Role of the Main Access Channel of Catalase-Peroxidase in Catalysis*

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Catalase-peroxidases (KatG) are bifunctional heme peroxidases with an overwhelming catalatic activity. The structures show that the buried heme b is connected to the exterior of the enzyme by a main channel built up by KatG-specific loops named large loop LL1 and LL2, the former containing the highly conserved sequence Met-Gly-Leu-Ile-Tyr-Val-Asn-Pro-Glu-Gly. LL1 residues Ile248, Asn251, Pro252, and Glu253 of KatG from Synechocystis are the focus of this study because of their exposure to the solute matrix of the access channel. In particular, the I248F, N251L, P252A, E253Q, and E253D mutants have been analyzed by UV-visible and resonance Raman spectroscopies in combination with steady-state and pre-steady-state kinetic analyses. Exchange of these residues did not alter the kinetics of cyanide binding or the overall peroxidase activity. Moreover, the kinetics of compound I formation and reduction by one-electron donors was similar in the variants and the wild-type enzyme. However, the turnover numbers of the catalase activity of I248F, N251L, P252A, E253Q, and E253D were only 12.3, 32.6, 25, and 42% of the wild-type activity, respectively. These findings demonstrate that the oxidation reaction of hydrogen peroxide (not its reduction) was affected by these mutations. The altered kinetics allowed us to monitor the spectral features of the dominating redox intermediate of E253Q in the catalase cycle. Resonance Raman data and structural analysis demonstrated the existence of a very rigid and ordered structure built up by the interactions of these residues with distal side and also (via LL1) proximal side amino acids, with the heme itself, and with the solute matrix in the channel. The role of Glu253 and the other investigated channel residues in maintaining an ordered matrix of oriented water dipoles, which guides hydrogen peroxide to its site of oxidation, is discussed.

Catalase-peroxidases (KatG) are present in prokaryotes and fungi. These bifunctional enzymes have a predominant catalase activity together with a substantial peroxidatic activity with broad specificity. KatG enzymes are the only heme peroxidases that exhibit a catalatic activity comparable with that of the classical monofunctional heme catalases. On the basis of sequence similarity, KatG enzymes have been recognized as part of the class I superfamily of plant, fungal, and bacte-rial peroxidases (1). Moreover, the four available crystal structures of KatG enzymes from Halobacula marismortui (Protein Data Bank code 1ITK), Burkholderia pseudomallei (code 1MWY), Mycobacterium tuberculosis (code 1SIF), and Synechococcus PCC 7942 (code 1UB2) (2–5) revealed that the heme pocket contains catalytic residues virtually identical to those of other peroxidases belonging to class I, such as cytochrome c peroxidase and ascorbate peroxidase. In particular, the distal and proximal heme pockets contain the amino acid triads His-Arg-Asp and His-Trp-Asp, respectively. Moreover, features unique to catalase-peroxidases were found. (a) Compared with cytochrome c peroxidase or ascorbate peroxidase, they are double in length, with the N-terminal half, which includes the heme b-binding site, being more conserved. This has been ascribed to gene duplication (6). (b) They have an unusual covalent adduct consisting of the distal side tryptophan, tyrosine, and methionine (Trp212, Tyr249, and Met275 in Synechocystis numbering) (2–5). (c) They have one short stretch and three large loop (LL)4 insertions (LL1–LL3) that are not found in either ascorbate peroxidase or cytochrome c peroxidase (2). (d) They have a more deeply buried active site, and the proposed access route for H2O2 is provided by a channel that is similar but longer and more constricted than that in the other heme peroxidases (3). The constriiction of the access channel results from the LL1 and LL2 insertions, as the former is positioned at one edge of the heme between helices D and E (see Fig. 1A). It also connects the distal and proximal catalytic domains. The LL1 insertion is of particular interest, as it contains a number of residues neighboring the covalently linked tyrosine (Tyr249 in Synechocystis numbering) that are highly conserved in KatG enzymes, viz. Ile248, Asn251, Pro252, and Glu253 (see Fig. 1B). This latter residue creates an acidic entrance to the channel, which is characterized by a pronounced funnel shape and a continuum of water (2–5). After passing the narrowest part of the channel, corresponding to the two KatG-specific residues Asp152 and Ser335, substrates come immediately into contact with the distal active-site residues Arg119, His123, and Trp122. Recently, it has been demonstrated that Asp152 is hydrogen-bonded to the LL1 residue Ile248 (7). This hydrogen bond is important for the stability of the heme architecture, and its substitution markedly changes the proximal His–Asp hydrogen bond interaction (7), underlining the role of the LL1 insertion in linking the distal and proximal heme sites. Moreover, Asp152 has been shown to be essential for the catalase (but not peroxidase) activity of KatG enzymes (8).

To date, only two KatG-specific amino acids of the substrate channel have been studied in detail, viz. Asp152 of Synechocystis (8) and Ser335 of M. tuberculosis KatG (9, 10), which constitute the narrowest part of the

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4 The abbreviations used are: LL, large loop; RR, resonance Raman; MES, 2-2-(N-morpholino)ethanesulfonic acid; WT, wild-type; 5-c, five-coordinate; 6-c, six-coordinate; HS, high-spin; LS, low-spin; mW, milliwatt.
access channel. Exchange of Asp\textsuperscript{152} significantly decreases the catalatic activity (8), whereas exchange of Ser\textsuperscript{315} in \textit{M. tuberculosis} KatG only moderately reduces the rate of oxygen release from H\textsubscript{2}O\textsubscript{2} (9, 10).

Recent studies have unequivocally demonstrated that the catalase (but not peroxidase) activity is very susceptible to exchange of amino acids at both the distal and proximal heme sites (8, 11–15). These findings suggest a correlation between the loss of catalase activity and the disruption of the particularly extensive hydrogen-bonding network typical of KatG (7, 15) as well as the formation and stability of protein radicals (14, 15). Therefore, to establish the role of the main access channel in KatG catalysis, we have extended the study to four conserved amino acids belonging to LL1 of \textit{Synechocystis} KatG, viz. Ile\textsuperscript{248}, Pro\textsuperscript{252}, Asn\textsuperscript{251}, and Glu\textsuperscript{253}, the latter three being on the surface of this channel (Fig. 1). In particular, the I248F, N251L, P252A, E253Q, and E253D variants were investigated by combining pre-steady-state and steady-state kinetics with electronic absorption and resonance Raman (RR) spectroscopies.

**MATERIALS AND METHODS**

**Reagents**—Standard chemicals and biochemicals were obtained from Sigma at the highest grade available.

**Mutagenesis**—A pET-3a expression vector containing the cloned catalase-peroxidase gene from the cyanobacterium \textit{Synechocystis} PCC 6803 (16) was used as the template for PCR. Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described previously (17). Unique restriction sites flanking the region to be mutated were selected. The flanking primers were 5’\text{/H11032}-AAT GAT CAG GTA CCG GCC AGT AAA TG-3’ (containing a KpnI restriction site) and 5’\text{/H11032}-AGT GCA GAC TAG TTC GGA AAC G-3’ (containing an SpeI restriction site). The internal 5’\text{/H11032}-primer was 5’\text{/H11032}-GAG GGG GTC GAC GGG CAC CCG GAT C-3’ and possessed an SalI restriction site. The following mutant primers with the desired mutation and a silent mutation introducing the SalI restriction site were constructed (with point mutations in italics and restriction sites underlined): 5’\text{/H11032}-GCC CGT CGA CCC CCT CAG GGT TAA CGT AAA TTA ATC CCA TTT G-3’ changed Pro\textsuperscript{252} to Ala, 5’\text{/H11032}-GCC CGT CGA CCC CCT CAG GGT TAA CGT AAA TTA ATC CCA TTG G-3’ changed Ile\textsuperscript{248} to Phe, 5’\text{/H11032}-GCC CGT CGA CCC CCT CAG GGT TAA CGT AAA TTA ATC CCA TTG G-3’ changed Asn\textsuperscript{251} to Leu, and 5’\text{/H11032}-GCC CGT CGA CCC CCT CAG GGT TAA CGT AAA TTA ATC CCA TTG G-3’ changed Glu\textsuperscript{253} to Gln. To substitute Glu\textsuperscript{253} with Asp, the following internal primers containing an AccIII restriction site were used: 5’\text{/H11032}-TAA TCC GGA CGG GGT GGA TGG-3’ and 5’\text{/H11032}-CGT CGG (G/A) T TAA CGT AAA TTA ATC CC-3’. The fragment defined by the KpnI and SpeI restriction sites was replaced with the new construct containing the point mutation. All constructs were sequenced to verify DNA changes by thermal cycle sequencing.

**Electronic Absorption**—Absorption spectra were measured with a Cary 5 spectrophotometer at room temperature. Absorption spectra were measured prior to and after Raman experiments. No degradation was observed under the experimental conditions used. Kinetic measurements were performed using a Zeiss Specord S10 diode array spec-
Substrate Channel in Catalase-Peroxidase

trophotometer and a Hitachi U-3000 spectrophotometer equipped with a thermostatted cell holder.

Circular Dichroism—CD studies were carried out using a Jasco J-600 spectropolarimeter. Far-UV (190–260 nm) experiments were carried out using a protein concentration of 1.5 μM and a cuvette with a 1-mm path length. A good signal-to-noise ratio in the CD spectra was obtained by averaging 12 scans (resolution, 1 nm; bandwidth, 1 nm; response, 2 s; and scan speed, 10 nm/min). The protein concentration was calculated from the known amino acid composition and absorption at 280 nm according to Gill and Hipel (18).

Resonance Raman Spectroscopy—The RR spectra of the ferric and ferrous forms were obtained with excitation from the 406.7-nm line of a Kr⁺ laser (Coherent Innova 302) and the 441.6-nm line of a helium/cadmium laser (Kimmon IK4121R-G), respectively. To minimize the local heating of the sample, a gentle flux of N₂ passed through liquid N₂ was used. The sample was cooled to a temperature of ~15 °C. The backscattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034A). Polarized spectra were obtained by inserting a Polaroid analyzer between the sample and the entrance slit of the monochromator. The depolarization ratios ($\rho = I_\perp/I_\parallel$) of the bands at 314 and 460 cm$^{-1}$ of CCl₄ were measured to check the reliability of the polarization measurements. The values obtained (0.73 and 0.00, respectively) compare well with the theoretical values of 0.75 and 0.00, respectively.

The RR spectra were calibrated with indene and CCl₄ as standards to an accuracy of 1 cm$^{-1}$ for intense isolated bands. In the figures, the relative intensities of the high- and low-frequency RR bands of the ferric form were normalized on the $v_4$ and $v_3$ bands at 1373 and 679 cm$^{-1}$, respectively. In the case of the ferrous form, the low-frequency region was normalized on the $v_5$ band at 347 cm$^{-1}$. Peak intensities and positions of the $\pi(=\pi)$ vinyl stretches in the 1600–1650 cm$^{-1}$ frequency region of the RR spectra of ferric KatG enzymes and mutants were determined by a curve-fitting program (Lab Calc, Galactic Industries Corp.) (TABLE ONE).

The following buffers were used: 100 mM MES and 100 mM citric acid at pH 6.0, 100 mM KH₂PO₄ and 100 mM citric acid at pH 6.5, 100 mM sodium phosphate at pH 7.0 and 7.5, and 100 mM Tris-HCl at pH 8.0. The ferrous form of the protein was obtained by adding a small volume (2–5 μl) of fresh sodium dithionite solution (10–20 g/liter) to a deoxygenated protein solution. The sample concentrations were ~80–150 μM for electronic absorption spectra and ~30–80 μM for RR experiments.

Steady-state Kinetics—Catalase activity was determined polarographically in 50 mM phosphate buffer using a Clark-type electrode (YSI 5331 oxygen probe) inserted into a thermostatted water bath (YSI 5301B). All reactions were performed at 30 °C and started by addition of KatG. One unit of catalase is defined as the amount that decomposes 1 μmol of H₂O₂/min at pH 7 and 30 °C. To cover the pH range 4.0–9.0, 50 mM citrate/phosphate or 50 mM Tris-HCl buffer was used.

Peroxidase activity was monitored spectrophotometrically using 1 mM H₂O₂ and 5 mM guaiacol ($\epsilon_{26}_{0} = 26.6 \text{ mmol}^{-1} \text{ cm}^{-1}$) or 1 mM o-dianisidine ($\epsilon_{46}_{0} = 11.3 \text{ mmol}^{-1} \text{ cm}^{-1}$). Alternatively, 1 mM peroxyacetic acid and 5 mM guaiacol were used. One unit of peroxidase is defined as the amount that oxidizes 1 μmol of electron donor/min at pH 7 and 30 °C.

Transient-state Kinetics—Transient-state measurements were made using a Model SX.18MV stopped-flow spectrophotometer and a PInStar-180 circular dichroism spectrometer (Applied Photophysics Ltd.) equipped with a 1-cm observation cell thermostated at 15 °C. Calculation of pseudo first-order rate constants ($k_{\text{obs}}$) from experimental traces at the Soret maximum was performed with a SpectraKinetic work station (Version 4.38) interfaced to the instrument. The substrate concentrations were at least five times that of the enzyme to allow determination of pseudo first-order rate constants. Second-order rate constants were calculated from the slope of the linear plot of the pseudo first-order rate constants versus substrate concentration. To follow spectral transitions, a Model PD.1 photodiode array accessory (Applied Photophysics Ltd.) connected to the stopped-flow machine together with XScan diode array scanning software (Version 1.07) were utilized. The kinetics of oxidation of ferric catalase-peroxidase to compound I by peroxyacetic acid or hydrogen peroxide as well as the kinetics of cyanide binding to ferric KatG were followed in the single mixing mode. Catalase-peroxidase and the peroxides or cyanide were mixed to give a final concentration of 1 μM enzyme and 5–250 μM peroxide or 20–500 μM cyanide. The first data point was recorded 1.5 ms after mixing, and 2000 data points were accumulated. Sequential mixing stopped-flow analysis was used to measure compound I reduction by one-electron donors. In the first step, the enzyme was mixed with peroxyacetic acid, and after a defined delay time, the formed compound I was mixed with the electron donor aniline, ascorbate, or o-dianisidine. All stopped-flow determinations were made in 50 mM phosphate buffer at pH 7.0 and 15 °C, and at least three determinations were performed per substrate concentration.

RESULTS

Electronic Absorption and Resonance Raman Spectra of Ferric KatG Variants—The far-UV (190–250 nm) CD spectrum is a sensitive probe of protein secondary structure. The CD spectra of all variants (data not shown) were similar to that of wild-type KatG and showed the typical features of α-helical protein structure with the 222 and 208 nm dichroic bands. Therefore, mutations did not induce changes in the overall secondary structure, and if conformational changes did occur, they must be very localized and minimal.

Fig. 2 (A and B) compares the UV-visible and RR spectra of ferric KatG and its variants at pH 7.0. The UV-visible absorption spectra of recombinant wild-type KatG and the four variants exhibited the typical bands of heme b-containing peroxidases in the visible and near-UV region. It was recently shown that, in the UV-visible spectrum of wild-type (WT) KatG, a Soret band at 407 nm, Q-bands at 502 and 542 nm, and a CT1 band (long wavelength (>600 nm) porphyrin-to-metal charge transfer band) at 637 nm are indicative of a five-coordinate (5-c) high-spin (HS) heme coexisting with a six-coordinate (6-c) HS heme and a 6-c low-spin (LS) heme (19). The RR spectrum in the high-frequency region confirmed that the predominant form is a 5-c HS heme ($v_5$ at 1493 cm$^{-1}$, $v_{13}$ at 1551 cm$^{-1}$, $v_4$ at 1571 cm$^{-1}$, $v_{37}$ at 1594 cm$^{-1}$, and $v_{56}$ at 1631 cm$^{-1}$), coexisting with a 6-c LS heme ($v_5$ at 1501 cm$^{-1}$ and $v_{13}$ at 1639 cm$^{-1}$) and a 6-c HS heme ($v_5$ at 1485 cm$^{-1}$, $v_4$ at 1545 cm$^{-1}$, and $v_5$ at 1566 cm$^{-1}$). Furthermore, by means of polarized RR spectra, two distinct vinyl stretches have been identified at 1624 and 1629 cm$^{-1}$ (19). The different frequencies correspond to different torsion angles (π, i.e. the relative orientation of the vinyl $C_\alpha=C_\beta$, π-bond with respect to one of the two pyrrole $C_\alpha=C_\beta$ π-bonds) of the 2-vinyl...
group with respect to the 4-vinyl group (20) resulting from interaction with the protein matrix.

The UV-visible and RR spectra of E253Q and E253D were identical to those of the WT protein (data not shown). The replacement of Glu253 affected neither the spin state of the iron atom nor the vinyl stretches. On the contrary, mutation of Pro252, Asn251, and Ile248 led to the conversion of the 6-c HS form to both 5-c HS and 6-c LS hemes. The replacement had minor effects in the P252A mutant, but changes were progressively more evident in the order P252A, N251L, and I248F. The substitution of Pro252 with Ala only slightly affected the coordination of the iron atom. The maximum of the Soret band did not change with respect to WT KatG. However, the amount of the LS form increased, as indicated by the red shift in the Q-band to 507 nm and by the intensity increase of the band at 542 nm. Moreover, the increase of the shoulder at 380 nm and the red shift of the CT1 band to 643 nm indicate that the mutation increased the 5-c HS form at the expense of the aquo 6-c species, as confirmed by the intensity decrease of the ν2 band at 1485 cm⁻¹ in the corresponding RR spectrum (Fig. 2B). The replacement of Asn251 with Leu gave rise to similar effects. However, the amount of the 6-c LS form was even larger, as shown by the red shift of the Q-band to 512 nm and the intensity increases of the 542 nm band (Fig. 2A) and of the RR bands at 1501 (ν5) and 1639 (ν6) cm⁻¹ (Fig. 2B). Finally, the substitution of Ile248 with Phe led to the disappearance of the 6-c HS heme with the concomitant increase of the 5-c HS heme. In fact, in the UV-visible spectrum, the shoulder at 380 nm markedly increased, and the CT1 band shifted to 643 nm. Accordingly, the RR spectrum showed core size marker bands of 5-c HS and 6-c LS forms only.

Because we observed an intensity variation in the 1600–1650 cm⁻¹ region in the N251L and I248F RR spectra compared with the WT protein spectrum (Fig. 2B), we collected polarized RR spectra (Fig. 3) to determine if and how the frequencies of the vinyl stretches differ from that of the WT protein. In fact, in this region, upon Soret excitation, the polarized bands due the ν(C=C) vinyl stretching modes overlap with the ν5 depolarized bands due to the non-totally symmetric modes (B5). The curve-fitting analysis (supported by measurement of the depolarization ratios, ρ = I⊥/I∥) showed that the two ν(C=C) vinyl stretching modes of the N251L mutant downshifted by 2–3 cm⁻¹ with respect to the WT protein (TABLE ONE). Therefore, it appears that, compared with the WT protein, the N251L mutation changed the vinyl group orientation with respect to the heme plane.

The conclusions drawn from the analysis of the UV-visible and RR core size marker bands were confirmed by the RR spectra in the low-frequency region (Fig. 4). The low-frequency region spectra of the E253Q and E253D mutants were identical to that of WT KatG. The spectra were analyzed following the previous assignment proposed for WT KatG (19) and the assignment of myoglobin by Hu et al. (30), which was based on isotopically labeled hemes. The band at 336 cm⁻¹ (assigned to the out-of-plane γCC mode) is active only in the 5-c HS form and is thus present in the spectra of all mutants. The band at 349 cm⁻¹ (ν5) in the spectrum of WT KatG was shifted to 347 cm⁻¹ in the spectra of P252A, N251L, and I248F. The band at 376 cm⁻¹ (assigned to the porphyrin propionate bending mode β(C2pC3p)) was relatively less intense in the spectra of P252A and N251L compared with that of WT KatG. The variation of the intensity of this band may be related to conformational changes of the propionate substituents. The band
The presence of a shoulder at 385 nm indicated the presence of free heme in the ferrous mutants. The high-frequency RR spectra of the mutants confirmed these data.

The low-frequency spectrum of a 5-c HS heme protein is characterized by the presence of a strong band due to the iron–imidazole stretching mode (ν(iron–imidazole)), which occurs in the 200–250 cm⁻¹ region. Its frequency correlates with the bond strength between the iron atom and the imidazole ring, and a shift of this band has been interpreted to derive from a change in the hydrogen-bonding status of the proton on the proximal imidazole. Therefore, in peroxidases, the low-frequency region RR spectrum gives important information on the strength of the hydrogen bond between the N⁸ atom of the imidazole ring of the axial ligand and the oxygen atom of the nearby aspartate carboxylate group (which is conserved in peroxidases from plants, fungi, and bacteria) (Ref. 21 and references therein). In WT KatG, a hydrogen bond is formed between the imidazole ring of the proximal ligand (His²⁹⁰) and the carboxylate group of Asp⁴⁰², which strengthens the bond between the heme iron and the proximal histidine. In the low-frequency spectrum of Synechocystis KatG, two iron–imidazole frequencies have been identified (7, 19): at pH 6.5, a band at 203 cm⁻¹, corresponding to a species in which the proton resides on the imidazole, and a band at ~251 cm⁻¹, corresponding to a species in which the proton is almost completely transferred to the carboxylate group. Upon mutation of Asp¹⁵² to Ser (D152S), an intense band at 237 cm⁻¹ (which became more intense at alkaline pH) grew at the expense of the band at 251 cm⁻¹. These two bands were assigned to two tautomers of the hydrogen bond between the His²⁹⁰ and Asp⁴⁰² carboxylate side chains: in one tautomer (237 cm⁻¹), the proton is closer to the imidazole, whereas in the other (251 cm⁻¹), the proton is almost transferred to the carboxylate group (7). As mentioned above, Asp¹⁵² is a KatG-specific residue at the distal heme site hydrogen-bonded to Ile²⁴⁸, which is part of LL1, which is connected to helices E and F, the latter accommodating the proximal histidine. Therefore, we concluded that the hydrogen bond between Asp¹⁵² and Ile²⁴⁸ was altered by mutation and that the variation of the hydrogen bond between Asp⁴⁰² and His²⁹⁰ caused a change in the ν(iron–imidazole) frequencies. Moreover, previous data indicate that displacement of LL1 may also give rise to changes in iron–imidazole bond strength (7). To confirm this conclusion, we studied the effect of the mutation of the residues of the substrate channel on the proximal side of the heme and, in particular, on the iron–imidazole bond strength.

At pH 6.5, the low-frequency RR spectra of E253Q and P252A were characterized by two ν(iron–imidazole) bands at the same frequencies as in the WT protein (203 and 251 cm⁻¹). Upon increasing the pH, no frequency shifts or intensity variations were observed (data not shown). On the contrary, the N251L and I248F variants exhibited marked changes in the low-frequency RR spectra (Fig. 5). A band at 237 cm⁻¹ (which overlapped with the porphyrin mode at 233 cm⁻¹) grew at the expense of the band at 251 cm⁻¹. This band (weak in the N251L spectrum) was clearly detected in the I248F spectrum at pH 6.0. Its intensity increased at alkaline pH at the expense of the band at 251 cm⁻¹. Therefore, it was assigned to ν(iron–imidazole) in analogy with the previous results on the D152S mutant (7).

**Bifunctional Activity**—The kinetic parameters for the catalase and peroxidase activities of WT KatG and the variants are presented in TABLE TWO. WT KatG exhibited an overwhelming catalase activity with an apparent kcat of 3500 s⁻¹ and a Km of 4.1 mM. Similar values were obtained for P252A; however, significant changes in the catalatic activities were observed for the other variants. The apparent turnover numbers (kcat) for E253D, N251L, E253Q, and I248F were 42, 33, 25, and 12%
of cyaniide. From the slope of the plots (data not shown), the apparent second-order rate constants for cyanide binding ($k_{on}$) were calculated. The binding rates for all variants were very similar to that for WT KatG ($4.8 \times 10^4$ M$^{-1}$ s$^{-1}$); I248F, $2.8 \times 10^4$ M$^{-1}$ s$^{-1}$; N251L, 4.5 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$; P252A, 7.8 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$; E253Q, 8.2 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$; and E253D, 3.4 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$.

Moreover, within experimental error, only minor variations were observed in the calculated dissociation constant ($15.8 \pm 3.1$ $\mu$M) for the corresponding cyanide complexes, i.e. $k_{off}/k_{on}$, with $k_{off}$ being the intercept of the plots of pseudo first-order rate constants against cyanide concentration. Only the dissociation constant for N251L was decreased by ~45%.

**Reaction of the Ferric Enzymes with Peroxoacetic Acid**—Because of the overwhelming catalatic activity in monofunctional catalases and WT catalase-peroxidases, the formation of a classical compound I (iron-oxoporphyrin $\pi$-cation radical species) mediated by H$_2$O$_2$ cannot be followed even by stopped-flow spectroscopy. As a consequence, organic peroxides (e.g. pero xoacetic acid) have to be used to monitor the formation of this type of compound I, which is typically characterized by a 40—50% hypochromicity in the Soret region. Addition of pero xoacetic acid to *Synechocystis* WT KatG results in the formation of compound I characterized by a 45% hypochromicity at 407 nm and the appearance of two prominent peaks around 600 and 650 nm (8, 12, 20, 28). Both P252A and E253Q gave a similar compound I spectrum when the ferric proteins were mixed with a 50-fold excess of pero xoacetic acid. In variants I248F, N251L, and E253D, the hypochromicity at the Soret band was less pronounced.

The reaction between WT KatG and pero xoacetic acid was monophasic and could be fitted using a single exponential equation. The obtained pseudo first-order rate constants plotted against the pero xoacetic acid concentration yielded a bimolecular rate constant of $(3.9 \pm 0.3) \times 10^9$ M$^{-1}$ s$^{-1}$ at pH 7.0. The reactions of variants I248F, P252A, E253Q, and E253D with pero xoacetic acid were also monophasic, and the corresponding rate constants were similar to that of WT KatG, ranging from $3.0 \times 10^8$ to $6.7 \times 10^8$ M$^{-1}$ s$^{-1}$ (TABLE THREE). Variant N251L was an exception because the reaction with pero xoacetic acid was biphasic. The corresponding time trace at 408 nm was fitted with a double exponential fit and yielded two rate constants: a first fast phase responsible for ~80% of the absorbance decrease at 408 nm and a bimolecular rate constant of $3.2 \times 10^4$ M$^{-1}$ s$^{-1}$, followed by a slower reaction with a rate constant of $3.5 \times 10^3$ M$^{-1}$ s$^{-1}$, very similar to that of WT KatG (TABLE THREE).

**Reaction of the Ferric Enzymes with Hydrogen Peroxide**—No spectral changes could be monitored when ferric WT KatG and I248F were mixed with a small excess of hydrogen peroxide (1—200 $\mu$M) in the stopped-flow apparatus. By contrast, upon adding a 50—100-fold excess of H$_2$O$_2$ to ferric P252A, N251L, and E253D, a 5—9% hypochromicity in the Soret region was observed, followed by a slow increase in absorbance at 406 nm back to the ferric protein (data not shown).

When E253Q was mixed with hydrogen peroxide, the spectral changes were interesting. Upon addition of a 5-fold excess of H$_2$O$_2$, only a re-increase in absorbance at 406 nm could be observed, whereas upon addition of higher hydrogen peroxide concentrations (50-fold excess and higher), a new spectroscopic intermediate accumulated with a red-shifted Soret band, the appearance of a broad peak around 521 nm, and a decrease in absorbance at 640 nm. It has to be noted that the formation of this intermediate was extremely fast and could not be followed by the stopped-flow technique. The first spectrum obtained after 1.3 ms depended on the amount of H$_2$O$_2$ added to ferric E253Q. Spectra monitored after 1.3 ms and obtained when 5 $\mu$M E253Q was mixed with various concentrations of hydrogen peroxide in the conventional
H₂O₂ had its Soret band at 418 nm. Not only the position of the Soret spectrum monitored after addition of a higher excess (1000-fold) of the formation a species with its Soret band at 412 nm, whereas the first peroxide (\(\text{mM} \)) are shown in Fig. 7.

A low excess (50-fold) of H₂O₂ immediately resulted in the monitored spectrum strongly depended on the amount of hydrogen peroxide added. A low excess (50-fold) of H₂O₂ immediately resulted in the formation a species with its Soret band at 412 nm, whereas the first peroxide (\(\text{mM} \)) are shown in Fig. 7.

FIGURE 6. pH dependence of the catalase activity determined polarographically. Black line, WT enzyme; gray line, E253Q. The conditions were as follows: 5 mM hydrogen peroxide, 50 mM citrate/phosphate, or 50 mM Tris-HCl buffer and 30 °C.

The spectrum monitored 1.3 ms after mixing still resembled that of conventional compound I. The subsequent spectra taken at 11 and 42 ms (bold lines) indicate a clear transition with isosbestic points detected at 399, 476, and 548 nm. The redox intermediate formed with absorbance maxima at 418 and 521 nm (thus identical to that described above) was stable for ~500 ms, and after the complete depletion of hydrogen peroxide, it was transformed again to the conventional compound I because of the excess of peroxoacetic acid still present in the assay (Fig. 8D).

Based on these interesting findings, we returned to WT KatG compound I and incubated it with millimolar concentrations of hydrogen peroxide. Fig. 9A shows what happened when 3 mM WT compound I (preformed with 100 mM peroxoacetic acid) was mixed with 6 mM hydrogen peroxide in the sequential stopped-flow mode. Again, the spectrum taken at 1.3 ms after mixing resembled that of conventional compound I, and a redox intermediate with peak maxima at 418 and 521 nm was formed within 40 ms and disappeared after 700 ms, whereas with E253Q, it disappeared after 1.9 s (Fig. 9B). In both cases, the kinetics corresponded to that of H₂O₂ dehydration (data not shown) and very well reflected the differences in the overall catalytic activity (TABLE TWO).

Finally, we probed the reactivity of this redox intermediate with cyanide and the one-electron donors ascorbate (Fig. 10) and tyrosine (data not shown). In each case, the intermediate was formed by mixing 3 mM E253Q with 5 mM hydrogen peroxide, and after a delay time of 100 ms, cyanide (200 mM), ascorbate (2 mM), or tyrosine (1 mM) was added. Upon addition of cyanide, the LS complex was formed (Fig. 10A), whereas upon addition of the one-electron donors, the ferric enzyme was formed (Fig. 10B). However, detailed data analysis revealed an important difference between these two reactions in Fig. 10C, the time...
traces for the reaction with 200 μM cyanide followed both at 422 nm (formation of the LS complex) and at 521 nm (disappearance of the intermediate) are shown. Both the formation of the LS complex and the decay of the intermediate started immediately after mixing. By contrast, upon addition of ascorbate or tyrosine within the first 500 ms (corresponding to the time needed for H₂O₂ depletion), no absorbance changes were observed. After this period, the intermediate was directly transformed to the ferric protein (see time traces at 521 and 407 nm in FIGURE 7.

FIGURE 7. Reaction of ferric E253Q with hydrogen peroxide. A, absorption spectra of 5 μM ferric enzyme (thick gray line) and the first detectable spectra (after 1.3 ms) in the reaction with 25 μM (thin dark gray line), 250 μM (thin light gray line), 1 mM (thin black line), and 5 mM (thick black line) hydrogen peroxide. The conditions were as follows: 50 mM phosphate buffer at 25 °C. B, time traces at 521 nm of the reactions in A. C, reaction of 5 μM ferric E253Q with 5 mM hydrogen peroxide. The reaction was recorded at 240 nm (black line) and at 521 nm (gray line). Conditions were as described for A. Abs, absorbance.

FIGURE 8. A, absorption spectra of ferric E253Q (thin line) and compound I (thick line). The conditions were as follows: 2 μM E253Q, 200 μM peroxoacetic acid, and 50 mM phosphate buffer (final concentrations) at pH 7.0 and 25 °C. B, time trace and single exponential fit of the reaction of 1 μM E253Q with 50 μM peroxoacetic acid. The inset depicts the obtained pseudo first-order rate constants plotted against peroxoacetic acid (POA). The conditions were as follows: 1 μM E253Q, 25-200 μM peroxoacetic acid, and 50 mM phosphate buffer at pH 7.0 and 15 °C. C, reaction of preformed E253Q compound I with hydrogen peroxide. Compound I was formed by mixing 3 μM ferric E253Q with 50 mM hydrogen peroxide. The reaction was recorded at 240 nm (black line) and at 521 nm (gray line). Conditions were as described for A. Abs, absorbance.

Substrate Channel in Catalase-Peroxidase

TABLE THREE

Bimolecular rate constants of compound I formation and reduction for wild-type Synechocystis KatG and variants I248F, N251L, P252A, and E253Q

|                      | WT        | I248F     | N251L                 | P252A                  | E253Q                  | E253D                  |
|----------------------|-----------|-----------|-----------------------|------------------------|------------------------|------------------------|
| Compound I formation |           |           |                       |                        |                        |                        |
| Hydrogen peroxide    | ND        | ND        | ND                    | ND                     | ND                     | ND                     |
| Peroxoacetic acid    | 3.9 × 10⁴ M⁻¹ s⁻¹ | 6.7 × 10⁴ M⁻¹ s⁻¹ | 31.6 × 10⁴ M⁻¹ s⁻¹ | 5.7 × 10⁴ M⁻¹ s⁻¹ | 3.0 × 10⁴ M⁻¹ s⁻¹ | 4.7 × 10⁴ M⁻¹ s⁻¹ |
| mCPB                 | 5.3 × 10⁴ M⁻¹ s⁻¹ | 74.5 × 10⁴ M⁻¹ s⁻¹ | 43.2 × 10⁴ M⁻¹ s⁻¹ | 21.9 × 10⁴ M⁻¹ s⁻¹ | 4.7 × 10⁴ M⁻¹ s⁻¹ | 12.8 × 10⁴ M⁻¹ s⁻¹ |
| Compound I reduction |           |           |                       |                        |                        |                        |
| Ascorbate            | 0.7       | 1.8       | 0.6                   | 0.8                    | 0.6                    | 0.8                    |
| α-Dianisidine        | 271       | 297       | 564                   | 105                    | 476                    | 476                    |
| Aniline              | 1.4       | 9.7       | 1.5                   | 1.2                    | 0.5                    | 1.6                    |

The conditions were as follows: 50 mM phosphate buffer at pH 7 and 15 °C. Compound I was formed with peroxoacetic acid. For details, see “Materials and Methods.” ND, not detectable; mCPB, m-chloroperbenzoic acid.
compound I was formed in the reaction of 3 μM ferric KatG with 100 μM peroxoacetic acid. The conditions were as follows: 50 mM phosphate buffer at pH 7.0 and 25 °C. Spectra were recorded 1.2, 9, and 37 ms (thick line) after adding 6 mM hydrogen peroxide. B, time traces at 427 nm for the reactions of 3 μM preformed WT (thick line) and E253Q (thin line) compound I with 6 mM hydrogen peroxide. The conditions were as follows: 50 mM phosphate buffer at pH 7.0 and 25 °C. Absorbance.

Reduction of Conventional Compound I with One-electron Donors—In catalase-peroxidases, conventional compound I formed with peroxoacetic acid can be reduced by various one-electron donors in two steps via compound II back to the ferric enzyme. In contrast to the other peroxidases, in which compound II exhibits a red-shifted Soret band, in WT KatG, the Soret band of compound II is very similar to that of the resting state (12–14). This was also the case in I248F, N251L, P252A, E253D, and E253Q. The reaction of one-electron donors with compound I was biphasic, with the fast phase suggested to represent compound II formation. The variants investigated in this study exhibited similar rate constants for the reduction of compound I (TABLE THREE). Also the hierarchy of the rate constants of the different substrates was the same as in WT KatG, with o-dianisidine being the fastest, followed by aniline and finally ascorbate. Only in N251L was compound I reduction with o-dianisidine too fast to determine an accurate rate constant, which fits well with the higher overall peroxidase activity with this one-electron donor.

**DISCUSSION**

The structures of catalase-peroxidases from four different sources have been reported (2–5), revealing several KatG-specific peculiarities that distinguish these enzymes from other homologous peroxidases. The buried heme b is connected to the exterior of the enzyme by a main channel that could provide the route for peroxide ingress and product egress. A second access route normally found in peroxidases approximately in the plane of the heme is blocked by loops in KatG enzymes (2–5). Another potential route that provides access to the core of the protein between the two domains of the subunits has been described, and it was speculated that this could be the binding site for substrates with extended, possibly even polymeric character (3). The *in vivo* peroxidatic substrate of KatG enzymes is still unknown.

In this work, we have focused on the highly conserved residues that are situated in the main channel of all catalase-peroxidases that have been sequenced (Figs. 1B and 11). They all belong to the KatG-specific insertion loop LL1, which is positioned at one edge of the heme and connects the distal and proximal catalytic domains. Closer inspection of this loop revealed several interesting connections to the distal heme site and to the main access channel. First, the distal tyrosine (Tyr249 in *Synechocystis* numbering), which is part of the peculiar Trp-Tyr-Met adduct found in all crystal structures, is part of this loop. Second, the neighboring LL1 amino acid isoleucine (Ile248) hydrogen bonds to a highly conserved aspartate (Asp152), which, together with serine (Ser335), forms the narrowest part of the funnel-shaped main channel. It is noteworthy that the neighboring amino acid of Asp152 is Asn153, which is found in all peroxidases and hydrogen bonds to the distal histidine, thereby controlling its basicity (7). Third, the conserved LL1 amino acid glutamate (Glu253) located at the entrance of this access channel provides a negative charge. This is very similar to monofunc-
Substrate Channel in Catalase-Peroxidase

The RR data underline the existence of a very rigid and ordered structure built up by interactions of LL1 residues with both distal and proximal residues and the heme itself. Exchange of Ile248 with Phe significantly altered the heme pocket. The mutation induced an increase of the 5-c HS form at the expense of the 6-c HS form. Because this effect was similar to that previously observed for the D152S variant (7), it can be ascribed to the alteration of the hydrogen bonding between Ile248 and Asp152 and, as a consequence, to a change in the position of Asp152. This may cause the rupture of the hydrogen bonds between Asp152 and three water molecules in the distal cavity, which probably leave the cavity (Fig. 11). This disruption significantly reduced the catalase activity by 87%, which is very similar to the recently described effect of exchanging Asp152 with Ser (96%) (8).

The mutation of Ile248 also had a significant effect on the proximal heme site as revealed by analysis of the low-frequency RR spectra of the ferrous forms. This confirms that perturbation of the hydrogen bond between Asp152 and Ile248 leads to changes at the proximal heme site mediated by LL1 and its connection with helices E and F, with helix F accommodating the proximal histidine. Recently, we reported that exchange of Asn153 gives rise to similar but less extensive effects on both the coordination state of the ferric atom and the proximal iron–imidazole bond strength (7). The changes on the proximal side observed for I248F are less evident if compared with the D152S variant (7). One reason might derive from the fact that replacement of Ile248 with Phe could possibly alter the hydrogen bond between the carboxylate group of Asp152 and the nitrogen atom of Ile248 as a consequence of the steric hindrance of the encumbered phenylalanine residue. Nevertheless, we cannot rule out that the more pronounced alteration of the proximal iron–imidazole bond strength induced by the D152S variant is also determined by a concomitant change of the hydrogen bond interaction between the neighboring Asn153 and His123. In fact, we have previously observed that mutation of the distal Asn153 residue has selective marked effects on the proximal side; the rupture of the hydrogen bond between Asn153 and His123 in the N153A variant relaxes the protein structure and weakens the hydrogen bond between the proximal His129 and Asp402 residues (7).

Mutation of Asn251 and Pro252 also affected the coordination of the iron atom, although the variations were less pronounced. By contrast, both E253Q and E253D exhibited RR spectral features very similar to those of WT KatG in both the ferric and ferrous states. Glu253 is located at the entrance of the funnel-shaped access channel (Fig. 11A) and is ~9 Å from the conformation formed by Asp152 and Ser335 (Fig. 11B). Although its exchange did not alter the spin and coordination states of the heme iron, it significantly altered the bifunctionality of the enzyme. In E253Q, the catalase activity was reduced by 75%, whereas in E253D, the decrease was less pronounced (58%). In N251L, the $k_{cat}$ was reduced by 68%, whereas P252A exhibited the same catalase activity as the WT enzyme. A closer inspection of the published structures of Burkholderia and Mycobacterium KatG enzymes clearly shows that both Asn251 and Glu253 are connected by a hydrogen bond between the amide oxygen of Asn251 and the main chain nitrogen of Glu253 as well as via at least two water molecules in their vicinity (Fig. 11B). The hierarchy of the $k_{cat}$ values for E253Q, N251L, and E253D most probably reflects the impact of the corresponding mutations on the extent of changes and disruption of this hydrogen-bonding network. This assumption implies that this network is essential for the catalase activity of KatG enzymes. Indeed, we have shown here that, with the exception of P252A (which is definitely not involved in the direct stabilization of the water matrix), all variants exhibited a reduced catalase activity and an unaffected or even increased peroxidase activity with two different one-electron donors (TABLE TWO). Moreover, the kinetics of cyanide binding as well as compound I formation clearly indicated that the mutations altered neither heme accessibility nor the coordinates of the distal site histidine.

![FIGURE 11. A, surface structure of the main channel of KatG from B. pseudomallei. The amino acid numbering is for Burkholderia KatG, and the numbers in parentheses denote numbering for Synechocystis KatG. Yellow, Glu253 (Glu254); cyan, Asn251 (Asn253); purple, Pro252 (P252); blue, Ser335 (Ser337) and Asp152 (Asp154); green, heme. Water (W) molecules are numbered 1–13 and correspond to the following structural water molecules of Burkholderia. W1, position 3588; W2, position 2472; W3, position 3149; W4, position 2880; W5, position 3629; W6, position 4107; W7, position 3898; W8, position 3086; W9, position 3180; W10, position 2653; W11, position 2511; W12, position 3770; and W13, position 3138. B, detailed view of the main channel showing the residues investigated in this study, the amino acids (aspartate and serine) that form the narrowest part of the channel, and the interacting water molecules. W1–W9 and W11–W13 correspond to the positions in A. W14, position 3071; W15, position 2764. C, detailed view of the water molecules in the active site starting at the constriction of the channel. W16, position 3897; W17, position 3085; W18, position 2229.](image-url)
which is known to stabilize the cyanide complex in heme peroxidases and to promote heterolytic cleavage of \( \text{H}_2\text{O}_2 \) in compound I formation (23).

These findings suggest that the first reaction in the catalase cycle, viz. the two-electron oxidation of the ferric enzyme to the redox intermediate compound I (Reaction 1), was not affected by these mutations. However, they decelerated the hydrogen peroxide oxidation reaction, i.e. the formation and release of dioxygen. Thus, it is this reactivity that demands a defined and extended hydrogen bonding network that helps to guide the two-electron donor hydrogen peroxide through the access channel to the active site of KatG in its compound I state to deliver its electrons.

Compound I in Synechocystis KatG has the heme initially oxidized to the oxoiron(IV) state (Fe(IV)\(=\)O) and a porphyrin cation \( \pi \)-radical (Por\( ^+ \)) (15). With organic peroxides (ROOH), this compound I species can be easily trapped with the stopped-flow technique (Reaction 1), and this study underlines previous observations (11–14) that this compound I species can be directly reduced with one-electron donors, forming compound II (Reaction 2) and, finally, ferric KatG (Reaction 3). The exact nature of compound II in KatG is still not clearly understood, but the spectral features suggest the existence of an iron(III) species plus a protein radical (aa\( ^+ \), not yet identified protein radical) (8, 11–14) rather than the classical oxoiron(IV) form (23). Its absorption spectrum is only slightly perturbed from that of the resting enzyme (27, 28). In the absence of one-electron donors, intramolecular electron transport to the porphyrin (Reaction 4) has been shown to form Trp and Tyr radicals (15).

**Substrate Channel in Catalase-Peroxidase**

\[ \text{KatG (Por—Fe(III)) + ROOH} \rightarrow \text{compound I (Por} ^+ \text{—Fe(IV)=O + ROH)} \]

**REACTION 1**

\[ \text{Compound I (Por} ^+ \text{—Fe(IV)=O + AH} \rightarrow \text{compound II (Por—Fe(III)aa} ^+ \text{) + A} \cdot \text{ + H}_2\text{O)} \]

**REACTION 2**

\[ \text{Compound II (Por—Fe(III) aa} ^+ \text{) + AH} \rightarrow \text{KatG (Por—Fe(III)) + A} \cdot \]

**REACTION 3**

\[ \text{Compound I (Por} ^+ \text{—Fe(IV)=O} \rightarrow \text{compound I (Por—Fe(IV)=O Trp} ^+ \text{)} \rightarrow \text{compound I (Por—Fe(IV)=O Tyr} ^+ \text{)} \]

**REACTION 4**

When hydrogen peroxide \((R = \text{H})\) was used to initiate Reaction 1, a classical Por\( ^+ \text{—Fe(IV)=O} \) compound I could be trapped only in mutants that (almost) completely lost the catalytic activity (13, 14). This could mean that the reaction of the classical compound I of WT KatG with \( \text{H}_2\text{O}_2 \) back to ferric KatG is so fast that it obviates monitoring of Reaction 1 with the stopped-flow technique. In monofunctional catalases, in which compound I formed via Reaction 1 is known to serve as a two-electron oxidant of \( \text{H}_2\text{O}_2 \), affording \( \text{O}_2 \) (i.e. the catalatic reaction), the high rate of \( \text{H}_2\text{O}_2 \) oxidation also precludes compound I accumulation (29). However, it was recently proposed that the catalatic cycle in KatG enzymes could be different from that in monofunctional catalases (27). The kinetic and spectroscopic data of our work now clearly demonstrate the existence of a redox intermediate that dominates in the catalatic cycle and does not resemble ferric KatG, classical compound I (23), or compound III (27, 28). The design of E253Q was a fluke because the variant still had catalase activity but with decelerated \( \text{H}_2\text{O}_2 \) oxidation kinetics. Mixing E253Q with excess \( \text{H}_2\text{O}_2 \) formed this intermediate with its Soret band at 418 nm and a prominent peak around 521 nm within the mixing time of the stopped-flow apparatus \((1.5 \text{ ms})\). Most interestingly, this intermediate could not be formed with peroxoacetic acid, whereas the Por\( ^+ \text{—Fe(IV)=O} \) compound I obtained with the organic peroxide was rapidly transformed to it within milliseconds upon incubation with \( \text{H}_2\text{O}_2 \). Finally, we demonstrated that the dominating redox intermediate in the catalatic cycle of WT KatG has the same spectral features. At the moment, we do not know the electronic nature of this intermediate. The spectral features indicate the existence of an LS species and thus of an oxoiron(IV) form plus a protein radical (aa\( ^+ \)) (Reaction 5). Clearly, further experiments are needed. Nevertheless, its transformation to the resting state by \( \text{H}_2\text{O}_2 \) (Reaction 6) seems to be the rate-limiting step in the catalase cycle, and it is this reaction that strongly depends on an ordered matrix of water dipoles. By contrast, the impact of the performed mutations on Reactions 1–3 was small.

\[ \text{KatG (Por—Fe(III)) + HOOH} \rightarrow \text{compound I (Por—Fe(IV)=O aa} ^+ \text{) + H}_2\text{O)} \]

**REACTION 5**

\[ \text{Compound I (Por—Fe(IV)=O aa} ^+ \text{) + HOOH} \rightarrow \text{KatG (Por—Fe(III)) + O} \cdot + \text{H}_2\text{O)} \]

**REACTION 6**

Recent findings on the kinetic isotope effect of the catalase reaction of a native monofunctional catalase and myoglobin mutants indicate that there could be two different mechanisms for \( \text{H}_2\text{O}_2 \) oxidation to \( \text{O}_2 \), i.e. ionic and radical mechanisms, depending on the presence and absence of an acid/base catalyst (29). Monofunctional catalases follow an ionic reaction, which is initiated by a proton abstraction from the second \( \text{H}_2\text{O}_2 \) with the help of the distal histidine, followed by the two-electron reduction of compound I back to ferric catalase. In some myoglobin mutants without an acid/base catalyst located at the proper position, the reaction has been demonstrated to start by a hydrogen atom transfer from \( \text{H}_2\text{O}_2 \) to the ferryl species to yield a radical intermediate (29). The actual site of \( \text{H}_2\text{O}_2 \) oxidation in KatG is unknown, but recent findings (27) as well as the data of this work are compatible with a radical mechanism that includes Reactions 5 and 7.

\[ \text{Compound I (Por—Fe(IV)=O aa} ^+ \text{) + HOOH} \rightarrow \text{compound I (Por—Fe(III)—OH aa} ^+ \text{) + HO}_2\cdot} \]

**REACTION 7**

A “molecular ruler” mechanism that selects the neutral and polar hydrogen peroxides through the long 30–50-Å access channel in mono-
Substrate Channel in Catalase-Peroxidase

functional catalases has been proposed recently based on the structure of human catalase (25), although the authors did not discriminate between the role of hydrogen peroxide as oxidant and as reductant. In monofunctional catalases, the access channel is typically constricted to 2–3 Å, and at either end of this hydrophobic constriction, water molecules form hydrogen bonds to the protein and are well ordered. The constriction allows only small molecules like water or hydrogen peroxide to enter the active site. Moreover, water molecules with short occupancy times that cannot bridge the channel with water—water hydrogen bonds have been described (25). It has been suggested that H_2O_2 would be able to satisfy this hydrogen-bonding network with water. This mechanism fits well with the observation that mutations which widen the hydrophobic constriction possess lower catalase activity (25, 26).

In *Escherichia coli* hydroperoxidase II, which is also a monofunctional catalase, it has been demonstrated that a negatively charged side chain in the main channel enhances water occupancy in the access channel, and it has been proposed that an electrical potential (between the negatively charged carboxylate and the positively charged heme iron) will influence the orientation of both the water molecules and the substrate hydrogen peroxide (24). The common orientation of water molecules will favor the formation of an ordered hydrogen bond matrix as well as orientation of the H_2O_2 molecule for reaction. It should be mentioned that, also in this work, the authors did not discriminate between compound I formation and reduction. In the case of catalase-peroxidases, there is the advantage that the enzyme is bifunctional and thus allows a comparison of both radicals with an altered pH profile of the catalase (but not peroxidase) activity. However, the reports on monofunctional catalases also suggest that disruption of the water matrix concerns H_2O_2 oxidation and not compound I formation, which is underlined by the fact that, in catalase variants with increased channel dimensions, the peroxidase activity increases (25, 26).

In KatG enzymes, the access channel is more restricted compared with monofunctional peroxidases, but is not as pronounced as in monofunctional catalases. Similar to monofunctional catalases, there are conserved acidic amino acids in the main access channel: Glu^{253} at the entrance and Asp^{152} at the ~3–4 Å wide constriction (2–5). Exchange of both residues significantly reduced the catalase activity, but did not alter the peroxidase activity (TABLE TWO). Both residues seem to be critical for stabilizing the solute matrix in the channel and for orienting the water dipoles. In addition, Asp^{152} has been shown to be essential for maintaining the heme iron spin state and the iron–imidazolate bond strength (7). It controls the immediate access to the distal residues Arg^{119}, His^{123}, and Trp^{122}. Most interestingly, both D152S (8) and E252Q (but not E253D) are the only KatG variants investigated so far with an altered pH profile of the catalase (but not peroxidase) activity. Normally, KatG enzymes have a pH optimum for the catalase activity of 6.5 (8). In the E253Q variant, the pH optimum was shifted to 5.5 (Fig. 6). These findings suggest that both negative residues not only participate in the stabilization and orientation of the solute matrix, but also may modify the actual transformation reaction of H_2O_2 to molecular oxygen. A mechanism that involves Asp^{152} directly in the hydrogen peroxide oxidation reaction has been proposed previously (8). It could also be possible that the negatively charged carboxylate residues modify the protonation status of a catalytically active residue via the highly oriented water dipoles. Because it has been shown that protein radicals are also formed in KatG enzymes (15), it is feasible that the ordered hydrogen-bonding network plays an important role in maintaining a defined reduction potential at catalytically important redox-active amino acid(s).

In conclusion, we have shown here that, in bifunctional catalase-peroxidases, the hydrogen peroxide oxidation (but not reduction) reaction depends strongly on a defined and oriented water matrix maintained by the channel architecture and highly conserved residues at the KatG-specific loop LL1. Two carboxylate groups at two strategically important locations are crucial for oxygen release from H_2O_2.

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