

# Inter- and intramolecular domain interactions of the catalase-peroxidase KatG from *M. tuberculosis*

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**Abstract** The inter- and intramolecular interactions between the different domains of the catalase-peroxidase KatG from *Mycobacterium tuberculosis* were analyzed using the two-hybrid assay. It was shown that the dimerization of the enzyme is due to a strong interaction of the first 99 amino acids of the N-terminal domain whereas the C-terminal domain does not play a role in the dimerization. In addition, an intramolecular interaction between the N- and C-terminal domains was detected which might play a functional role in the mechanism of the enzyme. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Bacterial catalase-peroxidase; *katG* gene product; Catalase HPI; Cytochrome *c* peroxidase; Two-hybrid assay; Isoniazid

## 1. Introduction

Catalase-peroxidases are an important class of enzymes involved in the response of bacteria and fungi to oxidative stress [1–4]. They belong to the superfamily of heme-containing bacterial, plant and fungal peroxidases [5]. The main activity of catalase-peroxidases is their catalase activity, with the catalase-peroxidase KatG from *Mycobacterium tuberculosis* possessing a  $k_{\text{cat}}$  of  $\sim 1 \times 10^4 \text{ s}^{-1}$  against hydrogen peroxide [6]. The catalase mechanism can be divided into two steps. First, the enzyme reacts with one molecule of hydrogen peroxide to yield an oxyferryl ( $\text{Fe}^{\text{IV}}=\text{O}$ ) porphyrin  $\pi$  cation radical which is called compound I [7]. In the second step, compound I reacts with another molecule of hydrogen peroxide, transforming the enzyme back to the resting state and liberating one equivalent of oxygen and water. Catalase-peroxidases differ from other catalases by possessing substantial peroxidase activity against a variety of substrates [3,6]. The reaction also passes through compound I as an intermediate, but is followed by two subsequent one-electron transfer reactions from two substrate molecules to the enzyme. However, the natural substrates are not yet characterized and the physiological significance of the peroxidase activity is not well understood.

Of particular interest among the different catalase-peroxi-

dases is KatG from *M. tuberculosis*, as it plays a key role in the mechanism of action of the tuberculosis drug isoniazid [8]. In short, oxidation of isoniazid by KatG transforms the drug into a nucleophilic radical which reacts with the cofactor  $\text{NAD}^+$ , yielding a potent inhibitor of the enoyl-ACP reductase InhA [9,10]. The inhibition of InhA affects the mycolic acid synthesis pathway of the mycobacterium [11]. Mutations in KatG are one of the major mechanisms of the mycobacterium to acquire isoniazid resistance [8,12]. In addition to its role in the mechanism of action of isoniazid, KatG from *M. tuberculosis* has been shown to be an important virulence factor, as KatG-deficient mycobacterial strains are susceptible to the oxidative stress imposed on them by the host organism [13–15].

Catalase-peroxidases have evolved by gene duplication from an ancestral peroxidase: Both halves of the catalase-peroxidases show sequence homology to each other and to other members of the superfamily of bacterial, plant and fungal peroxidases such as cytochrome *c* peroxidase (CCP) (Fig. 1) [5]. However, the sequence alignments indicate that only the N-terminal domain of the enzyme harbors a functional heme binding site, as a number of the amino acids involved in heme binding and important for catalysis are mutated in the C-terminal domain [5]. In addition, catalase-peroxidases are in general dimers or tetramers, although monomeric forms have been reported [16,17]. Whether the oligomerization affects the activity of the enzymes is not known.

Investigations concerning mechanistic questions, drug resistance, the function of the individual domains and the role of the formation of multimers are extremely difficult since there is no detailed structural information available on KatG [18,19]. In order to learn more about the structure-function relationship of KatG from *M. tuberculosis*, we have investigated the interaction between the individual domains using the yeast two-hybrid assay [20]. The results show that the dimerization of KatG is largely mediated by the first 100 amino acids of the N-terminal domain. In addition, the measured intramolecular interaction between the N- and C-terminal domains might point to a regulatory function of the C-terminal domain.

## 2. Materials and methods

### 2.1. Reagents, media, plasmids, strain

Standard chemicals, yeast nitrogen base, 3-aminotriazole (3-AT) and amino acids were purchased from Fluka AG or Sigma-Aldrich AG. Enzymes for recombinant DNA works were purchased from MBI Fermentas or New England Biolabs. Zeocin was purchased from Cayla and used at a concentration of 100  $\mu\text{g/ml}$ . Antibodies

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**Abbreviations:** CCP, cytochrome *c* peroxidase; 3-AT, 3-aminotriazole; ONPG, *o*-nitrophenyl- $\beta$ -D-galactopyranoside

KatG	1	MPEQHPPITETTTGAASNGCPVVGHMKYPVEGGGNQDWWPNRLNLKVLHQNPV
		#S # S # # ## SS# **# # # * * * * * # *
CCP	1	TTPLVHVASVEKGRSYEDFQKVYNA-IALKLREDDEY--DNYIGYGPVLVRLAWITSGTWKDHNTGGSYGGTYRFKKEFNDPSN
KatG	55	ADPMGAADFYAEEVATIDVDALTRD-IEEVMTTSPQWVPADYGHYGPLFIEMAWIAAGTYRIHDGRGGAGGGMQRFAPLNSWPDN
KatG	425	ARYLGLPLVPKQTLWQDPVPAVSHDLVGEAEIASLKSQIRASGLTVSQLVSTAWAAASSFRGSDKRGANGGRIQLQPQVGWEVN
		# # *S#S # * ** SS* * # \$ *** # \$
CCP	84	AGLQ---NGFKFLEPIHKE-----FP-WISSGDLFSLGGVTAVQEMQGP-----KIPWRCGRVDTPEDTTPDN-----
KatG	139	ASLD---KARRLLWPVKKK-----YGKKLSWADLIVFAGNCALESMTGFK-----TFGFGFGRVDQWEVD-EVYWGKEATWLGD
KatG	510	DPDGLRKRKIVIRLTLEEIQESFNSAAPGNI-KVSFADLVVLGGCAAIEKAAKAAGHNITVPFTPGRTDASQEQTDE-----
		\$ ## # # # \$ ## * * * # \$###
CCP	142	-----GRLPDADKADYVTRTFQRLNMNDREVVVALM-GAIALGKTHLK-----
KatG	208	ERYSGKRDLLENPLAAVQMLIYVNPEAPNGNDPMAAAVDIRETFRMMAMNDVETAAALIVGGITFGKTHGAGPADLVGPEPEAAP
KatG	584	-----SFAVLEPKADGFRNYLGKG-NPLPAEYMMLDKANLLTSLAEMTVLVGGLRVLGANY-----
		# \$S # S* * S*S # #S# # #
CCP	184	-----NSGYEGPWAANNVFTNEFYLNLLNEDWKLEKNDANNEQWDS-----KSG
KatG	293	LEQMGLGWKSSYGTGTGKDAITSPGIEVWNTNTPTKWDNSFLEILYGYEWELTKSPAGAWQYTAKDG--AGAGTIPDPFGGPGRS
KatG	640	-----KRLPLGVFTEASESLTNDFFVNLLDMGITWEPSPADDGTQYQKD-GSG--KV-----
		## ## *# ## # # ## ## #
CCP	229	YMLPTDYSLIQDPKYLSIVKEYAND--QDKFFKDFSKAFEKLENGITFPKDAPSPFIKTLLEEQGL.
KatG	376	PTMLATDLRLVDPIYERITRRWLEH--PEELADEFAKAWYKLIHRDMG-PV
KatG	689	----KWTGSRVDLVFGSNSELRALVEVYGADDAQPKFVQDFVAADWKVMNLDLDR-FDVR.

Fig. 1. Sequence alignment of the N- and C-terminal domains of KatG from *M. tuberculosis* and CCP from *S. cerevisiae* according to Welinder [5]. The following residues important for heme binding and catalysis are boxed: Arg48, His52, His175 and Trp191 (numbering according to CCP). Sequence identities between CCP and both domains of KatG (\*), between CCP and the N-terminal domain (#) and between CCP and the C-terminal domain (\$) are highlighted.

were purchased from Invitrogen (anti-LexA antibody (R990-25) and anti-V5 antibody (R960-25)) and Sigma AG (anti-rabbit HRP antibody conjugate (A6154) and anti-mouse HRP antibody conjugate (A4416)). The plasmids pHybLex/Zeo (LexA, bait) and pYESTrp2 (B42, prey) were purchased from Invitrogen and used for the construction of fusion proteins. All two-hybrid experiments were done in *Saccharomyces cerevisiae* L40 (*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3)URA3::(8lexAop-lacZ)GAL4*).

## 2.2. Oligonucleotides

### 2.2.1. Primers used for sequencing and single colony PCR.

pHybLex/ZeoNEW\_forward, 5'-CAATAAAGTCGAACGTGTG-3'; pHybLex/Zeo\_reverse, 5'-GAGTCACTTTAAAAATTGTGATACAC-3'; pYESTrp2\_forward, 5'-GATGTTAACGATACCA-GCC-3'; pYESTrp2\_reverse, 5'-GCGTGAATGTAAGCGTGAC-3'.

2.2.2. Construction of fusion proteins. The following primers have been designed to possess either an *Eco*RI or a *Not*I restriction site. Numbering is according to the corresponding amino acids of KatG, restriction sites are marked in *italics* and stop codons are underlined.

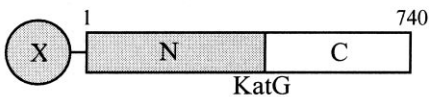
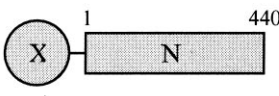
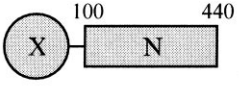
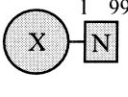
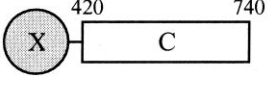
prey\_(N<sub>1</sub>)\_forward, 5'-GTTATTGAATTCAGATGCCCGAGCA-ACACCCAC-3'; prey\_(C<sub>420</sub>)\_forward, 5'-CGATACGAATTCCTG-GTCCCGTTGCGAGATACCTTG-3'; prey\_(N<sub>100</sub>)\_forward, 5'-GT-TATTGAATTCAGGCGCTGTTATCCGGATG-3'; bait\_(N<sub>1</sub>)\_forward, 5'-GTTATTGAATTCAGTCCCGAGCAACACCCAC-3'; bait\_(C<sub>420</sub>)\_forward, 5'-CGATACGAATTCGGTCCCGTTGCGAG-ATACCTTG-3'; bait\_(N<sub>100</sub>)\_forward, 5'-GTTATTGAATTCGCC-GCTGTTATCCGGATG-3'; bait/prey\_(C<sub>740</sub>)\_reverse, 5'-TTCA-TACGGGCGCTCAGCGCACGTCGAACCTGTC-3'; bait/prey\_(N<sub>440</sub>)\_reverse, 5'-TGAAACGCGCGCTCAATCCTGCCACAG-CAGGGTCTG-3'; bait/prey\_(N<sub>99</sub>)\_reverse, 5'-TGACACGCGGCC-GCTCACCGTAGTGGCGTAGTCGGCG-3'.

The desired KatG fragment was amplified from pET-15b containing *katG* of *M. tuberculosis* by PCR using Vent DNA-polymerase and the appropriate primers. The fragments containing the mutation Cys20Ser were amplified from the plasmid pKAT-Cys20Ser. The PCR products were digested with *Eco*RI and *Not*I and subsequently ligated into the vectors pHybLex/Zeo and/or pYESTrp2. After electroporation into *Escherichia coli* XL1-blue, the nature of all constructs was verified by DNA sequencing. In the resulting constructs, the fusion proteins carry a linker of 1 amino acid (bait) and 19 amino acids (prey), respectively.

## 2.3. Two-hybrid assay

*S. cerevisiae* L40 was transformed with the appropriate combinations of plasmids (Table 1) and grown on plates lacking tryptophan and uracil, but containing zeocin (100 μg/ml). The presence of the correct plasmids was verified by single colony PCR. The expression

Table 1  
Fusion proteins of KatG used in the two-hybrid assay

Fusion proteins	
	LexA-KatG B42-KatG
	LexA-N <sub>1-440</sub> LexA-S <sup>20</sup> N <sub>1-440</sub> B42-N <sub>1-440</sub> B42-S <sup>20</sup> N <sub>1-440</sub>
	LexA-N <sub>100-440</sub> B42-N <sub>100-440</sub>
	LexA-N <sub>1-99</sub> LexA-S <sup>20</sup> N <sub>1-99</sub> B42-N <sub>1-99</sub> B42-S <sup>20</sup> N <sub>1-99</sub>
	LexA-C <sub>420-740</sub> B42-C <sub>420-740</sub>

X indicates LexA and B42, respectively; numbering according to the amino acid sequence of KatG.

of each fusion protein after transformation of *S. cerevisiae* L40 with the appropriate plasmid was verified by Western blotting using a combination of anti-LexA antibody and anti-rabbit HRP antibody conjugate for the LexA fusion proteins and a combination of anti-V5 antibody and anti-mouse HRP antibody conjugate for the B42 fusion proteins, respectively.

Screening for interaction of fusion proteins was performed by plating transformed *S. cerevisiae* L40 onto plates lacking tryptophan, histidine, uracil and lysine, but containing zeocin (100 µg/ml) and 3-AT (5 µM). The pH of all plates was adjusted to 5.2.

A qualitative membrane colony lift assay for β-galactosidase activity was performed for the resulting growth-positives using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate [18]. β-Galactosidase activity was quantified using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) liquid culture assay [19]. One Miller unit of β-galactosidase as reported in Table 2 is defined as the amount which hydrolyzes 1 µmol of ONPG to *o*-nitrophenol and D-galactose per min per cell. All experiments have been done at least as triplicates, all interactions have been verified as double-positives (growth and β-galactosidase).

### 3. Results and discussion

#### 3.1. Construction of fusion proteins

An overview of the constructed fusion proteins is given in Table 1. The N- and C-terminal domains of KatG were both fused to LexA as well as B42. Based on the sequence alignment, amino acids 1–440 were chosen as the N-terminal domain, yielding fusion proteins LexA-N<sub>1–440</sub> and B42-N<sub>1–440</sub>. As C-terminal domain the amino acids 420–740 were selected, yielding fusion proteins LexA-C<sub>420–740</sub> and B42-C<sub>420–740</sub>. The two selected fragments are overlapping, as the sequence alignment does not allow to predict precisely the beginning and the end of the individual domains. Furthermore, KatG from *M. tuberculosis* possesses an N-terminal extension of about 50 residues compared to CCP (Fig. 1). In general, sequence identities among the first 100 residues of catalase-peroxidases with other members of the superfamily of bacterial, plant and fungal peroxidases are very low. In order to investigate the role of these first 99 residues of KatG, the N-terminal domain was further split into two different fragments, N<sub>1–99</sub> and N<sub>100–440</sub>, and fused to LexA and B42, yielding fragments LexA-N<sub>1–99</sub>, B42-N<sub>1–99</sub>, LexA-N<sub>100–440</sub> and B42-N<sub>100–440</sub>. It has been shown that Cys20 of KatG from *M. tuberculosis* is forming a disulfide bond in the dimeric structure, indicating that this region of the enzyme might be important for its dimerization [21]. To address the role of the disulfide bond in the dimerization, we also constructed fusion proteins carrying the muta-

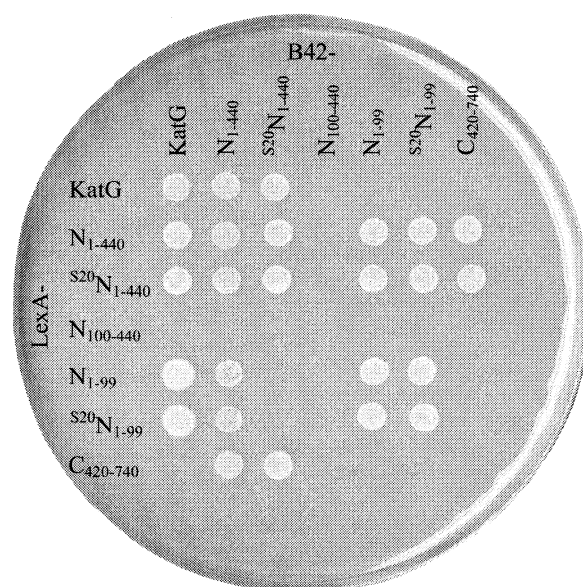


Fig. 2. Screening for interactions of LexA and B42 fusion proteins. Transformants were plated on plates lacking tryptophan, histidine, uracil and lysine, supplemented with zeocin (100 µg/ml) and 3-AT (5 mM) and incubated for 5 days. Furthermore, all positives possess β-galactosidase activity as verified by a colony lift assay [18].

tion Cys20Ser, i.e. LexA-S<sup>20</sup>N<sub>1–440</sub>, B42-S<sup>20</sup>N<sub>1–440</sub>, LexA-S<sup>20</sup>N<sub>1–99</sub> and B42-S<sup>20</sup>N<sub>1–99</sub>.

Combinations of prey and bait plasmids were transformed into *S. cerevisiae* L40 and screened for interactions of fusion proteins by growth on selective plates (Fig. 2). Expression of the fusion proteins was verified for each construct by Western blotting. Yeast cells expressing only the bait construct were His<sup>–</sup>. As a further control for non-specific interactions, all prey plasmids were co-transformed with a plasmid encoding a LexA-lamin fusion protein. No growth was observed on plates lacking histidine, indicating that the observed His<sup>+</sup> phenotypes in Fig. 2 were due to an interaction of KatG domains. To quantify the interactions, β-galactosidase activity was measured for suspensions of cells expressing pairs of interacting fragments using the ONPG liquid culture assay (Table 2).

#### 3.2. Interactions of the N- and C-terminal domains in the quaternary structure of KatG

The known dimerization of KatG was verified by the interaction of LexA-KatG and B42-KatG in our screening assay. This conclusion is based on the assumption that in full-length KatG intramolecular domain interactions will not give rise to intermolecular interactions.

Furthermore, the results obtained with fusion proteins containing the N-terminal domain or fragments thereof strongly suggest that the dimerization of KatG is mediated by the N-terminal domain. In particular, the strong interactions of N<sub>1–99</sub> with itself, N<sub>1–440</sub> and KatG suggest that the first 99 amino acids of KatG are responsible for the dimerization. The fragment N<sub>100–440</sub> does not show interactions to any other fragment or domain, indicating that it does not fold autonomously or is not involved in binding. Consequently, we can not rule out that N<sub>100–440</sub> also participates in the dimerization. The mutation Cys20Ser did not affect the dimerization of

Table 2  
Quantitative β-galactosidase assay using ONPG

LexA-	B42-	Miller units
KatG	KatG	2.0 ± 0.1
KatG	N <sub>1–440</sub>	6.2 ± 0.5
N <sub>1–440</sub>	KatG	7.5 ± 0.6
N <sub>1–440</sub>	C <sub>420–740</sub>	8.7 ± 0.5
N <sub>1–440</sub>	N <sub>1–440</sub>	17.0 ± 3.3
S <sup>20</sup> N <sub>1–440</sub>	S <sup>20</sup> N <sub>1–440</sub>	16.5 ± 1.9
N <sub>1–440</sub>	N <sub>1–99</sub>	9.7 ± 0.4
C <sub>420–740</sub>	N <sub>1–440</sub>	7.8 ± 0.4
N <sub>1–99</sub>	N <sub>1–440</sub>	124.5 ± 50.3
N <sub>1–99</sub>	KatG	437.2 ± 31.0
N <sub>1–99</sub>	N <sub>1–99</sub>	26.6 ± 1.2
S <sup>20</sup> N <sub>1–99</sub>	S <sup>20</sup> N <sub>1–99</sub>	24.8 ± 0.4
C <sub>420–740</sub> <sup>a</sup>	C <sub>420–740</sub>	0.02 ± 0.01

<sup>a</sup>Was used for determination of background activity.

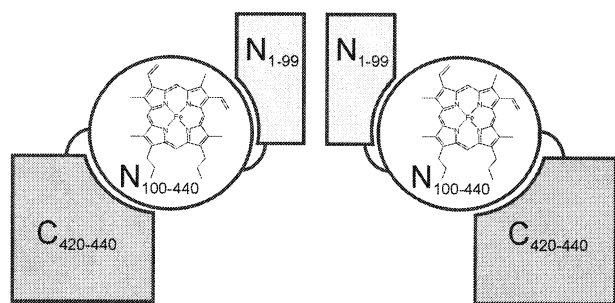


Fig. 3. Proposed domain interactions within KatG of *M. tuberculosis*.

either  $N_{1-99}$  and  $N_{1-440}$  with itself, respectively. The  $\beta$ -galactosidase activities of the mutants were not significantly different from wild-type (Table 2). The interpretation of the data is hampered by the fact that we do not know if and to what extent the disulfide bridge of the wild-type is formed in the nucleus of yeast. However, the detection of interactions essentially dependent on the formation of disulfide bridges by the two-hybrid assay has been reported [22]. In any case, the data are in agreement with earlier observations that disulfide bonds are not a prerequisite for the formation of dimeric KatG.

No interactions among C-terminal domains were detected, indicating that they do not participate in the dimerization of the enzyme. In contrast, there is an interaction between the C-terminal domain  $C_{420-740}$  and the complete N-terminal domain  $N_{1-440}$ . This interaction apparently does not involve the first 99 residues of the N-terminal domain, as an interaction between  $C_{420-740}$  and  $N_{1-99}$  was not detected. Since no interaction between  $C_{420-740}$  and  $N_{100-440}$  was measured by this assay, we have to assume that the corresponding binding site for  $C_{420-740}$  is either composed of  $N_{1-99}$  and  $N_{100-440}$  or that the binding site is only formed within  $N_{100-440}$  upon contact with  $N_{1-99}$ , i.e. that  $N_{100-440}$  does not fold autonomously (see above). Interestingly, no interaction between  $C_{420-740}$  and KatG can be detected, indicating that in KatG the binding site for  $C_{420-740}$  is already blocked by the C-terminal domain of KatG. We therefore conclude that the observed interaction between  $C_{420-740}$  and  $N_{1-440}$  corresponds to an intramolecular interaction in KatG. These results provide for the first time direct evidence for an intramolecular interaction between the N- and the C-terminal domains of KatG.

It should be noted that not all interactions are observed in both orientations of the two-hybrid system (Fig. 2) and that different orientations of the same interaction partners can result in significantly different transcription levels (Table 2). Similar effects have been previously reported in yeast two-hybrid assays and an explanation for this observation has been suggested. In general, the two-hybrid system works best if the fusion protein with the activation domain is present in excess over the fusion protein with the DNA binding domain [24]. Consequently, if protein fragments with different stabilities will be examined, different orientations will give rise to significantly different transcription levels.

The observed interactions and the resulting conclusions concerning the quaternary structure of KatG from *M. tuberculosis* are summarized in Figs. 2 and 3. An important question emerging from these studies is if the interactions between the N- and C-terminal domains affect the catalytic properties of the enzyme. It has been noted earlier that the deletion of

the C-terminal domain results in inactive enzyme [2]. However, it was not clarified if this is due to a resulting instability of the truncated enzyme or due to the removal of an interaction essential for activity. Attempts in our laboratory to express and purify sufficient amounts of the isolated domains were unsuccessful so far, indicating that the isolated domains are less stable than in KatG. Preliminary experiments on the catalase-peroxidase CpeB from *Streptomyces reticuli* suggest that deletion of 195 amino acids from the C-terminal domain affects the catalase and the manganese-peroxidase activity to different extents [23]. However, more data are needed to confirm this conclusion.

The functional role of the C-terminal domain in KatG thus remains unclear. Although the C-terminal domain has lost its ability to bind heme, it appears reasonable that it functions as a redox-active domain and that this function is related to that of the N-terminal domain. It appears also possible that the detected intramolecular interaction between the N- and C-terminal domains might play a functional role in the mechanism of the enzyme. Fine-mapping the interaction between the N- and the C-terminal domains using the two-hybrid system and the kinetic characterization of mutants with altered interactions between the two domains might yield further insight into the structure–function relationship of this puzzling class of enzymes.

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