protein moiety as compared to native ferricatalase, see below). In the presence of NADPH, the rate of compound II formation was largely (but not completely) suppressed, presumably due to the reaction of NADPH with the intermediate already described above (Reaction 4), in line with previous reports [7–9,13]. Therefore, reactions involving compound II (Reactions 5 and 6) then should contribute to the regeneration of ferricatalase to a smaller degree, i.e. the degradation of H_2O_2 is switched from Reactions 3 + 5 + 6 to 3 + 4 under these conditions. NADPH consumption via Reactions 3 + 4 has been studied intensively, but only with focus on its protective function against the inactivation of catalase as compound II [7–9,11,13]. Since the binding site for NADPH is at the surface of the enzyme, i.e. NADPH cannot get into close contact with the heme group which is buried deep inside the enzyme, the electron transfer between NADPH and the enzyme's active site has been proposed to occur by electron tunnelling [10,20,21].

Alternative pathways, especially those involving compound III [6] are less likely to participate in the reductive metabolism of H_2O_2. Compound II can be converted into compound III by reaction with H_2O_2 (Reaction 8). However, the rate of this reaction is too low to allow its occurrence at a significant rate at nanomolar H_2O_2 concentrations. Accordingly, no noticeable formation of compound III could be observed in the present experiments. On the other hand, we cannot exclude contributions of other, yet unknown pathways, such as those participating in an internal reductase and transhydrogenase reaction where unbound or loosely bound NADH or NADPH are used as substrates for the reduction of tightly bound $NADP^+$, as reported recently [12].

In the literature, Reactions 3–6, suggested here as viable catalytic pathways at low H_2O_2 fluxes, are usually assumed as "inactivation" and "reactivation" pathways, respectively. Furthermore, compound II is usually referred to as inactive form of catalase [5,6]. Both, however, are true only if one considers the conversion of H_2O_2 to O_2 and H_2O as the only and decisive catalytic function of catalase. If one accepts that H_2O_2 degradation without formation of molecular oxygen according to Reactions 3–6 is a true catalytic pathway, then compound II may no longer be regarded as an inactive but as an active intermediate of catalase.

So far, we discussed the reductive degradation of H_2O_2 as if there was only one turn of the cyclic process. However, the discussed pathways of H_2O_2 degradation obviously proceed for several cycles even in the absence of NADPH. In the presence of NADPH there is little problem in maintaining a catalytic cycle. The reducing equivalents may be provided by NADPH and, in addition to the still possible Reaction 2, compound I should return to native ferricatalase via Reactions 3 + 4 at each cycle. In the absence of NADPH, however, the ultimate source of the reducing equivalents is less clear. Assuming that the protein moiety is the ultimate electron donor means that at each catalytic cycle the protein moiety would be consecutively oxidized by two electrons. Although such a process should lead to an irreversible oxidative modification of the protein backbone, there are some arguments in favour of this concept. The supply of reducing equivalents by reducing groups within the catalase protein has repeatedly been reported. Already 40 years ago Nicholls and Schonbaum [17] suggested that "catalase is endowed with a good supply of reducing equivalents". Some support for this concept arises from preliminary experiments which demonstrate that bovine liver catalase can be confronted with very high amounts of the oxidant peroxynitrite without losing its activity (unpublished results) in line with the behaviour of other catalases [22,23]. On the other hand, we cannot exclude that glucose and xanthine, the substrates of the respective H_2O_2 generating systems, may also provide at least a fraction of the required reducing equivalents. Although both glucose and xanthine cannot react directly with the oxyferryl group at the active site of the enzyme because of steric reasons, they may re-reduce oxidized residues of the enzyme at its surface. At present we do not know which kind of molecular alterations of the protein moiety are accomplished by oxidation. Additional crosslinking covalent bonds may be formed, but oxygenation by reaction of intermediate protein radicals with molecular oxygen is also likely to occur. The latter may be indicated by the observation that at very low $\nu(H_2O_2)$/[catalase] ratios the O_2 consumption indeed exceeds 100% (Figure 2).

For the rate of H_2O_2 generation *in vivo*, values between *ca.* 1 and close to 1000 $\mu M\,min^{-1}$ have been estimated [4]. Taking these values and a typical catalase concentration in tissue of 2 μM [7], H_2O_2 generation to catalase concentration ratios of 0.5–500 min^{-1} can be calculated. Thus, the range of $\nu(H_2O_2)$/[catalase] ratios which can be expected *in vivo* includes the whole range of ratios studied here (compare Figure 3). On the other hand, in living systems, enzymes such as glutathione peroxidase may contribute to H_2O_2 degradation in addition to catalase. Further, catalase is usually compartmentalized, and in the presence of suitable, small substrates for compound I, such as ethanol, peroxidatic pathways of catalase have also to be taken into consideration. Thus, an extrapolation of the results presented here to the *in vivo* situation would be too far-reaching.

To summarize, 2 μM bovine liver catalase degrades H_2O_2 via the catalatic cycle at high H_2O_2 fluxes, i.e. at 20 $\mu M\,min^{-1}$ and above. Under these conditions, pathways involving compound II and NADPH do not significantly contribute to H_2O_2 degradation, and compound II formation results in a decrease in the catalytic activity of the enzyme which may be considered as (reversible) inactivation. At lower H_2O_2 fluxes, the pathways of reductive H_2O_2 metabolism to H_2O increasingly gain significance. In these pathways, at least some of the reducing equivalents may be provided by the protein moiety of the enzyme. So far, these conclusions only apply to bovine liver catalase. However, other NADPH-binding heme catalases, such as erythrocytic or yeast catalase, behave similar with respect to compound II formation and its suppression by NADPH [8,24]. Therefore, it can be assumed that the conclusions drawn here may apply to other heme catalases as well.

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