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REPORT

Exploring substrate binding in homoprotocatechuate 2,3-dioxygenase using isothermal titration calorimetry

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Abstract Homoprotocatechuate 2,3-dioxygenase (HPCD) is a member of the extradiol dioxygenase family of non-heme iron enzymes. These enzymes catalyze the ring-cleavage step in the aromatic degradation pathway commonly found in soil bacteria. In this study, isothermal titration calorimetry (ITC) is used to measure the equilibrium constant ($K = 1.1 \pm 0.6 \times 10^6$) and enthalpy change ($\Delta H = -17.0 \pm 1.7$ kcal/mol) associated with homoprotocatechuate binding to HPCD. The ITC data are consistent with the release of approximately 2.6 protons upon binding of the substrate to HPCD. These results raise new questions regarding the relationships between substrate, protein, and the oxygen activation mechanism for this class of non-heme metalloenzymes.

Keywords Isothermal titration calorimetry · Extradiol dioxygenase · Substrate binding · Two histidine/one carboxylate facial triad · Non-heme iron

Abbreviations

ACES	*N*-(2-acetamido)-2-aminoethanesulfonic acid
CTD	Catechol 2,3-dioxygenase
DHBD	2,3-Dihydroxybiphenyl 1,2-dioxygenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPCA	Homoprotocatechuate
HPCD	Homoprotocatechuate 2,3-dioxygenase
ITC	Isothermal titration calorimetry
MOPS	3-Morpholinopropane-1-sulfonic acid
4-NC	4-Nitrocatechol
PIPES	1,4-Piperazinediethanesulfonic acid

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Extradiol dioxygenases are a family of bacterial metalloenzymes that catalyze the ring-opening step in the biodegradation of aromatic compounds [1–3]. The active site of these enzymes contains a non-heme Fe^{2+}, which is bound to the enzyme through two histidine and one glutamate side-chain residues that occupy one face of the octahedral coordination environment, leaving the three adjacent labile sites for substrate coordination (Fig. 1, top). In this two histidine/one carboxylate facial triad binding motif [4, 5], the reactive metal site is partially buried within the protein, allowing the protein structure to play a significant role in stabilizing the enzyme/substrate complex (Fig. 1, bottom). These enzyme/substrate interactions are likely to be a significant factor in determining enzyme selectivity and may contribute to efficient dioxygen activation in these enzymes [6–8].

The proposed oxygen activation mechanism associated with the extradiol dioxygenases is based on kinetic, structural, and computational data collected on the reaction of the *Brevibacterium fuscum* homoprotocatechuate 2,3-dioxygenase (HPCD) with 4-nitrocatechol (4-NC) [7, 9–11]. Although 4-NC is not the native substrate for HPCD, it is cleaved in a similar fashion as the natural substrate, homoprotocatechuate (HPCA), and has been commonly used as an HPCA surrogate. 4-NC exhibits characteristic visible absorption bands that are sensitive to its ionization and oxidation states [7, 12]. These spectral changes have

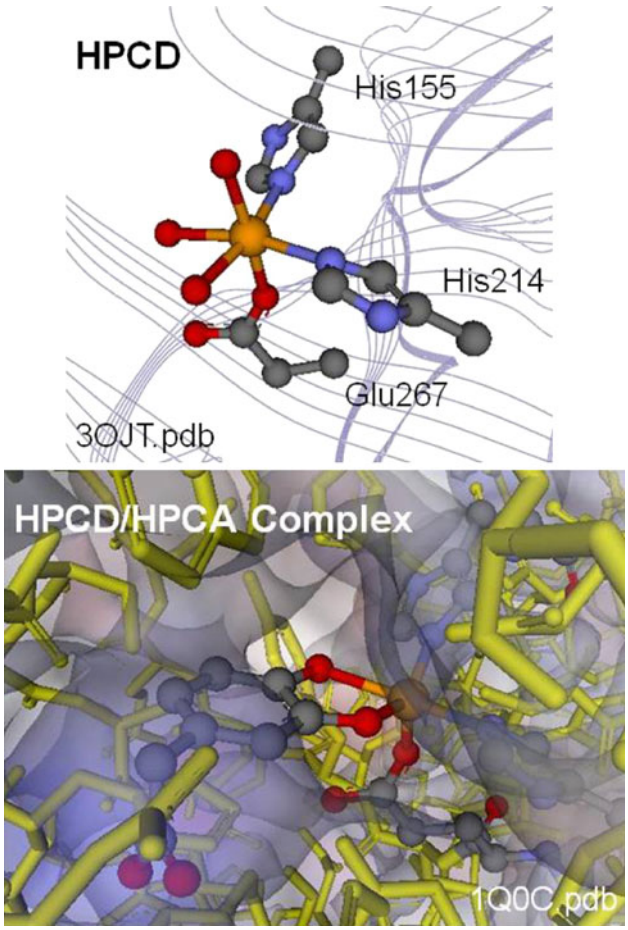


Fig. 1 Active-site structure of *HPCD* from *Brevibacterium fuscum* (*top*) and the enzyme/substrate complex (*bottom*). The non-heme iron center (*orange sphere*) is coordinated by *HPCA* and the two histidine/one carboxylate facial triad as described by coordinates from Protein Data Bank file 3OJT.pdb. The HPCD/HPCA complex is shown with the surrounding protein side chains (*yellow structure*) along with the atomic surface of the protein. The image was generated using Accelrys Discoversy Studio Visualizer 2.5

been used to elucidate the mechanistic steps associated with dioxygen activation and the aromatic ring-opening chemistry in HPCD. However, the protonation state of the enediol unit in HPCA differs from that of 4-NC in that at neutral pH, 4-NC is monoanionic whereas HPCA is a neutral molecule [13]. 4-NC has been shown to bind to the Fe^{2+} in HPCD as a dianion [7]. This bound dianionic form of 4-NC will lower the redox potential of the ferrous iron, effectively inducing its dioxygen reactivity. HPCA is proposed to lose a single proton when forming the enzyme/substrate complex [1, 8, 14, 15], although there is currently limited evidence supporting this notion in the literature. The crystal structures of the HPCD/HPCA complex show HPCA binding in an asymmetric fashion, supporting a monoanionic binding mode [8]. However, in related HPCD/4-NC structures where the substrate is known to be in a dianionic form, the corresponding bond lengths appear to be asymmetric as well [16]. In related catechol dioxygenases, spectroscopic evidence of monoanionic substrate binding to similar non-heme iron sites has been reported. In catechol 2,3-dioxygenase, X-ray absorption spectroscopic studies report a short Fe–O/N bond (approximately 1.9 Å) in the enzyme/substrate complex, which was assigned as a deprotonated metal-bound hydroxide from the catechol [15]. In the dihydroxybiphenyl dioxygenase system, a series of resonance Raman experiments yielded vibrational data supporting the catecholate substrate bound in a monoanionic form [14].

In this study, we provide the first thermodynamic data on HPCA binding to the active site of HPCD, which also yields information regarding the number of protons released as the substrate is bound. A series of isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC instrument (MicroCal, Northampton, MA, USA) under anaerobic conditions. These steps are necessary in order to separate the substrate binding interactions from the accompanying heat effects of further reactions associated with dioxygen activation and ring fission. ITC experiments directly measure the observed change in enthalpy (ΔH_{obs}), the stoichiometry, and the equilibrium constant (K) for formation of the HPCA/HPCD complex [17.] ΔH_{obs} is a summation of all enthalpy changes related to the ligand binding interaction, including terms associated with solvent dissociation, substrate binding, dilution of the substrate and protein, and the change in ionization state of the buffer. A representative baseline-corrected raw data set and the related isotherm associated with HPCA binding to HPCD are shown in Fig. 2 (top and bottom panels, respectively). Average ITC data for binding of HPCA to HPCD in a range of buffers are summarized in Table 1.

The number of protons lost by HPCA upon binding to HPCD was calculated from the ΔH_{obs} values for HPCA binding to HPCD in various buffers at pH 7.2. The heats of buffer ionization were plotted against ΔH_{obs} (Fig. 3), for which the number of protons released (n) and the intrinsic enthalpy change (ΔH^*) for substrate binding can be extracted [18, 19]. The related thermodynamic parameters, change in entropy (ΔS) and free energy (ΔG), were calculated from K and ΔH^* using the following relationships: $\Delta G = -RT \ln K$ and $\Delta G = \Delta H - T\Delta S$.

As expected, the binding of HPCA to HPCD is well described by a single-site binding equilibrium linked with proton release. From the observed enthalpy change (ΔH_{obs}) associated with substrate binding in a range of Good's buffers, we calculate approximately 2.6 ± 0.1 protons are released as each HPCA molecule binds to HPCD. The ΔH_{obs} values measured for HPCA binding to HPCD in phosphate and *N*-(2-acetamido)-2-aminoethanesulfonic

acid (ACES) are anomalously low, suggesting the there is an additional enthalpy term of approximately +9 kcal/mol masking the heat associated with substrate binding. We attribute this additional enthalpy to buffer interactions with the enzyme, either nonspecific in nature or buffer–metal interactions within the active site. In any case, the thermodynamic data reported here are fully consistent with the notion that HPCA is at least singly deprotonated when coordinated to the iron center of HPCD. However, the additional 1.6 protons released upon HPCA binding raises new questions regarding the mechanism of substrate binding. This additional proton loss could be accounted for by a shift in the pK_a value of a side-chain amino acid positioned in or near the substrate binding pocket to help stabilize the enzyme/substrate complex. Reasonable candidate residues include local histidine residues known to be involved in the dioxygen activation mechanism, such as His200 and His248 [6, 20]. Alternatively, the additional 1.6 protons could also result from the complete deprotonation of HPCA as it binds to HPCD, as shown in Scheme 1, and further deprotonation of a local amino acid residue. This

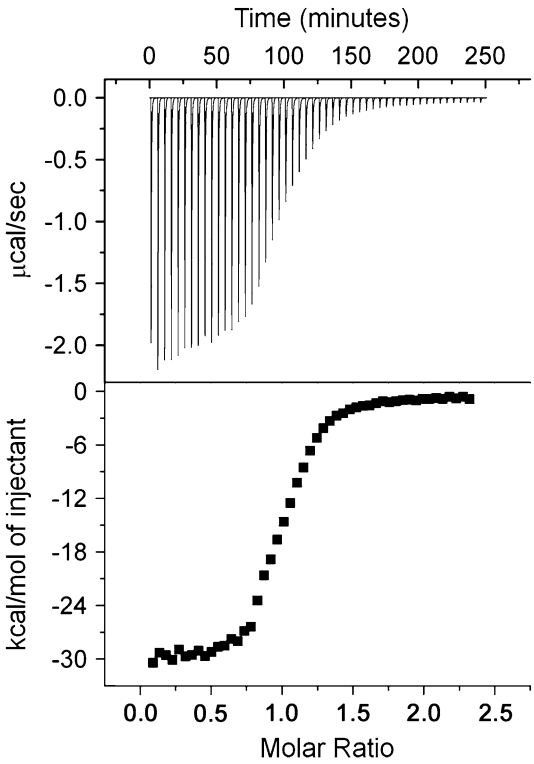


Fig. 2 Isothermal titration calorimetry (ITC) data of HPCA titrated into HPCD in 25 mM 3-morpholinopropane-1-sulfonic acid (MOPS) pH 7.2 at 25 °C. *Top panel*: The raw baseline-smoothed ITC data plotted as heat flow versus time. *Bottom panel*: The integrated and concentration-normalized heat for each injection. The nonlinear least-squares analysis of the data was done using a one-site model to generate the best-fit values reported in Table 1

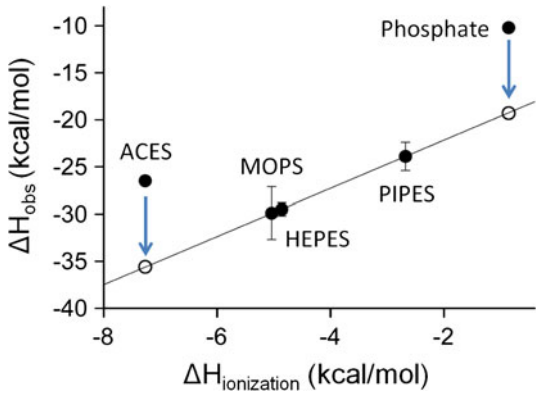
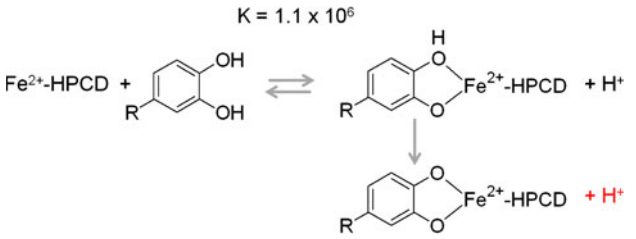


Fig. 3 Observed change in enthalpy (ΔH_{obs}) versus the enthalpy change associated with ionization of the buffer ($\Delta H_{ionization}$) for experiments associated with HPCA binding to HPCD. *Error bars* are one standard deviation unit associated with at least two, and more commonly three trials. The correlation line was generated from MOPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (*HEPES*), and 1,4-piperazinediethanesulfonic acid (*PIPES*) data with $r^2 = 0.99$. The slope of this line, 2.6 ± 0.1, represents the number of protons released and the *y* intercept is −17.0 ± 1.7 kcal/mol, which is the enthalpy change of substrate binding (ΔH^*). Phosphate and *N*-(2-acetamido)-2-aminoethanesulfonic acid (*ACES*) data fit to the line when adjusted for an additional +9 kcal/mol process (*open circles*)

Table 1 Thermodynamic parameters associated with homoprotocatechuate (HPCA) binding to homoprotocatechuate 2,3-dioxygenase (HPCD)

25 mM buffer pH 7.2	*K*	ΔH_{Obs} (kcal/mol)	$\Delta H_{ionization}$ (kcal/mol)	ΔH^* (kcal/mol)	ΔG (kcal/mol)	ΔS (cal/mol K)
MOPS	$1.6 \pm 0.5 \times 10^6$	−29.9 ± 2.8	−5.04	−15.5 ± 2.8	−8.4 ± 0.2	−28.7 ± 9.3
HEPES	$3.3 \pm 0.1 \times 10^5$	−29.5 ± 0.7	−4.86	−17.0 ± 0.7	−7.5 ± 0.02	−31.8 ± 2.2
PIPES	$1.2 \pm 0.3 \times 10^6$	−23.9 ± 1.5	−2.68	−17.0 ± 1.5	−8.3 ± 0.2	−29.3 ± 5.3
Phosphate	$1.2 \pm 0.6 \times 10^6$	−10.2 ± 0.4	−0.86	−7.9 ± 0.4	−8.3 ± 0.3	−0.8 ± 0.4
ACES	$5.5 \pm 1.9 \times 10^5$	−26.5 ± 0.3	−7.27	−7.9 ± 0.3	−7.8 ± 0.2	−0.2 ± 0.2

Equilibrium constants (*K*) and observed changes in enthalpy (ΔH_{obs}) are the average of multiple titrations and the error reported is plus or minus one standard deviation unit. HPCD concentrations associated with the isothermal titration calorimetry data were adjusted on the basis of occupancy of the metal site in HPCD. The change in enthalpy for HPCA binding (ΔH) was calculated by subtracting the heat of ionization of the buffer from the ΔH_{obs} term.



Scheme 1 Proposed mechanism for homoprotocatechuate binding to homoprotocatechuate 2,3-dioxygenase (*HPCD*)

suggests that the pK_a of the singly protonated hydroxide moiety in HPCA will become more acidic as the enediol unit binds iron. Theoretical studies on the enzyme/substrate complex of HPCD report there is a relatively low energy barrier (approximately 2 kcal/mol) between the monoanionic and dianionic forms of HPCA bound to HPCD [11]. This lends credibility to the idea that the double deprotonated state may be possible for the enediol in the enzyme/substrate complex of HPCD.

The ITC data for binding of HPCA to HPCD yields an equilibrium constant for HPCA binding of 1.1 ($\pm$0.6) $\times$ 10^6. This is consistent with the estimated $1/K_d$ values previously reported by Groce et al. [7] and the $1/K_M$ values for HPCA binding to HPCD presented throughout the literature [21–23]. The calculated enthalpy change (ΔH^*) for HPCA binding to HPCD is -17.0 ± 1.7 kcal/mol from the data in Fig. 3, where ΔG and ΔS were calculated to be -8.1 ± 0.4 kcal/mol and -29.7 ± 0.6 cal/mol K, respectively. These values indicate that HPCA binding is favorable and that the large exothermic enthalpy change (ΔH) is largely offset by the unfavorable change in entropy ($-T\Delta S \approx 8.9$ kcal/mol). This enthalpy change must result from substrate binding and a number of weaker interactions between HPCA and the substrate binding pocket. The entropy change then must reflect restricted substrate mobility, and more importantly suggests significant protein reorganization associated with HPCA binding to HPCD and stabilizing the enzyme/substrate complex.

Clearly the ionization state of the substrate plays a significant role in forming and stabilizing the enzyme/substrate complex in HPCD. However, it also plays a major role in determining the one-electron redox potential of catechols and other substituted aromatic compounds [24], which makes the protonation state of the HPCD/HPCA complex mechanistically important. We believe our results are most consistent with HPCA binding to HPCD as a dianionic species. The high substrate affinity and the large exothermic enthalpy change for the formation of the enzyme/substrate complex must include a large contribution from Coulombic interactions between the dianionic substrate and the divalent iron. This thermodynamic model is influenced by the fact that we measure the release of 2.6 protons on formation of the HPCD/HPCA complex. This new model associated with the enzyme/substrate complex warrants further exploration into the substrate binding interactions and the role the substrate plays in controlling dioxygen reactivity in the extradiol dioxygenases and in other non-heme iron oxygenases.

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