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XAS characterization of the active sites of novel intradiol ring-cleaving dioxygenases: hydroxyquinol and chlorocatechol dioxygenases

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Abstract The intradiol cleaving dioxygenases hydroxyquinol 1,2-dioxygenase (HQ1,2O) from *Nocardiodes simplex* 3E, chlorocatechol 1,2-dioxygenase (ClC1,2O) from *Rhodococcus erythropolis* 1CP, and their anaerobic substrate adducts (hydroxyquinol-HQ1,2O and 4-chlorocatechol-ClC1,2O) have been characterized through X-ray absorption spectroscopy. In both enzymes the iron(III) is pentacoordinated and the distance distribution inside the Fe(III) first coordination shell is close to that already found in the extensively characterized protocatechuate 3,4-dioxygenase. The coordination number and the bond lengths are not significantly affected by the substrate binding. Therefore it is confirmed that the displacement of a protein donor upon substrate binding has to be considered a general step valid for all intradiol dioxygenases.

Key words: Chlorocatechol 1,2-dioxygenase; Extended X-ray absorption fine structure; Hydroxyquinol 1,2-dioxygenase; Intradiol ring-cleaving dioxygenase; *Nocardiodes simplex*; *Rhodococcus erythropolis*

1. Introduction

Ring-cleaving dioxygenases represent essential enzymes in the aerobic pathways of degradation of aromatic substances. The microbial catabolic strategies for utilizing aromatic compounds as carbon sources consist essentially in mono- and/or di-hydroxylation of the aromatic substrates until all the derivatives are converted into a few key chemical structures like catechol, pyrogallol, hydroxyquinol, protocatechuic and gentisic acids [1,2]. These aromatic derivatives then undergo ring cleavage in the presence of oxygen under the action of enzymes usually mentioned as ring-cleaving dioxygenases. These enzymes form two distinct classes called intradiol and extradiol dioxygenases, depending on the position of cleavage in the aromatic ring [3–8]. Both classes of enzymes contain iron ions in their active site with different oxidation states: Fe(II) in the extradiol, Fe(III) in the intradiol dioxygenases [3–8]. The structural information available are still scanty, and the question of how oxygen is activated is a matter of debate. Whereas in the case of the extradiol enzymes the iron(II) center can directly interact with the dioxygen molecule, a more indirect mechanism has to be devised for the iron(III) containing enzymes [4–8].

Focusing on the intradiol enzymes, structural information is, at the moment, available for only one enzyme, the one specific for cleaving protocatechuic acid. The X-ray structure of protocatechuate 3,4-dioxygenase from *Pseudomonas putida* (previously *aeruginosa*), available since 1988, shows that the iron(III) ion is coordinated to two histidines, two tyrosines and a water molecule/hydroxide ion in a distorted trigonal bipyramidal geometry [9,10].

All the spectroscopic and mechanistic studies available up to now on intradiol dioxygenases have been performed only on this enzyme, although from different sources (*Pseudomonas putida*, *P. cepacia* and *Brevibacterium fuscum*), and therefore it has been regarded as the archetype of all intradiol dioxygenases [4,11–17]. Only a few spectroscopic studies have been performed on a different enzyme, the catechol 1,2-dioxygenase from *Pseudomonas putida* C1 (previously *Pseudomonas arvilla*), with results in some cases diverging from those on the protocatechuate enzyme [18,19].

An interesting aspect of the protocatechuate enzyme, first revealed through EXAFS spectroscopy, was that the binding of the substrate induced the displacement from the iron center of an endogenous protein ligand [20]. Very recently the X-ray structures of the anaerobic adducts with the substrates protocatechuic acid and 3,4-dihydroxyphenylacetate have been reported definitely showing that one of the tyrosine ligands is no longer bound to the iron center [21,22].

This competition between the endogenous tyrosine ligand and an OH group of the chelating substrate for the same iron coordination site has been proposed to be an important factor in the catalytic mechanism since it favors the quinonization of the substrate phenyl ring, which in turn is essential in order to promote dioxygen attack on the ring C1 position [21,22].

In order to validate the generality of the above reported mechanism for molecular oxygen activation it seems appropriate to investigate other dioxygenases of this class. Since intradiol enzymes generally have strict substrate specificity, a specific enzyme is necessary for each different key chemical structure. For instance, the substrate of HQ1,2O might appear very similar to protocatechuate considering also that both rings are cleaved at the same position, i.e. between the two ortho-hydroxy groups. However, protocatechuate is not cleaved at all by HQ1,2O, but it rather acts as a strong inhibitor towards the enzyme ($K_i = 74$ μM). This indicates that the reactivity of even strictly related chemical structures is finely tuned into the active sites of these enzymes and it is

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Abbreviations: ClC1,2O, chlorocatechol 1,2-dioxygenase; EXAFS, extended X-ray absorption fine structure; HQ1,2O, hydroxyquinol 1,2-dioxygenase; Hydroxyquinol, 1,2,4-trihydroxybenzene; XAS, X-ray absorption spectroscopy

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surely important to ascertain if this property depends on the metal center structure.

Recently we have isolated and characterized a hydroxyquinol (1,2,4-trihydroxybenzene) 1,2-dioxygenase (HQ1,2O) from *Nocardiodes simplex* 3E and a chlorocatechol 1,2-dioxygenase (ClC1,2O) from *Rhodococcus erythropolis* 1CP [23,24]. We report here the XAS characterization of these enzymes, both in the native forms and in the presence of their specific substrates, in order to increase the base of comparison among different intradiol dioxygenases.

2. Materials and methods

2.1. Sample preparation

HQ1,2O and ClC1,2O dioxygenases were purified to homogeneity using previously reported procedures [23,24].

Samples for the XAS measurements were concentrated under nitrogen to about 2–3 mM Fe(III) and introduced into 1 mm thick plastic cells (75 μl) covered with kapton windows. The substrate-enzyme adduct samples were prepared by anaerobically adding about 30 molar excess substrate over the respective measured K_m to the enzyme solution.

2.2. XAS measurements and data analysis

X-ray absorption measurements were performed at the EMBL EXAFS beamline (c/o DESY, Hamburg) [25,26]. During the experiments the DORIS III storage ring was operating in dedicated mode at 4.5 GeV with ring currents ranging from 55 to 100 mA. A Si(111) double crystal monochromator with an energy resolution of 1.8 eV at 7250 eV was used. The second monochromator crystal was detuned to 50% of peak intensity in order to reject higher harmonics. The monochromator angle was converted to an absolute energy scale using a calibration technique [27].

The sample fluorescence was detected with an energy discriminating 13 element Ge solid state detector. The data were collected at the iron edge (7124 eV at the edge jump inflection point). The spectra were recorded from 6940 to 8050 eV with variable step widths. In the XANES and EXAFS regions steps of 0.3 and 0.5–1.2 eV were used respectively. Series of 24–30 spectra were collected on the frozen samples at 20 K. After inspection of each scan for edge consistency, the data were normalized by the edge jump and averaged. The EXAFS was then extracted by subtracting the slowly varying atomic background fitted with a cubic spline. Data reduction based on standard procedures was performed with the local set of programs [28]. The pre-edge peak (1s–3d electronic transition at ~7113 eV) areas were calculated by subtracting an arctangent function from the normalized edge spectra and integrating over the range 7108–7118 eV. The analysis of the EXAFS data by utilizing the rapid curved single and multiple scattering theory was performed with the set of programs EXCURVE88 [29–31].

The spectra were analyzed by filtering out the contribution of the iron atomic environment up to about 0.20 nm by backtransforming the Fourier transforms in the 0.08–0.20 nm range. The filtered data were then simulated by varying the atom types and the coordination numbers (as integers) and iteratively refining the distance (R) and the Debye-Waller factor ($2\sigma^2$) for each atomic shell. The quality of the fit obtained was assessed by the fit index (FI) as defined within EXCURVE88:

$$FI = \Sigma_i[(\chi_o^{(i)} - \chi_c^{(i)})k^3]^2/n \times 100$$

The parameter AFAC in EXCURVE88 represents the energy-independent amplitude factor introduced to compensate the reduction in amplitude due to multiple excitations. This corresponds to the S(k) factor in the standard EXAFS formula.

3. Results and discussion

3.1. Edge data

Fig. 1 shows the edge region of the native (A) and substrate-complexed (B) HQ1,2O and the edge data for ClC1,2O (C) from *Rh. erythropolis* and its substrate adduct (D).

Inspection of the edge and XANES regions shows significant changes in the formation of the substrate adducts, qualitatively indicating the binding of the substrate to the metal center. The pre-edge absorption feature (at about 7113 eV) is usually observed in K-edge spectra of iron complexes and it is assigned to a dipole forbidden 1s–3d transition which becomes allowed by admixture of 4p and 3d metal orbitals. The more the metal complex deviates from centrosymmetry, the more the mixing is pronounced. Consequently, the $1s \rightarrow 3d$ intensity increases in going from octahedral to pyramidal and tetrahedral geometry of iron complexes and can be correlated to the metal coordination number [32–34]. However, it should be remembered that the intensity of the pre-edge peak depends directly on the geometry and not on the coordination number of the complex. The measured pre-edge peak intensities for native HQ1,2O and ClC1,2O are 11.6×10^{-2}, and 11.0×10^{-2} eV respectively. These values fall in the characteristic range observed for five-coordinated Fe(III) ions [32–34]. Since square-planar Fe(III) complexes are not known and tetrahedral complexes show intensities higher than about 23×10^{-2} eV, the pre-edge data strongly support a five-coordinated

Table 1
One and two distance fits of the first iron coordination shell of native and substrate complexed HQ1,2O and ClC1,2O enzymes

Sample	Shell 1			Shell 2			
		R (nm)	$2\sigma^2 \times 10^4$ (nm^2)		R (nm)	$2\sigma^2 \times 10^4$ (nm^2)	FI
HQ1,2O[a]	6 O/N	0.193	2.7				0.968
	3 O	0.192	1.7	3 N	0.208	5.3	0.585
	3 O	0.191	1.4	2 N	0.210	2.2	0.445
	2 O	0.190	1.0	3 N	0.206	2.6	0.397
HQ1,2O+substrate[a]	6 O/N	0.196	1.9				0.600
	3 O	0.192	1.0	2 N	0.208	0.7	0.467
	2 O	0.191	0.5	3 N	0.206	0.9	0.439
ClC1,2O[b]	6 O/N	0.196	2.5				0.952
	3 O	0.192	0.9	2 N	0.211	0.6	0.190
	2 O	0.190	0.6	3 N	0.208	1.2	0.212
ClC1,2O+substrate[b]	6 O/N	0.199	1.8				0.418
	3 O	0.195	0.6	2 N	0.211	0.3	0.238
	2 O	0.194	0.3	3 N	0.210	0.7	0.232

ESD on distances = ±0.002 nm. AFAC = 1.0, k range of the fits 35–115 nm^{-1}.
[a]$\Delta E_0 = 27$ eV.
[b]$\Delta E_0 = 23$ eV.

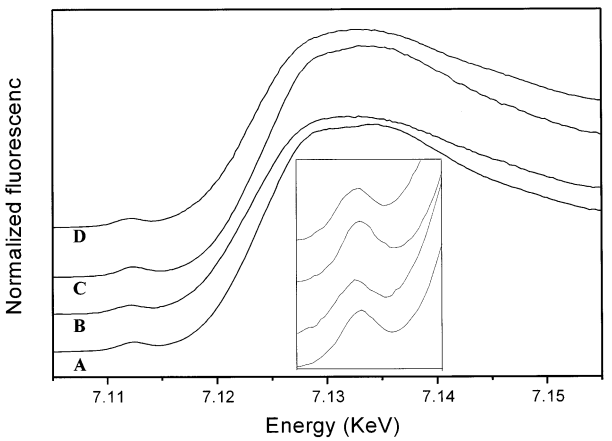

Fig. 1. Edge spectra of native (A) and substrate complexed (B) HQ1,2O from *Nocardioides simplex* and of native (C) and substrate complexed (D) ClC1,2O from *Rhodococcus erythropolis* (ClC1,2O) samples. In the inset are shown enlargements of the pre-edge peak regions.

chromophore in both native enzymes. This is also confirmed by the EXAFS investigation (see below). Furthermore, the analysis of the pre-edge peak areas reveals that the coordination number of the ferric ion does not change upon substrate binding in both samples; in fact the spectra of the anaerobic substrate adducts of HQ1,2O and ClC1,2O show pre-edge peak intensities of 10.8×10^{-2} and 10.3×10^{-2} eV respectively implying that the substrates displace ligands from the iron coordination sphere without increasing the coordination number.

3.2. EXAFS data

The k^3 weighted experimental EXAFS spectra of native (A) and substrate complexed (B) samples of HQ1,2O and the corresponding ones for ClC1,2O (C and D) are reported for comparison in Fig. 2.

The EXAFS spectrum of the native enzyme is typical of a metal bound to oxygen and nitrogen ligands with the features appearing at 0.40–0.65 nm^{-1} being typical of histidine imidazole backscattering ([6,35,36] and references therein). It is noteworthy that these features are less pronounced in the spectra of the respective substrate-bound samples. Due to different beam conditions, the spectra of substrate complexed samples show a worse signal/noise ratio and hence their analysis has a higher degree of uncertainty. Fig. 2 shows that the presence of the substrate visibly perturbs the EXAFS spectra of the enzymes. The features observed between 40 and 100 nm^{-1} change intensities and shapes upon substrate binding. These changes together with the changes observed in the XANES region (Fig. 1) indicate a direct interaction of the substrate with the metal ion.

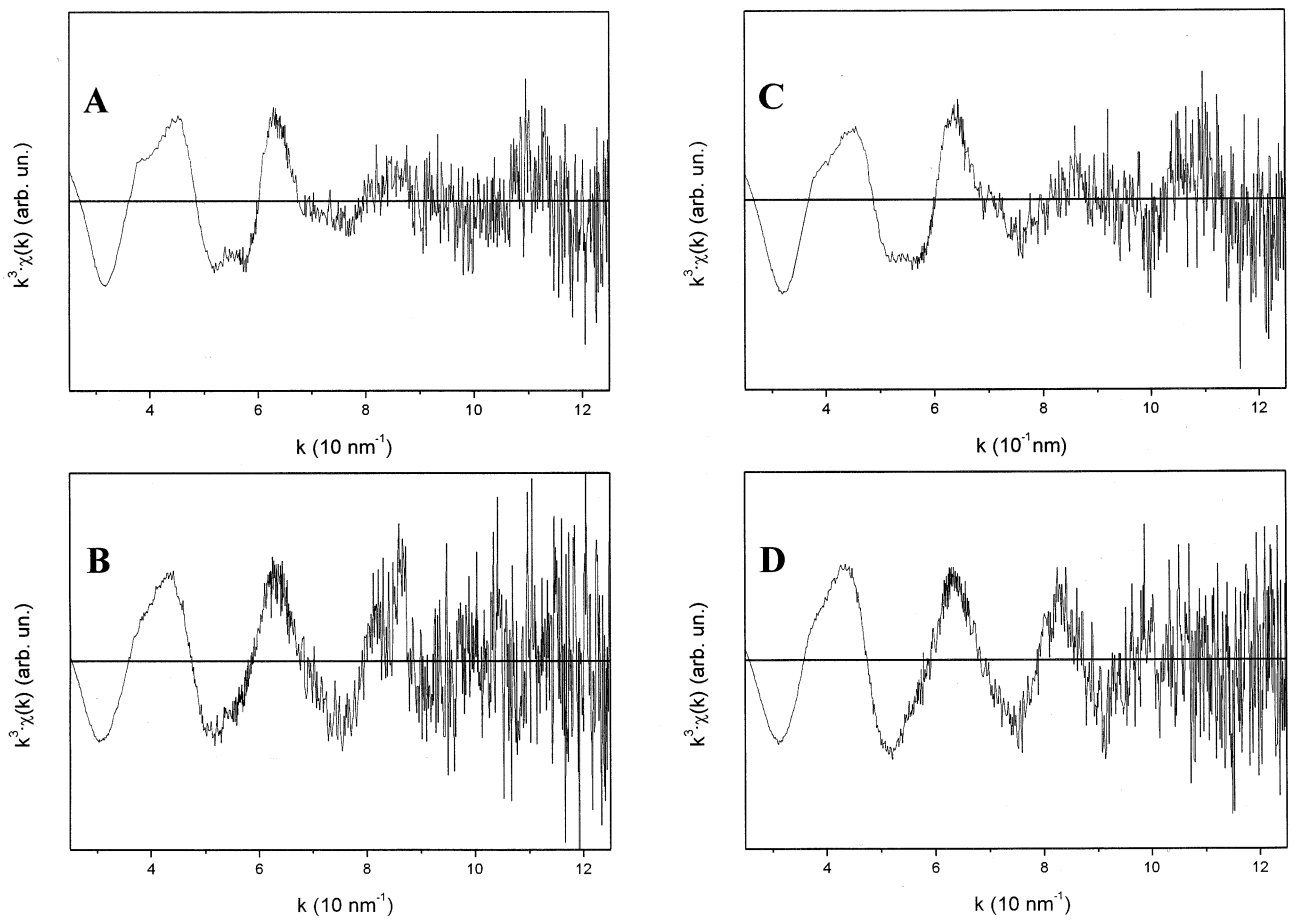

Fig. 2. Experimental k^3 weighted EXAFS spectra for the (A) native HQ1,2O enzyme, (B) substrate complexed HQ1,2O enzyme and for the (C) native ClC1,2O enzyme, and the (D) substrate complexed ClC1,2O enzyme.

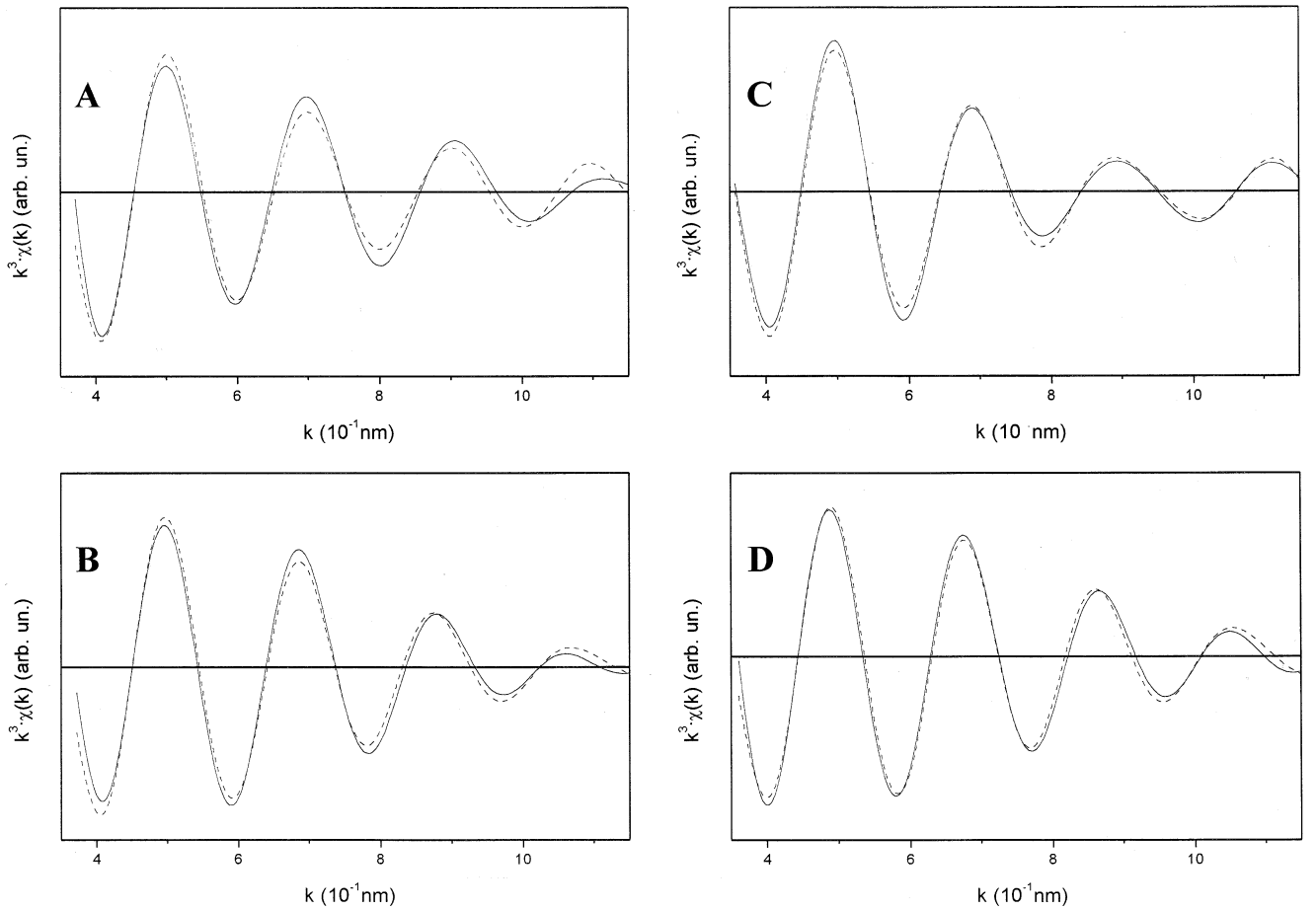

Fig. 3. Experimental (solid line) and calculated (dotted line) k^3 weighted filtered EXAFS spectra for the native (A) and the substrate complexed (B) HQ1,2O enzyme and for the native (C) and the substrate complexed (D) ClC1,2O enzyme.

3.3. First shell analysis

The iron first coordination shells in the native and substrate-bound HQ1,2O and ClC1,2O were analyzed by Fourier filtering the nearest neighbor contribution and by backtransforming them to k space. The results of the fits are reported in Table 1 and in Fig. 3. Attempts to simulate the first shell of each sample by a single shell of O/N ligands always failed to reproduce the signal. On the contrary, double shell fits using any combination of O/N ligands summing up to five were able to satisfactorily reproduce the first coordination shell signal. Trials with higher coordination numbers of the iron did not improve the fits. Table 1 shows that the Fe(III) ions in the two native enzymes have the same coordination environment within the experimental error. In both cases two different combinations of O/N ligands, divided in two shells of coordination numbers 2 and 3, at distances differing by $\sim$0.018 nm result in good quality fits. The distances of about 0.190 nm obtained for the iron nearest neighbors correspond perfectly to the Fe–$O_{phenolate}$ distances found in the protocatechuate dioxygenase structure [9,10,21,22] whereas the longer distances measured of about 0.210 nm correspond to the Fe–$N_{histidine}$ bond lengths observed in the same enzyme. The same set of two tyrosines and two histidines then most likely provides the protein ligands to iron in the present enzymes. The nature of the fifth ligand remains uncertain as it could be either a water molecule or a hydroxyl ligand since the first iron coordination shell was equally well simulated by a set of three close and two far, or by a set of two close and three far ligands (see Table 1).

The analysis of Fourier filtered first shell contributions to the EXAFS spectra of the substrate adducts of the two enzymes results in a shell characterized by two sets of distances centered at about 0.191 and 0.210 nm and 0.192 and 0.211 nm for HQC1,2O and ClC1,2O respectively (see Table 1). As indicated by the pre-edge analysis, the overall iron coordination number remains unchanged upon substrate binding. It is now widely accepted that the substrates of the intradiol dioxygenases characterized by the presence of two ortho-hydroxyl groups bind to the metal center as bidentate catecholate anions, this representing a prerequisite for dioxygen attack to the aryl moiety [21,22]. Therefore, since the iron ion remains pentacoordinated upon substrate binding also in our enzymes, one of the protein ligands, in addition to the water/hydroxide moiety, is displaced from the metal coordination as already observed for the protocatechuate enzyme [9,10,21,22].

4. Conclusions

The present investigation extends the knowledge on the active site iron(III) coordination environment of intradiol ring cleaving dioxygenases to enzymes with very different and highly specific substrate requirements.

The iron(III) active site ion appears to be pentacoordinated for both the HQ1,2O and ClC1,2O enzymes and this situation does not change upon substrate binding. The substrate coordination leaves also unperturbed, within the experimental error, the distance distribution inside the Fe(III) first coordination shell. The nature of the ligands and the iron coordination geometry appear to be the same as those found in the protocatechuate 3,4-dioxygenase and the displacement of a tyrosine and of a water molecule/hydroxide ion upon substrate binding observed by X-ray diffraction analysis of the above enzyme [21,22] is entirely consistent with the EXAFS data of the present study.

The current data clearly indicate that substrate specificity does not depend on the iron coordinated ligands. Specific interactions with residues in the active site cavity should be responsible for the modulation of the catalytic cycle and for the recognition of each different key chemical structure. Therefore detailed X-ray structural data on the different intradiol ring-cleaving dioxygenases will be necessary in order to fully rationalize the interrelationship between the substrate specificity and the catalytic mechanisms of such enzymes.

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