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PROTOCATECHUATE 3,4-DIOXYGENASE

INHIBITOR STUDIES AND MECHANISTIC IMPLICATIONS

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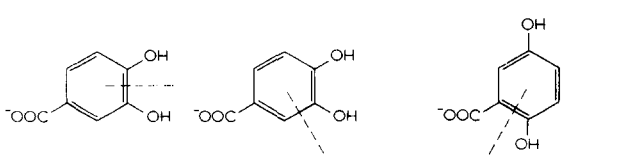
Summary

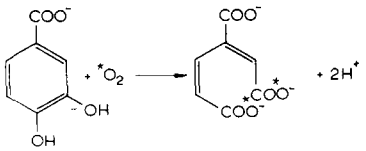
Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) from *Pseudomonas aeruginosa* catalyzes the cleavage of 3,4-dihydroxybenzoate (protocatechuate) into β-carboxy-*cis*,*cis*-muconate. The inhibition constants, K_i, of a series of substrate analogues were measured in order to assess the relative importance of the various functional groups on the substrate. Though important for binding, the carboxylate group is not essential for activity. Compounds with para hydroxy groups are better inhibitors than their meta isomers. Our studies of the enzyme · inhibitor complexes indicate that the 4-OH group of the substrate binds to the active-site iron. Taken together, Mössbauer, EPR, and kinetic data suggest a mechanism where substrate reaction with oxygen is preceded by metal activation of substrate.

Introduction

Bacterial non-heme iron dioxygenases are an essential component in the metabolism of many aromatic compounds [1]. These enzymes cleave aromatic rings in natural products such as flavanoids, alkaloids, and lignin by incorporating two atoms of molecular oxygen into dihydroxybenzenes [2] to yield aliphatic products which are, in turn, metabolized via the tricarboxylic acid cycle. Several modes of ring fission are known: intradiol and extradiol cleavage of *o*-dihydroxybenzenes, and *p*-dihydroxybenzene cleavage.



Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) from *Pseudomonas aeruginosa* catalyzes the intradiol cleavage of protocatechuate to β-carboxy-*cis,cis*-muconate.



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Though this enzyme has been studied extensively in the past decade [3—6], little is known of its catalytic mechanism. Several schemes for intradiol cleavage have been proposed [7,8], all postulating activation of molecular oxygen by iron.

Spectroscopic investigations [4,5] on protocatechuate 3,4-dioxygenase show that the enzyme can be prepared in three distinct states: (i) the native enzyme, (ii) enzyme · substrate complex, and (iii) a ternary complex of enzyme, 3,4-dihydroxyphenylpropionate, and oxygen. Recent Mössbauer [9] studies have established the iron site to be high-spin ferric in all three states. EPR spectroscopy shows changes in the ligand field environment of the iron in each of the three states as expressed in the zero-field splitting parameters (D,λ) of the ^6S ground state. The native enzyme exhibits an EPR signal at $g = 4.3$, typical of high-spin ferric iron in a "rhombic" environment ($D = 1.6$ cm^{-1}, $\lambda = 0.28$) [6]. Upon substrate binding, the rhombic signal is decreased concomitant with the rise of axial signals around $g = 6$. In all enzyme · substrate complexes studied, at least two sets of signals can be discerned ($D > 0$; $\lambda = 0.02, 0.12$); the cause of the multiplicity of these signals is not understood at the present time [9]. The ternary oxy complex exhibits signals at $g = 6.7$ and $g = 5.3$. These signals arise from high-spin ferirc iron in an "axial" environment characterized by a negative zero-field splitting ($D = -2$ cm^{-1}, $\lambda = 0.03$) [9], which is unique among biological iron complexes.

In this communication, we report the results of a kinetic and spectroscopic study of the enzyme with inhibitors and propose a mechanism of action for protocatechuate 3,4-dioxygenase; the distinctive feature of this mechanism is the activation of the substrate by the metal prior to oxygen attack.

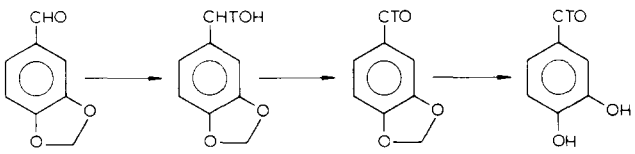
Experimental Methods

The enzyme was prepared from *Pseudomonas aeruginosa* (ATCC 23975) as previously reported [9]. The inhibitors were obtained commercially and used without further purification. The fluorinated compounds were a generous gift of Professor P.J. Chapman of the Department of Biochemistry, University of Minnesota, St. Paul, Minn. 55108.

The inhibition kinetics experiments were performed on a Gilson Oxygraph K-1C. Measurements of the rate of oxygen uptake by the enzyme solution were made in the presence of various concentrations of protocatechuate and inhibitor. A Dixon plot [10] ($1/v$ vs. [I]) of the data yielded the type of inhibition and the K_i value for the inhibitor. Optical experiments were performed on

a GCA-McPherson double beam spectrophotometer. The binding constants were calculated from double reciprocal plots of changes in absorbance and inhibitor concentration.

Protocatechualdehyde ^{3}H-labelled at the aldehyde proton was synthesized by reducing piperonal with [^{3}H]$NaBH_4$ and oxidizing the alcohol back to the aldehyde with Collin's reagent [11] and removing the CH_2 blocking group with PCl_5 [12].



The radioactive labelling experiment was performed on 0.2 ml of 40 μM enzyme. 10 μl of a 50 mM ethanol solution of labelled protocatechualdehyde was added to the enzyme solution, followed by 10 μl of a 1 M ethanol solution of $NaBH_4$. The reaction mixture was incubated at 0° C for 18 h. The solution was then run through a Biogel P-10 Column (10 × 1 cm) and the eluate was counted and assayed for enzyme activity.

EPR spectroscopy was performed at X-band on a Varian E-9 spectrometer in Dr. Orme-Johnson's laboratory with provisions for 100 kHz modulation. The samples were immersed in liquid helium for the measurements at 4.2 K and helium boil-off gas was used as coolant in the temperature range of 5.5—50 K [12]. Temperatures were routinely monitored via a calibrated carbon resistor below the sample in the gas stream. Spectrometer conditions are given in the figure captions.

Kinetic Studies

Substrate analogues are frequently used to assess the relative importance of various functional groups on the substrate in binding. Several modes of substrate binding to protocatechuate 3,4-dioxygenase and, in particular, to the iron active site are possible since there are various functional groups on the aromatic ring of protocatechuic acid (I). The iron could bind to any one of the substituent groups, chelate to both hydroxy groups, or not bind at all.

Inhibition kinetic studies as well as EPR and optical spectroscopic experiments were conducted on a series of substrate analogues where one of the original functional groups is modified or missing. The results of the inhibition kinetics experiments are presented in Table I. All inhibitors tested were found to be competitive with protocatechuate. Some of the K_i values were cross-checked with optical titration experiments to determine the K_d of inhibitor binding to the enzyme. The K_d values were found to be consistent with the K_i values.

It is clear from the inhibition data for II, III, and IV that the carboxylate group is important for binding. All three compounds are substrates for the enzyme, though their turnover rates are much smaller than for protocatechuate (I); as such, they can be considered competitive inhibitors to the protocatechuate cleavage reaction. The fact that all three are pseudosubstrates [3,5]

TABLE I

DISSOCIATION CONSTANTS FOR PROTOCATECHUATE 3,4-DIOXYGENASE

	Substrate analogue	K_I^*	K_D^{**}
II	3,4-Dihydroxyphenylacetate	13 μM	
III	3,4-Dihydroxyphenylpropionate	18 μM	
IV	Catechol	700 μM	
V	Protocatechualdehyde	14 μM	
VI	3,4-Dihydroxyacetophenone	130 μM	130 μM
VII	Protocatechuic acid, methyl ester	400 μM	300 μM
VIII	*p*-Hydroxybenzoate	240 μM	180 μM
IX	*m*-Hydroxybenzoate	4 mM	
X	Vanillate	3 mM	
XI	Isovanillate	20 mM	
XII	3-Fluoro-4-hydroxybenzoate	1 μM	
XIII	4-Fluoro-3-hydroxybenzoate	800 μM	
XIV	2-Fluoro-4-hydroxybenzoate	1 μM	

* Obtained from inhibition kinetic data.
** Obtained from optical titration experiments.

suggests that the carboxylate group is not essential for activity. Furthermore, the optical absorption spectra of the enzyme with each of the three inhibitors are similar to the spectrum of the enzyme · protocatechuate complex; in all cases, λ_{max} shifts from 450 nm in the native enzyme to 480 nm with a concomitant increase in the absorption at 600 nm [5]. The above observations all suggest that while the carboxylate is important for binding, it does not bind to the iron.

The carboxylate binding site is most probably a positively charged moiety capable of electrostatic interaction with negatively charged groups, probably the ε-amino group of a lysyl residue. Compounds (V, VI, and VII) with carbonyl functional groups replacing the carboxylate can interact with this group and are indeed inhibitory to the enzymatic reaction. In particular, protocatechualdehyde (V) could react with the amino group to form a Schiff's base which could then be reduced by sodium borohydride. To test this, ^{3}H-labelled protocatechualdehyde was synthesized (specific activity $1.31 \cdot 10^9$ dpm/mmol), then added in excess to the enzyme. The enzyme · inhibitor complex formed, as evidenced by the change of color from the pink of the native enzyme to the yellow of the inhibitor complex. The complex was then reduced with $NaBH_4$ and the reaction was allowed to proceed overnight at 0° C. Gel filtration of the reaction mixture on a Biogel P-10 column yielded a protein (specific activity $1.56 \cdot 10^9$ dpm/mmol) with 1.2 mol of ^{3}H incorporated per mol enzyme *. Efforts to increase the amount of ^{3}H incorporation by varying the concentration of the various reactants were unsuccessful. The enzyme has been reported to have 8 active sites, although we have more recent evidence suggesting that there may be 10 (J.D. Lipscomb, L. Que and J.M. Wood, in preparation). Thus, the incorporation of 1.2 mol ^{3}H per mol enzyme represents only a 12% labeling

* Schiff's base formation with surface lysyl residues does not appear to occur under our reaction conditions. The addition of a 10-fold excess of benzaldehyde over protocatechualdehyde to some experiments did not affect the amount of radioactive label incorporated.

of the active sites. Enzymatic activity measurements on the labelled enzyme show a 10% reduction in specific activity, consistent with the above observation.

During the course of the reaction, the enzyme solution became purple (λ_{max} = 480 nm), indicating the formation of an enzyme · substrate complex [4]. This can arise because the borohydride reduction product of protocatechualdehyde is 3,4-dihydroxybenzyl alcohol, a pseudo-substrate. The rate of Schiff's base reduction appears to be less favorable than that of aldehyde reduction and during the reaction period enough of the alcohol is produced to displace the aldehyde from the active site. This suggests that the active site pocket may not be easily accessible to the borohydride. Alternatively, Schiff's base formation may not readily occur if the pK_a of the ϵ-amino group is too high. Currently work is in progress to further characterize the carboxylate binding site.

The other substrate analogues in Table I are essentially grouped in pairs of meta and para hydroxy isomers. The data clearly indicate that the binding of the para hydroxy group to the enzyme is more important than that of the meta hydroxy group. The binding of *p*-hydroxybenzoate is at least an order of magnitude tighter than that of the meta isomer. More striking is the difference between XII and XIII, where binding of XII is almost three orders of magnitude tighter. The enhancement of binding of the fluoro-derivatives over the unsubstituted hydroxybenzoates is probably due to the electron-withdrawing effect of the fluorine group making the phenolic proton more acidic. The lack of an effect on K_i when the 3-fluoro (XII) isomer is replaced with 2-fluoro (XIV) isomer suggests that there are no specific interactions of the fluoro group and the protein.

EPR Studies

Because of its high resolution and sensitivity to changes in ligand environment, EPR spectroscopy has been a very potent tool for exploring the high-spin ferric ion. We have obtained EPR spectra of the enzyme complexed with inhibitors with either meta or para hydroxy groups. Before presenting the data, we briefly discuss the spin Hamiltonian

$$\hat{\mathcal{H}} = D[S_z^2 - \tfrac{35}{12} + \lambda(S_x^2 - S_y^2)] + g_0\beta\vec{S}\cdot\vec{H}\,, \tag{1}$$

which, in general, quite adequately describes the EPR spectra of high-spin ferric compounds (S = 5/2). In Eqn. 1, the parameters D and λ in the first term describe the fine structure splitting of the spin sextet into three Kramers doublets. The last term describes the interaction of the electronic magnetic moment $g_0\beta S$ with the applied magnetic field. We assume this interaction to be isotropic and take the g value of a free spin, g_0 = 2.00. For applied magnetic fields such that $\beta H << |D|$, it is customary to describe the magnetic properties of each Kramers doublet by an effective S' = 1/2 spin Hamiltonian.

$$\hat{\mathcal{H}}_i = E_i + \beta\vec{S'}\cdot\tilde{g}_i\cdot\vec{H} \tag{2}$$

where E_i gives the energy of the ith doublet (i = 1, 2, 3). The g_i-tensors in Eqn. 2 are the quantities commonly reported from EPR experiments. They can

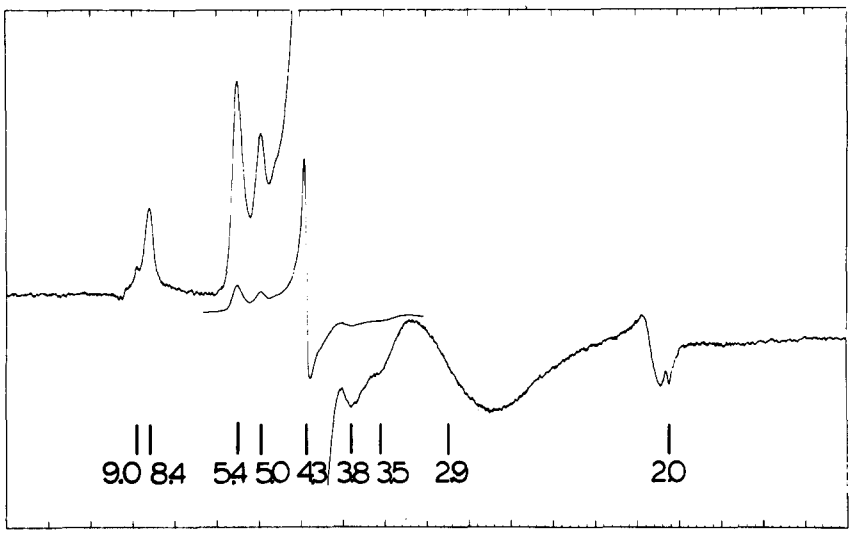


Fig. 1. EPR spectrum of protocatechuate 3,4-dioxygenase complexed with 3-fluoro-4-hydroxybenzoate (XII). Conditions: T = 9.6 K; microwave frequency, 9.113 GHz; microwave power, 3 mW; modulation amplitude, 10 G; sweep rate, 1000 G/min; time constant, 0.3 s; receiver gain, 3200 for the whole spectrum and 320 for the inset. Abscissa: magnetic field strength, increasing to the right; ordinate: an arbitrary linear function of the first derivative of microwave absorption with respect to the field. Similar spectra are obtained with other *p*-hydroxybenzoates.

be computed from Eqn. 1 for each doublet; the g-values depend essentially on λ, which reflects the presence of low symmetry components (lower than tetragonal or trigonal) of the ligand field *.

Fig. 1 shows the EPR spectrum of the enzyme complexed with 3-fluoro-4-hydroxy-benzoate (XII). Two sets of signals, originating from two species, are observed; one with g-values at 8.4, 5.4, and 2.9 resulting from Eqn. 1 with λ = 0.13, and another with g-values at 9.0, 5.0, 3.8, and 3.4 consistent with λ = 0.21. (There is also a remnant g = 4.3 signal in the EPR spectrum due to adventitiously bound iron.) A titration of the enzyme with XII shows that the species with λ = 0.13 is the more tightly bound. The spectrum with λ = 0.21 is not observed until after stoichiometric amounts of XII have been added. Using the procedure described by Aasa and Vanngard [14], the concentration of the minority species is estimated to be at most 15% of the concentration of the majority species. At this point, we do not understand the nature of this minority species.

The signals of the λ = 0.13 species arise from a high-spin ferric ion with a negative zero-field splitting. The pertinent energy level diagram is shown in Fig. 2. The signal at g = 8.4 results from the upper Kramers doublet, while that

* The use of the terms "axial" and "rhombic" refer to λ values around 0 and 1/3, respectively. Although tetragonal and trigonal symmetries require λ to be equal to zero, the converse is not necessarily true. Thus, the terms "axial" and "rhombic", as they are used here, are more descriptive of the type of EPR spectrum observed, rather than the geometry around the iron site of the complex in question.

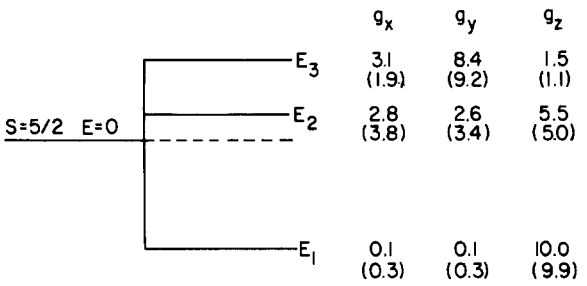


Fig. 2. Zero-field splitting diagram of an $S = 5/2$ system, according to Eqn. 1, with $D < 0$ and $g_0 = 2.0$. The effective g-values computed for Eqn. 1 for $\lambda = 0.13$ are given for each doublet. Those computed for $\lambda = 0.21$ are given in parentheses.

at $g = 5.4$ arises from the middle doublet. The signal around $g = 2.9$ is a composite of resonances from both the upper and middle doublets; the ground doublet is EPR silent. The temperature dependences of the signal at $g = 5.4$ and 8.4 are shown in Fig. 3. Note that the $g = 5.4$ signal reaches maximum intensity near 7 K, while the $g = 8.4$ signal peaks around 11 K. The observed temperature dependence can be described by

$$I_i(T) \propto \frac{1}{T} \frac{\exp(-E_i/kT)}{\exp(-E_1/kT) + \exp(-E_2/kT) + \exp(-E_3/kT)} \tag{3}$$

where $i = 2, 3$. The $1/T$ dependence describes the population difference within the Kramers doublet; the exponential factor takes the Boltzmann distribution

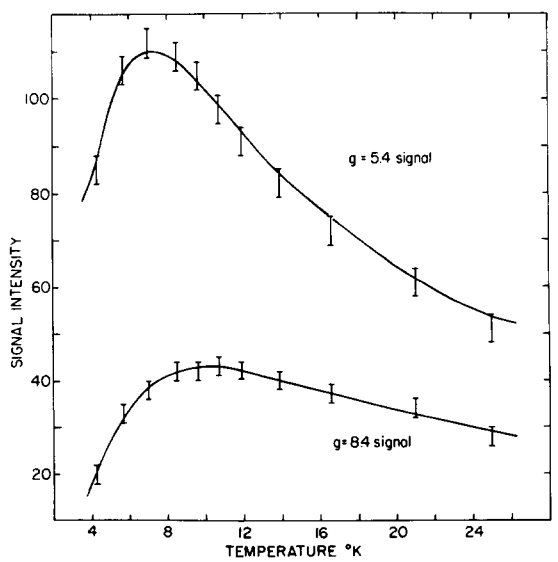


Fig. 3. The signal intensities under the $g = 8.4$ and the $g = 5.4$ resonances of the Enzyme-XII complex are plotted in arbitrary units for each resonance as a function of temperature. The solid lines were generated from Eqn. 3.

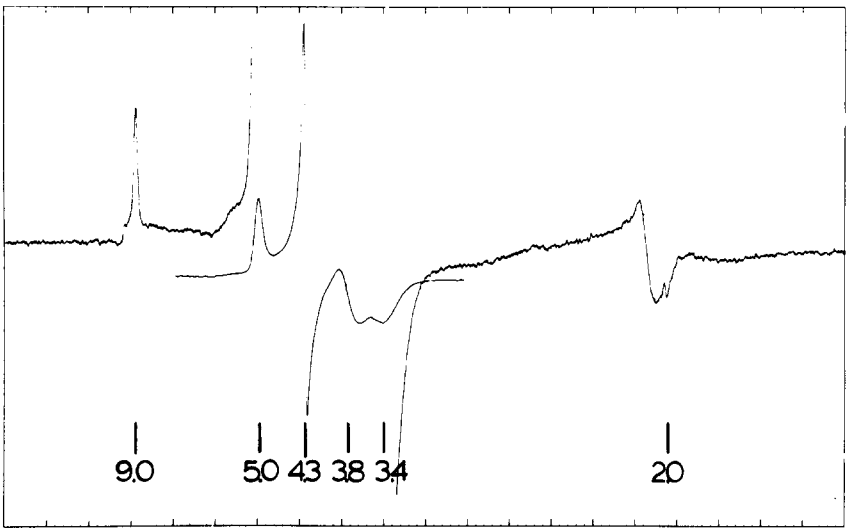


Fig. 4. EPR spectrum of the complex of protocatechuate 3,4-dioxygenase with 3-hydroxy-4-fluorobenzoate (XIII). Conditions: T = 12.7 K; 9.114 GHz, 3 mW; modulation amplitude, 10 G; sweep rate, 1000 G/min; receiver gain, 3200 and 320 for the inset. Magnetic field increases linearly to the right.

of the three doublets into account. For $\lambda = 0.13$, one obtains $E_1 = 3.37D$, $E_2 = -0.53D$, and $E_3 = -2.84D$. The best individual fits of the experimental data to Eqn. 3 are as follows: for the $g = 5.4$ signal, $D = -(1.82 \pm 0.07)$ cm^{-1} and $D = -(1.63 \pm 0.07)$ cm^{-1} for the $g = 8.4$ resonance.

An EPR spectrum of protocatechuate 3,4-dioxygenase with 4-fluoro-3-hydroxybenzoate (XIII) is shown in Fig. 4. The signals at $g = 9.0$, 5.0, 3.8, and 3.4 are consistent with $\lambda = 0.21$ for the quadratic spin Hamiltonian (Eqn. 1) where $E_1 = 3.42D$, $E_2 = -0.33D$, and $E_3 = -3.09D$. This complex also exhibits a negative zero-field splitting. An analysis of the temperature dependences of the resonances at $g = 9.0$ and $g = 5.0$ yields $D = -(0.94 \pm 0.07)$ cm^{-1} for the $g = 9.0$ resonance and $D = -(1.22 \pm 0.07)$ cm^{-1} for the $g = 5.0$ resonance.

Our analysis of the temperature dependence of the EPR signals of the two inhibitor complexes reveals a disagreement in their respective D values when the upper and the middle doublets are fitted individually. This discrepancy may possibly be ascribed to the inadequacy of the spin Hamiltonian, Eqn. 1, in describing the relative energies of the Kramers doublets. Spartalian et al. [15] have recently pointed out the utility of quartic terms in the spin Hamiltonian description of enterobactin, a high-spin ferric iron transport complex. The augmented Hamiltonian,

$$\hat{\mathcal{H}} = D[S_z^2 - \tfrac{35}{12} + \lambda(S_x^2 - S_y^2) + \frac{\mu}{6}(S_x^4 + S_y^4 + S_z^4 - \tfrac{707}{16})] + g_0\beta\vec{H}\cdot\vec{S} \qquad (4)$$

improved the agreement between the EPR and Mössbauer data. The EPR spectra of enterobactin exhibit an isotropic signal at $g = 4.3$. The quadratic spin Hamiltonian (Eqn. 1), predicts $\lambda = 1/3$ and the ratio of the relative energies of the Kramers doublets, $E_{31}/E_{21} = 2$ (where $E_{ij} = E_i - E_j$).

Analysis of the Mössbauer spectra revealed $E_{31}/E_{21} = 1.55$. This seeming

TABLE II

EPR PARAMETERS FOR PROTOCATECHUATE 3,4-DIOXYGENASE AND ITS COMPLEXES

Enzyme complex	D (cm^{-1})	λ	μ
Native enzyme	1.6 *	0.28 *	
Enzyme-substrate	>0	0.02	
		0.12	
Enzyme-substrate-O_2	−2.0	0.03	
Enzyme-XII	−1.72	0.13	
	−1.66	0.11	0.16
Enzyme-XIII	−1.08	0.21	
	−1.00	0.13	0.28

* Ref. 6.

contradiction of experimental observations could be reconciled by choosing $\lambda = 0.46$ and $\mu = -0.27$ for Eqn. 4. Similarly, Debrunner and Schulz [16], in a Mössbauer analysis of ferric rubredoxin (which exhibits an EPR signal at $g = 4.3$) found $E_{31}/E_{21} = 3.3 \pm 0.5$. The quartic spin Hamiltonian parameters consistent with the data are $\lambda = 0.17 \pm 0.01$ and $\mu = 0.3 \pm 0.1$ [16].

The two enzyme inhibitor complexes provide a good opportunity to explore the utility of the quartic spin Hamiltonian (Eqn. 4). Because of the negative zero-field splitting and the particular λ-values, resolved signals from both upper and middle Kramers doublets can be observed allowing a reasonable determination of their relative energies to the ground state. For the E-XII complex, the quadratic spin Hamiltonian (Eqn. 1) predicts $E_{31}/E_{21} = 1.59$, while the best experimental fits show $E_{31}/E_{21} = 1.42 \pm 0.11$. Inclusion of a quartic term improves the agreement between theory and experiment. With $\mu = 0.16$ and $\lambda = 0.11$ in Eqn. 4, parameters which generate g values consistent with the observed EPR spectrum, $E_{31}/E_{21} = 1.43$ and a fit of both signals gives $D = -(1.66 \pm 0.07)$ cm^{-1}. For the E-XIII complex, a similar analysis yields $E_{31}/E_{21} = 1.38$ and $D = -(1.00 \pm 0.07)$ cm^{-1} with $\mu = 0.28$ and $\lambda = 0.13$, consistent with the experimental results. Although our evidence is not compelling enough to necessitate a quartic term in the spin Hamiltonian, it does point out the need for a more thorough investigation of ferric systems using EPR, Mössbauer and far-infrared spectroscopic techniques.

Let us consider the EPR information obtained so far on protocatechuate 3,4-dioxygenase and its complexes with substrates and inhibitors (Table II). Judged from the zero-field splitting parameters we find three situations. The native enzyme is characterized by $D = 1.6$ cm^{-1} and $\lambda = 0.28$. Enzyme · substrate complexes yield two species with λ-values around 0.02 and 0.12, respectively. Because of the complexity of the spectra we have not been able yet to obtain D values with reasonable accuracy; D, however, is positive *. Finally, inhibitor

* The EPR spectra of the complexes of protocatechuate 3,4-dioxygenase with various substrates are quite similar. The spectra, however, are very complex. In particular, they all show two major species and their relative concentrations do not seem to be dependent on the substrate concentration. Our assertion that the zero-field splitting parameter D is positive for both species follows from a series of Mössbauer studies performed in our laboratory. These studies show that the major resonances seen in the EPR spectra result from the ground state Kramers doublet.

complexes as well as the ternary oxy-complex [9] are characterized by negative values for D.

Some remarks are in order here regarding the coordinate frames used to express the zero-field splittings. We have described our data in a coordinate frame for which the z and the x axes are the ones of maximum and minimum ligand field distortion, respectively. In this frame, termed a "proper" frame by Blumberg [17], all possible eigenvalues and eigenfunctions can be obtained for $0 < \lambda < 1/3$, and D can take either positive or negative values. (Improper frames allow $\lambda > 1/3$.) Since we have studied samples with randomly oriented molecules, our measurements do not provide any information on the spatial correlation between the zero-field splitting tensor and some frame fixed to the molecule. From the wide range of λ values observed for the various enzyme complexes (see Table II), we suspect that the "proper" frames are indeed quite differently oriented. Thus, in protocatechuate 3,4-dioxygenase, we face a situation quite different from that in high-spin ferric hemes. In the latter compounds, the strength of the tetragonal ligand field provided by the pyrrole nitrogens causes the z-axis of the "proper" frame to coincide fairly well with the heme normal. No set of ligands in protocatechuate 3,4-dioxygenase appears to exert such a dominating influence on the iron ligand field.

Inspection of Table II shows that the magnitude of the zero-field splitting parameter D in the E-XIII complex is noticeable smaller than those for the native enzyme, the ternary oxy complex, and the E-XII complex. The data may suggest the following interpretation: NMR water relaxation experiments * indicate that water is ligated to the iron of the native enzyme. In the E-XII complex, the para hydroxy group of the inhibitor binds to the iron, displacing the water ligand. Similarly, in the ternary oxy complex, the intermediate peroxide (see Mechanstic implications) occupies the water coordination site. Thus, in all three states, this very site has an oxygenous ligand. 4-Fluoro-3-hydroxybenzoate (XIII) also displaces the water ligand. We suggest that the decreased zero-field splitting in the E-XIII complex reflects a loss of the water ligand, without the concomitant coordination of the 3-hydroxy group of the inhibitor to the iron.

Mechanistic implications

Various mechanisms for the enzyme action of dioxygenases have been postulated in the past few years. There have been suggestions that the active site ferric iron is reduced upon substrate binding, which then sets the stage for oxygen binding to the iron [7]. Hamilton [8], from a series of bond-energy calculations on the possible intermediates of the dioxygenase reaction, favors a peroxy-type (XVI) intermediate over a dioxetane (XVII).

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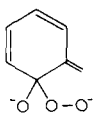
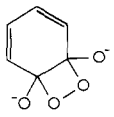
* We have performed NMR water relaxation experiments at 30 MHz on the native enzyme and its complexes with XII, XIII, and 4-nitrocatechol. The molar relaxivity of the native enzyme ($9.8 \cdot 10^3$ M^{-1} s^{-1} at 22°C) decreases by a factor of 3 upon binding of the inhibitors. These results compare favorably with those obtained for transferrin at 5 MHz. Koenig and Schillinger [18], from a comprehensive NMR study on transferrin, suggest that a water molecule is bound about 2 Å from the ferric ion. Since we have not yet performed NMR dispersion experiments, our conclusions are, at best, tentative.



XVI



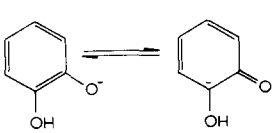
XVII

The dioxetane formation is considerably endothermic and its ring opening is so exothermic that analogous compounds in organic systems react with the emission of light. On the other hand, the formation of peroxide is thermodynamically more reasonable.

Our Mössbauer and EPR study on protocatechuate 3,4-dioxygenase [9] shows that the iron retains its high-spin ferric character throughout the enzymatic reaction cycle. This ferric center, however, is distinctly different in each of the three states. The iron in the native enzyme reflects a "rhombic" environment. Upon substrate binding, this environment becomes "axial", characterized by a positive zero-field splitting. Oxygen binding yields another "axial" environment but reverses the sign of the zero-field splitting. These observations preclude earlier suggestions that the iron is reduced when substrate binds [7]. How then does the oxygen bind? To date, molecular oxygen has been found to bind only high-spin ferrous iron (e.g. hemoglobin, hemerythrin, synthetic heme complexes). Though this does not necessarily exclude oxygen binding to the high-spin ferric iron in the enzyme-substrate complex, there is an alternative to this formulation which may be more reasonable, namely an initial attack of oxygen on the substrate.

The reaction of catechols with oxygen is well documented. The initial products of catechol autoxidation as observed by EPR spectroscopy are the corresponding semiquinone [19] and presumably superoxide. Kinetic studies of this reaction show that the autoxidation rate of catechol increases with the extent of dissociation, the dianionic catecholate having a greater reactivity towards oxygen [20]. Grinstead [21] has observed that in fairly alkaline solution (pH 12—14), the reaction of oxygen with 3,5-di-*t*-butylcatechol yielded ring cleavage products as well as the corresponding quinone.

Taking the Mössbauer, EPR, and inhibition kinetic data into consideration, we can propose a mechanism for the enzyme action of protocatechuate 3,4-dioxygenase (Fig. 5). As the substrate enters the active site, the carboxylate end is anchored by an ϵ-amino group of a lysyl residue. The iron binds the 4-OH group of the substrate and acts as an electrophile promoting electron migration toward C-4. This is, in effect, a keto-enol tautomerization of the catechol.



XVIII XIX

This reaction has been observed in 3,6-di-*t*-butyl-4-methoxycatechol at −50° C (Baldwin, J., private communication). Furthermore, the organic system reacts

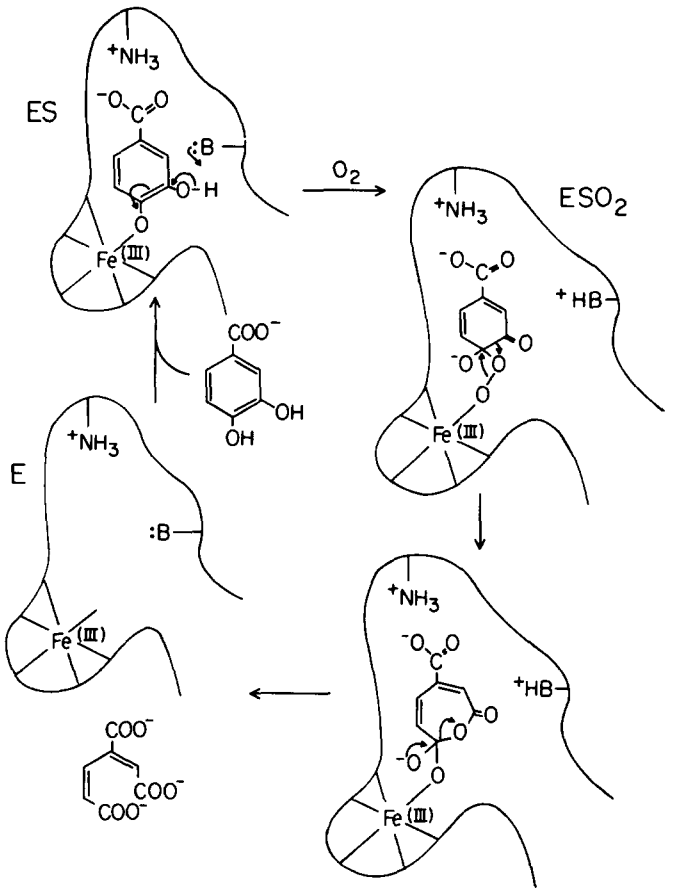
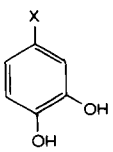


Fig. 5. Proposed mechanism of action for protocatechuate 3,4-dioxygenase. It should be noted that (a) the nature and the number of ligands that comprise the iron site are not known; (b) in the native enzyme, the vacant site may be occupied by a water molecule (see footnote p. 69); and (c) B is a hypothetical base to facilitate the ionization of the 3-OH group of the substrate.

TABLE III

RELATIVE RATES OF REACTION FOR SUBSTITUTED CATECHOLS



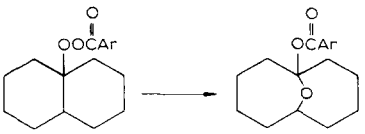
X—	Relative rate *	σ_p**
—$CH_2CH_2COO^-$	0.02	—0.13
—H	0.4	0
—COO^-	100	+0.13
—COOMe	0	+0.39
—CHO	0	+0.45
—NO_2	0	+0.78

* From refs. 3 and 5.
** From ref. 26.

with oxygen to yield ring fission products. Similarly, in the enzyme-catalyzed reaction, the substrate, thus activated into the ketoform (XIX) reacts with oxygen to form the semiquinone and superoxide. The superoxide binds to the iron and the two radicals couple to form the peroxy intermediate. This would then be the oxygenated intermediate [5,9] which gives rise to the transient optical spectrum (λ_{max} = 520 nm) and the EPR center with $D = -2$ cm^{-1}, $\lambda = 0.03$. Supportive evidence for a peroxide-iron complex is provided by comparing the optical spectrum of the ternary oxy-complex of the enzyme with hemerythrin. The latter protein, in its oxy form, also exhibits strong absorption, a charge-tranfer band, around 500 nm; resonance Raman and Mössbauer studies implicate a peroxide-iron (III) complex [22,23]. Furthermore, it is interesting to note that soybean lipoxygenase-1, a non-heme iron dioxygenase, exhibits a purple-colored species (λ_{max} = 570 nm, ϵ_M = 1000 M^{-1} cm^{-1}) in the presence of a three molar excess of 13-L-hydroperoxylinoleic acid, which is the product of the lipoxygenase reaction [24].

In the light of this mechanism, it is thus not surprising that the addition of metal ions to alkaline catechol solutions retarded the formation of ring fission products and promoted the formation of the corresponding quinones. This observation made by several workers (ref. 21 and Baldwin, J., private communication) reflects the tendency of metal ions to chelate onto the catechol in free solution and thereby reduce the concentration of the keto form. In the enzyme, the iron cannot bind to both hydroxy groups. Rather, it binds the 4-OH group, activates the substrate to react with O_2, and acts as a template to keep the initial products together to form the oxygenated intermediate.

The resulting peroxide adduct then rearranges to give an anhydride, which in turn opens up to yield the product *cis,cis*-muconate. Such rearrangements involving peroxides are known in organic chemistry, the best example being the rearrangement [25] of trans-decalin peresters (XX) into XXI.



XX XXI

This trans-decalin reaction is enhanced by electron-withdrawing groups in the aryl radical and retarded by electron donating groups.

Table II shows a series of catechols with a variety of substituents together with their Hammett σ_p constants [26] and their rates of reaction [3,5]. It is interesting to note that compounds with good electron withdrawing groups do not react at all and are inhibitors. The best substrate has an intermediate Hammett σ_p constant, just slightly electron withdrawing, and therefore, the reaction appears to be quite finely tuned. The oxygenation step requires electron donation to the oxygen while the rearrangement of the peroxide intermediate is enhanced by electron withdrawal. Thus, the nitro, the formyl, and the carbomethoxyl substituents are too electron-withdrawing to allow reaction with oxygen, while the carboxyethyl and unsubstituted catechols react to form

the peroxide intermediate. The latter substituents, however, are now too electron-donating and, consequently, stabilize the peroxide intermediate and retard the rate of reaction. The carboxyl substituted catechol is the only substrate with the appropriate electron balance to make effecient use of this enzyme.

Several spectroscopic experiments are suggested by the mechanism proposed here. Resonance Raman studies on the oxygenated intermediate using $^{16}O_2$ and $^{18}O_2$ should provide evidence for the peroxide complex. ENDOR studies and EPR experiments with ^{17}O-labelled compounds could further elucidate the nature of the iron coordination site. If this mechanism is borne out by further experiments, protocatechuate 3,4-dioxygenase provides a unique example of a metalloenzyme reaction where oxygen activation is preceded by metal activation of substrate.

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