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Aliphatic and aromatic inhibitors binding to the active site of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2

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Abstract

The interaction of different classes of inhibitors with the extradiol cleaving catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 was monitored by longitudinal and transverse proton relaxation measurements as well as by kinetic activity studies in order to characterize the type of interaction of such inhibitors with the active site of the enzyme. The average distances of the inhibitors from the catalytic iron(II) ion have been estimated from the ^1H longitudinal relaxation rates. Phenols and aliphatic ketones appear to be coordinated to the iron(II) ion in the active site.

Key words: Catechol; Dioxygenase; Extradiol cleavage; *Pseudomonas putida*

1. Introduction

Extradiol dioxygenases are enzymes which play essential roles in the biodegradation of most aromatic compounds in the biosphere [1,2]. In particular catechol 2,3-dioxygenase (C2,3O, metapyrocatechase, catechol:oxygen 2,3-oxidoreductase, EC 1.13.1.2) catalyzes the dioxygenation of catechol to form α-hydroxymuconic ε-semialdehyde.

The enzyme consists of four identical subunits (M_r 32,000) containing one Fe(II) ion each, $[\alpha Fe(II)]_4$, and is encoded by the *xylE* gene on the TOL plasmid (pWW0) contained in the bacterial strain mt-2 of *Pseudomonas putida* [3,4]. The amino acid sequence predicted by the nucleotide sequence consists of 307 residues [5].

First detected by Dagley and Stopher in 1959 [6], the enzyme was isolated and found to be strongly sensitive to oxygen by Kojima et al. [7]. A few years later it was observed that the presence of an organic solvent like ethanol or acetone could stabilize the enzyme against inactivation [8] but only in 1968 was it shown that the iron(II) ions were essential for activity and that the inactive oxidized enzyme could be reactivated by incubation with reducing agents [9].

The characterization of the active site of extradiol dioxygenases is still limited, mainly due to the difficulties in the spectroscopic investigation of high spin iron(II). Some progress has recently been obtained by using EPR to study the NO derivative (S=3/2) of such enzymes [1,10–13]. Hyperfine broadening of the EPR resonances by ^{17}O-enriched H_2O shows that water is bound to iron in the native enzyme and is displaced by the substrate catechol [11]. EPR studies together with a combination of optical absorption, circular dicroism and magnetic circular dicroism techniques also revealed that the binding of catechols to the active site Fe(II) ion of extradiol dioxygenases occurs in a bidentate fashion and simultaneously to NO [11–13].

The extradiol insertion of molecular oxygen into catechol catalyzed by C2,3O proceeds by an ordered bi-uni mechanism where catechol binds first followed by oxygen [14]. This process is inhibited to different degrees by a series of aromatic compounds which resemble catechol and also by aliphatic compounds like alcohols and ketones. Although since 1968 it was shown that acetone inhibited the enzyme [9], the mechanism of protection against oxidation accomplished by such organic solvents has been scarcely investigated.

In this study we performed kinetic and NMR relaxation measurements utilizing different aromatic and aliphatic inhibitors in order to better understand the nature of their interactions with the active site of C2,3O and to shed some light onto its catalytic mechanism.

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Abbreviations: C2,3O, Catechol 2,3-dioxygenase; EPR, Electron Paramagnetic resonance; NMR Nuclear Magnetic Resonance; XAS, X-ray Absorption Spectroscopy.

2. Experimental

2.1. Bacterial growth

The *Pseudomonas putida* bacterial strain utilized was mt-2 paW1 (ATCC 23973) which contains the TOL plasmid, pWW0, encoding the enzymes involved in the degradation of toluene and xylenes [4].

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The bacteria were maintained as pure colonies on a minimal medium with *meta*-toluate as the principal carbon source. Batch cultures was grown in a medium containing 0.3% sodium benzoate, 0.3% NaCl, 0.5% meat extract, and 0.5% peptone brought to pH 7.2 before sterilization [3] using a 1% fresh inoculum grown on *meta*-toluate. The cells were cultured with vigorous areation in 10 l bottles immersed in a 30 °C water bath for 36–48 h.

The bacteria were harvested using a Beckman J-ZCF continuous flow rotor running at 10,000 rpm with a 500 ml/min flow. The bacterial paste was then stored at −70°C until utilized. The yield was 5–7 g wet wt. of bacterial paste per l of medium.

2.2. Enzyme purification

The enzyme purification was performed following the procedures reported by Nakai et al. [3] and the modifications made by Arciero et al. [11]. All the steps were carried out at 4°C using freshly de-gassed 50 mM potassium phosphate buffer, pH 7.5, supplemented with 10% acetone.

2.3. Activity measurements

C2,3O activity was routinely assayed by monitoring the absorbance increase at 375 nm due to the formation of the product α-hydroxymuconic ε-semialdehyde ($\varepsilon_{375} = 4.4 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 24°C and pH 7.5). All the kinetic assays were performed at normal buffer saturations of oxygen (about 260 μM).

One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmol of the product per min at 24°C [15], and corresponds to a 44.1 optical density change per min in a 1 ml assay volume.

The specific activity, defined as U/mg of protein, was determined in a 0.05 M phosphate buffer, pH 7.5, containing 333 μM catechol [15]. The enzyme solution, prepared in 0.05 M phosphate buffer plus 10% acetone to obtain 0.5–1.0 U/ml, was added to start the reaction at 24° C.

The protein concentration was determined using the Coomassie blue G-250 binding method [16–18] with crystalline ovalbumin as the reference protein. The specific activity of the enzyme used in this study was always greater than 300 U/mg.

The presence of traces of iron(III) ions in the protein solutions was checked by differential titrations with *o*-phenanthroline just before the performing of the NMR experiments [9]. No relevant traces of iron(III) were observed in the freshly prepared samples.

The pH readings in D_2O solutions are reported uncorrected for the isotope effect (pH*).

Steady-state kinetic experiments were performed with varying substrate, enzyme and inhibitor concentrations at 25°C. In the enzyme concentration range investigated the rates are directly proportional to the enzyme concentration. Data were collected on a Cary 3 Varian UV-Visible Spectrophotometer, interfaced with a personal computer for data acquisition.

Data analyses were performed using linear or non linear regression programs. The initial rates of the enzyme-catalyzed reactions were obtained by fitting the linear initial part of the kinetic curves (first 5–30 s after mixing). The Michaelis–Menten constants were determined fitting the data to:

$$V = \frac{V_{max}}{1 + K_M/[S]} \tag{1}$$

where v is the initial reaction velocity, V_{max} is the maximal velocity, K_M is the Michaelis–Menten constant and $[S]$ is the substrate concentration.

Inhibition constants were determined by plotting $1/v$ vs. $1/[S]$ (double reciprocal or Lineweaver–Burk plot) for each inhibitor concentration and then plotting the calculated slopes of the lines determined for each inhibitor concentration vs. the inhibitor concentration (slope vs. $[I]$) or using the Dixon plot ($1/v$ vs. $[I]$). The data for competitive inhibitors were fitted to:

$$v = \frac{V_{max}}{1 + K_M(1 + [I]/K_I)/[S]} \tag{2}$$

where $[I]$ is the inhibitor concentration and K_I is the inhibition constant.

2.4. NMR measurements

^1H nuclear magnetic resonance spectra were collected utilizing a Bruker MSL 200 spectrometer operating at 200 MHz proton frequency. T_1 measurements were performed using the inversion recovery pulse sequence (180-τ-90). T_2 values were calculated from the linewidths as $T_2 = 1/(\pi\Delta\nu)$ where $\Delta\nu$ is the line-width at half peak height.

In the presence of chemical exchange the observed relaxation rates T_{iObs}^{-1} are enhanced in such a way that:

$$T_{iObs}^{-1} = T_{id}^{-1} + T_{ip}^{-1} \tag{3}$$

where i = 1 or 2, T_{id}^{-1} and T_{ip}^{-1} are the diamagnetic and paramagnetic contributions to the relaxation rates, respectively. Therefore the paramagnetic contributions can be determined by subtraction of the diamagnetic effect from the observed rates. The longitudinal relaxation rate enhancement, T_{1p}^{-1}, in the presence of chemical exchange changes in accordance to the following equation:

$$T_{1p}^{-1} = \frac{f_M}{T_{1M} + \tau_M} \tag{4}$$

where f_M is the molar fraction of protons sensing the paramagnetic center, T_{1M} is the paramagnetic relaxation time of such protons and τ_M is the chemical exchange time. From the nuclear relaxation rate enhancement T_{1M}^{-1}, assuming that the theory originally developed by Solomon and Bloembergen for the dipolar contribution is valid also for Fe(II), it is possible to estimate the average Fe(II)–inhibitor distance r:

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_o}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \tag{5}$$

where μ_o is the permeability of vacuum, μ_B is the Bohr magneton, S is the electron spin multiplicity, γ_N is the nuclear magnetogyric ratio, ω_I and ω_S are the nuclear and electronic Larmor frequencies at the magnetic field of the experiment, respectively, τ_c is the correlation time usually defined as:

$$\tau_c^{-1} = \tau_s^{-1} + \tau_r^{-1} + \tau_M^{-1} \tag{6}$$

where τ_s^{-1} is the electronic relaxation rate, and τ_r^{-1} is the rotational correlation rate [19–21].

The line-width of the resonances of the inhibitors (in large excess with respect to the enzyme) in the presence of a known amount of enzyme can be described by:

$$T_{2p} = \frac{(T_{2M} + \tau_M)}{[E]_0}(K_{diss} + [I]_0) \tag{7}$$

where K_{diss} is the dissociation constant of the complex. This equation is valid in the case of fast exchange and under the condition of intermediate exchange provided the chemical shift difference between the paramagnetic and the bulk sites is small [22–24]. K_{diss} can be evaluated by plotting the paramagnetic contribution to the transverse relaxation time, T_{2p}, as a function of $[I]_o$.

3. Results and discussion

The inhibition constants determined through kinetic activity measurements for a series of aromatic and aliphatic compounds using catechol as a substrate are reported in Table 1. It can be observed that almost all the aromatic inhibitors investigated have a K_I below 1 mM. In particular, the phenols substituted in the *ortho* position, which structurally mimic catechols, are good inhibitors of C2,3O, as previously observed by Arciero et al. [11].

More interesting is the effect of aliphatic alcohols and ketones on the catechol dioxygenation. As suggested from the studies of Nozaki et al. [9], these organic solvents are inhibitors of C2,3O. Competitive, or almost competitive inhibition towards catechol is observed for

Table 1
Kinetic inhibition constants for the extradiol insertion of molecular oxygen into catechol catalyzed by C2,3O

	K_I (mM)
Aromatic compounds	
Phenol	7.1
2-Methoxy-phenol	0.23
2-Fluoro-phenol	0.56
2-Chloro-phenol	0.52
Benzyl-alcohol	1.4
2-Hydroxy-benzylalcohol	5.3
Acetophenone	0.21
2-Hydroxy-acetophenone	0.23
Aliphatic compounds	
Alcohols	
Methanol	480
Ethanol	147
n-Propanol	21
i-Propanol	90
n-Butanol	1.7
t-Butanol	720
n-Pentanol	0.58
Ketones	
Acetone	22
2-Butanone	0.35
2-Pentanone	0.15
3-Pentanone	3.0

all the compounds checked. Table 1 shows that the inhibition constants decrease considerably with increasing length of the alcohol chain. Exceptions to this observation occur when, instead of compounds with linear chains and having the functional group on one side (see for example *n*-propanol or *n*-butanol), we checked more voluminous and hindering branched analogues (like *i*-propanol or *t*-butanol). In all cases the kinetic inhibition constant K_I increased substantially passing from the *normal-* to the *iso-* and then to the *tert*-isomer, suggesting that steric factors are also important for the positioning of the substrate/inhibitor molecules into the catalytic cavity of C2,3O.

Similar observations can be made in the case of the investigated ketones, where K_I decreases with increasing chain length but increases in the case of bulky analogues.

Aromatic inhibitors which resemble the natural substrate do not seem to interact more strongly with the catalytic site than the aliphatic alcohols and ketones. In any case, we can observe that the hydrophobicity of the inhibitors is inversely related to their inhibition constant.

^{1}H NMR experiments have been performed in order to clarify the mode of interaction and the possible differences among the various classes of inhibitors. T_{1Obs}^{-1} and T_{2Obs}^{-1} proton relaxation rates were determined for 2-methoxy phenol and 2-pentanone as examples of aromatic and aliphatic inhibitors, respectively, in the absence or in the presence of known amounts of active C2,3O enzyme. Such compounds have similar inhibition constants although their structures are completely different.

The effects of the paramagnetic center both on the longitudinal (T_1) and the transverse (T_2) relaxation times of the proton NMR resonances of the inhibitors are reported in Table 2. The addition of the enzyme results in a differential change in the T_{1Obs}^{-1} rates of the various proton resonances of the inhibitors. In particular, for the 2-pentanone we observe that the proton resonances exhibit decreasing longitudinal relaxation rates going from the methyl group in position 1 to that in position 5 (Table 2). This suggests that the 2-pentanone binds in the environment of the iron ion and is oriented with the functional group towards the metal ion.

Regarding the transverse relaxation rates, we focused our attention on the isolated -CH_3 proton spin systems of each inhibitor. For free 2-pentanone at 25°C and pH 7.5 the T_{2d}^{-1} is 4.81 s^{-1}, whereas the same inhibitor (16.5 mM) in the presence of C2,3O (0.205 mM in Fe(II)) shows a $T_{2p}^{-1} = 28.0$ s^{-1}. Therefore a strong paramagnetic enhancement of the nuclear relaxation rate is observed. For free 2-methoxy phenol at 25°C and pH 7.4 the T_{2d}^{-1} is 3.36 s^{-1} whereas in the presence of C2,3O (0.205 mM in Fe(II)) the inhibitor (16.1 mM) shows a $T_{2p}^{-1} = 39.8$ s^{-1}. For both the inhibitors tested T_{2M}^{-1} is much larger than T_{1M}^{-1}, showing that T_1 is in the fast exchange region ($T_{1p}^{-1} = f_M \cdot T_{1M}^{-1}$) on the NMR time scale. The effect is not due to unspecific binding because up to fivefold dilution of the enzyme–inhibitor solution did not lead to relevant line-width changes, as expected in the case of exchange controlled line-width.

The temperature dependence of the proton transverse relaxation rate T_{2Obs}^{-1} of the -OCH_3 group of 2-methoxy-phenol in the presence of C2,3O is reported in Fig. 1. A linear decrease of the line-width as the temper-

Table 2
^{1}H NMR longitudinal and transvers relaxation times determined at 200 MHz and 25°C for different inhibitors in the absence or presence of C2,3O

Inhibitor	Group identity	T_{1Obs} [T_{2Obs}] (s) for free inhibitor[a]	T_{1Obs} [T_{2Obs}] (s) for inhibitor + C2,3O[b]
2-Methoxy-phenol	-OCH_3	2.4 ± 0.07 [0.298]	0.37 ± 0.006 [0.0232]
2-Pentanone	3-CH_2	4.9 ± 0.06	0.40 ± 0.01
	1-CH_3	6.3 ± 0.04 [0.208]	0.17 ± 0.002 [0.0305]
	4-CH_2	5.0 ± 0.08	0.47 ± 0.006
	5-CH_3	4.7 ± 0.1	0.63 ± 0.004

[a] 2-Methoxy-phenol (0.91 mM) in D_2O, pH* 7.4; 2-pentanone (0.94 mM) in D_2O, pH* 7.5.
[b] 2-Methoxy-phenol (16.1 mM) + C2,3O (0.205 mM Iron(II)) in D_2O, pH* 7.4; 2-pentanone (16.5 mM) + C2,3O (0.205 mM Iron(II)) in D_2O, pH* 7.5.

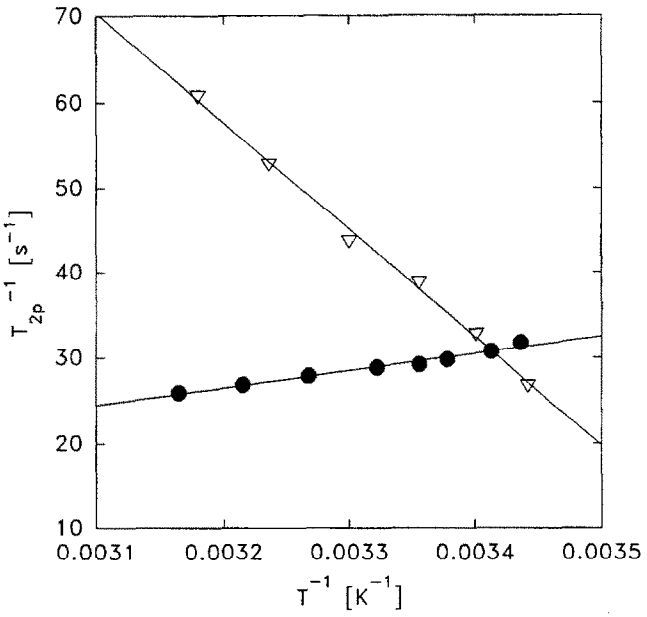

Fig. 1. Temperature dependence of the paramagnetic contribution to the proton transverse relaxation rates T_{2p}^{-1} for 2-methoxy-phenol (▽) and 2-pentanone (●) in the presence of C2,3O.

ature is decreased is observed, indicating that T_2 is under chemical exchange control. The fitting of such experimental behavior allows us to estimate an exchange time, τ_M ranging from 2×10^{-4} to 5×10^{-4} s over the investigated temperature range. In the case of 2-pentanone, in contrast, we observe an increase of T_{2p}^{-1} as the temperature is decreased (Fig. 1), indicating that T_1 and T_2 are both in the fast exchange region.

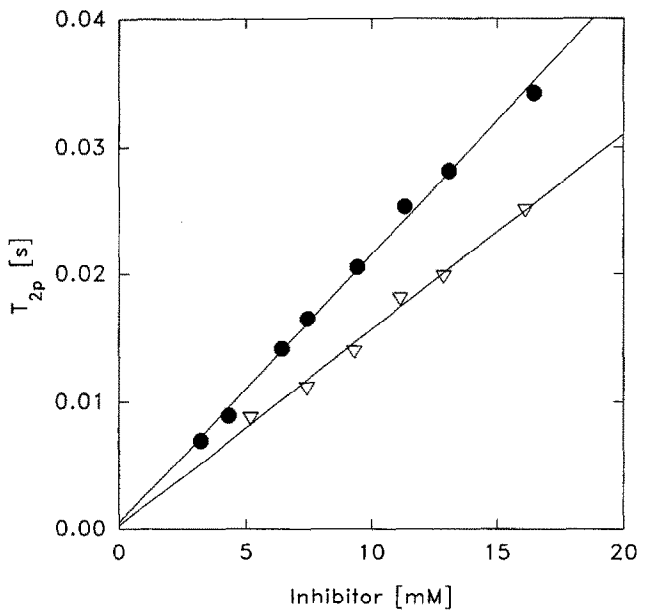

Fig. 2. Paramagnetic proton transverse relaxation times T_{2p} for the -OCH_3 group of 2-methoxy-phenol (▽) and for the 1-CH_3 group of 2-pentanone (●) at different concentrations of each inhibitor in the presence of C2,3O (0.2 mM Fe(II)).

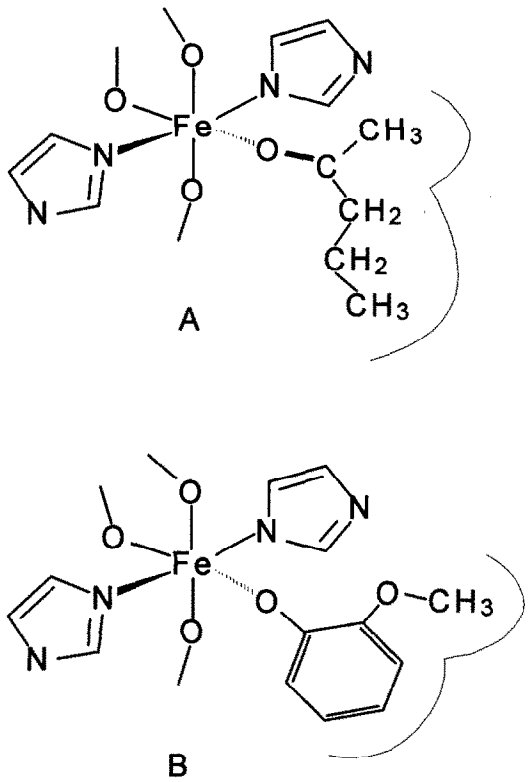

Fig. 3. Schematic representations of (A) 2-methoxy-phenol and (B) 2-pentanone binding to the active site iron(II) ion of C2,3O. The coordination geometry and the ligands of the active site iron(II) ion are deduced from unpublished XAS data from our laboratory.

The effect of the paramagnetic iron(II) center in C2,3O on the transverse relaxation rate, T_{2p}^{-1}, of the ^{1}H NMR resonances of 2-methoxy phenol (-OCH_3 group) and 2-pentanone (1-CH_3 group) inhibitors, obtained by subtracting the rates measured on the diamagnetic blank, is reported in Fig. 2, as a function of inhibitor concentrations. The data are corrected for dilution effects. The best fittings of Eq. 7 to the experimental data (Fig. 2) provide dissociation constants of about 0.2 mM for both 2-methoxy phenol and 2-pentanone, which appear to be comparable to the inhibition constants, K_I, obtained through activity measurements. This observation suggests that the rate-limiting step in the enzyme–inhibitor interaction equilibrium corresponds to the inhibitor dissociation process.

From the experimental T_{1M}^{-1} values it is possible to evaluate, using the Solomon–Bloembergen equation, the average distance between the catalytic Fe(II) ion and the methyl groups for both the inhibitors. In the case of iron(II) ions in macromolecules τ_c is clearly dominated by τ_s which for Fe(II) has been estimated to be of the order of 10^{-12}-10^{-13} s, the aquoion being 4.7×10^{-13} s [25]. Utilizing a $\tau_s = 1 \times 10^{-12}$ s in the case of 2-methoxy-phenol the calculated average distance between the

methyl protons of the *ortho*-methoxy group and the active site iron(II) ion is 0.45 nm, whereas in the case of 2-pentanone the estimated average distance between the methyl protons of the 1-CH_3 group and the active site iron(II) ion is 0.38 nm. The distances, calculated through ^1H NMR T_1 measurements, are consistent with the inhibitor molecules being directly bound to the active site metal ion both in the case of aromatic and aliphatic compounds as observed by inspection of molecular models (see Fig. 3A,B) considering a Fe(II)-OC bond of 2.0 Å. The direct binding of 2-methoxy phenol to the iron(II) active site ion seems to occur in a monodentate fashion and is quite consistent with the property of those ligands to complex iron ions in solution. In fact, a large number of iron(II) and iron(III) ions complexes with phenols and catechols have been characterized for long time [26]. More surprising is, on the other hand, the fact that 2-pentanone binds directly to the iron(II) ion with such a high affinity because aliphatic ketones or alcohols are known to have low affinity for iron(II) ions in aqueous solutions. Hydrophobic interactions in the active site of this enzyme seem to play a very relevant role in governing the binding of inhibitors.

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