Catecholato Complexes of *o*-Phenylenebis(salicylideneiminato)iron(III) and *meso*-Tetra(parasulphonatephenyl)porphyrinatoferrate(III). A Comment on the Structure of the Active Site in Catechol-1,2-dioxygenase

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Abstract

Formation of catecholato complexes of Fe(saloph)$^{+}$ and Fe(TPPS)$^{3-}$ in solution is studied. Fe(saloph)(cat)$^{-}$ contains a cat^{2-} bidentate ligand. Its formation in solution competes efficiently with the hydrolysis and dimerization of Fe(saloph)$^{+}$ to give $Fe_2(saloph)_2O$. This behaviour shows that the planar saloph^{2-} ligand, as the analogous salen^{2-}, is easily distorted, and is not as rigid as generally considered. Iron(III) porphyrin Fe(TPPS)$^{3-}$ with catechol gives the complex [Fe(TPPS)(Hcat)]$^{4-}$. Deprotonation of the unidentate Hcat^{-} ligand cannot be studied because the smaller stability of the complex, and the dimerization of the metalloporphyrin dominates in basic medium. The strong tendency of the cat^{2-} anion to be coordinated to Fe(III) in chelate form only can be sterically hindered. On the basis of these results the suggested structure of the active site of catechol-1,2-dioxygenase is discussed.

Introduction

Catechol-1,2-dioxygenase [1] is a non-heme iron protein which catalyzes catechol (benzene-1,2-diol) oxidation by dioxygen to give *cis,cis*-muconic acid. The two oxygen atoms are inserted in the oxidized substrate as in other reactions which are catalyzed by dioxygenases [2]. The nature of the active site of this metalloprotein, which contains a high-spin iron(III) ion, has been exhaustively investigated in the last years by different spectroscopic techniques such as ESR [3], Raman [4], EXAFS [5], and the near edge X-ray absorption [6]. As a result of these studies it is thought that the metal ion is linked to two tyrosinate ligands, two histidines, and a water molecule, having a square pyramidal structure. Moreover, an unknown weaker ligand is linked to Fe(III), in *trans* position to a tyrosine group, yielding a highly distorted octahedral structure. This environment is similar to those observed for other iron(III)–tyrosinate proteins [7].

The studies with model compounds such as Fe(salen)$^{+}$, salen = ethylenebis(salicylideneimine), and other iron(III) complexes with tetradentate Schiff-bases, have significantly contributed to the elucidation of the nature of the active site of these metalloproteins [1, 8, 9].

Catechol-1,2-dioxygenase forms with catechol an enzyme-substrate complex which contains a unidentate cat^{2-} ligand which is bound to the metal ion by only one of its phenolate oxygens [1]. It is thought that this kind of coordination, which is unknown in simple metal complexes, activates the ligand towards the dioxygen. Although catecholate bridged ligands in metal complexes are known [10], cat^{2-} ligand is usually and almost exclusively coordinated to transition metal ions as a bidentate ligand, *i.e.* in the complex $[Fe(cat)_3]^{3-}$ [11].

Recently, the complex [Fe(saloph)(Hcat)], saloph = *o*-phenylenebis(salicylideneimine), which contains a unidentate Hcat^{-} ligand, has been isolated and characterized [12]. However, deprotonation of the compound [Fe(salen)(Hcat)], which would have the same structure as [Fe(saloph)(Hcat)], yields the compound K[Fe(salen)(cat)], whose structure reveals the presence of a bidentate catecholate ligand [13]. A study of the interaction of Fe(salen)$^{+}$ with catechol in solution shows that the generally planar salen^{2-} ligand is easily distorted enabling the coordination of a bidentate ligand. Although distorted salen^{2-} ligand is a poorer ligand than when it has its usual planar geometry, the stability loss is largely compensated by the binding of cat^{2-} to Fe(III) in the chelate form [14]. At this point it is interesting to study the catechol interaction with complexes

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of iron(III) and tetradentate ligands having a higher rigidity than salen^{2-}. In this work we investigate the formation of catecholate complexes of Fe(saloph)$^{+}$ and Fe(TPPS)$^{3-}$, TPPS^{6-} = tetra(*para*-sulphonatephenyl)porphyrinate.

Experimental

Reagents and Solvent

Fe(saloph)Cl [15] and Na_3[Fe(TPPS)] [16] were synthesized and purified as reported in the literature. Catechol (Merck, synthesis grade) was recrystallized by sublimation before use. Because of catechol sensitivity to oxidation in basic medium, O_2 traces in the N_2 used as inert atmosphere were carefully eliminated. Dimethylsulphoxide (DMSO) was purified by distillation under reduced pressure and stored in a dark bottle over 4 Å molecular sieve. From this solvent the DMSO–H_2O (80:20 *w/w*) mixture was obtained. Carbonate-free potassium hydroxide (0.1 mol dm^{-3}) and perchloric acid (0.1 mol dm^{-3}) solutions were used in the potentiometric and spectrophotometric studies. All the other chemicals were Merck reagents.

Physical Techniques

Potential measurements, in DMSO–H_2O mixture, were performed with a Radiometer 84 pH-meter using a GK-2401C combined glass electrode, at 25.0 ± 0.1 °C and in 0.1 mol dm^{-3} $KClO_4$. Electrode calibration and the experimental procedure were described previously [17]. Absorption spectra were recorded with a UV–Vis Pye Unicam SP 100–8 spectrophotometer using 0.1 cm length cuvettes. The diffuse reflectance spectra were measured using magnesium oxide as the reference. Conductivity measurements were performed with a Radiometer CDM3 conductimeter. The magnetic susceptibility measurements were made by the Evans NMR method [18]. We used the paramagnetic shift of the methyl protons of t-butanol which was previously added to the solutions.

Synthesis of [Fe(saloph)]$_2$O

20 ml of 0.1 mol dm^{-3} KOH aqueous solution were added with continuous stirring to 0.8 g of the complex Fe(saloph)Cl dissolved in 40 ml of DMSO. The yellow crystalline precipitate was filtered off and washed with a few ml of DMSO–H_2O (80:20) mixture and then exhaustively with acetone and ether. The crystals were oven-dried at 70 °C and stored in a $CaCl_2$ desiccator. The yield was almost quantitative (>90%). *Anal.* Found: C, 63.3; Fe, 14.7; N, 7.4; H, 3.8. Calc. for $C_{40}H_{28}Fe_2N_4O_5$, C, 63.5; Fe, 14.8; N, 7.4; H, 3.7%.

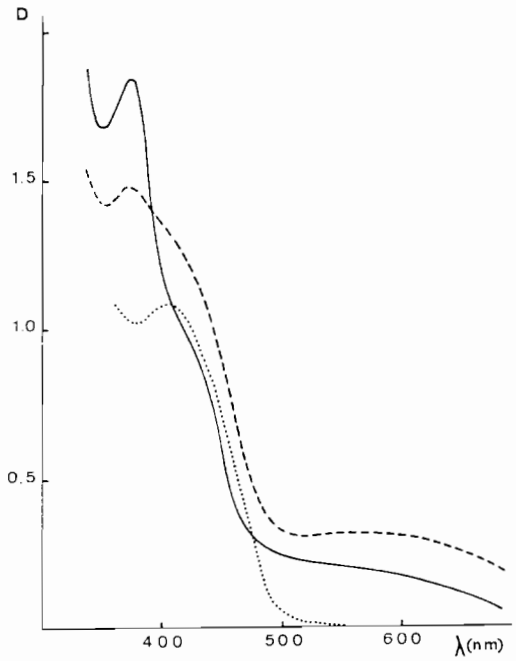

Fig. 1. UV–Vis absorption spectra of Fe(saloph)$^{+}$ (——), [Fe(saloph)(cat)]$^{-}$ (-----), and [Fe(saloph)]$_2$O (·····) in DMSO–H_2O (80:20) solution with $C_M = 10^{-4}$ mol dm^{-3}. The spectra were recorded with 1 cm cells.

Results

(1) Solutions of Fe(saloph)Cl in DMSO–H_2O. Formation Equilibrium of μ-oxo-bis[N,N'-o-phenylenebis(salicylideneiminato)iron(III)]

The compound Fe(saloph)Cl dissolves easily in DMSO–H_2O (80:20) yielding reddish brown solutions. Conductivity measurements show that the chloride ligand is uncoordinated, so the existing species in solution must be the complex cation Fe(saloph)$^{+}$. The UV–Vis absorption spectra display two absorption bands at 380 nm (ϵ *ca.* 1.6×10^4 dm^3 mol^{-1} cm^{-1}) and 520–550 nm (ϵ *ca.* 1.7×10^3 dm^3 mol^{-1} cm^{-1}) (Fig. 1). These solutions are stable and do not undergo any decomposition by addition of strong acids. Therefore, the absorption spectra of one of these solutions to which an excess of perchloric acid has been added, do not show any change after 24 h. Moreover, when KOH is added a colour change from reddish brown to yellow occurs. The absorption spectra of a series of solutions in which the Fe(saloph)Cl concentration was kept constant and the concentration of KOH was varied, displayed an isosbestic point at 486 nm which indicated two absorbing species in solution. Reaction was complete when 1 mol of OH^- for each mol of Fe(saloph)$^{+}$ had been added, and the absorption spectrum is identical to the reflectance spectrum of the solid [Fe(saloph)]$_2$O. The magnetic moment of the Fe(saloph)$^{+}$ solutions is 5.9 μ_B, but it is substantially

decreased by addition of KOH. All these data enable us to conclude that the reaction in eqn. (1) occurs.

$$2\text{Fe(saloph)}^{+} + \text{H}_2\text{O} \overset{K_D}{\rightleftharpoons} [\text{Fe(saloph)}]_2\text{O} + 2\text{H}^{+} \quad (1)$$

The μ-oxo dimer precipitates if the initial concentration of the monomer is higher than 5×10^{-4} mol dm^{-3}. In order to determine the value of the dimerization constant, K_D (eqn. (1)) Fe(saloph)$^{+}$ solutions were titrated potentiometrically with KOH. The initial concentration, C_M, was varied in the range 3×10^{-4}–10^{-3} mol dm^{-3}. Precipitation of the μ-oxo dimer compound occurred for solutions with $C_M > 5 \times 10^{-4}$ mol dm^{-3}. Consequently, potentiometric titrations were stopped when the precipitate appeared. Potentiometric curves displayed a steep inflexion for $C_B/C_M = 1$ ratio, in agreement with the spectrophotometric data.

The value of K_D was determined using the previously reported graphical method [14] being also computed by the program Miniquad [19]. The same value, $K_D = (1.50 \pm 0.02) \times 10^{-12}$ (25 °C, 0.1 mol dm^{-3} $KClO_4$), was obtained by both procedures. In this calculation 90 experimental points from five different titrations were used. The dimerization reaction was almost complete for pH ≥ 9.5 in the concentration range studied (see Fig. 2).

(2) Interaction of Fe(saloph)$^{+}$ with Catechol. Stability Constant of the Complex [Fe(saloph)(cat)]$^{-}$

Solutions of Fe(saloph)$^{+}$ of variable concentration, C_M = *ca.* 10^{-3}–5×10^{-3} mol dm^{-3}, with an excess of catechol, C_L/C_M = *ca.* 5–20, were titrated with KOH. The potentiometric curves obtained showed that 2 mol of OH^{-} were spent for each mol of Fe(saloph)$^{+}$, according to the following equation,

$$\text{Fe(saloph)}^{+} + \text{H}_2\text{cat} \rightleftharpoons [\text{Fe(saloph)(cat)}]^{-} + 2\text{H}^{+} \quad (2)$$

The absorption spectra of the green solutions of the catecholate complex display absorption bands at 380 nm (ϵ *ca.* 1.4×10^4 dm^3 mol^{-1} cm^{-1}) and 570 nm (ϵ *ca.* 2.8×10^3 dm^3 mol^{-1} cm^{-1}) (see Fig. 1). Measurements of μ_{eff} of the solutions over the whole titration process indicate first a progressive decreasing value, increasing later to reach at the end point the normal value, *ca.* 5.9 μ_B, for a high-spin Fe(III). These data show that, the dimerization process occurs in the formation of the catecholate complex (eqn. (1)). The UV–Vis absorption spectra registered throughout the titration process also reveal the transitory formation of the dimer $[\text{Fe(saloph)}]_2\text{O}$. The formation degree of the μ-oxo complex is very dependent on the C_L/C_M ratio value. Thus, when solutions containing a large excess of catechol are used, the concentration of the dimer becomes negligible for the whole pH range for which the formation of the catecholate complex occurs. However, the μ-oxo complex precipitates if the C_L/C_M ratio value is small. From potentiometric data and K_D value, we obtain the formation curve, $\bar{n}$ *versus* $-\log[\text{cat}^{2-}]$, by using previously reported eqns. [14]. The value of the stability constant, K, was obtained from this formation curve by means of the usual graphical methods. In addition, we have simultaneously determined the K_D and K values by electronic computation using the program Miniquad. The same results were obtained by both methods, $K = (1.09 \pm 0.02) \times 10^{14}$ (25 °C, 0.1 mol dm^{-3} $KClO_4$). In the calculation 120 experimental points from six different experiments were used. The theoretical curve fits the experimental data well (see Fig. 3). Figure 4 shows the distribution

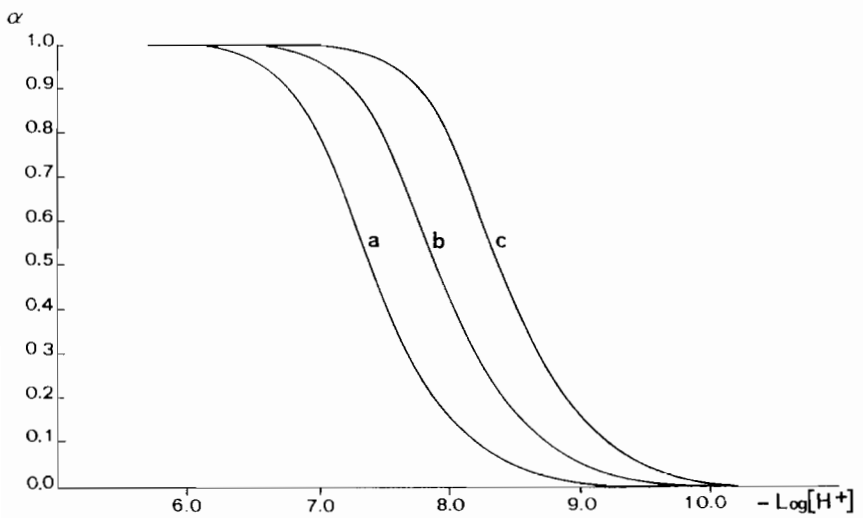

Fig. 2. Monomer fraction in a Fe(saloph)$^{+}$ solution (α) as a function of pH for three total concentration values: $C_M = 10^{-3}$ (a), 10^{-4} (b), and 10^{-5} mol dm^{-3} (c).

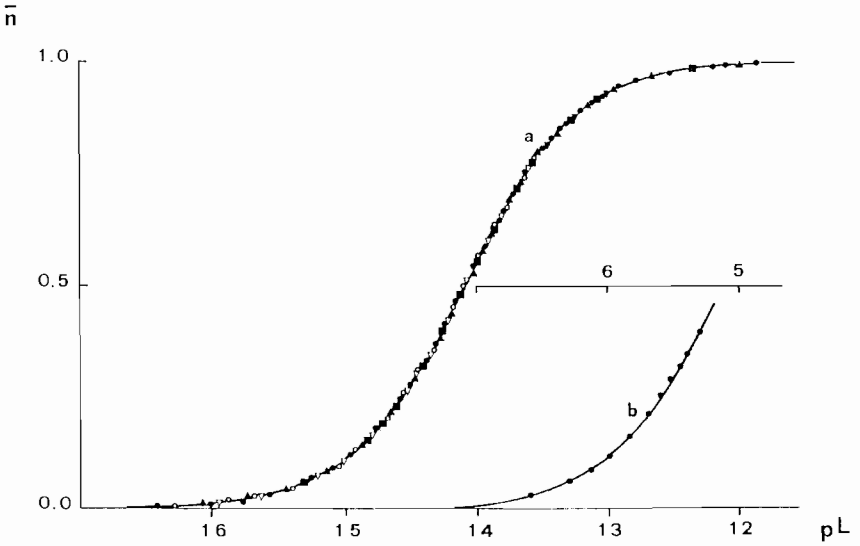

Fig. 3. (a) Formation curve of Fe(saloph)(cat)$^-$: $C_M = 10^{-3}$, $C_L = 5 \times 10^{-3}$ (○); $C_M = 2 \times 10^{-3}$; $C_L = 2 \times 10^{-2}$ (△); $C_M = 10^{-3}$, $C_L = 2 \times 10^{-2}$ (■); $C_M = 10^{-3}$, $C_L = 10^{-2}$ (▲); $C_M = 5 \times 10^{-3}$, $C_L = 10^{-1}$ mol dm^{-3} (●). (b) Formation curve of Fe(TPPS)-(Hcat)$^{4-}$: $C_M = 3.13 \times 10^{-5}$ mol dm^{-3}. Top scale is for the curve (b).

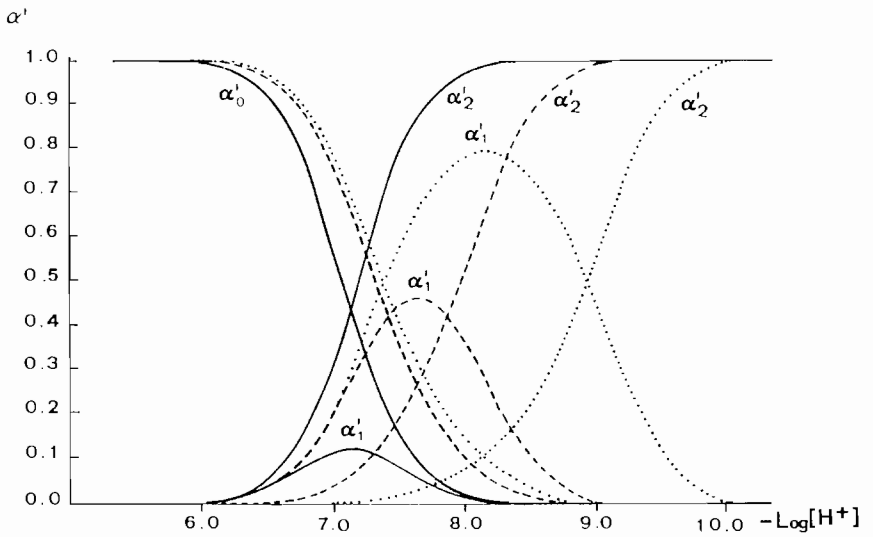

Fig. 4. Distribution diagram (α' *vs.* pH) for the system Fe(saloph)$^+$ –cat^{2-}: α'_0 = Fe(saloph)$^+$, α'_1 = [Fe(saloph)]$_2$O, α'_2 = [Fe-(saloph)(cat)]$^-$ (α' is the molar fraction of metal ion). Concentrations $C_M = 10^{-3}$ mol dm^{-3} and $C_L = 10^{-1}$ (——), 10^{-2} (- - - - -), 10^{-3} mol dm^{-3} (· · · · ·).

diagram of the existing species in solution for several C_L/C_M ratio values.

(3) Protonation of o-phenylenediamine in DMSO–H_2O (80:20 w/w)

o-phenylenediamine solutions were titrated with $HClO_4$ by potentiometry. The experimental data show that this aromatic diamine is not easily protonated, so at pH *ca.* 2, the average number of protons bound to the diamine, $\bar{j}$, is smaller than 0.3. The experimental data fit only one protonation equilibrium with β_{j1} ca. 40.

(4) Interaction of Fe(TPPS)$^{3-}$ with Catechol in Aqueous Solution. Stability Constant of the [Fe-(TPPS)(Hcat)]$^{4-}$ Complex

We have spectrophotometrically studied the interaction between the metalloporphyrin, Fe(TPPS)$^{3-}$, and the catechol ligand. We could not carry out a potentiometric study similar to the preceding one because in this case the dimerization equilibrium to yield $\{[Fe(TPPS)]_2O\}^{8-}$ predominates over the catecholate complex formation. This fact shows that the stability of the catecholate com-

plex of this metalloporphyrin is smaller than the one of $Fe(saloph)^+$.

We recorded the UV-Vis absorption spectra of a series of solutions at pH = 4.8 (NaAc, 0.1 mol dm^{-3}// HAc) keeping constant the $Fe(TPPS)^{3-}$ concentration, C_M = *ca.* 3×10^{-5} mol dm^{-3}, and varying the catechol concentration, C_L, in the range $C_L/C_M = 2 \times 10^3$–8×10^3. At this pH value, the metalloporphyrin exists in monomeric form, but a very large excess of catechol is required to form the catecholate complex. A colour change from reddish brown to green–yellow occurs by adding catechol to the $Fe(TPPS)^{3-}$ solutions. For this spectrophotometric study we have chosen the Soret band which is shifted from 393 to 412 nm exhibiting also a decrease of its extinction molar coefficient (see Fig. 5). The application of the Asmus method and Generalized Asmus method [20] shows the formation of only one 1:1 monomeric complex (see Fig. 6).

Although the protonation state of the coordinated catecholate ligand is not inferred from the spectrophotometric study at a constant pH value, the relative low stability of the mentioned complex enables us to conclude that the catechol ligand must be a unidentate $Hcat^-$ anion. By application of the Generalized Asmus method we determine the value of the stability constant, $K = (1.40 \pm 0.07) \times 10^5$, and the molar extinction coefficient at the wavelength of the study. From the value of the molar extinction coefficient we can obtain the formation curve of the system, ($\bar{n}$, $-\log[Hcat^-]$) which shows that the complex formation is not yet total ($\bar{n}_{max}$ (*ca.* 0.4) in spite of the very large excess of ligand in the solutions. The value of the stability constant obtained from the formation curve is identical to the one found by the Asmus method (see Fig. 3).

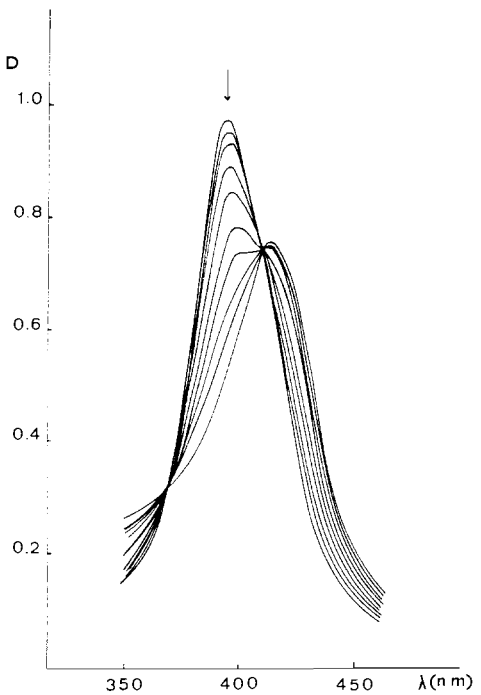

Fig. 5. Visible absorption spectra of $Fe(TPPS)^{3-}$–$Hcat^-$. C_M = 3.13×10^{-5} mol dm^{-3} and different C_L:C_M ratios. (pH = 4.8).

Discussion

$Fe(saloph)^+$ is insoluble in water but easily soluble in the DMSO–H_2O (80:20 *w/w*) mixture to yield the cationic complex $Fe(saloph)^+$; a solvent molecule replaces the halide ion in the axial position. The μ oxo dimer, $[Fe(saloph)]_2O$, is formed by adding stoichiometric quantities of KOH. This same behaviour has previously been reported for the complex $Fe(salen)^+$ [14]. The equilibrium constant of the dimerization reaction is somewhat greater for $Fe(saloph)^+$ (see Table I) pointing out that the electron density donation to the metal ion decreases from salen to saloph. The tendency toward hydrolysis and dimerization is higher for the metalloporphyrin, $Fe(TPPS)^{3-}$ [21]. The solubility of the dimer $[Fe(saloph)]_2O$ in DMSO–H_2O (80:20) is smaller than the one with salen, precipitating if the initial concentration of the monomer is higher than 5×10^{-4} mol dm^{-3}. This fact provides a simpler and better procedure than the previously reported one [22] for isolating the μ-oxo dimer with a high purity grade.

$Fe(saloph)^+$ is completely stable in acid medium and does not undergo any decomposition. This behaviour is very different to the observed one for the complex $Fe(salen)^+$ which is quantitatively decomposed by hydrolysis to give salicylaldehyde and ethylenediammonium [17]. Salen is not hydrolyzed in the DMSO–H_2O mixture, the protonation of the diamine in acid medium being the determining factor of its spontaneous decomposition [17]. Therefore the stability of the complex $Fe(saloph)^+$ in acid

TABLE I. Formation Constants of μ-oxo Complexes at 25 °C

Compound	Medium	log K_D	References
$Fe(salen)^+$	DMSO–H_2O (80:20) ($KClO_4$ 0.1 mol dm^{-3})	−12.30	[14]
$Fe(saloph)^+$	DMSO–H_2O (80:20) ($KClO_4$ 0.1 mol dm^{-3})	−11.82	this work
$Fe(TPPS)^{3-}$	H_2O (KNO_3 0.1 mol dm^{-3})	−7.46	[21]

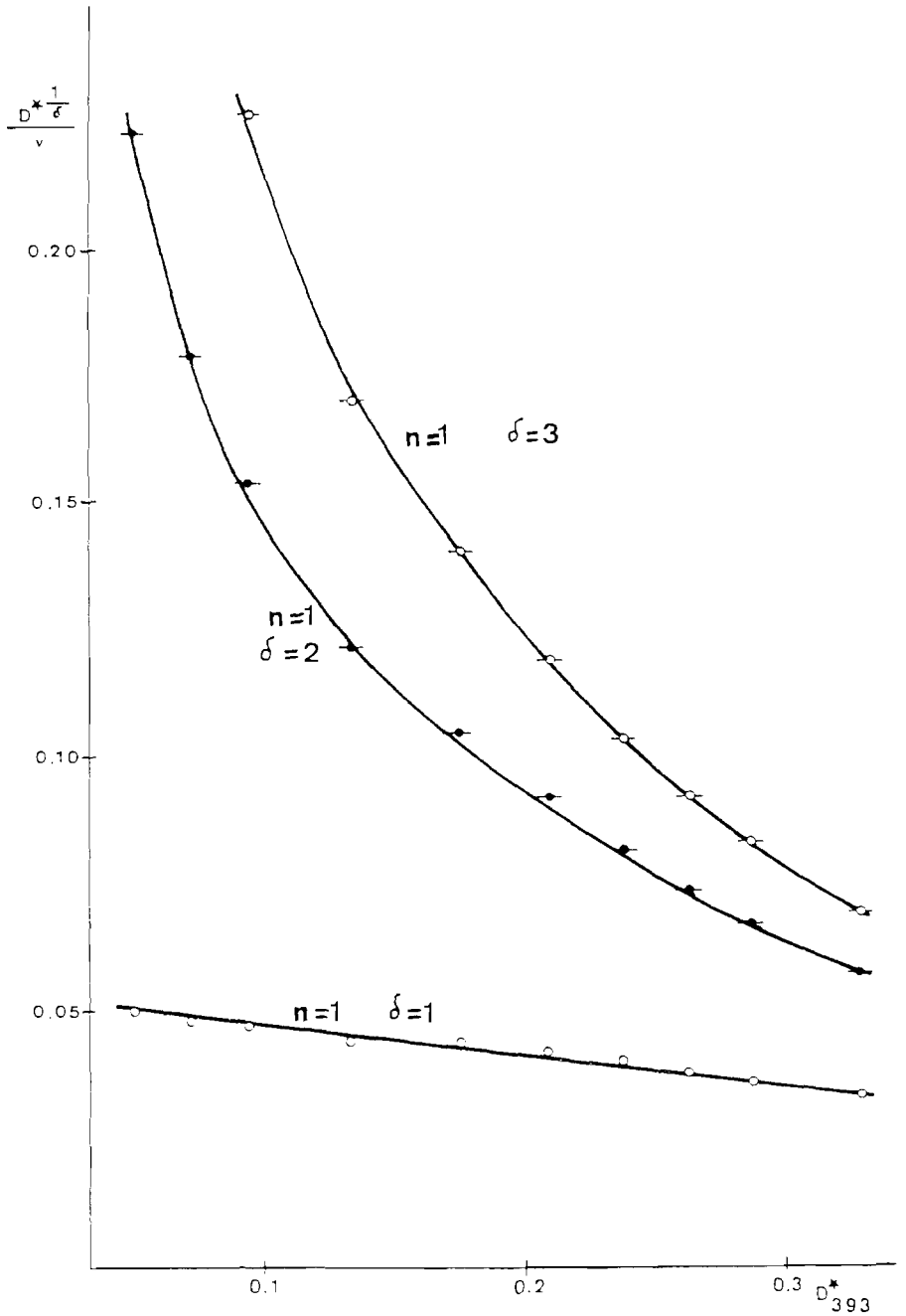

Fig. 6. Generalized Asmus plot for the system $Fe(TPPS)^{3-}$–H_2cat. Absorbance values at 393 nm. A straight line is only obtained for $n = 1$ and $\delta = 1$.

media is attributed to the smaller basis character of *o*-phenylenediamine which remains unprotonated in the media where ethylenediamine is completely protonated (see Table II).

$Fe(saloph)^+$ reacts with catechol in solution to form $[Fe(saloph)(cat)]^-$. According to Fig. 4, the formation of the catecholate complex competes efficiently with the dimerization reaction. Since

TABLE II. Protonation Constants in DMSO–H_2O (80:20) (I = 0.1 mol dm^{-3}, 25 °C)

Compound	K_1	K_2	References
Ethylenediamine	1.0×10^{10}	9.1×10^{16}	[17]
o-phenylenediamine	*ca.* 40		this work

TABLE III. Stability Constants of Catecholate Complexes at 25 °C

Compound	Medium	K	References
$[Fe(salen)(cat)]^-$	DMSO–H_2O (80:20) ($KClO_4$ 0.1 mol dm^{-3})	4.8×10^{14}	[14]
$[Fe(saloph)(cat)]^-$	DMSO–H_2O (80:20) ($KClO_4$ 0.1 mol dm^{-3})	1.1×10^{14}	this work
$[Fe(TPPS)(Hcat)]^{4-}$	H_2O (KNO_3 0.1 mol dm^{-3})	1.4×10^5	this work

the value of its stability constant is close to the previously determined one for the analogous complex $[Fe(salen)(cat)]^-$ (see Table III), we can conclude that cat^{2-} is coordinated to the metal ion as a bidentate ligand [7, 14]. This fact involves a considerable distortion on the $saloph^{2-}$ ligand giving a *cis*-octahedral conformation which allows the coordination of a bidentate ligand. At first sight, this result may be rather surprising. Although the coordination chemistry of 'saloph' has been less investigated than that of the 'salen', it is generally thought that the former ligand is much more rigid than the latter, and it has been suggested that this well known kind of distortion is not possible for 'saloph' [8]. Our results show that the $saloph^{2-}$ ligand is rather less rigid than it was thought, and that at least its behaviour towards catechol is very similar to that reported earlier for the salen complex. The somewhat reduced value of the stability constant of the $[Fe(saloph)(cat)]^-$ complex may reveal a higher energetic cost for the saloph distortion. On the other hand, the formation and dissociation equilibrium is instantaneously reached as well as for $Fe(salen)^+$. This fact indicates that there is no significant kinetic hindrance at room temperature.

The potentiometric and spectrophotometric data show that the formation of the protonated species, [Fe(saloph)(Hcat)], which has been isolated by Que in acetonitrile [23], does not exist in our solutions. The stability of this species is very dependent upon solvent polarity; therefore it can be stabilized in less polar solvents which do not stabilize ionic species. In our solvent mixture DMSO–H_2O it would undergo quantitative disproportion to yield $[Fe(saloph)(cat)]^-$ and H_2cat, due to the greater stability of the chelate complex.

$$2\,[Fe(saloph)(Hcat)] \rightleftharpoons [Fe(saloph)(cat)]^- + Fe(saloph)^+ + H_2cat \qquad (3)$$

$Fe(TPPS)^{3-}$ reacts with catechol in aqueous solution to give $[Fe(TPPS)(Hcat)]^{4-}$ which contains a unidentate ligand, $Hcat^-$. The stability constant of this complex is roughly of the same order of magnitude as that expected for a unidentate phenolic ligand. In this case the peculiar bonding mode of the catecholate ligand is determined by the rigidity of the tetrapyrrolic macrocycle of the porphyrin which cannot be distorted as $salen^{2-}$ and $saloph^{2-}$ ligands. At the pH of this study, the noncoordinated phenolic group remains protonated. Unfortunately it is not possible to study its deprotonation, which would give a unidentate cat^{2-} ligand, because the formation of the μ-oxo metalloporphyrin dimer predominates in basic medium, and the less stable catecholate complex is dissociated. It would be very interesting to study the interaction of catechol with the iron complexes of both-faces hindered porphyrins such as basket-handle porphyrins [24] which are unable to form μ-oxo dimers.

We can conclude that although it is possible to stabilize a unidentate $Hcat^-$, the catecholate dianion cat^{2-} will be coordinated as bidentate ligand whenever two contiguous coordination positions are available on the metal ion.

Taking into account our results it is apparent that the proposed reaction mechanism for catechol 1,2-dioxygenase does not agree well with the suggested structure for the active site. In brief, the reaction mechanism involves the coordination of cat^{2-} as a unidentate ligand which would react quickly with O_2 to give superoxide anion, O_2^-. This latter would be coordinated to iron(III) undergoing a subsequent reaction with the coordinated semiquinone to yield *cis,cis*-muconic acid [1].

On the other hand, if the suggested structure for the active site of catechol 1,2-dioxygenase is correct, the metal ion has two coordination positions easily accessible, the positions occupied by the water molecule and by the unknown ligand X. This latter, shown by near edge X-ray absorption spectroscopy [6], has been described as a more weakly bound or more easily dissociable ligand.

In these circumstances the occurrence of a unidentate catecholate ligand in the enzyme-substrate complex cannot be understood. We think that iron(III) can indeed be pentacoordinated in the metalloprotein, with a square pyramidal structure, and that the X group is not close enough to iron(III) to be considered a ligand, remaining not far from the metal ion but uncoordinated. Its role could be to block the access to the vacant axial position, hindering the coordination of cat^{2-} as a chelate ligand even allowing the binding of the superoxide anion O_2^- (see Fig. 7).

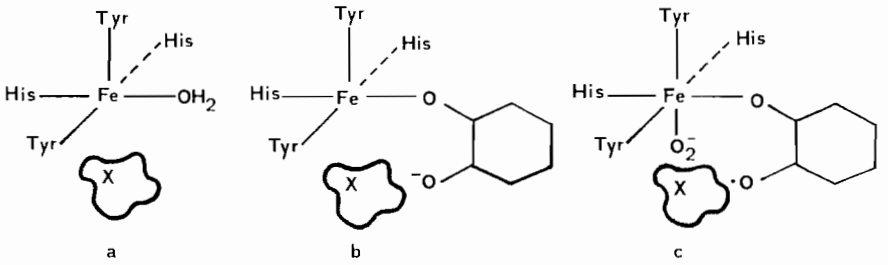

Fig. 7. A possible role for the unknown X group in the reaction mechanism of catechol-1,2-dioxygenase. (a) Structure of active site: Fe(III) is pentacoordinated (C_{4v}) and X is a distal group uncoordinated to the metal ion. (b) Enzyme–substrate complex: The anion cat^{2-} binds Fe(III) as an unidentate ligand. The steric bulk of X does not allow the preferred chelate form. (c) However X does not hinder the coordination of superoxide anion, O_2^-, formed by a fast electron transfer reaction between O_2 and the coordinated cat^{2-}.

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