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Role of Active Site Residues on Catalytic Activity of Catalase with Oxidase Activity from *Scytalidium Thermophilum*

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Abstract

Scytalidium thermophilum produces a catalase with phenol oxidase activity (CATPO) that catalyses the dismutation of hydrogen peroxide (H_2O_2) to dioxygen and water and also oxidizes several phenolic compounds in the absence of hydrogen peroxide. It comprises 717 amino acids with a 19 amino acid signal sequence, and a 17 amino acid prosequence. It is a homotetrameric protein of molecular mass 320 kDa and subunit molecular mass 80 kDa. Although catalases have been studied for many years, a peroxide independent oxidative activity of catalases has recently been recognized. 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. The goals of our current studies are to investigate the main and lateral channels known that connect the deeply buried active site to the exterior of the enzyme. We have introduced a number of mutations into these regions and analyzed their specific activities.

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Keywords: Catalase, hydrogen peroxide, oxidase, *Scytalidium thermophilum*

1. Introduction

Catalases (hydrogen peroxide:hydrogen peroxide oxidoreductases; (EC 1.11.1.6) are haem-containing enzymes that are present in most aerobic organisms (Goldberg & Hochman,1989; Nicholls et al., 2001). They serve to protect

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cells against reactive oxygen species by degrading hydrogen peroxide to water and oxygen (1) (Switala & Loewen, 2002; Chelikani et al., 2004; Maté´ et al., 2001; Nicholls et al., 2001):

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

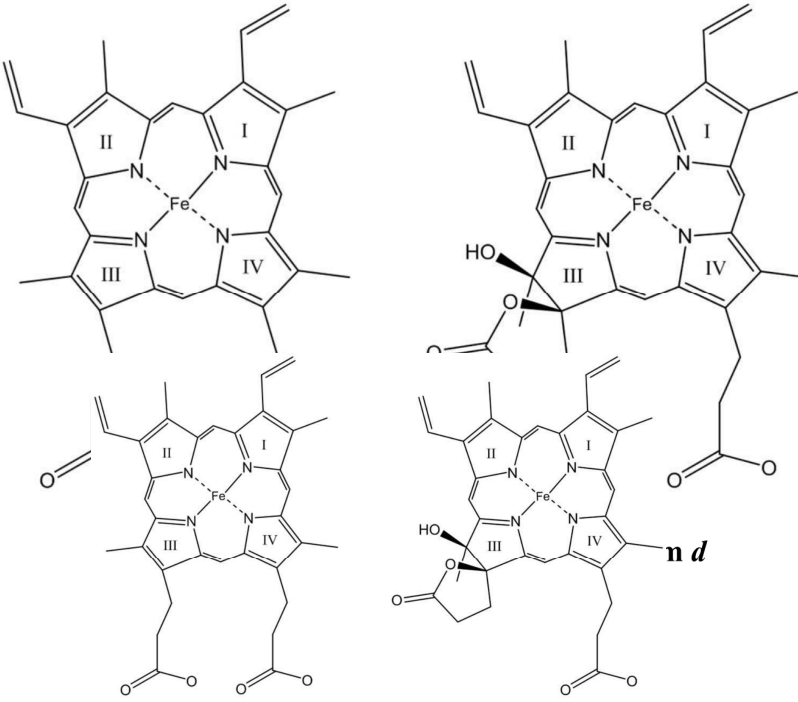
This catalytic reaction occurs in two distinct stages (Chelikani et al., 2004). The first stage involves the oxidation of the haem by the first hydrogen peroxide to form an oxyferryl species and a porphyrin cation radical (2). In the second stage, this radical intermediate, known as compound I, is reduced by a second hydrogen peroxide to regenerate the resting-state enzyme, water and oxygen (3) (Switala & Loewen, 2002; Nicholls et al., 2001):

$$\text{Enz (Por-Fe}^{III}\text{)} + H_2O_2 \rightarrow \text{Cpd I (Por}^{+\bullet}\text{-Fe}^{IV}\text{=O)} + H_2O \quad (2)$$
$$\text{Cpd I (Por}^{+\bullet}\text{-Fe}^{IV}\text{=O)} + H_2O_2 \rightarrow \text{Enz (Por-Fe}^{III}\text{)} + H_2O + O_2 \quad (3)$$

At limiting H_2O_2 concentrations and in the presence of a suitable organic donor, catalases may function as peroxidases by catalyzing the one-electron reduction of compound I to form compound II (4):

$$\text{Cpd I (Por}^{+\bullet}\text{-Fe}^{IV}\text{=O)} + 2AH_2 \rightarrow \text{Cpd II (Por-Fe}^{III}\text{)} + 2AH^{\bullet} + H_2O \quad (4)$$
$$\text{Cpd II (Por-Fe}^{III}\text{)} + 2AH_2 \rightarrow \text{Enz (Por-Fe}^{III}\text{)} + AH^{\bullet}$$

The catalase reaction has evolved in four phylogenetically unrelated enzyme classes: the monofunctional or typical catalases, the bifunctional catalase–peroxidases, the nonhaem manganese-containing catalases and the minor catalases (Maté et al., 2001; Nicholls et al., 2001). The largest and most extensively studied group are the monofunctional cat[illegible] which can be subdivided into those with large (75–84 kDa) subunits containing haem *d* and those with s[illegible] s are generally homotetramers hav[illegible]; Nicholls et al., 2001; Klotz & Loe[illegible]ristic Soret band at 406 nm and sma[illegible]xhibit an almost identical Soret ban[illegible]998). The haem prosthetic groups i[illegible]) or an oxidized form of protoporph[illegible]es of *Penicillium vitale* catalase (PV[illegible]ne that is rotated 180 degrees about [illegible]ıtation found for haem *b* in bovine li[illegible]



Haem *b* **Haem *d***

Fig. 1. Structures of haem *b* and haem *d*

Three channels, the main channel oriented perpendicular to the plane of haem, the lateral channel approaching in the plane of haem and the central channel leading from the distal side of the haem to the central cavity, connect the deeply buried active site to the exterior of enzyme (Díaz et al., 2004; Chelikani et al., 2003). The main channel is believed to be a primary route for peroxide access to the active site (Chelikani et al., 2003; Switala & Loewen, 2002) and studies with various catalase variants from different sources mutated in residues lining the channel (Zámocký et al., 1995) as well as molecular dynamics simulations (Kalko et al., 2001; Amara et al., 2001) support this idea. The lateral, or minor channel approaches haem from above, adjacent to the essential asparagine and emerges on the molecular surface at a location corresponding to the NADP(H) binding pocket in catalases that bind a nicotinamide cofactor (Maté et al., 2001). The function of this channel remains unknown (Díaz et al., 2004), although molecular dynamics simulations suggest that water can exit the protein through this channel (Sevinc et al., 1999).

Although catalases have been studied for many years, a peroxide independent oxidative activity of catalases has been recently recognized (Vetrano et al., 2005; Sutay Kocabas et al., 2008; Yuzugullu et al., 2013). For example, the *Scytalidium thermophilum* catalase (CATPO) has been shown to oxidize *o*- and some *p*-diphenolic compounds in the absence of hydrogen peroxide (Ögel et al., 2006; Sutay Kocabas et al., 2008; Yuzugullu et al., 2013). This and other studies have led to the proposal that this secondary oxidative activity may be a general characteristic of catalases.

2. Methodology

2.1. Research Goal

As part of an ongoing study of haem catalases, CATPO variants with mutations of Val228, Thr188 and Glu484 were produced. Variants at other positions were also created in order to further investigate the role of the protein structure and molecular channels in catalysis. In total, 10 mutant variants of CATPO have been biochemically characterized.

2.2. Materials

Standard chemicals and biochemicals were obtained from Sigma. Molecular-size markers and DNA ladder were obtained from BioLabs.

2.3. Strains and Plasmids

The plasmid pET28a-CATPO (Yuzugullu et al., 2013) including a hexa-His tag and a TEV cleavage site was used as the source of the *catpo* gene. *Escherichia coli* XL-1 Blue was used as a general cloning strain and the BL21 Star (DE3) strain was used for expression of the mutant *catpo* constructs and isolation of the mutant CATPO proteins.

2.4. Site-directed mutagenesis

Single-point mutations were introduced into the *catpo* coding region by a QuikChange approach with mutagenic primer pairs using the approach described by Stratagene but with KOD DNA polymerase (Novagen) for 25 cycles before *Dpn*I digestion (Yuzugullu et al., 2013). The PCR primers containing the desired mutations were purchased from Sacem and Sentegen and are listed in Table 1.

Table 1. Oligonucleotides used in site-directed mutagenesis of catpo.

Mutant	Sequence change	Oligonucleotide*
V228A	GTT→GCT	5′-GCACATGGACGGCTTCGGT**GCT**CACACTTTCCGTTTC
V228C	GTT→TGC	5′-GCACATGGACGGCTTCGGT**TGC**CACACTTTCCGTTTC
V228G	GTT→GGT	5′-GCACATGGACGGCTTCGGT**GGT**CACACTTTCCGTTTC

V228I	GTT→ATC	5′-GCACATGGACGGCTTCGGT**ATC**CACACTTTCCGTTTC
T188A	ACC→GCC	5′-CCCGCAGGCTGCT**GCC**GCTCACGACTCTGCTTGGG
T188D	ACC→GAC	5′-CCCGCAGGCTGCT**GAC**GCTCACGACTCTGCTTGGG
T188F	ACC→TTC	5′-CCCGCAGGCTGCT**TTC**GCTCACGACTCTGCTTGGG
E484A	GAA→GCA	5′-CCTGGTTAACGCTATGCGTTTC**GCA**ATCTCTCTGGTTAAATCTG
E484D	GAA→GAC	5′-CCTGGTTAACGCTATGCGTTTC**GAC**ATCTCTCTGGTTAAATCTG
E484I	GAA→ATC	5′-CCTGGTTAACGCTATGCGTTTC**ATC**ATCTCTCTGGTTAAATCTG

*The sequence in bold is the codon that has been modified.

2.5. Protein expression and purification

pET28a-CATPO was freshly transformed into *E. coli* BL21 Star (DE3) cells and a single colony was inoculated into 10 ml LB medium supplemented with 50 mg ml^{-1} kanamycin and incubated overnight at 310 K with shaking (200 rev min^{-1}) (Yuzugullu et al., 2013). This overnight culture was used to inoculate 1 l LB with 50 mg ml^{-1} kanamycin in a 2.4 l conical flask and was grown at 310 K (200 rev min^{-1}) to an OD600 of 0.6–0.8. IPTG was added (to a final concentration of 0.1 mM) and incubation was continued at 303 K (120 rev min^{-1}) for 24 h in order to achieve semi-anaerobic conditions. The cells were then harvested by centrifugation for 10 min at 9000 rev min^{-1} and the pellets were frozen at 193 K until use. Following thawing of the pellet, the cells were lysed using 100 ml lysis buffer [50 mM Na HEPES, 25% (w/v) sucrose, 1% (v/v) Triton-X 100, 5 mM $MgCl_2$] per litre of original culture. The resulting suspension was centrifuged at 10 000 rev min^{-1} for 30 min and the supernatant was dialyzed overnight against 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. The dialyzed fraction was filtered (0.2 mm) and loaded onto ProtinoNi-NTA column (1 ml; ProtinoNi-NTA, Germany) precharged with Ni^{2+} and pre-equilibrated with 20 mM sodium phosphate,0.5 M NaCl, 20 mM imidazole pH 7.4 using a Akta Prime (GE Healthcare, USA). Recombinant hexa-His-CATPO was purifed by affinity chromatography by applying a wash step (20 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole pH 7.4) followed by elution as 1 ml fractions over a 30 ml linear gradient from 0.05 to 0.2 M imidazole. Each fraction was tested for protein purity by SDS–PAGE and the concentration of the pooled fractions was determined by the Bradford assay using bovine serum albumin as a standard.

2.6. Enzyme activity assay

Catalase and phenol oxidase activity assays were performed using a temperature-controlled spectrophotometer (Cary 60 UV-Vis, Ailgent). All assays were performed in triplicate in 100 mM sodium phosphate buffer pH 7.0 at 333 K. Specific activity assays for catalase activity were carried out using 10 mM H_2O_2 as the substrate and monitoring the decrease in absorbance at 240 nm. Enzyme activity was determined using the initial rate of the reaction and an extinction coefficient at 240 nm for H_2O_2 of 39.4 M^{-1} cm^{-1} (Merle et al., 2007). One unit of activity was defined as the amount of enzyme that catalyzed the decomposition of 1 μmol of H_2O_2 per minute. Phenol oxidase activity was determined by monitoring the increase in absorbance at 420 nm using 100 mM catechol as the substrate. Enzyme activity was determined using the initial rate of the reaction and an extinction coefficient at 420 nm of 3450 M^{-1} cm^{-1} for oxidized catechol (Ogel et al., 2006), where one unit of activity corresponded to the formation of 1 nmol of product per minute.

2.7. UV-Vis spectra

All spectroscopic measurements were performed on a Cary 60 UV-Vis (Agilent, USA) spectrophotometer at room temperature in a 1 cm quartz cuvette between 400 and 750 nm.

3. Results and Discussion

3.1. QuikChange application to generate mutational variants

Following mutation by thermal cycling and *Dpn*I digestion according to the procedure given in Methodology section, *E. coli* XL-1 Blue competent cells were transformed with the plasmids carrying desired mutations (V228A, V228C, V228G, V228I, T188A, T188D, T188F, E484A, E484D and E484I). The bands with expected size of approximately 7500 bp were visualized for different CATPO variants by agarose gel electrophoresis (Figure 2). The introduction of mutations was confirmed by sequencing.

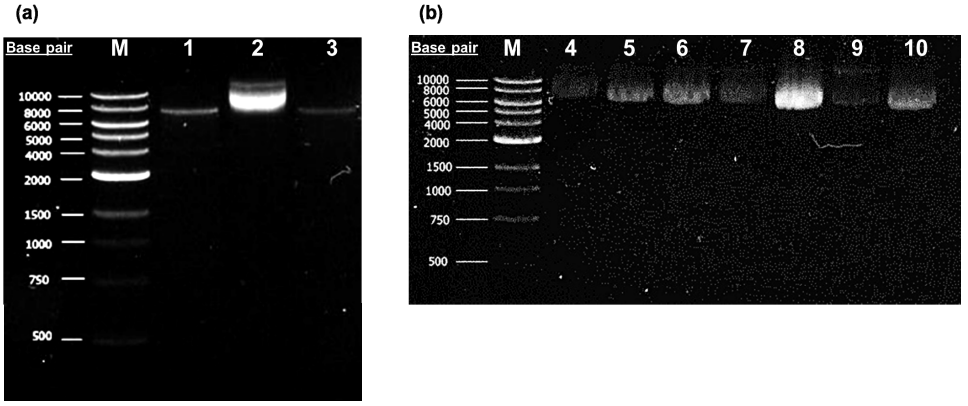


Fig. 2. Isolated plasmids from different CATPO variants. M, molecular size marker (1 kbp DNA Ladder, NEB); Lane 1, V228G; Lane 2, T188F; Lane 3, E484A; Lane 4, V228A; Lane 5, V228I; Lane 6, V228C; Lane 7, T188A; Lane 8, T188D; Lane 9, E484I; Lane 10, E484D. Annealing temprature for Lane 1-3 was 55.9 °C; for Lane 4-6 and Lane 9-10 was 63 °C; for Lane 7 was 60 °C, for Lane 8 was 55-60 °C.

3.2. Characterization of CATPO variants

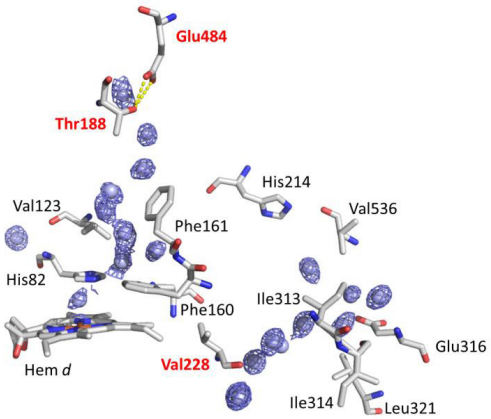
All mutational variants of CATPO appeared as single bands at an apparent molecular weight of 79 kDa as determined by SDS–PAGE.

Residue Val228 in *S. thermophilum* CATPO is situated adjacent to the haem edge at the entrance to the lateral channel (Figure 3), an access route to the haem cavity that also parallels an electron transfer route between NADPH and the haem in some small subunit clade 3 enzymes (Olson et al., 1995; Sicking et al., 2008). Only five different amino acids are found at the location among all catalases, including Val (62%), Ser (32%), Gly (3%), Ile (2%), and Ala (1%) (Jha et al., 2011). To investigate the integrity of this residue in the catalytic function of the enzyme, it was mutated to residues found at the same location in other catalases. Specifically, plasmids harboring mutated *catpo* genes encoding V228A, V228G and V228I were constructed, along with genes encoding V228C, to provide a sulfhydryl group for the introduction of nonstandard amino acid side chains. Characterization of mutants containing smaller uncharged residues showed that V228A has 15% of wild type catalase activity and 7% of oxidase activity (Table 2). V228G has a similar catalatic activity profile as V228A does but has 86% of wild type oxidase activity. Change of Val to Ile counterpart in HPII resulted in significant increase in catalase activity (174%) however; oxidase activity was reduced to 24%. V228C exhibited a similar catalytic activity profile, 137% and 3% of wild type catalase and oxidase activities, respectively. Purified V228C and V228I were green in colour, indicative of a *d*-type haem prosthetic group while V228A and V228G were yellow colour, indicative of a *b*-type haem prosthetic group, which was also confirmed by spectroscopic measurements (Figure 4).

Changing the side chain of Val228 in CATPO elicits a number of changes in the activity of the enzyme. Enlarging the entrance to the lateral channel resulted in decreased catalatic efficiency and explanation to this may lie in the recent prediction arising from a computational study that the haem cavity has evolved to restrict efflux of H_2O_2, thereby extending its residency time in the cavity (Dominguez et al., 2010). Thus, the bulky valine or isoleucine restricts movement of H_2O_2 out of the cavity via the lateral channel, and the longer occupancy in the cavity increases its chances of participating in the reaction. Conversely, opening the lateral channel (with smaller side chains at residue 228) facilitates diffusion of H_2O_2 out of the cavity, leading to a shorter residency time, poorer substrate binding, and a slower reaction (Jha et al., 2011).

The residues equivalent to Thr188 and Glu484 in *N. crassa* CAT-1 (S198 and E489) were identified as components of a putative gate controlling access of H_2O_2 to the haem cavity (Jha et al., 2012). They are also notable because of their conservation, their location at the junction between the hydrophobic inner and hydrophilic outer sections of the main channel with unobstructed paths between their side chains and the haem iron 17–20 Å distant. To investigate the influence of the side chains on the catalatic reaction, mutations to acidic, uncharged polar, and aliphatic side chains were introduced. Specifically, plasmids harboring mutated *catpo* genes encoding T188A, T188D, T188F, E484A, E484D and E484I were constructed. Changing threonine to a bulkier residue, phenyl alanine, resulted in enzyme with slightly increased catalatic activity but reflected two-fold higher oxidase activity for larger substrates (Table 2). This was attributed to easier access for larger oxidative substances. On the other hand, introducing acidic side chain with T188D variant resulted in reduced catalase (20%) and oxidase (50%) activities of wild type enzyme. Increasing the volume of main channel by changing glutamic acid to alanine enhanced the catalase activity (139%) but revealed minor change in oxidase activity compared to wild type enzyme. Spectroscopic measurements show that E484Aand T188A contained haem *d*, while T188F had haem *b* in the active site (Figure 4).

The A_{406}/A_{280} ratio or R_Z is commonly used as a measure of protein purity and haem content. For recombinant wild type CATPO, the pure enzyme exhibits an Rz of 0.8, consistent with its aromatic amino acid content. However,



the Rz value exhibited by V228A is much more lower, 0.2. Two explanations for such low value, impure protein or low haem content, are inconsistent with the >95% purity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the integrity of the haem in the core of the enzyme that precludes protein folding in its absence.

Fig. 3. Active site of recombinant CATPO. (PDB code: 4aum; designed by PyMOL).

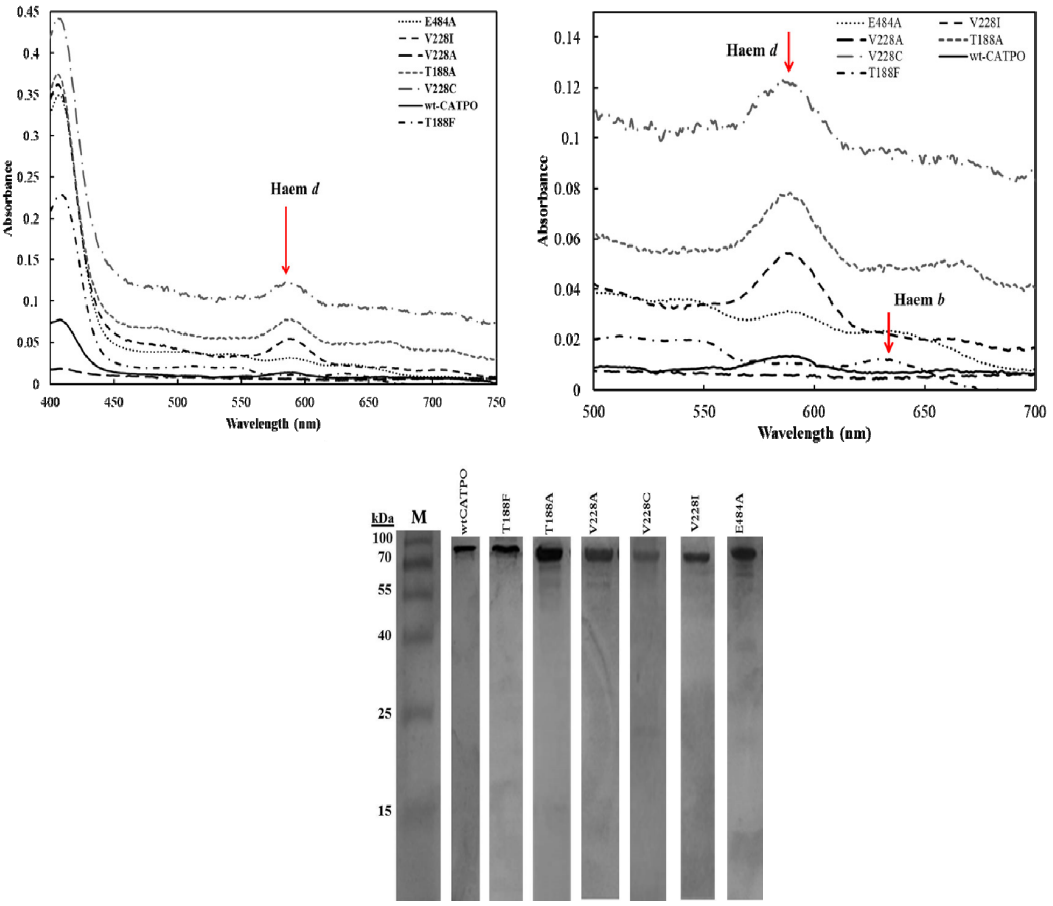


Fig. 4. (a) UV-Vis spectra of CATPO variants. The spectra were obtained with 1 mg ml-1 catalase in 20 mM sodium phosphate pH 7.4 at room temperature. The wild-type CATPO (wt_CATPO) spectrum is shown in each panel, adjusted to have an equivalent value at the Soret peak for each set of mutants. (b) SDS–PAGE gels of the CATPO variants stained with Coomassie Blue, showing that each mutant was purified to homogeneity.

Table 2. Comparison of specific activities of purified CATPO variants

	% Relative Activity	
Variant	*CAT*	*PO*
wt_CATPO	100	100
V228A	15	7
V228C	137	3
V228G	2	86
V228I	174	24
T188D	20	50
T188F	106	204
E484A	139	87

Percent relative activities were based on the highest initial reaction rate of the enzyme observed on the substrates analyzed

4. Conclusion

We report here the biochemical characterization of a series of main and lateral channel mutants of the *S. thermophilum* catalase. Differences in the volume of two channels, main and lateral, caused the altered catalase and phenol oxidase activities of the mutants that may be caused by a differential disruption of the access of dioxygen species to the active site, as has previously been proposed for equivalent mutations in monofunctional catalases derived from other sources (Carpena et al., 2003; Chelikani et al., 2003; Maté et al., 1999; Melik-Adamyan et al., 2001).

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Abbreviations

CATPO, catalase-phenol oxidase; R_Z, A_{406}/A_{280}; PDB, Protein Data Bank; HPII, hydroperoxidase II; PVC, *Penicillum vitale* catalase.

References

Amara, P., Andreoletti, P., Jouve, H. M. & Field, M. J. (2001). Protein Sci. 10, 1927–1935.
Carpena, X., Soriano, M., Klotz, M. G., Duckworth, H. W., Donald, L. J., Melik-Adamyan, W., Fita, I. & Loewen, P. C. (2003). Proteins, 50, 423–436.
Chelikani, P., Carpena, X., Fita, I. & Loewen, P. C. (2003). J. Biol. Chem. 278, 31290–31296.
Chelikani, P., Fita, I. & Loewen, P. C. (2004). Cell. Mol. Life Sci. 61,192–208.
Díaz, A., Horjales, E., Rudin˜ o-Pin˜ era, E., Arreola, R. & Hansberg,W. (2004). J. Mol. Biol. 342, 971–985.
Domínguez, L., Sosa-Peinado, A., and Hansberg, W. (2010) Arch. Biochem. Biophys. 500, 82–91.
Ferrer, J. C., Switala, J., Loewen, P. C. & Fita, I. (1996). J. Biol. Chem. 271, 8863–8868.
Goldberg, I. & Hochman, A. (1989). Biochem. Biophys. Acta, 991,330–336.
Jha, V., Louis, S., Chelikani, P., Carpena, X., Donald, L. J., Fita, I. & Loewen, P. (2011). Biochem. 50, 2101–2110.
Jha, V., Chelikani, P., Carpena, X., Fita, I. & Loewen, P. C. (2012). Arch. Biochem. Biophys. 526, 54–59.
Kalko, S. G., Gelpı´, J. L., Fita, I. & Orozco, M. (2001). J. Am. Chem. Soc. 123, 9665–9672.
Klotz & Loewen, (2003). Mol. Biol. Evol. 20, 1098–1112.
Maj, M., Loewen, P. C., Nicholls, P., (1998). Biochim. Biophys. Acta, 1384, 209–222.
Maté, M. J., Murshudov, G., Bravo, J., Melik-Adamyan, W., Loewen, P. C. & Fita, I. (2001). Encyclopedia of Inorganic and Bioinorganic Chemistry, edited by R. A. Scott, pp. 486–502. New York: John Wiley & Sons.
Maté, M. J., Zamocky, M., Nykyri, L. M., Herzog, C., Alzari, P. M., Betzel, C., Koller, F. & Fita, I. (1999). J. Mol. Biol. 286, 135–149.
Melik-Adamyan, W., Bravo, J., Carpena, X., Switala, J., Maté, M. J., Fita, I. & Loewen, P. C. (2001). Proteins, 44, 270–281.
Merle, P. L., Sabourault, C., Richier, S., Allemand, D. & Furla, P. (2007). Free Radic. Biol. Med. 42, 236–246.
Murshudov, G. N., Grebenko, A. I., Barynin, V., Dauter, Z., Wilson, K. S., Vainshtein, B. K., Melik-Adamyan, W., Bravo, J., Ferra´ n, J. M., Ferrer, J. C., Switala, J., Loewen, P. C. & Fita, I. (1996). J. Biol. Chem. 271, 8863–8868.
Nicholls, P., Fita, I. & Loewen, P. C. (2001). Adv. Inorg. Chem. 51,51–106.
Ogel, Z. B., Yuzugullu, Y., Mete, S., Bakir, U., Kaptan, Y., Sutay, D. & Demir, A. S. (2006). Appl. Microbiol. Biotechnol. 71, 853–862.
Olson, L. P. & Bruice, T. C. (1995). Biochemistry, 34, 7335–7347.
Sevinc, M. S., Ens, W. & Loewen, P. C. (1995). Eur. J. Biochem. 230, 127–132.
Sicking,W., Korth, H. G., de Groot, H. & Sustmann, R. (2008). J. Am. Chem. Soc. 130, 7345–7356.
Sutay Kocabas, D., Bakir, U., Phillips, S. E. V., McPherson, M. J. & Ogel, Z. B. (2008). Appl. Microbiol. Biotechnol. 79, 407–415.
Switala & Loewen, (2002). Switala, J. & Loewen, P. C. (2002). Arch. Biochem. Biophys. 401, 145–154.
Vetrano, A. M., Heck, D. E., Mariano, T. M., Mishin, V., Laskin, D. L. & Laskin, J. D. (2005). J. Biol. Chem. 280, 35372–35381.
Yuzugullu, Y., Trinh, C. H., Smith, M. A., Pearson, A. R., Phillips, S. E. V., Sutay Kocabas, D., Bakir, U., Ogel, Z. B. & McPherson, M. J. (2013). Acta Cryst. D69, 398–408.
Zámocký, M. & Koller, F. (1999). Prog. Biophys. Mol. Biol. 72, 19–66.