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Arene hydroxylases: metalloenzymes catalysing dioxygenation of aromatic compounds[1]

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Contents

Abstract

Some properties of hydroxylating enzymes able to add the two oxygen atoms of molecular oxygen into aromatic substrates such as benzene and phthalate are reviewed. In particular the non-haem single iron dioxygenases capable of incorporating two hydroxyl groups are presented as well as their up-to-date biophysical characterization.

Keywords: Hydroxylating enzymes; Dioxygenation; Metalloenzymes

1. Dioxygenases

Many aerobic bacteria possess enzymes which, through different catabolic pathways, are able to catalyse the degradation of aromatic compounds. Some steps such as ring hydroxylation and ring cleavage are common for all the pathways [1]: these steps are catalysed by different kinds of dioxygenases. On the basis of the type of reaction that they perform on the substrate, they can be divided into two types

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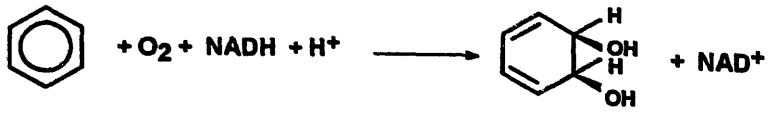
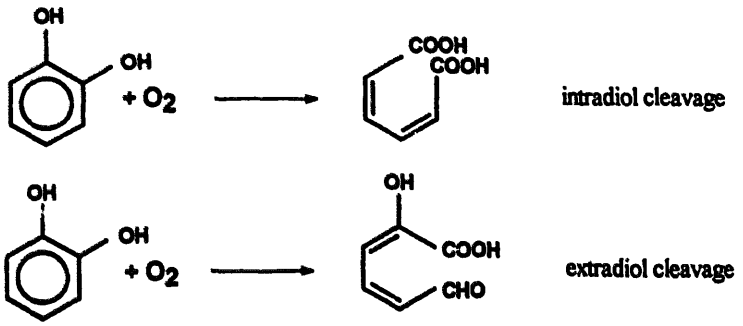
(i) The first type consists of dioxygenases which incorporate two hydroxyl groups into the aromatic ring of the substrate with oxidation of NAD(P)H to $NAD(P)^+$, such as benzene dioxygenase:



the obtained product is a *cis*-dihydro diol, which is then converted into catechol by a dehydrogenase. (ii) The other dioxygenases open the aromatic ring by incorporating two atoms of oxygen into the substrate [2], such as 1,2-catechol dioxygenase. Substrates of this type of dioxygenase are catechol and other aromatic compounds that already possess two hydroxyl groups in the *ortho* position on the aromatic ring.



The present paper will deal with the former type, i.e. with hydroxylating dioxygenases. They are a consortium of two or three proteins. They constitute an electron transfer chain which transfers electrons from NADH to a terminal oxygenase. A scheme of the electron transfer chain for benzene dioxygenase is reported in Fig. 1. The NADH molecule, oxidized at the beginning of the electron transfer process, is formed again by the subsequent dehydrogenation of the dihydrodiol (not shown) [1].

The proteins belonging to the group of hydroxylating dioxygenases are commonly divided into three classes (Fig. 2 and Table 1) [3,4]. In class I there are two-

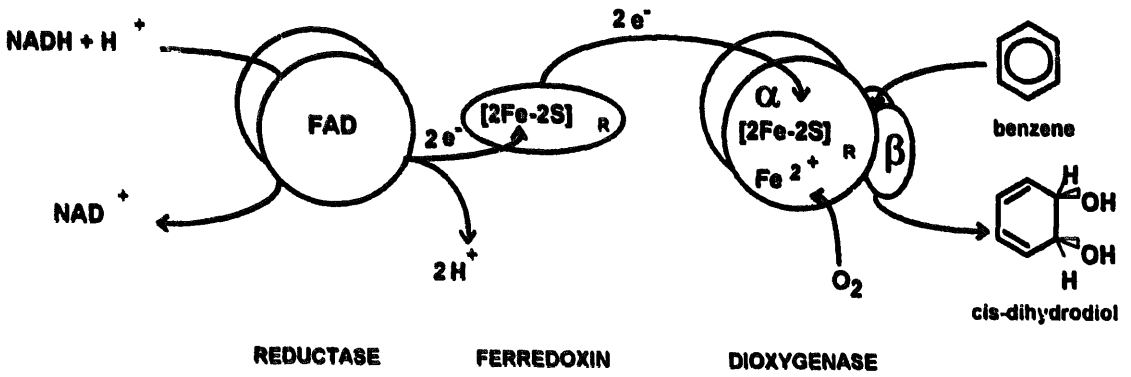


Fig. 1. Scheme of the electron transfer chain for benzene dioxygenase. $[2Fe–2S]_R$ indicates a Rieske-type cluster.

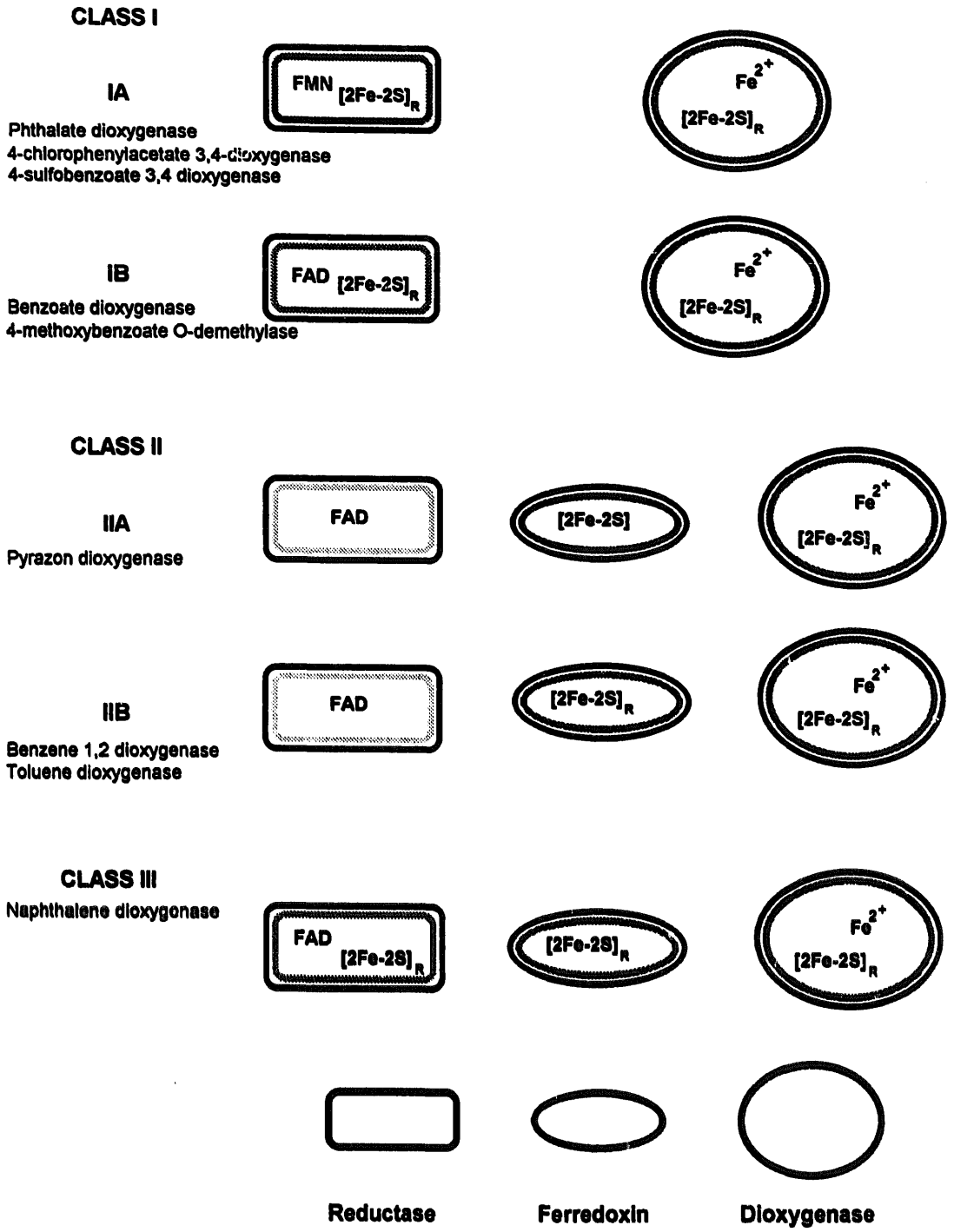


Fig. 2. Classification of dioxygenases. [2Fe–2S] indicates a plant-type cluster. $[2Fe–2S]_R$ indicates a Rieske-type cluster.

component dioxygenases: the former component is a reductase containing a flavin and a [2Fe–2S] cluster, the latter is an oxygenase; in class II there are three-component dioxygenases constituted by a reductase which contains only a flavin, a [2Fe–2S] ferredoxin, and a terminal oxygenase; class III contains three-component

Table 1
Hydroxylating oxygenase [4]

Enzyme system	Components	Prosthetic groups	Class	Ref.
Phthalate dioxygenase	Reductase	FMN, [2Fe–2S]	I	[5, 6]
	Oxygenase (α_4)	4[2Fe–2S]$_R$, 4Fe		[5, 6]
4-chlorophenylacetate-3,4-dioxygenase	Reductase		I	[7]
	Oxygenase (α_3)	3[2Fe–2S]		[7, 8]
4-sulphobenzoate-3,4-dioxygenase	Reductase		I	[9]
	Oxygenase (α_2)			[9]
Benzoate dioxygenase	Reductase	FAD, [2Fe–2S]	I	[10–12]
	Oxygenase ($\alpha_3\beta_3$)	3[2Fe–2S], 3Fe		[10, 11]
4-methoxybenzoate-O-demethylase	Reductase	FMN, [2Fe–2S]	I	[13, 14]
	Putidamonooxin (α_3)	[2Fe–2S], Fe		[13, 15]
Benzene-1,2-dioxygenase	Reductase$_{BED}$ (α_2)	FAD	II	[16–19]
	Ferredoxin$_{BED}$	[2Fe–2S]$_R$		[16–19]
	ISP$_{BED}$ ($\alpha_2\beta_2$)	2[2Fe–2S]$_R$, Fe		[16–20]
Toluene dioxygenase	Reductase$_{TOL}$	FAD	II	[21–23]
	Ferredoxin$_{TOL}$	[2Fe–2S]$_R$		[21, 22]
	ISP$_{TOL}$ ($\alpha_2\beta_2$)	2[2Fe–2S]$_R$, Fe		[21, 22, 24]
Pyrazon dioxygenase	Reductase	FAD	II	[24]
	Ferredoxin	[2Fe–2S]		[24]
	Oxygenase	[2Fe–2S]		[24]
Naphthalene dioxygenase	Reductase$_{NAP}$	FAD, [2Fe–2S]	III	[25, 26]
	Ferredoxin$_{NAP}$	[2Fe–2S]		[26–28]
	ISP$_{NAP}$ ($\alpha_2\beta_2$)	2[2Fe–2S], 2Fe		[26, 28, 29]

dioxygenases constituted by a reductase, which contains again a flavin and a [2Fe–2S] cluster, a [2Fe–2S] ferredoxin and a terminal oxygenase. The only member of class III known to date is naphthalene dioxygenase [25,27].

The first step in the electron transfer chain is represented by the two-electron reduction of the prosthetic group, FAD or FMN of the reductase, at the expenses of NAD(P)H. In the three-component dioxygenase the FAD-containing flavoprotein has molecular weight of 42000–67000 [21,25]. The iron–sulphur flavoproteins of the two-component dioxygenases have molecular weights in the range between 34000 and 38000. In the reduced form they have an electron paramagnetic resonance (EPR) spectrum typical of plant-type [2Fe–2S] clusters containing an $Fe_2S_6^{3-}$ centre [5,30].

Ferredoxins perform the intermediate step in the electron transfer chain, transferring electrons from the flavoprotein to the terminal dioxygenase. They are small proteins (molecular weight, 12000–15000) containing a [2Fe–2S] cluster and they cannot be replaced, in their function, by other ferredoxins of different type [22,27,31]. The majority have spectroscopic and redox properties close to those of Rieske-type [2Fe–2S] clusters [32], containing an $Fe_2S_4N_2$ centre in which one of the iron ions is coordinated to the protein by two His residues and the other by two Cys residues [33,34]. A typical example is the ferredoxin of benzene dioxygenase: the visible spectrum is red shifted with respect to that of plant-type ferredoxins with a maximum

at 460 nm (plant-type ferredoxins have absorption maxima at 415 nm), the redox potential is less negative (−155 mV) [35] and the EPR spectra have an average g value of 1.92, which is closer to the average g value of Rieske-type ferredoxins (1.91) [34,36] than to that of plant-type ferredoxins (1.96) [37].

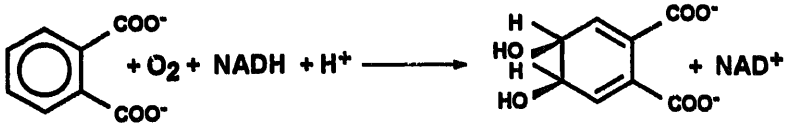
The terminal dioxygenases are responsible for the insertion of the two hydroxyl groups in the aromatic substrate. They are large protein aggregates (molecular weight, 150000–200000) containing two to six subunits. These subunits can be of two different types, named α and β, and organized as either $\alpha_2\beta_2$ tetramers (benzene, toluene and naphthalene dioxygenase) [20,24,38] or $\alpha_3\beta_3$ hexamers (benzoate dioxygenase) [10], or of the same type, and organized as α_2 (4-sulphobenzoate-3,4-dioxygenase) [9], α_3 (4-chlorophenylacetate-3,4-dioxygenase) [8], or α_4 (phthalate dioxygenase (PDO)) aggregates [5]. α subunits contain a Rieske-type cluster which accepts electrons from the ferredoxin [5,35,39]. β subunits do not seem to be involved in the catalytic function; on the basis of the genetic studies, they have been proposed to be involved in recognizing the substrate structure, thereby ensuring specificity to the enzyme [11,40].

The presence of Fe^{2+} ions is essential for the catalytic activity. The latter ions are bound to a specific site which is supposed to be the site where oxygen activation occurs [4]. Not much is known about the coordination of this site; from a comparison among the amino acid sequences which are conserved in different dioxygenases it has been speculated that two histidines and two tyrosines are involved as ligands [11,16]. A possible mechanism for dioxygen activation was proposed by Twilfer et al. [41]. On the basis of this mechanism the active species would be an iron(III)–peroxo complex which reacts with the substrate. An expanded version of the sequence of electron transfer events is proposed in Fig. 3. Possible substrate hydroxylation mechanisms (steps G→I or H→I) have been discussed elsewhere; the actual mechanism is not yet known in detail.

Specific examples of dioxygenases are discussed below.

2. Phthalate dioxygenase

PDO catalyses the reaction of dihydroxylation of phthalate to form *cis*-1,2-dihydroxy-4,5-dicarboxy-3,5-cyclohexadiene:



It is a dioxygenase of class I composed of two different proteins, namely the NAD(H)-dependent phthalate dioxygenase reductase (PDR) and the terminal dioxygenase (PDO) [5].

PDR is a monomer of molecular weight 34000, which contains one FMN and one plant ferredoxin per molecule [5]. Its structure has been solved to 2.0 Å in both oxidized and reduced forms [42], with and without pirydine nucleotide bound.

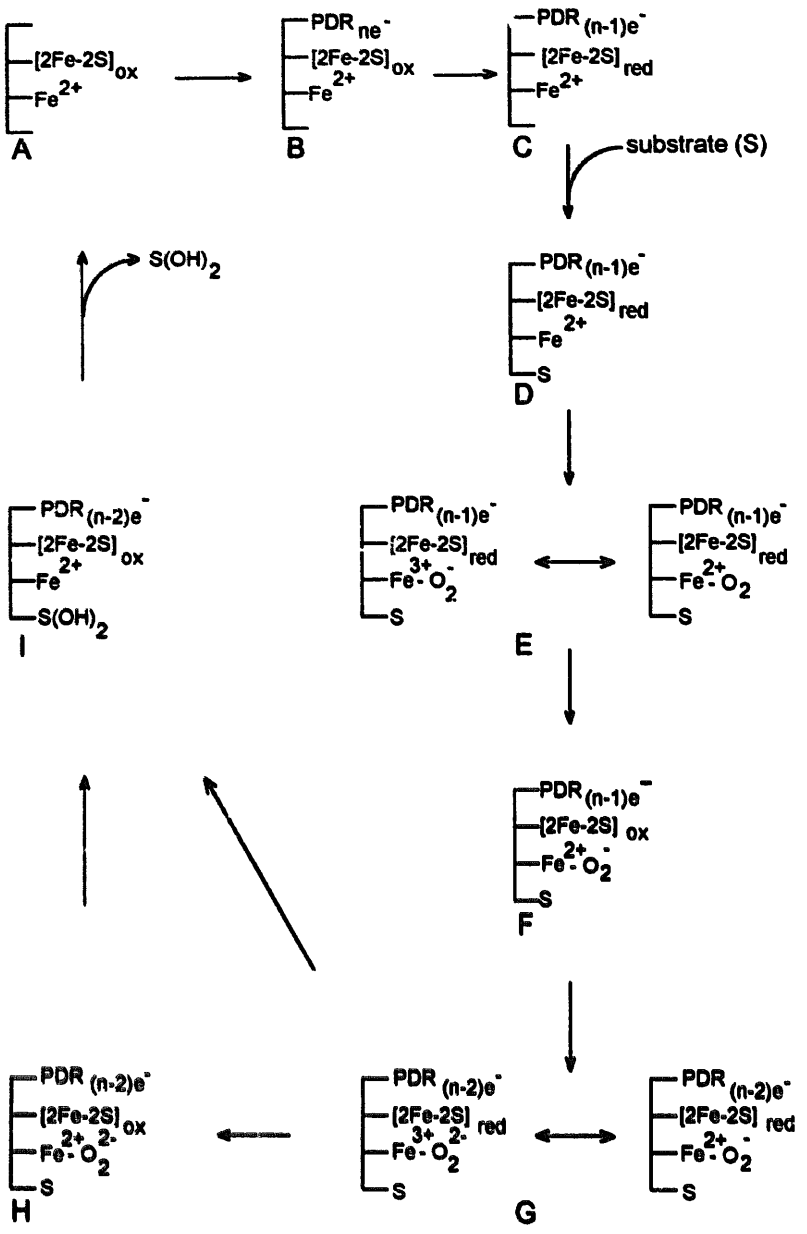


Fig. 3. A possible sequence of electron transfer events in aromatic dioxygenase activity. The example illustrates the case of PDO, but it can be extended to all the aromatic dioxygenase systems. The resting enzyme (A) reversibly binds the electron transfer protein (or the reductase itself) (B) accepting one electron, which reduces the Fe_2S_2 centre (C). Then substrate binds (D), and then dioxygen binds the Fe^{2+} ion (E). The resulting adduct, that can be viewed as Fe^{2+}–O_2^- or Fe^{3+}–O_2^{2-}, accepts a further electron from the Fe_2S_2 centre (F), which is again reduced by a second external reductant. The resulting species, viewed as either Fe^{2+}–O_2^- or Fe^{3+}–O_2^{2-} (G) can be the active species or can accept a further electron (H) before hydroxylating the substrate (I). In both cases the resting species (A) is reconstituted after product dissociation. PDR indicates the reductase component of PDO enxymatic system. $[2Fe–2S]_{ox}$ and $[2Fe–2S]_{red}$ indicate the Fe_2S_2 centre in the oxidized and in the reduced states respectively.

PDO is a tetramer of identical α monomers (molecular weight, 48 000), as confirmed by denaturing gel electrophoresis analysis, gel filtration chromatography and amino terminal sequencing [5]. Each monomer contains a Rieske-type [2Fe–2S] cluster and a mononuclear Fe^{2+} ion, which is essential for the whole system to maintain the catalytic activity.

Many spectroscopic works have been published in the last 10 years on the PDO from *Pseudomonas cepacia*, especially from Ballou and coworkers. The Fe_2S_2 cluster in PDO was actually the first Rieske-type cluster to be characterized. Electron nuclear double resonance (ENDOR) studies have been carried out on four different samples of PDO obtained from the DB01 strain and from a mutant (DB0110), which is not auxotrophic for histidine: (a) natural abundance PDO, (b) ^{15}N-labelled His in a ^{14}N background, (c) ^{14}N His in a ^{15}N-labelled background and (d) fully ^{15}N-labelled protein. X band and Q band spectra indicate the presence of two nitrogen atoms, belonging to imidazole rings of His residues, directly bound to the [2Fe–2S] cluster [34,43]. Since Mössbauer spectra have previously suggested that two non-cysteine residues were coordinated to the cluster Fe^{2+} moiety [36], it was possible to propose that both histidines were coordinated to the same (more reducible) iron, and to sketch an idealized geometry for the cluster as shown in Fig. 4.

The Rieske-type [2Fe–2S] cluster in PDO has been also characterized by using extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge structure (XANES) spectroscopy [44].

EXAFS experiments on both oxidized and reduced protein, after substitution of mononuclear iron(II) with cobalt(II) or zinc(II), with or without bound phthalate, showed (i) that there was a small change in the EXAFS spectra on reduction of the Rieske centre, showing that there was a modification of the protein structure surrounding the cluster, and (ii) that the presence of bound substrate or of metals at the mononuclear site had no detectable effects on the spectra, leading to the conclusion that there was no modification of the protein structure. Moreover, the EXAFS experiments carried out on the oxidized PDO containing iron(II) at the mononuclear site showed that these spectra were almost identical with that obtained from the oxidized iron-depleted protein, suggesting that the mononuclear site ligands were low *Z* ligands (oxygen and/or nitrogen), since these atoms are weaker backscatterers than iron and sulphur.

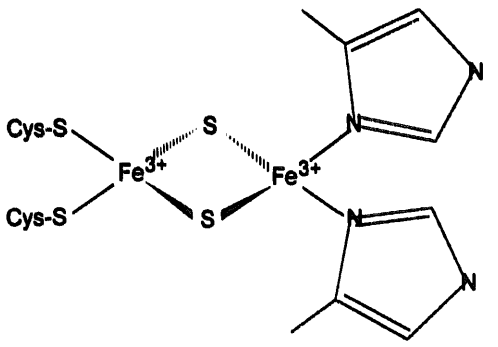


Fig. 4. Scheme of the Rieske-type cluster of PDO.

From the Fourier transform of the EXAFS spectrum of the above samples it was possible to calculate the average distances among the atoms of the cluster. The Fe–Fe distance was 2.63 Å for both oxidized and reduced cluster and the measured average Fe–S distance was 2.24 Å in the oxidized cluster and 2.28 Å in the reduced form; the calculated value for the S–Fe–S and Fe–S–Fe angles were ca. 105° and 75° respectively, with only slight variations between the oxidized and reduced forms of the protein.

These values show that the Rieske cluster has structural parameters similar to those obtained for other similar Fe–S clusters, and therefore that the unusual high redox potential of this class of ferredoxins is not due to structural differences from the plant-type [2Fe–2S] clusters, but only arises from the substitution of the cysteine thiolates with two histidine nitrogen atoms as ligands of one of the two iron ions.

By the analysis of the EXAFS spectrum an average value of 2.05 Å for the Fe–N distances in the oxidized protein and an average value of 2.09 Å for the same distance in the reduced form was also calculated.

The XANES spectra obtained for the iron-depleted dioxygenase showed that there is a 2.0 eV shift to lower energy on reduction, in agreement with the values reported in the literature for other Rieske clusters. Moreover, the fact that the edge shape is identical for different oxidized and reduced iron-depleted PDO samples, with or without bound substrate and with or without metal occupancy of the mononuclear binding site, suggests that there are no structural changes on binding of phthalate and/or metal ions, in agreement with the EXAFS results.

In the XANES spectrum the 1s→3d low energy transition of the Rieske-type centres decreases on reduction, and the reduced iron ion is characterized by a very low 1s→3d intensity, less than 40% with respect to the oxidised iron. This low value was interpreted by these researchers as due to a possible increase in the coordination number in the reduced cluster and/or to a change in geometry at one of the iron sites. A possible interpretation could be that, on reduction, one of the iron ions of the cluster would gain an additional ligand, on passing from 4-coordination to 5-coordination. Since the ENDOR spectra showed that only two nitrogen atoms are coordinated in the reduced protein, the additional ligand could be an oxygen atom from an amino acid side chain or from the peptide backbone. Another interpretation could be a distortion of the symmetry of one of the iron sites, which would distort from a tetrahedral towards a square planar coordination. In any case, both EXAFS and XANES data seem to indicate that the Rieske cluster undergoes structural changes on reduction.

The EPR spectrum of reduced PDO is typical of a Rieske type cluster with g values of 2.016, 1.915, 1.773. Addition of the essential Fe^{2+} ion at the mononuclear site produces some changes in the EPR spectrum of the reduced Rieske dimetallic centre yielding g values of 2.011, 1.900, 1.726. Similar effects are caused by the addition of metal ions such as Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}. Therefore the perturbation in the EPR spectrum does not seem to be due to magnetic interactions, but rather to slight structural changes in the cluster [45].

The coordination geometry of Fe^{2+} in the mononuclear site, both in the absence and in the presence of the substrate, was monitored through low temperature

magnetic circular dichroism (MCD) measurements [45]. Two overlapping bands, near 1000 nm, were observed in the substrate-free enzyme, which are presumably indicative of an octahedral 6-coordination. A larger splitting of the two bands (about 6000 cm^{-1}) is observed in the substrate-bound enzyme and interpreted as typical of 5-coordination. A similar effect was observed when the mononuclear site binds Co^{2+}: indeed, from MCD spectra, a partial interconversion between 6-coordinated Co^{2+}, in the absence of substrate, and 5-coordinated Co^{2+}, in the presence of substrate, was proposed. Therefore, substrate binding was supposed to perturb the Fe^{2+} site with displacement of a metal ligand preparing, in such a way, the ferrous centre for dioxygen binding and activation.

Derivatives containing Mn^{2+} and Cu^{2+} at the catalytic iron site have been also investigated by water ^1H NMRD [46]. There is evidence of one or two metal-coordinated water molecules, one of which remains coordinated also on binding of the phthalate substrate. This finding may be consistent with the 6- to 5-coordination transition on substrate binding proposed from MCD studies.

3. Benzene dioxygenase

From samples of refinery soil some strains which are capable to convert benzene into catechol were isolated [47]. One of these strains, identified as *Pseudomonas putida*, was demonstrated to be able to grow in salts medium containing benzene as the major carbon source and to possess a soluble dioxygenase system which is stable at 4 °C for several weeks [48]. Three components were identified in the system, and a procedure to isolate and to purify them was set up by Geary and coworkers [17,18]. One of the components, the reductase, has the typical electronic absorption spectrum of a flavoprotein from which it was possible to extract the flavin component, FAD [17]. It is constituted by two subunits, each of them having a molecular weight of 43 800. Its role is that of transferring electrons from NADH to the second component of the system, an iron–sulphur protein, ferredoxin. The latter has a molecular weight of 11 900 and contains a [2Fe–2S] cluster. From the midpoint redox potential (−155 mV) and from the features of the electronic spectrum which has a maximum at 460 nm, we can ascribe this ferredoxin to the class of Rieske-type iron–sulphur proteins, with histidines and cysteines as ligands. The reduced protein gives an EPR spectrum, observable up to 155 K, with g values of 2.026, 1.890 and 1.834 [35]. From kinetic studies it seems that the ferredoxin functions as an electron shuttle which binds reductase and accepts one electron from reduced reductase. Then, the reduced ferredoxin binds to the terminal dioxygenase component, ISP, transfers its electron to it, and dissociates from the reduced ISP.

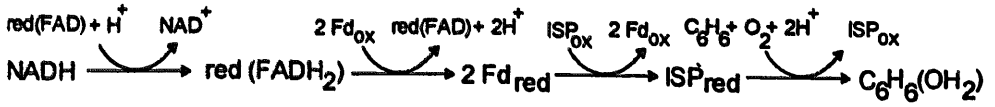
The terminal dioxygenase represents the catalytic site of the oxygen insertion reaction. It has a molecular weight of 147 000 and it is constituted by four subunits, two with molecular weight 51 100 (α subunit), and two with molecular weight 22 250 (β subunit). It was reported to contain four iron atoms and four inorganic sulphur atoms per molecule [35] which were demonstrated, by cluster extrusion experiments and by Mössbauer spectroscopy, to be arranged in two [2Fe–2S] clusters [49,50].

In the oxidized form the two iron ions are high spin Fe(III) whereas in the reduced form there is one high spin Fe(II).

For oxygenase activity the protein requires two additional Fe^{2+} per molecule [35]. Indeed, in the absence of added Fe^{2+} it was demonstrated, by using NADH and the complete enzyme system, that reduction of the iron–sulphur cluster occurred, but no oxygenase activity was present, i.e. the conversion of benzene to *cis*-dihydrodiol does not occur [49]. It seems therefore that the additional iron-binding site acts as an oxygen-binding and activation centre.

The protein exhibits an electronic spectrum with a maximum at 450 nm (ε/[Fe] = 4200 M^{-1} cm^{-1}) and a shoulder at 560 nm (ε/[Fe] = 2200 M^{-1} cm^{-1}). These absorption bands are typical of a Rieske-type cluster.

The order in which electrons flow from NADH to the terminal dioxygenase (ISP) was demonstrated spectrophotometrically by following the bleaching of the absorbance at 450 nm of ISP, caused by reduction of the cluster. From oxygen electrode experiments it was observed that the stoichiometry of the electron transfer process should be the following [49]:



1 mol of NADH reduces 2 mol of ferredoxin. Two electrons are then transferred to ISP, one for each cluster. From the clusters the electrons could be involved in the activation of oxygen at the iron mononuclear site with the formation of a peroxo complex which reacts with one molecule of substrate (see Section 1).

The oxidized protein does not exhibit any EPR signal. However, the reduced protein gives a signal characteristic of non-haem-iron proteins and typical of a Rieske protein with $g_z = 2.018$, $g_y = 1.914$, $g_x = 1.758$. The spectrum is observable up to 120 K [17,35]. This temperature dependence is typical of 2Fe–2S ferredoxins. The EPR spectrum reveals the presence of only one paramagnetic species, thus supporting the hypothesis that the coordination geometry of both clusters is identical.

The redox potential was determined through titration of the protein with different mediators, following the method of Dutton [51]. The titration was followed through EPR and a midpoint redox potential of −110 mV was found. This value is not affected by the presence of Fe^{2+} [35].

The protein, reactivated with Fe^{2+}, was demonstrated to bind two Fe^{2+} ions per molecule. These iron ions are EPR silent, either in the presence or in the absence of the substrate [35].

In order to investigate the iron(II) binding site and its relationship with the iron–sulphur cluster some metal-substituted derivatives (Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}) have been studied [46]. The interaction has been monitored through spectroscopic techniques (CD, EPR, EXAFS).

The iron content of all the samples isolated and purified in our laboratory was checked through atomic absorption analysis. In the majority of the cases, the results indicate an Fe:ISP ratio around 2. That can be explained by assuming the loss of

one of the two clusters, apart from the mononuclear iron ions. Many attempts were made during the purification procedure to obtain a protein with four iron ions. It seems that the tetrameric $\alpha_2\beta_2$ species with only one cluster in one of the two α subunits is the most stable form.

The CD spectrum of the enzyme is reported in Fig. 5, spectrum A. It displays two positive bands, centred at 310 and 455 nm, and three negative bands, one below 300 nm, one centred at 375 nm and the last centred at 600 nm. The two bands at lower energy are due to the [2Fe–2S] cluster.

The sample was titrated with Zn^{2+}. The spectrum of the final adduct, reported in Fig. 5, spectrum B, exhibits a decrease in intensity of the bands of the cluster in the region between 400 and 640 nm. The above effect indicates that Zn^{2+} binding to the mononuclear site induces some slight changes in the cluster structure, thus suggesting that the [2Fe–2S] cluster and the mononuclear site belong to the same subunit.

A similar behaviour was observed by adding Cu^{2+} to the iron(II)-depleted protein (Fig. 5, spectrum C). Furthermore we observe a new band, in the region of the spectrum where typically copper(II) complexes absorb. The difference spectrum (also shown in Fig. 5) obtained by subtracting the spectrum of the Zn^{2+} adduct from the spectrum of the Cu^{2+} adduct, displays the new band, with a maximum at 620 nm, which is assigned to Cu^{2+} in the mononuclear site. The EPR spectrum of copper

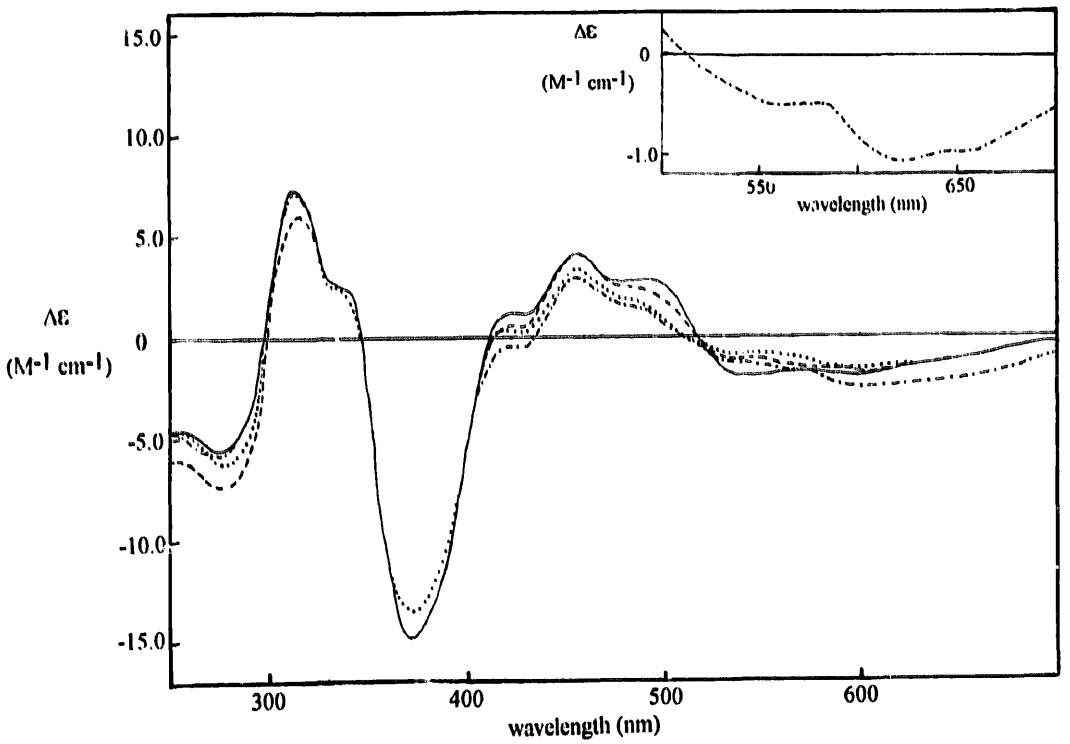


Fig. 5. CD spectra: spectrum A, benzene dioxygenase (—); spectrum B, Zn^{2+} adduct of benzene dioxygenase (···); spectrum C, Cu^{2+} adduct of benzene dioxygenase (–·–·–); spectrum D, Co^{2+} adduct of benzene dioxygenase (– – –). The samples are in 25 mM phosphate, pH 7.2. Protein concentration is 0.3 mM. The difference spectrum obtained by subtracting the spectrum of the Zn^{2+} adduct from the spectrum of the Cu^{2+} adduct is also shown.

in the catalytic site of BDO has been obtained on a sample containing the oxidized, EPR-silent, Rieske protein (Fig. 6). The spectrum exhibits an $A_{\|}$ value of 197×10^{-4} cm^{-1} and $g_z = 2.245$, $g_y = 2.058$, $g_x = 2.010$.

The titration with Co^{2+} (Fig. 5, spectrum D) confirmed the binding of the metal ion at the mononuclear site and the effect of this interaction on the cluster. However, no absorption of cobalt(II) was observed, probably on account of hexacoordination. Six-coordinated cobalt(II) complexes are expected to display very low absorption. The same effect is observed with the addition of Ni^{2+} (data not reported): the bands due to the cluster were affected by the addition of the metal ion, but no new band was observed.

By following the changes in intensity of the Rieske dimetallic centre as a function of the metal ion added, a stoichiometry of 1 metal ion per Rieske centre was found. As an example the titration with Ni^{2+} is reported in Fig. 7. A reasonable explanation for this behaviour could be that only the mononuclear site of the subunit containing the cluster is able to bind the metal ion; as a consequence of the loss of the cluster, the α subunit could presumably modify its structure, preventing the binding of the metal ion in the mononuclear site.

In order to confirm a possible interaction between the cluster and the mononuclear site, we have titrated the reduced protein with the following metal ions: Zn^{2+}, Co^{2+}, Ni^{2+}. In all cases, by following the EPR spectrum of the cluster we observed some slight changes in the spectrum of the cluster, especially as far as the g_x and g_z features are concerned. The titration with Zn^{2+} is reported in Fig. 8. The final adduct, obtained at a metal:ISP ratio of 1, is characterized by $g_z = 2.022$, $g_y = 1.910$, $g_x = 1.764$. The same effect was observed with Co^{2+} and Ni^{2+}. These results confirm

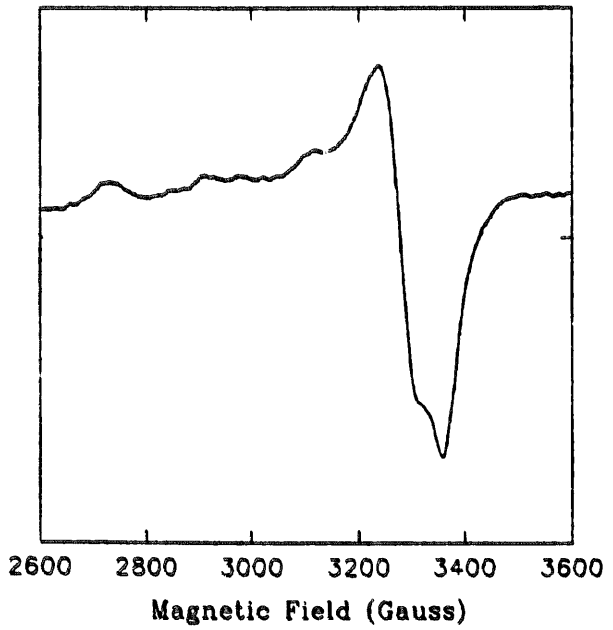


Fig. 6. EPR spectrum of the copper(II) adduct of benzene dioxygenase in 25 mM phosphate, pH 7.2. Protein concentration is 0.9 mM. Conditions: temperature, 135 K; microwave power, 67 mW; frequency, 9.54 GHz.

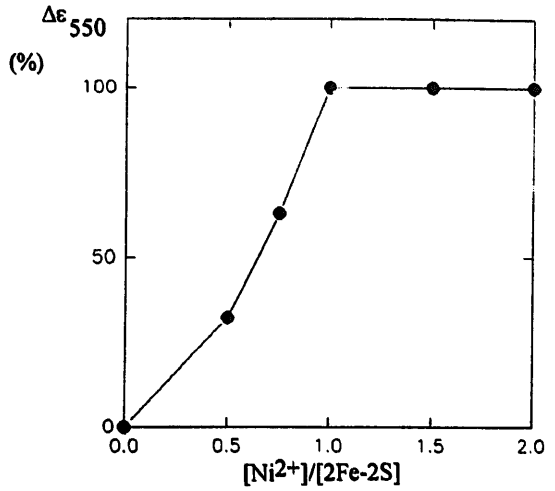


Fig. 7. Variation in intensity of the benzene dioxygenase CD band at 550 nm as a function of Ni^{2+}/[2Fe–2S] ratio.

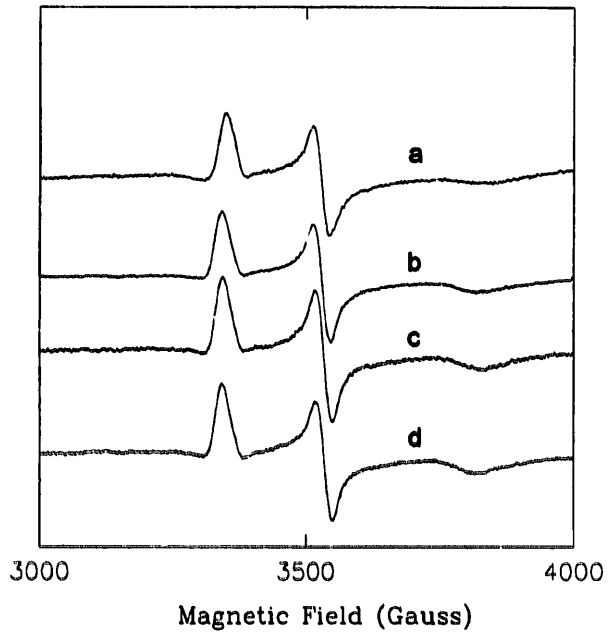


Fig. 8. EPR spectra of benzene dioxygenase with different Zn^{2+}:[2Fe–2S] ratios, in 25 mM phosphate, pH 7.2: spectrum a, Zn^{2+}:[2Fe–2S]=0; spectrum b, Zn^{2+}:[2Fe–2S]=0.25; spectrum c, Zn^{2+}:[2Fe–2S]=0.5; spectrum d, Zn^{2+}:[2Fe–2S]=1.0. No further change is observed at higher Zn^{2+}:[2Fe–2S] ratios. Conditions: 4 K; microwave power, 20.0 μW; frequency, 9.57 GHz.

what was previously observed through CD spectroscopy: as a consequence of the binding of a metal ion in the mononuclear site, the cluster structure is affected by slight distortions. This confirms the hypothesis that the mononuclear site is in the same subunit as the cluster (subunit α).

The Ni^{2+} adduct of ISP_{bed} has been also investigated through EXAFS [46]. The spectrum (Fig. 9) is typical of hexacoordinated Ni^{2+}. The shoulders, labelled as a

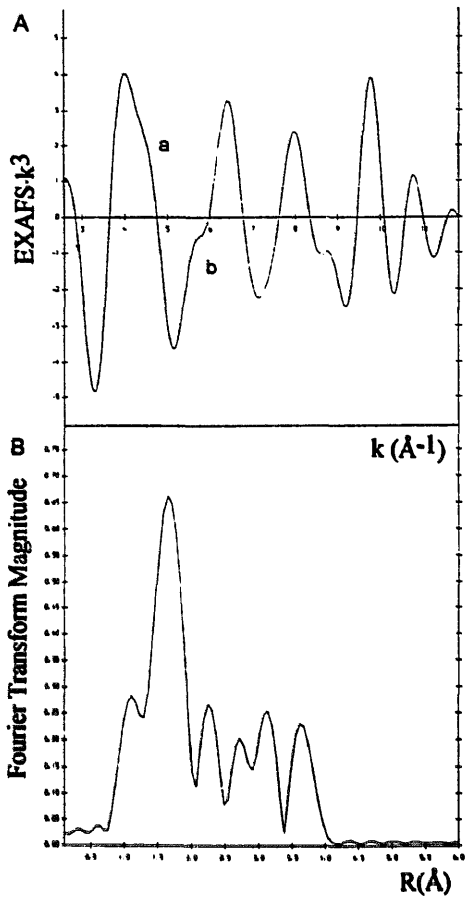


Fig. 9. EXAFS spectrum (spectrum A) and its Fourier transform (spectrum B) of the Ni^{2+} adduct of benzene dioxygenase.

and b in the spectrum, are indicative of the presence of two histidine residues, coordinated to the metal ion. Furthermore, in the proximity of the Ni^{2+} edge an intense backscattering signal was observed, probably due to iron or sulphur of the cluster, thus confirming that the mononuclear site and the cluster should be close to one another.

4. Perspectives

Similarly to benzene dioxygenase, naphthalene dioxygenases and 1,2,4-trichlorobenzene dioxygenase are also reported to possess an $\alpha_2\beta_2$ terminal dioxygenase component, in addition to the reductase and the ferredoxin, which incorporates both atoms of molecular oxygen into the substrate, yielding (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene and 1,2,4-trichloro-5,6-*cis*-dihydrodiol respectively [26,28,29,52].

Their investigation is essential to have a complete view of these non-haem single-

iron dioxygenases which represent a novelty in the field of oxygen activation and provide unique reactions.

One aim of future research in the field is to understand the interactions between the enzyme and the substrate. What is attracting a substrate such as benzene inside the cavity? NMR investigations of the substrate may provide evidence on this as well as information on the affinity constants. Kinetics measurements may also provide the value of the Michaelis–Menten dissociation constant and provide information on the ability of the enzyme to activate the organic substrate.

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