Journal of Molecular Catalysis B: Enzymatic 64 (2010) 53–59

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Experimental and theoretical affinity studies of substituted phenols to chlorocatechol 1,2-dioxygenases: A step toward the comprehension of inhibitor/substrate binding to intradiol dioxygenases

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ARTICLE INFO

Article history:
Received 11 September 2009
Received in revised form 1 January 2010
Accepted 2 February 2010
Available online 7 February 2010

Keywords:
Chlorocatechol 1,2-dioxygenase
Enzyme kinetics
Substrate analogue reactivity
Molecular orbital calculations
Quantitative structure/activity relationship (QSAR)

ABSTRACT

The inhibition kinetics of 4- and 3-chlorocatechol 1,2-dioxygenases from *Rhodococcus opacus* 1CP were investigated using 14 different substituted phenols. The obtained experimental data were analyzed against experimental (pK_a) and theoretical reactivity parameters of the phenols calculated by semi-empirical AM1 method (DPE, E_{HOMO}, charge on oxygen atom of the reactive hydroxyl group, van der Waals surface areas and partial charges of substituents on aromatic ring). From these comparisons it appears that the main factor determining the inhibitors binding in the active center of these enzymes is the deprotonation ability of their reactive hydroxyl group. The analysis also allowed to detect, in 3-CCD, enzyme factors influencing on the interactions with the investigated phenols. Some correlations between the calculated van der Waals surface areas as well as the partial charges of substituents on the aromatic ring of the phenols and their inhibition effect on the enzymes were found.

1. Introduction

A great variety of natural and xenobiotic aromatic compounds are biodegraded by microorganisms which first convert them into a small number of central intermediates, generally containing two adjacent hydroxyl groups in the aromatic ring, such as protocatechuate, catechol, chloro- and dichlorocatechols, hydroxy- and chlorohydroxyquinols [1,2]. The subsequent degradation of such intermediates usually occurs through the cleavage of the aromatic ring via insertion of both atoms of molecular oxygen catalyzed by ring-cleaving dioxygenases. When this process is catalyzed by non-heme Fe(III)-dependent dioxygenases the insertion takes place between two adjacent hydroxyl groups of the substrate molecules, therefore this class of enzymes is called intradiol ring-cleaving dioxygenases.

In all known intradiol dioxygenases the active Fe(III) ion is coordinated to four endogenous ligands: 2 Tyr and 2 His residues and one water molecule/hydroxide ion [2–6]. Currently the catalytic mechanism of intradiol ring-cleaving dioxygenases has been hypothesized to occur through initial deprotonation of exogenous catechol-like molecule with concomitant dissociation of endogenous axial Tyr-ligand and exogenous hydroxide ion from ferric coordination sphere, and following formation of the Fe(III)–catecholate radical and electrophilic attack of molecular oxygen on it [3,4].

Substrate selection and conversion should be mainly controlled by the ring substituents effects on the electron density of the carbon atoms exposed to the molecular oxygen attack as well as by the interactions of ring substituents with the surrounding active site amino-acidic residues. As an example in protocatechuate 3,4-dioxygenase from *Acinetobacter* sp. ADP1 the carboxyl group of protocatechuate forms a hydrogen bound to Tyr324 and electrostatic interactions with Arg133, Arg330 and Arg450 that allows the correct positioning of the substrate molecule into the active cavity [7]. Vetting and Ohlendorf [8] showed that the active site of catechol 1,2-dioxygenase from the same strain is more hydrophobic than that of protocatechuate 3,4-dioxygenase. Additionally mutagenesis studies demonstrated the critical role of several amino acid residues

Abbreviations: CCD, chlorocatechol 1,2-dioxygenase; K_i, inhibition constant; pK_a, acid–base dissociation constant; AM1, Austin model 1; DPE, deprotonation enthalpies; H_f, heat of formation; E_{HOMO}, energy of the highest occupied molecular orbital; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; QSAR, quantitative structure/activity relationship; r^2, coefficient of determination.

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doi:10.1016/j.molcatb.2010.02.001

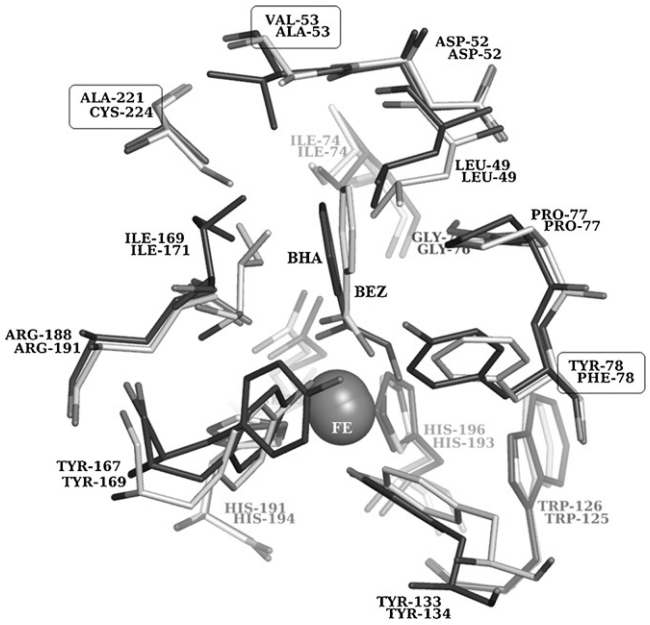

Fig. 1. Superimposition of 3-CCD (dark) and 4-CCD (light) active centers. BHA, benzohydroxamate molecule; BEZ, benzoate molecule.

in position 48, 52, and 73, forming the active center cavity, in differentiating the substrate specificities in two highly homologous chlorocatechol 1,2-dioxygenase mutants from *Ralstonia eutropha* NH9 and *Pseudomonas* sp. strain P51 [9].

The kinetic properties of chlorocatechol 1,2-dioxygenases are not studied in detail, however the high selectivity of the enzyme to chloro- and methyl-substituted catechols was observed [10–19]. Recently the crystal structure of two chlorocatechol 1,2-dioxygenases from *Rhodococcus opacus* 1CP grown on 2- and 4-chlorophenols as a sole source of carbon was reported [5,6]. Investigated CCDs exhibited different substrate specificity relative to 3- and 4-substituted catechols [6]. 3-CCD was more active toward 3-methyl- and 3-chlorocatechols, whereas 4-CCD showed high selectivity to 4-methyl- and 4-chlorocatechols. As all known intradiol dioxygenases [2–4,20] the chlorocatechol 1,2-dioxygenases from *R. opacus* 1CP contain a ferric ion coordinated by two Tyr and two His residues [5,6]. Furthermore, the active sites of 3- and 4-chlorocatechol 1,2-dioxygenases (3-CCD and 4-CCD) differ by only three residues: Val53/Ala53, Tyr78/Phe78 and Ala221/Cys224 for 3-CCD and 4-CCD, respectively (Fig. 1) [5,6].

In spite of the existing data, however, there is no clear interpretation for the chlorocatechol 1,2-dioxygenases functional selectivity.

To better understand the nature of interactions between substrate and the active center cavity of chlorocatechol 1,2-dioxygenases, in 3- and 4-chlorocatechol 1,2-dioxygenases from *R. opacus* 1CP, as well as the role of substrate reactivity in this process the following approaches were applied: (1) a more detailed investigation of the enzyme kinetic data using a large variety of substituted phenols, probably having different ways of binding into the active site, achieved due to the diverse nature and number of substituent groups onto the aromatic ring; (2) the study of the structural and electronic properties of the phenols and their influence on the enzyme functioning.

2. Experimental

2.1. Chemicals

Phenol, 2-methyl-, 3-methyl- and 4-methylphenols, 2-chloro-, 3-chloro-4-chloro-, 2,4-dichloro-, 2,5-dichloro-, 2,6-dichloro-, 3,4-dichloro-, 3,5-dichloro-, 2,4,5-trichloro- and 2,4,6-trichlorophenols were from Sigma, Fluka and Merk.

2.2. Microorganism and growth conditions

3-Chlorocatechol and 4-chlorocatechol 1,2-dioxygenases were purified from *R. opacus* 1CP grown on 2-chlorophenol or 4-chlorophenol correspondingly as a sole carbon and energy source.

Biomasses for enzyme purification were obtained by cultivation of the strain in a 10-l bioreactor with 7 l of mineral medium [21] and constant addition of 0.3 mM 2- or 4-chlorophenols at 29 °C. Substrate exhaustion was indicated by the decrease in oxygen uptake. The cells were harvested in the exponential growth phase by centrifugation at 3000 × *g*, washed twice with 50 mM Tris/HCl, pH 7.2, and stored at −20 °C.

2.3. Preparation of crude extract and enzyme purification

Frozen cells were suspended in an equal volume of 50 mM Tris/HCl, pH 7.2, and disrupted by French press. After the treatment with 0.01 mg/ml DNAase (Sigma, USA), the cell extract was clarified by centrifugation for 30 min at 31,000 × *g*. The supernatant was used for further investigations.

All the procedures for enzyme purification were performed in the presence of 50 mM Tris/HCl pH 7.2.

4-Chlorocatechol 1,2-dioxygenase (4-CCD) was purified by a modified version of the earlier used method [16] including 5 steps of purification: anion-exchange chromatography on Q-Sepharose with linear gradient 0–0.5 M NaCl, hydrophobic chromatography on Phenyl-Sepharose CL-4B with linear gradient 1.6–0 M $(NH_4)_2SO_4$, gel-filtration on Superdex 200 prep grade, anion-exchange chromatography on Resource-Q with step gradient 0–1 M NaCl (0–10%, 10–20%, 20–100%) and hydrophobic chromatography on Resource ISO with linear gradient 1.6–0 M $(NH_4)_2SO_4$.

3-Chlorocatechol 1,2-dioxygenase (3-CCD) was purified by the modification of method [17] containing four following steps with Q-Sepharose Fast Flow, Phenyl-Sepharose CL-4B, Superdex 200 prep grade and Resource Q similarly to scheme for 4-CCD.

For estimation of enzyme homogeneity electrophoresis of the purified enzymes in 12% polyacrylamide gel in the presence of SDS was performed according to the modified Laemmli method [22] using low molecular weight kit SigmaMarker (6.5–66 kDa) as a standard.

2.4. Enzyme assay and analysis of the kinetic data

Activity of chlorocatechol 1,2-dioxygenases was measured spectrophotometrically with Shimadzu UV-160 spectrophotometer (Japan) in 1 cm quartz cuvettes at 25 °C with catechol as a substrate at 260 nm [10] in the reaction mixture containing 50 mM Tris/HCl pH 7.2 and inhibitor. The reaction was started by adding the enzyme. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol product/min under the described conditions. Inhibition constants were determined graphically utilizing the method of Dixon [23] using three fixed catechol concentrations: for 4-CCD—10 μM, 20 μM and 30 μM; for 3-CCD—3 μM, 6 μM and 10 μM. The enzyme activity was measured at least 4 times for each of the 10 increasing concentrations of an inhibitor for each catechol concentration. For calculation of each kinetic constant the data of at least three experiments were used.

2.5. Calculation of reactivity parameters of phenols

All molecular orbital calculations were performed using the semi-empirical AM1 method implemented in HyperChem6.03 [24].

In view of the fact that influence of solvation effects and a different dielectric constant into the active site of the enzyme on the frontier orbital characteristics of the substrate analogues compared to the *in vacuo* situation are not significant when the relative differences between parameters of a series of closely related compounds are appreciated [25], all the reported quantum chemical calculations were performed *in vacuo*.

The starting molecular geometries of the phenols were calculated by molecular mechanics method using the Amber2 force field. Searches for the optimized molecule conformations with minimal energy (optimal torsion angles of hydroxyl-substituents) were performed with 1000 iterations of the semi-empirical method AM1 *in vacuo* using a spin-restricted Hartree–Fock (for the protonated molecule) and unrestricted Hartree–Fock (for the anionic state of the molecule) calculations (RHF and UHF wave functions, respectively). Closed and open-shell calculation of Mulliken charges on oxygen atom of the hydroxylic group of phenols in protonation and anionic states were also performed using the AM1 method after searching for the optimal conformation of molecule. The values of total charges of fully protonated and deprotonated molecules were 0 and −1 corresponding to a spin multiplicity value equal to 1 [26].

Deprotonation enthalpies (DPE) of phenols in the reaction $HB \rightarrow H^+ + B^-$ were calculated using the equation [27]:

$$DPE(HB) = \Delta H_f(H^+) + \Delta H_f(B^-) - \Delta H_f(H^+)$$

where the experimental value of the enthalpy of formation of H^+($\Delta H_f(H^+)$) is equal to 367.2 kcal/mol. The values of the enthalpy of formation of fully protonated and deprotonated molecules were calculated using the semi-empirical method AM1 as mentioned above.

van der Waals surface areas and partial charges (based on current charges) of substituents on the aromatic ring of phenols were calculated after preliminary conformational search as described above, with the QSAR properties module of HyperChem6.03 program using the grid method described by Bodor et al. [28] and the atomic radii of Gavezotti [29], and utilizing the empirical model based on the Partial Equalibration of Orbital Electronegativity (PEOE) approach of Gasteiger and Marsili [30].

The energy of the highest occupied molecular orbital (E_{HOMO}) of the phenols (i.e. the energy of the most reactive π electrons in the aromatic system) was calculated after preliminary conformational search as described above using a semi-empirical method AM1. E_{HOMO} values were calculated for substituted phenol molecules in anion state.

The values of pK_a of the phenols were calculated as average of experimental values of the pK_a presented in "Chemical database, Department of Chemistry at the University of Akron" [31], "Footprint pesticide database" [32], and in the studies by Dean [33] and Lepri et al. [34].

2.6. Enzyme structure analysis

Protonation of the chlorocatechol 1,2-dioxygenase molecules was performed at 300 K, pH 7.20 and 0.00 mol/l of salt by Protonate 3D module of MOEv.2008.10 [35] using the Generalized Born/Volume Integral (GB/VI) formalism [36]. The pK_a values of amino acid residues were predicted by MOEv.2008.10 [35] using a method based on PROPKA [37]. Hydrophobic isosurface (−2.5 kcal/mol) and hydrophylic isosurface (−5.5 kcal/mol) inside active centers of the preliminary protonated enzymes were visualized using the Interaction Potential map representation based on the GRID method [38,39] implemented in MOEv.2008.10 [35]. Electrostatic coloring of molecular surfaces of the preliminary protonated molecules was performed by MOEv.2008.10 [35] using the analytical method of surface calculation described by Connolly [40].

Structure analysis of the chlorocatechol 1,2-dioxygenases was accomplished using PyMolV0.95 [41]. Superimposition of the enzyme active centers was performed utilizing ProFit2.3 [42] using the McLachlan algorithm [43], specifying the amino acid iron ligands as the fitting subset.

3. Results and discussion

3.1. Affinity of chlorocatechol 1,2-dioxygenases for phenols

The inhibition kinetics of 3-CCD and 4-CCD with different substituted phenols as the reduced model of chlorocatechol 1,2-dioxygenases substrate analogues containing only one reactive hydroxyl group and various substituents on the phenolic ring were investigated.

Phenol and its chloro- and methyl-substituted analogues act as competitive inhibitors for these enzymes, the inhibition constants are reported in Table 1.

Both enzymes show a comparable behavior with respect to monochloro- and monomethyl-substituted phenols. The closer the chloro-substituent to the hydroxyl group the higher the inhibition of the enzymes. On the contrary the presence of a methyl-substituent in *ortho*-position to the hydroxyl group on the aromatic ring led to a significant decrease of the enzyme affinity for the corresponding phenol. This fact is evidence of the preferable formation of electrostatic interactions between the *ortho*-substituent on the aromatic ring of phenols and the inner surface of the active center cavity of chlorocatechol 1,2-dioxygenases. Such electrophylic determinants of the active center can be iron ion as well as the nearest amino acid residues of the iron ion environment.

On the whole, increasing the number of chloro-substituents on the aromatic ring led to a strengthening of the enzyme binding (Table 1). Furthermore, the values of the inhibition constants for 3-CCD are generally lower than the corresponding for 4-CCD (Table 1). That indicates a stronger binding the phenols to 3-CCD and can be explained by a more hydrophobic inner surface of 3-CCD active center than 4-CCD (Fig. 2). Among the different residues placed inside active centers of the CCDs (Figs. 1 and 2) Val53 of 3-CCD is more hydrophobic than Ala53 of 4-CCD correspondingly.

The influence of a number of experimental and calculated reactivity parameters of the phenols on the affinity of the enzyme active centers was investigated in order to try to comprehend the origins of such effects.

3.2. Correlation between the inhibition constants of the enzymes and the charge values of oxygen atom of hydroxyl group of phenols

Since the reactive hydroxyl groups of catechol-like molecules after their deprotonation are vital for their binding to the iron ion of the active center of intradiol dioxygenases [4], the oxygen charge values on hydroxyl group of the phenols in both protonation and anionic states were calculated using the semi-empirical AM1 method.

As it can be noted from the comparison of the data reported in Table 1 and in Fig. 3A the inhibition of the dioxygenases by phenols increases with the reduction of the negative charge on the deprotonated oxygen atom of the hydroxyl group (r^2 = 0.908 for 4-CCD). This dependence appears to be more pronounced for 3-CCD than for 4-CCD although exceptions are observed with 2,6-di-, 2,4,5-tri- and 2,4,6-trichlorophenols (Fig. 3A).

A similar dependence of the inhibitory effect is also observed for the protonated oxygen atom (Table 1).

Since the inverse correlation between the oxygen charge of hydroxyl group and inhibition effect of the phenols was unexpected, other parameters for deprotonation process of the hydroxyl group of the phenols were also analyzed.

Table 1
Experimental and theoretical reactivity parameters of substituted phenols and kinetic data for 4-chlorocatechol 1,2-dioxygenase (4-CCD) and 3-chlorocatechol 1,2-dioxygenase (3-CCD) from *R. opacus* 1CP toward the same phenols.

Inhibitors	Reactivity parameters							Inhibition constants (exp.), K_i (μM)	
	pK_a (exp.)	Oxygen atom charge		Heat of formation (ΔH_f), kcal/mol		DPE, kcal/mol	E_{HOMO}, eV	4-CCD	3-CCD
		HB	B^-	HB	B^-				
2-Methylphenol	10.27 ± 0.06	−0.254	−0.526	−29.6534	−48.5298	348.32	−2.7005	1566 ± 65.041	535 ± 13.796
4-Methylphenol	10.22 ± 0.05	−0.253	−0.527	−29.9962	−49.3899	347.81	−2.7142	878 ± 21.502	65.4 ± 2.829
3-Methylphenol	10.07 ± 0.05	−0.253	−0.532	−30.0303	−48.4715	348.76	−2.7252	943 ± 31.765	4.6 ± 0.208
Phenol	9.98 ± 0.06	−0.253	−0.533	−22.4085	−41.1200	348.49	−2.7023	1243 ± 40.079	91 ± 3.055
4-Chlorophenol	9.33 ± 0.11	−0.248	−0.514	−29.4722	−55.4596	341.21	−3.0403	184 ± 6.245	30.7 ± 1.054
3-Chlorophenol	9.02 ± 0.12	−0.248	−0.514	−29.2059	−54.8654	341.54	−3.1079	90 ± 3.606	1.02 ± 0.030
3,4-Dichlorophenol	8.55 ± 0.12	−0.244	−0.498	−34.6411	−66.2572	335.58	−3.3647	20 ± 0.252	0.44 ± 0.021
2-Chlorophenol	8.52 ± 0.03	−0.245	−0.494	−28.8394	−53.2575	342.78	−3.0177	41 ± 1.793	0.55 ± 0.025
3,5-Dichlorophenol	8.18 ± 0.00	−0.243	−0.497	−35.5578	−67.3246	335.43	−3.4737	50 ± 1.000	0.026 ± 0.001
2,4-Dichlorophenol	7.93 ± 0.11	−0.241	−0.476	−35.3146	−66.4828	336.03	−3.3207	6.5 ± 0.154	0.23 ± 0.009
2,5-Dichlorophenol	7.51 ± 0.01	−0.240	−0.477	−35.2698	−66.1221	336.35	−3.3871	5.7 ± 0.157	0.127 ± 0.003
2,4,5-Trichlorophenol	7.21 ± 0.33	−0.237	−0.462	−40.2086	−76.5974	330.81	−3.6190	1.1 ± 0.035	0.297 ± 0.014
2,6-Dichlorophenol	6.86 ± 0.14	−0.230	−0.456	−32.5605	−64.1586	335.60	−3.3029	9.9 ± 0.127	1.43 ± 0.066
2,4,6-Trichlorophenol	6.41 ± 0.18	−0.226	−0.440	−38.4919	−76.3547	329.34	−3.5757	0.9 ± 0.035	34 ± 1.650

exp., experimental data (the mean values and the standard deviation of mean are listed).

3.3. Relationship between dioxygenase inhibition by phenols and their pK_a values

In Table 1 and Fig. 3B it can be noticed that the inhibition strength is directly dependent on the acidic strength of the phenols (r^2 = 0.928 for 4-CCD).

Even for this property the greater deviation from the linear dependence is observed for 3-CCD inhibition by 2,6-di-, 2,4,5-tri- and 2,4,6-trichlorophenols. This is probably due to the fact that the pK_as for these phenols are equal or less than the operational pH (7.2), therefore they are mainly in the anionic form under the testing conditions thus probably affecting their approach to the 3-CCD active site metal ion. The lack of a linear correlation for the phenols-3-CCD affinity is possibly caused by the fact that those phenols that are presented in an anionic form at the working pH probably have some electrostatic repulsion from the active site residues, such as Tyr78 at the 3-CCD active site entrance (Fig. 1). To check this hypothesis we calculated electrostatic potential on the Connolly surface of the both preliminary protonated native enzymes (Fig. 4). In spite of protonated Tyr78 (pK_a = 11.68) in 3-CCD molecule the region of surface formed by Tyr78 is negatively charged (Fig. 4B) in contrast to the similar region generated by Phe78 of 4-CCD (Fig. 4A). On the whole, the external surface of 3-CCD active center entrance is more negatively charged than 4-CCD one that confirms our assumption. Furthermore, the neck located between the entrance and the inner cavity of the active center in 4-CCD is more hydrophobic than in 3-CCD (Fig. 2), that can additionally promote entering the inhibitors into the active center cavity of 4-CCD.

3.4. Influence of deprotonation enthalpies of phenols on dioxygenase inhibition

The calculated deprotonation enthalpies (DPE) shown in Fig. 3C and Table 1 reveal a similar dependence: the inhibition effect of the phenols on both enzymes is linearly increasing as DPE decreases (r^2 = 0.917 for 4-CCD) with the same exceptions previously observed in the 3-CCD inhibition by trichlorophenols.

As a result, the strength of CCDs inhibition by substituted phenols generally depends on the ease of hydroxyl deprotonation directly linked to the electron density effect of substituents on the aromatic ring of phenols. Dorn and Knackmuss [10] made a similar assumption observing the decrease of the K_m values when the pH was increased in the case of catechol cleavage catalyzed by pyrocatechase from *Pseudomonas* sp. B13. Furthermore they also showed that electron-withdrawing substituents decreased the K_m values for catechols. Though, it should be noted that in the case of CCD kinetics with catechols the K_m is not a parameter reflecting only the binding substrate analogues in the enzyme active center. This can be related to the fact that, in contrast to the K_i values observed for the phenols, several steps can affect the K_m for catechols: deprotonation of both hydroxyl groups for their binding to the Fe(III) active center, the axial Tyr dissociation from the iron-coordination sphere as well as the following steps of the catalytic process [4].

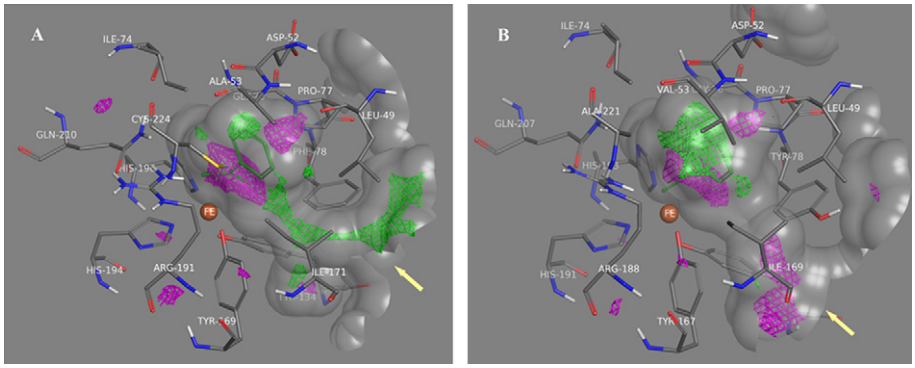

Fig. 2. Hydrophobic (green meshes) and hydrophilic (magenta meshes) isosurfaces inside active centers of 4-chlorocatechol 1,2-dioxygenase (A) and 3-chlorocatechol 1,2-dioxygenase (B). The entrance in the active center of the enzymes is indicated by yellow arrow. Green structures inside active centers are the molecules of benzoate (A) and benzohydroxamate (B) correspondingly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

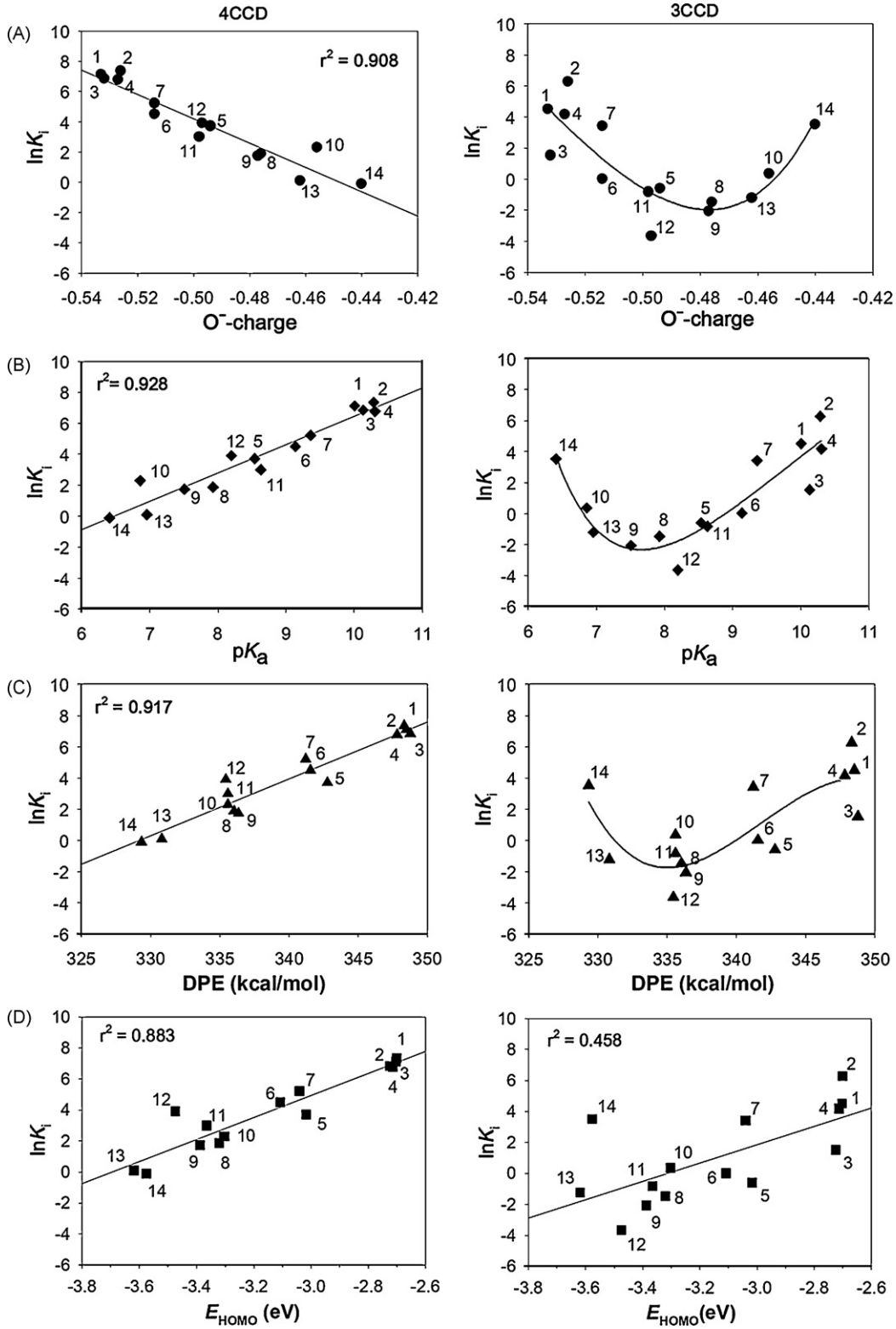

Fig. 3. Correlation between the reactivity parameters of substituted phenols and their inhibitory effect on 4-chlorocatechol 1,2-dioxygenase (4-CCD) and 3-chlorocatechol 1,2-dioxygenase (3-CCD) from *R. opacus* 1CP: 1: phenol; 2: 2-methylphenol; 3: 3-methylphenol; 4: 4-methylphenol; 5: 2-chlorophenol; 6: 3-chlorophenol; 7: 4-chlorophenol; 8: 2,4-dichlorophenol; 9: 2,5-dichlorophenol; 10: 2,6-dichlorophenol; 11: 3,4-dichlorophenol; 12: 3,5-dichlorophenol; 13: 2,4,5-trichlorophenol; 14: 2,4,6-trichlorophenol.

3.5. Contribution of the energy of the reactive HOMO electrons of phenols to their inhibitory effect

The comparison of the $\ln K_i$ of the phenols for 4-CCD with the energies of the reactive HOMO electrons in the corresponding aromatic ring (E_{HOMO}) in the anion state shows a linear correlation ($r^2 = 0.883$); on the contrary 3-CCD shows a limited correlation ($r^2 = 0.458$) (Fig. 3D). It is known that the interaction between the frontier orbitals, the highest occupied molecular orbital (HOMO) of the nucleophile and the lowest unoccupied molecular orbital

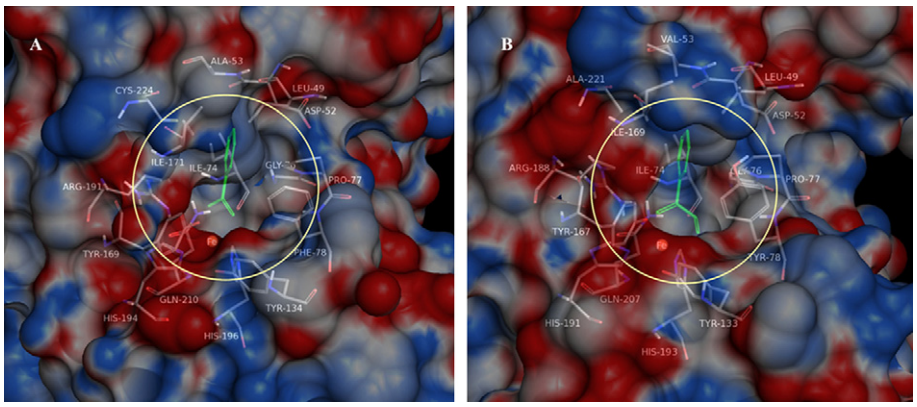

Fig. 4. Connolly accessible surface of the active centers of the native 4-chlorocatechol 1,2-dioxygenase (A) and 3-chlorocatechol 1,2-dioxygenase (B). Green structures inside active centers are the molecules of benzoate (A) and benzohydroxamate (B) correspondingly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
van der Waals surface areas and partial charges of the substituents on the aromatic ring of the phenols in protonation and anionic states. S1, S2, and S3—the subsequent substituents after hydroxyl group.

Inhibitors	van der Waals surface area (Å)/atom charge					
	HB			B^-		
	S1	S2	S3	S1	S2	S3
2-Methylphenol	32.268/0.071	–	–	33.427/0.014	–	–
3-Methylphenol	33.733/0.074	–	–	33.671/−0.004	–	–
4-Methylphenol	33.601/0.072	–	–	33.483/−0.005	–	–
2-Chlorophenol	27.801/−0.018	–	–	29.016/−0.120	–	–
3-Chlorophenol	28.824/−0.014	–	–	29.143/−0.146	–	–
4-Chlorophenol	28.888/−0.017	–	–	29.016/−0.135	–	–
2,4-Dichlorophenol	27.801/−0.007	28.824/−0.006	–	28.952/−0.105	28.952/−0.121	–
2,5-Dichlorophenol	27.801/−0.009	28.952/−0.002	–	28.952/−0.107	29.016/−0.131	–
2,6-Dichlorophenol	27.610/−0.010	28.824/0.016	–	28.888/−0.106	28.888/−0.106	–
3,4-Dichlorophenol	28.057/0.013	28.185/0.009	–	28.249/−0.107	28.185/−0.099	–
3,5-Dichlorophenol	28.824/−0.002	29.016/0.000	–	29.080/−0.127	29.016/−0.127	–
2,4,5-Trichlorophenol	27.801/0.000	28.057/0.018	28.057/0.023	28.952/−0.094	28.313−0.086	28.185/−0.095
2,4,6-Trichlorophenol	27.610/0.001	28.824/0.003	28.824/0.027	28.888/−0.091	28.888/−0.107	28.952/−0.091

(LUMO) of the electrophile, is essential and lowers the activation barrier [44]. Electron-donating substituents (methyl-group) on the aromatic ring raise the energy level of HOMO leading to an increase of the aromatic π electron reactivity, whereas electron-withdrawing substituents (such as chlorine) induce a decrease of the nucleophilic reactivity of the phenols. The chlorine residues therefore cause a decrease of the electronic density on the oxygen atom of the hydroxyl group of the phenols thus facilitating the deprotonation process. Thereby the energy of the reactive HOMO electrons in aromatic ring of the phenols indirectly contributes to the strength of phenols/ferric ion interactions.

As shown by Ridder et al. [25] the E_{HOMO} values of substituted catechols directly influence the catalytic activity of catechol 1,2-dioxygenase from *Pseudomonas putida* (*arvilla*) C1. The authors explained this effect considering that the E_{HOMO} values reflect the ability of the aromatic π electrons of the iron(III)-bound catecholate to accept a nucleophilic attack by the oxygen molecule, an important step in the proposed reaction mechanism for intradiol dioxygenases [1,3,4]. In our case the variations of the catalytic activities of 4-CCD and 3-CCD with a number of mono- and dichloro-substituted catechols were earlier demonstrated too [6,16,17].

3.6. Role of van der Waals surface areas and partial charges of substituents on phenol ring in inhibitory effect

The weaker 3-CCD inhibition observed for 2,6-di-, 2,4,5-tri- and 2,4,6-trichlorophenols cannot be either simply explained by the high van der Waals surface areas of chloro-substituted phenols or their increase due to phenols deprotonation (Table 2), since similar areas are observed for other chlorophenols such as 2,4-di-, 2,5-di- and 3,5-dichlorophenols which on the contrary are excellent inhibitors for 3-CCD. Additionally the van der Waals surface areas of the chloro-substituents are decreasing when their number on the aromatic ring of the phenols is increased (Table 2).

It is therefore evident that the simple surface area of the binding inhibitors cannot explain the observed dependences but it also strongly rely on the relative spatial orientation of the inhibitor into the active site and their steric interactions into the 3-CCD active site cavity.

On the contrary, for the methyl-substituted phenols the significantly decreased inhibition effect detected for the investigated CCDs (Table 1) can be simply associated to the large values of the van der Waals surface areas of the methyl-substituent together with their inability to form strong electrostatic interactions (Table 2).

Furthermore, the presence of additional electrostatic interactions between the active cavity and chloro-substituents is not excluded as a consequence of the increase of negative charge on chlorine atoms during phenol deprotonation.

4. Conclusions

Thus, the present study reveals that in the active center of the chlorocatechol 1,2-dioxygenases the binding strength of the phe-

nols possessing one reactive hydroxyl group directly depends on the ease of its deprotonation that is determined by substituent effect in the aromatic ring of the inhibitors. On the other hand such analysis allowed to evidence in 3-CCD the presence of enzyme factors influencing on the interaction with the investigated phenols.

As a conclusion the analysis of the experimental and calculated reactivity parameters for a series of inhibitors of chlorocatechol 1,2-dioxygenases not only clarifies the mechanisms of substrate analogues binding in CCD active center but can be employed in future to predict inhibition effects of new compounds for the investigated enzymes.

Further investigations through structural and theoretical approaches on the reactivity of Fe(III)–catecholate complexes are needed in order to elucidate the complete catalytic mechanism for this class of enzymes.

Acknowledgements

The work was supported by INTAS YS Fellowship Ref. Nr 06-1000014-5954 and Russian Science Support Foundation. The Italian "Ministero dell'Istruzione, dell'Università e della Ricerca" PRIN 2007 project funding is acknowledged.

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