Chapter

Typical Catalases: Function and Structure

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Abstract

Catalase (EC 1.11.1.6) is a heme-containing enzyme ubiquitously present in most aerobic organisms. Although the full range of biological functions of catalase still remains unclear, its main function is the decomposition of hydrogen peroxide into water and oxygen. Catalases have been studied for over 100 years, with examples of the enzyme isolated, purified, and characterized from many different organisms. The crystal structures of 16 heme-containing catalases have now been solved, revealing a common, highly conserved core in all enzymes. The active center consists of a heme with a tyrosine ligand on the proximal side and a conserved histidine and an aspartate on the distal side. Although catalases have been studied for many years, additional functions of catalases have recently been recognized. For example, *Scytalidium thermophilum* catalase (CATPO) has been shown to oxidize o-diphenolic and some p-diphenolic compounds in the absence of hydrogen peroxide. This and other studies have led to the proposal that this secondary oxidative activity may be a general characteristic of catalases. The present chapter will focus on the function and structure of monofunctional heme catalases, emphasizing the information obtained in the last few years mainly in relation to the secondary activity of these enzymes.

Keywords: catalase, oxidase, heme, NADPH, channel, secondary activity

1. Introduction

Catalases are one of the most studied groups of enzymes. The term catalase was first identified by Loew as hydrogen peroxide (H_2O_2) degrading enzyme in 1901, and the protein has been the focus of study for biochemists and molecular biologists ever since. The overall reaction for catalase can simply be described as the degradation of two molecules of hydrogen peroxide to water and oxygen (reaction 1). This catalytic reaction occurs in two distinct stages, but what each of the stages includes is mainly based on the kind of catalase [1]. The first stage involves oxidation of the heme using first hydrogen peroxide molecule to form an oxyferryl species in which one oxidation equivalent is taken off from the iron and one from the porphyrin ring to make a porphyrin cation radical (reaction 2). In the second stage, this radical intermediate, known as compound I, is reduced by a second hydrogen peroxide to regenerate the resting state enzyme, water and oxygen (reaction 3) [2, 3]. Catalases can also function as peroxidases, in which suitable organic compound is used as an electron donor. During peroxidase reaction, compound I is converted to compound II (reaction 4), which can be oxidized by another hydrogen peroxide to produce the inactive compound III (reaction 5). For NADPH-binding catalases, it has been

suggested that enzyme inhibition through the appearance of compound III can be prevented by the NADPH blocking or releasing compound II generation [4–6].

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (1)$$

$$\text{Enz}\,(\text{Por}-\text{Fe}^{\text{III}}) + H_2O_2 \rightarrow \text{Cpd I}\,(\text{Por}^{+\bullet}-\text{Fe}^{\text{IV}}=\text{O}) + H_2O \quad (2)$$

$$\text{Cpd I}\,(\text{Por}+\bullet-\text{Fe IV}=\text{O}) + H_2O_2 \rightarrow \text{Enz}\,(\text{Por}-\text{Fe}^{\text{III}}) + H_2O + O_2 \quad (3)$$

$$\text{Cpd I}\,(\text{Por}+\bullet-\text{Fe IV}=\text{O}) + AH_2 \rightarrow \text{Cpd II}\,(\text{Por}-\text{Fe}^{\text{IV}}-\text{OH}) + AH^{\bullet} \quad (4)$$

$$\text{Cpd II}\,(\text{Por}-\text{Fe}^{\text{IV}}-\text{OH}) + H_2O_2 \rightarrow \text{Cpd III}\,(\text{Por}-\text{Fe}^{\text{III}}-\text{O2}^{-\bullet}) + H_2O \quad (5)$$

Catalases have been classified into three groups: monofunctional heme-containing catalases, heme-containing catalase-peroxidases, and manganese-containing catalases [7]. Among them, monofunctional catalases constitute the largest and most extensively studied group of catalases [1, 2]. They all possess two-step mechanism for dismutation of hydrogen peroxide. Members of this largest class of catalases can be biochemically subdivided based on having large (75–84 kDa) subunits with heme *d* associated or small (55–69 kDa) subunits with heme *b* associated. All small subunit enzymes so far characterized, unlike larger enzymes, have been found with NADP(H) bound [1, 8]. In turn, larger subunit enzymes have been shown to exhibit significantly enhanced stability against high temperatures and proteolysis [1, 9]. The catalase-peroxidases, less widespread class, exhibit significant peroxidatic activity in addition to catalytic activity [2]. They are found in bacteria, archaebacteria, and fungi. Catalase-peroxidases have a molecular mass in the range of 120–340 kDa [10, 11]. Manganese-containing catalases are not as widespread as the heme-containing catalases, and there are only three of them so far characterized, one from lactic acid bacteria (*Lactobacillus plantarum*) and two from thermophilic bacteria (*Thermus thermophilus* and *Thermoleophilum album*) [1, 2]. These enzymes are also called pseudo-catalases as their active site contains a manganese-rich reaction instead of heme group [12, 13]. Crystal structures of two manganese catalases, one from *T. thermophilus* and the other from *L. plantarum*, show the presence of dimanganese group in the catalytic center [1].

Although monofunctional catalases are described as such due to the prolonged-agreed belief that their only role is hydrogen peroxide removal, this rather limited catalytic role has recently been questioned. Vetrano et al. expressed a novel oxidase activity in the absence of hydrogen peroxide [14]. Later, a catalase from *S. thermophilum* was shown to have an unselective phenolic oxidase activity in the absence of hydrogen peroxide [15–17]. It is thought that such bifunctional enzymes might be more common due to the evidence on the presence of oxidase/peroxidase activity in catalase enzymes from different organisms such as *Bacillus pumilus* [18], *Thermobifida fusca* [19], and *Amaranthus cruentus* [20]. Such studies are likely to give evidence that translates from various sources to a great deal of catalases. Bifunctional enzymes can be advantageous in many industrial applications including the removal of toxic chemicals and/or chemoprotective agent activity especially when the oxidase activity is enhanced by directed evolution or engineering.

2. Regulation of catalase gene expression

The study of the bacterial response to oxidative stress has given insights into how catalase synthesis is controlled in different cells. Studies with *E. coli* and *Salmonella typhimurium* have shown that there are two regulatory pathways available in bacterial catalase expression [9, 21].

E. coli produces two catalases or hydroperoxidases, the bifunctional catalase-peroxidase HPI and the monofunctional catalase HPII. These two types of catalases are induced independently; HPI synthesis is promoted by H_2O_2 added to a medium, and HPII synthesis is induced during growth into stationary phase [22]. The katG gene, encoding HPI, has been found to be regulated by the OxyR regulon which responds to oxidative stress [9, 21, 22]. OxyR protein is a member of LysR family of regulatory proteins that respond to oxidant levels in the cell [9]. OxyR protein undergoes a conformational change during its transition from the reduced (transcriptionally inactive) to the oxidized (transcriptional active) form. This protein directly senses the oxidative stress by becoming oxidized, and that oxidation results in conformational change by which it transduces oxidative stress to RNA polymerase [21].

The regulatory mechanism of the katE gene, encoding HPII, is quite different and requires a functional katF gene as a positive effector [22]. HPII levels are expressed at high levels when cells enter stationary phase and are unaffected by hydrogen peroxide and/or anaerobiosis [9, 22]. The most important factor for HPII induction seems to be σ^S, as concluded from studies related with the involvement of additional transcription factors [22, 23].

3. Catalase cofactors

The prosthetic group of horse liver catalase enzyme was first isolated by Stern in 1935 [24]. This non-covalently bound component was identified as protoheme (also called hematin), consisting of an iron atom and a porphyrin ring.

The heme prosthetic group has been found to be buried inside the protein, approximately 20 Å from the surface in almost all hem-containing catalases whose structures have been dissolved [25–28]. Despite the similarities in heme-binding pocket, catalases from different sources contain different prosthetic groups [29]. All small subunit size catalases have been shown to include a non-covalently bound iron protoporphyrin IX (heme *b*) as prosthetic group per subunit [29, 30]. Consecutively, an oxidized form of protoporphyrin IX, heme *d*, has been found in almost all large subunit size catalases [30]. The heme *d* group characterized in the active sites of crystal structures of two large subunit size catalases, *Penicillium vitale* catalase (PVC) and HPII from *E. coli*, has the structure of the cis-hydroxy γ-spirolactone and is rotated 180 degrees about the axis defined by the α-γ-meso carbon atoms, with regard to the orientation found for heme *b* in small subunit size catalases like bovine liver catalase (BVC) [29]. **Figure 1** shows the structural differences between *b*-type and *d*-type heme.

The γ-spirolactone ring and additional hydroxyl group make heme *d* more asymmetric with respect to heme *b*. The conversion of heme *b* to heme *d* has been studied in *E. coli* by many scientists, and it is proposed that the oxidation of heme in HPII may be catalyzed by HPII itself. Loewen and colleagues [32] also reported this conversion in the presence of hydrogen peroxide. However, the modification takes place on the proximal side of ring III opposite to the essential distal histidine [29, 33]. Díaz et al. proposed another possible change of protoheme to heme,

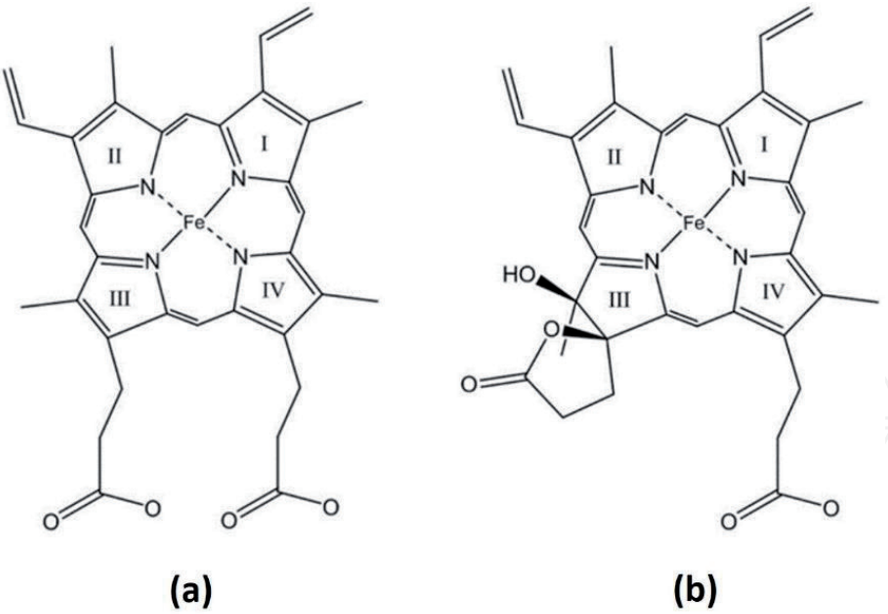

Figure 1.
Structures of heme b (a) and heme d (b), taken from the study reported by Yuzugullu et al. [31].

where γ-spirolactone is formed either by a singlet oxygen or in a light-mediated mechanism [34].

The residues in a contact with heme in the active center are shown to be different for protoheme and heme d enzymes. Such residues for BLC include Met60, Ser216, Leu298, and Met349, whereas analogous residues for PVC involve Ile41, Val209, Pro291, and Leu342 and for HPII contain Ile114, Ile279, Pro356, and Leu407 [29, 35].

Small subunit size catalases have the ability to bind NADP(H) cofactor which is not essential for the activity of catalase [36], but it is believed to have a role in protecting the enzyme from the formation of catalytically inactive intermediate (cpd II) by promoting its reduction to resting state (Fe^{3+}) during catalytic cycle [37, 38]. According to this hypothesis, large subunit enzymes, whose catalytic cycle lacks compound II formation, do not require to bind NADP(H) [38]. It has also been found that NADP(H) is essential for the dismutation of small peroxides, other than hydrogen peroxide [37]. Instead, large subunit size catalases possess the extra C-terminal domain with a flavodoxin-like topology [29, 30]. Despite this difference, residues defining the NADPH pocket in the bovine liver catalase appear to be well preserved in HPII. Only two residues that interact ionically with NADP(H) in the bovine catalase (Asp212 and His304) differ in HPII (Glu270 and Glu362), but it has been proven that their mutation to the bovine sequence does not promote nucleotide binding [4].

4. Catalase catalytic cycle

As described previously, catalytic reaction occurs in two steps [1–3]. The first phase of catalytic cycle involves reaction of ferric enzyme and hydrogen peroxide molecule to generate compound I and water. In the second stage, compound I combines with a second molecule of hydrogen peroxide molecule to regenerate the ferric enzyme, molecular oxygen, and water [2].

Paulos and Kraut firstly proposed the formation of compound I using crystal structure of cytochrome c peroxidase in 1980 [39]. According to this mechanism,

proton transfer takes places from hydrogen peroxide to distal imidazole group, and iron-oxygen bond is generated [40]. The studies of water release or rebinding to the coproduct formation site have shown that compound I intermediate might exist in two forms either in a wet form in which a water molecule is present at or near the site of coproduct water formation or dry form where the coproduct water formation site is dry. It is assumed that the presence of water may play a significant role in both substrate selectivity and the variety of redox pathways available in the donor oxidation phase of the catalytic cycles [40, 41].

Compound I intermediate is also perceived in the presence of organic peroxides as substrate, and the reaction rate of compound I production decreases with an increase in the molecular size of the leaving group such as H— > CH_3— > $HOCH_2$— > CH_3CH_2— > $CH_3C(=O)$— > $CH3(CH_2)_2$— > $CH_3(CH_2)_3OOH$— [42]. At low hydrogen peroxide concentrations and in the presence of suitable organic electron donors, compound I can be reduced by one-electron addition leading to the formation of compound II (a formal Fe^{4+} state) which can cause enzyme inactivation. In this reaction, the porphyrin accepts one electron, therefore losing its radical character [43, 44].

5. Kinetics

The proposed catalytic mechanism supports that catalase enzyme is never saturated with its substrate, H_2O_2, and that turnover of enzyme increases indefinitely as substrate concentration increases [2]. Apparently, catalases have been recognized with a rapid turnover rate and the maximum observed velocities ranging between 54,000 and 833,000 reactions per second [3].

The classical kinetic parameters, Vmax, kcat, and Km, cannot be directly applied to the observed data as catalases do not follow Michaelis-Menten kinetics except at very low substrate concentrations. However, at concentrations below 200 mM, all small subunit size catalases show Michaelis-Menten-like dependence of velocity. At concentrations above 300–500 mM, most small subunit size catalases suffer inactivation. Conversely, large subunit size catalases begin to suffer inhibition above 3 M hydrogen peroxide concentrations [1, 3].

6. Overall structure of catalases

All catalases, whose structure have been dissolved, exhibit highly conserved β-barrel core structure [45]. Their structure is composed of four domains (**Figure 2**) [26, 30, 46, 47]:

a. An amino-terminal arm

b. An anti-parallel eight-stranded β-barrel domain

c. Wrapping domain

d. α-helical domain

The amino-terminal domain is an extended arm and is quite variable in length ranging from 53 residues in *Proteus mirabilis* catalase (PMC) to 127 in HPII [30, 47]. This domain is shown to constitute expanded intersubunit interactions, and residues from this region confer us to describe the heme pocket of a

symmetry-associated subunit. The frequency of intersubunit interactions increases with the length of the domain demonstrating catalases' molecular stability [30].

The second domain, referred to as β-barrel domain, is the central feature of catalase. Most of the residues involved in forming the cavity on the distal side of the heme are placed in the first half of the β-barrel. On the other hand, the second half corresponds to the NADP(H)-binding pocket in small subunit catalases. This domain also involves at least six helices situated in two long insertions between β-strands along the polypeptide chain [30, 47].

The wrapping loop is an extended region of almost 110 residues that link the β-barrel and α-helical parts. This region, residues from 366 to 420, does not have any secondary structure except the essential helix (α9) stating the proximal side of heme with tyrosine residue. This part of the polypeptide chain is involved in different interdomain and intersubunit interactions especially with residues from the amino-terminal arm region from another subunit [30, 47].

The α-helical region contains four anti-parallel helices that are close to some of the helices from the β-barrel domain [30, 47].

Unlike BLC, the structures of PVC and HPII present an extra carboxy-terminal domain including roughly 150 residues with a high content of secondary structure elements organized with a "flavodoxin-like" topology [30, 46, 47]. The possible role of this extra domain in PVC remains unknown [30]. In BLC, prior to the flavodoxin-like domain is occupied by an NADP(H) molecule [48].

Although PVC and HPII share common structural similarities, HPII differs in the existence of 60 residues at N-terminal end that increase the contact area between subunits [25].

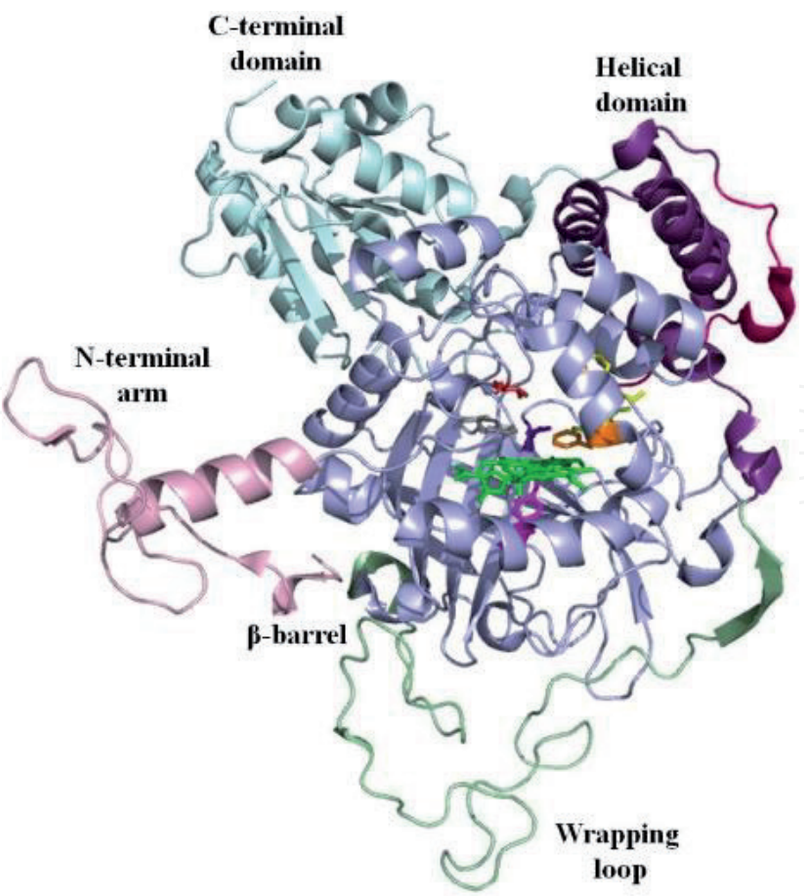

Figure 2.
Schematic drawing of the polypeptide chain and elements of secondary structure in a S. thermophilum *catalase subunit. The heme is colored green, Tyr369 magenta, His82 gray, Asn155 purple blue, Val123 red, Phe160 lemon, Phe161 yellow, and Phe168 orange. This figure is taken from the report of Yuzugullu et al. [17].*

6.1 Heme pocket

In all catalases, the heme group is deeply buried in the core structure, and its distance from the nearest part of the molecular surface is about 20 Å [9, 30]. Three residues, tyrosine on the proximal side of the heme (Tyr415 in HPII) and histidine and asparagine on the distal side (His128 and Asn201 in HPII), are believed to be essential for catalysis [30]. The oxygen of phenolic hydroxyl group in tyrosine residue is the proximal ligand of heme iron and is probably deprotonated with negative charge, so that it can lead to the stabilization of iron's high oxidation states. The imidazole ring of distal histidine is placed almost parallel to the heme at a mean distance of about 3.5 Å above either pyrrole ring III in PMC or pyrrole ring IV in PVC and HPII [9]. The histidine and asparagine residues on the distal side of the heme make the environment strongly hydrophobic [30]. A conserved serine residue (Ser167 in HPII) is also found to be hydrogen bonded to the N^{δ} of the essential histidine and might facilitate the enzymatic mechanism [46].

Despite possessing the same type of heme in active site, PVC and HPII differ in the presence of covalent bond between tyrosine and histidine residues. HPII contains a novel type of covalent bond joining the C^{β} of the essential Tyr415 and the N^{δ} of His392 but not in PVC [33, 44, 46, 49].

6.2 Channels to the heme group

The limited accessibility to heme grouping catalases requires the presence of channels [30]. The heme of the enzyme is connected to the exterior surface by three channels, namely, the main channel, the lateral channel, and the central channel. Among them, the main channel is placed perpendicular to the surface of the heme. The lateral channel approaches horizontal to the heme and the central one heading from the distal side [34, 45].

The main channel is considered to be the primary route for substrate movement to the active site [1, 3]. It is funnel-shaped with 30 Å long in small catalases [30, 48], while in large catalases that channel is replaced by an elongated, constricted, and possibly bifurcated channel that includes the C-terminal domain of adjacent subunit [3, 30].

The conserved residues in the main channel are shown in **Figure 3** including the essential histidine, a valine, and an aspartate (His82, Val123, and Asp135 in CATPO) situated 4, 8, and 12 Å from the heme, respectively [17]. The histidine residue is essential for catalysis in HPII, and the side chain of valine residue makes the channel narrower to a diameter of about 3 Å that prevents any molecule larger than H_2O and H_2O_2 from gaining access to the active site. The role of aspartate has not been investigated in any catalase, but the presence of negatively charged side chain has been found to be critical for catalysis [45].

The lateral or minor channel approaches heme above and below the essential asparagine and emerges in the molecular surface at location corresponding to the NADP(H)-binding pocket in catalases that bind a cofactor (**Figure 4**) [30, 50]. The function of this channel remains unknown [34]. Molecular dynamics analysis indicates that water can exit the protein through this channel [4].

The main channel is a preferred route for substrate entry, but it might be too long and narrow for the release of reaction products (water and molecular oxygen). As the central channel is mainly hydrophilic and leads to the central cavity that is contiguous to the bulk water, this could be a way out for O_2. However, substitutions of amino acid residues extending the major channel in large catalases might allow the exit of oxygen through the main channel. In fact, oxygen preferentially exits

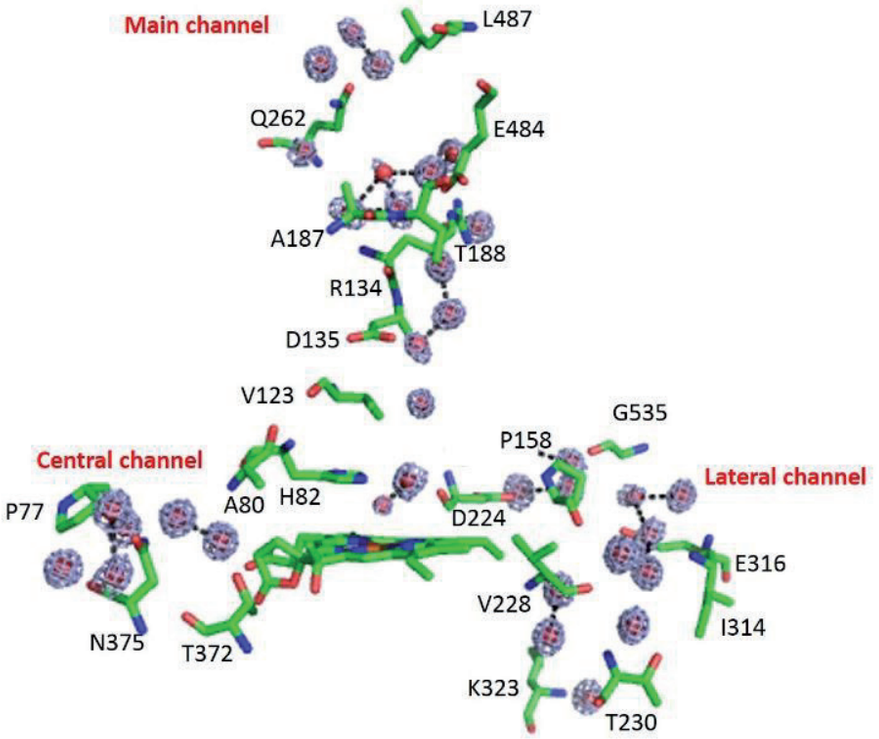

Figure 3.
Channels in CATPO of S. thermophilum.

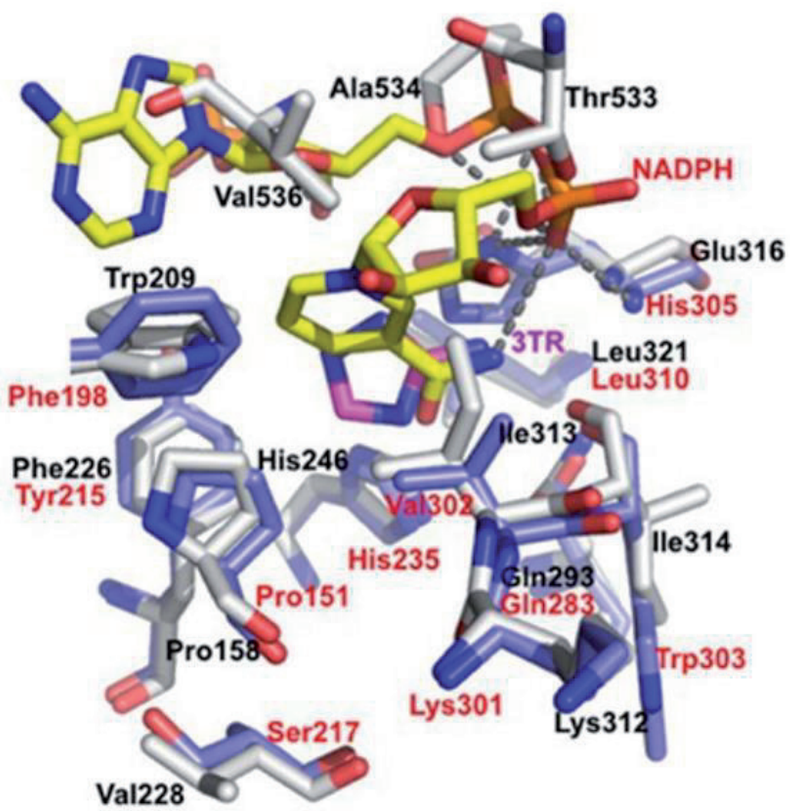

Figure 4.
View of chain A of CATPO complex with 3TR (PDB 5ZZ1, gray) superposed onto human catalase (PDB 1DGH7, blue). CATPO loop 533–537 lies across the top of the NADPH-binding pocket, clashing with the position of the NADPH in the human enzyme [50].

through the main channel instead of central one in all catalases having b-type heme in the active site. Thus, the presence of minor channels might be an alternative

mechanism for a fast release of products under the condition of high H_2O_2 stress. These results indicate that O_2 can exit the enzyme through different channels although the main exit in large catalases might be through the central channel and in small catalases through the major channel [34, 51].

6.3 Bifunctionality of catalase and phenol oxidase

Many reports on catalase and phenol oxidase enzymes suggest that the activities may flap in some way that catalases exhibit additional oxidase activity and phenol oxidases present further catalase activity. This relationship can be explained by the release of H_2O_2 due to polyphenol oxidation [52]. Hydrogen peroxide generation by phenol oxidation was also reported by Aoshima and Ayebe [53]. They observed high concentrations of H_2O_2 in beverages like tea or coffee directly after opening caps as a result of oxygen.

Jolley et al. first developed mushroom tyrosinase with catalase activity in the presence of hydrogen peroxide [54]. Garcia-Molina et al. [55] and Yamazaki et al. [56] also studied this bifunctional behavior of tyrosinase. In addition to this novel tyrosinase, a catalase-like process was found to have one isozyme of catechol oxidase from sweet potatoes (*Ipomoea batatas*) [57].

In literature, the first report on catalase known as a monofunctional enzyme but possessing secondary activity (oxidase) was introduced for mammalian catalase. This enzyme has been reported to present oxidase activity when hydrogen peroxide is absent or levels of H_2O_2 are low. As mentioned previously, the main function of catalase is the decomposition of hydrogen peroxide into water and oxygen (catalytic activity). Moreover, it is known that catalases can oxidize low molecular weight alcohols in the presence of low concentrations of H_2O_2 (peroxidatic activity). The catalytic mechanism of catalases is a two-step process in which catalase heme Fe^{3+} reduces one hydrogen peroxide molecule to water and generates a porphyrin cation radical called compound I, which is then oxidized by a second hydrogen peroxide to give molecular oxygen and water. The peroxidase activity stems from the oxidation of alcohols by compound I through single-electron transfer. Vetrano et al. expressed a novel oxidase activity in the absence of hydrogen peroxide. This oxidase reaction involves the interaction of catalase heme with a strong reducing agent like benzidine (HB) and molecular oxygen leading to the formation of a compound II-like intermediate. The subsequent electron transfer causes substrate oxidation and regeneration of resting enzyme. An incomplete reaction may result in the formation of radical centered intermediates and the production of superoxide [14].

Later, catalase from the thermophilic fungus *S. thermophilum* has been reported to possess additional phenol oxidase activity [16]. This enzyme, named as CATPO, is the first bifunctional catalase-phenol oxidase in the literature that is characterized in detail. *S. thermophilum* CATPO is a homotetramer with a molecular mass of 320 kDa. Based on the amino acid sequence and preliminary three-dimensional structure [58], CATPO is classified as a large heme catalase with the highest structural homology (77%) to catalase of *Penicillium vitale* [16]. CATPO can oxidize *o*-diphenols such as catechol, caffeic acid, and L-DOPA in the absence of hydrogen peroxide, and the highest oxidase activity is observed against catechol. This enzymatic activity is oxygen-dependent and is inhibited by classic catalase inhibitors, including 3-amino-1,2,4-triazole (3TR). The peroxide-independent secondary activity has also been identified in other catalases [14, 19, 20] and has been presumed to also occur at the heme active site.

There are a great number of reports available describing the structural and biochemical characterization of catalases. However, basic questions related to

substrate and product flow remain unanswered, particularly related to the oxidase activity. Therefore researchers have recently focused on the investigation of the region of CATPO that corresponds to the NADPH-binding region of bovine liver catalase (BLC) and the lateral channel. A number of mutations were introduced into this region, and the properties of these mutant variants, including their specific activities and sensitivities to various inhibitors, are interpreted in terms of a role for the lateral channel in CATPO. The structural, mutation, and kinetic evidences suggested that this pocket at the entrance to the lateral channel, captured by NADPH's nicotinamide moiety in mammalian catalases, should be the site of both oxidase substrate and 3TR binding. The promiscuous nature of CATPO oxidase is clarified by the presence of numerous ordered water molecules which facilitate substrate binding through hydrogen bond formation and can be transferred to accommodate various size and shaped substrates. Peroxide-independent phenolic substrate oxidation is then likely to happen in a similar manner to NADPH oxidation, by electron transfer from the substrate to a high-valent iron-oxo intermediate, apparently arisen through reaction with oxygen [50].

7. Conclusions

Catalases have been studied for over 100 years, with examples of the enzyme isolated, purified, and characterized from many different organisms. The crystal structures of 16 monofunctional catalases have been solved at high resolution. These structures show that they are tetramers, and each of the four active sites consists of a pentacoordinated-iron protoporphyrin IX prosthetic group with a tyrosinate axial ligand. Some also contain a NADPH cofactor tightly bound at the periphery of each subunit. Recently, it has been found that these enzymes exhibit an oxidase activity in addition to their H_2O_2 degrading activity. Although they are old enzymes, a peroxide-independent oxidase activity of catalases is new in the literature. Such studies have led to the proposal that this secondary oxidative activity may be a general characteristic of catalases.

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Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

BLC	bovine liver catalase
CATPO	catalase-phenol oxidase
Cpd	compound

3TR	3-amino-1,2,4-triazole
H_2O_2	hydrogen peroxide
HB	benzidine
HPI	hydroperoxidase I
HPII	hydroperoxidase II
L-DOPA	L-3,4-dihydroxy-phenylalanine
NADP(H)	nicotinamide adenine dinucleotide phosphate
PVC	*Penicillium vitale* catalase
PMC	*Proteus mirabilis* catalase
POR	porphyrin

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