Catalytic mechanism for the conversion of salicylate into catechol by the flavin-dependent monooxygenase salicylate hydroxylase

Débora M.A. Costa a,1, Stefanya V. Gómez b,1, Simara S. de Araújo a, Mozart S. Pereira b, Rosemeire B. Alves b, Denize C. Favaro c, Alvan C. Hengge d, Ronaldo A.P. Nagem a*, Tiago A.S. Brandão b,*

a Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil
b Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil
c Instituto de Química, Universidade Estadual de Campinas, Campinas, SP 13083-970, Brazil
d Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300, USA

Abstract

Salicylate hydroxylase (NahG) is a flavin-dependent monooxygenase that catalyzes the decarboxylative hydroxylation of salicylate into catechol in the naphthalene degradation pathway in Pseudomonas putida G7. We explored the mechanism of action of this enzyme in detail using a combination of structural and biophysical methods. NahG shares many structural and mechanistic features with other versatile flavin-dependent monooxygenases, with potential biocatalytic applications. The crystal structure at 2.0 Å resolution for the apo form of NahG adds a new snapshot preceding the FAD binding in flavin-dependent monooxygenases. The kcat/Km for the salicylate reaction catalyzed by the holo form is \( \sim 10^4 \text{M}^{-1} \text{s}^{-1} \) at pH 8.5 and 25 °C. Hammett plots for \( K_c \) and \( K_a \) using substituted salicylates indicate change in rate-limiting step. Electron-donating groups favor the hydroxylation of salicylate by a peroxylavin to yield a Wheland-like intermediate, whereas the decarboxylation of this intermediate is faster for electron-withdrawing groups. The mechanism is supported by structural data and kinetic studies at different pHs. The salicylate carboxyl group lies near a hydrophobic region that aids decarboxylation. A conserved histidine residue is proposed to assist the reaction by general base/general acid catalysis.

© 2019 Elsevier B.V. All rights reserved.

1. Introduction

Salicylate hydroxylase (NahG, EC 1.14.13.1) is a flavin-dependent monooxygenase that catalyzes the decarboxylative hydroxylation of salicylate into catechol, CO2, and water, with stoichiometric consumption of NADH and O2 (Scheme 1) [1]. This reaction links the upper and lower pathways of naphthalene degradation by soil pseudomonads [2], a bacterial genus encompassing many species that can use naphthalene or salicylate as sole carbon sources [3–5].

A number of nahG genes and encoded proteins have been identified in several naphthalene-degrading strains, such as P. putida G7 [6,7], which harbors the archetype catabolic plasmid NAH7 [8]; P. putida S-1 [9–11]; P. putida KF715 [12]; P. stutzeri AN10 [13], whose dissimilatory genes are chromosomally encoded [14]; and Pseudomonas sp. ND6 [15], among others [16]. Other salicylate hydroxylase genes situated outside, but in close proximity, to the transcriptional unit forming the naphthalene degradation lower pathway, i.e. nahW and nahU, have been reported for P. stutzeri AN10 [17,18] and Pseudomonas sp. ND6 [19], respectively. Interestingly, the coexistence of the classic NahG with NahW was shown to improve cell growth in salicylate-supplemented media [17]. The structures and catalytic mechanisms of NahW and NahU are unknown. NahG is the most studied salicylate hydroxylase, though poorly understood.

NahG hydroxylates and decarboxylates the substrate at the same aromatic carbon atom (ipso substitution) [11,20], an exquisite feature that is remarkable from a synthetic perspective. NahG is one of the few examples of a flavin enzyme that catalyzes an ortho hydroxylation relative to the substrate phenol group, which is usually required for efficient catalysis. Other monooxygenases exhibit different regioselectivities. For example, the oxidative decarboxylation of 6-hydroxynicotinate catalyzed by 6-hydroxynicotinate 3-monooxygenase (NicC) occurs para to the substrate phenol group [21]. In the reaction catalyzed by 3-hydroxybenzoate 6-hydroxylase (3HB6H), which the substrate...
carboxylate group is meta to the phenol, the 3-hydroxybenzoate is converted to 2,5-dihydroxybenzoate through a hydroxylation followed by a deprotonation [22].

Catalysis in these enzymes involves a C(4a)-hydroperoxyflavin species, which provides a powerful peroxo electrophile tuned for oxygen insertion at nucleophilic carbons and soft centers. Examples include hydroxylation coupled with different kinds of substitution, epoxidation, Baeyer-Villiger oxidation, and oxidation of heteroatoms (B, S, Se, N, and P) [23-26]. Formation of the C(4a)-hydroperoxyflavin species from the oxidized flavin and NAD(P)H requires two reactions coupled with dynamics of the isoalloxazine group between two positions [27]. In an external position, aside from the hydroxylation site, the oxidized isoalloxazine group is reduced by NAD(P)H. Then, the reduced isoalloxazine swings to an internal position, where it reacts with molecular oxygen to yield the C(4a)-hydroperoxyflavin species. This reaction is fast in enzymes, with rate constants typically between 10^4 and 10^8 M^−1 s^−1 at 4 °C [28-31]; in contrast, the rate constant for the corresponding reaction in water is about 250 M^−1 s^−1 at 30 °C [32].

Herein, we explore the catalytic mechanism of NahG, that, like those other monoxygenases, remains a subject of intense debate [10,20]. Particular goals are to identify the catalytic groups involved in catalysis, the reactivity of reaction intermediates, and the reaction pathway that affords the products. The mechanistic proposal resulting from this work is supported by kinetics and x-ray crystallography data that provide a close look into the active site of NahG. In addition, we report the first structure to date of an apo flavin enzyme, providing a new snapshot to the set of events that culminate with the conversion of the apo to the peroxflavin-bound protein.

2. Experimental section

2.1. Materials

Synthesis of 4-nitrosalicylic acid was as reported in the literature [33]. All other compounds were analytical grade and used without further purification. Doubly de-ionized water with a conductivity of <17.3 μS/cm was used to prepare all aqueous solutions.

2.2. Cloning, protein expression and purification

The coding sequence for salicylate hydroxylase NahG (NCBI Gene ID: 3974216) was amplified from P. putida G7 NAH7 plasmid (NCBI Accession No. AB237655) and cloned as described in the Supporting Information. Protein expression in Escherichia coli Rosetta (DE3) cells was carried out at 18 °C for 16 h by induction with IPTG. The recombinant histidine-tagged NahG was purified by nickel affinity chromatography prior to a final size exclusion chromatography. The 6xHis-tag was removed for binding and kinetic studies using TEV protease prior a second nickel affinity chromatography step. In the text, we refer to the histidine-tagged form as 6xHis-NahG, while the tag-free enzyme is termed NahG. The protein concentration was estimated using the molar extinction coefficient of 76,890 M^−1 cm^−1 at 280 nm, calculated with the ProtParam tool [34] from the amino acid composition, and corrected for the amount of FAD. The concentration of FAD was estimated at 450 nm (ε = 11,300 M^−1 cm^−1) [11], and used to calculate its absorption contribution at 280 nm with ε = 23,000 M^−1 cm^−1. Residual FAD (<30%) from protein expression was found in all purified protein samples. Detailed information regarding NahG production is provided in the Supporting information.

2.3. Dynamic light scattering and size exclusion chromatography

The hydrodynamic radius of NahG molecules and polydispersity of the purified sample were estimated using dynamic light scattering (DLS) assays performed on a Zetasizer Nano ZS system (Malvern Instruments). Protein samples were centrifuged at 17,000g for 10 min at 4 °C prior to any measurement. The final protein concentration for DLS measurements was about 1.5 mg/mL in buffer C (50 mM Tris-HCl buffer at pH 7.4, 50 mM NaCl). Each sample was measured in a 1 cm path-length quartz cuvette at 25 °C. Data were collected and analyzed using the Malvern Zetasizer software v. 6.01, in which the “Protein Utility” routine was used to estimate particle mass based on the hydrodynamic radius.

The oligomeric state of the enzyme was also estimated by size exclusion chromatography, using a calibrated HiLoad 16/60 Superdex 200 column (GE Healthcare) previously equilibrated with buffer C at a flow rate of 1.0 mL/min. The following calibration standards were used: β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); albumin (66 kDa); carbonic anhydrase (29 kDa); and cytochrome C (12.4 kDa) (from Sigma-Aldrich).

2.4. Crystallization, data collection, and structure determination

Pure and monodisperse 6xHis-NahG samples were concentrated to 30 mg/mL and 65 mg/mL were used in crystallization trials. Initial conditions were identified by high-throughput robotic screening [35] (SaltRX, Crystal Screen, and Crystal Screen 2 from Hampton Research; Precipitant Synergy, Wizard I, and Wizard II from Emerald BioSystems; PACT and JCSG+ from Qiagen) using the sitting drop vapor diffusion method at 18 °C.

Diffraction-quality crystals were obtained by the hanging drop vapor diffusion method at 18 °C after manual optimization of the condition 1.5 M ammonium sulfate, 0.1 M Tris at pH 8.5, and 12% v/v glycerol by grid screening of salt and precipitant concentrations, and buffer pH. Crystallization drops were formed by mixing equal volumes (1 μL) of protein and reservoir solutions. The crystal dimensions were approximately 0.38 × 0.18 × 0.06 mm. An iodide derivative was obtained using the quick cryo-soaking approach [36-38]. Essentially, a native crystal was soaked for about 30 s in a cryoprotectant solution – formed by the reservoir solution supplemented with 10% (v/v) ethylene glycol – additionally containing 0.06 M sodium iodide. Prior to data collection, crystals were flash-frozen in a nitrogen stream.

X-ray diffraction data were collected at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas-SP, Brazil) at the W10B-MX2 beamline [39]. X-ray diffraction images were recorded on a Rayonix MarMosaic 225 CCD detector and processed using the HKL-2000 software suite [40]. The 6xHis-NahG 3D structure was solved by the Single-wavelength Anomalous Dispersion method. Eight iodide sites were located by SHELXC/D [41]. AutoSol in the PHENIX software suite [39]. X-ray diffraction images were recorded on a Rayonix MarMosaic 225 CCD detector and processed using the HKL-2000 software suite [40]. The 6xHis-NahG 3D structure was solved by the Single-wavelength Anomalous Dispersion method. Eight iodide sites were located by SHELXC/D [41]. AutoSol in the PHENIX software suite [39]. X-ray diffraction data were collected at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas-SP, Brazil) at the W10B-MX2 beamline [39].
in the Protein Data Bank (entry 6BZ5). Pictures were generated with the program UCSF Chimera [45].

A three-dimensional search using the DALI server (http://ekhidna.biocenter.helsinki.fi/dali_server/) [46] was performed to identify proteins structurally similar to NahG. The primary structures of selected flavoprotein monooxygenases were aligned, and their crystallographic structures were superimposed, allowing a comparison between the apo and the holo forms of the related enzymes.

### 2.5. Fluorometric titrations

Experiments were carried out on a Cary Eclipse fluorometer fitted with a magnetic stirring mechanism and a Peltier single cell holder at 25 °C. Quartz cuvettes of 1 cm pathlength and 3 mL capacity were used. Solutions containing the enzyme were buffered with 55.6 mM Hepes, 181 mM NaCl and 1.11 mM EDTA ( ). The initial concentration of salicylate and 3-methylsalicylate were 200 μM and 800 μM, respectively. The reactions were initiated by addition of NahG in the same buffer as described above to give a final concentration of 0.32 μM.

### 2.6. Product identification by NMR

Reactions were followed at 25 °C by 1H NMR spectroscopy on Bruker spectrometers at 400 MHz (256 transients) and 600 MHz (128 transients), equipped with room temperature probes. The experiments were performed using 600 μL of aqueous solutions at pH 8.2 containing 10% D2O. The reaction mixture consisted of 56 mM Hepes, 150 μM FAD, 1.0 mM NADH, and an equivalent amount of NaCl to give an ionic strength of 0.222. The initial concentration of salicylate and 3-methylsalicylate were 200 μM and 800 μM, respectively. The reactions were initiated by addition of NahG in the same buffer as described above to give a final concentration of 0.32 μM.

### 2.7. Kinetics

Reactions were followed on a Cary50 spectrophotometer at 25.0 °C by monitoring NADH consumption at 340 nm. For substrates (5-NH2 and 5-CH2O substituted salicylates) that absorbed appreciably at this wavelength, reactions were followed by fluorimetry using the NADH emission at 472 nm (excitation at 340 nm). The initial rate (v) was calculated from v = D[NADH]/Δt. The NADH concentration was determined using a molar absorptivity of 6220 M⁻¹ cm⁻¹ in the spectrophotometric studies, or a standard curve for NADH in the fluorometric studies. Quartz cuvettes of 1 cm pathlength were used in all studies. The reaction mixture consisted of 56 mM Buffer, 1.1 mM EDTA, and 171 mM NaCl to give an ionic strength of 0.222. The following buffers were used according to the indicated pH ranges: Bis-Tris (pH 5.3–7.3); Hepes (pH 6.7–8.5); Bicine (pH 7.4–9.4); Chex (pH 8.6–9.9); and Caps (pH 9.7–10.3). Reactions were initiated by addition of enzyme (10 μL) using an add-mixer device. The steady-state kinetic parameters (Km and Vmax) for substrates and cofactors were determined by non-linear regression of rate vs [Substrate] or [Cofactor] using the Michaelis-Menten equation. The kcat value was calculated from kcat = Vmax/[E]o, where [E]o is the total enzyme concentration. Studies in different [O2] were carried under argon. Samples were prepared from dilution of an O2-saturated solution with a previously degassed solution obtained by three freezing-thaw cycles under vacuum. The concentration of O2 in the saturated solution was determined by the Winkler method [47].

### 2.8. Hydrogen peroxide determination

 Peroxide concentrations produced by the uncoupled path were quantified in triplicate using a modification of the Trinder method [48]. NADH oxidation (400 μM) was monitored by UV–Vis spectroscopy at 25 °C under aerobic conditions in the presence of 20 μM FAD, 0.3 μM NahG, and 120–600 μM salicylates. The pH and ionic strength were kept constant as described above for the kinetic experiments. After complete oxidation of NADH, the reaction mixture (1 mL) was mixed with a solution (1 mL) containing horseradish peroxidase (700 U/L), 20 mM phenol, and 5 mM 4-aminoantipyrine in 50 mM sodium phosphate, pH 7.2, 0.02% (w/v) sodium azide, and 0.5% (w/v) BSA (stored at 4 °C). After incubation at 37 °C for 10 min, the absorbance was measured at 505 nm. A standard curve for H2O2, previously standardized with a standard KMnO4 solution, was used to calculate the amount of H2O2 formed in the reactions catalyzed by NahG. Control experiments showed that the presence of salicylates does not affect the determination of H2O2. In addition, benzoate was used as pseudosubstrate to evaluate the efficiency of the H2O2 determination. We measured the formation of 100 ± 3% H2O2, in agreement with previous studies in which benzoate stimulates NADH oxidation in salicylate hydroxylase without substrate oxygenation [49,50].
3. Results and discussion

3.1. The overall structure of 6xHis-NahG

The 6xHis-NahG exists as a monomer in solution according to size exclusion chromatography (Fig. S1) and dynamic light scattering assays (Fig. S2), in agreement with literature observations for the native enzyme [6]. The crystal structure of 6xHis-NahG has been determined at 2.0 Å resolution using SAD phasing, and represents the first structure in the apo form for a flavin-dependent monooxygenase. Refinement statistics are summarized in Table 1.

The asymmetric unit contains two monomers of apo 6xHis-NahG, and the final model consists of residues 7–248 and 251–424 (native NahG numbering) (Fig. 1). The first six residues of the native protein, together with the earlier 16 residues for the 6xHis-affinity tag and TEV protease cleavage site, were not visible in the electron density map and therefore were not included in the final model [51]. Two other regions, corresponding to residues 249–250 and 425–434, are also lacking in the final 6xHis-NahG apo model due to weak electron density. As expected, NahG exhibits an overall α/β fold similar to that observed for other flavin-dependent monooxygenases [25,52], which consists of three domains. The central part of the first domain includes the nucleotide-binding motif or Rossman fold. The main feature of the second domain is a large β-sheet comprised by mostly antiparallel β-strands that defines a significant portion of the substrate binding cavity, while the C-terminal domain is an α-helical region arranged next to the first two domains. In some flavoprotein monooxygenases the C-terminal domain is involved in oligomerization [53–56], which seems not to be the case for NahG, given that the enzyme is a monomer in solution.

Although cells were disrupted in a lysis buffer with an excess of salicylate, no electron density for the aromatic substrate was found in the active site. Despite numerous attempts, NahG crystals could not be obtained from cocrystallization trials with salicylate, FAD, or NADH. Soaking experiments were equally unsuccessful with these ligands. A number of variables and issues influence ligand co-crystallization and soaking which can hinder formation of protein-ligand complex crystals [57].

3.2. Comparison of NahG with others flavoprotein monooxygenases

According to the DALI server [46], the apo NahG model displays good structural agreement with the salicylate hydroxylase from P. putida S-1.
and substrate-binding sites, which are in equivalent positions among structures as shown in Fig. 1 for the superimposition with the is found in a distinct closed position compared to FAD-bound (G313-D314), in which the highly conserved aspartate residue forms Rhodococcus jostii

3

A comparison of NahG with the structurally related enzymes provides insight into the active site architecture, including the FAD and substrate-binding sites, which are in equivalent positions among all the aligned structures. A remarkable exception is made for the loop comprised by the residues 38–53 in the apo 6xHis-NahG, which is found in a distinct closed position compared to FAD-bound structures as shown in Fig. 1 for the superimposition with the holo 3-hydroxybenzoate 6-hydroxylase (3HB6H, PDB entry 4BJZ, green) from Rhodococcus jostii RHA1 [22].

The N-terminal GXGXXG fingerprint sequence (G14-X-G16-XX-G19), comprising the secondary structure elements β1 and α1, represents the common [αβ]2-fold of the dinucleotide-binding domain, known to interact with the ADP moiety of the FAD cofactor [59]. The second FAD binding fingerprint consists of the GD sequence (G313-D314), in which the highly conserved aspartate residue forms hydrogen bonds with the O-3 hydroxyl group of the ribityl chain of the flavin moiety, while the glycine accommodates the PO4 group of FAD [60]. Besides, a strictly conserved DG sequence motif (D162-G163) on the large turn (residues 161–166) that connects strand β11 and helix α5 is involved in binding the pyrophosphate moieties of both FAD and NADH [61].

The putative substrate-binding pocket, inferred from superimposition of NahG structure to that of the ligand-bound structures of 4-hydroxybenzoate hydrolase (PHBH, PDB entry 1DOD) [62] and P. putida S-1 SALH (PDB entry SEVY) [10], is clearly divided into hydrophobic and hydrophilic regions. The aromatic residues F85, W87, F230, F243, and W293 are positioned on the antiparallel (i-sheet opposite from the FAD isoalloxazine ring and likely form a hydrophobic environment facing the salicylate (Fig. 2). In addition, a number of nonpolar amino acid residues, including A190, M194, M219, L221, L228, and V241, also compose the substrate-binding pocket, whereas the charged residues D224, H226, R247, H322, E381, and R383 are distinctly grouped near the active site, laterally positioned on the re-side of the isoalloxazine and leading to the solvent-accessible protein surface. Curiously, in the second closest relative, urato oxidase (HpxO, PDB entry 3RP7) [58], the charged residues R204, D293, and H348 (equivalent to H226, H332 and E381, respectively, in NahG) take part in an unusual hydrogen-bonding network which is mostly buried in a hydrophobic environment, while the catalytic residue R204 is proposed to deprotonate the substrate [58]. Indeed, a remarkable feature of the p-hydroxybenzoate hydrolase (PHBH, PDB entry 1PBE) [63] from Pseudomonas fluorescens, the best known member of this family of enzymes, is a hydrogen-bonding network connecting the phenolic group of the substrate p-hydroxybenzoate (pOHB) in the buried active site to the surface of the protein, which lowers the pKa of pOHB and favors proton exchange between the substrate and the external solvent and regulates the coordination of conformational states for efficient catalysis [54]. A similar mechanism of substrate activation has been proposed for the structural homologue 3-hydroxybenzoate 6-hydroxylase (3HB6H; PDB entry 4BJZ) [22], in which mutagenesis of the basic residue H213 has been shown to impair substrate hydroxylation without preventing substrate binding and C4a-hydroperoxyflavin formation. The role of H213 has been suggested to facilitate the deprotonation of the substrate's phenol group during the electronophilic aromatic substitution [22,64].

Several reports on PHBH have concluded that the internal proton-transfer network and substrate activation through phenolic ionization constitute a "password" or "signal", for molecular recognition, in which the enzyme tests for the presence of a suitable aromatic substrate before allowing the FAD's isooalloxazine ring to assume the "out" conformation, which is solvent accessible and required for reduction by NADPH [65–67]. Furthermore, regions of positive electrostatic potential in the active site would situate the anionic reduced flavin to the "in" position adjacent to the substrate, in addition to previously promoting the formation of the substrate phenolate [68]. Interestingly, a hydrogen-bonding network is not found in the active site of kynurenine 3-monooxygenase (KMO), in which conformational changes and flavin reduction are triggered by π-π interactions between the substrate and the loop above the re-side of the isoalloxazine group [69].

It has been established that the rearrangement of FAD between the "out" and "in" positions is required in the catalytic mechanism comprised of reductive and oxidative reactions [55,67], which are shared among NahG and other flavin-dependent monooxygenases. Unlike all known enzyme structures in this class, there is no ligand in the structure of NahG. Interestingly, a notable structural feature is a large loop connecting strand β2 and helix α2, which exhibits a conformation markedly different from that in other homologs (Fig. 3). This segment of 16 residues spanning from E38 to G53 stretches over the FAD-binding cleft, instead of being laterally positioned on the si-side of the isoalloxazine ring, as observed in the homologs. This loop is suggested to control the tunnel access that allows the substrate to enter the active site of the HpxO homologue [58]. A similar flexible loop in the phenol hydrolase from Trichosporon cutaneum moves during catalysis to accommodate changes in FAD conformation, shielding the catalytic site during hydroxyl transfer or opening a channel for substrates and products to enter or leave the active site [55].

3.3. Stoichiometry and binding constants

Formation of holo NahG and its interaction with salicylate (Sal) followed tight-binding profiles as observed by fluorometric titrations at pH 8.0 and 25 °C (Fig. 4). In holo NahG formation, the excitation of apo NahG at 292 nm gives the characteristic emission for the tryptophan residues at 340 nm (F340 at 340 nm), which is quenched by FAD binding to the enzyme (Fig. 4a). This event is solely caused by holo NahG (SALH, PDB entry 5EVY) [10], in addition to the homologs urato oxidase from Klebsiella pneumoniae (HpxO, PDB entry 3RP7) [58], 3-hydroxybenzoate 6-hydroxylase from Rhodococcus jostii RHA1 (3HB6H, PDB entry 4BJZ) [22], 6-hydroxynicotinate 3-monoxygenase from P. putida KT2440 (NicC, PDB entry 5EOW) [21], and flavin-containing monooxygenase from Pseudomonas aeruginosa (PhzS, PDB entry 2RG) [53]. The root mean square deviation for these enzymes with respect to NahG is <3.2 Å for at least 332 Cα atoms with Z-scores of z32.4 (Fig. S3).

Fig. 2. Active-site of 6xHis-NahG showing the putative positions of FAD isooalloxazine ring and salicylate (Sal) relative to the hydrophobic region formed by the residues F85, M219, H226, L228, F230, V241, and F243. The ligands FAD and Sal were modeled using superpositions with the respective structures of 3HB6H (PDB entry 4BJZ) [22] and PHBH bound to 2,4-dihydroxybenzoate (PDB entry 1DOD) [62], in which the 4-OH group was suppressed to give Sal. The hydrophobic region depicts only residues within 5 Å of distance from the plane formed by all Sal atoms.
formation, since FAD shows no substantial effect in the $F_{\text{obs}}$ at 340 nm in absence of enzyme and the self-inner filtering effect due to FAD absorption accounts for ~2% of the fluorescence quenching as estimated according to the method of Birdsall et al. [70].

The tight-binding profile in holo NahG formation (Fig. 4b) fits to Eq. (1), in which the holo NahG molar fraction ($\chi_{\text{bound}}$) is calculated by Eq. (2). The $F_{\text{obs}}$, $F_{\text{free}}$, and $F_{\text{bound}}$ represent the fluorescence intensities, respectively, for the observed, free, and fully bound enzyme, and $[L]_o$

![Fig. 3](image)

**Fig. 3.** FAD binding cleft showing three conformational states: (a) the apo state is represented by 6xHis-NahG, in which the loop from E38 to G53 is found in a closed conformation; (b) and (c), the equivalent loop lies in an open conformation upon FAD binding, and the isoalloxazine ring swings between the “out” (PDB entry 3C96 for the flavin-containing monooxygenase phzS from *Pseudomonas aeruginosa*) and “in” (PDB entry 4BJZ for the 3HB6H) states.

![Fig. 4](image)

**Fig. 4.** Formation of holo NahG and its interaction with salicylate (Sal) at pH 8.0, $I = 0.222$ (NaCl) and 25 °C: (a) and (c), fluorescence spectra of apo (0.74 μM) and holo NahG (1.0 μM) as a function of FAD and salicylate concentrations, respectively; (b) and (d), fluorescence intensity at the indicated wavelengths as a function of ligand concentration. Arrows in each fluorescence spectra indicate the direction of an increasing ligand concentration. Solid lines are fits according to Eq. (1) and the following parameters: (b) $F_{\text{free}} = 566$, $F_{\text{bound}} = 203$, $[E]_o = 0.74$ μM and $K_d = 2 \times 10^{-9}$ M, (d) $F_{\text{free}} = 0$, $F_{\text{bound}} = 285$, $[E]_o = 1.0$ μM and $K_d = 2.2 \times 10^{-7}$ M.
and \([E]_0\) are the total concentrations of the ligand and enzyme, respectively. The data provides an estimated dissociation constant \((K_d)\) of \(2 \times 10^{-9} \text{ M}\) for the holo NahG, which is in the same range reported for the salicylate hydroxylase from \(P.\ putida\ S-1\) \((K_d = 45 \times 10^{-9} \text{ M})\) \cite{71}. The inflection point occurs near to equivalent concentrations of FAD and apo NahG as expected for a 1:1 complex.

\[
F_{\text{obs}} = F_{\text{free}} + (F_{\text{bound}} - F_{\text{free}})K_d/E_0
\]

\[
X_{\text{bound}} = \frac{(E_0 + E_0 + K_d) - \sqrt{((L)_0 + [E]_0 + K_d)^2 - 4[E]_0 K_d}}{2[E]_0}
\]

The interaction of salicylate with holo NahG was followed by excitation at 292 nm and emission at 410 nm \((F_{\text{obs}} \text{ at } 410 \text{ nm, Fig. 4c})\). The emission is associated with intramolecular proton transfer in the excited state (ESIPT), which is common for \(o\)-substituted phenols with acidic groups \cite{73}. The fluorescence intensity for salicylate follows a tight-binding profile for a 1:1 complex \((\text{Fig. 4d})\). The \(K_d\) calculated by non-linear curve fitting with Eq. (1) is \(2.2 \times 10^{-7} \text{ M}\) for the salicylate- holo NahG complex, about 10 times lower than reported by Suzuki et al. \((K_d = 3.5 \times 10^{-6} \text{ M})\) for the corresponding substrate-enzyme complex using the salicylate hydroxylase from \(P.\ putida\ S-1\) \cite{71}. The fluorescence increase is consistent with salicylate binding to NahG, which hinders the substrate from interaction with the solvent and results in efficient radiative decay.

### 3.4. Identification of reaction products

We probe the regioselectivity of the oxidative decarboxylation of salicylate catalyzed by holo NahG using 3-methylsalicylate as substrate. Comparing the \(1^H\ NMR\ spectra \((\text{Fig. 5})\) before and after partial substrate conversion shows that 3-methylsalicylate signals disappear at 2.14 ppm \((3H, \text{ singlet}), 6.79 \text{ ppm} \,(1H, \text{ triplet}), 7.26 \text{ ppm} \,(1H, \text{ doublet})\) and 7.59 ppm \((1H, \text{ doublet})\) and new signals for 3-methylcatechol appeared at 2.13 ppm \((3H, \text{ singlet})\) and 6.67-6.70 ppm \((3H, \text{ multiplet})\) in agreement to a pure sample. The conversion of salicylate to catechol catalyzed by NahG shows a similar profile \((\text{Fig. 5})\). These results are strong evidences of \(ipso\) substitution in the salicylate reaction catalyzed by NahG, as the hydroxylation of 3-methylsalicylate in a carbon site different from C-1 would not afford 3-methylcatechol. Similar observation is reported for salicylate hydroxylase from \(P.\ putida\ S-1\), which also catalyzes the conversion of 3-methylsalicylate into 3-methylcatechol \cite{74}. The \(ipso\) substitution is also supported by computational studies \cite{20} and by early experimental studies \cite{11}, which elegantly showed that salicylate hydroxylase catalyzes the conversions of 2,3- and 2,6-dihydroxybenzoates to the same product pyrogallol, while 2,4- and 2,5-dihydroxybenzoates are converted to different products. These observations do not support hydroxylation at the C-3 atom of salicylate followed by decarboxylation of a 2,3-dihydroxybenzoate intermediate as previously reported \cite{10}.

In general, aromatic hydroxylations by flavin-monooxygenases occur via electrophilic aromatic substitution \((\text{SaAr})\). The \(ipso\) substitution is favored by phenol groups at ortho and para positions of the aromatic ring \cite{75}. Salicylate undergoes decarboxylation in the presence of salicylate hydroxylase, but there are several other examples, including NahG itself \cite{76,77}, which the step following the hydroxylation involves deprotonation, denitration, dehalogenation, among others \cite{25,75}. In some cases, the hydroxylation is followed by deprotonation, for instance, 3-hydroxybenzoate is converted to 2,5- or 3,4-dihydroxybenzoate in the presence of 3-hydroxybenzoate 6-hydroxylase \((3\text{HBBH})\) \cite{29,64} or 3-hydroxybenzoate hydroxylase \((\text{MHBH})\) \cite{56}, respectively. On the other hand, 3,4-dihydroxybenzoate can also be obtained from 4-hydroxybenzoate in the presence of 4-hydroxybenzoate hydroxylase \((\text{PHBH})\) \cite{62}. The reasons that control the regioselectivity in these enzymes are still not well understood, nonetheless, hydroxylation seems to be the key step \cite{79}. Literature reports show that mutation of nonpolar residues at the active-site \cite{80} and the presence of co-substrates \cite{22} may change the orientation and proximity of the substrate to the C4a-hydroxyperoxyflavin group in the hydroxylation step.

### 3.5. Kinetic studies

The Michaelis-Menten parameters shown in Table \(2\) were determined at pH 8.0 and 25 °C for salicylate, dioxygen \((O_2)\), and NADH. The \(v/\text{[E]}_0\) \((\text{s}^{-1})\) measured at different concentrations of salicylate and dioxygen were determined in the presence of 200 \(\mu\text{M}\) NADH, 84% of saturation based on the \(K_m\) value for NADH \((\text{Fig. 5S})\). The same experiments under different concentrations of dioxygen and NADH were carried out in the presence of 192 \(\mu\text{M}\) salicylate, which represents 87% of saturation regard the \(K_m\) value for salicylate. The FAD and dioxygen concentrations were kept under saturation conditions, except when varied. The kinetic data under increasing concentrations of FAD fitted to a 1:1 binding model with a \(K_d\) value of 150 ± 30 \(\text{nM}\) \((\text{Fig. 5Sc})\). Therefore, \(k_{\text{cat}}\) represents the product of \(k_{\text{cat}}\) and the molar fractions for salicylate.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sal</th>
<th>(O_2)</th>
<th>FAD</th>
<th>NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) ((\text{mM}))</td>
<td>28 ± 3</td>
<td>3.2 ± 0.4</td>
<td>0.15 ± 0.03</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>(k_{\text{cat}}) ((\text{s}^{-1}))</td>
<td>3.3 ± 0.1(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) In 56 mM Buffer HEPES, 1.1 mM EDTA and \(I = 0.222\) (\(\text{NaCl}\)), [NahG] = 16–64 \(\text{nM}\). Except where the concentration was varied, salicylate, FAD, and NADH concentrations were 192 \(\mu\text{M}\), 31.5 \(\mu\text{M}\) and 200 \(\mu\text{M}\), respectively. Errors are standard deviations.

\(^b\) Corrected for the uncoupled path \((g = 2\%);\) uncorrected \(k_{\text{cat}} = 3.5 ± 0.1 \text{ s}^{-1}\).
dioxygen, FAD, and NADH. Because the C(4a)-hydroperoxylvlin may generate H2O2 without causing substrate oxygenation (uncoupled path) [49,50], the $k_{\text{cat}}$ was also corrected to the amount of H2O2 (5.5%) determined after complete NADH oxidation in the presence of excess substrate.

The $k_{\text{cat}}$ and $K_m$ values for salicylate and NADH catalyzed by NahG at pH 8.0 and 25 °C (Table 2) have the same magnitude reported for the salicylhydroxylase from *P. putida* S-1, in which the $k_{\text{cat}}$ is about 21 s⁻¹ and $K_m$ values are 1.6–1.9 μM for salicylate and 2.6–8.2 μM for NADH [11,72,81]. Stopped-flow kinetic data for salicylate hydroxylase from *P. putida* S-1 indicated that salicylate binds prior NADH, and its oxidation to afford catechol is rate-limiting [72].

### 3.6. Linear free energy relationships

Further details about the mechanism of catalysis were determined in the presence of 200 μM NADH (84% saturation) and increasing concentrations of substituted salicylates (Fig. S6 and S7). The $k_{\text{cat}}$ values were corrected for the uncoupled path, estimated from H2O2 determinations after complete NADH oxidation in excess of each substrate. We observed that $k_{\text{cat}}$ varies systematically for salicylates substituted with electron-donating and electron-withdrawing groups (Table 3). This observation is consistent with formation and cleavage of covalent bonds affected by the electronic demand in the substrate and inconsistent with a rate-limiting step controlled only by diffusion of substrate, products, or cofactors.

The kinetic parameters for the substituted salicylates were fitted to Hammett relationships (Fig. 6) as described by Eq. (3). Interested readers may find background information in chemistry textbooks [82–85] and reviews [86,87]. The $\sigma_k$ (x-axis) corresponds to the values of $\sigma_{\text{carboxy}}$ and $\sigma_{\text{ap}}$ [86] for the respective substituents at positions 4 and 5 of salicylate, $\rho$ is the sensitivity factor as commonly employed in linear free energy relationships.

$$\log k_{\text{cat}}^\text{ap/(X)} \left( K_m^{(X)} \right) = \rho \sigma + \log k_{\text{cat}}^\text{ap/(H)} \left( K_m^{(H)} \right)$$

(3)

Linear Hammett relationships provide information for rate-limiting steps that are affected by the electronic demand in the substrate. The presence of two intersecting straight lines indicates a change in the rate-limiting step. This effect is found for both $K_m$ and $k_{\text{cat}}$ near $\sigma_{\text{carboxy}} = 0$. Linear behaviors are evident for both parameters at $\sigma_{\text{carboxy}} > 0$ and for $K_m$ at $\sigma_{\text{carboxy}} < 0$. The $k_{\text{cat}}$ values for 5-amino and 5-methoxy salicylates do not correlate with the others and are lower than expected. These deviations are discussed later in the text. Poor relationships are observed with $\sigma_{\text{phenol}}$ (i.e., $\sigma_{\text{meta}}$ and $\sigma_{\text{para}}$ of the respective substituents at positions 4 and 5 of salicylate, Fig. S8).

The Hammett plot observations fit the mechanism proposed in Scheme 2, an aromatic electrophilic substitution (S_{Ar}) through a Wheland-type intermediate, in which either hydroxylation or decarboxylation is rate-limiting, depending on the electronic character of the aryl substituent. The S_{Ar} mechanism proposal is also supported by a recent theoretical study [20].

Analysis of the Hammett plot for $k_{\text{cat}}$ considers only the rate-limiting step. The hydroxylation step is facilitated by electron donating groups ($\sigma_{\text{carboxy}} < 0$) that increase the nucleophilicity of the aryl nucleophile, but when $\sigma_{\text{carboxy}} > 0$ the hydroxylation step is slower and rate-limiting. The converse is observed when $\sigma_{\text{carboxy}} < 0$, in which the decarboxylation is the rate-limiting step and faster towards electron withdrawing groups.

The log $K_m$ values correlate well with $\sigma_{\text{carboxy}}$ (Fig. 6). In the hydroxylation step, the less steep $\rho$ of –2.6 for log $k_{\text{cat}}$ compared to the $\rho$ of –3.5 for log $K_m$ represents the additive effects of electron-withdrawing groups to the formation of the ES complex. Because $K_m = (k_{\text{cat}} + k_{\beta\text{hyd}})/k_{\beta\text{hyd}}$, in the hydroxylation step, when the equilibrium is shifted towards ES, $K_m$ decreases and $k_{\beta\text{hyd}}$ increases with electron-withdrawing groups, resulting in a lower $K_m$. When the decarboxylation step is rate limiting, $K_m = (k_{\text{cat}} + k_{\beta\text{dec}\text{hyd}})/k_{\beta\text{dec}\text{hyd}}$, where $k_{\beta\text{dec}\text{hyd}}$ in the case is $\rho$ less steep than in the hydroxylation step because $k_{\beta\text{dec}\text{hyd}}$ decreases with electron-donating groups in contrast to the effect of electron-withdrawing groups in formation of the ES complex.

The driving-force for decarboxylation of the Wheland-type intermediate is provided by the hydrophobic pocket formed by the residues P56, L228, F230, V241 and F243 (Fig. 2). When the salicylate’s C-1 atom is hydroxylated at the re-face by the C4a-hydroperoxylvlin, the carboxyl group is pushed towards the hydrophobic pocket situated at the si-face. The resulting electrostatic destabilization of the Wheland-type intermediate favors the more neutral transition state leading to catechol and carbon dioxide formation (Scheme 2). It is worth mentioning that such assistance of a hydrophobic pocket for decarboxylation is a common feature of decarboxylases [88–91].

NahG activity requires the presence of a hydroxyl group ortho to the carboxyl group, as shown by the fact that NahG does not catalyze the decarboxylation of benzoate. The hydroxyl group allows the generation of the aryl nucleophile in the S_{Ar} step, and, as a carboxyl group in the second step, assists the oxidative decarboxylation of salicylate as an electronic relay providing charge dispersion in the transition state. Thus, substituents simultaneously influence the hydroxyl and carboxyl groups on the salicylate. Therefore, it is not surprising that the $k_{\text{cat}}$ values for 5-amino and 5-methoxy are lower than predicted by the linear fit at $\sigma_{\text{carboxy}} < 0$ (Fig. 6). In these cases, the rate-limiting step is decarboxylation, in which the amino and methoxy groups at position 5 of salicylate contribute by resonance in the Wheland-type intermediate and increase the electron density at the carbon neighboring the site of decarboxylation. This slows down the decarboxylation because the restoration of the resonance in the transition state is impaired in higher extent compared to groups that show only electron-donation by inductive effects.

The Jaffé relationship (Eq. (4)) [87,92] separates the electronic effects over both groups in the salicylate and for each rate-limiting step. Scheme 3 explains the effect of the aryl substituent on each step. In the hydroxylation step, $r_x$ and $r_{\beta\text{hyd}}$ arise from groups para-positioned relative to the carboxyl and phenol groups, respectively, which activate these positions by resonance. On the other hand, activation by resonance in the decarboxylation step is provided by groups meta-positioned relative to the carboxyl and carbonyl sites, which give $r_x$ and $r_{\beta\text{dec}\text{hyd}}$ as sensitivity factors, respectively. It is important to note that the distance between the substrates and the reaction centers in the decarboxylation step is shorter than in the hydroxylation step, and therefore, the influence
of substituents on the $\rho$ values is expected to be higher in the decarboxylation step.

$$\frac{1}{\sigma_{\text{carboxy}}} \log \frac{k_{\text{cat}}^\text{ap (X)}}{k_{\text{cat}}^\text{ap (H)}} = \sigma_{\text{phenol}} + \rho_Y + \rho_X$$

(4)

Fig. 7 results from application of Eq. (4) to the data in Table 3. Well-defined linear relationships were obtained for each rate-limiting step, sorted according to the electronic character of the salicylate substituent, as discussed above for the Hammett relationships. The hydroxylation or decarboxylation step becomes rate-limiting when salicylate is substituted with electron-withdrawing or electron-donating groups, respectively.

The $\rho_X = -2.0$ in the hydroxylation step results from the effect of electron-donating groups that increase the nucleophilicity of the salicylate C-1 atom. This effect is smaller than observed for non-enzymatic SEAr’s, where $\rho$ values range from $-3$ to $-12$ [93,94]. The $\rho_Y$ of 0.1 is consistent with minimal to no charge formation on C-2 or phenolic oxygen atoms in the transition state (Scheme 2), a situation satisfied by a concerted proton transfer from the phenol group to a nearby general-base. The most likely general base in NahG is H226, which is structurally equivalent to H211 and H213 in 6-hydroxynicotinate 3-monoxygenase (NicC) [21] and 3-hydroxybenzoate 6-hydroxylase (3HB6H) [22,64], respectively. In both enzymes, these residues have been proposed to promote substrate activation by the same mechanism. As observed for H213A 3HB6H mutant [22,64], the H226A mutation in NahG also resulted in an inactive enzyme for NADH oxidation and catalysis.

The $\rho_Y = 1.9$ in the decarboxylation step is consistent with the effect of electron-withdrawing groups that weaken the bond between the C-1 atom and the carboxyl group and stabilize the developing charge in the transition state. In contrast, the $\rho_X = -2.3$ denotes the effect of electron-donating groups over the carbonyl position of the Wheland-type intermediate, which is converted to catechol by general-acid catalysis of the protonated H226 generated in the hydroxylation step (Scheme 2).

3.7. pH-rate profile

The $k_{\text{cat}}^\text{ap}$ and $K_m$ values for the oxidative decarboxylation of salicylate catalyzed by NahG were determined from pH 3.8 to 11 in the presence of 200 $\mu$M NAD$^+$ at 25 °C (Table S1). The $K_m$ values are

Scheme 2. Proposed mechanism for NahG catalyzed the oxidative decarboxylation of substituted salicylates.
lower under acidic conditions (Fig. S9), an effect that is not caused by protonation of salicylate to give salicylic acid, which has \( pK_a \) values of 3.0 and 13.7 at 25 °C [95] and is found in the monoanionic state over the entire pH range of these kinetic experiments. The effect on \( K_m \) is ascribed to protonation of active site groups, most likely histidine residues (viz. H110, H226 or H322) as revealed by inspection of the x-ray structure.

The \( \log k_{cat}^{ap} \) follows a bell-shaped profile as a function of pH (Fig. 8) and is uncorrected for the contribution of the uncoupled path, which routine \( H_2O_2 \) measurements between pH 5–10 showed to account for ~6% of the overall NADH consumption. A reaction pathway that accounts for the pH effect on the oxidative decarboxylation of salicylate is shown in Scheme 4. On the basic limb of the bell, \( \log k_{cat} \) decreases with a slope of \(-1\) as result of a single deprotonation of \( ESAH_1 \) to afford the catalytically inactive \( ESA \) species. On the acidic limb, the \( \log k_{cat} \) decreases as result of acid-base equilibria for two active species of the enzyme. The \( ESAH_2 \) deprotonation provides the \( ESAH_3 \) species that converts the substrate to product by \( k_i \), whereas, under slightly basic conditions, the \( ESAH_2 \) deprotonates to afford the \( ESAH_1 \) species, which is the most active species and catalyzes the reaction through \( k_{max} \). These rate constants correlate with \( k_{cat}^{ap} \) according to Eq. (5), which gives Eq. (6) when the molar fractions of the catalytic species \( ESAH_1 \) and \( ESAH_2 \) are expressed in relation to \( K_{ES1}, K_{ES2}, \) and \( K_{ES3} \). The logarithmic form of Eq. (6) was used to fit the data in Fig. 8, which afforded the kinetic and equilibrium parameters shown in Table 4.

\[
k_{cat}^{ap} = k_i X_{ESA} + k_{max} X_{ESA1}
\]  

\[
k_{cat} = \frac{\frac{H^+}{K_{ES1}} + \frac{K_{ES1}}{[H]} + \frac{K_{ES1}K_{ES2}}{[H]^2} + \frac{H^+}{K_{ES2}} + 1}{K_{ES3}}
\]

We attribute the \( pK_a \) of 10.6 to the hydroxyl peroxide in the C4a-hydroperoxoflavin, which is the electrophile in the hydroxylation step. Deprotonation of the C4a-hydroperoxoflavin would render it catalytically inactive. This \( pK_a \) is in the range observed for other peroxides. Specifically, the \( pK_a \) for \( H_2O_2 \) is 11.6 [95], and free C4a-hydroperoxoflavin has a \( pK_a \) of about 9.2 [96]. The acidity of enzyme-bound C4a-hydroperoxoflavin may vary. A \( pK_a > 9.4 \) has been estimated for the reaction catalyzed by p-hydroxyphenylacetate 3-hydroxylase [97], while a \( pK_a = 8.4 \) has been reported for cyclohexanone monoxygenase [98].

The rate increase from pH 4 to 8 is consistent with the role of basic residues in catalysis. We attribute the \( pK_a \) of 6.6 to the conjugate acid of H226, general-base in hydroxylation step, and directly related to the formation of most active species \( ESAH_1 \). The identity of the residue responsible for the \( pK_a \) of 4.2 is still uncertain. Our best assumption is H110, which is near the NADH- and FAD-binding domains and close to the cationic side chain of R111 that may alter the acidity of H110 from the \( pK_a \) of 6.5 for free histidine. Mutagenesis studies are underway to test this hypothesis and will be reported in a future communication.

4. Conclusions

The crystal structure for the FAD-unbound NahG from P. putida G7 adds a new snapshot for the events that culminate with conversion of the apo to the peroxyflavin-bound protein. The loop of 16 residues

---

**Scheme 3.** Guide for analysis of substituent effects on the oxidative decarboxylation of salicylate.

**Scheme 4.** Species in the reaction pathway of NahG.
from E38 to G53 stretches over the FAD-binding cleft. Its closed conformation in the apo form must change to an open conformation for FAD binding. In the holo protein, the E38-G53 loop forms a lid over FAD and contributes to strong interactions responsible for the tight binding of FAD. The formation of the peroxyflavin species is fast and highly favorable in NahG. Our results provide compelling evidence and new information on the catalytic groups involved in catalysis, the reactivity of reaction intermediates, and the reaction pathway. Catalysis by salicylate hydroxylase (NahG), and very likely by other flavin-dependent monooxygenases, occurs through a SEAr mechanism involving a Wherland-like intermediate. This step aids by H226, that, in proximity to the phenol group, drives the decarboxylation of the Wheland-like intermediate. Intermediate formation is favored by electron-withdrawing groups. The Wheland-like intermediate is favored by the peroxo moiety in the peroxyflavin species. Intermediate formation is favored by the transposition of the generally encoded catalytic base by a tyrosine recombinase (J. Bacteriol. 188 (2006) 4057–4067).

Conflicts of interest

The authors declare no competing interests.

Acknowledgment

We thank Dr. Masataka Tsuda and Dr. Masahiro Sota for kindly providing us the P. putida G7 strain, Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil), Laboratório Nacional de Biociências (LNBio, Campinas, Brazil), INCT-Catalysis, VALE S.A., and the Brazilian Foundations CNPq, CAPES and FAPEMIG for financial support.

Author contributions


Appendix A. Supplementary data

Experimental procedures for cloning, expression and protein purification. Size exclusion chromatogram and dynamic light scattering for apo NahG. Sequence alignment of NahG with other flavin-dependent monooxygenases. NMR spectra for the oxidative decarboxylation of salicylate catalyzed by NahG. Kinetic data for the reaction of NahG-catalyzed oxidative decarboxylation of substituted salicylates.

Table 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_0$ (s$^{-1}$)</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>$k_{max}$ (s$^{-1}$)</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>$pK_{cat}$</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>$pK_{cat}$</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>$pK_{cat}$</td>
<td>10.6 ± 0.1</td>
</tr>
</tbody>
</table>

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiomac.2019.01.135.

References

1. Introduction

The mechanism of oxygen activation in biological systems is a fundamental question in biochemistry and enzymology. The oxygen activation process typically involves the formation of superoxide anion, which can then be further oxidized to peroxide and finally to water. This process is catalyzed by various enzymes, each with a specific mechanism and function.

2. Oxygen Activation in Enzymes

Oxygen activation in enzymes is a complex process that involves the interaction of the enzyme with the substrate and the solvent. The enzyme-substrate complex is stabilized by non-covalent interactions, such as hydrogen bonding and van der Waals forces, which facilitate the oxygen activation process.

3. Protein Structures and Enzymes

The structures of enzymes, such as superoxide dismutase and hydrogen peroxidase, have been studied extensively. These enzymes have been characterized at the atomic level, and their structures provide insights into the functional and mechanistic aspects of oxygen activation.

4. Conclusion

The oxygen activation process is a fundamental aspect of biological systems, and understanding it is crucial for the development of new drugs and therapies. Further research is needed to elucidate the molecular mechanisms of oxygen activation in different enzymes and to identify new targets for the development of therapeutic agents.


