4-Nitrocatechol as a Colorimetric Probe for Non-heme Iron Dioxygenases

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SUMMARY

4-Nitrocatechol is examined as an active site probe for non-heme iron dioxygenases and found to be of value, particularly with those containing iron in the Fe(III) oxidation state. 4-Nitrocatechol is a strong competitive inhibitor of substrate oxygenation by protocatechuate 3,4-dioxygenase, forming a reversible complex with this enzyme, and by pyrocatechase. The number of binding sites per enzyme molecule titrated spectrophotometrically with 4-nitrocatechol agrees with results from previous studies with either the principal substrate or other analogues, as expected of an effective probe.

Despite these facts and the observation that both enzymes cleave the same substrates at the same carbon-carbon bond, the optical and electron paramagnetic resonance (EPR) spectra of their 4-nitrocatechol complexes are remarkably different. The 4-nitrocatechol-protocatechuate 3,4-dioxygenase optical spectra resemble that of the 4-nitrocatecholate ion shifted 20 to 30 nm to longer wavelength. Concomitant with this change the EPR signal centered at $g = 4.28$ shows increased rhombicity ($g$ values at 4.74, 4.28, and 3.74). In contrast, the spectrum of the 4-nitrocatechol-pyrocatechase complex has a maximum at the same wavelength as that of a 1:1 solution of free Fe(III) and 4-nitrocatechol in the absence of enzyme after titration of the catecholic protons with base and the $g = 4.28$ EPR signal is not resolved at liquid N$_2$ temperature.

These changes are interpreted as resulting in part from a pronounced change in the ligand fields about the iron at the active sites which in the case of protocatechuate 3,4-dioxygenase leads to enzyme inactivation. The results also are the first indication that substrate analogues change their ionization form upon complexation with Fe(III) dioxygenases.

The interaction of the probe with metapyrocatechase, an Fe(II) containing dioxygenase, and with several additional oxygenases and hydroperoxidases is also briefly examined. The probe is not specific for any particular class of non-heme iron dioxygenases.

No reliable chemical reagent exists for detecting kinetic and structural differences in the active sites of non-heme Fe(III) dioxygenases. 3,5-Disulfocatechol, or Tiron, a commonly used Fe(III) reagent and substrate analogue of these dioxygenases, is ineffective as an inhibitor of pyrocatechase and of protocatechuate 3,4-dioxygenase (1-4). Both are Fe(III)-containing enzymes and cleave pyrocatechol rings in an intradiol manner. Protocatechualdehyde and some other pyrocatechol analogues produce minor perturbations of the $d ightarrow d^*$ transition associated with iron in the Fe(III) oxidation state (5). These changes are not sufficiently dramatic to permit use of the analogues as colorimetric agents for Fe(III).

The notion that such a probe might exist was predicated on the proposition that Fe(III) and the catechol substrate form a chelate at the active site prior to activation. Pronounced and predictable changes in the substrate spectrum should result if this occurs (6). Because 4-nitrocatechol (4.NC) has strong color in neutral solutions, more intense than the dioxygenases, has a lower degree of steric hindrance and is stable toward O$_2$ in mildly alkaline solutions, it was a logical candidate (7). Upon combination with protocatechuate 3,4-dioxygenase in stoichiometric amounts, a pronounced change in the 4.NC optical spectrum was in fact observed. In addition, the EPR spectrum of protocatechuate 3,4-dioxygenase was markedly altered. These findings prompted a more extensive investigation of the interaction of this probe with other O$_2$ catabolizing enzymes. The phenomenology of these interactions and possible interpretations of the results with non-heme iron dioxygenases form the basis of this report.

EXPERIMENTAL PROCEDURE

Chemicals—4-Nitrocatechol was a product of Aldrich Chemical Co., recrystallized once from water. Guanidine hydrochloride was supplied by Nakarai Chemical and was pure by spectrophotometric examination and by titration with NaOH (8). Flavin adenine dinucleotide was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. All other chemicals were reagent grade from Fisher Scientific.

Enzyme Preparations—Protocatechuate 3,4-dioxygenase (65 units per mg), pyrocatechase (20 units per mg), metapyrocatechase (100 units per mg), and salicylate hydroxylase (A$_{400}$ = 0.15 per min-mg) were prepared as described (9-12). Catalase and lipoxidase (6000 units per mg) were obtained from Sigma Chemical Co. (13-14). Tryptophan pyrrolase (6 units per mg) was donated by Dr. Y. Ishimura and H. Nakano, Kyoto University (15); lysine monoxygenase (10 units per mg) by Dr. T.

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Nakazawa, Kyoto University (16); horseradish peroxidase by Dr. I. Yamazaki, Hokkaido University (15); bacterial P-450 by Dr. I. C. Gunsalus, Illinois University (17), and chloroperoxidase by Dr. L. P. Hager, Illinois University (18).

The rate of the horseradish peroxidase-catalyzed oxidation was followed at 430 nm for 4-NC and at 343 nm for protocatechualdehyde. Experimental concentrations after mixing were as follows: enzyme, 5.6 \mu M; \text{H}_2\text{O}_2, 22 \text{mM}; catecholic substrate, 0.34, 1.02, or 1.62 \mu M in a total volume of 9.0 ml; all other conditions being standard (19). Other assay procedures were followed as previously described (9-12, 14-18).

RESULTS

Non-heme Fe(III) Dioxygenases: Protocatechuate 3,4-Dioxygenase; Optical Studies—Protocatechuate 3,4-dioxygenase has a molecular weight of 700,000, has 8 subunits and 7 iron atoms per molecule, and appears under the electron microscope as spheres arranged in an octahedron (4, 5, 19). The iron atoms are in the Fe(III) oxidation state and are probably bound to sulfur atoms in tetrahedral configurations at the active sites (20).

Upon mixing 4-NC and the oxygenase in either the presence or absence of air, an entirely new spectrum with peaks at 408 and 550 nm appeared (Fig. 1). A progressive titration of 4-NC with a concentrated oxygenase solution (also Fig. 1) had an isosbestic point at 465 nm up to a fairly sharp end point at 1.0 mol of protein for 7.9 mol of 4-NC. This end point agrees fairly well with that derived from a previous titration of the oxygenase with protocatechualdehyde, a competitive inhibitor of protocatechue acid oxygenation (5, 21). Spectral experiments in which the addition of 10 mM protocatechue acid to a solution containing the 4-NC-oxygenase complex substantially restored the 4-NC monoanion peak at 432 nm showed that the complex was reversible.

4-Nitrocatechol strongly inhibited the rate but not the total amount of O_2 consumed by protocatechue acid in a regular assay mixture. The inhibition was competitive with a K_i of 0.3 \mu M, an order of magnitude lower than that for any other analogue (4). When protocatechuate 3,4-dioxygenase was preincubated with stoichiometric amounts of 4-NC for 10 min and an aliquot added to a standard assay solution, the O_2 uptake rate exhibited a short induction period during which time the rate rose to the same level as that in a control solution containing the same concentrations of the oxygenase and 4-NC added separately at the start. This experiment shows that dissociation of the 4-NC-oxygenase complex leaves the catalytic capability of the enzyme unimpaired.

Although the 4-NC-protocatechuate 3,4-dioxygenase spectrum resembled more that of the 4-NC dianion displaced to longer wavelength than that of an Fe(III)-4-NC complex (Fig. 2), the latter possibility was not entirely ruled out. Attempts to duplicate the 4-NC-oxygenase spectrum by modification of the ligand field around non-protein-bound, or free, Fe(III) solutions, however, were unsuccessful. Auxiliary ligands with a variety of O, N, and S binding atoms, amino acids, available polypeptide fragments, including both reduced and oxidized glutathione, different buffer systems or their absence, other trivalent and divalent metal ions including Mn(II), isoelectronic with Fe(III), and solvent mixtures simulating more hydrophobic environments produced either the ordinary Fe(III)-4-NC spectrum (Fig. 2) or one completely unlike that of 4-NC-protocatechuate 3,4-dioxygenase. When the enzyme was denatured with 6 M guanidinium chloride in aqueous solution, the spectrum was the same as that of a free 1:1 Fe(III)-4-NC solution at the same pH.

An alternative approach for detecting formation of a chelate at the active site is through potentiometric titration of the liberated intradiol proton of 4-NC upon complexation with the enzyme. This was impractical because of the large number of accessible protons on the enzyme and its low solubility below

\[ \text{Fe}^{3+} \text{O}_2 + 4 \text{NC} \rightarrow \text{Fe}^{2+} \text{O}_2 \text{NC}_4 \]

\[ \text{K}_i = 0.3 \mu \text{M} \]

\[ \text{Ki} \text{PCNase} = 0.020 \text{ml} \text{each; I and J, 0.020 ml each of } 9.4 \times 10^{-4} \text{ M dioxygenase, in that order. K, dioxygenase at same concentration as in G. Inset, absorbance (A) at 550 and 432 nm versus molar ratio of dioxygenase titrant to 4-NC; 24°C; under air.} \]
0.60 ml of 9.5 X 10^-4 M pyrocatechase 3,4-dioxygenase (PCase) + 0.10 ml of 1.0 X 10^-3 M 4-NC in 2.50 ml of 0.05 M Tris-acetate buffer, pH 7.5, same concentration of 4-NC only at pH 4.0, 8.5, and 12.0, respectively. Right hand side: ---, 4.2 X 10^-3 M 4-NC in mixture of 1.00 ml of 0.05 M Tris-acetate and 1.00 ml of 0.05 M Tris-HCl buffers, pH 8.0, to which 0.40 ml of 1.35 X 10^-4 M pyrocatechase (PCase) was added. ---, pyrocatechase only at same concentration; ---, 0.010 ml 1.0 X 10^-5 M 4-NC, 0.60 ml 2.0 X 10^-4 M Fe(III) sulfate, and 2.50 ml of same buffer mixture; ---, same as preceding experiment but in 0.10 M KCl medium, adjusted to pH 3.9 with NaOH; 24°; under air.

**Fig. 2.** Spectra of 4-NC and complexes. Left hand side: ---, 0.20 ml of 9.5 X 10^-4 M protecatechuate 3,4-dioxygenase (PCase) + 0.10 ml of 1.0 X 10^-3 M 4-NC in 2.50 ml of 0.05 M Tris-acetate buffer, pH 7.5; ---, same concentration of 4-NC only at pH 4.0, 8.5, and 12.0, respectively. Right hand side: ---, 4.2 X 10^-3 M 4-NC in mixture of 1.00 ml of 0.05 M Tris-acetate and 1.00 ml of 0.05 M Tris-HCl buffers, pH 8.0, to which 0.40 ml of 1.35 X 10^-4 M pyrocatechase (PCase) was added. ---, pyrocatechase only at same concentration; ---, 0.010 ml 1.0 X 10^-5 M 4-NC, 0.60 ml 2.0 X 10^-4 M Fe(III) sulfate, and 2.50 ml of same buffer mixture; ---, same as preceding experiment but in 0.10 M KCl medium, adjusted to pH 3.9 with NaOH; 24°; under air.

**Fig. 3.** Pyrocatechase (PCase) titration of 4-NC. Spectrum A is that of 1-cm quartz cell containing 4.2 X 10^-4 M 4-NC in a mixture of 1.00 ml of 0.05 M Tris-acetate, and 1.00 ml of 0.05 M Tris-HCl buffers at pH 8.0. To this solution were added: B, 0.10 ml; C to J, 0.05 ml each of 1.35 X 10^-4 M pyrocatechase, in that order. K, pyrocatechase at same concentration as in G. Inset, absorbance (A) at 450 nm versus molar ratio of pyrocatechase titrant to 4-NC; 24°; under air.

pH 6.0. A way around this difficulty is to titrate the sulphydryl groups with p-chloromercuribenzoate, but unfortunately only 12 sulphydryl protons are readily neutralized in this way (4), less than 10% of the total released by the enzyme in the neutral pH region.

The 4-NC-protecatechuate 3,4-dioxygenase spectrum was remarkably stable and remained unchanged after more than 1 year of standing at 4° in pH 8.5 Tris-HCl buffer. The enzyme still retained catalytic activity at the end of this period.

**Pyrocatechase; Optical Spectra—**Pyrocatechase performs the same intradiol type cleavage on catecholic rings as protecatechuate 3,4-dioxygenase but with pyrocatechol as substrate. The enzyme has a molecular weight of 90,000 and contains 2 atoms of iron, at least one of which is in the Fe(III) oxidation state (1-3).

Titration of 4-NC with a concentrated pyrocatechase solution gradually converted the 4-NC absorbance peak at 432 nm and the enzyme peak at 450 nm into a new spectrum having a maximum at 392 nm and a broadened tail into the visible region (Fig. 3). An isosbestic point at 480 nm maintained up to a ratio of 2:1 unbuffered.

**TABLE I**

Absorption maxima of 4-NC and its dioxygenase complexes

| Species                  | pH | Absorption maxima | ε X 10^-4 | Absorption maxima | ε X 10^-4 |
|-------------------------|----|-------------------|----------|-------------------|----------|
| 4-NC, undissociated     | 4.0| 345               | 0.56     | 520               | 1.25     |
| 4-NC monomeric          | 8.5| 428               | 1.47     | 625               | 0.27     |
| 4-NC dianion            | 12.5| 390               | 0.82     | 650               | 1.25     |
| Fe(III)-4-NC (1:1)      | 4.0| 390               | 0.83     | 625               | 0.27     |
| Fe(III)-4-NC (1:1)      | 7.5| 428               | 1.65     | 590               | 1.25     |
| Fe(III)-4-NC (1:1)      | 8.5| 450               | 1.65     | 590               | 1.25     |
| Pyrocatechase, 3,4-dioxygenase | 7.5| 450 | 0.37     | Pyrocatechase, 4-nitrocatechol | 7.5| 410 | 0.85 |
| Pyrocatechase, 4-nitrocatechol | 7.5| 440 | 0.24     | Pyrocatechase, 4-nitrocatechol | 7.5| 392 | 0.92  |

a Tris-HCl buffer, 0.05 M.
b Unbuffered.
c Fe(III)-4-NC, 1 mM, KCl, 0.10 M, brought to pH with NaOH. Fe(II)-4-NC maximum and absorptivity estimated from studies with Mn(II) and Co(II)-4-NC at pH 8.5. See, for example Ref. 19.
d Per atom of iron, present in enzyme molecule; 7 g atoms for pyrocatechuate 3,4-dioxygenase, 2 g atoms for pyrocatechase.

2 4-nitrocatechol molecules to 1 pyrocatechase (based on protein). This ratio is the same as that obtained from titration of pyrocatechase with its substrate under anaerobic conditions (19).

The maximum at 392 nm is at the same wavelength as that of a solution of free Fe(III)-4-NC in 1:1 ratio at pH 3.9 (right hand side, Fig. 2). The latter spectrum is tentatively attributed to the 1:1 chelate of the two reagents, since a potentiometric titration with base carried out concurrently on this system indicated that up to pH 4.0 only two protons per 4-NC molecule are neutralized. Above this pH competition by hydroxyl ions for the metal, disproportionation and probably polynucleation complicate the spectra. Since the observed spectrum of the 4-NC-pyrocatechase complex does not resemble that of the free Fe(III)-4-NC system at pH 7.5, it is likely that the metal ions remain bound to the enzyme. Table I compares the spectral properties associated with these complexes.

4-Nitrocatechol was a competitive inhibitor of pyrocatechol with a K_i = 2.7 μM, slightly lower than the 6.0 μM value for the K_m of this substrate. The probe was not readily metabolized by pyrocatechase since the spectrum of the complex was stable for more than 12 hours. After standing 3 weeks at 4° in 0.05 M Tris-acetate buffer, pH 7.5, however, the absorption maximum at 392 nm had disappeared and only a gradual tailing off of adsorption toward the infrared was observed. Addition of a fresh solution of the same amount of 4-NC did not restore the spectrum of the complex. Since a solution of Fe_2(SO_4)_3 and 4-NC was spectrally stable under the same conditions and, since the original 4-NC spectrum was not recovered upon loss of enzyme color during storage, the possibility obtains that 4-NC may have been slowly metabolized during standing but the product from this reaction remains to be isolated and identified.
Fig. 4. EPR spectra of protocatechuate 3,4-dioxygenase (PCAase) and enzyme with 8-fold excess of 4-NC at X-band and 77°, in 0.05 m Tris-HCl buffer, pH 8.5.

**TABLE II**

*Apparent g values of Fe(III)-containing complexes*

The derivative spectrum of the 4-NC-protocatechuate 3,4-dioxygenase complex was taken at X-band, 77°, in a Nihondekahi ESR spectrometer. The concentration of the oxygenase, 4-NC, and Tris-HCl buffer, pH 8.5, were 0.11, 1.0, and 50 mm, respectively. For the experimental conditions used for obtaining the other spectral parameters in the table, see the references given in parentheses.

| Substance                                      | Apparent g values |
|-----------------------------------------------|-------------------|
| Fe(III)-transferrin-bicarbonate (21)          | 4.15, 4.40, 4.30  |
| Fe(III)-transferrin, pH 5.4 (22)              | 3.61, 4.77, 4.30  |
| PCAase-protocatechualdehyde (5)               | 3.80, 3.75, 4.31  |
| PCAase-protocatechualdehyde (6)               | 3.07, 4.68, 4.31  |
| PCAase-3,4-dihydroxyphenylacetic acid (5)     | 4.07, 4.78, 4.31  |
| PCAase-4-NC                                   | 3.74, 4.74, 4.28  |

*PCAase, protocatechuate 3,4-dioxygenase.

**Fe(III) Dioxygenases; EPR Spectra—**Both Fe(III) dioxygenase solutions give a single EPR signal at $g = 4.28$ at X-band and liquid $N_2$ temperature (1-3). Upon addition of excess substrate or either of the inhibitor analogues, protocatechualdehyde or o-protocatechuic acid, to protocatechuate 3,4-dioxygenase the signal intensity diminished, but never to zero. When excess 4-NC was added to this enzyme a signal comprised of three bands with apparent $g$ values of 4.74, 4.28, and 3.74 appeared (Fig. 4). Resolution of the $g = 4.28$ signal was more complete and the bands more symmetric than obtained with other analogues in the past (5, 20). In contrast, 4-NC did not resolve the $g = 4.28$ signal of pyrocatechase (although it did decrease its intensity significantly). The $g$ values of biological materials in which similar resolution of the $g = 4.28$ signal has been observed at X-band are compared in Table II.

A plot of either total area under the central derivative curve or the derivative curve at $g = 4.74$ against the molar ratio of 4-NC to protocatechuate-3,4-dioxygenase showed an apparent end point at about 9:1, beyond which no significant change in signal intensity occurred. Total signal intensity under all the curves at this point was roughly 15% of the original value.

**Non-heme Fe(II) Dioxygenases: Metapyrocatechase—**The interaction of 4-NC with other non-heme iron-containing enzymes in which the metal ions are thought to be in the Fe(II) oxidation state was of interest for comparative purposes. These enzymes have no color or EPR signal in the absence of substrate turnover. They cleave aromatic rings at a carbon-carbon bond involving only one of the hydroxyl groups to form semialdehydes (23, 24). Metapyrocatechase, which utilizes pyrocatechol as substrate, has a molecular weight of 140,000, 1 to 3 g atoms of Fe(II) and three substrate binding sites per protein molecule (25, 26).

4-Nitrocatechol was a substrate for metapyrocatechase with $K_m^e$ for $k^{NC} = 0.06$ where $k = V_{max}$ (under air) for metapyrocatechase, the initial total analytical enzyme concentration and PC is pyrocatechol. The Michaelis constants for 4-NC and for pyrocatechol were 6.8 and 7.8 $\mu$M, respectively. At saturating levels of 4-NC, enzyme deactivation occurred, as evidenced by deterioration of the oxygen consumption rate to zero before depletion of available O$_2$ and failure of additional substrate to re-stimulate the rate. The spectrum of 4-NC, however, was not appreciably altered by the enzyme in the absence of O$_2$. The disappearance of 4-NC also was studied by following the decrease in optical density at 432 nm in a stopped flow apparatus but no evidence of spectral intermediates was obtained.

The reaction product from 4-NC oxidation had a peak at 398 nm, $\epsilon = 2.5 \times 10^4$ cm per mol in 50 mm K$^+$-phosphate buffer at pH 7.5. The product was stable relative to other semialdehydes at concentrations under 1.0 mm (no significant spectral change after 2 hours). Conversion of the product to the pyridine derivative gave a spectrum with peaks at 285 nm (shoulders at 349 and 252 nm) and 229 nm at pH 7.4, characteristic of semialdehydes (23, 24). The melting point of the product after crystallization from pyridine was 209°, corresponding to that for 5-nitropicolinic acid, the expected product from 2,3-cleavage (27, 28).

The kinetics of the interaction of 4-NC and protocatechuate 4,5-dioxygenase, another extradiol cleaving enzyme and counterpart to the 3,4-cleaving dioxygenase, has been studied (29). In contrast to pyrocatechase 4-NC is a competitive inhibitor but not a substrate for this enzyme. Table III compares the $K_i$ and $K_m$ for 4-NC with all four dioxygenases with the $K_m$ values for their natural substrates. Whereas the $K_m$ values for the natural substrates of these enzymes are within a factor of 3 of

**TABLE III**

*Ki and Km values for 4-NC with dioxygenases*

The $K_i$ and $K_m$ values were determined from Lineweaver-Burk plots in the standard manner using two different inhibitor concentrations in the inhibition studies and the principal substrate of the enzyme. The following buffered media were used: 0.05 m Tris- acetate, pH 7.5, for pyrocatechase and metapyrocatechase; 0.05 m Tris-HCl, pH 8.5, for protocatechuate-3,4-dioxygenase; and 0.05 m K$^+$-phosphate, pH 7.5, for protocatechuate 4,5-dioxygenase, all at 25°. The protocatechuate 4,5-dioxygenase results are taken from Ref. 30.
**TABLE IV**
Survey of effects of 4-NC on oxygenases and oxidation-reduction enzymes
The numbers in parentheses are reference numbers.

| Oxygenase                  | Prosthetic Group | Effect* |
|----------------------------|------------------|---------|
| Dioxygenase                |                  |         |
| Pyrocatechase              |                  |         |
| Pseudomonad                | Fe(III) non-heme | S, P    |
| Brevibacterium (30)        | Fe(III) non-heme | NS      |
| Proteocatechase 3,4-dioxygenase | Fe(III) non-heme | I       |
| Proteocatechase 4,5-dioxygenase | Fe(III) non-heme | 4       |
| Metapyrocatechase          | Fe(II) nonheme   | S'      |
| Low [S]                    |                  |         |
| High [S]                   |                  |         |
| Tryptophan pyrrole (pseudo-monad) |                  |         |
| Peroxidase                 |                  |         |
| Horseradish peroxidase     | Fe(III) heme     | S''     |
| activity, DHF-O2 system (32) |               |         |
| Chloroperoxidase (18)      | Fe(III) heme     | A, S'   |
| Catalase                   | Fe(III) heme     | T'      |
| Monoxygenase               |                  |         |
| Cytochrome P=450 (33)      | Fe(III) heme     | F       |
| Lysine monooxygenase       | No metal cofactor| None    |
| Salicylate hydroxylase     | No metal cofactor| D''     |
| Tyrosinase (34)            | Cu(II)           | S       |
| Polyphenoloxidase          |                  |         |
| Tealeaf (35, 30)           | Cu(II)           | I, NS   |
| Potato (37)                | Cu(II)           | S       |
| Tryptophan hydroxylase     | Fe(II) non-heme  | I''     |
| Electron transferase       |                  |         |
| Laccase (39)               | Cu(II)           | S       |
| Cytochrome c oxidase (40)  | Fe(III) heme + Cu(II) | I |

* The abbreviations used are: S, substrate; I, inhibitor; D, deactivator; A, activator; NS, not substrate, only effect tested. See "Experimental Procedures" and all other footnotes for standard conditions under which these assays were run.

**DISCUSSION**

Information on the nature of the interaction of catechol substrates with dioxygenases has been virtually nonexistent. Apart from the detection of a reactive amine group in protocatechuate 3,4-dioxygenase and evidence from optical rotary dispersion studies, Mössbauer, and low temperature EPR studies that the ligand fields surrounding the irons are perturbed during complexation with substrate, little more is known (20, 26, 29). This lack of definitive information, despite several years of experimentation on these enzymes, attests in part to the difficulty in finding probes with properties which can be measured and interpreted in their presence. 4-Nitrocatechol appears to go some distance toward satisfying this need.

Certainly the most interesting result from the use of 4-NC has been the contrast in spectral and kinetic effects observed when it interacts with non-heme iron dioxygenases. Most noteworthy are the results with pyrocatechase and proteocatechuate 3,4-dioxygenase each of which has its iron in the Fe(III) oxidation state. Both are broad spectrum, intradial-cleaning enzymes which can utilize either pyrocatechol or protocatechol acid as substrate though of course with different efficiencies. 4-Nitrocatechol is a strong competitive inhibitor of substrate oxygenation by either enzyme and it binds to both in the same ratios as substrates do. For these reasons it was somewhat unexpected to find that the optical and EPR characteristics of the 4-NC complexes differ so remarkably.

A reasonable interpretation of the 4-NC-pyrocatechase optical spectrum is that it derives from a 1:1 Fe(III)-4-NC chelate in which, on the basis of the continued presence of the d→d* absorption from the enzyme-bound Fe(III), all of the metal protein linkages have not been broken. Supporting evidence for this interpretation might be derived from low temperature EPR and Mössbauer studies with a series of more labile analogues and 4-NC. In marked contrast, the spectrum of the 4-NC-proteocatechuate 3,4-dioxygenase complex resembles that of the 4-NC diiron shifted 20 to 30 nm to higher wavelength. It would appear from this that the binding sites in this oxygeonase are either sufficiently basic to promote displacement of the intramolecular hydroxyl proton of 4-NC (pKₐ = 10.75) or that the iron atoms themselves can assist in this process without chelation with the molecule. Regardless of the explanation it is clear that the orientation of 4-NC relative to the iron atoms at the active sites in both enzymes is different. Coincidental with this different orientation are changes in the ligand field arrangement about the iron atoms in protocatechuate 3,4-dioxygenase, as reflected in the profoundly modified EPR spectrum of its 4-NC complex.

While it is natural to consider that these different ligand field configurations and molecular orientations at the active sites...
might account for differences in reactivity of the complexes, such a conclusion is premature. It has not been established that the complex observed immediately after adding 4-NC to protocatechuate leads directly to the disintegration of the probe or that such a complex is a kinetic intermediate in the degradative process. Indirect evidence that this complex is such an intermediate would have to come from ancillary biophysical studies such as EPR or Mössbauer in which the ligand field configurations of the iron atoms in 4-NC and more labile analogue-protocatechuate complexes are compared and shown to be essentially the same. However, it is clear that the different configuration and the particular molecular orientation of 4-NC in protocatechuate 3,4-dioxygenase results in inactivation. This is of considerable value when it is noted that almost all other protocatechueic acid analogues can be metabolized by the enzyme.2 Thus the 4-NC-protocatechuate 3,4-dioxygenase complex may serve as a marker for determining what changes in the ligand fields about the iron atoms result in inactivation of the enzyme.

The studies on the 4-NC-metaprotocatechase system are included in this work for contrast with the Fe(III)-containing dioxygenases. Steady state kinetics on this system indicated that the $K_a$ values for 4-NC and for protocatechol are a close approximation of the binding constants for these analogues with the enzyme.2 No pronounced perturbation of the 4-NC spectrum was seen, however, either under anaerobic conditions or aerobically in a stopped flow apparatus. It would appear that the iron atoms do not come in direct contact with the substrate and that the nitrocatechol molecule is bound as the monoanion (as great as with 4-NC) with these enzymes, indicating that 4-NC is probably not interacting in a unique way with them.

Further appreciation of the sensitivity of 4-NC in detecting differences in the active site environments of non-heme iron dioxygenases derives from examination of the kinetic parameters in Table III. Although the $K_a$ values for the natural substrates with each of these four enzymes are within a factor of 3 of 3 each other, the $K_i$ values for 4-NC with protocatechueic acid cleaving dioxygenases differ by more than 3 orders of magnitude, even though these enzymes are specific for the same substrate. In both cases the inhibition is competitive. Protocatechualdehyde, another competitive inhibitor for these enzymes, had $K_i$ values of similar magnitude (although the spread in values is not as great as with 4-NC) with these enzymes, indicating that 4-NC is probably not interacting in a unique way with them.

The original motivation behind this study, to find a chemical reagent for non-heme Fe(III) dioxygenases, which would assist in discriminating between substrates in their active site makeup, has been achieved. Because of the favorable attributes of 4-NC, intense color, resistance to oxidation, good chelating properties, minimal steric interference from the substituent group, and a structural similarity to the substrates, it may be the best single one available for this purpose. A survey of its effects in Table IV suggests that more detailed examination of its interaction with other enzyme systems may also prove informative.

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