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Inhibitory effects of a novel Val to Thr mutation on the distal heme of human catalase

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Abstract

True catalases efficiently breakdown hydrogen peroxide, whereas the catalase-related enzyme allene oxide synthase (cAOS) is completely unreactive and instead metabolizes a fatty acid hydroperoxide. In cAOS a Thr residue adjacent to the distal His restrains reaction with H₂O₂ (Tosha et al (2006) *J. Biol. Chem.* 281:12610; De Luna et al (2013) *J. Phys. Chem. B* 117: 14635) and its mutation to the consensus Val of true catalases permits the interaction. Here we investigated the effects of the reciprocal experiment in which the Val74 of human catalase is mutated to Thr, Ser, Met, Pro, or Ala. The Val74Thr substitution decreased catalatic activity by 3.5-fold and peroxidatic activity by 3-fold. Substitution with Ser had similar negative effects (5- and 3-fold decreases). Met decreased catalatic activity 2-fold and eliminated peroxidatic activity altogether, whereas the Val74Ala substitution was well tolerated. (The Val74Pro protein lacked heme). We conclude that the conserved Val74 of true catalases helps optimize catalysis. There are rare substitutions of Val74 with Ala, Met, or Pro, but not with Ser or Thr, possibly due their hydrogen bonding affecting the conformation of His75, the essential distal heme residue for activity in catalases.

Keywords

Human catalase; Val74; mutation; catalatic activity; peroxidatic activity; proximal heme

1. Introduction

The classical mono-functional catalases, typified by human catalase, are hemoproteins with tetrameric structures having one molecule of heme per 56 kD protomer [1]. These Fe heme catalases efficiently dismutate hydrogen peroxide to water and oxygen (2H₂O₂ → 2H₂O + O₂), the “catalatic” reaction. In addition to the major catalatic activity, catalases also exhibit a minor peroxidatic activity using H₂O₂ to oxidize small molecules that can access the distal

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heme, ethanol for example ($\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{CHO} + 2\text{H}_2\text{O}$) [2]. In certain cyanobacteria and invertebrate animals, small catalase-related proteins of ~40 kD are known that react with fatty acid hydroperoxides rather than H_2O_2 [Ph, Am, Anab] [3-6]. Analysis of this unexpected lack of reaction with H_2O_2 raises questions concerning the reactions of true catalase itself.

The prototypical enzyme that reacts with fatty acid hydroperoxide, characterized catalytically and by X-ray crystallography, is the catalase-related allene oxide synthase (cAOS) from the coral *Plexaura homomalla* [3, 7]. Although sharing less than 20% sequence identity to true catalases, the X-ray crystal structure clearly shows retention of a central catalase fold with particularly striking conservation around the heme (Fig. 1). In cAOS, as in true catalases, a tyrosine (Y353) residue serves as the proximal heme ligand. The distal heme cavity also shows sequence conservation, with retention of the two main residues crucial for catalysis (His67 and Asn137). Given the close resemblance in structure to true catalases, it is remarkable that *P. homomalla* cAOS shows no reaction whatsoever on exposure to hydrogen peroxide. Investigation of this phenomenon identified a seemingly minor distal heme amino acid substitution as critical in preventing the reaction of cAOS with H_2O_2 . Thr66 in cAOS, immediately adjacent to the distal heme His67, is typically a Val residue in true catalases (never threonine) (Fig. 1). The Thr66Val mutation in cAOS allowed reaction with H_2O_2 and promoted the fast inactivation of the enzyme [8]. This background prompted the studies reported here, analysis of the effects of the reciprocal mutation (Val to Thr, and other residues) on the catalatic and peroxidatic activities of human catalase.

2. Materials and methods

All the chemicals, NAD^+ , aldehyde dehydrogenase, and glucose oxidase were purchased from Sigma Aldrich. H_2O_2 was purchased from Fisher.

2.1. Human catalase expression and purification

Human catalase cDNA was amplified by RT-PCR from tonsil tissue mRNA using forward 5' CAT ATG GCT GAC AGC CGG GAT CCC GCC 3' and reverse 5' GAT ATC TCA GTG ATG GTG ATG GTG ATG CAG ATT TGC CTT CTC CCT TGC CGC 3' oligonucleotide primers. The primers introduce an *NdeI* and *EcoRV* restriction sites, with a C-terminal (His)₆-tag at the 5'- and 3'-ends, respectively. Products which had sequence identical to human catalase was digested, purified, and ligated into compatible sites of pET17b vector. Five mutations of catalase, Val74Thr, Val74Ala, Val74Met, Val74Pro, and Val74Ser were generated using the QuikChange site-directed mutagenesis kit. The recombinant plasmids were transformed into BL21 (DE3) competent cells. The catalase and its mutants were expressed by inoculating Terrific Broth (TB) medium containing 100 µg/ml Ampicillin and shaking at 28 °C and 250 rpm for 24 h. Cells were harvested and resuspended in BugBuster Protein Extraction Reagent followed by sonication. The clarified cell lysate were applied to a Ni-NTA column. Catalase and its mutants were eluted with 250 mM imidazole. The last step of purification was performed with gel filtration chromatography on a 100 × 1.5 cm column packed with Superdex 200 prep grade resin

developed with buffer containing 50 mM K-phosphate pH 7.0 and 150 mM NaCl. The purity of the eluted proteins was analyzed by SDS-PAGE.

2.2. Catalatic activity of catalase and its mutants

Catalatic activity was measured via oxygen electrode (YSI model 5300 Biological Oxygen monitor) by monitoring the production of oxygen. The assay was performed in a 2 ml cell containing 50 mM potassium phosphate buffer pH 7.2 at 25°C, using concentrations of H₂O₂ of 0, 5, 10, 15, 20, 25, 50, 100, 200, 300, 500, and 1000 mM. The reaction was started by adding either wild-type catalase or mutants at a final concentration of 0.75 nM.

2.3. Peroxidatic activity of catalase and its mutants

Peroxidatic activity was measured as the oxidation of ethanol to acetaldehyde; a low concentration of H₂O₂ was generated in situ using glucose and glucose oxidase and the peroxidatic reaction was followed as the reduction of NAD⁺ to NADH catalyzed by aldehyde dehydrogenase [2]. The assay was performed in 1 ml of 50 mM potassium phosphate buffer pH 7.2, 10 mM glucose, 0.1 mM NAD⁺, 0.05 unit of aldehyde dehydrogenase, 50 mM ethanol, and 20 nM of catalase or the mutants. The reaction was initiated by addition of 1.0 µg of glucose oxidase (21200 units/g) and the formation of NADH was monitored at 340 nm for 3 min.

3. Results

3.1. Catalatic activity of human catalase and its mutants

Initial rates were obtained by measuring evolution of O₂ with H₂O₂ concentrations ranging from 0 to 1000 mM. The rates increase with H₂O₂ concentration up to 200 mM and then decrease in higher H₂O₂ concentrations probably due to toxicity of the H₂O₂ [9]; the Val74Met mutant was almost resistant to this effect with only a small drop at 1000 mM, Fig. 2A. To exclude the decrease in activity of the enzymes in higher concentrations of H₂O₂, only data points up to 200 mM H₂O₂ were used to fit to the Michaelis-Menten curve (solid lines, Fig. 2A). As summarized in Fig. 2B, the catalatic efficiency (kcat/Km) of the Val74Thr mutant is 30% of the wild-type, and the Val74Ser mutation has a similar negative effect (20% of the wild-type). In comparison, the kcat/Km of the Val74Ala and Val74Met mutants are about 140% and 60% of the wild-type, respectively. The Val74Pro substitution was not tolerated within human catalase, and the protein expressed weakly with no heme (Fig. 2B); the wild-type and other mutants expressed with A₄₀₆/A₂₈₀ ratios of the purified proteins of 1.05–1.27 (Fig. 2B), similar to the reported values for 95% pure human catalase [10].

3.2. Peroxidatic activity of human catalase and its mutants

Peroxidatic properties of wild-type catalase and its mutants (Val74Thr, Val74Ala, Val74Met, Val74Ser, and Val74Pro) were compared in the oxidation of ethanol to acetaldehyde at a fixed rate of H₂O₂ generation by 10 mM glucose and 1.0 µg glucose oxidase, monitored at 340 nm in the conversion of NAD⁺ to NADH by aldehyde dehydrogenase. The mutation of Val74 to Thr and Ser reduced the activity of the enzyme by ~70%. The Val74Ala mutation had only a minor affect on the peroxidatic activity (~20% decrease) while the Val74Met enzyme was devoid of peroxidatic activity, Fig. 2B.

4. Discussion

The effects of mutating several distal heme residues in catalase are well described [11-13], but the Val74 position has not been studied previously. We became interested in the potential effects of such an apparently simple change to the distal heme structure of catalase through results with the catalase-related cAOS. The equivalent Thr66 residue in wild-type cAOS is critically important in blocking reaction with hydrogen peroxide. Whereas the wild-type Thr66 cAOS is completely unresponsive to H₂O₂, the Thr66Val mutant reacts instantly with H₂O₂ to form Compound I, followed within seconds by bleaching of the heme with rapid loss of the main Soret band in the UV-Vis spectrum [8]. The crystal structure of the cAOS indicated that the position of the distal heme residues were compatible with direct hydrogen bonding between His67 and Thr66 [7], whereas a recently reported molecular dynamics (MD) study came to a different conclusion [14]. The MD simulations predict that in wild-type cAOS the distal heme Thr66 forms a strong hydrogen-bonding interaction with H₂O₂ which in cooperation with His67 keeps the H₂O₂ away from the heme iron. By contrast, the Thr66Val mutation allows H₂O₂ to approach much closer to the iron and permits a productive Fe-O₂H₂ interaction.

Here we show that Val74, a highly conserved residue on the catalase distal heme, plays an important role in optimizing the catalatic and peroxidatic activities with hydrogen peroxide. The Val74Thr (and Val74Ser) mutation inhibits catalatic and peroxidatic activities to a similar extent, compatible with a diminished capacity to form Compound I. This could be investigated experimentally by spectral analysis of the rates and characteristics of Compound I formation using peracetic acid to avoid return of the enzyme to its resting state [15]. Potentially, the mutant Thr74 can form a hydrogen bond with the distal heme His75 and compete with hydrogen bonding to Ser114 on the opposite side of the imidazole ring. This Ser residue is 100% conserved in true catalases (an Ala is in this position in cAOS). The native His-Ser interaction may stabilize the imidazole ring in relation to the distal heme Asn147 and heme iron in the correct orientation for reactivity. A competing interaction with the mutated Thr requires a rotation of the imidazole with the result that the His is no longer in an optimal catalytic conformer, (a scenario that is open to mutational and computational analysis). Finally, we note that although the 70-80% decrease in activity in the Val74Thr mutant is a profound loss, the effects are not so all-or-none as occurs with the equivalent Thr66Val substitution in the *P. homomalla* cAOS enzyme [8].

There were some interesting findings with the other Val74 mutations we tested. The Val74Pro substitution (occurring naturally in *Saccharomyces cerevisiae* [16]) was not acceptable within the framework of human catalase and the expressed protein lacked heme. The Val74Met mutation (found naturally in *Neisseria meningitidis* and *Yersinia pestis*) causes a 40% decrease in catalatic activity of human catalase and complete loss of peroxidatic activity, the latter possibly associated with the larger Met residue or its sulfinic acid oxidation product interfering with the binding of the peroxidatic substrate, ethanol; the structure of *P. mirabilis* catalase shows an oxidized Met residue in close proximity to the distal His [17]. As apparent from the kinetic analyses in Fig 2A, this Met mutant was more resilient to very high concentrations of H₂O₂ compare to wild-type catalase or the other mutations in this study. Substitution of Val74 with Ala results in a 40% increase in catalatic

activity and a slight (~20%) decrease in peroxidatic activity. Alanine in this position is found in a few catalase-related proteins (e.g. in *Acinetobacter baumannii*, *Xanthomonas campestris*, *Sclerotinia sclerotiorum*) that to the best of our knowledge have yet to be characterized.

4.1. Conclusion

The conserved Val74 adjacent to the distal heme His75 of catalase is important in optimizing both catalytic and the peroxidatic activity. Mutation of Val74 to Thr, the corresponding residue in catalase-related proteins involved in fatty acid hydroperoxide metabolism, reduces catalytic and peroxidatic activities by 70%, possibly due to T-H hydrogen bonding producing a sub-optimal conformer of His75.

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Abbreviations footnote

cAOS	catalase-related allene oxide synthase
LOX	lipoxygenase
HPETE	hydroperoxy-eicosatetraenoic acid
NAD⁺/NADH	nicotinamide adenine dinucleotide
CAT	catalase
MD	molecular dynamics

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Highlights

- A distal heme Thr in catalase-related AOS is known to preclude reaction with H_2O_2 .
- We mutated the equivalent Val74 in human catalase to Thr and other residues.
- The Val74Thr mutant remained active, but exhibited only ~30% catalytic efficiency.
- The mutant Thr may hydrogen bond to the catalytic His75 and impair activity
- We conclude that the conserved Val74 is important for optimal catalase activity.

Figure 1.

Structural and sequence comparison of the distal heme in human catalase and cAOS. **A)** Sequence alignment of residues around the catalytic His. In catalases the residue preceding the distal His is conserved as Val, while the Thr-His sequence is a signature of cAOS-related proteins. **B)** Key residues in the heme center of human catalase, and **(C)** in *P. homomalla* cAOS. Val74 in human catalase is substituted by Thr66 in cAOS as shown in **bold**.

Figure 2.

Activities of WT-CAT and its mutants. **A)** Catalatic activity of WT-CAT (black), Val74Ala (green), Val74Met (magenta), Val74Thr (red), and Val74Ser (blue) measured as initial rate of oxygen production versus H_2O_2 concentration. Activity decreases at higher H_2O_2 concentrations due to enzyme inactivation (cf. [9]). Kinetic parameters were calculated using data points on the solid lines. Rates at the high concentrations of H_2O_2 may be underestimated due to the slow response time of the O_2 electrode. **B)** The ratios of A_{406}/A_{280} for the purified enzymes, apparent Km, kcat, and kcat/Km of the catalatic activities, and rate of ethanol oxidation in the peroxidatic assay are summarized. ND = not determined.