The Catalase Activity of Catalase-Peroxidases Is Modulated by Changes in the pK_a of the Distal Histidine

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ABSTRACT: The unusual Met-Tyr-Trp adduct composed of cross-linked side chains along with an associated mobile Arg is essential for catalase activity in catalase-peroxidases. In addition, acidic residues in the entrance channel, in particular an Asp and a Glu ~7 and ~15 Å, respectively, from the heme, significantly enhance catalase activity. The mechanism by which these channel carboxylates influence catalase activity is the focus of this work. Seventeen new variants with fewer and additional acidic residues have been constructed and characterized structurally and for enzymatic activity, revealing that their effect on activity is roughly inversely proportional to their distance from the heme and adduct, suggesting that the electrostatic potential of the heme cavity may be affected. A discrete group of protonable residues are contained within a 15 Å sphere surrounding the heme iron, and a computational analysis reveals that the pK_a of the distal His$_{112}$, alone, is modulated within the pH range of catalase activity by the remote acidic residues in a pattern consistent with its protonated form having a key role in the catalase reaction cycle. The electrostatic potential also impacts the catalatic reaction through its influence on the charged status of the Met-Tyr-Trp adduct.
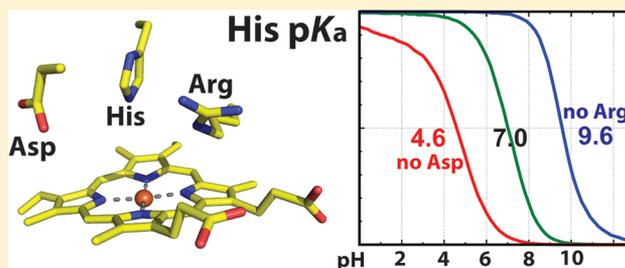
Catalase-peroxidases or KatGs are fascinating proteins with a peroxidase-like core, catalyzing a broad substrate-range peroxidase reaction, that have evolved to support a robust catalase

reaction, dismutating hydrogen peroxide to oxygen and water,[1] and an oxygenase function that generates superoxide.[2−4] The *in vivo* roles of the peroxidase and oxidase reactions are as yet undefined. Furthermore, a combination of the oxygenase and peroxidase reactions of KatG in *Mycobacterium tuberculosis* facilitates the conversion of the prodrug isoniazid into isonicotinyl-NAD, an active anti-tubercular drug.[5]

Rationalizing the overlapping mechanisms of these seemingly disparate reactions has been a challenge. The key lies in a unique intramolecular adduct of the cross-linked side chains of Met_{264}, Tyr_{238}, and Trp_{111} [residue numbering corresponds to that of *Burkholderia pseudomallei* KatG (BpkatG)], hereafter termed the M-Y-W adduct, its reversible association with a mobile Arg_{426}, and a reversible perhydroxy modification on the indole of the M-Y-W adduct (M-Y-W_{111}-OOH), with two orientations, A and B, all in the proximity of the heme (Figure 1). Computational
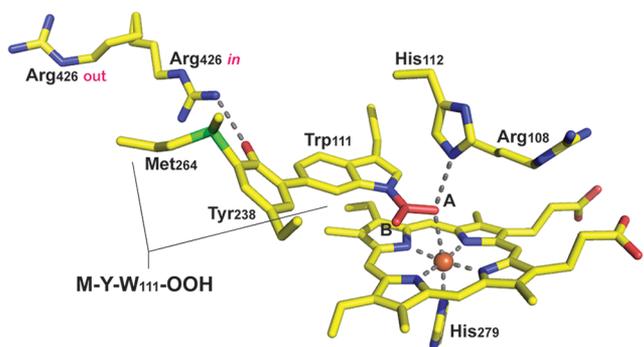
Figure 1. Orientation of the key residues surrounding the heme in BpKatG. The side chains of the three residues, Met_{264}, Tyr_{238}, and Trp_{111}, are cross-linked to form the M-Y-W adduct that can be reversibly perhydroxylated on the indole nitrogen of Trp_{111} to create the M-Y-W_{111}-OOH modification. The terminal -OH of the perhydroxy modification is found in two orientations, A, in contact with the heme iron and His_{112}, and B, having no interactions in the protein. The mobile Arg_{426} can also adopt two conformations, *in*, forming an ionic interaction with a tyrosinate ion on Tyr_{238}, and *out*, rotated away from the adduct.

studies have implicated the transient formation of a radical on the M-Y-W adduct[6] as a possible intermediate in the catalatic reaction and in the reversible formation of the M-Y-W_{111}-OOH modification.[7,8] In addition, electron paramagnetic resonance (EPR) studies have identified two sites of stable radical accumulation, Trp_{139} and Trp_{153},[9] thereby defining a possible electron transfer pathway from isoniazid to the M-Y-W adduct and heme for the generation of superoxide, required for isoniazid activation.[9,10]

A comprehensive mechanism (Figure 2) for the catalatic process has been proposed,[8,11,12] and while the process is cyclic, a starting point is defined at RS 1 (resting state 1), the predominant species that accumulates in protein crystals actively metabolizing H_2O_2.[8] RS 1 must be converted to RS 2, with the release of O_2, before H_2O_2 can bind for Compound I (Cpd I) formation. The subsequent reduction of Cpd I may in fact be concerted, but discrete intermediates are shown to define more clearly the molecular changes. A transient radical is formed on the M-Y-W adduct with the transfer of an electron to the porphyrin (I_A), followed by the binding of H_2O_2 to Cpd I (I_B). The transfer of a hydrogen atom to the ferryl−oxo species yields a two-radical ferryl−hydroxo intermediate (I_C), and the transfer of a proton from the Trp_{111} indole produces water, leaving the two radicals (I_D) to combine into RS 1. All species in this cycle

have been characterized by either X-ray crystallography or quantum mechanical/molecular mechanical calculations.

Despite this detailed understanding of the inner workings of KatGs, a perplexing question regarding the influence of acidic residues on catalase activity remains. Removal of an aspartate side chain ~7 Å from the heme iron (Asp_{141} in Figure 3) reduces catalase activity by >95% with little change in peroxidase activity.[13,14] Even more surprising is the fact that removal of a glutamate side chain ~15 Å from the heme iron (Glu_{242} in Figure 3) reduces catalase activity by >70%.[15] An electrical potential between the negatively charged carboxylate and a positively charged iron, previously proposed in classical catalases,[16] was suggested as a possible explanation,[15] but restoration of catalase activity in the D141A/R108A double variant suggested a more complex situation.[14]

In this paper, we confirm the importance of channel carboxylates for the catalase activity of catalase-peroxidases with a combination of crystallographic, computational, and biochemical approaches and demonstrate that native enzyme activity can actually be enhanced by the introduction of additional carboxylates in the entrance channel. Seventeen new BpKatG variants with changes in channel residues are characterized biochemically and structurally to demonstrate the scope of the carboxylate influence. In parallel, the pK_a values for all ionizable residues in the protein are calculated revealing a correlation among increasing numbers of channel carboxylates, increasing His_{112} pK_a, and catalase activity. The impact of these results on the catalase reaction mechanism is discussed.

■ EXPERIMENTAL PROCEDURES

Chemicals and Media. All chemicals were obtained from Sigma-Aldrich or Fisher unless otherwise stated. All components of media were obtained from Becton-Dickson, and all restriction enzymes were obtained from Invitrogen.

Enzyme Assays. Catalase activity was determined by the method of Rørth and Jensen[17] in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min in a 60 mM H_2O_2 solution at pH 7.0 and 37 °C. Peroxidase activity was determined using ABTS [2,2′-azinobis(3-ethylbenzothiazoline)-sulfonic acid] (ε_{405} = 36800 M^{-1} cm^{-1}),[18] and one unit is defined as the amount that decomposes 1 μmol of ABTS in a solution of 0.4 mM ABTS and 2.5 mM hydrogen peroxide at pH 4.5 and 25 °C.[19] Assays were performed in triplicate and results averaged.

Variant Protein Construction, Purification, and Characterization. The following oligonucleotides were purchased from Invitrogen and used to mutate pBpG-KC, containing the *Kpn*I−*Cla*I fragment (base pairs 1−1006 of BpKatG), or pBpCH, containing the *Cla*I−*Hin*dIII fragment (base pairs 1003−1731), following the Kunkel[20] procedure: CCCGACAA-CGACAACCTCGAC (A143D), AAGATCTGGGACGAACT-GAGC (L209D), ATCTGGCTGCAACTGAGCGGC (E210Q), CTGGCTGGAAGACAGCGGCGGC (L211D), GCCGCCGTGGAGATGGGCCTCA (Q233E), CAGATGG-GCCGACTCTACGTGA (L236D), ATCTACGTGAGATCG-GAAGGCC (N240D), TGAATCCGGCAAGCCCGGTGC (E242Q), GCGGGGCCCGACTCGAACGTC (A290D), GCGTCGAACGACGGCGCCGAG (V293D), GTCGGCGC-CGCGCCGGAGGCC (E296A), ACGAGCGGGGACGAAG-TCACG (L326D), GAAGAGCCCGGACGGCGCGCACC (A357D), and CCGGCGGGCGACCACCAGTGG (A359D). The mutant sequences were confirmed and used to generate plasmids pA143D, pL209D, pE210Q, pL211D, pQ233E, pL236D,
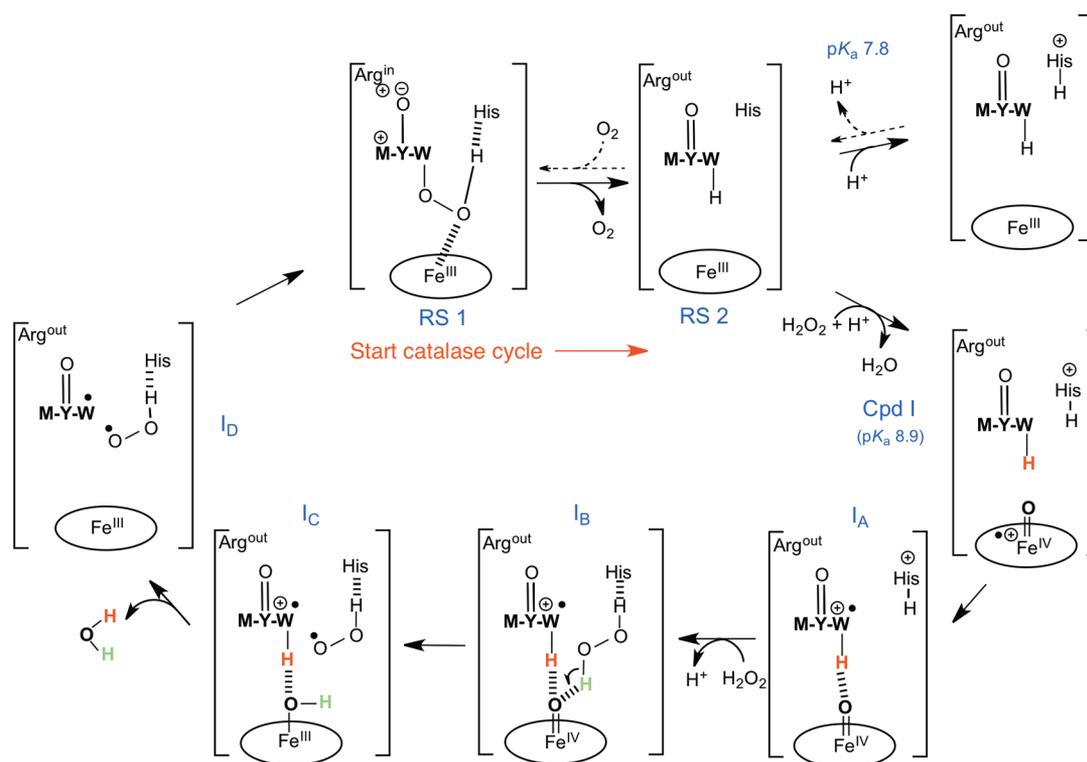
Figure 2. Catalase reaction scheme originally proposed in ref 8 but modified to include the possible roles of distal His₁₁₂ protonation. RS 1 predominates in the crystal structure, suggesting an energy barrier for O_2 removal to generate RS 2 and Cpd I formation. For the purposes of discussion, we have indicated that the catalase cycle begins with RS 1.

pN240D, pE242Q, pA290D, pV293D, pE296A, pL326D, pA357D, pA359D, and pQ233E/N240D by reincorporating the fragment into the full-length *katG* gene. Plasmid pD141A has been constructed previously[14] and was used for the construction of multiple-variant plasmids pD141A/Q233E and D141A/Q233E/N240D. The native and variant proteins were expressed and purified as described previously.[9]

Crystallization of the KatG Variants. Crystals were obtained at room temperature by the vapor diffusion hanging drop method at 20 °C over a reservoir solution containing 16−20% (w/v) PEG 4000, 20% 2-methyl-2,4-pentanediol, and 0.1 M sodium citrate (pH 5.6).[10,21] Crystals were in primitive orthorhombic space group $P2_12_12_1$ with two subunits in the crystal asymmetric unit. Diffraction data were collected using synchrotron beamline CMCF 08ID-1 at the Canadian Light Source in Saskatoon, SK, from crystals flash-cooled in reservoir buffer and cooled with a nitrogen cryostream. Data were processed and scaled using XDS[22] and SCALA[23] (Table 1). The structural refinement starting with the native BpKatG structure [Protein Data Bank (PDB) entry 1MWV] was completed using REFMAC[24] and manual modeling with the molecular graphics program COOT.[25] Figures were generated using PYMOL (The PYMOL Molecular Graphics System, Schrodinger, LLC). The cavities associated with the main channel were visualized with HOLLOW.[26]

pK_a Calculations. The distribution of all charged or potentially charged residues in KatG was mapped, revealing the isolation of a subset of charged side chains in the heme cavity (Figure 4). A summation of the charges within the heme vicinity (Figure 4 C) reveals a net negative charge. A sphere of 15 Å centered at the heme iron contains only the charged side chains implicated in the catalytic mechanism or interacting with the heme, the Lys₂₈₃-Glu₃₂₇ pair, and Glu₂₄₂. The mobile Arg₄₂₆ is

outside the sphere when in the out (or R)[27] conformation and inside the sphere when in the in (or Y)[27] conformation interacting with the adduct Tyr₂₃₈, which requires the tyrosine to be unprotonated. The pK_a changes in the side chains of all residues found in this ∼15 Å coordination sphere of the iron atom of native BpKatG and its D141A, E242Q, Q233E, and N240D variants were calculated at different stages of the catalytic cycle, including resting state 1 [RS 1 with Fe^{III} and W₁₁₁-OOH (Figure 2)], resting state 2 (RS 2 with Fe^{III} and M-Y-W₁₁₁-H), Compound I (with Fe^{IV}=O and W₁₁₁-H), and in the presence of acetate. In addition, both RS 1 and RS 2 were considered with Tyr₂₃₈ in its neutral hydroxyl form and in its anionic form, with and without interaction with the mobile Arg₄₂₆. The structures for each state were derived from crystal structures, including RS 1 of native BpKatG from 5SXR (3N3O),[10] D141A from 5SYH (4KA5),[28] D141A with acetate bound from 5KSF, E242Q from 5SYU (4QZK), S324T from 5L02 (1X7U),[29] Q233E from 5SYW (4QZN), N240D from 5SYV (4QZL), BpKatG Cpd I from 5SW8 (2B2R),[27] D141A Cpd I, generated *in silico* from 5SYH and 5SW8, and RS 2 of BpKatG from 5SXS (3N3P).[10] The first 14 residues of all BpKatG structures are not resolved and were not included in the calculations.

The charge parameters were taken from a previous work.[30] For M-Y-W₁₁₁-OOH, we started with the coordinates from 5SXR (3N3O)[10] and the geometry was optimized with the wB97X functional[31] and the 6-31+G** basis set. The electrostatic potentials were calculated on the optimized conformations following a previous protocol.[30] The parameters for acetic acid were easily derived from the side chains of aspartate or glutamate found in the Gromos 54a7 force field.[32,33]

We used Poisson−Boltzmann (PB) calculations to compute the individual and pairwise terms needed to obtain the changes in
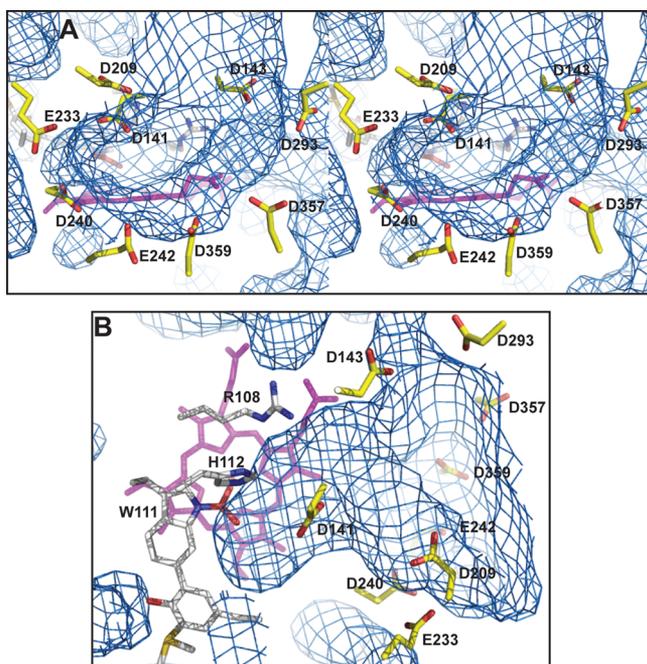
Figure 3. Distribution of carboxylates that enhance the catalase reaction. (A) Stereoview looking into the entrance channel illustrating the distribution around the opening. The heme is colored mauve for the sake of visibility. (B) View of the channel extending into the heme cavity looking down on the heme to illustrate the relative distances between the carboxylates and the heme. The locations of the various carboxylates are derived from the structures outlined in Table 1, including Asp$_{357}$ from 5KQ2, Asp$_{359}$ from 5KQ6, Asp$_{293}$ from 5KQH, Asp$_{141}$ and Glu$_{242}$ from 5LO5, Glu$_{233}$ from 5SYW, and Asp$_{240}$ from 5SYV. The location of the mutated side chain of L209D was fit *in silico*.

the free energies of protonation. These were then used to perform Monte Carlo (MC) sampling of the protonation states. pK_a values for protonable groups were obtained from the corresponding titration curves.[34] All calculations were performed considering alternative proton positions (tautomers) for all titrable sites.[34,35] The atomic charges and radii used in the PB calculations were derived from the Gromos 54a7 force field,[32,33] with the exception of the heme and M-Y-W peptide adduct, for which we used our QM-derived charges. All PB calculations consisted of finite-difference linear Poisson–Boltzmann calculations performed with MEAD,[36,37] using a temperature of 300 K, an ionic strength of 100 mM, a molecular surface defined with a solvent probe radius of 1.4 Å,[38] and a Stern (ion exclusion) layer of 2.0 Å. A two-step focusing procedure[39] was used, with consecutive grid spacings of 1.0 and 0.25 Å. The dielectric constants of the solvent and protein were set to 80 and 20, respectively.[34] The value of 20 has been shown to minimize the error in pK_a predictions in a set of protein X-ray structures with known experimental pK_a values.[34] The MC simulations were performed using PETIT[35,40] with multistate titrations by performing trial changes of both single and pair sites.[35,40] MC simulations were performed at intervals of 0.2 pH unit, each using 10^5 MC cycles. Each cycle consisted of sequential state changes over all individual sites and also pairs of sites with at least one interaction term above 2.0 pK_a units.

For more details about pK_a calculations, please refer to refs 34 and 35. Reference 34 also provides a comprehensive study of pK_a values on several protein X-ray structures, which served as a benchmark for our methodology and revealed a typical error of ~0.8 pK unit.

RESULTS

Acidic Residues in the Entrance Channel Are Important for Catalase Activity. Mutation of acidic residues in the entrance channel of KatGs causes a decrease in catalase activity with little or no effect on peroxidase activity. For example, removal of the carboxylate of Asp$_{152}$ in *Synechocystis* KatG (SyKatG) or Asp$_{141}$ in BpKatG, ~7 Å from the heme iron, reduces activity by >95%.[13,14] A direct catalytic role of the carboxylate, although proposed,[13] seemed unlikely because simultaneous removal of the nearby Arg$_{108}$ in the BpKatG double variant, D141A/R108A, returned catalytic activity to 80% of native levels.[14] The alternative explanation that Asp$_{141}$ and Arg$_{108}$ were contributing to a charge balance in the heme cavity was proposed, although how the charges were influencing catalase activity was not clear. The puzzle of channel carboxylates deepened with the observation that removal of Glu$_{253}$ (Glu$_{242}$ in Figure 3) in SyKatG, situated 14 Å from the heme, reduced catalase activity by ~70%.[15] In this instance, an analogy was drawn with monofunctional catalases, where it was suggested that an electrical potential between an aspartate in the entrance channel and the heme acted on the electrical dipoles of incoming H_2O_2 to create a favorable or reactive orientation.[16] However, the more open architecture of catalase-peroxidase entrance channels and the dramatic influence of Asp$_{141}$ suggested that other mechanisms should be considered.

To investigate further the role of channel carboxylates, a series of variants with acidic residues added and removed from various locations in the entrance channel have been constructed and characterized structurally (Table 1) and for activity (Table 2). Of the existing acidic residues, removal of Glu$_{210}$, Glu$_{296}$, and Glu$_{242}$ (see Figure 3 for location) causes a range of levels of inhibition, 20, 40, and 85%, respectively, roughly inversely correlated with their distance from the heme (24.1, 21.3, and 14.2 Å, respectively). Surprisingly, the insertion of additional acidic residues in a number of locations, including Gln$_{233}$ (to Glu) and Ala$_{143}$, Asn$_{240}$, Val$_{293}$, Ala$_{357}$, and Ala$_{359}$ (all to Asp), causes varying increases in catalase activity, again roughly inversely correlated with distance from the heme, although a 2-fold increase in activity is the maximum regardless of the number of carboxylates introduced. By contrast, replacement of Ala$_{290}$, Leu$_{236}$, and Leu$_{326}$ (all with Asp), the latter two within 12 Å of the heme, caused a small decrease in catalase activity for which an explanation is not obvious.

A survey of the location and orientation of the acidic residues relative to the cavity entrance and the heme (Figure 3) reveals that carboxylates in all quadrants surrounding the entrance are effective in causing activation. In other words, variations in activity (Table 2) could not be correlated with any changes in side chain location or water matrix in the entrance channel among the variants, including A143D (5TXQ), A290D (5KQ0), A357D (5KQ2), A359D (5KQ6), V293D (5KQH), L326D (5KQI), E242Q (5SYU), N240D (5SYV), Q233E (5SYW), D141A/Q233E (5KQ3), D141A/Q233E/N240D (5KQ7), and Q233E/N240D (5KQK).

Acetate Activates Catalase Activity. The pH profile of catalase activity of catalase-peroxidases with a broad maximum around pH 6.5 differs from the even broader and very flat plateau of monofunctional catalases.[19] The determination of pH profiles usually demands the use of several different buffers to span the pH range of 4–10, and the transition between buffers was accompanied by discontinuities in the activity profile. The discontinuities were eliminated by using a mixture of phosphate

Table 1. Data Collection and Refinement Statistics of Various BpKatG Variants

PDB entry	5KQ0	5KQ2	5KQ3	5KQ6	5V53	5KQH	5KQI	5KQK
variant	A290D	A357D	D141A/Q233E	A359D	D141A/Q233E/N240D with 25 mM acetate	V293D	L326D	Q233E/N240D
Data Collection								
unit cell parameters								
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a (Å)	100.76	100.83	101.04	100.58	100.84	100.79	100.79	100.72
b (Å)	113.49	114.13	114.03	115.79	115.82	113.43	115.23	115.05
c (Å)	174.51	174.99	174.51	174.73	174.77	174.68	174.54	174.51
α, β, γ (deg)	90	90	90	90	90	90	90	90
resolution[a] (Å)	47.57–1.80 (1.90–1.80)	47.80–1.90 (2.00–1.90)	47.76–1.85 (1.95–1.85)	48.33–1.62 (1.71–1.62)	48.27–1.70 (1.79–1.70)	47.57–1.82 (1.92–1.82)	48.42–1.87 (1.97–1.87)	48.39–1.75 (1.84–1.75)
no. of unique reflections	181938 (26569)	154155 (22788)	152779 (23222)	251711 (35905)	214623 (32021)	178897 (25952)	165881 (24178)	199866 (29240)
completeness (%)	98.7 (99.5)	97.3 (99.3)	89.3 (93.5)	97.7 (96.3)	96.1 (98.7)	99.9 (100.0)	98.9 (99.8)	98.4 (99.2)
R_{merge}	0.059 (0.474)	0.070 (0.474)	0.076 (0.487)	0.044 (0.520)	0.061 (0.538)	0.071 (0.475)	0.092 (0.478)	0.062 (0.413)
R_{pim}	0.041 (0.330)	0.047 (0.326)	0.042 (0.283)	0.022 (0.268)	0.034 (0.310)	0.038 (0.256)	0.050 (0.256)	0.044 (0.298)
$\langle I/\sigma I \rangle$	12.3 (2.4)	10.6 (2.3)	9.8 (2.3)	20.8 (2.7)	11.7 (2.4)	14.2 (3.0)	9.3 (2.6)	12.8 (3.0)
$CC_{1/2}$	0.998 (0.811)	0.998 (0.784)	0.998 (0.802)	0.999 (0.832)	0.998 (0.814)	0.998 (0.794)	0.992 (0.808)	0.997 (0.819)
multiplicity	2.9 (2.9)	2.9 (2.9)	3.7 (3.6)	4.7 (4.2)	3.9 (3.8)	4.3 (4.4)	4.1 (4.2)	2.7 (2.7)
Model Refinement								
no. of reflections	172743	146428	145121	238907	203772	169937	157637	189792
R_{cryst} (%)	15.2	15.6	15.9	14.5	14.3	15.4	16.4	15.2
R_{free} (%)	18.5	19.1	19.5	17.0	17.2	18.4	19.8	18.2
no. of non-H atoms	12876	12676	12481	12835	12697	12795	12535	12860
no. of qater molecules	1625	1425	1255	1579	1465	1550	1271	1629
average B factor (Å²)								
protein	24.4	27.0	28.3	23.2	25.3	22.1	27.5	20.1
heme	17.2	20.6	21.3	16.4	18.9	15.4	20.4	13.1
waters	33.8	34.9	35.1	32.9	33.9	31.7	34.3	29.4
other								
coordinate error (Å)[b]	0.073	0.089	0.086	0.048	0.0.52	0.072	0.088	0.066
root-mean-square deviation for bonds (Å)	0.028	0.023	0.023	0.034	0.028	0.027	0.028	0.028
root-mean-square deviation for angles (deg)	2.27	2.03	2.01	2.66	2.29	2.26	2.32	2.23

Table 1. continued

	5KSF	5TXQ	5KSK	5SYU	5SYV	5SYW	SV4O
PDB entry							
variant	D141A with 25 mM acetate	A143D	native with 50 mM acetate	E242Q	N240D	Q233E	D141A/Q233E/N240D
Data Collection							
unit cell parameters							
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a (Å)	100.69	100.67	100.71	100.82	101.79	101.02	100.83
b (Å)	115.47	115.18	115.48	113.71	113.30	114.19	115.45
c (Å)	174.59	174.74	174.97	174.62	174.52	174.67	175.58
α, β, γ (deg)	90	90	90	90	90	90	90
resolution[a] (Å)	48.37–1.75 (1.84–1.75)	48.37–1.90 (2.00–1.90)	48.39–1.69 (1.78–1.69)	57.08–1.80 (1.90–1.80)	48.42–1.75 (1.84–1.75)	47.81–1.85 (1.95–1.85)	48.23–1.95 (2.06–1.95)
no. of unique reflections	203501 (29608)	159525 (22983)	225720 (32915)	166673 (24661)	197714 (29084)	170242 (24810)	141391 (21006)
completeness (%)	99.6 (99.9)	99.8 (99.6)	99.4 (99.8)	90.3 (92.2)	98.4 (99.9)	99.1 (99.5)	95.0 (97.3)
R_{merge}	0.062 (0.481)	0.077 (0.563)	0.070 (0.484)	0.095 (0.457)	0.069 (0.426)	0.075 (0.503)	0.074 (0.438)
R_{pim}	0.030 (0.235)	0.038 (0.276)	0.040 (0.291)	0.052 (0.251)	0.040 (0.244)	0.045 (0.291)	0.043 (0.262)
$\langle I/\sigma I \rangle$	14.7 (3.1)	17.5 (2.9)	10.8 (2.4)	8.3 (2.7)	15.1 (3.0)	11.1 (3.1)	9.8 (2.5)
$CC_{1/2}$	0.998 (0.872)	0.998 (0.824)	0.996 (0.807)	0.994 (0.819)	0.998 (0.831)	0.997 (0.847)	0.997 (0.833)
multiplicity	4.9 (5.0)	5.0 (5.0)	3.7 (3.7)	3.8 (3.5)	3.9 (3.9)	3.6 (3.7)	3.4 (3.4)
Model Refinement							
no. of reflections	193124	151540	214411	157558	187721	161698	134265
R_{cryst} (%)	14.8	15.7	15.2	13.9	16.0	15.2	14.9
R_{free} (%)	17.5	19.1	18.0	17.2	18.9	18.2	18.1
no. of non-H atoms	12667	12726	12836	12746	12807	12643	12310
no. of water molecules	1439	1683	1608	1507	1598	1420	1096
average B factor (Å²)							
protein	27.4	26.3	25.3	21.9	18.5	25.4	31.7
heme	21.0	20.2	18.6	15.0	12.0	18.1	24.9
waters	35.3	33.8	34.3	30.7	27.2	33.5	36.9
other							
coordinate error (Å)[b]	0.060	0.088	0.058	0.058	0.071	0.074	0.084
root-mean-square deviation for bonds (Å)	0.031	0.027	0.030	0.027	0.019	0.018	0.026
root-mean-square deviation for angles (deg)	2.44	2.22	2.40	2.27	1.77	1.76	2.14

[a]Values in parentheses correspond to those of the highest-resolution shell. [b]Based on maximum likelihood.

Figure 4. Location of protonable residues within a 15 Å radius of the heme iron. Panel B is rotated approximately 90° compared to panel A. In panel C, the charges in the vicinity of the heme in RS 1 illustrate the net negative charge of the region.

Table 2. Catalase and Peroxidase Activities of BpKatG Variants

variant	catalase[a] [μmol of H_2O_2 (μmol of heme)$^{-1}$ s^{-1}]	peroxidase[b] [μmol of ABTS (μmol of heme)$^{-1}$ s^{-1}]
WT	9450 ± 520	19.3 ± 0.3
WT with acetate	10500 ± 430	19.3 ± 0.3
WT with formate	10300 ± 420	nd[c]
WT with propionate	10130 ± 390	nd[c]
A143D	17660 ± 630	13.9 ± 0.2
L209D	11010 ± 90	16.2 ± 0.4
E210Q	7730 ± 320	16.3 ± 0.3
L211D	10370 ± 380	19.2 ± 0.3
Q233E	17530 ± 740	17.6 ± 0.3
L236D	6560 ± 120	6.3 ± 0.1
N240D	18990 ± 780	21.0 ± 0.4
E242Q	1380 ± 40	12.5 ± 0.3
A290D	6410 ± 180	11.2 ± 0.2
V293D	14260 ± 620	18.3 ± 0.2
E296A	5990 ± 230	16.9 ± 0.2
L326D	6706 ± 280	17.6 ± 0.3
A357D	12130 ± 350	14.0 ± 0.2
A359D	14570 ± 150	17.5 ± 0.3
Q233E/N240D	18920 ± 550	19.8 ± 0.2
D141E	5910 ± 160	18.2 ± 0.2
D114N	1060 ± 50	13.7 ± 0.2
D141A	420 ± 30	16.4 ± 0.2
D141A/Q233E	533 ± 30	18.7 ± 0.4
D141A/Q233E/N240D	530 ± 20	15.8 ± 0.2
D141A with acetate[d]	5840 ± 180	16.4 ± 0.2
D141A with formate[d]	1550 ± 110	nd[c]
D141A with propionate[d]	2860 ± 210	nd[c]

[a]Catalase activity was determined at pH 7.0 with 60 mM H_2O_2. [b]Peroxidase activity was determined at pH 4.5 with 2.5 mM H_2O_2 and 0.4 mM ABTS. [c]Not determined. [d]Acetate, formate, and propionate at 50 mM.

(plotted on a separate axis with a dashed line in Figure 5A) appears to plateau at lower pHs. For comparison, the optimal pHs for E240Q and N240D are 5.5 and 7.0, respectively. The apparent K_M values of BpKatG for H_2O_2 are unchanged by acetate from those reported previously (25 mM at pH 6.5 and 62 mM at pH 5.0).[19] D141A differed slightly in acetate, its apparent K_M for H_2O_2 decreasing from 65 to 30 mM at both pH 5.0 and 6.5. Formate and propionate also at 50 mM induce smaller increases in the activity of D141A (Table 2), leading to the conclusion that carboxylate-containing molecules external to the protein can also enhance the catalase reaction.

To investigate if acetate binds to the protein, crystals of the native enzyme and the D141A variant were grown in the presence of 50 and 25 mM acetate, respectively, and X-ray diffraction data sets were collected. The electron density maps of the native enzyme contain no evidence of acetate binding anywhere on the protein (5KSK), whereas the maps of the D141A variant contain new regions of density, well satisfied by acetate, deep in the heme cavity, associated with the heme iron, distal Trp_{111} and His_{112} (Figure 6 and 5KSF). Acetate was also found in a similar location in the heme cavity of triple variant D141A/Q233E/N240D (5V53). Thus, acetate enters the access channel and heme cavity in the absence of Asp_{141}, and its presence facilitates the catalase reaction.

Entrance Channel Carboxylates Influence M-Y-W$_{111}$-OOH. The perhydroxy modification, M-Y-W$_{111}$-OOH, is found

and acetate across the whole pH range, revealing a 10−15% increase in the catalase activity of native BpKatG at pH 6.5, and even more strikingly a 10-fold increase in the catalase activity of the D141A variant at pH 5.0 compared to those for buffers without acetate present (Table 2 and Figure 5). The pH optima of both enzymes appear to be unchanged by the acetate, pH 6.5 for the native enzyme and pH 5.0 for D141A (Figure 5A), although the very low activity of D141A in the absence of acetate

Figure 5. Effect of acetate on the catalase activity of WT BpKatG (blue) and its D141A variant (red). The catalase activity was determined without (filled circles) and with (filled squares) 50 mM sodium acetate added to the buffer. The activity of D141A is ∼10% of the activity with acetate present and is plotted using a dashed line and the right axis. Error bars are shown for all points but in some cases are not visible because they are smaller than the size of the marker.
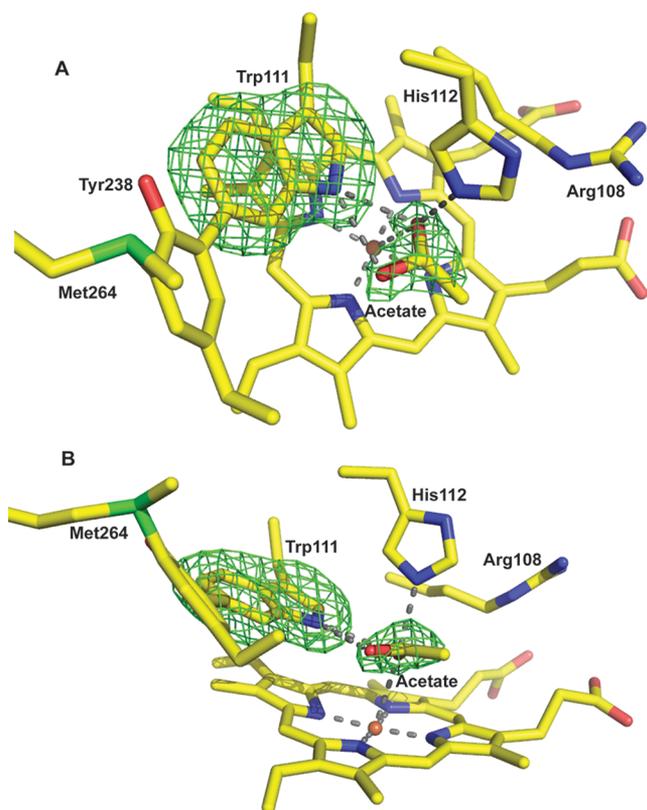
Figure 6. Two views of acetate bound in the heme cavity of D141A. The $F_o − F_c$ omit electron density maps at 7.0σ were calculated without Trp$_{111}$ or acetate in the model.

in the native enzyme at pH 6.1 with ∼60% occupancy split in two orientations. The predominant A orientation in the native enzyme and most variants is hydrogen bonded with the imidazole of His$_{112}$ (Figure 1); the less common B orientation is rotated ∼180° from His$_{112}$ (Figure 1). Removing the carboxylate of Asp$_{141}$ (in D141A) causes a dramatic change in the orientation of the perhydroxy modification from a predominantly A orientation to a predominantly B orientation,[8] but with similar ∼60% occupancy (5SYH), suggesting a correlation

between the B orientation and the inactivity of D141A.[8] However, the introduction of a channel carboxylate into the double and triple variants, D141A/Q233E (5KQ3) and D141A/Q233E/N240D (5V53), respectively, led to an increase in the A orientation, but without an increase in activity.

Computation of pK_as of Protonable Side Chains in BpKatG. To understand more clearly the interplay between Asp$_{141}$ and Arg$_{108}$, wherein both must be present or absent for catalase activity, and the impact of the channel carboxylates on charged residues in the vicinity of the heme, the pK_as of ionizable residues in the 15 Å sphere centered on the heme iron (Figure 4) were computed looking, in addition, at the influence of externally added acetate, the perhydroxy modification on the M-Y-W adduct (M-Y-W$_{111}$-OOH), the mobile Arg$_{426}$, and conversion to Cpd I (Table 3). Remarkably, the only residue in the heme cavity

Table 3. Computed pK_a Values for Selected Protonable Groups in BpKatG and Selected Variants

variant	MYW-OOH conformation	His$_{112}$	Asp$_{141}$	Asp$_{389}$	Glu$_{242}$	PD[a]
E242Q	A	5.8	<0[b]	<0	−	1.1
WT RS 1	A	6.2	<0	<0	3.0	1.1
Q233E	A	6.6	<0	<0	3.1	1.1
N240D	A	6.7	<0	<0	3.8	1.2
E242Q	B	6.6	<0	<0	−	1.1
WT RS 1	B	7.0	<0	<0	3.0	1.1
Q233E	B	7.5	<0	<0	3.1	1.2
N240D	B	7.5	<0	<0	3.8	1.3
D141A with acetate	−	8.9	−	<0	2.7	2.3
D141A with acetate at 2.5 Å	−	8.2	−	<0	2.9	2.2
D141A with acetate at 5.0 Å	−	7.0	−	<0	3.1	1.9
D141A with acetate at 7.5 Å	−	5.8	−	<0	2.8	1.0
D141A (no acetate)	B	4.6	−	<0	2.2	0.4
R108A	B	9.6	<0	<0	3.3	3.6
D141A/R108A	B	7.3	−	<0	2.7	2.8
WT RS 2 Y$_{238}$-O⁻	−	7.8	<0	<0	3.0	1.6
WT RS 2 Y$_{238}$-O⁻⁺R$_{426}$	−	7.2	<0	<0	2.9	1.3
WT RS 2 Y$_{238}$-OH	−	6.1	<0	<0	2.8	1.0
Cpd I D141A	−	6.3	−	<0	2.5	2.3
Cpd I E242Q	−	8.5	<0	<0	−	2.9
WT Cpd I	−	8.9	<0	<0	3.2	3.1
Cpd I Q233E	−	9.3	<0	<0	3.2	3.1
Cpd I N240D	−	9.3	<0	<0	3.7	3.1
Cpd I D141A/Q233E	−	6.5	−	<0	2.7	2.4
Cpd I D141A/N240D	−	6.6	−	<0	3.7	2.4

[a]PD, heme propionate D. [b]The reported values of <0 correspond to those of acidic residues that have their charged species stabilized by a nearby positively charged residue and should be interpreted as nontitrable in the physiological pH range.

that exhibits a change in pK_a within the pH range of catalase activity (pH 4.0−8.0) in response to changes in nearby residues is His$_{112}$, and the changes are substantial from 4.6 in D141A to 9.6 in R108A (Figure 7 and Table 3). Within this range, the pK_a of His$_{112}$ is very sensitive to its surrounding electrostatic environment being influenced by M-Y-W$_{111}$-OOH, by the orientation of Arg$_{426}$, by the protonation of Y$_{238}$, and by carboxylates in the entrance channel.
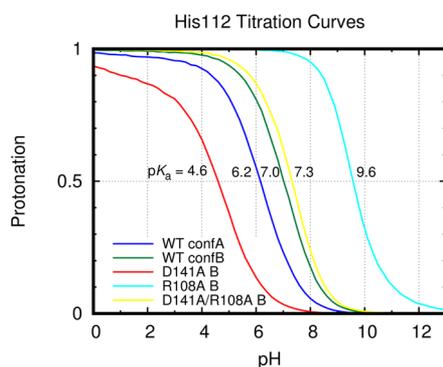
Figure 7. Titration curves of His_{112} in different variants of BpKatG, including the pK_a values determined for each. conf A and conf B refer to the two orientations of the perhydroxy modification on W_{111} (Figure 1).

Protonation of His_{112} is precluded by its hydrogen bonding interaction with M-Y-W_{111}-OOH in the A orientation, and the pK_a values reported are generally for M-Y-W_{111}-OOH in the B orientation, for example, 7.0 in the native enzyme with Arg_{426} in ionic contact with Tyr_{238}. Removal of the perhydroxy from M-Y-W_{111}-OOH (as O_2 or superoxide after INH treatment) and the shift of Arg_{426} to the out conformation increase the pK_a of His_{112} to 7.8, and protonation of Tyr_{238} lowers it to 6.1 (Table 3). Conversion of the heme iron of native BpKatG to an oxo−ferryl species introduces an electronegative atom (oxygen) next to His_{112}, which increases its pK_a to 8.9, which is consistent with the reported increase in the pK_a of the distal His in Cpd I of *Coprinus cinereus* peroxidase.[41]

Arguably, the most striking correlation is between catalase activity and changes in the His_{112} pK_a of both the resting state and Compound I species in the series of variants D141A, E242Q, native BpKatG, and N240D wherein the resting state pK_a of His_{112} changes from 4.6 to 6.6 to 7.0 to 7.5 and the Compound I pK_a of His_{112} changes from 6.3 to 8.5 to 8.9 to 9.3 as the catalase activity increases from 0.05 to 0.15 to 1.0 to 2.0 times the native level, respectively (Table 3). A similar correlation is evident in the increase in the catalase activity of D141A from 0.5 to 0.5 of native levels caused by acetate and the calculated pK_a of His_{112} that increases from 4.6 to 8.2 as acetate is moved progressively closer to the heme (Table 3). Similarly, the single variants, D141A and R108A, have low activity (0.05 and 0.10 of native levels, respectively) correlated with extreme pK_a values (4.6 and 9.6, respectively), whereas double variant D141A/R108A with 0.8 of native activity has a pK_a of 7.3.

DISCUSSION

Considerable progress has been made in our understanding of the mechanism underlying the catalase reaction in catalase-peroxidases, but the basis for how distant charged species and acetate in the buffer can influence the catalase reaction, but not the peroxidase reaction, still lacks a clear explanation. The hypothesis pursued in this work is that the influence of remote negative charges is mediated through a charged or protonable species in the vicinity of the heme. Protonable species include Arg_{108}, His_{112}, Asp_{141}, Trp_{111}, and Y_{238}, and the M-Y-W adduct, as a whole, may carry a positive charge while interacting with the mobile Arg_{426} and as the transient radical in the catalatic cycle (Figure 2). However, the direct involvement of Arg_{108} and Asp_{141} can be eliminated by the observation that double variant D141A/R108A, lacking both side chains, exhibits near normal catalase activity. Similarly, an increased level of protonation of

Tyr_{238} would weaken its interaction with Arg_{426}, essential for catalase activity, and more difficult deprotonation of Trp_{111} would interfere with the process of Compound I reduction. Therefore, by a process of elimination, His_{112} and the M-Y-W adduct are the most likely candidates to mediate remote charge effects.

To test the hypothesis that His_{112} might be responding to remote negative charges, the pK_as of all protonable residues near the heme were computed, revealing that only His_{112} responds to environmental changes in the pH range of catalase activity. The resting state pK_a of His_{112} of 7.0 (with M-Y-W_{111}-H) is higher than expected for a normal histidine, consistent with the predicted negative electrostatic environment in the heme cavity (Figure 4C). Significantly, there is a correlation of increasing His_{112} pK_as in the narrow range of 6.5 and 7.5 with increasing catalase activity and with increasing numbers of channel carboxylates. Removal of either Arg_{108} or especially Asp_{141} close to His_{112} causes more extreme changes in His_{112} pK_a outside of the normal activity pH range of the native enzyme, for example 4.6 in D141A and 9.6 in R108A, and these changes are associated with greatly decreased catalase activity. A similar correlation is also evident among the Compound I species of the variants ranging from 6.3 to 9.3. The optimal pHs of the catalase reactions of the variants are also correlated with the pK_a of His_{112}. The involvement of a protonated histidine is also consistent with the more rapid disappearance of catalase activity above pH 8 than below pH 6.[19]

What then is the role of protonated His_{112}? At pH 6.5, the optimum for catalase activity of native BpKatG, >80% of the M-Y-W adduct has the perhydroxy modification (M-Y-W_{111}-OOH) hydrogen bonded to His_{112} (RS 1 in Figure 2A),[42] precluding its protonation. However, RS 1 and RS 2 are in equilibrium, and increases in the pK_a of His_{112} will shift the protonation equilibrium of RS 2 (Figure 2) toward the more protonated species, which, in turn, will pull the resting state equilibrium toward RS 2 required for continuation of the catalase reaction cycle (Figure 2). The fact that RS 1 predominates in a catalytically active crystal suggests that it is at an energy minimum in the reaction pathway and anything that facilitates the release of O_2 and the conversion of RS 1 to RS 2, including enhanced protonation of His_{112}, will enhance this step of the catalase reaction. The large variations in the pK_a of His_{112} in Compound I suggest that protonated His_{112} may also be a key determinant in the catalatic reduction of Compound I by the second H_2O_2 molecule (Figure 2).

The very low catalase activity of D141A, which is unaffected by carboxylates in the channel, is relatively insensitive to pH but is activated by acetate (Figure 5), which seems to suggest that, besides protonation of His_{112}, the stability of a charged species is critical to the catalase cycle. A likely candidate is the transient adduct cation radical formed during Compound I reduction (Figure 2), which would be less stable with a less electronegative potential as in D141A and more stable with acetate present.

In summary, the catalase activity of catalase-peroxidases is dependent on a negative electrostatic potential in the vicinity of the heme, which is enhanced by carboxylate side chains in the entrance channel and by acetate approaching the heme. The negative potential influences catalase activity by modulating the pK_a of His_{112} and by stabilizing the transient charged M-Y-W adduct radical that is integral to the catalase reaction.

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Notes

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