J. Biochem. **135**, 721–730 (2004)
DOI: 10.1093/jb/mvh089

Quantitative Structure-Activity Relationship for the Cleavage of C3/C4-Substituted Catechols by a Prototypal Extradiol Catechol Dioxygenase with Broad Substrate Specificity

Tetsuo Ishida*, **Hiroyuki Tanaka and Kihachiro Horiike**

Department of Biochemistry, Shiga University of Medical Science, Seta, Ohtsu, Shiga 520-2192

Received March 31, 2004; accepted April 21, 2004

Catechol 2,3-dioxygenase [EC 1.13.11.2] from *Pseudomonas putida* mt-2 (Mpc) catalyzes the extradiol cleavage of catechol to produce 2-hydroxymuconate semialdehyde. The K_m values for the catecholic substrate (K_{mA}) and O_2 (K_{mO2}), and catalytic constants (k_{cat}) were kinetically determined for eight C3/C4-substituted catechols at 25°C and pH 6.5 or 7.5. The first pK_a values (pK_1) were determined for eleven catechols (pK_1 = 7.26–9.47), correlated with Hammett substituent constants, and electron-withdrawing substituents significantly stabilized the monoanionic species of free catechols. Mpc preferred catechols with non-ionic substituents at the C3 or C4 position. 3-Phenylcatechol, a biphenyl, was cleaved, while 4-*tert*-butylcatechol was not. The logarithm of k_{cat}/K_{mA} (substrate specificity constant) exhibited a good linear correlation with pK_1, with the exception of those for 4-halocatechols. The logarithm of k_{cat}/K_{mO2} showed a good linear correlation with pK_1, with the exception of that of 3-phenylcatechol. These results demonstrate that catechol binding to the Mpc active site, the following O_2 binding, and the activation of the bound O_2 are all sensitive to electronic effects of the substituents. However, k_{cat} did not correlate significantly with pK_1. The present study distinguishes clearly between the electronic and the steric effects of catecholic substrates in the reactivity of Mpc, and provides important insight into the mechanistic basis for a vast range of substrate specificities of extradiol dioxygenases.

Key words: catechols, dioxygenase, non-heme iron, oxygen, substituent effects, pK_a values of catechols.

Abbreviations: Mpc, catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2; HOMO, the highest occupied molecular orbital; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. KKS102.

Many aerobic microorganisms can degrade aromatic compounds to aliphatic compounds using a *meta*-cleavage pathway (*1–3*). In the biodegradation, aromatics are first converted to 1,2-dihydroxybenzene (catechol) derivatives, and then the catechols are subjected to oxidative cleavage of the aromatic ring adjacent to the vicinal hydroxyl groups (*meta* or extradiol cleavage) to yield 2-hydroxy-6-keto-2,4-dienoic acid products (substituted 2-hydroxymuconate semialdehydes) (Fig. 1A). The extradiol catechol dioxygenases catalyze the cleavage using mostly Fe(II) ions or sometimes Mn(II) ions (*4–6*) as the sole cofactors. A large number of extradiol dioxygenases have been isolated for bioremediation from various aerobic microorganisms, and are classified into two large groups (Types I and II) based on primary and tertiary structural data (*7–9*). Since the extradiol cleavage step is often a bottleneck step in the complete degradation of aromatic pollutants such as alkylbenzoates and polychlorobiphenyls (PCBs) (*10–12*), it is important to understand the structural and mechanistic basis of the substrate specificity of this large enzyme family. In natural environments, the availability of O_2 is usually limited due to the low solubility and slow diffusion rate of O_2 in aqueous solutions. Therefore, the dependence of the enzyme activity on O_2 concentration is an especially important factor in the efficiency of the enzymes.

The catalytic mechanism of extradiol dioxygenases has been actively investigated. Figure 1 is a summary of proposed reaction mechanisms as reported in recent reviews (*13–16*). In the reaction, catechol first binds to the active site with bidentate coordination to the Fe(II) ion in a monoanionic manner (*17*, *18*) (Fig. 1B). Then, dioxygen binds directly to the metal center of the enzyme-substrate complex (EA) to form a ternary enzyme-substrate-O_2 complex (*18*) (EAO_2) in which O_2 is activated by accepting one electron from the bound catechol via the Fe(II) center (*19*) (Fig. 1C). A recombination in the Fe(II)-superoxide-semiquinone complex results in a proximal hydroperoxide intermediate (*20*), and then alkenyl migration in the intermediate gives a α-keto lactone and a hydroxide ion bound to the Fe(II) center (*21*) (Fig. 1D). The requirement of a proton donor for this series of reactions has been proposed based on both experimental and theoretical studies (*22*, *23*). The lactone is attacked by the hydroxide ion to give the opened ring product (EP).

Most of the reaction steps proposed for extradiol dioxygenases (Fig. 1) have not been experimentally substantiated, because reaction intermediates other than the EA complex are invisible and not isolatable under physiologically realistic conditions. In addition to this difficulty in

*To whom correspondence should be addressed. Tel: +81-77-548-2158, Fax: +81-77-548-2157, E-mail: teishida@belle.shiga-med.ac.jp

© 2004 The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

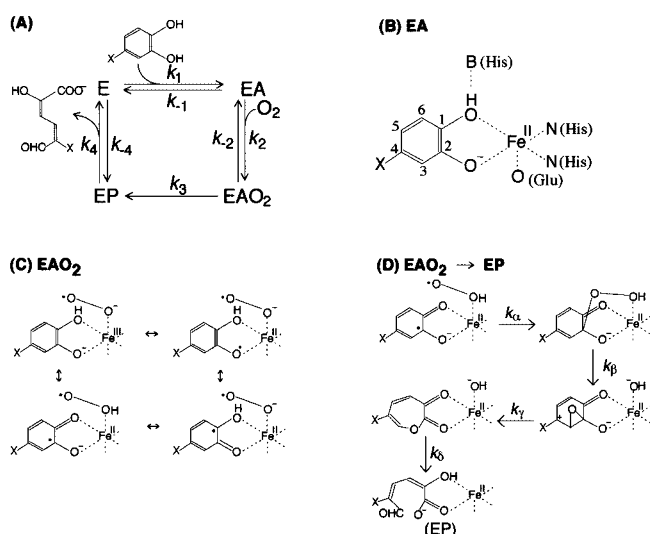

Fig. 1. **A summary of reaction mechanisms proposed for extradiol catechol dioxygenases.** (A) The reaction cycle of enzymatic cleavage of catechols. The relationship between the rate constants and steady-state kinetic parameters are given in Appendix. X denotes a substituent. (B) A schematic active site structure of the EA complex (see Fig. 7). (C) An O_2 activation mechanism in the EAO_2 complex. (D) Proposed intermediates for the conversion from the EAO_2 to EP complex. The rate constant k_3 in Fig. A has the following relation to the elementary rate constants shown in the figure: $1/k_3 = 1/k\alpha + 1/k\beta + 1/k\gamma + 1/k\delta$.

the transient kinetics, steady-state kinetic experiments are also difficult due to the extreme instability of the enzymes in the presence of O_2. Therefore, in most cases only the apparent values of the catalytic constant (k_{cat}) have been examined, and analyses of steady-state kinetic parameters, including K_m for O_2 (K_{mO2}), have been carried out for only very few enzymes (*12*, *24*, *25*). We describe here the quantitative analysis of the substrate specificity of recombinant catechol 2,3-dioxygenase [EC 1.3.11.2] from *Pseudomonas putida* mt-2 (metapyrocatechase, Mpc). Using improved assay methods, we were able to measure the kinetic parameters of Mpc for various C3/C4-substituted catechols with exceptional precision compared to other enzymes belonging to the same enzyme family. To discriminate between the electronic and steric effects of the substituents on the Mpc reaction, we determined the first pK_a (pK_1) of eleven catechols as a quantitative measure of the electronic nature of the substituents. We show that the substrate specificity of Mpc in terms of k_{cat}/K_{mA} and k_{cat}/K_{mO2} is significantly affected by the electronic nature of the C3- or C4-substituents. Unexpectedly, the k_{cat} values are less sensitive to the electronic nature of the substituents.

EXPERIMENTAL PROCEDURES

Materials—Catechol, 4-bromocatechol, 4-chlorocatechol, 3-chlorocatechol, 3,4-dihydroxybenzyl alcohol (4-hydroxymethycatechol), 3-methycatechol, 4-ethycatechol, 4-nitrocatechol, and 4-*tert*-butylcatechol were purchased from Tokyo Kasei Kogyo (Tokyo); protocatechualdehyde (4-formylcatechol), 4-methylcatechol and L-ascorbic acid were from Nacalai Tesque (Kyoto); 2,3-dihydroxybiphenyl (3-phenylcatechol), protocatechuic acid (4-carboxycatechol), 3,4-dihydroxyphenylacetate (4-carboxymethylcatechol), and ascorbate oxidase [EC 1.10.3.3] were from Wako Pure Chemical Industries (Osaka); and 2,3-dihydroxybenzaldehyde (3-formylcatechol) was from Sigma-Aldrich Japan (Tokyo). All other chemicals were of analytical grade and used without further purification.

Recombinant Mpc was expressed in *Escherichia coli*, purified to homogeneity, and stored in crystalline form in the presence of 10% (v/v) acetone as described previously (*24*).

Spectroscopic Titration of Catechols—Catechols were titrated with HCl and NaOH at 25°C in 0.15 M NaCl under anaerobic conditions according to the method described previously (*26*). A freshly prepared catechol solution (90–200 μM) was vigorously bubbled with argon for 5 min. An aliquot of the argon-saturated solution (3.0 ml) was transferred to a cuvette. The cuvette was then sealed with a silicone plug and set in a thermo-jacketed cell holder equipped with a magnetic stirrer in a Shimadzu UV-2200 spectrophotometer (Shimadzu, Kyoto). Throughout the titration experiment, moisturized argon was continuously flushed over the surface of the solution via a 19-guage needle through the plug. The argon was allowed to pass out through a thin hole in the plug, through which aliquots of argon-saturated aqueous NaOH or HCl solution (0.5–2.0 μl) were added to the catechol solution with a gas-tight syringe.

Spectra were recorded over the wavelength range from 220 nm to 600 nm. The pH was measured using a thin pH electrode (6029–10T, Horiba, Kyoto) inserted through the hole in the plug. The pH-dependence of the observed molar absorption coefficient (ε) at the absorption peak of the monoanionic species of a catechol was fitted to Eq. 1 by nonlinear regression,

$$\varepsilon = (\varepsilon_H + \varepsilon_10^{\mathrm{pH}-\mathrm{p}K_1})/(1 + 10^{\mathrm{pH}-\mathrm{p}K_1}) \quad (1)$$

where pK_1 is the first pK_a of a catechol, ε_H and $\varepsilon_$ are the molar absorption coefficients of its neutral and monoanionic species, respectively.

Handling of Mpc Samples—Mpc is a homotetramer and each subunit has a single active site in the C-terminal-domain (*24*, *27*). No substantial cooperativity has been found among the active sites. Therefore, hereafter, the enzyme concentration is always described as the Mpc subunit concentration. The enzyme subunit concentrations were determined spectrophotometrically using a molar absorption coefficient of 43,830 M^{-1} cm^{-1} at 280 nm (*24*).

A concentrated Mpc solution was prepared as follows. Mpc crystals were collected from an aliquot of a crystalline Mpc stock solution stored at 4°C (about 200 μl, specific activity ≥ 300 U/mg) by centrifugation, and dissolved in a minimum amount of 50 mM HEPES, pH 7.5, ionic strength = 0.15 M adjusted with NaCl (HEPES buffer). The concentration of this concentrated Mpc stock solution was 1–2 mM and its specific activity remained greater than 100 U/mg for several weeks at 4°C under aerobic conditions.

A dilute Mpc stock solution (2–6 μM) for steady-state kinetic experiments was prepared in a sealed vial in the presence of an O_2-eliminating system composed of L-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

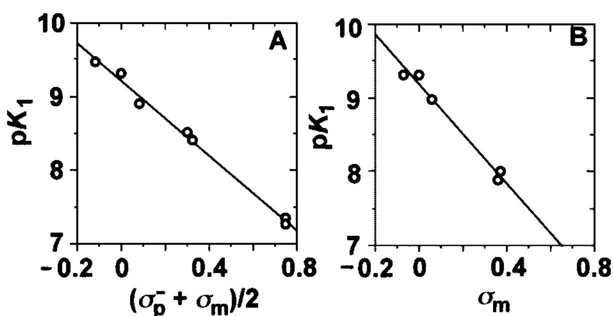

Fig. 2. **Linear free energy relationships for substituted catechols.** The pK_1 values were determined at 25°C in 0.15 M NaCl (Table 1). (A) Correlation between pK_1 of C4-substituted catechols and $(\sigma_p^- + \sigma_m)/2$. (B) Correlation between pK_1 of C3-substituted catechols and σ_m. The σ values are taken from Ref. *28*.

ascorbate and ascorbate oxidase. About 2 mg of solid L-ascorbic acid and 3 ml of argon-saturated HEPES buffer were placed into a 3.0-ml glass vial (Maruemu, Osaka). After sealing the vial with a PTFE/butyl rubber septum and aluminum cap using a clamp, the ascorbic acid was completely dissolved by gentle rotation of the vial. Then, an aliquot of ascorbate oxidase solution (10 µl, 20 U) was added to the vial with a micro-syringe through the septum. After incubation for more than 5 min at room temperature, an aliquot of the concentrated Mpc solution (5–10 µl) was added to the vial through the septum with a micro-syringe. The activity of the resultant dilute Mpc stock solution (50–170 U/mg) was stable for several days, and no significant difference in the enzyme activity was found before and after a series of kinetic experiments.

Kinetic Assays—Kinetic experiments were carried out at 25°C in 50 mM MES, pH 6.5, ionic strength = 0.15 M and in 50 mM HEPES, pH 7.5, ionic strength = 0.15 M. The dependence of the initial velocity on the O_2 concentration was measured in 2.9 ml of reaction mixture containing a constant concentration of a catecholic substrate (10–100 fold higher than the apparent K_m value determined for the substrate under air-saturation) by following the consumption of O_2 using a Model 5331 Clark-type polarographic O_2 electrode (Yellow Springs Instrument, Yellow Springs, OH, USA). The O_2 concentration was controlled over the range of 1–1,000 µM as described previously (*26*). The signal from the O_2 electrode was recorded with a Unicorder U-228 analogue recorder equipped with an accurate amplifier (Pantos, Kyoto). The signal was calibrated by recording the rapid and complete cleavage of a known amount of a relevant substrate in a specified buffer with a small aliquot of the concentrated Mpc stock solution (0.5–2.0 µl). The 1:1 stoichiometry of O_2 consumption and product formation for each substituted catechol was confirmed by comparing the calibration signal with the signal recorded for the complete cleavage of a known amount of catechol. The dependence of the initial velocity on catecholic substrate concentration was measured in 3.0 ml of reaction mixture under air-saturation ($[O_2]$ = 240–260 µM) by following the formation of the product using a UV-140 UV/VIS spectrophotometer (Shimadzu, Kyoto) equipped with a thermo-jacketed cell holder. The signal from the spectrophotometer was recorded with a Unicorder U-228 analogue recorder, and the signal was calibrated as described above for the calibration of the signal of the O_2 electrode.

The enzymatic reaction was started by the addition of a small aliquot of the dilute Mpc stock solution (0.5–5 µl), which was removed from the vial with a micro-syringe just before addition.

To determine the value of the catalytic constant (k_{cat}) of Mpc, the amount of the active ferrous center (E_t) should be known for the relevant kinetic assay conditions. E_t was estimated as follows. First, using the same amount of dilute Mpc stock solution as used for the kinetics experiments, the activity was measured at 25°C in 50 mM HEPES buffer (pH 7.5, ionic strength = 0.15 M) containing 100–200 µM catechol under air-saturation (standard assay conditions). Because there is a linear relationship between iron content and specific activity (*24*), the E_t value was calculated by multiplying the total Mpc subunit concentration by the ratio of the measured specific activity and the specific activity of fully active Mpc. We assumed that fully active Mpc shows a specific activity of 477 U/mg (*24*).

Analysis of Steady-State Kinetic Data—Steady-state kinetic data were analyzed using Eq. 2 on the basis of an ordered Bi Uni mechanism in which the binding of the catecholic substrate, A, precedes that of O_2 (Fig. 1A),

$$v = k_{cat}E_tAO_2/(K_{dA}K_{mO2} + K_{mA}O_2 + K_{mO2}A + AO_2) \quad (2)$$

where v is the initial velocity, K_{mA} and K_{mO2} represent the K_m for catecholic substrate and O_2, respectively, K_{dA} represents the dissociation constant for the binding of the substrate to the enzyme, A and O_2 in italic letters are the molar concentrations of the substrate and O_2, respectively. E_t is the molar concentration of the active ferrous center. Based on Eq. 2, the dependence of the initial velocity on the catechol concentration under fixed O_2 concentration and on the O_2 concentration under a fixed catechol concentration is described by the following Michaelis-Menten equations, respectively.

$$v = V_{max}{}^{app}A/(K_{mA}{}^{app} + A) \quad (3)$$

$$v = V_{max}{}^{app}O_2/(K_{mO2}{}^{app} + O_2) \quad (4)$$

The relations between the actual and apparent values of V_{max} and K_m are summarized in Appendix. All kinetic data obtained fitted well to Eq. 3 or 4. We first determined the apparent V_{max} and K_m values by linear regression to *s*/*v* versus *s* plots with equal weighting, where *s* is the concentration of the catecholic substrate or O_2. Then, to confirm the goodness of the fit, the data were plotted on the theoretical *v versus s* curve drawn using the fitted values of V_{max} and K_m.

pH-Dependence of k_{cat} Values—The effects of pH on the k_{cat} of Mpc for catechol and 4-chlorocatechol were examined using the following buffers: 50 mM MES-NaOH (pH 5.5–6.5), 50 mM HEPES-NaOH (pH 6.5–8.0), 50 mM Tris-HCl (pH 8.0–8.5), 50 mM glycine-NaOH (pH 9.0–10.0), 50 mM CHES-NaOH (pH 9.0–10.0), and 50 mM CAPS-NaOH (pH 10–11). The ionic strength of all buffers was adjusted to 0.15 M with NaCl. The relative k_{cat} values of Mpc for catechol, 4-methylcatechol, 4-chlorocatechol, 4-bromocatechol, 4-formylcatechol, and 3-formyl-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

Table 1. **Properties of C4/C3-substituted catechols.**

Substituent	pK_1[a]	Wavelength[b] (nm)	
		Neutral form	Monoanion
H	9.32 ± 0.02	274.8	288.4
4-CH_3	9.47 ± 0.01	279.8	294.8
4-CH_2OH	8.91 ± 0.03	279.3	292.7
4-Cl	8.51 ± 0.02	284.5	296.0
4-Br	8.41 ± 0.02	284.0	296.8
4-NO_2	7.35 ± 0.09	345.6	429.2
4-CHO	7.26 ± 0.02	309.3	346.3
3-CH_3	9.32 ± 0.02	273.8	286.5
3-Phenyl	8.98 ± 0.06	282.9	304.5
3-Cl	8.00 ± 0.01	275.4	291.7
3-CHO	7.89 ± 0.04	344.6	388.4

[a]pK_1 (the first pK_a) was determined by UV-VIS spectral titration in 0.15 M NaCl at 25°C as described under "EXPERIMENTAL PROCEDURES". [b]The wavelength values listed are those of the absorbance peak observed for the lowest electronic transition of the neutral and monoanionic forms of catechols.

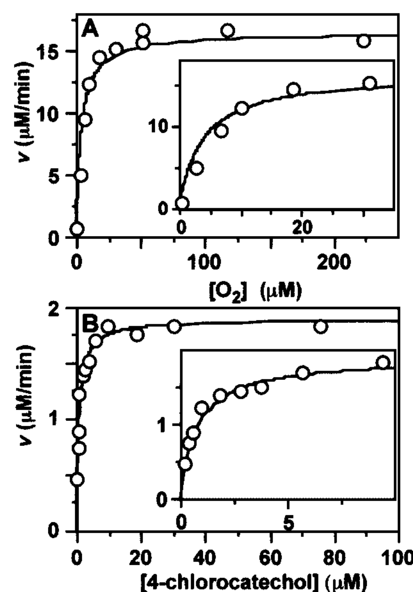

Fig. 3. **Typical steady-state kinetic behavior of Mpc reactions with catechols.** The experiments were performed using 50 mM MES, pH 6.5, ionic strength = 0.15 M (adjusted with NaCl), at 25°C. (A) The dependence of the initial velocity of the Mpc reaction with 4-methylcatechol (fixed concentration of 179 μM) on O_2 concentration. The inset is an enlargement of the data in the low O_2 concentration region. The total concentration of active enzyme subunit used was 2.96 nM. The line represents the best fit of the data to the Michaelis-Menten equation (K_{mO2} = 3.80 ± 1.8 μM, V_{max} = 16.4 ± 0.3 μM/min). (B) The dependence of the initial velocity on the concentration of 4-chlorocatechol under air-saturation ([O_2] = 240–260 μM). The inset is an enlargement of the data in the low substrate concentration region. The total concentration of active enzyme subunit used was 0.76 nM. The line represents the best fit of the data to the Michaelis-Menten equation (K_{mA} = 0.79 ± 0.52 μM, V_{max} = 1.89 ± 0.01 μM/min).

catechol were measured using saturating concentrations of catecholic substrates in air-saturated MES (pH 6.5), HEPES (pH 7.5), and Tris-HCl (pH 8.5) buffers and the same amount of Mpc (the final Mpc concentration of 11.2 nM, E_t = 4.73 nM).

The pH-dependence of the k_{cat} values for catechol and 4-chlorocatechol was simulated with Eq. 5 supposing that the Mpc-substrate-O_2 ternary complex has three ionizable groups involved in the rate-determining step or steps,

$$k_{cat} = (k_{cat,1}10^{pK_{II}-pH} + k_{cat,2})/(1 + 10^{pK_{II}-pH} + 10^{pK_I + pK_{II}-2pH} + 10^{pH-pK_{III}}) \quad (5)$$

where K_I, K_{II}, and K_{III} ($K_I > K_{II} > K_{III}$) are the respective dissociation constants of the three ionizable groups of the ternary complex, and $k_{cat,1}$ and $k_{cat,2}$ are the catalytic constants for the singly and doubly deprotonated forms of the ternary complex, respectively.

RESULTS

Properties of Substituted Catechols—Upon titration over the pH range 3.5–11, the absorbance due to the lowest energy transition of each catechol showed a red shift (11.5–83.6 nm) and an increase in absorption as the pH increased. Isosbestic points were observed for the absorption band of all catechols examined except 3-methylcatechol and 4-nitrocatechol. In this pH range, we did not observed any tendency for further deprotonation of the monoanionic species to the dianionic species for any catechol. The observed reversible pH-dependent changes in the absorption fitted Eq . 1 well. The pK_1 values obtained are summarized in Table 1 together with the λ_{max} values of the lowest energy transition of the neutral and monoanionic species of catechols. Electron-withdrawing substituents decreased the pK_1 of catechol (9.32) maximally to 7.26.

The pK_1 values for C4-substituted catechols showed the best correlation with the mean of the σ_p^- and σ_m values (*28*) (ρ = –2.55, Fig. 2A), while those of the C3-substituted catechols showed a good correlation with σ_m values (ρ = –3.77, Fig. 2B). These results suggest that the monoanion of a C4-substituted catechol exists in equilibrium between the 1-hydroxy-2-phenolate- and 2-hydroxy-1-phenolate-forms of the catechol, and that the monoanion of a C3-substituted catechol exists predominantly in the 2-hydroxy-1-phenolate form. The obtained ρ value for C4-substituted catechols is comparable to that reported for the linear relationship between the pK_a values of C4-substituted phenols and Hammett σ_p^- values (ρ = –2.23 in H_2O and = –2.67 in 50% ethanol) (*29*).

Steady-State Kinetic Analyses—3-Chlorocatechol, 4-ethylcatechol, and 4-hydroxymethycatechol rapidly inactivated Mpc during turnover, although significant amounts of products were formed before complete inactivation of Mpc. Therefore, we could not obtain reliable kinetic parameters for these three catechols. 4-Nitrocatechol was cleaved by Mpc, but the K_{mO2} value was very large (more than 1 mM), and we could not obtain precise kinetic parameters for this catechol. Catechols with anionic substituents such as 3,4-dihydroxybenzoate (4-carboxycatechol) and 3,4-dihydroxyphenylacetate (4-carboxymethylcatechol) were not cleaved by Mpc. The enzyme could not cleave 4-*tert*-butylcatechol, while 2,3-dihydroxybiphenyl (3-phenylcatechol) was a relatively good substrate of Mpc. Therefore, we carried out detailed kinetic analyses for the eight catechols listed in Table 2 at pH 6.5 and 7.5.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

Table 2. **Steady-state kinetic parameters for extradiol cleavage of substituted catechols by Mpc at 25°C.**[a]

Substituent	K_{mA} (μM)	K_{mO2} (μM)	k_{cat} (s^{-1})	k_{cat}/K_{mA} (M^{-1} s^{-1})	k_{cat}/K_{mO2} (M^{-1} s^{-1})
	pH 6.5				
H	2.54 ± 0.46	10.6 ± 0.4	402 ± 7	1.58×10^8	3.79×10^7
4-CH3	1.22 ± 0.31	3.80 ± 1.8	248 ± 5	2.03×10^8	6.53×10^7
4-Cl	0.79 ± 0.52	7.11 ± 0.31	150 ± 4	1.90×10^8	2.11×10^7
4-Br	1.17 ± 0.19	10.4 ± 0.3	143 ± 3	1.22×10^8	1.38×10^7
4-CHO	15.1 ± 0.3	492 ± 73	202 ± 20	1.34×10^7	4.11×10^6
3-CHO	1.79 ± 0.22	259 ± 8	98.9 ± 3.5	5.53×10^7	3.82×10^6
	pH 7.5[b]				
H	1.87	7.5	278	1.49×10^8	3.71×10^7
4-CH3	1.29	10	257	1.99×10^8	2.57×10^7
4-Cl	1.02	16	211	2.07×10^8	1.32×10^7
4-CHO	22.8	313	112	4.91×10^6	3.58×10^5
3-CHO	1.97	25	58.2	2.95×10^7	2.33×10^6
3-CH3	1.64	11.0	221	1.35×10^8	2.01×10^7
3-Phenyl	0.26	190	24	9.23×10^7	1.26×10^5

[a]4-Carboxycatechol, 4-carboxymethylcatechol, and 4-*tert*-butylcatechol are not substrates for Mpc. 3-Chlorocatechol, 4-ethylcatechol, and 4-hydroxymethylcatechol rapidly inactivate Mpc during turnover, and making it impossible to obtain reliable kinetic parameters for these two catechols. The K_{mO2} value for 4-nitrocatechol is more than 700 μM; therefore, precise kinetic parameters could not be obtained for this catechol.[b]The kinetic parameters for 3-formylcatechol and 3-phenylcatechol were determined in the present study; other parameters are taken from the previous study (*24*).

Figure 3 shows typical kinetic data obtained in the present study. All kinetics data fitted the Michaelis-Menten equation well. The best-fit steady-state kinetics parameters are provided in Table 2. The K_{mA} value of Mpc was mostly in the range of 0.2–3 μM; for 4-formylcatechol only it was 15.1 and 22.8 μM at pH 6.5 and 7.5, respectively. On the other hand, the K_{mO2} values of Mpc were in the range of 3–20 μM except for catechols with formyl or phenyl substituents. For 4-formylcatechol the K_{mO2} value was 492 and 313 μM at pH 6.5 and 7.5, respectively. For 3-formylcatechol the K_{mO2} value was 259 μM at pH 6.5, whereas it was 25 μM at pH 7.5. For 3-phenylcatechol the K_{mO2} value was 190 μM at pH 6.5.

Because the O_2 concentration is 240–260 μM in air-saturated buffers at 25°C, the apparent K_{mA} values obtained under air-saturation for these catechols with high K_{mO2} values were possibly not good approximations of the actual values (Eq. A3 in "APPENDIX").

The largest k_{cat} value was found to be 402 s^{-1} for catechol at pH 6.5, and the smallest was 24 s^{-1} for 3-phenylcatechol at pH 7.5. The difference in the k_{cat} values of Mpc among eight catechols examined was maximally about 17-fold, while the largest differences in the K_{mA} and K_{mO2} values were 88- and 129-fold, respectively.

Specificity Constants as a Function of Catecholic pK_1—The specificity constant k_{cat}/K_{mA} of Mpc was maximally about 2.0×10^8 M^{-1} s^{-1} for 4-methylcatechol and 4-chlorocatechol, slightly greater than that for catechol (about 1.5×10^8 M^{-1} s^{-1}, Table 2). The smallest k_{cat}/K_{mA} was 4.9×10^6 M^{-1} s^{-1} for 4-formylcatechol.

Figure 4A shows the dependence of the logarithm of the relative k_{cat}/K_{mA} on pK_1. With exception of 4-chlorocatechol, there was a good linear correlation between log k_{cat}/K_{mA} and pK_1 (slope = 0.66, r^2 = 0.96) at pH 7.5. The data point obtained for 3-phenylcatechol, a bulky bicyclic catechol, was on the line. A good linear correlation was also found between log k_{cat}/K_{mA} and pK_1 (slope = 0.48, r^2 = 0.94) at pH 6.5 when the data for 4-chloro- and 4-bromocatechols were excluded.

According to the reaction mechanism illustrated in Fig. 1A, the specificity constant of k_{cat}/K_{mA} is equal to the binding rate constant of the catecholic substrate to the enzyme active site (k_1, see Eq. A7 in Appendix). Therefore, the results indicate that electron-withdrawing substituents reduce the binding rate of catechol. The catechol binding process seems to be less sensitive to steric factors, such as the position of the substitution (C3 or C4) and the bulkiness of the substutients. Chlorine and bromine substituents at the C4 position showed halogen-specific effects to compensate for their electron-withdrawing effects on the substrate binding process. It is well known that 3-halocatechols rapidly inactivate many

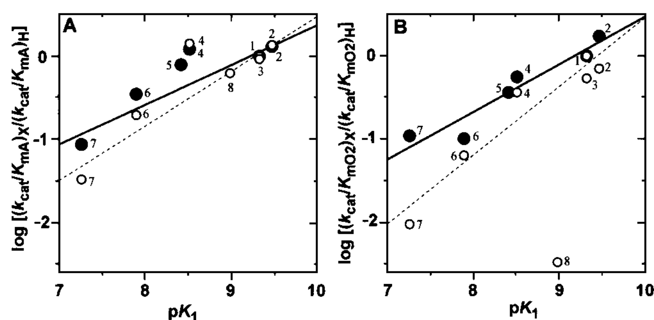

Fig. 4. **Substituent effects on the specificity constants of Mpc at 25°C.** (A) Plot of the logarithm of the relative k_{cat}/K_{mA} at pH 6.5 (solid circles) and 7.5 (open circles) against pK_1. The $(k_{cat}/K_{mA})_x$ and $(k_{cat}/K_{mA})_H$ denote the k_{cat}/K_{mA} values for substituted catechol and catechol, respectively. (B) Plot of the logarithm of the relative k_{cat}/K_{mO2} at pH 6.5 (solid circles) and 7.5 (open circles) against the pK_1. The $(k_{cat}/K_{mO2})_x$ and $(k_{cat}/K_{mO2})_H$ denote the k_{cat}/K_{mO2} values for substituted catechol and catechol, respectively. 1, catechol; 2, 4-methylcatechol; 3,3-methylcatechol; 4,4-chlorocatechol; 5,4-bromocatechol; 6,3-formylcatechol; 7,4-formylcatechol; 8,3-phenylcatechol.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

Table 3. **Effects of pH on k_{cat} for substituted catechols at 25°C.**

Substituent	k_{cat} (s^{-1})		
	pH 6.5	pH 7.5	pH 8.5
H	402	278	128
4-CH3	248	257	254
4-Cl	150	211	248
4-Br	143	207	256
4-CHO	202	112	20.3[a]
3-CHO	98.9	58.2	37.1[a]

[a] Apparent values obtained under air-saturation.

extradiol dioxygenases including Mpc in a mechanism-based manner (*30–32*). There may be some common mechanism in which chlorine and bromine interact with the active site of Mpc to induce halogen-specific effects.

The k_{cat}/K_{mO2} values decreased in the order 4-methy-catechol > catechol > 4-chlorocatechol > 4-bromocatechol > 4-formylcatechol > 3-formylcatechol at pH 6.5. The plot of the logarithm of the relative k_{cat}/K_{mO2} *versus* pK_1 revealed a good linear correlation (slope = 0.57, $r^2 = 0.91$) at pH 6.5 without any outlier (Fig. 4B). At pH 7.5, the k_{cat}/K_{mO2} values decreased in the order catechol > 4-methyl-catechol > 3-methylcatechol > 4-chlorocatechol > 3-formylcatechol > 4-formylcatechol > 3-phenylcatechol. With exception of 3-phenylcatechol, there was a good linear correlation between log k_{cat}/K_{mO2} and pK_1 (slope = 0.83, $r^2 = 0.93$) at pH 7.5. The aberrant behavior observed for 3-phenylcatechol suggests that bulky substituents at the C3 position inhibit sterically the binding of O_2. For all other catechols, the specificity constant k_{cat}/K_{mO2} decreased as the electron-withdrawing nature of the substituents increased.

The specificity constant of k_{cat}/K_{mO2} is proportional to the rate constant of O_2 binding to the EA complex (k_2) and intimately related to the activation of the bound O_2 (Fig. 1A and Eq. A8 in Appendix). The ternary enzyme-catechol-O_2 complex is committed to ring cleavage (k_3) or dissociates O_2 (k_{-2}). When k_{-2} is much smaller than k_3 or of the same order of magnitude as k_3, then k_{cat}/K_{mO2} reflects mainly the rate constant of O_2 binding (k_2). The observed linear free energy relationship suggests that electron-withdrawing substituents suppress the binding of O_2 molecules to the enzyme-catechol complex.

Substituent Effects on the Overall Reaction Rate—Figure 5 shows the plot of the logarithm of the relative k_{cat} versus pK_1. Although there seems to be a weak tendency for the k_{cat} value to decrease as the pK_1 value decreases, no clear correlation between the k_{cat} and pK_1 values was observed. The results suggest that after the proper activation of O_2 in the enzyme active site, the irreversible steps to the enzyme-product complex (Fig. 1D) are less sensitive to the electronic nature of the substituent compared to the preceding two steps of catechol and O_2 binding.

pH-Dependence of the k_{cat} Values for Catechol and 4-Chlorocatechol—Substituents effects on the k_{cat} values were further examined as a function of pH. The k_{cat} of Mpc for six catechols was determined at pH 6.5, 7.5, and 8.5 (Table 3). The k_{cat} value for 4-methylcatechol did not changed as pH increased from 6.5 to 8.5 and remained about 250 s^{-1}, while the k_{cat} values for catechol, 4-formylcatechol, and 3-formylacatechol decreased as the pH increased from 6.5 to 8.5. On the other hand, the k_{cat} value for 4-chlorocatechol and 4-bromocatechol increased as the pH increased from 6.5 to 8.5.

To investigate the different pH dependences of the k_{cat} values, those for catechol and 4-chlorocatechol were measured over the pH range of 5–10. The results are shown in Fig. 6. The k_{cat} values at pH < 5.5 could not be measured due to rapid enzyme inactivation, and those at pH > 10.5 could not be measured because significant autooxidation of catechols occurs at high pH values. The pH dependence of the k_{cat} values of the two catechols could be simulated with a triple ionization curve (Eq. 5) using the same set of three pK_a values of 5.5, 7.5, and 9.8.

Since the pH profile of k_{cat} reflects the ionization state of the ternary EAO_2 complex, the results suggest that a deprotonated group with an approximate pK_a value of 5.5 and a protonated group with an approximate pK_a value of 9.8 in the EAO_2 complex are essential for ring cleavage. In addition to these two groups, it is suggested that another ionizable group with a pK_a of 7.5 in the ternary complex affects the rate-determining step relatively weakly in a substrate-dependent manner; deprotonation of this group disfavors the enzymatic cleavage of catechol but facilitates the cleavage of 4-chlorocatechol.

DISCUSSION

In the present study, we show for the first time that the electronic nature of substituents is a major factor in determining the specificity constants of k_{cat}/K_{mA} and k_{cat}/K_{mO2} of Mpc for a series of C4/C3-substituted catechols whose pK_1 values range from 7.26 to 9.47. Exceptions are 4-halocatechols in the case of k_{cat}/K_{mA}, and 3-phenylcatechol in the case of k_{cat}/K_{mO2}. The overall turnover rate (k_{cat}) of Mpc is less sensitive to the electronic nature of substituents than the specificity constants.

Cleaving the Catechol Ring—There have been two opposing proposals for the electronic effects on the irreversible ring cleavage step of the enzymatic reaction ($EAO_2 \rightarrow EP$). One considers that electron-withdrawing groups on the catechol ring support the nucleophilic attack of the O_2 molecule on the C3 carbon atom to form a peroxy intermediate (*13*, *33*). The other considers that a peroxy intermediate is formed by a radical mechanism (see Fig. 1D) and that electron-withdrawing substituents inhibit the alkenyl migration in the peroxy intermediate (*22*). If these reaction processes are the rate-determining step in the overall reaction, then electron-withdrawing substituents are expected to increase or decrease the k_{cat} value, respectively. However, we could not observe a significant correlation between k_{cat} and pK_1. The pH dependence of the k_{cat} of Mpc suggests the existence of an ionizable residue with a pK_a of about 7.5 that is not essential for ring cleavage but affects the overall reaction rate. Thus, the present results on k_{cat} values suggest that steric and/or group-specific factors of the substituents significantly affect the irreversible ring cleavage step to obscure the electronic effects. Of course, there is a possibility that the release of the product from the enzyme-product complex is the rate-determining step in the overall reaction and determines the k_{cat} value. Experimental determination of the rate of product release (k_4) is

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

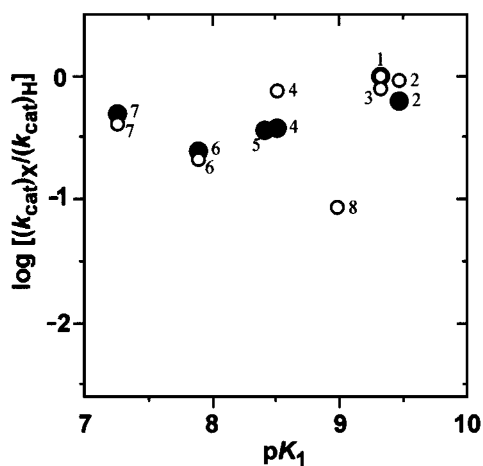

Fig. 5. **Substituent effects on the overall reaction rate at 25°C.** The logarithm of the relative k_{cat} at pH 6.5 (solid circles) and 7.5 (open circles) was plotted against pK_1. The $(k_{cat})_H$ and $(k_{cat})_X$ denote the k_{cat} for catechol and 4-substituted catechols, respectively. The numbers show the same catechol compounds as indicated in Fig. 4.

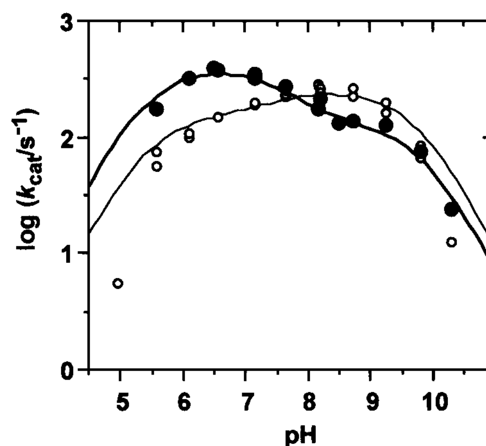

Fig. 6. **pH Dependence of the k_{cat} value of Mpc with catechol and 4-chlorocatechol at 25°C.** The thick and thin lines were drawn according to Eq. 5 for catechol (solid circles) and 4-chlorocatechol (open circles) using the values pK_I = 5.5, pK_{II} = 7.5, pK_{III} = 9.8, and the following respective values: $k_{cat,1}$ = 402 s^{-1} and $k_{cat,2}$ = 128 s^{-1} for catechol; $k_{cat,1}$ = 150 s^{-1} and $k_{cat,2}$ = 248 s^{-1} for 4-chlorocatechol (see Table 3).

needed, but it is difficult to measure the k_4 value because it is difficult to discriminate spectrophotometrically between the EP complexes and free products.

Substrate Binding—Surprisingly, there is a good linear free energy relationship between the k_{cat}/K_{mA} of Mpc and the pK_1 of catechols, including 3-phenylcatechol. Because the specificity constant of k_{cat}/K_{mA} is equal to the rate constant of the binding of substrate to the enzyme (k_1), this result suggests that the Mpc active site is large enough to accommodate these substituents at the C4 or C3 position, and that a substituent at the C4 position that is smaller than or similar to a formyl group does not give rise to steric hindrance with chemical groups in the active site, but a substituent at the C4 position that is larger than or similar to a nitro group gives rise to significant steric hindrance for the binding of catechol derivatives to the Mpc active site. It is intriguing that electron-withdrawing substituents decrease the substrate binding rate of Mpc. Mono-substituted phenols are competitive inhibitors of Mpc for catecholic substrates, and electron-withdrawing substituents increase the stability of the enzyme-phenol complex (our unpublished results). One possible mechanism to explain the electronic effects on the catechol binding rate is as follows. The binding of catechol to the active site consists of at least two steps. First, an intermediate is formed in which the catechol is monodentately bound to the Fe(II) center, as with phenols. The intermediate is stabilized by electron-withdrawing substituents, and the transition from the intermediate to the final EA complex is the rate-determining step in the substrate binding. To test this possible mechanism for substrate binding, the substituent effects on the binding energy between catecholic substrates and the enzyme are now investigated in our laboratory.

O_2 Binding and Activation—A significant linear free energy relationship was found between the specificity constant of k_{cat}/K_{mO2} for Mpc and pK_1. The HOMO energies (E_{homo}) of free catecholate monoanions have been calculated for catechol and 4-methyl-, 4-chloro- and 4-bromo-catechols (*34*). We found a linear relationship between the reported E_{homo} (eV) and pK_1 values with slope of 0.41. The HOMO energy of the catecholate monoanion bound to the active site Fe(II) center is expected to be reduced by electron-withdrawing substituents. If one electron transfer from the bound catechol monoanion to the O_2 via the Fe(II) center is the rate-determining step in the O_2 binding, then electron-withdrawing substituents inhibit O_2 binding by reducing the nucleophilicity of the catechol in the active site. Because k_{cat}/K_{mO2} is proportional to the rate of O_2 binding to the EA complex (k_2), the observed electronic effects on the k_{cat}/K_{mO2} value is possibly due to effects on the k_2 value.

Comparison of Substituent Effect between Intradiol- and Extradiol-Cleaving Enzymes—Intradiol-cleaving enzymes catalyze the cleavage of catechols between the vicinal hydroxyl groups using Fe(III) ions. The effects of C4- and/or C3-substituents on the k_{cat} values have been investigated for intradiol dioxygenases (*34*, *35*). A linear correlation between the logarithm of the k_{cat} values and Hammett σ values has been found for pyrocatechases I and II (*35*). A strong linear correlation between the logarithm of the k_{cat} values and the HOMO energies of C4-substituted catechol monoanions has been reported for catechol 1,2-dioxygenase from *Pseudomonas putida* (*arvilla*) C1 (*34*). It is considered that O_2 molecules cannot bind directly to the Fe(III) center of the enzyme-catechol complex (*13*, *15*). The attack of O_2 on the activated substrate to form the ternary enzyme-substrate-O_2 complex (peroxy intermediate) and subsequent acyl migration of the intermediate is proposed as the rate-determining step in intradiol cleavage (*15*, *22*, *34*).

In the case of Mpc, we did not observe significant electronic effects of substituents on the k_{cat} values. The present results suggest that in the ternary Mpc-catechol-O_2 complex the bound O_2 molecule is activated to a superoxide species prior to the formation of the alkyl-peroxy intermediate. In the case of intradiol dioxygenases, the electrophilic attack of O_2 on the activated substrate

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

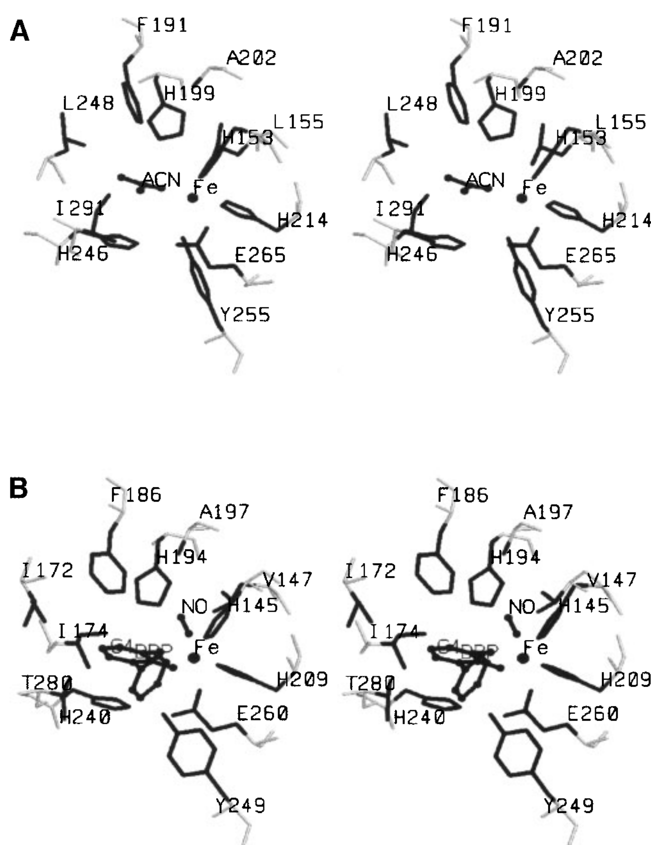

Fig. 7. **Stereo view of the active-site structures of (A) the Mpc-acetone complex (PDB code: 1MPY) (*27*) and (B) the BphC-DBP-NO complex (PDB code: 1KW8) (*18*).** BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. strain KKS102; DBP, 2,3-dihydroxybiphenyl. The bound substrates and the side chains are darkly shaded. Small black spheres are Fe(II) ions. These figures were prepared using the program RASMOL (*36*).

includes the activation of O_2 to a peroxy species. Thus, the electronic effects on the k_{cat} of intradiol dioxygenases may correspond to those on the k_{cat}/K_{mO2} of Mpc.

Active Site Structure—Crystal structures of Mpc in complexes with substituted catechols are essential for interpreting the present results, but are still not available despite our continued efforts. For 2,3-dihydroxybiphenyl 1,2-dioxygenass from *Pseudomonas* sp. strain KKS102 (BphC), a high-resolution structure of the BphC-2,3-dihydroxybiphenyl-NO complex has recently been determined (*18*). Since 2,3-dihydroxybiphenyl (=3-phenylcatechol) is a moderately good substrate for Mpc and the subunit structure of Mpc resembles that of BphCKKS102, as described previously (*27*), the active-site structure of the Mpc-acetone complex was compared to that of the BphC-2,3-dihydroxybiphenyl-NO complex (Fig. 7).

The molecular plane of acetone, a competitive inhibitor of catechol (*24*), is parallel to the imidazole ring of His246 (Fig. 7A), just as the catechol ring of the bound substrate is parallel to the imidazole ring of His240 (Fig. 7B). The space occupied by the 3-phenyl group of the substrate bound to the BphC active site is wide open at the corresponding position in the Mpc active site, consistent with the fact that Mpc can catalyze the extradiol cleavage of 3-phenylcatechol. On the other hand, BphC cannot catalyze the extradiol cleavage of 4-substituted catechols (manuscript in preparation). In fact, the C4 atom of the catechol ring is in contact with the side chain of Thr280 and cannot accept even relatively small substituents such as chlorine, whereas there is a relatively wide space to accept C4-substituents in the corresponding region of the Mpc active site. In fact, the binding of 4-formylcatechol to the Mpc active site is sterically well tolerated. Steric hindrance becomes apparent for the binding of 4-nitrocatechol, and bulky *t*-butyl groups at the C4 position can not be accepted in the Mpc active site.

The pH-dependence of the k_{cat} values (Fig. 6) revealed that three ionizable residues of the Mpc-substrate-O_2 complex are catalytically important. There are only three ionizable residues at the active site of Mpc other than the three Fe(II) ligands; His199, His246, and Tyr255 (Fig. 7A). On the basis of the structural data for the BphCKKS102-substrate complex (*18*), BphC-substrate-NO complex (*18*), and BphCLB400-substrate complex (*17*), the following is suggested: The residue with a pK_a value of 5.5 is His199, and acts as a proton shuttle between the 1-OH group of the substrate and the proximal oxygen atom of the activated O_2 (Fig. 1, B and C). The residue with a pK_a value of 7.5 is His246, and affects the rate-determining step through a steric mechanism. (Fig. 1D). Tyr255 is a probable candidate for the residue with a pK_a value of 9.8, and forms a hydrogen bond with the 2-OH group of the catehol to contribute to the asymmetric binding of the substrate that is essential for the binding and activation of O_2 (Fig. 1, C and D).

In the ternary complex of the BphC-substrate-NO complex, the NO binding site (=O_2 binding site) is formed by the catechol ring of the substrate, the side chains of Phe186, Val147, Ala197 and His194, and iron-chelating His145 and His209 (*18*). In the Mpc active site, the Val147 of BphC is replaced by the more bulky Leu155, and the other residues are completely conserved. Therefore, it is expected that the C4-substituent of catecholic substrate does not contact the O_2 binding site of Mpc directly. On the other hand, the bulky C3-substituent may hinder O_2 binding sterically. In fact, only 3-phenylcatechol shows an aberrant low k_{cat}/K_{mO2} values, and the electronic effects of the substituents on k_{cat}/K_{mO2} were successfully observed without disturbance by steric effects.

Concluding Remarks—The reaction mechanisms so far discussed have been based only on the apparent k_{cat} values. In the present study, we determined kinetically the true values of k_{cat}, K_{mA}, and K_{mO2} for various catecholic substrates for the first time among extradiol dioxygenases, and analyzed the substrate specificity constants and the overall reaction rate in terms of the pK_1 values of catecholic substrates, *i.e.*, the electronic nature of the substrates.

All the processes of catechol binding to the Mpc active site, the subsequent O_2 binding, and the activation of the bound O_2 are sensitive to the electronic effects of the substituents. However, the overall reaction rate (k_{cat}) does not correlate significantly with pK_1. The present study distinguishes clearly between the electronic and steric effects of catecholic substrates on the reactivity of Mpc,

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

and sheds new light on the mechanistic basis for the different substrate specificities of extradiol dioxygenases.

To clarify further the reaction mechanisms of extradiol-type dioxygenases, the following investigations are now under way: 1) examination of substituent effects on the reaction process and the energy of Mpc-substrate complex formation, 2) kinetic and crystallographic studies of BphC (2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. KKS102) using C3/C4-substituted catechols.

APPENDIX

The dependence of the initial velocity (v) on the catecholic substrate concentration (A) at a constant concentration of O_2 (O_2) is expressed as follows:

$$v = k_{cat}{}^{app}E_tA/(K_{mA}{}^{app} + A) \quad (A1)$$

$$k_{cat}{}^{app} = k_{cat}O_2/(K_{mO2} + O_2) \quad (A2)$$

$$K^{app}{}_{mA} = (K_{dA}K_{mO2} + K_{mA}O_2)/(K_{mO2} + O_2) \quad (A3)$$

The dependence of the initial velocity on the O_2 concentration at a constant concentration of catechol is expressed as follows:

$$v = k_{cat}{}^{app}E_tO_2/(K_{mO2}{}^{app} + O_2) \quad (A4)$$

$$k_{cat}{}^{app} = k_{cat}A/(K_{mA} + A) \quad (A5)$$

$$K_{mO2}{}^{app} = K_{mO2}(K_{dA} + A)/(K_{mA} + A) \quad (A6)$$

To obtain actual k_{cat} values, the dependence of the initial velocity on the O_2 concentration at saturating concentrations of catecholic substrate should be examined (Eq. A5). When K_{mO2} is larger than the O_2 concentration in air-saturated buffer, the apparent k_{cat} value obtained by examining the dependence of the initial velocity on the catechol concentration using air-saturated buffer is approximately equal to $(k_{cat}/K_{mO2})O_2$.

The following are the relationships between the steady-state kinetic parameters and the rate constants described in Fig. 1:

$$k_1 = k_{cat}/K_{mA} \quad (A7)$$

$$k_2 / \left(1 + \frac{k_{-2}}{k_3}\right) = k_{cat}/k_{mO2} \quad (A8)$$

$$k_3 / \left(1 + \frac{k_3}{k_4}\right) = k_{cat} \quad (A9)$$

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (B, NO. 753) (NO. 13125202) (to K.H., T. I., and H. T.) and a Grant-in-Aid for Scientific Research (C) (NO. 15590485) (to T. I., and H. T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Worsey, M.J. and Williams, P.A. (1975) Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for a new function of the TOL plasmid. *J. Bacteriol.* **124**, 7–13
2. Greated, A., Lambertsen, L., Williams, P.A., and Thomas, C.M. (2002) Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. *Environ. Microbiol.* **4**, 856–871
3. Maeda, K., Nojiri, H., Shintani, M., Yoshida, T., Habe, H., and Omori, T. (2003) Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicity and the presence of large catabolic transposon Tn*4676*. *J. Mol. Biol.* **326**, 21–33
4. Que, L., Jr., Widom, J., and Crawford, R.L. (1981) 3,4-Dihydroxyphenylacetate 2,3-dioxygenase. A manganese(II) dioxygenase from *Bacillus brevis*. *J. Biol. Chem.* **256**, 10941–10944
5. Boldt, Y.R., Sadowsky, M.J., Ellis, L.B., Que, L., Jr., and Wackett, L.P. (1995) A manganese-dependent dioxygenase from *Arthrobacter globiformis* CM-2 belongs to the major extradiol dioxygenase family. *J. Bacteriol.* **177**, 1225–1232
6. Hatta, T., Murkerjee-Dhar, G., Damborsky, J., Kiyohara, H., and Kimbara, K. (2003) Characterization of a novel thermostable Mn(II)-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase from a polychlorinated biphenyl- and naphthalene-degrading *Bacillus* sp. JF8. *J. Biol. Chem.* **278**, 21483–21492
7. Eltis, L.D. and Bolin, J.T. (1996) Evolutionary relationships among extradiol dioxygenases. *J. Bacteriol.* **178**, 5930–5937
8. Spence, E.L., Kawamukai, M., Sanvoisin, J., Braven, H., and Bugg, T.D.H. (1996) Catechol dioxygenases from *Escherichia coli* (MhpB) and *Alcaligenes eutrophus* (MpcI): Sequence analysis and biochemical properties of a third family of extradiol dioxygenases. *J. Bacteriol.* **178**, 5249–5256
9. Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M., and Mitsui, Y. (1999) Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Structure* **7**, 953–965
10. Ramos, J.L., Wasserfallen, A., Rose, K., and Timmis, K.N. (1987) Redesigning metabolic routes: Manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science* **235**, 593–596
11. Cerdan, P., Wasserfallen, A., Rekik, M., Timmis, K.N., and Harayama, S. (1994) Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 of *Pseudomonas putida* and its relationship to cell growth. *J. Bacteriol.* **176**, 6074–6081
12. Dai, S., Vaillancourt, F.H., Maaroufi, H., Drouin, N.M., Neau, D.B., Snieckus, V., Bolin, J.T., and Eltis, L.D. (2002) Identification and analysis of a bottleneck in PCB biodegradation. *Nat. Struct. Biol.* **9**, 934–939
13. Que, L., Jr. and Ho, R.Y.N. (1996) Dioxygen activation by enzymes with mononuclear non-heme iron active sites. *Chem. Rev.* **96**, 2607–2624
14. Bugg, T.D.H., Sanvoisin, J., and Spence, E.L. (1997) Exploring the catalytic mechanism of the extradiol catechol dioxygenases. *Biochem. Soc. Trans* **25**, 81–85
15. Lange, S.J. and Que, L., Jr. (1998) Oxygen activating nonheme iron enzymes. *Curr. Opin. Chem. Biol.* **2**, 159–172
16. Bugg, T.D.H. (2001) Oxygenases: mechanisms and structural motifs for O_2 activation. *Curr. Opin. Chem. Biol.* **5**, 550–555
17. Vaillancourt, F.H., Barbosa, C.J., Spiro, T.G., Bolin, J.T., Blades, M.W., Turner, R.F.B., and Eltis, L.D. (2002) Definitive evidence for monoanionic binding of 2,3-dihydroxybiphenyl to 2,3-dihydroxybiphenyl 1,2-dioxygenase from UV resonance Raman spectroscopy, UV/Vis absorption spectroscopy, and crystallography. *J. Amer. Chem. Soc.* **124**, 2485–2496
18. Sato, N., Uragami, Y., Nishizaki, T., Takahashi, Y., Sazaki, G., Sugimoto, K., Nonaka, T., Masai, E., Fukuda, M., and Senda, T. (2002) Crystal structures of the reaction intermediate and its homologue of an extradiol-cleaving catecholic dioxygenase. *J. Mol. Biol.* **321**, 621–636
19. Spence, E.L., Langley, G.J., and Bugg, T.D.H. (1996) *Cis-trans* isomerization of a cyclopropyl radical trap catalyzed by extradiol catechol dioxygenases: Evidence for a semiquinone intermediate. *J. Amer. Chem. Soc.* **118**, 8336–8343
20. Winfield, C.J., Al-Mahrizy, Z., Gravestock, M., and Bugg, T.D.H. (2000) Elucidation of the catalytic mechanisms of the non-heme iron-dependent catechol dioxygenases: Synthesis of

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

carba analogues for hydroperoxide reaction intermediates. *J. Chem. Soc. Perkin Trans.* **1**, 3277–3289

21. Sanvoisin, J., Langley, G.J., and Bugg, T.D.H. (1995) Mechanism of extradiol catechol dioxygenases: Evidence for a lactone intermediate in the 2,3-dihydroxyphenylpropionate 1,2-dioxygenase reaction. *J. Amer. Chem. Soc.* **117**, 7836–7837
22. Lin, G., Reid, G., and Bugg, T.D.H. (2001) Extradiol oxidative cleavage of catechols by ferrous and ferric complexes of 1,4,7-triazacyclononane: Insight into the mechanism of the extradiol catechol dioxygenases. *J. Amer. Chem. Soc.* **123**, 5030–5039
23. Deeth, R.J. and Bugg, T.D.H. (2003) A density functional investigation of the extradiol cleavage mechanism in non-heme iron catechol dioxygenases. *J. Biol. Inorg. Chem.* **8**, 409–418
24. Kobayashi, T., Ishida, T., Horiike, K., Takahara, Y., Numao, N., Nakazawa, A., Nakazawa, T., and Nozaki, M. (1995) Overexpression of *Pseudomonas putida* catechol 2,3-dioxygenase with high specific activity by genetically engineered *Escherichia coli*. *J. Biochem.* **117**, 614–622
25. Vaillancourt, F.H., Han, S., Fortin, P.D., Bolin, J.T., and Eltis, L.D. (1998) Molecular basis for the stabilization and inhibition of 2,3-dihydroxybiphenyl 1,2-dioxygenase by *t*-butanol. *J. Biol. Chem.* **273**, 34887–34895
26. Nakajima, H., Ishida, T., Tanaka, H., and Horiike, K. (2002) Accurate measurement of near-micromolar oxygen concentrations in aqueous solutions based on enzymatic extradiol cleavage of 4-chlorocatechol: Applications to improved low-oxygen experimental systems and quantitative assessment of back diffusion of oxygen from the atmosphere. *J. Biochem.* **131**, 523–531
27. Kita, A., Kita, S., Fujisawa, I., Inaka, K., Ishida, T., Horiike, K., Nozaki, M., and Miki, K. (1999) An archetypical exradiol-cleaving catecholic dioxygenase: The crystal structure of catechol 2,3-dioxygenase (metapyrocatechase) from *Pseudomonas putida* mt-2. *Structure* **7**, 25–34
28. Hansch, C., Leo, A., and Taft, R.W. (1991) A survey of Hammett substituent constants and resonance and field parameters. *Chem. Rev.* **91**, 165–195
29. Cohen, L.A. and Jones, W.M. (1963) A study of free energy relationships in hindered phenols. Linear dependence for solvation effects in ionization. *J. Amer. Chem. Soc.* **85**, 3397–3402
30. Klecka, G.M. and Gibson, D.T. (1981) Inhibition of catechol 2,3-dioxygenae from *Pseudomonas putida* by 3-chlorocatechol. *Appl. Environ. Microbiol.* **41**, 1159–1165
31. Bartels, I., Knackmuss, H., and Reineke, W. (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. *Appl. Environ. Microbiol.* **47**, 500–505
32. Vaillancourt, F.H., Labbé, G., Drouin, N.M., Fortin, P.D., and Eltis, L.D. (2002) The mechanism-based inactivation of 2,3-dihydroxybiphenyl 1,2-dioxygenase by catecholic substrates. *J. Biol. Chem.* **277**, 2019–2027
33. Shu, L., Chiou, Y.M., Orville, A.M., Miller, M.A., Lipscomb, J.D., and Que, L., Jr. (1995) X-ray absorption spectroscopic studies of the Fe(II) active site of catechol 2,3-dioxygenase. Implications for the extradiol cleavage mechanism. *Biochemistry* **34**, 6649–6659
34. Ridder, L., Briganti, F., Boersma, M.G., Boeren, S., Vis, E.H., Scozzafava, A., Veeger, C., and Rietjens, I.M.C.M. (1998) Quantitative structure/activity relationship for the rate of conversion of C4-substituted catechols by catechol-1,2-dioxygenase from *Pseudomonas putida* (*arvilla*) C1. *Eur. J. Biochem.* **257**, 92–100
35. Dorn, E. and Knackmuss, H. (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochem. J.* **174**, 85–94
36. Bernstein, H.J. (2000) Recent changes to RasMol, recombining the variants. *Trends Biochem. Sci.* **25**, 453–455

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012