CHARACTERISATION OF A NOVEL NON-HEME DIOXYGENASE

Mary G. Wallis

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Finally, I would like to thank my family and friends for tremendous support, encouragement and many moments of madness, in particular during the preparation of this manuscript.
For my family,
especially

Jack Wallis
and
Campbell Beattie
Inspiration did not come easily to Mary:

'I thought and pondered - vainly. I felt that blank incapability of invention which is the greatest misery of authorship, when dull Nothing replies to our anxious invocations. "Have you thought of a story?" I was asked each morning, and each morning I was forced to reply with a mortifying negative'.

But revelation was at hand.

Maurice Hindle on Mary Shelley
Except where specific reference is made to other sources, the work presented in this thesis is the original work of the author. It has not been submitted, in whole or in part, for any other degree. Some of the results have already been published.

Mary G. Wallis
ABSTRACT

A purification procedure has been developed for a novel extradiol dioxygenase, designated as 3-methylcatechol 2,3-dioxygenase. The enzyme which is expressed in *Escherichia coli*, was originally derived from a *Pseudomonas putida* strain able to grow on toluidine. 3-Methylcatechol 2,3-dioxygenase was purified to homogeneity as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Physical and kinetic properties of the purified enzyme were investigated. The enzyme consists of a single subunit type of \( M_r = 33,500 \pm 2,000 \) by SDS-PAGE. Gel filtration indicated a molecular weight, under non-denaturing conditions, of 120,000 \( \pm 20,000 \) consistent with the native enzyme existing as a tetramer of identical subunits. The \( \text{NH}_2 \)-terminal sequence (35 residues) has been determined and shows 50% identity with other extradiol dioxygenases. The structural characterisation of 3-methylcatechol 2,3-dioxygenase at the primary, secondary and quaternary levels indicates that the enzyme is typical of the extradiol dioxygenases.

Evidence from several experiments, such as metal removal and replacement, oxidation and reduction, indicates that Fe(II) is essential for enzymatic function with the oxidised, ferric, form being inactive. No requirement for other cofactors was detected.
Thermal inactivation experiments demonstrated the complete stability of the enzyme up to 45°C for prolonged periods, however, above this temperature the enzymic activity was seen to decline.

The kinetics of 3-methylcatechol 2,3-dioxygenase were investigated using UV/visible spectrophotometry and oxygen electrode polarography. Measurements were made under both standard and modified conditions. Typical saturation kinetics were observed for catechol, 3-methylcatechol and 4-methylcatechol as substrates. Data were analysed to give values of $V_{\text{max}}$ and $K_m$. The substrate specificity for this enzyme was somewhat different from that seen for other catechol 2,3-dioxygenases, with 3-methylcatechol being cleaved at the highest rate. The $K_m$ values for the organic substrates were all around 0.3 $\mu$M, the lowest found for any dioxygenase to date. The $K_m$ for dioxygen was determined to be $< 10^{-6}$M. The enzyme consumed one mole of oxygen per mole of substrate in all three cases.

The dependence of enzyme activity on pH follows a classic bell-shaped curve with a pH optimum of about 7.5 and pKa values of 6.9 ($\pm$ 0.1) and 8.7 ($\pm$ 0.1). The possibility that the lower pKa value might be due to an active site histidine was investigated by attempted modification of this residue.

The implications of these results are discussed in relation to the structure and function of the enzyme.
UNITS AND ABBREVIATIONS

Abbreviations of units are of a standard form;

- dalton units: Da
- gram(s): g
- litre(s): l
- second(s): s

Other unit abbreviations include;

- molar absorption coefficient: \( M^{-1} \text{ cm}^{-1} \)
- first order rate constant: \( s^{-1} \)

Both the single and three letter codes have been used to denote the amino acid residues;

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<th>Amino Acid</th>
<th>Three Letter Abbreviation</th>
<th>One Letter Symbol</th>
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<td>Alanine</td>
<td>Ala</td>
<td>A</td>
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<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
</tr>
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</table>

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<table>
<thead>
<tr>
<th>Amino Acid</th>
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<th>One Letter Symbol</th>
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<tbody>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
</tr>
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<td>Serine</td>
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</tr>
<tr>
<td>Threonine</td>
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</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
</tr>
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</table>

The following nomenclature has been adopted in referring to the dioxygenases:

<table>
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<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
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<tr>
<td>Catechol 1,2-dioxygenase</td>
<td>C1,2D</td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase</td>
<td>C2,3D</td>
</tr>
<tr>
<td>1,2-Dihydroxybiphenyl 2,3-dioxygenase</td>
<td>1,2DHB2,3D</td>
</tr>
<tr>
<td>1,2-Dihydroxynaphthalene 2,3-dioxygenase</td>
<td>1,2DHN2,3D</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylacetate 2,3-dioxygenase</td>
<td>3,4DHPA2,3D</td>
</tr>
<tr>
<td>Gentisate 1,2-dioxygenase</td>
<td>G1,2D</td>
</tr>
<tr>
<td>3-Methylcatechol 2,3-dioxygenase</td>
<td>3MC2,3D</td>
</tr>
<tr>
<td>Protocatechuate 3,4-dioxygenase</td>
<td>P3,4D</td>
</tr>
<tr>
<td>Protocatechuate 4,5-dioxygenase</td>
<td>P4,5D</td>
</tr>
<tr>
<td>Steroid 4,5-dioxygenase</td>
<td>S4,5D</td>
</tr>
</tbody>
</table>

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The following abbreviations have been employed in referring to the biomimics:

<table>
<thead>
<tr>
<th>Chelating Ligand</th>
<th>Abbreviation</th>
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<tr>
<td>acetylacetonate</td>
<td>acac</td>
</tr>
<tr>
<td>nitrilotriacetic acid</td>
<td>NTA</td>
</tr>
<tr>
<td>N,N′-(3,3′dipropylamine)bis(salicylidene-amine)</td>
<td>saldpt</td>
</tr>
<tr>
<td>N,N′-ethylenebis(salicylideneamine)</td>
<td>salen</td>
</tr>
<tr>
<td>N,N′-1,2-benzenebis(salicylideneamine)</td>
<td>saloph</td>
</tr>
</tbody>
</table>

Other miscellaneous abbreviations include:

- Michaelis constant: $K_m$
- Limiting value for reaction rate: $V_{max}$
- Nicotinamide adenine dinucleotide: NAD
- Nicotinamide adenine dinucleotide phosphate: NADP
- Pounds per square inch: p.s.i.
- Revolutions per minute: r.p.m.
- Tris(hydroxymethyl)aminomethane: tris
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CHAPTER 1

INTRODUCTION
1.1 Historical Perspective

Studies on biological oxidation processes were first initiated by Lavoisier about two hundred years ago. The early investigators generally agreed that the combination of a substrate, \( S \), with oxygen was the essential characteristic of a biological oxidation process. The formation of an oxide was designated as "oxidation", and "reduction" was defined as the removal of oxygen from an oxide, Eqn. 1.1.

\[
\begin{align*}
\text{"oxidation"} & \quad S + O_2 \longrightarrow SO_2 \\
\text{"reduction"} &
\end{align*}
\]  

Biological tissues were found to contain a number of catalysts which facilitated this process by activating molecular oxygen.

It soon became apparent, however, as a result of an extensive series of studies by Wieland [1932] and others, that biological oxidation can proceed in the complete absence of oxygen. According to his view, biological oxidation proceeds exclusively by the removal of electrons or hydrogen atoms from substrate and the direct addition of molecular oxygen need not be assumed. Oxygen might still be incorporated into substrate by hydration reactions, but prior and subsequent oxidative processes would involve the removal of hydrogens and/or electrons.
This theory of biological oxidation was generally accepted until 1955, when the enzymic incorporation of molecular oxygen into substrates was established through the use of a heavy isotope of oxygen, \(^{18}\)O.

In 1955, Mason and his co-workers observed that an oxygen atom derived from molecular oxygen was incorporated into 4,5-dimethylcatechol when 3,4-dimethylphenol was oxidised by "phenolase", Eqn. 1.2. Oxygen from water was not used.

\[
\begin{align*}
\text{OH} & \\
\text{CH}_3 & + \frac{1}{2} \text{O}_2 & \rightarrow & \\
\text{CH}_3 & \text{OH} \\
\end{align*}
\]

Concurrently, Hayaishi and his associates [1955], using \(^{18}\)O \(_2\) and \(H_2^{18}O\), established that the two atoms of oxygen inserted into catechol (1,2-dihydroxybenzene) by the action of "pyrocatechase" [Hayaishi, 1957], were both derived exclusively from atmospheric oxygen, Eqn. 1.3. These findings were obviously in contrast to Wieland's concept that molecular oxygen could act only as an ultimate electron acceptor and that all oxygen atoms incorporated into substrates were derived from the oxygen.
Both these reactions (Eqns. 1.2 and 1.3) involve "oxygen fixation" into a substrate molecule, and are similar to oxygenation reactions known to occur by chemical or photochemical processes. Since the enzymes catalysing these reactions were different from previously known dehydrogenases and oxidases, it was inferred that molecular oxygen must be activated in some way prior to its incorporation into organic substrates. In view of these considerations, Bach and Chodat's [1903] term "oxygenases" was selected to designate a group of enzymes which catalyse the incorporation of molecular oxygen into various substrates [Hayaishi, 1956].

1.2 Distribution of Oxygenases

The oxygenases, which were first discovered in Pseudomonads and mushrooms, now appear to enjoy a
ubiquitous distribution in nature; they have been isolated from animals, plants and microorganisms. Aerobic microorganisms such as Pseudomonads, Mycobacteria and Nocardia, are rich sources of oxygenases while anaerobic microorganisms are devoid of this activity. Within the cells, oxygenases are not associated with any one specific constituent of the cell, but are distributed in various cellular fractions.

1.3 Physiological Significance of Oxygenases

In 1908, microbiologists first discovered bacteria which metabolised aromatic compounds [Buchanan, 1930], and since that time many different species have been isolated from soils which possess an impressive metabolic potential. In particular, the aerobic Pseudomonads, occasionally pathogenic to man, attracted attention as early as 1926 [den Dooren de Jong] due to their range of habitats, variety of biological types and biochemical versatility [Stanier, 1966; Holloway, 1969; Hashimoto, 1987]. Metabolically, they are among the most versatile organisms known, capable as a group of utilising over one hundred different organic compounds as their sole carbon and energy sources. Today a bewildering range of oxygenases are known to function in the biosynthesis, transformation and degradation of essential natural metabolites such as amino acids, lipids, sugars, nucleic acids, porphyrins, vitamins and hormones. They also play
a crucial role in the dissimilation of various synthetic aromatic compounds such as drugs, herbicides, insecticides and pesticides [Haller, 1979; Engesser, 1980] which are often carcinogenic [Parke, 1959; Patty, 1963; Miller, 1970; Wallcare, 1971]. Furthermore, they participate in the biological oxidation of inorganic sulphur. These degradative reactions are necessary for the recycling of various biologically important elements [Clarke, 1975; Leisinger, 1983]. Several reviews on the significance of oxygenases have been published and many of these can be found within the following references; Hayaishi [1962], Dagley [1972], Que [1980], Makino [1988] and Nozaki [1988].

With such an array of often recalcitrant [Haller, 1978], natural and synthetic aromatics present in the soil and water, resulting from natural decomposition, deliberate application or discharge of industrial waste [McKinney, 1956; Riddick, 1970], clearly it is important to know their fate in the environment and understand the demands and limitations of the processes involved in their biodegradation. Complex aromatic compounds are degraded by different catabolic pathways which are channelled towards a limited number (circa six) of key intermediates, simple aromatics such as catechol [Waite, 1981; Manthey, 1989] (Fig. 1.1). The pivotal ring-opening step is mediated by the enzymatic insertion of molecular oxygen.
Figure 1.1: The Role of Catechol as a Central Metabolite in the Bacterial Degradation of Benzenoid Components

Reactions opposite from:


Redrawn from Dagley [1972].
The cleavage of the catechol nucleus proceeds in two different ways. Either the ring is cleaved between the two neighbouring hydroxyl groups, "ortho"-fission, and cis,cis-hexa-2,4-diene-1,6-dioic acid is formed, or it is cleaved adjacent to one of the hydroxyl groups forming 2-hydroxy-6-oxohexa-2,4-dienoic acid, "meta"-fission (Fig. 1.2). The two modes of ring fission are the initial steps of two separate pathways which lead to the formation of products that can be fed into the citrate cycle. After ring-cleavage no further oxygen is incorporated.

1.4 **The Classification of Oxygenases**

As previously mentioned (Section 1.1), enzymes which catalyse the incorporation of molecular oxygen into various substrates are called "oxygenases". As in the cases of phenolase and pyrocatechase, respectively, oxygenases catalyse the incorporation of either one or two atoms of molecular oxygen into their substrates, therefore, two sub-classes are generally assigned; "monooxygenases" and "dioxygenases" [Hayaishi, 1964].

Enzymes which catalyse the addition of only one atom of oxygen into a substrate are called "monooxygenases" (Eqn. 1.4).

\[ S + 1/2 O_2 \rightarrow SO \]  

(1.4)

Phenolase, first examined by Mason, belongs to this group.
Figure 1.2: **Two Pathways for the Cleavage of Catechol**

Ring-opening (opposite) results from both "meta"-fission producing 2-hydroxymuconic semialdehyde (2-hydroxy-6-oxohexa-2,4-dienoic acid) and "ortho"-fission which affords cis,cis-muconate (cis,cis-hexa-2,4-diene-1,6-dioic acid). Redrawn from Dagley [1972].
2,3-OXYGENASE

1,2-OXYGENASE

catechol

ortho pathway

cis, cis-muconate

LACTONIZING ENZYME

muconolactone

ISOMERASE

β-ketoadipate

HYDROLASE

enol lactone

HYDROLASE

β-ketoadipate

TRANSFERASE

acetyl-CoA

formate

HCOO\

CH3

COO\-

2-hydroxymuconic semialdehyde

HYDROLYASE

4-hydroxy-2-ketovalerate

ALDO LASE

acetaldehyde

pyruvate

meta pathway

2-hydroxymuconic semialdehyde

It was soon realised that monooxygenases function only in the presence of an appropriate electron donor which serves to reduce the other oxygen atom to water. The simplest type of monooxygenase catalyses the incorporation of a single atom of molecular oxygen concomittant with the reduction of the other atom by electrons derived from the substrate. Thus, the overall reaction may be expressed by the following equation (Eqn. 1.5).

\[ \text{SH}_2 + O_2 \rightarrow \text{SO} + \text{H}_2\text{O} \]  

(1.5)

Since the reducing agent is internally supplied, these enzymes may be referred to as "internal" monooxygenases. All the internal monooxygenases that have so far been purified and characterised contain flavin coenzymes.

Most of the other monooxygenases, however, require various kinds of external hydrogen or electron donors and are therefore referred to as "external" monooxygenases. The stoichiometry of such a reaction is schematically represented in Eqn. 1.6.

\[ S + O_2 + \text{DH}_2 \rightarrow \text{SO} + \text{H}_2\text{O} + \text{D} \]  

(1.6)

One of the atoms of molecular oxygen is incorporated into a substrate molecule, \( S \), and the other is reduced to \( \text{H}_2\text{O} \) in the presence of an appropriate electron donor, \( \text{DH}_2 \). Monooxygenases were previously called "mixed function"
oxidases or oxygenases by Mason [1956, 1957] because they function both as oxidases and oxygenases [Mason, 1957]. These enzymes are also referred to as "hydroxylases" as a more descriptive nomer [Massart, 1959].

The external hydrogen donors include reduced NAD, reduced NADP, ascorbic acid and sulfhydryl compounds. Cofactors required for the external monooxygenases are flavin [Yamamoto, 1969], pteridine, copper [Nozaki, 1979], non-heme iron and heme. The monooxygenases are dominated by the protoheme containing cytochrome P-450 family, a group of proteins characterised by the Soret absorption maximum of their ferrous-carbon monoxide complexes at around 450 nm [Omura, 1964; Ortiz de Montellano, 1985].

Enzymes which incorporate both atoms of oxygen into a molecule of substrate are designated "dioxygenases". Synonyms include "true" oxygenases and "oxygen transferases" [Hayaishi, 1964]. Pyrocatechase, originally studied by Hayaishi is of this type. In most cases, one substrate acts as an oxygen acceptor and a single molecule of the substrate receives two atoms of oxygen as shown in Eqn. 1.7.

\[ S + O_2 \rightarrow SO_2 \]  \hspace{1cm} (1.7)

In some dioxygenase reactions, however, the two atoms of one dioxygen molecule are incorporated into two different molecules of one substrate, Eqn. 1.8 or Eqn. 1.9, into two
different substrate molecules, S and S'.

\[
\begin{align*}
2S + O_2 & \rightarrow 2SO \quad (1.8) \\
S + S' + O_2 & \rightarrow SO + SO' \quad (1.9)
\end{align*}
\]

The term "intramolecular" dioxygenases may be used to designate the dioxygenases catalysing the reaction shown in Eqn. 1.7, and "intermolecular" dioxygenases for those catalysing the reactions shown in Eqns. 1.8 and 1.9, respectively.

The dioxygenases are involved in different types of reactions and require a variety of cofactors. A major function of dioxygenases is the cleavage of the aromatic ring (Section 1.3) with the insertion of two oxygen atoms from molecular oxygen [Nozaki, 1963, 1968; Kojima, 1967; Nakazawa, 1969]. The indole ring-cleaving enzyme, tryptophan 2,3-dioxygenase contains heme as a cofactor [Tanaka, 1959; Ishimura, 1970]. A flavonol-cleaving enzyme, quercetinase has been reported to be a copper protein [Krishnamurty, 1969; Oka, 1971] and a pyridine ring-cleaving enzyme, 2-methyl-3-hydroxypyridine-5-carboxylate dioxygenase, has been reported to be a flavin-containing enzyme [Sparrow, 1969]. Recently, 3,4-dihydroxypyphenylacetate 2,3-dioxygenase from Bacillus brevis has been reported to be a Mn(II)-containing enzyme [Que, 1981]. With the exception of these enzymes, most of the other ring-cleaving dioxygenases, if not all
of them, contain non-heme iron as the sole cofactor. Among these, some enzymes contain the ferrous form of iron and some the ferric form [Nozaki, 1974, 1975, 1988].

Of about forty dioxygenases known to date, more than 80% have firmly bound non-heme iron (i.e., the metal atom of the active site is not at the centre of a tetrapyrrolic ring) or require added iron for full activity. The cleavage of aromatic rings, especially phenols and catechols, appear to depend almost entirely upon this type of enzyme, thus extensive studies on these non-heme iron dioxygenases have been carried out. Details of these studies have been recently reviewed by Nozaki [1988].

When catechol derivatives are cleaved by the action of individual dioxygenases, two modes of ring fission (briefly mentioned in Section 1.3) and consequently two classes of enzymes can be defined; the (a) intradiol and (b) extradiol (proximal/distal) dioxygenases (Fig. 1.3). As will be discussed more extensively in the following sections, differences between the intradiol and extradiol dioxygenases include not only their functions but also the oxidation state of the iron bound at the active site of the enzyme. This difference in oxidation state has had a profound influence on the characterisation of these enzymes and is of immense importance in determining their catalytic mechanisms.
Figure 1.3: Ring Fission Modes of Substituted Catechols

a: intradiol cleavage
b: proximal extradiol cleavage
c: distal extradiol cleavage
1.5 Structural Studies of Both the Intradiol and Extradiol Catechol Dioxygenases

This thesis is concerned with the study of 3-methylcatechol 2,3-dioxygenase, an extradiol dioxygenase. These Fe(II)-containing enzymes typically show no significant absorption in the visible range and are EPR silent. (Moreover, studies on their properties and mechanism have been greatly hampered by the fact that many of them are too unstable to be extensively purified). Due to their spectroscopic inaccessibility, strategies have had to be developed to probe the coordination of these ferrous centres. For example, by studying Fe(II) ligand-field bands in the CD and MCD spectra, Whittaker and Solomon gained insight into the geometries of the iron sites in soybean lipoxygenase and superoxide dismutase [1986]. Arciero and Lipscomb utilised the EPR properties of NO complexes of protocatechuate (3,4-dihydroxybenzoate) 4,5-dioxygenase together with ¹⁷O-labelled ligands to determine the number of available exogenous binding sites [1986]. On the other hand, the red Fe(III)-containing intradiol dioxygenases have proved amenable to study using several spectroscopic techniques and as a result they have been well characterised. Subsequently much of the information gathered on the latter has proved to be pertinent to the extradiol enzymes. The structure/function discussion will therefore rely heavily on information gained from the intradiol dioxygenases.
The elucidation of the structures of the intradiol catechol dioxygenases and their enzymatic mechanism(s) [Que, 1977] has come about as a result of spectroscopic and kinetic investigations coupled with comparative studies on model compounds. Central to these studies has been the phenolate-to-Fe(III) charge-transfer transition which persists in all states of the enzyme during catalytic turnover and determines the UV/visible, resonance Raman and NMR properties of the iron complex in these enzymes [Pyrz, 1985].

The two best-studied examples are catechol 1,2-dioxygenase (C1,2D), and protocatechuate 3,4-dioxygenase, (P3,4D), the discussion will therefore concentrate on these enzymes. C1,2D, from Pseudomonas arvilla grown on benzoate as its sole carbon source, has a molecular weight of 63,000 with two non-identical subunits, αβFe [Hayaishi, 1955]. P3,4D, from Pseudomonas aeruginosa which utilises 4-hydroxybenzoate as its sole carbon source, has a molecular weight of 783,000 and a composition of (α₂β₂Fe)₈ [Iwaki, 1979, 1981; Kohlmiller, 1979]. These intradiol enzymes thus appear to come in various forms and sizes unlike the extradiol catechol dioxygenases which almost exclusively have only one subunit type and the molecular form (αFe)₄.

With the intradiol enzymes, EPR spectroscopy reveals a signal characteristic of a high-spin ferric centre in a rhombic environment [Oosterhuis, 1974], similarly
Mossbauer spectroscopy reveals spectra parameters consistent with a high-spin ferric form [Que, 1976; Whittaker, 1984; Kent 1987].

The enzymes are most easily characterised by their visible spectroscopy which shows a broad absorption band with a maximum centred near 460 nm and a molar absorption coefficient of 3000 to 4000 M\(^{-1}\) cm\(^{-1}\) per iron [Que, 1983]. Since ligand-field bands of high-spin ferric ions are both spin and orbitally forbidden and thus have small absorption coefficients (0.1 to 1 M\(^{-1}\) cm\(^{-1}\)), the visible absorption band has been assigned to a ligand-to-metal charge-transfer transition. Considering the possible amino acid ligating groups, the two with high-lying filled orbitals to serve as donor orbitals are thiolate (cysteine) and phenolate (tyrosine). Indeed, early studies favoured the description of the dioxygenases as iron-sulphur proteins [Blumberg, 1973] because of EPR and visible spectra similarities to rubredoxin, a protein with a ferric centre tetrahedrally coordinated to four thiolate groups.

Resonance Raman spectroscopy is an excellent technique for probing charge-transfer transitions. Normal Raman scattering is weak and thus usually impractical for dilute biological samples. However, if the incident radiation approaches the energy of an allowed electronic transition, such as a charge-transfer band, the vibrational modes that are vibronically coupled to the electronic transition will
be enhanced by factors of $10^2$ to $10^6$, depending on the molar extinction coefficient of the transition. When the dioxygenases are probed with radiation within their absorption envelopes, resonance-enhanced Raman features at ~1170, 1270, 1500 and 1600 cm$^{-1}$ are observed [Felton, 1978; Keyes, 1978; Tatsuno, 1978; Bull, 1979; Que, 1979]. These have been identified as characteristic ring vibrations of tyrosinate, thus the visible bands have been assigned as tyrosinate-to-Fe(III) charge-transfer.

The vibrations have been assigned as principally $\nu_{\text{C-H}}$, $\nu_{\text{C-O}}$ and two $\nu_{\text{C-C}}$'s, respectively, based on a normal mode analysis [Tomimatsu, 1976]. In contrast, iron-sulphur proteins exhibit Raman features near 370 and 800 cm$^{-1}$ corresponding to $\nu_{\text{Fe-S}}$ and $\nu_{\text{C-S}}$ [Yachandra, 1983]. Resonance-enhanced tryptophan modes were also observed in spectra of the intradiol enzymes. Fluorescence measurements indicate that tryptophan is close to the active site [Hou, 1978; Nagami, 1973]. Tryptophan, however, is thought to be a poor ligand and the resonance-enhancement probably arises from coupling with tyrosine ring vibrations or from ring stacking interactions with the iron-tyrosinate chromophore. Comparative studies on other dioxygenase complexes provide additional insights into the coordination chemistry of the iron centre [Felton, 1978; Que, 1980, 1981]. Thus the iron centre is coordinated to two endogenous tyrosinates and has a site available for the coordination of exogenous
ligands. The remaining ligands on the iron are proposed to be histidines [Felton, 1982; Wu, 1989] and water, or hydroxide [Whittaker, 1984], giving rise to a five or six coordinated metal centre (Fig. 1.4). NMR resonances in the range 105 to -67 ppm have been assigned by Que [1987] as being β-CH₂ protons on the iron ligands, tyrosine and histidine, and interpretation of the X-ray absorption pre-edge features [Roe, 1984] imply flexibility between five and six coordination. Both of these observations lend credence to the active site proposals. The crystallographic study of P3,4D [Ohlendorf, 1988] further substantiates these proposed ligands and advocates an approximate trigonal bipyramidal geometry at the iron (Figs. 3.9 and 3.10).

Two ferric salen and saloph complexes (N₂O₂ ligand set, Fig. 1.5) [Lauffer, 1981; Heistand, 1982] have been shown to provide a reasonable approximation to the iron environment in the dioxygenases. The nature of the "water" site on these proteins has not yet been addressed. Specifically, given the high Lewis acidity of Fe(III), is the water bound as water or has it undergone hydrolysis to hydroxide? The true nature of the bound water is of considerable importance because this weakly occupied position is the apparent basis of interaction between the iron and external molecules such as substrates and inhibitors. The coordinative unsaturation at the iron provided by the labile water is clearly an important
X is postulated to be a weakly coordinated, easily displaceable ligand which is trans to one of the tyrosines. This proposal allows the complex to distort towards a square pyramidal structure with one apical and one basal tyrosine. The two tyrosines would thus exhibit charge-transfer bands of different energies in agreement with Raman excitation profiles. The absence of thiolate ligation has been deduced from EXAFS, Mössbauer and Raman studies. Redrawn from Roe [1984].
Figure 1.5: Nitrogen and Oxygen Ligand Set Complexes Which Mimic the Iron Environment

The model complexes above contain donor groups (N and O) similar to those implicated in the proteins (histidine and tyrosine) and are coordinatively unsaturated which permits solvent water binding.
feature with respect to its chemical reactivity. In order to investigate this, Spartalian [1983, 1989] and Patch [1983] made use of quinquedentate model complexes containing donor groups similar to those implicated in the proteins and having an open coordination site where solvent water can bind. The Fe-(EHGS), N-[2-((o-hydroxyphenyl)glycino)ethyl] salicyclideneamine, complex (Fig. 1.5) has already proved valuable in the study of the ionisation state of the bound water in the iron binding site of the transferrins because of the distinctive signatures in the optical and Mössbauer spectra of its aquo and hydroxo forms [Carrano, 1985].

Substrate binding in the dioxxygenases in the absence of oxygen results in the development of absorption in the long wavelength region [Kojima, 1967; Fujisawa, 1968]. The persistence of the visible spectrum suggests that the iron centre is not reduced in this complex, as had been suggested earlier. Reduction of the iron centre would have resulted in the bleaching of the charge-transfer band. EPR and Mössbauer studies corroborate the ferric nature of the iron in the enzyme-substrate (ES) complex [Que, 1976; Whittaker, 1984; Kent, 1987]. Raman studies on the ES complexes [Que, 1979] reveal new resonance-enhanced features at ~ 1260, 1320 and 1470 cm⁻¹. These are due only to the substrate, as indicated by spectral shifts when ring-deuterated substrate is used.

The various E, ES and EI (inhibitor) complexes show a
wide variation in colour. In all cases the tyrosinate-to-
Fe(III) charge-transfer band persists, as indicated by
the presence of tyrosinate vibrations in the Resonance
Raman spectra of the complexes [Felton, 1978; Que, 1980,
1981]. These observations indicate that the
charge-transfer transition is sensitive to the nature of
the other ligands in the iron complex and may be used to
deduce the identity of an unknown ligand.

The reaction of the dioxygenase ES complexes with
dioxygen has been investigated with stopped-flow kinetics
[Bull, 1981; Walsh, 1983]. In most cases, two transient
intermediates are observed and their rates of formation
and decomposition can be calculated. From these rate
constants, the visible spectra of the intermediates can be
constructed. The sequence of the reaction is E, ES, ESO,
and ESO₂*. The native enzyme is obtained upon the
decomposition of the second intermediate. The visible
absorption remains in these intermediates, though the
position of the band shifts from one species to the next.
This indicates the persistence of the tyrosinate-to-
Fe(III) charge-transfer band as the exogenous ligand is
transformed from substrate to product. The iron centre
would appear to be in the ferric state in these
intermediates. Mössbauer and EPR studies confirm this
conclusion [Kent, 1987]. Raman spectra [Keyes, 1979]
suggest structural alterations occurring at the active
site upon oxygenation corresponding to changes observed in
the EPR, Mössbauer and visible spectra [Fujisawa, 1972].
No vibrations assignable to either O-O or Fe-O-O modes were observed in experiments using $^{18}\text{O}_2$ and $^{16}\text{O}_2$. This rules out a proposed structure for ESO$_2$ involving a ferric-peroxide (Fe(III)-O$_2^-$) interaction [Que, 1977], since Resonance Raman data on other metal-peroxide complexes clearly indicate the presence of O-O vibrations [Dunn, 1973; Freedman, 1976; Eickman, 1978].

The observation of the ferric oxidation state in the native enzyme, the ES complex and all the transient intermediates suggest the possibility that the iron does not undergo reduction during the catalytic cycle. Indeed the presence of tyrosines in the metal coordination environment serves to stabilise the ferric oxidation state relative to ferrous state by lowering the redox potential [Pyrz, 1985]. The redox potential of the iron has not been accurately determined but preliminary measurements suggest below -300 mV [Whittaker, 1984]. This stabilisation fundamentally affects the mechanism of action for these enzymes [Cox, 1988].

1.6 Dioxygenase Biomimics

The instability of many dioxygenases, in particular those of the extradiol class of enzymes, along with their lack of spectroscopic handles, partially explains why their structures and catalytic mechanisms are as yet abstruse. Although many dioxygenases have been
crystallised, their crystals have not been of diffractable quality and only recently has the first dioxygenase crystal structure been published [Ohlendorf, 1988]. Consequentially a plethora of widely diverging models has emerged that endeavour to mimic the chemical behaviour of the dioxygenase systems.

The catalytic mechanisms of the dioxygenases would seem prone to biomimetic study since no cofactors other than the iron are required for enzyme action. In recent years various kinds of approaches have been carried out. These have involved structural studies on catecholate complexes to elucidate the coordination mode of substrate and other ligands [Lauffer, 1981, 1983; Heistand, 1982; Pyrz, 1985], mechanistic studies to elucidate the active oxygen species, the steps of activation of both catechol and oxygen, and the nature of the reaction intermediates, and catalytic studies to realise the oxygenations in an analogous way to the enzymes. Some of these models test the reactivities of singlet oxygen [Matsuura, 1972; Sternson, 1973], superoxide ion [Moro-Oka, 1976; Müller, 1989], peracid [Pandell, 1983], oxygen in alkaline solution [Grinstead, 1964] and oxygen in the presence of metal complexes. It is known for the most part, that the dioxygenases require either copper or iron for maximum activity and as a result, the majority of model compounds retain this feature from the enzymes. Much time has been devoted to the study of a dimeric Cu(I) species involving
methoxy and hydroxy bridges and complexed pyridine [Tsuji, 1974, 1975, 1978; Rogić, 1978; Demmin, 1981]. However, it has been demonstrated that dioxygen is not required for the cleavage reaction but for regeneration of the cupric ion which is in fact the active oxidising reagent, hence the mechanism of oxygen incorporation is essentially different from that of the iron enzymes. Brown [1979, 1981] successfully catalysed the first full insertion of molecular oxygen using a Cu(II)-bidentate nitrogen ligand species, but by an unspecified mechanism. From the viewpoint of the model, Funabiki thought it important to find iron complexes to catalyse the oxygenation of catechols in a mechanism analogous to that of the enzyme. Initial studies used Fe(II) coordinated by 2,2'-bipyridine and pyridine since the oxygen activation by coordination to Fe(II) seemed to be essential for oxygenation, but he later found that the reaction proceeded in a similar way by using Fe(III)-Cl3 [1983], suggesting a ferric complex as an active species. It was also found that both intra- and extra-diol oxygenations could be catalysed [1986]. Other iron-catalysed oxygenations have been reported to employ Fe(III)-NTA [Weller, 1982, 1985] and Fe(III)-Hbpnp (2-[bis(2-pyridylmethyl)aminomethyl]-4-nitrophenol) [Nishida, 1984]. Other workers in the field of dioxygenase biomimicry have developed catechol-cleavage systems with ruthenium(II) [Matsumoto, 1982], and vanadium(III, IV and V) complexes [Tatsuno, 1982, 1984,
1987; Nishida, 1989], although oxygen incorporation in these systems will be mechanistically distinct from that in the dioxygenases.

1.7 Mechanistic Studies of the Catechol Dioxygenases

The most striking feature of this group of enzymes is their utilisation of molecular oxygen, in contrast to other enzyme classes which reduce dioxygen to either superoxide, peroxide or water. The dioxygenases must therefore have a mechanism for bringing dioxygen into direct interaction with the carbon atoms of the aromatic rings being cleaved. Furthermore, dioxygen exists in a triplet ground state whereas carbon compounds exist in a singlet state. Thus concerted reactions between them, which are kinetically unfavourable, are difficult without some kind of activation to circumvent the formal spin conservation forbiddenness. In general, understanding of the mechanism for oxygen activation by non-heme oxygenases is rudimentary compared to that for the heme-containing dioxygenases because of the dearth of information regarding the nature of their active sites. Since the intradiol dioxygenases contain exclusively ferric iron [Que, 1976], and since no complexes containing an Fe(III)-O$_2$ bond are known, it is unlikely that the spin inversion occurs by direct interaction with the metal. More plausibly, the initial interaction with dioxygen is directly with enzyme-bound substrate which would lead to
radical substrate and oxygen intermediates. Despite the fact that Fe(II) shares the same preference for coordination number and geometry as ferric iron, it possesses an outstanding ability to form coordination complexes with molecular oxygen. Resultant dioxygen activation would therefore precede ionic attack on the substrate in the ferrous-containing extradiol dioxygenases. Thus mechanistic differences appear to arise as a consequence of the redox state of the iron in the enzyme. Other salient and controversial mechanistic features include the mode of substrate binding, the nature of the intermediate(s) and the putative alteration in the coordination sphere of the metal during enzymatic turnover.

Early mechanistic postulates, by analogy with the oxygenation of hemoglobin, required the binding of dioxygen to a ferrous centre to serve as the oxygen activation step. Since the native intradiol enzymes were known to have a ferric centre, it was proposed that substrate binding resulted in the reduction of the ferric centre followed by oxygen binding and product formation. However, the spectroscopic data accumulated to date argues against such a mechanism, since the enzyme-substrate complexes are clearly ferric complexes. This data has forced a re-evaluation of the postulated mechanism and has resulted in a different proposal of substrate rather than oxygen activation [Que, 1977, 1987]. Based on several
studies a mechanistic cycle has been proposed in which deprotonated substrate hydroxyl groups initially chelate to the active site occupying both of the iron sites available for exogenous ligands [Que, 1981; Lauffer, 1982; Whittaker, 1984; Orville, 1989]. The high affinity of catecholate for ferric ion has been demonstrated by Raymond [1976], however, it is this stabilisation due to chelation that one would want to avoid in order to obtain a reactive species. Model studies indicate that a chelated substrate is a poor reductant for dioxygen [Lauffer, 1981; White, 1984; Que, 1987]. Support for the monodentate mode of catecholate coordination has been obtained from NMR study of the catechol 1,2-dioxygenase enzyme-substrate complexes [Heistand, 1982; Que, 1987] and models [Que, 1979; Lauffer, 1983]. The observations of Que and Lauffer suggest that the preference for substrate oxygen (deprotonated hydroxyl) is presumably dictated by steric factors in the active site. Similar conclusions have been drawn from Mössbauer, EPR [Pyrz, 1985] and UV/visible spectrophotometric [Kent, 1987] studies, where the dissociation of the second, uncoordinated hydroxyl proton, partially if not wholly, is implicated as enhancing the tendency of the substrate to react with dioxygen.

The Lewis acidity of the ferric centre (as modulated by the active site ligands [Cox, 1988]), acts to effect ketonization of the hydroxyl group of the enzyme-bound
substrate to form a species of carbanion. Tautomerisation of the substrate is thought to be critical since this would "activate" the substrate for electrophilic attack provided that the enzyme structure prevents the substrate from forming the more stable chelate complex. Studies with a transition state analog [May, 1982; Whittaker, 1984], led to the prediction that ketonized substrate was contained in the first oxy complex [Bull, 1981] since similar spectra were not observed in the native reaction prior to dioxygen addition. Therefore, it is probable that molecular oxygen is required to promote and stabilise the keto tautomer of the substrate, forcing the carbon (C*, Fig. 1.6) atom to become tetrahedral.

This carbanion, which acts as the reductant for molecular oxygen, generates an intermediate radical pair. Recoupling of these radicals is proposed to lead to an intermediate peroxide as the substrate-oxygen adduct. The formation of this intermediate is preceded in the literature and is analogous to the reaction of reduced flavin with oxygen [Walsh, 1977]. This peroxide has also been proposed to be the first intermediate (ESO₂) observed in the stopped-flow studies. The blue shift of the charge-transfer band would be consistent with the coordination of peroxide to the ferric ion, since the alkyl peroxide would have a basicity similar to that of phenolate. Studies of Roe [1984] and Whittaker [1984] independently point to the flexibility of the active site.
The dioxygenase mechanism for intradiol cleavage (opposite) is shown to proceed via the seven-membered, Hamilton intermediate, however, the cyclic four-membered dioxetane (Hayaishi intermediate, below) would also afford the product.
An easily dissociable ligand would provide a readily available site during the catalytic cycle to bind an intermediate species. Such a site could serve to coordinate the distal oxygen of the organic peroxide formed during the postulated structural rearrangement. Observations from inhibitor binding studies of Orville [1989], substantiate such an organisation. Kinetic studies [Cox, 1988] show that the rate-determining step of carbon-carbon oxidative cleavage involves the attack of dioxygen on model enzyme-substrate complexes.

The intermediate peroxide subsequently rearranges with insertion of dioxygen into the ring system. The decomposition affords either a cyclic four-membered ring dioxetane (Hayaishi intermediate [1955]) or the more thermodynamically favourable seven-membered anhydride (Hamilton intermediate [1974]), both of which lead to product upon opening. The formation of the latter would be a result of Crigee rearrangement (Baeyer-Villiger reaction) while the highly-strained dioxetane is similar to intermediates observed in chemiluminescent reactions [Hiatt, 1971]. Evidence for the intermediacy of both the dioxetane [Hayaishi, 1957; Grinstead, 1964; Pandell, 1983] and the anhydride [Que, 1980; Saeki, 1980; Matsumoto, 1982; Weller, 1982; Tatsuno, 1982, 1984, 1987; Mayer, 1984; Funabiki, 1986; Nishida, 1989] have been amply demonstrated, however, the proposal of the one-step oxygenation via the dioxetane seems to have been
superseded by that of the stepwise insertion process via the anhydride.

Arciero [1986] postulated that extradiol dioxygenases activate molecular oxygen by directly coordinating it to the iron in the active site (Fig. 1.7). His studies showed that there is a special ligand position reserved on the active site Fe$^{2+}$ which is accessible to nitric oxide, an oxygen analog [Arciero, 1985]. Thus, in the extradiol dioxygenases there appears to be three iron ligand sites available to exogenous ligands, one of which is particularly sensitive to small oxygen-like molecules. These conclusions are opposite to those reached from related studies [Whittaker, 1984; Orville, 1989] of the active site structure of intradiol dioxygenases where substrate and oxygen are mutually exclusive ligands. However, in the proposed intradiol mechanism there is no need to have a separate site because the substrate is activated through the formation of a complex with the Fe$^{3+}$ for direct attack by dioxygen.

Although the quaternary structures of the Fe(III)-containing intradiol and Fe(II)-containing extradiol dioxygenases vary significantly, the iron sites appear to be remarkably well conserved and the two distinct enzyme classes appear to share major catalytic strategies. The iron serves an organisational role, assuring that the organic substrate and dioxygen are held in close proximity and in the proper alignment for the
The proposed mechanism for the extradiol cleaving catechol dioxygenases (e.g., P4,5D opposite redrawn from Arciero [1986]) shows at least two and probably three iron coordination sites being vacant or occupied by displaceable ligands. R₁ and R₂ may be additional water, an amino acid side chain or an unoccupied ligand position. The most important aspect of the model is that the organic substrate and molecular oxygen bind directly to the iron.
highly site specific ring-opening reactions. Additionally, by simultaneously coordinating both species in the critical intermediates of the dioxygenase reactions, the iron may serve to facilitate a net shift in electron density from the aromatic ring towards oxygen, thus establishing conditions for facile oxygen-oxygen bond cleavage.
1.8 References


Mason, H.S., (1957), Science, 125, 1185-1188.

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Interactions, 7, 143-150.


CHAPTER 2

ISOLATION AND PURIFICATION OF 3-METHYLCATECHOL

2,3-DIOXYGENASE FROM *ESCHERICHIA COLI*
2.1 Introduction

Recently, McClure and Venables have described a *Pseudomonas putida* strain, UCC2, able to utilize meta- or para-toluidine as sole carbon and nitrogen source [McClure, 1986, 1987]. Strain UCC2 expresses a novel extradiol dioxygenase which is plasmid encoded, Fig. 2.1, [McClure, 1987]. A DNA fragment containing the structural gene for this dioxygenase has been cloned into vectors for direct expression in *Escherichia coli* (*E. coli*) [McClure, 1987; McClure, unpublished results]. In an effort to gain insights into the chemical and physical properties of this enzyme, it was necessary first to purify it, therefore, the following sections describe our first attempts to manipulate this novel enzyme, which we have called 3-methylcatechol 2,3-dioxygenase (3MC2,3D) [Wallis, 1990].

2.2 Strains, Media and Growth of Bacteria

3-Methylcatechol 2,3-dioxygenase was isolated from *E. coli* JM107 [Yanisch-Perron, 1985], containing pNMN24 (a gift from Dr. N.C. McClure, School of Pure and Applied Biology, University of Wales, Cardiff), which is an expression vector containing the structural gene encoding the enzyme [McClure, unpublished results]. Plasmid-bearing *E. coli* cells were grown aerobically in Luria Broth (LB) [Maniatis, 1982] supplemented with 100 µg/ml ampicillin (Sigma, to induce synthesis of 3MC2,3D, Section 6.1.1) at 37°C on an orbital shaker (Gallenkamp incubator,
Figure 2.1: An Expression Vector for 3-Methylcatechol 2,3-Dioxygenase in E. coli

Abbreviations:
- C2,30, the gene encoding for 3-methylcatechol 2,3-dioxygenase
- bla, the β-lactamase gene which allows ampicillin resistance
- ori, the origin of replication

The diagram shows pNMN24, which is an expression vector containing the structural gene encoding the enzyme.
250 r.p.m.) for about 12 hours. Cells were harvested at early stationary phase (Fig. 2.2, $A_{650} = 1.35$ to 1.45) by centrifugation (15,000 g, 5 min, Sorvall RC-5B refrigerated superspeed centrifuge, Section 6.2) at 0 to 4°C for 3 minutes. On average 5 to 6 g of cells were obtained from 1 l of liquid culture originally inoculated with one bacterial colony. This is approximately 20 times the amount of Pseudomonas putida cells which results from toluidine-induced growth (Section 6.1.3). These beige coloured cells were stored wet at -20°C until required. The cells could be stored frozen for several months without any noticeable effect on enzyme yield or activity.

2.3 Enzyme Isolation

The isolation procedure was derived from the original extradiol dioxygenase preparation published by Nozaki in 1963, however, substantial modifications were made to tailor the procedure for purification of 3-methylcatechol 2,3-dioxygenase. All subsequent extraction and purification procedures were carried out between 0 and 4°C. The buffer used throughout was phosphate buffer (0.05 M, pH 7.50) containing 10% acetone (Sections 2.4.3 and 6.3.3), hereafter referred to as acetone-phosphate buffer.

2.3.1 Step 1 : Crude Extract

50 g (wet weight) of frozen cells were thawed and
The increase in concentration of bacterial cells was followed by spectrophotometry, although at the wavelength used (650 nm), scattering accounted for all of the apparent absorbance. Each point (half hour intervals) represents the average of eight separate determinations. Little sign of a lag phase is detectable and the curve shows a gradual tapering off of growth as stationary phase is approached.
suspended in 500 ml of acetone-phosphate buffer. Lysozyme (from Chicken Egg White, Grade II, Sigma, Section 2.4.1) was added to approximately 0.2 mg/ml along with a small amount (1 mg) of deoxyribonuclease I (from Bovine Pancrease, Sigma, Section 2.4.2). The cell suspension was then left standing for 30 minutes. The mixture was centrifuged (15,000g, 10 minutes, Section 6.2) to remove cell debris and any unbroken cells.

2.3.2 Step 2 : Ammonium Sulphate Fractionation

The resulting supernatant was brought to 80% ammonium sulphate (Fisons, SLR, Section 6.4) saturation in 20% stages. These suspensions were centrifuged (39,000g, 15 minutes) and the precipitates were resuspended in a minimum volume of acetone-phosphate buffer. The 60% ammonium sulphate pellet contained 99% of the 3NC2,3D activity, therefore the other fractions and the remaining supernatant were discarded at this point. Any residual insoluble material was removed by centrifugation (as above) and the resulting clear supernatant solution was dialysed (Sections 2.4.5 and 6.5) against two changes (11) of acetone-phosphate buffer overnight. The volume increase on dialysis was slight (approximately 10%).

2.3.3 Step 3 : Ion Exchange Chromatography

Dialysed material was applied to a DE52 (Whatman) ion exchange column (30 cm x 3 cm, Section 6.6.2) previously
equilibrated with acetone-phosphate buffer. After washing
the column with one column-volume of the same buffer, to
remove any unbound protein, the enzyme was eluted with a
linear gradient established between acetone-phosphate
buffer (mixing chamber) and acetone-phosphate buffer
containing 5% ammonium sulphate (reservoir). 500 ml of
both the mixing and reservoir solutions were employed,
respectively. Fractions (50 ml) were collected manually
from the column and assayed immediately for enzyme
activity (Section 6.7). The activity of 3MC2,3D was
assayed by measuring the increase in absorbance centred
around 375 nm. The yellow colouration is due to the
accumulation of the product, 2-hydroxy-6-oxohexa-2,4-
dienoic acid, resulting from the extradiol ring fission of
the substrate, catechol. The desired enzyme, 3MC2,3D, was
eluted at a salt concentration of between 2.5 and 3.5%
(Fig. 2.3) from the column which exhibited a flow rate of
between 120 and 135 ml/hr, hence this chromatographic step
alone required 7 to 8 hours. Fractions containing active
enzyme were combined and brought to 70% ammonium sulphate
saturation. After centrifugation (39,000 g, 15 minutes)
the resulting precipitate was dissolved in acetone-
phosphate buffer and concentrated to less than 5 ml using
pressure dialysis (Sections 2.4.7 and 6.8). This
procedure necessitated the use of an Amicon
ultrafiltration cell and Diaflo membrane.
Figure 2.3: DE52 Elution Profile Showing the Emergence of 3-Methylcatechol 2,3-Dioxygenase Activity

The profile shows that 3-methylcatechol 2,3-dioxygenase was eluted as a single peak of activity, centred at approximately 3% ammonium sulphate.
2.3.4  **Step 4 : Gel Filtration**

Aliquots (1 ml) of this concentrated, partially purified, enzyme solution were applied to a Sepharose S300 (Pharmacia LKB) gel filtration column (150 cm x 3 cm, Sections 2.4.8 and 6.6.3) previously equilibrated with acetone-phosphate buffer. The enzyme was washed through the column with the same buffer at a flow rate of between 13 and 14 ml/hr. Fractions (200 drops, 6 ml) were collected overnight, using either a Biorad 2110 or a Gilson Microcol TDC80 fraction collector, and assayed for enzyme activity. 3MC2,3D emerged from the column as a single peak of activity, Fig. 2.4. Active fractions were pooled and concentrated to less than 5 ml using the aforementioned pressure concentration method.

2.3.5  **Isolation and Purification Summary**

This preparation produced concentrated, purified enzyme in 4 to 5 days, which, in the presence of 10% acetone could be stored at -20°C, with no detectable loss of activity, over a period of several months. The yield of enzyme, which was a sufficient quantity to support a substantial amount of work, and specific activity at each stage in the procedure was determined and a summary of a typical purification protocol is presented in Table 2.1. Following these stages the overall purification was 30 fold with a yield of 40%, these findings agree with
The profile obtained following elution showed at least three major peaks. To isolate the peak corresponding to 3-methylcatechol 2,3-dioxygenase, fractions were assayed before the peaks were pooled. The active fractions were combined separately.
Table 2.1: Purification Details for the Isolation of 3-Methylcatechol 2,3-Dioxygenase from *E. coli*

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Purity (mg)</th>
<th>Specific Activity (milliunits)</th>
<th>Recovery Purifn. (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP 1: Crude Extract Supernatant after Cell Lysis</td>
<td>4,180</td>
<td>245</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>STEP 2: Ammonium Sulphate Fraction after Dialysis</td>
<td>3,370</td>
<td>275</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>STEP 3: After DE52 Ion Exchange Chromatography</td>
<td>915</td>
<td>870</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>STEP 4: After S300 Gel Filtration</td>
<td>55</td>
<td>7,630</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

The table is based on an extraction from 50 g, wet weight, of cells.

Specific activity is expressed as the number of μmoles of product produced per minute per mg of protein, at pH 7.00 and 25°C.
results observed for other dioxygenases (Table 2.2). The specific activity of the final preparation was 7,630 milliunits.

2.4 Discussion of Isolation Techniques Employed and Comparison with Other Dioxygenase Preparations

2.4.1 Cell Lysis

In general, the first step towards enzyme isolation is cell lysis. One of the most common methods of breaking cells is to expose them to high intensity sonic fields. Cavitation is caused by the dissolved gases coming out of solution as bubbles and the disruption of cells results from the violent collapse of these bubbles. The literature preparations of several types of dioxygenases, from differing bacterial species, showed that sonic oscillation was frequently employed for lysis. However, this sometimes causes damage to the cell contents resulting in loss of enzyme activity. Alternative reported protocols include acetone powder treatment [Kita, 1965] and 'crushing' in a bacterial press with the abrasive, aluminium oxide [Takeinori, 1971; Crawford, 1975].

From the experience of earlier work (results not shown) it was decided to use lysozyme treatment, which is another alternative method for rupturing cells, in preparation for the isolation of internal components. Lysozyme is a degradative enzyme which catalyses the
The table opposite summarises final purification data for homogeneous preparations of several extradiol and two typical intradiol dioxygenases. The average purification fold (excluding *) was 36 and the average percentage yield was 29.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacterial Source</th>
<th>Number of Purification Steps</th>
<th>Purification Yield (Fold)</th>
<th>Percentage Yield (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td>P.arvilla</td>
<td>6</td>
<td>29</td>
<td>15.0</td>
<td>Nozaki, 1963</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P.putida mt-2</td>
<td>5</td>
<td>28.9</td>
<td>29</td>
<td>Nakal, 1983</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P.putida mt-2</td>
<td>5</td>
<td>53.3</td>
<td>45</td>
<td>Nakal, 1983</td>
</tr>
<tr>
<td>P4,5D</td>
<td>P.testosteroni</td>
<td>5</td>
<td>21</td>
<td>36</td>
<td>Dagley, 1968</td>
</tr>
<tr>
<td>P4,5D</td>
<td>Pseudomonad</td>
<td>4</td>
<td>44.9</td>
<td>26.5</td>
<td>Ono, 1970</td>
</tr>
<tr>
<td>P4,5D</td>
<td>P.testosteroni</td>
<td>8</td>
<td>37.2</td>
<td>50.3</td>
<td>Arclero, 1983</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td>P.ovalis</td>
<td>4</td>
<td>47.7</td>
<td>41.5</td>
<td>Kita, 1965</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td>B.Stearothermophilus</td>
<td>6</td>
<td>8.1</td>
<td>1.9</td>
<td>Jamaluddin, 1977</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td>B.brevis</td>
<td>4</td>
<td>18</td>
<td>57</td>
<td>Que, 1981</td>
</tr>
<tr>
<td>G1,2D</td>
<td>P.acidovorans</td>
<td>5</td>
<td>61.3</td>
<td>31</td>
<td>Harpel, 1990</td>
</tr>
<tr>
<td>G1,2D</td>
<td>P.testosteroni</td>
<td>6</td>
<td>791*</td>
<td>23</td>
<td>Harpel, 1990</td>
</tr>
<tr>
<td>S4,5D</td>
<td>N.restrictus</td>
<td>5</td>
<td>41.8</td>
<td>14</td>
<td>Tai, 1970</td>
</tr>
<tr>
<td>C1,2D</td>
<td>P.arvilla Cl</td>
<td>6</td>
<td>39.5</td>
<td>13.5</td>
<td>Kojima, 1967</td>
</tr>
<tr>
<td>C1,2D</td>
<td>P.putida mt-2</td>
<td>6</td>
<td>45.8</td>
<td>24.4</td>
<td>Nakal, 1988</td>
</tr>
<tr>
<td>P3,4D</td>
<td>P.aeruginosa</td>
<td>6</td>
<td>30</td>
<td>30</td>
<td>Fujisawa, 1970</td>
</tr>
</tbody>
</table>
hydrolysis of polysaccharide that is the major constituent of the cell walls of certain bacteria. Our experience has shown both sonic oscillation and lysozyme treatment to be equally effective; however, the latter technique has several advantages including simplicity, speed, availability and it presents no restriction on sample size. None of the other aforementioned methods surpass lysozyme treatment, which was employed in all enzyme preparations (Section 2.3.1).

2.4.2 Nucleic Acid Treatment

Deoxyribonuclease (Section 2.3.1) was incorporated to reduce the viscosity of the working material, caused by released DNA, and thus provided a substantially concentrated and more manageable enzyme extract to manipulate. The nucleic acids were removed early on in the preparation to prevent difficulties arising in the chromatographic steps.

Reports of other dioxygenase preparations from Bacillii, Nocardii and Pseudomonads have tackled this viscosity problem by a variety of methods including; streptomycin sulphate treatment [Kojima, 1967], freeze-thawing [Tai, 1970], ultracentrifugation [Ono, 1970], protamine sulphate treatment [Fujisawa, 1970; Jamaluddin, 1977; Lee, 1977; Arciero, 1983], and heat treatment [Que, 1981; Harpel, 1990], nevertheless, deoxyribonuclease was found to be the most convenient.
2.4.3 **Acetone-Phosphate Buffer**

The presence of a low concentration of an organic solvent such as acetone [Nozaki, 1963], ethanol or glycerol [Tai, 1970] is known to protect Fe(II) dioxygenases from inactivation by aerial oxidation to the Fe(III) form. Although the mechanism of this protection is not yet fully understood, extensive studies with metapyrocatechase (C2,3D) have shown that these organic solvents inhibit competitively with respect to substrate [Nozaki, 1968; Kachhy, 1976] thereby suggesting that their interaction with enzyme is at the active site.

Acetone-phosphate buffer was employed throughout the 3-methylcatechol 2,3-dioxygenase preparation in order that aerial inactivation was minimised. Acetone, present as 10%, did not appear to interfere with either the action of the degradative enzymes or with the chromatography. The Fe(II)-containing dioxygenases have been repeatedly handled in a low concentration of acetone, moreover, [Que, 1981] acknowledged that 3,4-dihydroxyphenylacetate 2,3-dioxygenase (3,4 DHPA 2,3D) from *Bacillus brevis*, the only reported dioxygenase utilising Mn(II), was also stabilised by this means. Que, however, opted to omit the organic solvent from elution buffers in order to be able to monitor UV absorption, arguing that no significant deterioration of the enzyme was observed during the time the enzyme was on the columns. A number of investigators

2.4.4 Ammonium Sulphate Fractionation

In the course of isolating and purifying macromolecules, solutions often become very dilute. Salting out with ammonium sulphate, which is dependent on relative hydrophobicity (Section 6.4), is an easy way to precipitate proteins which can be redissolved in a smaller volume of whatever buffer is required, after centrifugation. 3-Methylcatechol 2,3-dioxygenase was treated in this way, as in numerous other intra- and extra-diol dioxygenase isolations, not to achieve a large purification, but mainly as a step by which the working volume of enzyme could be dramatically reduced (Sections 2.3.2 and 2.3.3) making column loading much quicker. Several laboratories have employed acetone [Nozaki, 1963; Kita, 1965; Tai, 1970] or ethanol [Ono, 1970], present at 66% for protein fractionation, in spite of experiments [Nozaki, 1963] showing these solvents to be potent dioxygenase inhibitors.
2.4.5 Dialysis

Dialysis (Section 6.5) was employed to exchange solute, as in most other literature preparations.

2.4.6 Ion Exchange Chromatography

Ion exchange chromatography on diethylaminoethyl (DEAE) cellulose (Section 6.6.2), a weak anionic exchanger, was chosen as the first stage of 3MC2,3D purification due to its effective separation of a plethora of different dioxygenases. After the application of crude extract to the DEAE cellulose, the unbound material was washed out with a salt-free buffer solution before development of the column with a linear gradient of ammonium sulphate. The salt displaced the desired protein, by competing for the positively charged sites, at a characteristic ammonium sulphate percentage (Section 2.3.3) which separated it from similarly charged impurities. 3MC2,3D did not bind to the cationic exchanger, carboxymethyl (CM), suggesting that the dioxygenase was overall negatively charged (Section 3.3.2).

2.4.7 Pressure Dialysis

The partially purified protein was subjected to pressure dialysis (Section 6.8) before application to the molecular filtration column (Section 2.3.3). This was necessitated on two accounts; firstly to remove ammonium
sulphate, and secondly to reduce the volume of enzyme in order to maximise the final resolution. Pressure dialysis was also utilised to concentrate the final purified protein (Section 2.3.4). A significant proportion of published dioxygenase isolation procedures did not employ gel filtration as a purification step and therefore did not require pressure dialysis as a consequence. In those preparations that did, however, desalting was alternatively achieved by dialysis or by passage through an extra column.

2.4.8 Gel Filtration

The native molecular weights of the majority of extradiol dioxygenases lie in the range 100,000 to 150,000, subsequently Sepharose S300 was chosen to serve as a molecular filtration network providing a molecular weight cut off of 300,000 Da. Other laboratories preferred gels with a limit of 200,000 Da [Kojima, 1967; Dagley, 1968; Kachhy, 1976; Que, 1981; Nakai, 1988; Harpel, 1990]. The elution profile of the collected fractions was obtained by monitoring spectrophotometrically at 300 nm (Section 6.9) for the protein content in conjunction with assaying (Section 6.7) at 375 nm for any dioxygenase activity. The combination of these two measurements allowed the 3MC2,3D compositions of each fraction to be determined.
2.5 **Discussion of a New Dioxygenase Purification Protocol**

It was decided to develop a purification procedure for this novel catechol dioxygenase since existing procedures, all seemingly derived from Nozaki [1963], were unsuitable or ineffective.

Initially, as discussed fully in Section 2.4.1, the limitations imposed by sonic oscillation meant that cell lysis was lengthy which increases the likelihood of protein denaturation. Secondly, the acetone precipitation step led to a drastic inactivation of the enzyme, a problem also encountered by Kachhy [1976], and consequently this stage was abandoned in favour of the 'harmless' fractionation by ammonium sulphate (Section 2.4.4). Furthermore, the recommended procedure involved a final stage of three crystallisations with finely powdered ammonium sulphate which is reported to induce the formation of white needlelike crystals after several hours at 0°C. However, failure to crystallise any active 3-methylcatechol 2,3-dioxygenase after two days in two separate attempted preparations, also necessitated modification at this stage. Endeavours to crystallise other dioxygenases were similarly unsuccessful [Dagley, 1968; Arciero, 1983] or extremely time consuming [Kita, 1965]. Moreover, this procedure does not increase the specific activity of the enzyme [Nozaki, 1963; Kita, 1965; Arciero, 1983].
In conclusion, we have developed a protocol allowing efficient purification of the enzyme 3-methyl catechol 2,3-dioxygenase to homogeneity (as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis) within four to five days and in good yield.
2.6 References


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CHAPTER 3

PRELIMINARY CHARACTERISATION OF
3-METHYLCATECHOL 2,3-DIOXYGENASE
3.1 Introduction

The structure of a metalloprotein may be elucidated by employing a number of experimental techniques. The following chapters describe our attempts to characterise 3-methylcatechol 2,3-dioxygenase. This chapter is concerned with the physical properties of the protein component of the enzyme namely the molecular weight, the amino acid sequence and the protein stability.

3.2 Experimental

3.2.1 Subunit Molecular Weight Determination

In the presence of the ionic detergent sodium dodecyl sulphate (SDS) most proteins are dissociated and denatured. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Section 6.10) was employed to determine the molecular weight of the denatured protein, i.e., the relative molecular weight ($M_R$) of the subunit. The $M_R$ was determined by linear regression from a 12% polyacrylamide gel calibrated with six standards of known molecular weight, ranging between 12,300 and 78,000 (Section 6.10), Fig. 3.1.

3.2.2 Native Molecular Weight Determination

The molecular weight of the enzyme in the native state was determined by gel filtration (procedure as described earlier, Sections 2.3.4 and 2.4.8, see also Section 6.6.3) from a mixture of solubilised Blue Dextran added to a
Figure 3.1: Subunit Molecular Weight Determination of 3-Methylcatechol 2,3-Dioxygenase by SDS-PAGE

$D_r$ represents the distance migrated by the protein of unknown molecular weight (denaturing conditions), $M_r$ is the relative subunit molecular weight which is ascertained from the calibration by linear regression.

Calibration curve for the determination of subunit molecular weight from a 12% acrylamide gel in 1% SDS. The method is described in the text (Sections 3.2 and 6.10) and the protein molecular weight markers were as follows; cytochrome C (12,300 Da), myoglobin (17,200 Da), carbonic anhydrase (30,000 Da), ovalbumin (42,700 Da), albumin (66,250 Da) and ovotransferrin (76-78,000 Da).
sample of concentrated, purified enzyme using a Sepharose S300 gel filtration column previously calibrated with molecular weight markers (Section 6.6.3), Fig. 3.2.

3.2.3 NH₂-Terminal Amino Acid Sequence Analysis

The amino acid sequence of the NH₂-terminal portion of 3-methylcatechol 2,3-dioxygenase (1000 p.mol., > 90% pure) was determined by automated Edman degradation [Edman, 1967], Fig. 3.3. The analysis was performed on an Applied Biosystems 477A sequencer by the WELMET protein characterisation facility at the University of Edinburgh.

3.2.4 Thermal Stability

The time-dependence of thermal inactivation of the 3-methylcatechol 2,3-dioxygenase was determined by heating enzyme solutions (crude extract, 10 ml, approximately 5.6 μg/ml, dissolved in 10% acetone-phosphate buffer, 0.05M, pH 7.50) for a 1 hour period in a water bath. After a specific heating time (5 minutes) samples (25 μl) were withdrawn and assayed for remaining dioxygenase activity, as previously described (Section 6.7). Enzyme activity was measured before and after heating to determine residual activity at each temperature. This procedure was carried out with fresh samples of crude extract at 10°C intervals between 25 and 75°C.
Calibration for the determination of native molecular weight under non-denaturing conditions. The method is described in the text (Sections 2.3.4, 2.4.8, 3.2.2 and 6.6.3) and the protein standards were as follows; cytochrome C (M.Wt. 12,500 Da), myoglobin (17,500 Da), carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), alcohol dehydrogenase (150,000 Da) and \( \beta \)-amylase (200,000 Da).
The procedure for the sequential degradation of a protein from its NH$_2$-terminus (opposite), developed by Edman, can be summarised in three stages:

1. The COUPLING of phenylisothiocyanate (PITC) to the free NH$_2$-terminal amino group. This coupling takes place in alkaline conditions (8 < pH < 10), because the free, uncharged amino group is required.

2. The CLEAVAGE takes place under the influence of anhydrous strong acid. Cleavage yields an anilinothiazolinone (ATZ) amino acid residue and a new NH$_2$-terminal amino acid which can be coupled again.

3. The ATZ residue is unstable and cannot easily be identified as such, therefore CONVERSION to the more stable phenylthiohydantoin (PTH) amino acid has to be carried out using aqueous or methanolic acid.
3.3 Results and Discussion

3.3.1 Molecular Weight Determination

Fully purified 3-methylcatechol 2,3-dioxygenase, treated with SDS, revealed the presence of a single protein band on a Coomassie-stained polyacrylamide gel indicating the presence of only one type of subunit. The subunit molecular weight was determined, from the migration position, to be $33,500 \pm 2,000$.

The vast majority of extradiol dioxygenases exhibit only one subunit type, see Table 3.1, and generally the relative molecular weight of these subunits lies within the range 31,000 to 36,000. There are two notable exceptions. Firstly, gentisate 1,2-dioxygenase (G1,2D) [Harpel, 1990] shows a slightly larger subunit molecular weight of either 38,500 or 40,800 depending on the Pseudomonas species. Again, experimental evidence indicates the presence of only one subunit type. Secondly, protocatechuate 4,5-dioxygenase (P4,5D) from Pseudomonas testosteroni [Dagley, 1968; Arciero, 1983] possesses two non-identical subunit types. This is characteristic of intradiol dioxygenases [Nozaki, 1988], but is unique among the extradiol enzymes.

Comparison with the previously performed gel filtration calibration indicated that the native 3-methylcatechol 2,3-dioxygenase was eluting at a position corresponding to an $M_r$ of $120,000 \pm 20,000$, an average value seen for the native extradiol dioxygenases (Table
Table 3.1: Molecular Properties of Some Extradiol Dioxygenases

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BACTERIAL SOURCE</th>
<th>NATIVE MOLECULAR WEIGHT</th>
<th>SUBUNIT MOLECULAR WEIGHT</th>
<th>NUMBER OF SUBUNITS</th>
<th>MOLECULAR FORM</th>
<th>METAL REQUIREMENT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td><em>P. arvilla</em></td>
<td>140,000^o</td>
<td>35,000^o</td>
<td>4</td>
<td>α4</td>
<td>Fe^{2+}</td>
<td>Nozaki, 1963, 1983; Nakai, 1983; Ghosal, 1988</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em> mt-2</td>
<td>(±3,000)</td>
<td>35,155^o</td>
<td>4</td>
<td>α4</td>
<td>Fe^{2+}</td>
<td>Lee, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34,873^o</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em> U</td>
<td>116,000^o</td>
<td>31,000^o</td>
<td>4</td>
<td>α4</td>
<td>-</td>
<td>Lee, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(±2,000)</td>
<td>(±1,000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em> F1</td>
<td>-</td>
<td>27,200^*</td>
<td>8</td>
<td>α8</td>
<td>Fe^{2+}</td>
<td>Zylstra, 1989</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em> UCC2</td>
<td>120,000^*</td>
<td>33,000^*</td>
<td>4</td>
<td>α4</td>
<td>Fe^{2+}</td>
<td>Wallis, 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 20,000)</td>
<td>34,781^o</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P. testosterone</em></td>
<td>140,000^o</td>
<td>21,000α</td>
<td>4</td>
<td>α2β2</td>
<td>Fe^{2+}</td>
<td>Dagley, 1968; Zabinetski, 1972</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35,000β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>Pseudomonad</em></td>
<td>150,000^*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fe^{2+}</td>
<td>Ono, 1977</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P. testosterone</em></td>
<td>142,000^*</td>
<td>17,700α</td>
<td>4</td>
<td>α2β2</td>
<td>Fe^{2+}</td>
<td>Arcler, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33,800β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cont'd...
| 3,4DHPA₂,₃D | **P. ovalis** | 120,000⁺⁺ (±20,000) | 35,000 | 4 | α₄ | Fe²⁺ | Kita, 1977 |
| 3,4DHPA₂,₃D | **B. steare-thermophilus** | 106,000⁺⁺⁺ (±1,000) | 34,000 | 3 | α₃ | - | Jamaluddin, 1977 |
| 3,4DHPA₂,₃D | **P. putida U** | 103,000⁺⁺⁺ (±2,000) | 31,000 | 3 | α₃ | - | Lee, 1977 |
| 3,4DHPA₂,₃D | **B. brevis** | 130,000⁺⁺⁺ (±10,000)⁺⁺⁺ | 36,000 | 4 | α₄ | Mn²⁺ | Que, 1981 |
| G1,2D | **P. acidovorans** | 164,000 (±5,000) | 38,500 (±2,300) | 4 | α₄ | Fe | Harpel, 1990 |
| G1,2D | **P. testosteroni** | 158,000⁺⁺⁺⁺⁺ (±5,000) | 40,800 (±1,000) | 4 | α₄ | Fe²⁺ | Harpel, 1990 |
| 1,2DHN₂,₃D | **P. putida** | - | 33,882⁺⁺⁺⁺⁺ | - | - | - | Harayama, 1989 |
| 1,2DGB₂,₃D | **P. pseudoal-** | - | 33,074⁺⁺⁺⁺⁺ | 8 | α₈ | - | Furukawa, 1987 |
| 1,2DHB₂,₃D | **P. pauci-** | - | 32,964⁺⁺⁺⁺⁺ | - | - | - | Taïra, 1989 |

Native molecular weight (Mr) determination by gel filtration (*), polyacrylamide gel electrophoresis (#) and ultracentrifugation (o).

Subunit molecular weight determination from polyacrylamide gel electrophoresis (Mr, #) and nucleotide sequencing (MWt, o).
3.1). This result, in combination with the SDS-PAGE denatured molecular weight determination (Section 3.2.1), is consistent with the native enzyme existing as a tetramer of identical subunits, $\alpha_4$. This is similar to catechol 1,2-dioxygenase (C1,2D), 3,4-dihydroxyphenylacetate 2,3-dioxygenase (3,4 DHPA 2,3D) from *Pseudomonas ovalis* and *Bacillus brevis*, and two gentisate 1,2-dioxygenases, see Table 3.1 for references. It is therefore likely that these enzymes have similar quaternary structures. In stark contrast, the intradiol dioxygenases show remarkable diversity in physical terms, even within one enzyme type. For example, the isofunctional protocatechuate 3,4-dioxygenases (P3,4D) manifest their subunit compositions as $(\alpha\beta)_4$, $(\alpha\beta)_5$ and $(\alpha_2\beta_2)_8$ in *Pseudomonas cepacia* [Bull, 1979], *Brevibacterium fuscum* [Whittaker, 1984] and *Pseudomonas aeruginosa* [Fujisawa, 1968], respectively. Native intradiol dioxygenase molecular weights also vary widely between 60,000 [Nakai, 1979] and 700,000 [Fujisawa, 1968].

3.3.2 **Amino Acid Sequence Analysis**

The properties of a protein depend fundamentally on the order of amino acids that are polymerised to form its primary structure. It is the primary structure which determines the folding of a protein to attain its characteristic secondary and tertiary structures. Moreover, the chemical properties of the amino acid side-
chains form the basis for substrate specificity and a host of other properties. A knowledge of the amino acid sequence is therefore crucial to an understanding of a protein's function.

The NH$_2$-terminal amino acid residue of 3-methylcatechol 2,3-dioxygenase was found to be glycine. The high percentage recovery for this residue, based on the minimum Mr = 33,500, suggested that the NH$_2$-terminus was not blocked. The average amino acid repetitive yield was 98% which allowed the sequence to be established up to the 35th residue (Fig. 3.4) with confidence. This yield is typical of protein sequencing procedures in which only a single peptide is present. Thus this result suggests that the enzyme contains a single subunit type, a conclusion also arrived at in Section 3.3.

So far, the NH$_2$-terminal amino acid sequences of eight extradiol dioxygenases, all from Pseudomonads, have been determined (Table 3.2). The initial 35 residues of 3-methylcatechol 2,3-dioxygenase from Pseudomonas putida were compared with these others (Fig. 3.5).

Computer aided alignment (Appendix I) revealed immediately that two of the nine extradiol NH$_2$-terminal sequences were strikingly different; namely the two gentisate 1,2-dioxygenases [Harpel, 1990]. No homology was apparent either between these two proteins, from Pseudomonas acidovorans and testosteroni respectively, nor with any other dioxygenase for which the sequence is
Figure 3.4: The \( \text{NH}_2 \)-Terminal Sequence of 3-Methyl catechol 2,3-Dioxygenase Expressed in \( E. \) coli

Gly - Val - Leu - Arg - Ile -  
Gly - His - Ala - Ser - Leu -  
Arg - Val - Met - Asp - Ile -  
Ala - Ala - Ala - Val - Lys -  
His - Tyr - Glu - Glu - Val -  
Leu - Gly - Leu - Lys - Thr -  
Val - Met - Lys - Asp - Ser -

The \( \text{NH}_2 \)-terminal amino acid sequence was determined to the 35th residue by Edman degradation.
Table 3.2: Gene Designations for the Fully Sequenced Extradiol Dioxygenases

The gene designations listed opposite are used throughout Chapter 3 to refer to individual dioxygenases in an abbreviated and differentiable form. The extradiol dioxygenases, which cleave a diverse range of substrates, nevertheless consist of subunits of a comparable length.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacterial Source</th>
<th>Gene Designation of Amino Acids</th>
<th>Total Number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em> mt-2</td>
<td>Xyle</td>
<td>307</td>
<td>Nozaki, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zukowski, 1983</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em></td>
<td>NahH</td>
<td>307</td>
<td>Ghosal, 1987</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em> F1</td>
<td>TodE</td>
<td>290</td>
<td>Zylstra, 1989</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em></td>
<td>-</td>
<td>314</td>
<td>McClure, unpublished results</td>
</tr>
<tr>
<td>1,2DHB2,3D</td>
<td><em>P. pseudoalcaligenes</em></td>
<td>BpHC1</td>
<td>302</td>
<td>Furukawa, 1987</td>
</tr>
<tr>
<td>1,2DHB2,3D</td>
<td><em>P. paucimobilis</em> Q1</td>
<td>BpHC2</td>
<td>298</td>
<td>Taira, 1988</td>
</tr>
<tr>
<td>1,2DHN2,3D</td>
<td><em>P. putida</em></td>
<td>NahC</td>
<td>302</td>
<td>Harayama, 1989</td>
</tr>
</tbody>
</table>
The amino acid residues are represented by their single-letter abbreviations (opposite). The gene designations and enzyme abbreviations are those previously stipulated in Table 3.2. The numerals above the sequences refer to positions in the primary structure of the proteins, NahC serves as the reference sequence in this comparison. Gaps introduced in order to optimize alignment are represented by dashes. Asterisks indicate the positions at which the compared sequences (anomalous G1,2D’s excepted), contain identical residues.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MC2,3D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xy1E</td>
<td>G</td>
<td>V</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>NahH</td>
<td>G</td>
<td>V</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td>TodE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BpHC1</td>
<td>S</td>
<td>I</td>
<td>Q</td>
<td>R</td>
</tr>
<tr>
<td>BpHC2</td>
<td>V</td>
<td>A</td>
<td>V</td>
<td>T</td>
</tr>
<tr>
<td>NahC</td>
<td>M</td>
<td>S</td>
<td>K</td>
<td>Q</td>
</tr>
<tr>
<td>G1,2D1</td>
<td>S</td>
<td>L</td>
<td>V</td>
<td>Q</td>
</tr>
<tr>
<td>G1,2D2</td>
<td>M</td>
<td>Q</td>
<td>E</td>
<td>L</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>V</td>
</tr>
<tr>
<td>Xy1E</td>
<td>S</td>
<td>K</td>
<td>A</td>
<td>L</td>
</tr>
<tr>
<td>NahH</td>
<td>G</td>
<td>K</td>
<td>A</td>
<td>L</td>
</tr>
<tr>
<td>TodE</td>
<td>R</td>
<td>S</td>
<td>W</td>
<td>R</td>
</tr>
<tr>
<td>BpHC1</td>
<td>A</td>
<td>A</td>
<td>W</td>
<td>R</td>
</tr>
<tr>
<td>BpHC2</td>
<td>D</td>
<td>A</td>
<td>W</td>
<td>R</td>
</tr>
<tr>
<td>NahC</td>
<td>D</td>
<td>A</td>
<td>W</td>
<td>K</td>
</tr>
<tr>
<td>G1,2D1</td>
<td>N</td>
<td>N</td>
<td>L</td>
<td>V</td>
</tr>
<tr>
<td>G1,2D2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

continued on next page...
known. This lack of homology prevented computer alignment. Harayama [1989] has demonstrated the evolutionary relatedness among extradiol dioxygenases, from the different degrees of similarity the author divided the gene family for extradiol enzymes into two sub-families comprising dioxygenases for monocyclic and bicyclic aromatic compounds respectively. The author proposed the hypothesis that catabolic enzymes for oxidation of the polycyclic aromatic rings arose from those for oxidation of monocyclic aromatic compounds. The anomalous G1,2D's suggest, however, that this cannot be stated with complete certainty. The feature of these two enzymes, which may be significant, is that the two substrate hydroxyl groups are positioned para with respect to one another and not in an ortho arrangement as they are for the substrates of the enzymes for the other seven sequences presented here. It is clear from these sequences, however, that the ortho-dihydroxybenzene-utilising proteins, including ours, are evolutionary products of a common ancestor.

As briefly mentioned (this section) the NH$_2$-terminal amino acid of 3MC2,3D was found to be glycine. By comparison, five of the other NH$_2$-terminal sequences start with the amino acid methionine. Since the alignment placed our NH$_2$-terminal at position six of the consensus sequence (Fig. 3.5), this methionine, and perhaps other residues, are absent when 3-methylcatechol 2,3-dioxygenase
is expressed in *E. coli*. It is not uncommon for amino acids to be removed proteolytically by endogenous proteinases during extraction from *E. coli*. The determination of the DNA sequence encoding an enzyme permits the deduction of the entire amino acid sequence. This knowledge would verify either the presence or absence of this/these residue(s) in our case. The 3-methylcatechol 2,3-dioxygenase DNA sequence determination has very recently been achieved [McClure, N.C., personal communication] and will be discussed fully further on in this section.

Among the first 35 residues, three (2 glycine and 1 valine, 9%) are totally conserved and as such are implicated as fulfilling noteworthy roles in the enzyme. These roles are as yet undefined since the study of 3MC2,3D is still in its infancy, however, they may be structural or functional.

The 3-methylcatechol 2,3-dioxygenase sequence from *Pseudomonas putida*, perhaps not surprisingly, shows higher affinity with the monocyclic catechol dioxygenase; Xyle and NahH (gene designations, see Table 3.2, 49%) than with the polyaromatic cleaving enzymes; BpHCl, BpHC2 and NahC (23±3%). Unexpected, however, is the toluene-induced 3MC2,3D (TodE) which recognises the same substrate but shares only 17% identity with our toluidine-derived 3MC2,3D, indicating that the former, by sequence comparison (average identity 47%) belongs to Harayama's
[1989] polycyclic sub-family. The findings from NH$_2$-terminal studies of Zylstra, Harpel and ourselves point to the conclusion that the proposal of evolutionary relatedness of extradiol dioxygenases [Harayama, 1989] should be applied with caution until more evidence has been collated.

Investigations by several laboratories have been unsuccessful in discovering statistically significant sequence homology between intradiol and extradiol dioxygenases either macroscopically or after local examinations.

Frequently at the beginning of a project there is no knowledge of the DNA sequence, and thus an oligonucleotide probe must be designed on the basis of knowing a suitable short region of protein sequence. In practice a sequence of at least 30 to 40 residues is habitually used. Ideally sequences containing single codon amino acids, such as methionine and tryptophan, are most desirable. This length of sequence can be obtained quite readily from a single protein or peptide sample with the use of an automated protein sequencer. The usual strategy is to attempt initially to determine the NH$_2$-terminal sequence of the whole protein.

Thus the NH$_2$-terminal sequence was determined, not only to partially elucidate the primary structure, which in turn would throw some light on the secondary and tertiary structures of this novel dioxygenase, but also to
provide a probe to locate the correct open reading frame in the nucleotide sequence which is a prerequisite to the deduction of the complete amino acid sequence from the DNA sequence determination.

As previously mentioned, the complete nucleotide sequence of the structural gene for 3-methylcatechol 2,3-dioxygenase from *Pseudomonas putida*, has been determined [McClure, N.C., personal communication] (Fig. 3.6). The deduced amino acid sequence demonstrated that the molecular weight of the dioxygenase was 34,781, in agreement with that of SDS-PAGE analysis which showed the relative subunit molecular weight of 3MC2,3D to be 33,500 ± 2,000 (Section 3.2.1, Fig. 3.1). As briefly discussed in Section 3.3.2, the NH$_2$-terminal sequence determination indicated that the first residue was glycine. The DNA sequence data confirmed that methionine immediately preceeded glycine, but that glycine was indeed the true NH$_2$-terminus, indicating that no residues have been proteolysed during protein maturation, as originally suspected from comparison with other extradiol dioxygenase sequences (Section 3.3.2, Fig. 3.5).

Approximately one quarter of the residues are charged with equal numbers of positive (Arg, His, Lys) and negative (Asp, Glu) residues being present, which results in 3-methylcatechol 2,3-dioxygenase being neutral overall. These two features are common to other extradiol dioxygenases (Table 3.3). The enzyme was purified on an
The complete primary structure of 3-methylcatechol 2,3-dioxygenase has been determined and was found to consist of 314 residues in the following sequence from the NH$_2$-terminal end (opposite page, top line).

The annotation is identical to that previous designated in Fig. 3.5.
Table 3.3: Charge Comparison for the Extradiol Dioxygenases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene Designation</th>
<th>% of Residues Charged</th>
<th>Positively</th>
<th>Negatively</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td>XylE</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C2,3D</td>
<td>NahH</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>TodE</td>
<td>13</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>3MC2,3D</td>
<td></td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>1,2DHB2,3D</td>
<td>BpHC1</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>1,2DHB2,3D</td>
<td>BpHC2</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>1,2DHN2,3D</td>
<td>NahC</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

The charges were calculated from the amino acid sequences. In all seven cases the percentage of positively charged residues (R, H, K) was virtually identical to the percentage of those of a negative charge (D, E) resulting in the enzymes being formally neutral overall. The combined percentages of charged residues was also very similar among the different dioxygenases, 28 (±4)%.
anion-exchanger (Sections 2.3.3 and 2.4.6), which would suggest an overall negatively charged protein, however, in the light of the amino acid sequence it would have to be concluded that either the negatively charged residues appear on the enzyme surface in greater numbers than those of positive charge, or that the tertiary structure is such that it results in patches of negative charge on the surface through which the enzyme can bind to the positively charged column. The protein did not bind to a cation-exchanger suggesting that no such similar patches of positive charge exist on the surface. The charged residues were regularly spread throughout the sequence with no stretch of greater than eleven amino acids being uncharged (Fig. 3.7). The enzyme contained both a negatively charged patch (Asp-Glu-Asp-XXX-Glu) early on in the sequence, which may possibly end up on the surface after protein folding, and a positive patch (Lys-Lys-Lys-XXX-Lys) towards the end. The functional significance of these remain to be elucidated.

To date, the complete amino acid sequences of five extradiol enzymes have been determined [Nakai, 1983; Furukawa, 1987; Ghosal, 1987; Taira, 1988; Harayama, 1989]. The amino acid sequence of 3-methylcatechol 2,3-dioxygenase, deduced from the nucleotide sequence, was compared with these others (Fig. 3.6).

The enzymatic reactions catalysing the degradation of polycyclic aromatics such as biphenyl and naphthalene
Figure 3.7: Distribution of Charged Amino Acid Residues in 3-Methylcatechol 2,3-Dioxygenase

The figure opposite shows the complete amino acid sequence of 3-methylcatechol 2,3-dioxygenase where the positively charged residues (Arg, His, Lys), negatively charged residues (Asp, Glu) and neutral residues are denoted by the symbols +, - and 0 respectively.
<table>
<thead>
<tr>
<th>Line</th>
<th>String</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 0 0 0 + 0 0 + 0 0 0 + 0 0 - 0 0 0 0 0 + + 0 - - 0 0 0 0 +</td>
</tr>
<tr>
<td>31</td>
<td>0 0 0 + - 0 0 0 0 0 0 0 + 0 0 - - 0 - + 0 0 0 0 0 0 0 0 - +</td>
</tr>
<tr>
<td>61</td>
<td>0 0 0 0 + 0 0 0 + 0 - + - - 0 - 0 0 0 0 + 0 - 0 0 0 0 + 0</td>
</tr>
<tr>
<td>91</td>
<td>0 0 0 0 - 0 0 0 0 0 0 0 + 0 0 0 0 0 0 0 0 0 + - 0 + 0 0 0 0</td>
</tr>
<tr>
<td>121</td>
<td>+ - 0 0 0 0 - 0 0 0 0 0 0 0 - 0 0 0 + 0 0 0 0 + 0 0 - +</td>
</tr>
<tr>
<td>151</td>
<td>0 0 0 0 0 - 0 0 0 - 0 0 0 0 0 0 0 - 0 0 + 0 0 + - 0 0 - 0 0</td>
</tr>
<tr>
<td>181</td>
<td>0 0 - 0 0 0 0 0 0 0 0 - 0 0 0 0 0 0 0 + 0 0 0 0 + - 0 0 0</td>
</tr>
<tr>
<td>211</td>
<td>0 0 0 0 0 0 0 0 + + 0 0 0 0 0 - 0 0 - - 0 0 + 0 0 - 0 0 0 +</td>
</tr>
<tr>
<td>241</td>
<td>+ + 0 + 0 - 0 0 0 0 + + 0 0 0 + 0 - 0 0 0 0 0 - 0 0 0 0 + 0</td>
</tr>
<tr>
<td>271</td>
<td>- 0 0 0 0 0 0 0 0 0 0 - + 0 0 0 0 0 0 - - + 0 0 + 0 0 0 0</td>
</tr>
<tr>
<td>301</td>
<td>+ 0 0 - 0 0 0 0 0 0 - 0 0 0</td>
</tr>
</tbody>
</table>
resemble those involved in the meta-cleavage of monocyclic aromatic compounds (Fig. 3.8). Thus it is not surprising that the sequences of these enzymes may be aligned, demonstrating the evolutionary relatedness among these extradiol dioxygenases. A striking similarity was observed between the amino acid sequence of 3MC2,3D and the catechol 2,3-dioxygenases (~60%), the matching frequencies with the other sequences depicted in Fig. 3.6 were far less. Observation of the degrees of similarity among extradiol enzymes indicated that the bicyclic aromatics are more closely related to each other than to the monocyclics, likewise the monocyclic compounds form a closely related subgroup.

Amino acid sequences of several intradiol enzymes namely: protocatechuate 3,4-dioxygenase α [Kohlmiller, 1979] and β [Iwaki, 1979], and catechol 1,2-dioxygenase II [Frantz, 1987; Ghosal, 1988] and I [Neidle, 1988], have been determined and amino acid sequence similarity suggests that a common origin of these intradiol enzymes exists [Neidle, 1988]. Harayama [1989] tested the significance of all the intradiol and extradiol sequence similarities using the method of Needleman and Wunsch [1970] employing the Dayhoff matrix [1978]. The results showed that the sequence similarities between the intradiol and extradiol enzymes were not statistically significant.

Although the chemical structures of substrates for
The substrates of the sequenced extradiol dioxygenases (Fig. 3.6): catechol (I, R=H), 3-methylcatechol (I, R=CH₃), 2,3-dihydroxybiphenyl (II) and 1,2-dihydroxynaphthalene (III) each contain two hydroxyl groups on two adjacent aromatic carbons.
intradiol and extradiol enzymes are identical or similar, the reaction mechanisms are different. Extradiol enzymes are composed of single polypeptide species and contain ferrous ion as a prosthetic group, whereas intradiol dioxygenases are composed of either one or two subunits [Ludwig, 1984] and contain ferric ion in their catalytic centre. Although catechol 1,2-dioxygenase, an intradiol enzyme, can carry out both intradiol and extradiol cleavages [Fujiwara, 1975; Hou, 1977; Saeki, 1980], the site of ring-cleavage is usually specific for each type of enzyme. In agreement with the difference in enzymatic reactions, Harayama could not find any significant sequence homology between the two groups of enzymes.

Catechol 2,3-dioxygenases consist of four identical subunits (discussed in Section 3.3.1) and contain 1g atom of ferrous ion per subunit. Mössbauer data have suggested that the coordination environment of iron in this enzyme is very similar to that in the reduced form of protocatechuate 3,4-dioxygenase [Tatsuno, 1980]. The X-ray structure determination of P3,4D has revealed that the ferric ion in the active centre is coordinated with two tyrosine residues and two histidine residues (Figs. 3.9 and 3.10) [Ohlendorf, 1988], therefore tyrosine and/or histidine may also be the iron-binding residues of 3MC2,3D. Four histidines and one tyrosine residue, out of a total of nineteen, are conserved between all seven extradiol dioxygenases. These residues are therefore
Figure 3.9: Electron Density Map Around The Iron at the Active Site of Protocatechuate 3,4-Dioxygenase

In the figure opposite the iron atom and its ligand are shown in gold. Histidine and tyrosine amino acid residues are clearly visible in the vicinity of the active site. Electron density extending from the iron atom towards the viewer corresponds to an equatorially bound solvent molecule.

Figure 3.10: Ribbon Diagram of the P3,4D Protomer Main Chain

The α subunit is red and the β subunit is green. The α subunit secondary structural features are shown as magenta ribbons and those of the β subunit as cyan ribbons. The iron atom and its ligands are gold.

Figures 3.9 and 3.10 are reprinted by permission from D.H. Ohlendorf and Nature, 336, 403-405, Copyright (C) 1988 Macmillan Magazines Ltd.
obvious suspects for iron-binding.

The substrates of the extradiol enzymes generally contain two hydroxyl groups (Fig. 3.8) and the side-chains of these enzymes may hydrogen bond with one or both of these hydroxyl groups. Residues that are important for substrate binding may also be found among the conserved amino acids. This study therefore provides important information for future investigations to understand the structural and functional relationships of extradiol enzymes.

β-turns are stretches of tetrapeptides in which polypeptide chains fold back on themselves over an angle of 180°. Present predictive methods can locate these reverse turn regions in proteins with an 80% degree of accuracy. Proline and glycine are strong β-turn formers (Fig. 3.11). Proline is the only amino acid in which the side-chain loops back to reattach to the main polypeptide chain. One consequence of this is that proline forces a bend in the polypeptide backbone and can thus disrupt an α-helix and exert other constraints on the folding of a protein. At the other end of the spectrum, the tiny side chain of glycine introduces few constraints and this residue can be found in many types of structure, moreover, where the polypeptide backbone has a tight bend, glycine is the only residue that could be accommodated. The possible location of β-turns in the polypeptide chains of the extradiol enzymes have been predicted by the method of
It is noteworthy that six glycines and two prolines are among the nineteen amino acids conserved in all the sequences depicted in Fig. 3.6. The contribution of glycyl and prolyl residues is structural, and the high frequency of their conservation in the primary sequences suggests that the tertiary structures will prove to be highly conserved among these extradiol dioxygenases.
Chou and Fasman [1978]. Interestingly, when the secondary structure of five of these different extradiol dioxygenases were examined [Harayama, 1989], β-turns were implicated at the same four regions of the polypeptide; around the 115th, 139th, 188/189th and 266th residues, respectively. Many conserved amino acid residues were located in these areas, therefore, it is likely that these bends are essential for the active structure of the extradiol enzymes and that the amino acid residues that contribute to this secondary structure have been evolutionarily conserved. Similar β-turn prediction treatment (Appendix I) of 3-methylcatechol 2,3-dioxygenase, by computer, employing the methods of both Chou and Fasman and Garnier, Osguthorpe and Robson [1978] predicted bends to occur at positions coincident with those aforementioned. These regions are, not surprisingly, rich in proline and glycine residues.

3.3.3 Thermal Stability

The thermal inactivation of 3MC2,3D at 25, 35, 45, 55, 65 and 75°C is depicted in Fig. 3.12. The enzyme retained full activity after incubation (60 min) at 25, 35 and 45°C. Heating at 55, 65 and 75°C resulted in a time-dependent loss of dioxygenase activity over the 1 hour period. The decline in activity was more pronounced at elevated temperatures. By the end of the heating period, the crude extract incubated at 55°C showed 25%
Figure 3.12: The Thermal Inactivation of 3-Methylcatechol 2,3-Dioxygenase

Aliquots of crude 3MC2,3D extract were withdrawn at 5 minute intervals and assayed for dioxygenase activity at 375 nm. Enzyme activity was determined before heating to determine residual activities for each temperature.
residual activity, 5 to 10% of the original activity remained in the 65°C sample, and all activity was lost, after only 30 minutes, from the sample heated at 75°C.

Studies on thermal stability provides information concerning the molecular forces involved in stabilising enzymes. An input of thermal energy increases structural fluctuation in the protein, weakens or disrupts non-covalent bonds such as hydrogen bonds and van der Waal's forces in the transition state, hence denaturation of the enzyme ensues.

The reasons for using crude extract, i.e., only partially pure, 3-methylcatechol 2,3-dioxygenase protein samples were two fold; firstly, the nature of the experiment was such that a large quantity of enzyme was required, and secondly, the method employed was destructive. It would therefore have been expensive to study a fully purified sample. As a consequence of this, the crude extracts became turbid, during the course of the heating procedure, due to protein precipitation. No turbidity was seen at 25°C, however, at 35°C (t = ~ 60 min) and at 45°C (t = 15 min) gradual precipitation occurred, although the activity was largely unaffected. This observation suggests that proteins other than 3MC2,3D were responsible for the turbidity. At 55°C and above, protein precipitation was immediate, paralleling a decline in dioxygenase activity. A number of extradiol dioxygenase isolation procedures involve heat treatment as a
purification step (Table 3.4). The observations from these thermal inactivation experiments strongly suggest that the insertion of such a step in our protocol would also be suitable in the isolation of 3-methylcatechol 2,3-dioxygenase.

3.4 Summary of Physical Properties

The physical properties of 3-methylcatechol 2,3-dioxygenase have been investigated. Following purification, the enzyme consists of a single subunit type of $M_r = 33,500 \pm 2,000$ by SDS-PAGE. Gel filtration indicates a native molecular weight, under non-denaturing conditions, of $120,000 \pm 20,000$ consistent with the enzyme existing as a tetramer of identical subunits. The NH$_2$-terminus and subsequently the entire amino acid sequence have been determined. Both the sequence itself and the resultant $\beta$-turn predictions show significant homology with other extradiol dioxygenases.

The enzyme was found to be stable within the pH range 7 to 8 (Sections 2.3 and 5.3.2) and could be stored for a period of days between 0 and 4°C in acetone-phosphate buffer without any marked loss of activity. Further information about the optimal conditions for 3MC2,3D stability was gleaned from thermal inactivation and microcalorimetry experiments. The former demonstrated the complete stability of the enzyme up to 45°C for prolonged periods, however, above this temperature the enzymatic
Table 3.4: Heat Treatment of a Purification Step in the Isolation of Extradiol Dioxygenases

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>TEMPERATURE °C</th>
<th>INCUBATION TIME</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td>55</td>
<td>10</td>
<td>Murray, 1974; Kunz, 1981</td>
</tr>
<tr>
<td>C2,3D</td>
<td>55</td>
<td>10</td>
<td>Bartels, 1984</td>
</tr>
<tr>
<td>C2,3D</td>
<td>55</td>
<td>-</td>
<td>Engesser, 1988</td>
</tr>
<tr>
<td>P4,5D</td>
<td>-</td>
<td>-</td>
<td>Arciero, 1983</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td>55</td>
<td>5</td>
<td>Dagley, 1963</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td>63</td>
<td>10</td>
<td>Que, 1981</td>
</tr>
<tr>
<td>G1,2D</td>
<td>60</td>
<td>-</td>
<td>Harpel, 1990</td>
</tr>
<tr>
<td>P3,4D</td>
<td>60</td>
<td>5</td>
<td>Fujisawa, 1970</td>
</tr>
</tbody>
</table>
activity was seen to decline, commensurate with the unfolding, aggregation and resultant precipitation of the protein. This evidence for the thermal denaturation of 3MC2,3D was supported by microcalorimetry, the results of which hinted at a broad transition between 60 and 70°C due to a main change in the state of the protein. Unfortunately the exotic nature of the buffer precluded quantitation of these observations.

In conclusion, the structural characterisation of 3-methylcatechol 2,3-dioxygenase, at the primary, secondary and quaternary levels, suggested that the enzyme was typical of the extradiol dioxygenases.
3.5 References


CHAPTER 4

FURTHER CHARACTERISATION OF
3-METHYLCATECHOL 2,3-DIOXYGENASE
4.1 Introduction

The relationship between metal and protein, in a metalloprotein, is a reciprocal one. The presence of the metal ion can influence the electronic and structural arrangement of the protein and so affect its reactivity. In addition, the protein can enforce unusual stereochemistries upon the metal ion in that the protein internal structural requirements may not allow it to provide a normal symmetrical binding site, or even normal metal-ligand distances in some cases. This can in turn affect the reactivity of the metal.

Early investigators of catechol dioxygenases suggested that the role of the metal was in the association of the subunits [Senoh, 1966]. This assignment is contrary to more recent observations, which implicate a catalytic role for both the ferric iron-containing intradiol and the ferrous iron-containing extradiol enzymes [Nozaki, 1988].

This chapter reports the results of various experiments undertaken in an attempt to characterise the nature of the metal with a view to ascertaining its role in 3-methylcatechol 2,3-dioxygenase.

4.2 Experimental

4.2.1 Spectrophotometric Determination

The absorption spectrum of concentrated, purified 3-methylcatechol 2,3-dioxygenase was recorded on both Pye-Unicam SP8-400 and Perkin Elmer λ9 spectrophotometers
using acetone-phosphate buffer (0.05M, pH 7.50, Section 5.3.3) as a reference.

4.2.2 Fe(II) Removal to Prepare the 3MC2,3D Apo-protein and Reconstruction of the Holo-enzyme by Fe(II) Replacement

Fe(II) was removed from the active enzyme by treatment with ethylenediamine tetraacetate (EDTA, BDH Analar). EDTA (100-fold molar excess) was added to a 10–50 µM enzyme solution which was dialysed overnight against 0.05M phosphate buffer, pH 7.50 (Section 5.3.2, 100-fold volume excess), with no acetone present, between 0 and 4°C.

Replacement was achieved by addition of ferrous sulphate (10-fold molar excess) to the inactive apo-enzyme which was then re-dialysed overnight, this time against acetone-phosphate buffer (Section 5.3.3). Special care must be taken that the reconstitution procedure does not introduce metal ions in excessive amounts to avoid complications due to adventitious binding.

4.2.3 Inactivation of 3MC2,3D by Oxidation and Reactivation of the Inactivated Enzyme by Re-reduction

The oxidation of the Fe(II) was achieved by titration of an enzyme solution (10–50 µM) with either potassium ferricyanide or hydrogen peroxide until a complete loss of activity was achieved. Re-reduction was carried out by
the addition of a 100-fold molar excess of sodium dithionite.

4.2.4 **Metal for Metal Replacement**

The Fe(II) replacement studies (Section 4.2.2) were extended. Samples of apo-3MC2,3D were prepared in a manner entirely analogous to that previously described. Similarly, reconstruction of the enzyme was attempted employing the following substitutes for ferrous iron; Ca\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\).

4.3 **Results and Discussion**

A number of extradiol dioxygenases, isolated from *Pseudomonads*, reportedly contain ferrous ion as an essential cofactor (Table 3.1), in contrast, the intradiol cleaving enzymes such as catechol 1,2-dioxygenase [Nakazawa, 1969] and protocatechuate 3,4-dioxygenase [Que, 1976] contain iron only in the ferric form. It is also well documented that the presence of a low percentage of an organic solvent stabilises enzymes containing ferrous ions (Section 2.4.3 and references contained therein) and this was seen to be the case with 3-methylcatechol 2,3-dioxygenase. Thus, observations from the isolation protocol (Chapter 2) already suggest ferrous iron to be a cofactor for the enzyme.

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4.3.1 Spectrophotometric Determination

The spectrum (Fig. 4.1) was featureless in the visible range. The only significant peak occurred at ~280 nm due to $\pi-\pi^*$ transitions from the aromatic amino acids (Section 3.2). None of the extradiol dioxygenases exhibit evidence of a visible chromophore. This contrasts sharply with the red intradiol dioxygenases whose absorption around 460 nm arises from tyrosine-to-Fe(III) charge-transfer transitions. Our results are consistent with the colourless 3-methylcatechol 2,3-dioxygenase being an Fe(II) extradiol dioxygenase. Additionally, there was no indication of the existence of either a heme or a flavin prosthetic group in the molecule, from the UV/visible spectrum, suggesting that divalent iron was the sole cofactor.

4.3.2 Fe(II) Removal and Replacement

The removal of Fe(II) from active 3-methylcatechol 2,3-dioxygenase was concomitant with a loss of activity to less than 5% of the original value. Reactivation (maximally 76%) of the enzyme was achieved by the addition of Fe(II) ions to the inactivated apo-enzyme (Fig. 4.2).

3-Methylcatechol 2,3-dioxygenase in acetone-phosphate buffer can be stored for a period of days between 0 and 4°C without any marked loss of activity (Section 3.4). Treatment of the enzyme with EDTA caused a reversible loss.
The absorption spectrum of concentrated, purified 3MC2,3D was recorded using acetone-phosphate buffer (0.05M, pH 7.50) as a reference.
Figure 4.2: Preparation of the Apo-3-Methylcatechol 2,3-Dioxygenase and Reconstruction of the Holo-enzyme by Fe(II) Removal and Replacement

The figure shows that dialysis against acetone-free buffer (days 1, 2, 3) results in the production of virtually dead enzyme. Activity is partially restored (days 4, 5, 6) upon the addition of ferrous sulphate with re-dialysis in the presence of 10% acetone.
of enzymic activity. Activity was restored (76%) by incubation of the enzyme with ferrous ions, clearly demonstrating that activity is dependent on the presence of iron and also that metal removal and replacement is fairly easily achieved, which suggests that no gross modification of the apo-protein moiety had occurred.

Similarly, facile, ferrous iron removal and reconstitution of apo-extradial dioxygenases, often to their fully active holo-enzymes, has been observed for metapyrocatechase (C2,3D) [Taniuchi, 1962; Takemori, 1971 (60%); Nakai, 1983; Wallis, this work, 35%], protocatechuate 4,5-dioxygenase [Ono, 1970; Zabinski, 1972], protocatechuate 2,3-dioxygenase [Crawford, 1975] and 3-methylcatechol 2,3-dioxygenase [Klečka, 1981]. In several instances, the reconstruction involved the presence of a mild reducing agent (commonly ascorbate) and/or anaerobic conditions. That the ferrous ion is bound loosely to the protein in the extradial dioxygenases is supported by the ease with which it can be removed and replaced. This contrasts with both the Mn(II)-containing extradial 3,4-dihydroxyphenylacetate 2,3-dioxygenase, 3,4DHPA2,3D [Que, 1981] and the Fe(III)-containing intradiol dioxygenases, where tight binding is observed.

The trivalent iron which imparts the red colour to the type of enzyme represented by catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, and which functions as an integral part of these intradiol enzymes [Kojima, 1967,
was not liberated by dialysis in contrast to findings for
divalent iron-containing extradiol dioxygenases, by us and
others. Metal binding agents did not show appreciable
inhibition either by direct addition or by preincubation
with enzyme. These ferric atoms were therefore assumed to
be bound extremely tightly to the protein and their
removal necessitated treatment with a chelator (often
substrate analogues) in combination with a reducing agent.
The iron-chelate complex is then separated from the
apo-enzyme upon exhaustive dialysis. This treatment
affords colourless apo-proteins with no activity from
active intradiol holo-enzymes which are characteristically
red.

The apo-enzymes resulting from such treatment were
rapidly reconstructed to active holo-enzymes in the
presence of either ferrous iron and oxygen, or
alternatively by employing an oxidant under anaerobic
conditions. These observations suggest that the ferrous
iron added is bound to the apo-enzyme and is
simultaneously oxidised to the active ferric state.
Ferric ion was not effective for the reconstruction of the
enzyme, indicating that ferrous ion is favourably
incorporated into the enzyme. It is of interest that the
requirement of the reduced form of metal ion for the
reconstitution of some copper-depleted proteins was also
reported though the oxidised form, Cu(II), is present in
the native proteins [Morell, 1958; Blumberg, 1963; Nair, 1967].

As is the case with Fe(III)-containing intradiol dioxygenases, the Mn(II) enzyme produces the corresponding apo-protein only upon incubation with a metal chelator. Efforts to obtain holo-enzyme with reconstitution procedures [Harris, 1977; Ose, 1979] used for Mn-containing superoxide dismutase, however, have not been successful.

4.3.3 3MC2,3D Oxidation and Reduction

Treatment of the enzyme with oxidants, ferricyanide and hydrogen peroxide, also resulted in a rapid loss of activity. The oxidation with ferricyanide was found to be reversible with 60% of the original activity restored by reduction with sodium dithionite. Enzyme treated with hydrogen peroxide could not be reactivated at all by addition of sodium dithionite.

Oxidation of the Fe(II) cofactor, even in the presence of 10% acetone, by ferricyanide also resulted in a loss of enzymic activity. The fact that activity was restored following re-reduction indicates that Fe(II) is essential for enzyme function, with the oxidised Fe(III) state being inactive. Native 3MC2,3D was resistant to reducing agents, again indicating that the catalytically active enzyme contains divalent iron. The instantaneous irreversible loss of activity following treatment of the
enzyme with hydrogen peroxide suggests that this oxidant has effects other than a simple one-electron oxidation of the Fe(II) centre.

The extradiol dioxygenase literature reveals several reports of enzyme inactivation upon incubation with oxidants. Both fully reversible inactivation by ferricyanide, C2,3D [Taniuchi, 1962; Wallis, this work, 60%], P4,5D [Arciero, 1983] and partially reversible inactivation with hydrogen peroxide, C2,3D [Kojiina, 1967; Nozaki, 1968, 60%; Wallis, this work, 0%], P4,5D [Ono, 1970, 60%; Arciero, 1983, 50%], 3,4DHPA2,3D [Kita, 1965; Ono-Kamimoto, 1973], have been seen.

We failed to reactivate 3-methylcatechol 2,3-dioxygenase, oxidised by hydrogen peroxide, with sodium dithionite, unlike the other six examples quoted. However, the aforementioned authors, over and above the addition of Fe(II) ion, incubated in the presence of reductant. This evidence tends to support our suggestion, but does not provide conclusive proof, that hydrogen peroxide has other effects on the protein, for example a conformational change which may facilitate metal loss.

Not surprisingly, the effects of oxidants and reductants on the Fe(III)-containing intradiol dioxygenases directly oppose those observed for the Fe(II)-containing extradiol enzymes. In general the addition of reducing agents seems to accelerate the inactivation of the former dioxygenase class concomitant
with bleaching of the red colour. These colourless enzymes are considered to contain divalent iron. The intradiol enzymes are, however, resistant to oxidising agents. Restoration of enzymatic activity and reappearance of the red colour were both observed when the enzyme solution was exposed to air or by anaerobic incubation with potassium ferricyanide.

Again, in contrast to the Fe(II) extradiol dioxygenases, the Mn(II) enzyme [Que, 1981] is stable with respect to hydrogen peroxide oxidation. An interesting parallel can be drawn with the superoxide dismutases (SOD), where similarly hydrogen peroxide inactivates the Fe-SOD but not the Mn-SOD [Asada, 1975; Lumsden, 1976].

4.3.4 Metal for Metal Replacement, Metal Ions as Probes to Obtain Comparative Information

The metal ion in an enzyme may be replaced by a metal that has useful properties. The probe must be chosen with care, as ideally it should occupy exactly the same site as the native metal ion, i.e., the principle of isomorphous replacement must hold. The ionic charge and the radius of the metal ion are two important considerations. The probe metal should also have similar requirements to the native metal in terms of the preferred stereochemistry of the metal-binding site and the nature of the binding ligands. If the biological activity of the enzyme is maintained it may be assumed that the new metal ion has successfully
replaced the native metal ion.

If the criteria for isomorphous replacement are relaxed then a wide range of substitutions may be carried out. The divalent transition metals offer a series of probes that vary in size and geometry and provide access to a wide range of spectroscopic techniques. The catalytic efficiency of these cations may also be correlated with their changing geometrical and chemical properties and so give insight into kinetic factors.

It has been known for many years that the ferrous iron-containing heme group of proteins such as hemoglobin could be exchanged for other metalloporphyrins, for example cobalt, manganese and more recently the possibility of inserting even lanthanide-porphyrins has been demonstrated [Horrocks, 1976]. Hoffman [1971], in particular, has indicated that Co(II)-porphyrin can be effective as a dioxygen carrier.

The fact that the extradiol dioxygenase exhibit neither optical nor EPR spectra and the relative ease with which ferrous iron can be reincorporated into apo-3MC2,3D to form enzymatically active reconstituted protein, led to the undertaking of a more extensive metal replacement study. Unfortunately metals other than Fe(II) did not cause significant reactivation of 3-methylcatechol 2,3-dioxygenase. Other investigations have been similarly unsuccessful, Table 4.1, with other dioxygenases. There are numerous possibilities as to why substitution leads to
Table 4.1: Attempted Reactivation of Various Apo-Enzymes by the Substitution of Extraneous Metals

The table opposite shows that the reactivation of apo-dioxygenases, both intra- and extra-diol, was achieved only by the incorporation of ferrous iron. Successful and unsuccessful metal replacements are denoted by the symbols $\downarrow$ and $\times$ respectively.
<table>
<thead>
<tr>
<th>Apo-Enzyme</th>
<th>Mg$^{2+}$</th>
<th>Ca$^{2+}$</th>
<th>Cr$^{2+}$</th>
<th>Mn$^{2+}$</th>
<th>Fe$^{2+}$</th>
<th>Co$^{2+}$</th>
<th>Ni$^{2+}$</th>
<th>Cu$^{2+}$</th>
<th>Zn$^{2+}$</th>
<th>Cd$^{2+}$</th>
<th>Cr$^{3+}$</th>
<th>Fe$^{3+}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>Kojima, 1961</td>
</tr>
<tr>
<td>C2,3D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Taniuchi, 1962</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Wallis, this work</td>
</tr>
<tr>
<td>P4,5D</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>Ono, 1970</td>
</tr>
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<td>P4,5D</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Zabinski, 1972</td>
</tr>
<tr>
<td>C1,2D</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>/</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>Nakazawa, 1969</td>
</tr>
</tbody>
</table>
inactivation, however, in the case of P4,5D, Zabinski [1972] stated that the Co\(^{2+}\), Ni\(^{2+}\) and Cu\(^{2+}\) ions were indeed inserted at the iron binding site. Our investigations were not pursued further since the relevance of dead enzyme is debatable.

4.4 Summary

In order to elucidate the nature of the metal, with special emphasis on its valence state, removal/replacement, oxidation/reduction and metal substitution studies of 3-methylcatechol 2,3-dioxygenase were carried out.

In some cases, the removal of metal ions induce profound changes in the protein moieties which result in poor recovery of activity or require a long time for reconstitution [Ando, 1970]. This did not appear to be so for 3MC2,3D, however, the results suggest that some factors other than ferrous ions alone are required to produce completely active enzyme.

Oxidation of the colourless Fe(II)-containing enzyme, (which did not become red), by either potassium ferricyanide (outer-sphere electron-transfer) or hydrogen peroxide (inner-sphere electron-transfer), resulted in reversible and irreversible inactivation, respectively. This suggested that Fe(II) is essential for enzyme function with the oxidised, Fe(III) form being inactive, although the possibility that these observations are
caused by conformational changes of protein moiety cannot be completely ruled out. The difference in the restoration of activity levels, seen both by ourselves and others, presumably occurs as a result of secondary modification, perhaps in the vicinity of the metal-binding site of the enzyme, with varying degrees of severity depending on the experimental conditions. Several lines of evidence indicate the iron in the native enzyme to be in the divalent state. No other cofactor was demonstrated. From the above evidence it is premature to speculate as to how the presence of ferrous iron confers activity on 3-methylcatechol 2,3-dioxygenase, however, there is good evidence in the literature to propose that a linear relationship between the iron content of these extradiol dioxygenases and the activity exists [Takemori, 1971; Nakai, 1983].
4.5 References


Williams, R.J.P., (1978), Chem. in Britain, 14, 24-29.

CHAPTER 5

KINETIC CHARACTERISATION OF

3-METHYLCATECHOL 2,3-DIOXYGENASE
5.1 Introduction

McClure [1986] reported that a strain of *Pseudomonas putida* adapted to rapid growth on the aromatic amines; aniline and the toluidines (methylanilines). These growth substrates were directly converted to ammonia plus catechol or either of the methylated derivatives, depending on the initial toluidine. These catechols, formed by oxidative deamination, were in turn dissimilated by a meta-cleavage pathway [Fig. 1.2]. Reports of toluidine metabolism by other species of *Pseudomonas* [Latorre, 1984; Raabe, 1984] and *Rhodococcus* [Appel, 1984] also involved this mode of cleavage.

Meta-cleavage results in the formation of 2-hydroxy-6-oxohexa-2,4-dienoic acid or a methylated derivative, all of which exhibit a striking yellow colouration distinguishable by UV/visible absorption spectro-photometry. McClure [1986, 1987] carried out preliminary spectroscopic and polarographic studies on this meta-cleaving enzyme to discern the relative rates of oxygenation of catechol and the 3- and 4-methyl derivatives, which were ultimately derived from aniline, ortho- or meta-toluidine and meta- or para-toluidine, respectively.

In the reactions catalysed by dioxygenases, two substrates are involved, one is an organic substrate and the other is molecular oxygen. The aim of these studies was to quantitate the activity of this meta-cleaving
enzyme, 3-methylcatechol 2,3-dioxygenase, individually towards these two substrates. The kinetic studies measured either the formation of product (UV/visible spectrophotometry) or depletion of reactant (oxygen electrode work) under both standard and modified (pH or enzyme) conditions.

5.2 Experimental
5.2.1 Kinetic Studies

All kinetic measurements were carried out employing the standard assay protocol (Section 6.7) in 0.20M phosphate buffer at pH 7.00. pH profile experiments, which were conducted under steady-state conditions, were carried out in both phosphate and glycine buffers (Sections 6.3.2 and 6.3.4) between the pH values 5.00 and 11.00. All measurements were made at 25 (± 0.1)°C, the solutions being thermostatted beforehand. The substrates were obtained commercially from either Aldrich or Lancaster Synthesis and used without further purification. Enzyme activities were measured by monitoring the accumulation of reaction products using Pye-Unicam SP8-400 and Perkin Elmer λ9 spectrophotometers at the wavelengths shown in Table 5.1.

Experimental data were fitted to both the Michaelis-Menten and the Lineweaver-Burk equations (Appendix II, Eqns. VI and VII) using non-linear and linear least means squares computer programs respectively [Camp, 1980]. This
Table 5.1: **Substrates, Ring Fission Reaction Products and Spectroscopic Values Used in Monitoring 3-Methylcatechol 2,3-Dioxygenase Activity**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Wavelength (nm)</th>
<th>$\epsilon$ ($M^{-1} \text{cm}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>2-Hydroxy-6-oxohexa-2,4-</td>
<td>375</td>
<td>48,400</td>
</tr>
<tr>
<td></td>
<td>dienoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>2-Hydroxy-6-oxohepta-2,4-</td>
<td>390</td>
<td>19,800</td>
</tr>
<tr>
<td></td>
<td>dienoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>2-Hydroxy-5-methyl-6-oxohexa-2,4-dienoic acid</td>
<td>380</td>
<td>33,200</td>
</tr>
</tbody>
</table>

The molar absorption coefficients ($\epsilon$) were determined in a 0.20M phosphate buffer, pH 7.00.
treatment yielded two kinetic parameters; $K_m$, the Michaelis constant and $V_{\text{max}}$, the maximum reaction rate. Similarly, non-linear least squares analysis of the pH dependent kinetics afforded the $pK_a$ values. Any individual data point which was deemed to deviate by more than two standard deviations, from either of the graphical representations, was discarded.

5.2.2 Attempted Modification of Histidine

Commercially available diethylpyrocarbonate (DEPC, Aldrich) was used without further purification. This liquid was stored at 0 to 4°C and diluted with anhydrous ethanol immediately before use. Since the purity of the commercial reagent may be variable, owing to hydrolysis, the concentration of the dilution should be determined quantitatively. An aliquot (10 $\mu$l) of the dilution (25 mM) was added to 1 ml of 50 mM imidazole in a cuvette having a 1 cm path length, the increase in absorption at 240 nm due to N-carbethoxyimidazole ($\varepsilon_{240} = 3200 \text{ M}^{-1} \text{ cm}^{-1}$) was determined. The increase is rapid and quantitative since the imidazole is present as a 200-fold excess [Melchior, 1970]. It is desirable to use the lowest concentration of reagent necessary for modification to avoid the formation of disubstituted histidyl derivatives [Morris, 1972] and O-carbethoxylation of tyrosyl residues [Burstein, 1974], both of which complicate the interpretation of the results.
The protein concentration was determined by the Biuret Method (Section 6.9). Protein in the range 2 to 4 x 10^{-5}M was used to avoid the problems, discussed above. Two 1 ml aliquots of protein were required for the observation and reference cells respectively.

Several unsuccessful experiments revealed that acetone inhibited the alkylation of histidyl residues. This discovery necessitated a change of buffer system immediately prior to the experiment. This was achieved by diluting the protein, kept in 10% acetone-phosphate buffer to prevent inactivation (Sections 2.4.3 and 6.3.3), 10-fold with acetone-free phosphate buffer (0.05M, pH 7.50). The solution was re-concentrated to the initial volume using an Amicon concentrator (Section 6.8). This procedure was repeated resulting in a final acetone concentration of 0.1% which had a negligible effect on the modification reaction (Fig. 5.1). The most frequently used buffer, for modification studies, is potassium phosphate in the pH range 6.0 to 8.0, since this has a less deleterious effect on DEPC than other buffers [Berger, 1975].

Two quartz cuvettes containing 1.0 ml aliquots of protein (in 0.05M phosphate buffer, pH 7.50) were placed in the sample and reference compartments of a spectrophotometer maintained at room temperature. Spectra were recorded between 220 and 280 nm (Fig. 5.2) before and at 5 minute time intervals (over a 30 minute period) after the
The figure above shows that acetone, present in the buffer at 10%, considerably inhibits the carboxethoxylolation of imidazole. The level of inhibition (monitored at 238 nm) caused by the presence of 0.1% acetone, however, is shown to be negligible.
The figure shows the relationship between activity and the number of histidine residues modified. Activity at to was taken as 100%. After 120 minutes virtually 100% of the original activity remained even although several histidine residues were lost through modification (Table 5.6).
addition of DEPC in ethanol, present as a 20-fold excess, to the sample cuvette, and an equal volume of ethanol to the reference cuvette. A 20-fold excess of DEPC should ensure that all accessible histidines become modified immediately. An aliquot of treated enzyme was removed immediately after each spectrum was recorded and was assayed (Section 6.7) for dioxygenase activity.

5.2.3 Oxygen Consumption Studies

The concentration of dissolved oxygen in phosphate buffer (0.05M, pH 7.50) was measured by a Clark type oxygen electrode which consists of a Ag/AgCl reference anode joined to a Pt/O₂ cathode at which O₂ is reduced to O⁻. The polarizing voltage (ca. 0.6V) and the polythene or teflon membrane ensure that the electrode is specific for oxygen. Since both the characteristics of the electrode and the solubility of oxygen in water are temperature dependent, the electrode and sample must be thermostatted. Also the sample must be well stirred as oxygen will not readily diffuse to the electrode from an unstirred solution. Particular care is required to avoid the presence of air bubbles in the reaction chamber.

It was necessary to calibrate the instrument prior to use; firstly by measuring the electrode current for a known concentration of O₂ and, secondly, by establishing the ‘zero’ potential of the instrument, corresponding to no current flowing in the absence of O₂. The latter was
achieved by complete reduction with a small amount of solid sodium dithionite. The concentration of $O_2$ in deionised water when saturated with air at 25°C was taken to be 0.26 mM [Wasser, 1958].

The chamber was thermostatically maintained at 25 ($\pm$ 0.1)°C and contained, in a final volume of 3 ml, 0.05M phosphate buffer pH 7.50 and one of the three catechols used as assay substrates. This solution was first saturated with air and the reaction was initiated by the addition of 3-methylcatechol 2,3-dioxygenase (25 μl) in 10% acetone-phosphate buffer, which was introduced into the chamber using a syringe. The oxygen consumption analyses were performed in triplicate.

5.3 Results and Discussion

5.3.1 Kinetic Studies

During the development of the isolation procedure for 3-methylcatechol 2,3-dioxygenase (Chapter 2) crude assays were performed throughout in order to determine both the fate of the enzyme and the effectiveness of the individual purification steps, however if stringent assay conditions (such as concentration, ionic strength, pH and temperature) are employed, some kinetic parameters of the enzyme can be evaluated.

A full steady-state kinetic analysis (Appendix II) was carried out on catechol, 3-methylcatechol and 4-methylcatechol using both 3MC2,3D expressed in E.coli and the
same enzyme from *P. putida* UCC2 [McClure, 1987]. Steady-state analysis requires that the concentration of enzyme is negligible compared to that of the substrate. A series of initial rates, \( v \), (which show a linear dependence with respect to enzyme, provided short time intervals are measured) for product formation were collected over a range of substrate concentrations. At low substrate concentration, \( v \) increases linearly with substrate concentration. At sufficiently high, or saturating, substrate concentrations, \( v \) tends towards a limiting value, \( V_{\text{max}} \), i.e., the initial rate follows saturation kinetics with respect to substrate concentration [Fersht, 1985] as depicted in Fig. 5.3.

Typical saturation behaviour was observed with all three substrates, allowing determination of the specificity of the enzyme for these substrates. The steady-state kinetic measurements of an enzyme usually give two pieces of kinetic data, \( K_m \) and \( k_{\text{cat}} \); in ideal cases, the \( K_m \) is equivalent to the values for \( K_s \), where \( K_s \) is the dissociation constant of the enzyme-substrate complex and therefore reflects the strength of substrate binding, and \( k_{\text{cat}} \) is the first order rate constant for chemical conversion of enzyme substrate complex to enzyme product complex, i.e., it denotes the rate-determining step of formation product. For this system \( V_{\text{max}} \) values are compared as an indication of the rate of the cleavage reaction.
Reaction rate plotted against substrate (catechol, 3-methylcatechol and 4-methylcatechol) concentration for a reaction obeying Michaelis–Menten kinetics. Data were obtained at 25 (± 0.1)°C in phosphate buffer, 0.20M, pH 7.00. Redrawn from Fersht [1985].
Table 5.2: Substrate Binding Strength of 3MC2,3D Towards Catechol and Monomethyl-Derivatives Compared with Other Dioxygenases

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BACTERIAL SOURCE</th>
<th>GROWTH SUBSTRATE</th>
<th>Km x 10^6M</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>3MeC</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>P. putida</td>
<td>Toluidine</td>
<td>0.36*</td>
<td>0.35*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+0.08)</td>
<td>(+0.09)</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>P. putida</td>
<td>Toluidine</td>
<td>0.41#</td>
<td>0.25#</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+0.15)</td>
<td>(+0.05)</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida mt-2</td>
<td>Benzoate</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida</td>
<td>Toluene</td>
<td>22.0</td>
<td>10.6</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida</td>
<td>Benzoate</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida mt-2</td>
<td>3-Methylbenzoate</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida</td>
<td>Benzoate</td>
<td>2.6*</td>
<td>2.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+1.0)</td>
<td>(+0.4)</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida</td>
<td>Benzoate</td>
<td>2.8#</td>
<td>2.7#</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+0.8)</td>
<td>(+0.6)</td>
</tr>
<tr>
<td>S4,5D</td>
<td>N. restrictus</td>
<td>Progesterone</td>
<td>130</td>
<td>450</td>
</tr>
</tbody>
</table>

All kinetic measurements were carried out in 0.20M phosphate buffer at pH 7.00. C, 3MeC and 4MeC denote the organic substrates catechol, 3-methylcatechol and 4-methylcatechol, respectively. Linear (*) and non-linear (#) computer analysis was employed.
3-Methylcatechol 2,3-dioxygenase shows high affinity for catechol, 3-methylcatechol and 4-methylcatechol. The $K_m$ values are all around 0.3 µM and are the lowest values seen for any catechol dioxygenase (Table 5.2). The $K_m$ values for 3MC2,3D expressed in *P. putida* UCC2 (results not shown) were identical to those reported for the same enzyme from *E.coli*. Furthermore, both individual sets of results, analysed by non-linear and linear least squares computer programs, agreed within experimental error. These values are significantly lower than those quoted for other dioxygenases [Tai, 1970; Klećka, 1981; Arciero, 1983; Engesser, 1988]. All the data sets bar one, appear to show that the enzymes bind catechol at a similar level to both the monomethyl-substituted analogues, regardless of the methyl position. This observation suggests that the presence of a methyl group either ortho- and meta- or meta- and para- to the hydroxyl substituents causes neither an electronic nor steric influence which affects the strength of substrate binding to the enzyme. No explanations or conclusions were drawn by Klećka [1981] as to why the presence of a methyl substituent at position four results in the binding of substrate being 50-fold worse (with respect to catechol itself), whereas with the methyl group at position three there is largely no effect. The same question may be addressed among all the *Pseudomonas* species where the binding strength of the three catechols varies over a 100-fold range. The only
apparent difference lies in the original growth medium of the bacteria. Hughes [1984] reports that different isofunctional enzymes are induced under different growth conditions. A variation in the initial growth substrate leads to a difference in the amino acid sequence of the resulting enzymes, which have consequently developed with different enzyme/substrate interactions. A 10-fold difference in substrate $K_m$ values was calculated to correspond to a difference in binding energy of around 5 to 6 kJ mol$^{-1}$, ca. that amount required for one hydrogen bond [Fersht, 1985].

A quantitative conversion of the catechols into their respective ring fission products was obtained as calculated from the molar extinction coefficients determined (under the conditions employed) for these three compounds (Table 5.1). The substrates studied and their $V_{\text{max}}$ values, relative to catechol, are presented in Table 5.3. The substrate specificity of 3-methylcatechol 2,3-dioxygenase (as deduced from the relative rates of cleavage, towards catechol and methyl-substituted analogues) is different from that seen for most other enzymes exhibiting catechol 2,3-dioxygenase behaviour, with 3-methylcatechol cleaved at a higher rate than catechol or 4-methylcatechol. These $V_{\text{max}}$ values for the enzyme as isolated from E.coli are identical within experimental error to those seen for the same enzyme from P. putida UCC2 (results not shown).
Table 5.3: Substrate Specificity of 3MC2,3D Towards Catechol and Monomethyl Analogues Compared With Other Extradiol Dioxygenases

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BACTERIAL SOURCE</th>
<th>GROWTH SUBSTRATE</th>
<th>V_{max} C</th>
<th>3MeC</th>
<th>4MeC</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em></td>
<td>Toluidine</td>
<td>100</td>
<td>173</td>
<td>45</td>
<td>Wallis, 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. arvilla</em></td>
<td>Benzoate</td>
<td>100</td>
<td>62</td>
<td>100</td>
<td>Nozaki, 1970</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em></td>
<td>Toluene</td>
<td>100</td>
<td>291</td>
<td>78</td>
<td>Klécka, 1981</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida mt-2</em></td>
<td>Pseudocumene</td>
<td>100</td>
<td>74</td>
<td>114</td>
<td>Kunz, 1981</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida mt-2</em></td>
<td>Toluene</td>
<td>100</td>
<td>74</td>
<td>94</td>
<td>Kunz, 1981</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>Azotobacter vinelandii</em></td>
<td>Benzoate</td>
<td>100</td>
<td>11</td>
<td>39</td>
<td>Sala-Trepai, 1981</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>Alcaligenes eutrophus</em></td>
<td>m-Toluate</td>
<td>100</td>
<td>145</td>
<td>36</td>
<td>Hughes, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em></td>
<td>Benzoate</td>
<td>100</td>
<td>38</td>
<td>54</td>
<td>Wallis, unpublished results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4,5D</td>
<td><em>N. restrictus</em></td>
<td>Progesterone</td>
<td>100</td>
<td>1233</td>
<td>717</td>
<td>Tai, 1970</td>
</tr>
</tbody>
</table>

All values were determined from steady-state kinetic measurements at 25 (±0.1)°C in 0.20M phosphate buffer at pH 7.00. For the purposes of comparison all the V_{max} values have been normalised to a value of 100 for the catechol as substrate.
These results can be partly explained by considering the catabolic pathway in which the enzyme functions. The 3MC2,3D originates from *P. putida* UCC2 which can utilise meta- or para-toluidine as the sole carbon and nitrogen source. (This *Pseudomonad* is also capable of growth on ortho-toluidine, although it is not an inducer of the enzyme). Oxidative deamination leads to methyl-substituted catechols as ring-cleavage substrates [McClure, 1986]. Thus the enzyme might be expected to cleave a methyl-substituted derivative in preference to catechol itself.

There are many examples cited in the literature where the substrate specificity of enzymes (from several bacterial species) towards catechol and mono-methylated substrate analogues have been investigated. Specificity appears to vary with both the species and with the carbon and nitrogen source on which the organism was originally grown. All three catechols are substrates in all cases and the presence of a methyl group, either at the three or four position, is accommodated even when the original growth substrate is not itself substituted. One might have expected with the benzoate induced *Pseudomonas putida* [Nozaki, 1970], where the initial growth substrate is metabolised through catechol, that the introduction of a methyl group may provide a steric barrier to cleavage, however this does not appear to be the case. The other carbon and nitrogen sources, 3-methylbenzoate,
pseudocumene (1,2,4-trimethylbenzene), toluate and toluene, which contain a methyl substituent, have been reported to be metabolised via methylcatechols [Claus, 1964; Bayly, 1966; Ribbons, 1966; Gibson, 1970] and hence it is less surprising that all three catechols are substrates for these enzymes. There is no apparent trend inferrable from these data as to the preference of the enzyme for methyl position with respect to the ortho-dihydroxy groups. It is, however, interesting to note that the toluene-grown strain of *P. putida* [Klećka, 1981], but not the one of Kunz [1981], and also the meta-toluate-induced *Alcaligenes eutrophus* [Hughes, 1984] possess the same order of reactivity as was found with 3MC2,3D from *P. putida* UCC2, namely that 3-methylcatechol is cleaved at a significantly higher rate than catechol which is in turn cleaved at a higher rate than 4-methylcatechol.

The difference in the observed reaction rates may be due to a variation in the activation energy barrier for the cleavage reactions arising from the electron donating effects of the methyl substituent which either destabilise (3-methylcatechol) or stabilise (4-methylcatechol) the transition state.

A series of substrate analogues, where one of the original functional groups was modified or missing, was used to assess the relative importance of the functional groups on substrate binding to the enzyme. The possible 3-methylcatechol 2,3-dioxygenase-'catechol' complex could
result from binding of either one or both of the hydroxyl groups and/or perhaps from some methyl group interaction. Neither phenol nor methylphenol served as an oxygen acceptor with this enzyme. Among the three isomeric dihydroxybenzenes only the ortho-isomer, catechol, was substantially oxygenated, no activity (or activity less than 0.1% relative to catechol) could be detected for either the meta-isomer (resorcinol) or the para-isomer (hydroquinone).

This reflected both the steric as well as the electronic influence of the hydroxy substituents. Thus, the existence of an ortho-diol group in the aromatic ring was shown to be an essential structural requirement for substrate cleavage.

5.3.2 pH Dependence of Enzyme Kinetics

Although enzymes contain a multitude of ionising groups, it is usually found that a plot of the rate of reaction versus pH has the form of a simple single or double ionisation curve [Fersht, 1985]. This is because the only important ionisations are those of groups directly involved in catalysis at the active site, or those of groups elsewhere in the protein responsible for maintaining the active conformation of the enzyme. Four simplifying assumptions are generally made; 1) the groups act as perfectly titrating acids or bases, 2) only one ionic form of the enzyme is active, 3) all intermediates
are in protonic equilibrium and 4) the rate-determining step does not change with pH. Hence by studying the pH dependence of the reaction rate for 3-methylcatechol 2,3-dioxygenase we hoped to gain further information on the catalytic mechanism.

The enzyme exhibited a bell-shaped pH profile (under saturating conditions, Fig. 5.4). The pKa values from this were found to be 6.9 (± 0.1) and 8.7 (± 0.1) from non-linear least squares analysis. The pH optimum was around 7.5. These properties are typical of those seen for other dioxygenases (Table 5.4) perhaps indicating a similarity of both structure and reaction mechanism. Catechol was used as a substrate in these experiments, despite the suggestion from the $V_{\text{max}}$ results (Section 5.3.1) that 3-methylcatechol was the natural substrate, since the product of the latter was unstable in phosphate buffer above pH 7.6 and was seen to vary from pink to orange between pH 9 and 12. The two hydroxyl groups of catechol show pKa values of 9.28 (± 0.02) and 13.05 [Powell, 1982] which are too high to account for these experimentally determined values and they are therefore ascribed to be protein based protonations/deprotonations.

The lower pKa value of 6.9 is characteristic of the amino acid residue histidine which has a range of 5 to 8 in proteins [Fersht, 1985]. From this data in conjunction with the other values shown in Table 5.5, we can infer that in both the intradiol and extradiol mechanisms such a
The kinetic measurements at each pH value were determined under saturating conditions. The $V_{\text{max}}$ values were normalised to 100 for the value at pH 7.5 which corresponds to the pH optimum.
Table 5.4: **pH Optima Comparison**

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SOURCE</th>
<th>pH OPTIMUM</th>
<th>BUFFER</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td><em>P.putida</em> mt-2</td>
<td>6.5</td>
<td>P</td>
<td>Nozaki, 1963, 1968</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>Pseudomonas</em> OC1</td>
<td>7.5</td>
<td>P</td>
<td>Kojima, 1967</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.aeruginosa</em></td>
<td>7.5-8.0</td>
<td>T</td>
<td>Kachhy, 1976</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>Pseudomonas</em></td>
<td>7.6</td>
<td>P</td>
<td>Lee, 1977</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida</em> mt-2</td>
<td>6.5</td>
<td>P,A,T</td>
<td>Nakai, 1983</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida</em></td>
<td>7.0</td>
<td>P</td>
<td>Wallis, unpubished results</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P.testosteroni</em></td>
<td>7.0-7.5</td>
<td>P</td>
<td>Dagley, 1968</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P.testosteroni</em></td>
<td>7.0</td>
<td>P</td>
<td>Ono, 1970</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>P.ovalis</em></td>
<td>8</td>
<td>T</td>
<td>Kita, 1965</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>B.stearothermophilus</em></td>
<td>8.4-8.7</td>
<td>P</td>
<td>Jamaluddin, 1977</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>Pseudomonas</em></td>
<td>8.4</td>
<td>P</td>
<td>Lee, 1977</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>B.brevis</em></td>
<td>8</td>
<td>P</td>
<td>Que, 1981</td>
</tr>
<tr>
<td>S4,5D</td>
<td><em>N.restrictus</em></td>
<td>5.8-7.5</td>
<td>P</td>
<td>Tai, 1970</td>
</tr>
<tr>
<td>C1,2D</td>
<td><em>P.putida</em></td>
<td>7-9</td>
<td>P</td>
<td>Kojima, 1967</td>
</tr>
<tr>
<td>C1,2D</td>
<td><em>P.putida</em> mt-2</td>
<td>7.5</td>
<td>T</td>
<td>Nakai, 1988</td>
</tr>
<tr>
<td>C1,2D</td>
<td><em>P.arvilia</em> C-1</td>
<td>7.5</td>
<td>T</td>
<td>Nakai, 1988</td>
</tr>
<tr>
<td>P3,4D</td>
<td><em>P.aeruginosa</em></td>
<td>8.0</td>
<td>T</td>
<td>Fujisawa, 1968</td>
</tr>
</tbody>
</table>

A, P and T denote acetate, phosphate and tris-acetate buffers, respectively.
### Table 5.5: Catechol Dioxygenase pKₐ Comparison

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BACTERIAL SOURCE</th>
<th>SUBSTRATE</th>
<th>pKₐ₁</th>
<th>pKₐ₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MC2,3D E. coli</td>
<td>Catechol</td>
<td>6.9</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>P. putida</td>
<td>Catechol</td>
<td>6.9</td>
<td>-</td>
</tr>
<tr>
<td>3MC2,3D E. coli</td>
<td>3-Methylcatechol</td>
<td>7.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>P. putida</td>
<td>3-Methylcatechol</td>
<td>7.2</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td>P. putida</td>
<td>Catechol</td>
<td>6.2</td>
<td>8.5</td>
</tr>
<tr>
<td>C1,2D</td>
<td>P. putida</td>
<td>Catechol</td>
<td>6.1</td>
<td>10.0</td>
</tr>
</tbody>
</table>

All values were determined at 25 (±0.1)°C in 0.20M phosphate buffer.
residue is catalytically important. Theoretically this crucial protonation would be consistent with either a conformational change to an inactive form of the enzyme or it could correlate with an active site base which is essential to the cleavage mechanism. The second explanation was preferred due to the drastic nature of the observed effect and this possibility was investigated by its attempted chemical modification with diethyl pyrocarbonate (Sections 5.2.2 and 5.3.3), the most widely used reagent for the selective modification of histidine residues [Lungblad, 1984].

Although the pKa values differ slightly between catechol 2,3-dioxygenase (6.2) and 3-methylcatechol 2,3-dioxygenase (6.9) it is highly probable that these two values represent the 'same' catalytically important histidine in the two proteins but that the difference reflects a small change in the microenvironment of this amino acid residue, which may be easily explainable by sequence comparison (previously discussed at length, Section 3.3.2).

The other pKa of 8.7 (8.5 for catechol 2,3-dioxygenase) might possibly be a tyrosine residue, however, again chemical or genetic modification would be required before any conclusive mechanistic role could be attached to either of these proposals. It is also possible that these pKa's arise from ligands to the iron centre which become displaced during catalysis to
accommodate either of the two substrates. Evidence in the literature indicates that the dioxygenases undergo a change in coordination number at the active site during the catalytic cycle [Roe, 1984; Whittaker, 1984, Orville, 1989]. Furthermore, several publications provide evidence that would support the idea of histidine and tyrosine being present as active site ligands [Felton, 1978, 1982; Keyes, 1978; Tatsuno, 1978; Bull, 1979; Que, 1979, 1980, 1981, 1987; Ohlendorf, 1988; Wu, 1989].

5.3.3 Attempted Modification of Histidine

The dependence of the reaction rate on pH for 3-methylcatechol 2,3-dioxygenase gave a bell-shaped curve exhibiting pKa values of 6.9 and 8.7 (Section 5.3.2). The possibility that the lower pKa value might be due to an active site histidine was investigated by attempted modification of this residue.

The modification of amino acids in enzymes can be achieved in two different ways; one, by employing chemical reagents [Means, 1971], or two, by Site-Directed Mutagenesis which involves the use of recombinant DNA techniques [Smith, 1985]. It was not appropriate to undertake the latter method in this instance, and no further reference to it will be made.

Chemical modifications have been used for some time and a large series of reagents have now been developed [Means, 1971]. One such well-documented reagent DEPC,

\[
\text{DEPC} \quad \text{pH 7.5, RT} \quad 30 \text{ min.}
\]

\[
\begin{align*}
\text{R} & \quad \text{HN} & \quad \text{OC} & \quad \text{OC}_2\text{H}_5 \\
\text{N} & \quad \text{O} & \quad \text{C} & \quad \text{OC}_2\text{H}_5 \\
\text{N} & \quad \text{O} & \quad \text{C} & \quad \text{OC}_2\text{H}_5
\end{align*}
\]

The reaction is accompanied by an increase in absorbance, which has a maximum between 230 and 250nm, and may be conveniently followed spectrophotometrically. The extent of reaction, or quantification of successfully modified histidyl residues is calculated using the relevant molar extinction coefficient for N-carbethoxy-histidine around 240 nm [Ovádi, 1967; Tudball, 1972; Jackman, 1988]. Although it is likely that the extinction coefficient of modified histidyl residues varies slightly [Miles, 1977], consistent stoichiometry has been obtained using the spectrophotometric method [Ovádi, 1967; Setlow, 1970; Vincent, 1975]. Excess DEPC is hydrolysed slowly in water giving two equivalents each of ethanol and carbon dioxide.

The reliability of the extent of modification was
initially determined with commercially obtained bovine serum albumin prior to experimentation with fully purified catechol 2,3-dioxygenase and 3-methylcatechol 2,3-dioxygenase. The extinction coefficient of Jackman [1988], $\varepsilon_{238} = 2750 \pm 100$ M$^{-1}$ cm$^{-1}$ was employed throughout. All three proteins appeared to become modified (Table 5.6) suggesting that there are several histidines accessible to modifying reagent. This is consistent with the abundance of spectroscopic and chemical approaches which have shown that the 'catechol' dioxygenases are histidine rich proteins (Sections 1.5 and 3.3.2). However, treatment of the enzymes with DEPC had no discernable effect on dioxygenase activity and virtually 100% of the original activity was retained, even after two hours. This failure to inactivate enzyme does not in itself negate the role of an active site base, it may simply reflect the inaccessibility of such a residue to the modifying reagent. Alternatively, the mechanism might not depend on base-catalysis [Que, 1987] but perhaps the 'essential' residue is a displaceable ligand to the iron [Cox, 1988]. The pKa of an amino acid residue thus coordinated would be vastly shifted from the usual range observed in proteins.

5.3.4 Oxygen Consumption Studies

Oxygen electrode studies were undertaken firstly, in an attempt to confirm that molecular oxygen was indeed a substrate for this novel enzyme, designated as a
Table 5.6: Modification of Histidine with DEPC

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>NUMBER OF HISTIDINE RESIDUES MODIFIED</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>9(±1)*</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td>12</td>
</tr>
<tr>
<td>C2,3D</td>
<td>9-10</td>
</tr>
</tbody>
</table>

* Bovine serum albumin (BSA) was used as a control (six experiments) to test the reliability of the extent of carbethoxylation.
dioxygenase, and further, to quantify the dioxygenase activity of the enzyme.

3-Methylcatechol was found to consume one mole of dioxygen per mole of organic substrate (catechol, 3-methylcatechol and 4-methylcatechol) in all three cases (Fig. 5.5). The total amount of dioxygen consumed was shown to be independent of the amount of enzyme added (Fig. 5.6), however, a direct correlation was seen to exist between the rate of dioxygen consumption and the total amount of enzyme added (Fig. 5.7). These observations confirmed our expectations that 3-methylcatechol 2,3-dioxygenase is indeed a dioxygenase and the one mole to one mole, substrate to dioxygen ratio is consistent with the reaction mechanism proposed for such a class of oxygenases (Section 5.3.5). Unfortunately, it was not possible to determine the dioxygen binding constant which we would expect, by extrapolation from the literature (Table 5.7), to be in the order of $10^{-7}$ to $10^{-6}$ M. This was beyond the sensitivity limits of the oxygen electrode, nevertheless, we can conclude that the $K_m$ for molecular oxygen must be $< 10^{-6}$ M.

Substrate specificity for 3-methylcatechol 2,3-dioxygenase towards the three catechols was also determined by oxygen electrode polarography. The results (reproducible to within 10%) agreed with data previously published by McClure [1986] (Table 5.8). The trend observed is
The figure shows the number of moles of dioxygen consumed per mole of organic substrate. A 1:1 relationship is observed.
The figure shows that the total amount of dioxygen consumed was not dependent on the amount of enzyme added.
The figure shows that a direct correlation exists between the rate of dioxygen consumption and the total amount of enzyme added.
Table 5.7: **Substrate Binding Constant Comparison for Both Intra- and Extra-diol Dioxygenases**

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BACTERIAL SOURCE</th>
<th>$K_m$ (µM)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2,3D</td>
<td><em>Pseudomonas OC1</em></td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida mt-2</em></td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.arvilla</em></td>
<td>2.5</td>
<td>9</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.aeruginosa</em></td>
<td>3.3</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>Pseudomonad</em></td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida</em></td>
<td>22.0</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida mt-2</em></td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida</em></td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P.putida mt-2</em></td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P.testosteroni</em></td>
<td>46</td>
<td>303</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P.testosteroni</em></td>
<td>80</td>
<td>54</td>
</tr>
<tr>
<td>P4,5D</td>
<td><em>P.testosteroni</em></td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>P.ovialis</em></td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>B.stearothermophilus</em></td>
<td>3.4 (+0.2)</td>
<td>-</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>Pseudomonas</em></td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>3,4DHPA2,3D</td>
<td><em>B.brevis</em></td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>G1,2D</td>
<td><em>P.acidovorans</em></td>
<td>74</td>
<td>55</td>
</tr>
<tr>
<td>G1,2D</td>
<td><em>P.testosteroni</em></td>
<td>85</td>
<td>96</td>
</tr>
<tr>
<td>S4,5D</td>
<td><em>N.restrictus</em></td>
<td>2.5</td>
<td>175</td>
</tr>
<tr>
<td>C1,2D*</td>
<td><em>P.arvilla</em></td>
<td>8.8</td>
<td>200</td>
</tr>
<tr>
<td>C1,2D</td>
<td><em>Pseudomonas OC1</em></td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>C1,2D</td>
<td><em>P.putida</em></td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>P3,4D</td>
<td><em>P.aeuruginosa</em></td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>P3,4D</td>
<td><em>P.aeuruginosa</em></td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

The $K_m$ values, for the physiological organic substrate and dioxygen respectively, are shown for a variety of dioxygenases. * denotes extradiol cleavage.
Table 5.8: Substrate Specificity Determined by Oxygen Electrode Polarography

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SOURCE</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>3MeC</td>
</tr>
<tr>
<td>3MC2,2D</td>
<td><em>E. coli</em></td>
<td>100</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±12)</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>E. coli</em></td>
<td>100</td>
<td>206</td>
</tr>
<tr>
<td>3MC2,3D</td>
<td><em>P. putida</em></td>
<td>100</td>
<td>180</td>
</tr>
<tr>
<td>C2,3D</td>
<td><em>P. putida</em></td>
<td>100</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±9)</td>
</tr>
</tbody>
</table>

For the purposes of comparison all the V<sub>max</sub> values have been normalised to a value of 100 for catechol as substrate. The enzyme activities of McClure [1986, 1987] are the mean of at least three independent determinations, all of which were within 30% of the mean value.
reassuringly the same as that determined spectrophotometrically (Section 5.3.1) i.e., 3-methyl-catechol > catechol > 4-methyl-catechol, although the two data sets fail to agree in absolute terms. This disagreement between the absolute values may result from a weakness in both methods of experimentation. The spectrophotometric technique relies on the sensitivity of the product extinction coefficients [Pascal, 1986] which have inconsistent values reported in the literature, moreover, they are known to be extremely sensitive to pH [Nakai, 1983]. Generally this method is more accurate than following polarographic analysis of O₂ uptake rates [Engesser, 1988] which are open to error resulting from other oxygen consuming reactions, especially if crude extract rather than fully purified enzyme is used. Furthermore, traces run over long periods of time are more susceptible to drifting discrepancies.

5.3.5 Mechanistic Implications

From extensive kinetic studies on several extradiol dioxygenases [Nakazawa, 1965; Nozaki, 1968; Hayaishi, 1969; Tai, 1970; Hirata, 1971; Hori, 1973; Bartels, 1984], a Bi-Uni ordered reaction mechanism has been proposed in which the enzyme (E) combines first with the organic substrate (S), followed sequentially by the addition of dioxygen (O₂) to form a ternary complex (ESO₂), the product (P) is then released, Eqn. 5.2.
Mechanisms are called ordered if all the substrates combine with the enzyme, and the products dissociate, in an obligatory order. Furthermore, binding of the first substrate causes a conformational change that increases the affinity of the enzyme for the other substrate. One exception to this mechanism, which is thought to be applicable to dioxygenases in general, has been found. Tai [1970] presents good evidence for an extradiol steroid dioxygenase, which supports the postulation that oxygen is in fact added prior to the organic substrate, this was also the original hypothesis (later refuted) of Nakazawa [1965] studying catechol 1,2-dioxygenase.

The kinetic results here would be consistent with this Bi-Uni mechanism in which enzyme combines with one substrate molecule and one dioxygen molecule to form a ternary complex before product is released. More extensive studies will be necessary before the sequence of substrate addition can be determined for 3-methylcatechol 2,3-dioxygenase.

5.4 Summary

The kinetics of 3-methylcatechol 2,3-dioxygenase were investigated using UV/visible spectrophotometry and oxygen electrode polarography. Measurements were made under both
standard and modified conditions. Typical saturation kinetics were observed for catechol, 3-methylcatechol and 4-methylcatechol as substrates. Data were analysed to give values of $V_{\text{max}}$ and $K_m$. The substrate specificity for this enzyme was somewhat different from that seen for other catechol 2,3-dioxygenases, with 3-methylcatechol being cleaved at the highest rate. The $K_m$ values for the organic substrates were all around 0.3 μM, the lowest found for any dioxygenase to date. The $K_m$ for dioxygen was deduced to be $< 10^{-6}$ M. The enzyme consumed one mole of oxygen per mole of substrate in all three cases. The dependence of enzyme activity on pH follows a classic bell-shaped curve with a pH optimum of about 7.5 and pKa values of 6.9 (± 0.1) and 8.7 (± 0.1). The possibility that the lower pKa value might be due to an active site histidine was investigated by attempted chemical modification of this residue.
5.5 References


Melchior, W.B., Jr., and Fahrney, D., (1970), Biochemistry, 9, 251-258.


Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. and Hayaishi, O., (1968), J.Biol.Chem., 243 (10),
2682-2690.


Wasser, A., in Model 777 Laboratory Oxygen Analyzer, (1958), 12, Beckman Instruments Inc., Fullerton, California.


CHAPTER 6

MATERIAL AND METHODS
6.1 **Growth Media**

The following media were prepared for the growth and maintenance of the bacterial cultures. All solutions were autoclaved prior to use, using either an Amsco electric steam generator (121°C, 15 p.s.i., 20 min) or a Denley sovereign autoclave (122°C, 16 p.s.i., 20 min), in order that the solutions were sterile when used.

6.1.1 **Luria Broth (L.B.) for the Growth of *E. coli* Culture (pNMN 24)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Difco/Oxoid)</td>
<td>1.0%</td>
</tr>
<tr>
<td>Yeast Extract (Difco)</td>
<td>0.5%</td>
</tr>
<tr>
<td>NaCl (Fisons, SLR)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Ampicillin (Sigma)</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Fresh, sterile, ampicillin was added to the autoclaved L.B. immediately prior to inoculation. Ampicillin selects for cells that produce 3MC2,3D.

6.1.2 **L.B. for the Growth of *P. putida* Culture (pNCM 1)**

Procedure as above except resistance conferred by the antibiotic streptomycin (0.015%).
### 6.1.3 p-Toluidine Media for the Growth of *P. putida* (pNCM 1)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Toluidine (Fisons, SLR)</td>
<td>0.3%</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.05%</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{HPO}_4) (Sigma)</td>
<td>0.3%</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4) (Sigma)</td>
<td>0.12%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2%</td>
</tr>
<tr>
<td>(\text{FeSO}_4\cdot\text{7H}_2\text{O}) (Fisons, SLR)</td>
<td>0.01%</td>
</tr>
<tr>
<td>(\text{MgSO}_4\cdot\text{7H}_2\text{O}) (Fisons, SLR)</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

The metal salts are sterilised separately and added to the autoclaved medium only immediately prior to inoculation. Fe and Mg supplements are required for enzyme cofactors and for DNA replication respectively.

### 6.1.4 Sodium Benzoate Medium for the Growth of *Pseudomonas putida* (ATCC 23973 and consequent expression of the protein catechol 2,3-dioxygenase)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Benzoate (Fisons, SLR)</td>
<td>0.3%</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.05%</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{HPO}_4)</td>
<td>0.3%</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>0.12%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2%</td>
</tr>
<tr>
<td>(\text{FeSO}_4\cdot\text{7H}_2\text{O})</td>
<td>0.01%</td>
</tr>
<tr>
<td>(\text{MgSO}_4\cdot\text{7H}_2\text{O})</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

Procedure as above.
6.1.5 Plates and Stabs for the Maintenance of all the Aforementioned Bacteria

Agar (Difco) 3%
Required medium (as above) Twice the above %
Ampicillin, Streptomycin or Metal Salts % as above

All three components were sterilised separately. Equal volumes of agar and the required medium were mixed, resulting in a final agar strength of 1.5%.

6.2 Centrifugation and Rotor Specifications

All centrifugations were carried out between 0 and 4°C using a Sorvall RC-5B refrigerated superspeed centrifuge (Sections 2.2, 2.3 and 2.4.4). Centrifugations either employed the GSA rotor (for large volumes and slow speeds, i.e., 1200 ml maximum volume for one spin and less than 27,500g) or the SS34 rotor (for small volumes at high speeds, 320 ml maximum volume and maximum speed 47,800g).

6.3 Preparation of Buffer Solutions

6.3.1 General

The pH of buffers was measured using a WPA glass electrode. Calibration of the WPA CD620 digital pH meter assembly was carried out at pH 7.00 and either at pH 4.00 or pH 10.00 (± 0.02 at 20°C) using standard Colourkey buffer solutions (BDH, electrochemically checked). The standard solutions used for calibration and the buffer
solutions to be measured were all at the same temperature. Deionised water, purified to a resistivity of 18.3 MΩ by reverse osmosis and ion exchange (Millipore Milli-QSP reagent water system) was used throughout to make all buffers.

6.3.2 Phosphate Buffer

Monobasic and dibasic sodium phosphate, NaH$_2$PO$_4$ and Na$_2$HPO$_4$ respectively (Sigma, reagent grade), were used to prepare phosphate buffers for the pH range 5.8 to 8.0 at 25°C. An appropriate volume and concentration of NaH$_2$PO$_4$ was titrated with an appropriate amount of Na$_2$HPO$_4$ after the method of Gomori [1955]. This provided phosphate buffers of appropriate concentration and pH as required for individual experiments.

6.3.3 Acetone-Phosphate Buffer

Acetone-phosphate buffer was prepared by adding 10% (v/v) acetone to a solution of phosphate buffer (Section 6.1.2). This mixture, which was most often required at pH 7.5, was prepared as follows: 222 ml of acetone were added to 21 of phosphate buffer at pH 7.3 and provided a 10% acetone-phosphate buffer solution of pH 7.5. This was the only buffer used in the protein preparation procedures.

6.3.4 Glycine-NaOH Buffer

Glycine (aminoacetic acid)-NaOH was prepared after the
method of Gomori [1955] to provide a buffer in the pH range 8.6-10.6 solely for the purpose of the pH dependence experiments (Sections 5.2.1 and 5.3.2).

6.4 Ammonium Sulphate Fractionation

In the course of isolating and purifying macromolecules, solutions often become very dilute. Charged macromolecules in polar solvents are solvated and thereby rendered soluble. If high concentrations of electrolytes are added, the solvent molecules are bound so tightly by the ions that they are unable to solvate the macromolecules which aggregate together and consequently come out of solution, this is termed "salting out". Salting out with ammonium sulphate is an easy way to precipitate proteins which can be collected by centrifugation and then redissolved in a smaller volume of whatever buffer is required. Although salting out does not achieve a large purification, it does, however, offer a convenient step by which the working volume of the enzyme can be dramatically reduced (Sections 2.3.2 and 2.3.3.). Ammonium sulphate is highly soluble, highly pure, inexpensive and it does not significantly alter the pH. Additionally it has few harmful effects on proteins and indeed stabilises most enzymes. As such it is the electrolyte of choice [Dixon, 1979; Green, 1985].
6.5 **Dialysis**

Seamless, semi-permeable dialysis tubing (Sigma) was soaked in deionised water for about 30 minutes and then thoroughly washed with deionised water and the appropriate buffer solution. (Dialysis tubing contains small amounts of glycerol, included as a humectant to maintain the pliability, therefore pre-washing is necessary to avoid contamination). A knot was tied at one end of the tubing before the protein solution was carefully introduced. The tubing was then sealed by tying a second knot. The dialysis sack was in turn placed in a large reservoir of a given buffer at 0 to 4°C. Small solute molecules and ions freely pass through the membrane until equilibrium is reached. The tubing retains most proteins of molecular weight 12,000 or greater. The buffer solution (at least 100 times the volume of the dialysis sack) was changed at least thrice over a period of around 16 hours.

6.6 **Column Chromatography**

The following sections describe the preparation and the use of ion exchange and gel filtration materials. The procedures employed were in accord with the manufacturer's instructions.

6.6.1 **Columns**

Care was taken to ensure that the column was mounted vertically. A small amount of glass wool was positioned
at the bottom of the column to prevent leakage of the chromatographic material. A slurry of the material was poured carefully into the column, with the tap fully open, until the desired column height was obtained. All columns were equilibrated and used in a cold cabinet or cold room kept between 0 and 4°C. The column materials were prepared (Sections 6.6.2 and 6.6.3) and washed with the appropriate buffer until the effluent solution had the same pH as the elutant. The column dimensions are reported as diameter x height. Columns stored for long periods between use were equilibrated in the appropriate buffer containing 0.02% azide. Deionised water was used throughout. When required, fractions were collected manually or using either a Biorad 2110 or a Gilson Microcol TDC80 fraction collector.

6.6.2 Ion Exchange Chromatography

Diethylaminoethyl cellulose (DE52, Whatman, \(\text{P}-0\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NET}_2\)) was used in column chromatography to separate molecules according to charge. As a weak anion exchanger it also acts in the separation of highly charged molecules from those of a smaller charge because the latter usually fail to bind. DE52 was obtained pre-swollen and needed no preliminary treatment. The anion exchanger was ready for equilibration with the desired buffer. This was carried out by stirring with a concentrated solution (0.2-1.0 M) of the buffer to be used
in the initial separation stage, for 2 to 3 minutes. About 15 to 30 ml of buffer are recommended for every dry gram of cellulose initially taken. The pH of the buffer/ion exchanger slurry was adjusted to the desired pH value with the acidic or basic component of the buffer. The supernatant was decanted after the slurry had settled. The ion exchanger was redispersed in fresh buffer before pouring.

6.6.2.1 Recovery of DE52

The ion exchanger may be autoclaved for sterilisation purposes. This is best carried out as a slurry, buffered with a non-volatile buffer of pH between 6.5 and 7.5. Alternatively, DE52 may be chemically sterilised by dispersion in 0.5M NaOH followed by washing with sterile water.

6.6.3 Gel Filtration or Molecular Sieve Chromatography

A gel is an inert 3D random network, in this case, polymers of D-galactose and 3,6-anhydro-1-galactose, held together with hydrogen bonds, that contain small pores. If a solution containing molecules of various dimensions is passed through the column, molecules larger than the pore size move only in the space between the particles and hence are not retarded by the column material. However, smaller molecules are slowed down by diffusion in and out of the particles with a probability that increases with
decreasing molecular size. In this way the separation is based on molecular size and the molecules are eluted from the column in order of decreasing molecular weight. Gel chromatography is unsurpassed in molecular separation because it can be carried out under virtually all conditions, i.e., for enzymes, conditions of maximum stability can be maintained.

It has been observed for a variety of gel types that a plot of $V_e/V_o$ versus log molecular weight (M.Wt.) generally yields a straight line. The volume at which the most active fraction is eluted is called the elution volume, $V_e$. The void volume, $V_o$, is usually measured by passing the excluded high molecular weight material Blue Dextran (average M.Wt. 2,000 kDa, Sigma) through the column. Hence to determine the M.Wt. simply requires the inclusion of several molecules of known M.Wt. to define the straight line and the relative molecular weight, $M_r$, of the sample can be calculated by interpolation with a precision of 10%.

Sepharose S300 gel filtration beads (Pharmacia, LKB) were prepared by swelling the gel in excess buffer solution. After allowing a few hours for swelling the gel was packed in a similar manner to that described for ion exchange columns. When packed the column was tested by passing through it a sample of Blue Dextran. The passage of Blue Dextran through the gel column revealed any irregularities of packing such as air bubbles. When
required, fractions were collected using either a Biorad 2110 or a Gilson Microcol TDC80 fraction collector. All gel columns were run between 0 and 4°C. The column was calibrated using the following molecular weight markers (Sigma); β-amylase (M.Wt. 200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), myoglobin (17.5 kDa) and cytochrome c (12.5 kDa) to ensure that the column was sufficiently able to resolve 3-methylcatechol 2,3-dioxygenase.

6.6.3.1 Maintenance of S300

The column material was cleaned with 1 to 2 bed volumes of 0.2M NaOH for a contact time of 1 hour. Immediately afterwards the column was fully equilibrated with 2 to 3 bed volumes of eluent buffer. Sodium azide (NaN₃, 0.02%) was added to the column as a bacteriostatic agent. Alternatively the column material was recovered by washing with a large excess of deionised water and stored as a slurry, at 0 to 4°C containing 0.02% azide, after autoclaving.

6.7 Assay Procedures

The enzyme has been shown to cleave catechols [McClure, 1986] which produce derivatives of 2-hydroxy-6-oxo-2,4-dienoic acid, upon insertion of dioxygen. The substrates are colourless in a solution of phosphate buffer (0.05M, pH 7.50, Section 6.3.2), in contrast to
their products which are yellow and can be detected in the visible region around 380 nm. (The catechol absorbance at this wavelength is negligible). The appearance of a yellow colour in an assay vial therefore clearly results from the presence of enzyme and is then a measure of the extent of activity. The activity of 3-methylcatechol 2,3-dioxygenase at each stage of the preparation could be quantitatively measured by monitoring the increase in absorbance around 380 nm with either a Pye-Unicam SP8-400 or Perkin Elmer λ9 spectrophotometer. The reaction mixture, contained in a total volume of 3.0 ml; substrate (final concentration around 1 mM) and phosphate buffer (2.80 ml, 0.05M, pH 7.50) in a cuvette with a 1 cm light path at ambient temperature. The reaction was initiated by the addition of an adequate amount (µl quantities) of the enzyme which was dissolved in a buffer containing 10% acetone. Catechol itself was generally used as the assay substrate because, 2-hydroxy-6-oxohexa-2,4-dienoic acid, the corresponding product exhibits the largest of all the product extinction coefficients (Table 5.1), and consequently provides the most sensitive means of enzyme detection spectrophotometrically. The advantages of this method include speed and simplicity and hence is the preferred assay procedure for routine use.

There are two defined units of enzyme activity currently employed; firstly, one unit (U) of enzyme is defined as that amount which will catalyse the
transformation of 1 micromole (μmol) of substrate per minute (temperature, pH and substrate concentration should be stated), and secondly, specific activity is expressed as U per milligram (mg) of protein.

Alternatively, the activity of 3MC2,3D could have been determined polarographically by monitoring the rate of consumption of the second substrate, oxygen, with an oxygen electrode (see Sections 5.2.3 and 5.3.4). However, unless otherwise specified the spectrophotometric assay method, formerly described, was employed as standard.

6.8 Pressure Dialysis

In direct contrast to salting out, this method removes water from the protein solution. If a solution is placed in a chamber, one wall of which is a very thin (0.1-1.5 μm) semi-permeable Diaflo membrane, small molecules, but not macromolecules can pass through the pores. The flow rate through these membranes is so low that they require operation under pressure, the rate of passage of small molecules increasing with increasing pressure. Hence when pressure is applied to the chamber, water is forced through and the macromolecules are concentrated.

The preparation procedure of 3-methylcatechol 2,3-dioxygenase (Chapter 2) required that the protein collected from the DE52 anion exchange column (when partially pure, Section 2.3.3), and later from the Sepharose S300 gel filtration column (once fully purified,
Section 2.3.4), was concentrated to less than 5 ml using pressure dialysis (30 p.s.i.). This procedure necessitates the use of an Amicon (stirred cell) concentrator and Diaflo membrane (PM10, cut off M.Wt. greater than 10,000). Different Amicons were employed as dictated by the initial volume of material awaiting concentration. The recommended maximum operational pressure for all three stirred cells; Model 3 (3 ml), Series 8000 (10 and 50 ml), was 75 p.s.i. Pressure dialysis was also used to change the buffer system prior to the DEPC modification experiments in Chapter 4.

6.9 Protein Concentration Determination

The standard protocol for protein concentration determination involves spectrophotometric measurement at 280 nm. This distinct absorption maximum is due primarily to the presence of tryptophan and tyrosine. Since the content of these two amino acids in enzymes varies only within reasonably narrow limits [Warburg, 1941] then the absorption peak at 280 nm can be used at a rapid and fairly sensitive measure of protein concentration.

Lowry [1951] too has suggested a simple correlation based on optical density measurements at 280 and 260 nm, for the direct determination of protein concentration. This technique is extensively referenced in the extradiol dioxygenase literature. Unfortunately, in the case of 3MC2,3D it was not found to be practically feasible, since
the acetone, which is present in the phosphate buffer to stabilise the enzyme, has a large absorption centred near 280 nm. A similar problem, encountered with 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase from *Bacillus brevis*, has been highlighted by Que [1981]. As a result of the inaccessibility of the standard protocol, the Biuret method (Sections 6.9.1 to 6.9.5) of Gornall [1949] was employed throughout. This standardisation is, however, destructive, requires more material for assay, and cannot be used in the presence of ammonium salts.

6.9.1 Biuret Method for the Quantitative Colourimetric Determination of Protein.

**Background and Principle**

This describes a simple, convenient and accurate method of total protein estimation using the Gornall [1949] formulation that reacts as follows:

\[
\text{alkaline} \quad \text{Copper} + \text{Serum sulphate} \rightarrow \frac{\text{proteins}}{\text{pH}} \rightarrow \text{Copper-protein complexes}
\]

The copper in the biuret reagent (Sigma) reacts with peptide bonds of serum proteins to form a purple colour, having an absorption maximum at 545 nm. The intensity of the colour is proportional to the total protein concentration.

6.9.2 Protein Standard Solution (Sigma)

Albumin (human) 5g/dl
6.9.3 Manual Procedure

One reagent blank and several standards were prepared for each series of tests.

1. To the tube labelled 'Reagent blank' 0.1 ml of water was added. To the tube labelled 'Standard' 0.1 ml of protein standard solution was added and to the tube labelled 'Test' 0.1 ml of enzyme was added.

2. Biuret reagent (5.0 ml) was added to all tubes, mixed thoroughly and allowed to stand at room temperature (18 to 26°C) for 30 minutes.

3. The absorbances at 545 nm of the 'Standards' and 'Test' were read versus 'Reagent blank' as a reference.

Matched cuvettes were employed in conjunction with either a Pye-Unicam SP8-400 or Perkin Elmer λ9 spectrophotometer.

6.9.4 Calibration and Calculation

The linearity of the biuret reaction is well recognised [Layne, 1957]. A calibration curve (Fig. 6.1) was constructed from the protein standards. The 'unknown' enzyme concentration was subsequently determined by linear regression. Care was taken to ensure that all glassware was free of protein films. Endogenous and exogenous proteins are known to interfere [Crowley, 1969].
Figure 6.1: Biuret Calibration Curve

The linearity of the biuret reaction, plotted as the absorbance at 545 nm versus the amount of standard protein, is used to determine enzyme concentration by linear regression.
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Most biological polymers are electrically charged and will therefore move in an electric field. Weber [1969] showed that the molecular weights of most proteins could be determined by measuring their mobility in polyacrylamide gels containing the ionic detergent sodium dodecyl sulphate (SDS) which has long been recognised as a powerful protein denaturant [Steinhardt, 1969]. This results because the rate of movement depends on the frictional coefficient and is thus related to the size and shape of the molecule. At neutral pH, in 1% SDS and 0.1M mercaptoethanol most proteins bind SDS and dissociate, disulphide linkages are broken by the mercaptoethanol, secondary structure is lost and the complexes consisting of protein subunits and SDS assume a random coil configuration. Proteins treated in this way behave as though they have uniform shape and an identical charge-to-mass ratio. If a series of proteins of known molecular weight are electrophoresed, they will separate into a series of bands and a semilogarithmic plot of the distance migrated versus log M.Wt. gives a straight line. Hence if a protein of unknown molecular weight is electrophoresed, its relative molecular weight, Mr, can be calculated by linear regression. Greatest accuracy (5-10%) is obtained if the known molecular weight values
bracket the unknown. This is certainly the most common way of estimating the molecular weight of protein subunits. It can be carried out rapidly and reproducibly and it requires only microgram quantities of protein. There are a number of stable proteins, of accurately known molecular weight, commercially available, which may be used as standards (Table 6.1).

Table 6.1: Calibration Kit for SDS-PAGE, Molecular Weight Markers, BDH Chemicals Ltd.

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>M.Wt.(Da)</th>
<th>( \log_{10} \text{M.Wt.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>Equine</td>
<td>12,300</td>
<td>4.0899</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>&quot;</td>
<td>17,200</td>
<td>4.2355</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>Bovine Erythrocyte</td>
<td>30,000</td>
<td>4.4771</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Hen egg</td>
<td>42,700</td>
<td>4.6304</td>
</tr>
<tr>
<td>Albumin</td>
<td>Bovine Serum</td>
<td>66,250</td>
<td>4.8212</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>Hen egg</td>
<td>76-78,000</td>
<td>4.8808-4.8921</td>
</tr>
</tbody>
</table>

6.10.1 Procedure

Polyacrylamide gel electrophoresis was carried out using a vertical gel box system (BRL, V16) holding a (160 x 168 x 1) mm gel. A potential was applied across the gel and controlled either by an LKB 2103 power supply or a Biorad 200/2.0 power supply. The resolving gel (usually 12%) solution was prepared with resolving buffer, an
appropriate amount of acrylamide and deionised water. The solution was filtered and degassed then cross-linked using a 10% ammonium persulphate solution (2.5 μl/ml, Sigma) and N,N,N',N'-tetramethylethylenediamine (approximately 1 μl/ml, Sigma), hereafter referred to as TEMED. (This is sufficient catalyst for polymerisation in 3 to 5 min). The stacking gel solution was prepared from buffer, acrylamide (half the corresponding amount used for the resolving gel) and water. The gel solution was polymerised using ammonium persulphate and TEMED as with the resolving gel. The percentage of acrylamide used for the resolving gel depended on the protein molecular weight or protein separation required, typically 12% for 3MC2,3D.

Samples were prepared for electrophoresis by dissolving 110 μg in 100 μl of sample buffer. Samples were incubated at 100°C for 2 minutes and allowed to cool to room temperature before loading onto the gel. Sample load can be between 10 and 50 μl with a suitable amount of Bromophenol blue as an internal marker. Electrode buffer was added to the upper and lower reservoirs of the gel apparatus and subjected to electrophoresis at a constant current of 20 mA (variable voltage) for approximately 5 hours or until the Bromophenol blue dye had reached the end of the gel. After electrophoresis the gel slab was removed to a suitable container for staining (with Coomassie brilliant blue, Sigma, [Wilson, 1983]) overnight and subsequent destaining at room temperature.

190
6.10.2 **Calibration of Polypeptide Molecular Weights**

The location of each stained M.Wt. marker protein was determined and a plot of \( \log_{10} \) of M.Wts. of the marker standards against the distance moved by the proteins typically gives the straight line illustrated (Fig. 3.1). The M.Wts. of the unknown sample were then determined from these graphs.

6.10.3 **SDS-PAGE Recipes**

Polyacrylamide gel electrophoresis [Maizel, 1971; Swank, 1971] was carried out in denaturing conditions. The various buffers and solutions were prepared as follows:

6.10.3.1 **Resolving Buffer (Buffer A)**

was usually stored as a two fold concentrate;

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl pH 8.8</td>
<td>0.375M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

6.10.3.2 **Stacking Buffer (Buffer B)**

was usually stored as a two fold concentrate;

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl pH 6.8</td>
<td>0.125M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
</tbody>
</table>
6.10.3.3 Reservoir Buffer

was usually stored as a five fold concentrate and diluted immediately before use with deionised water;

Tris/HCl pH 8.8 0.025M
Glycine (Sigma) 0.19M
SDS 0.1%

6.10.3.4 Stain

Isopropanol (v/v) 25%
Acetic Acid (v/v) 10%
Coomassie Brilliant Blue 0.1%

6.10.3.5 Destains

1. Isopropanol 25%
   Acetic Acid 10%
2. Isopropanol 10%
   Acetic Acid 10%
3. Acetic Acid 10%

6.10.3.6 SDS Sample Buffer (Dye Front)

Tris pH 6.8 0.125M
SDS 2%
Glycerol 10%
β-Mercaptoethanol 10%
Bromophenol Blue 0.01%
6.10.3.7 Acrylogel 5 Premix (BDH)

was stored either as a solid or a 36% (w/v) solution.
6.11 References


APPENDICES
APPENDIX I: SEQUENCE ALIGNMENT AND SECONDARY STRUCTURE PREDICTION

The SEQNET facility at the Science and Engineering Research Council Daresbury Laboratory was used under the supervision of Dr. G.A. Reid (Department of Microbiology, School of Agriculture, University of Edinburgh). The sequence alignments employed the program CLUSTAL [Higgins, 1988]. The secondary structure prediction analysis used packages from the University of Wisconsin Genetics Computer Group (UWGCG) including PEPTIDESTRUCTURE [Chou, 1978; Devereux, 1984; Garnier, 1978].

Appendix References


Steady-state kinetics are important for the understanding of metabolism since they measure the catalytic activity of an enzyme in the steady state conditions in the cell. It should be stated, however, that it is an approximation since the substrate is gradually depleted during the course of an experiment. Nevertheless, provided that the rate measurements are restricted to a short time interval, over which the concentration of substrate does not vary greatly, it is a very good approximation.

The majority of enzymes, including 3-methylcatechol 2,3-dioxygenase, involve two substrates, however many principles developed for single substrate [Michaelis-Menten, M-M, 1913] systems may be extended to multi-substrate systems. Generally reactions obey M-M kinetics when the concentration of one substrate is held constant, at saturating conditions, and the other is varied.

The M-M mechanism is used for interpretation of kinetic phenomena for the type of reaction pathway represented below, Eqn. I.

\[
\begin{align*}
K_s & \quad k_{cat} \\
E + S & \leftrightarrow ES \rightarrow E + P
\end{align*}
\]

where E, S, ES and P denote enzyme, substrate, enzyme-substrate complex and product, respectively.
The catalytic reaction is divided into two processes. The enzyme and substrate first combine to give the enzyme-substrate complex, held together by physical forces. This step is assumed to be rapid and reversible with no chemical changes taking place. The chemical processes occur in the second step with the first order rate constant, $k_{cat}$. The rate equations are solved in the following manner:

From Eqn. I, \[ \frac{[E][S]}{[ES]} = K_s \] (II)

and the initial rate, \[ v = k_{cat}[ES] \] (III)

Also the total enzyme concentration $[E_0]$ and the free enzyme $[E]$ are related by,

\[ [E] = [E_0] - [ES] \] (IV)

Thus from (II) and (IV), \[ [ES] = \frac{[E_0][S]}{[K_s]+[S]} \] (V)

and substituting (III) into (V) gives,

\[ v = \frac{[E_0][S]k_{cat}}{K_s + [S]} \] (VI)
the Michaelis-Menten equation. Note that the concentration of substrate at which \( v = \frac{1}{2} V_{\text{max}} \) is termed \( K_m \), the Michaelis constant.

It is useful to transform the M-M equation into a linear form for analysing data graphically and detecting deviations from the ideal behaviour. One of the best known methods is the double-reciprocal or Lineweaver-Burk [1934] plot. Inverting both sides of equation [VI] and substituting \( V_{\text{max}} \) for \( k_{\text{cat}}[E_0] \) gives the Lineweaver-Burk plot:

\[
\frac{1}{v} = \frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}}
\]

(VII)

Plotting \( \frac{1}{v} \) against \( \frac{1}{[S]} \) gives an intercept of \( \frac{1}{V_{\text{max}}} \) on the y-axis as \( \frac{1}{[S]} \) tends towards zero, and of \( \frac{1}{[S]} \) equal to \( \frac{1}{K_m} \) on the x-axis. The slope of the line is \( \frac{K_m}{V_{\text{max}}} \). One disadvantage of linearising saturation data is the introduction of error as data points at high substrate concentration are compressed into a small region, while these points at lower concentrations are emphasised.

Appendix References


APPENDIX III : PUBLICATIONS
Isolation and partial characterization of an extradiol non-haem iron dioxygenase which preferentially cleaves 3-methylcatechol

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A purification procedure has been developed for an extradiol dioxygenase expressed in Escherichia coli, which was originally derived from a Pseudomonas putida strain able to grow on toluidine. Physical and kinetic properties of the enzyme have been investigated. The enzyme has a subunit $M_r$ of $33,500 \pm 2000$ by SDS/polyacrylamide-gel electrophoresis. Gel filtration indicates a molecular mass under non-denaturing conditions of $120,000 \pm 20,000$. The $N$-terminal sequence (35 residues) of the enzyme has been determined and exhibits 50% identity with other extradiol dioxygenases. Fe(II) is a cofactor of the enzyme, as it is for other extradiol dioxygenases. The reactivity of this enzyme towards catechol and methyl-substituted catechols is somewhat different from that seen for other catechol 2,3-dioxygenases, with 3-methylcatechol cleaved at a higher rate than catechol or 4-methylcatechol. $K_m$ values for these substrates with this enzyme are all around 0.3 $\mu$M. The enzyme exhibits a bell-shaped pH profile with $pK_a$ values of 6.9 $\pm$ 0.1 and 8.7 $\pm$ 0.1. These results are compared with those found for other extradiol dioxygenases.

INTRODUCTION

Dioxygenases incorporate both atoms of dioxygen into organic substrates [1]. They are involved in a variety of reactions, including the cleavage of aromatic rings. The best characterized of the non-haem iron dioxygenases are the bacterial enzymes which cleave the double bonds of aromatic compounds adjacent to, or in between, hydroxyl groups. These include the catechol dioxygenases [2], which divide into Fe(III)-containing enzymes which cleave in an intradiol fashion and Fe(II)-containing enzymes which cleave in an extradiol fashion [3].

Catechol 2,3-dioxygenase (or metapyrocatechase), which contains Fe(II) as a cofactor [3], cleaves catechol in an extradiol fashion as shown in eqn. (1). This enzyme is obtained from a Pseudomonas putida strain (A.T.C.C. 23973) grown on benzoate as the sole carbon source and inducer [4].

$$\text{OH} \quad \text{OH}$$
$$\text{OH} \quad \text{OH}$$
$$\text{CHO} \quad \text{CO}_2\text{H}$$
$$\text{OH} \quad \text{OH}$$

Catechol 2,3-dioxygenase is also capable of cleaving substituted catechols, such as 3-methylcatechol and 4-methylcatechol, in a proximal extradiol fashion as indicated in eqns. (2) and (3).

$$\text{OH} \quad \text{OH}$$
$$\text{OH} \quad \text{OH}$$
$$\text{CHO} \quad \text{CO}_2\text{H}$$

Recently, McClure & Venables have described a Pseudomonas putida strain, UCC2, able to utilize $m$- or $p$-toluidine as sole carbon and nitrogen source [5,6]. Strain UCC2 expresses a novel extradiol dioxygenase which is plasmid encoded [6]. A DNA fragment containing the structural gene for this dioxygenase has been cloned into vectors for direct expression in Escherichia coli ([6]; N. C. McClure, unpublished work). We report here the purification (from E. coli) and partial characterization of this non-haem iron dioxygenase which preferentially acts on 3-methylcatechol rather than catechol, and is thus referred to as 3-methylcatechol 2,3-dioxygenase throughout. Comparisons are made to the well-characterized catechol 2,3-dioxygenase from Pseudomonas putida (A.T.C.C. 23973).

MATERIALS AND METHODS

Strains, media and growth

3-Methylcatechol 2,3-dioxygenase was obtained from E. coli JM107 [7] containing pNMN24 (a gift from Dr. N. C. McClure), which is an expression vector containing the structural gene encoding for this enzyme (N. C. McClure, unpublished work). Plasmid-bearing E. coli cells were grown at 37°C in Luria broth [8] supplemented with 100 $\mu$g of ampicillin/ml.

Catechol 2,3-dioxygenase was obtained from Pseudomonas putida A.T.C.C. 23973. P. putida A.T.C.C. 23973 cells were grown using benzoate as inducer and major carbon source as previously described [4].
Table 1. Purification details for the isolation of the 3-methylcatechol 2,3-dioxygenase from *E. coli*

The Table is based on an extraction from 50 g (wet weight) of cells. Specific activity is expressed as the number of μmol of product produced/min per mg of protein at 25 °C and pH 7.00. A detailed description of the procedure can be found in the Materials and methods section. This Table can be compared with that previously reported for catechol 2,3-dioxygenase in [4].

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (munits)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant after cell lysis</td>
<td>4180</td>
<td>245</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>After (NH₄)₂SO₄ and dialysis</td>
<td>3370</td>
<td>275</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>After DE52 ion exchange</td>
<td>915</td>
<td>870</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>After S-300 gel filtration</td>
<td>55</td>
<td>7630</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

Enzyme isolation

The isolation procedure used was identical for both cell types and is substantially different to previously published methods. Frozen cells (50 g) were resuspended in 500 ml of acetone/phosphate buffer [0.05 m-phosphate buffer, pH 7.50, containing 10% (v/v) acetone] at 4 °C. A low concentration of an organic solvent (acetone or ethanol) is known to protect Fe(II)-dioxygenases from inactivation by oxidation [9]. Lysozyme (Sigma) was added to approx. 0.2 mg/ml and a small amount (1 mg) of DNAase (Sigma) was also added. The mixture was incubated on ice for 30 min, then centrifuged at 15000 g for 10 mins to remove cell debris. The supernatant which was then re-dialysed overnight, this time against acetone/phosphate buffer. The resulting precipitate was dissolved in acetone/phosphate buffer at pH 7.00 and concentrated to < 5 ml with an Amicon concentrator. Portions (1 ml) of this solution were passed through a Sepharose S-300 gel filtration column (150 cm x 3 cm) in acetone/phosphate buffer. Fractions containing active enzyme were combined and brought to 70% (NH₄)₂SO₄ saturation. After centrifugation at 39000 g for 15 min the resulting precipitate was dissolved in acetone/phosphate buffer and concentrated to < 5 ml with an Amicon concentrator. Portions (1 ml) of this solution were passed through a Sepharose S-300 gel filtration column (150 cm x 3 cm) in acetone/phosphate buffer. Fractions containing active enzyme were combined and concentrated to 5 ml using an Amicon concentrator. Fully purified enzyme showed a single band (Mr 33500 ± 2000) on a Coomassie-stained SDS/polyacrylamide gel. Further details of the purification procedure are given in Table 1.

Molecular mass determination

Subunit molecular masses were determined using SDS/PAGE (12% acrylamide). Native molecular masses were determined using a Sepharose S-300 column (150 cm x 3 cm) calibrated with the following molecular mass markers (Sigma): β-amyrase (200000), alcohol dehydrogenase (150000), bovine serum albumin (66000), carboxic anhydrase (29000), myoglobin (17500) and cytochrome c (12500).

N-Terminal sequencing

The N-terminal sequence was determined using an Applied Biosystems 477 sequencer by the WELMET protein characterization facility at the University of Edinburgh.

Fe(II) replacement and oxidation

Fe(II) was removed from the enzyme (with a concomitant loss of activity to less than 5% of the original value) by treatment with EDTA (BDH AnaR). EDTA (100-fold molar excess) was added to a 10–50 μM enzyme solution, which was then dialysed overnight against 0.05 m-phosphate buffer, pH 7.50, with no acetone present. Reactivation was achieved by addition of ferrous sulphate (10-fold molar excess) to the inactive enzyme which was then re-dialysed overnight, this time against acetone/phosphate buffer.

The oxidation of the Fe(II) cofactor was achieved by titration of an enzyme solution (10–50 μM) with either potassium ferricyanide or H₂O₂ until a complete loss of activity was achieved. Re-reduction was carried out by the addition of a 100-fold molar excess of sodium dithionite.

Kinetic studies

All kinetic experiments were carried out in 0.20 m-phosphate buffer at pH 7.00 and at 25 ±0.1 °C (except for the pH profile experiments which were carried out in phosphate buffer between pH 5.00 and pH 9.00). All

Table 2. Substrates and reaction products, and spectroscopic values used in monitoring enzyme activity

The molar absorption coefficients were determined in 0.20 m-phosphate buffer, pH 7.00.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Wavelength monitored (nm)</th>
<th>ε (M⁻¹·cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>Hexa-2,4-dienedioic acid</td>
<td>375</td>
<td>48400</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>2-Hydroxy-6-oxohepta-2,4-dienoic acid</td>
<td>390</td>
<td>19800</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>2-Hydroxy-5-methyl-6-oxohepta-2,4-dienoic acid</td>
<td>380</td>
<td>33200</td>
</tr>
</tbody>
</table>
Isolation of a 3-methylcatechol 2,3-dioxygenase

measurements were made under steady-state conditions with the substrates catechol, 3-methylcatechol and 4-methylcatechol. Enzyme activities were measured by following the formation of reaction products using Pye-Unicam SP800 and Perkin-Elmer λ9 spectrophotometers at the wavelengths shown in Table 2. Experimental data were fitted to a non-linear least-squares program and also represented as Lineweaver-Burk plots to yield $K_m$ values.

Modification with diethylpyrocarbonate

Protein to be modified (30 μM) was treated with a 20-fold molar excess of diethylpyrocarbonate (Sigma) using published procedures [10,11]. The diethylpyrocarbonate was standardized before use with imidazole. Bovine serum albumin was used as a control protein to verify successful modification of histidine residues by monitoring the increase in absorbance at 238 nm ($ε_{2750} = 1.1 m^{-1} cm^{-1}$ [11]).

RESULTS

Molecular mass determination

Purified 3-methylcatechol 2,3-dioxygenase showed a single band on SDS/PAGE corresponding to a subunit $M_r$ of 33 500±2000. The native molecular mass determined by gel filtration was found to be 120 000±20 000, consistent with the native enzyme being a tetramer of identical subunits.

N-Terminal amino acid sequence

The N-terminal sequence (35 residues) of the 3-methylcatechol 2,3-dioxygenase has been determined (Fig. 1) and shows around 50% identity to two published sequences of catechol 2,3-dioxygenases [12,13] deduced from the nucleotide sequences of the metapyrocatechase genes on the TOL plasmid of P. putida mt-2 [12] and the NAH7 plasmid of P. putida PpG7 [13].

Fe(II) replacement and oxidation

3-Methylcatechol 2,3-dioxygenase in acetone/phosphate buffer can be stored for a period of days at 4 °C without any marked loss of activity. Treatment of the enzyme with EDTA caused a reversible loss of enzymic activity. Activity was completely restored by incubation of the enzyme with excess ferrous sulphate. Treatment of the enzyme with oxidants, ferricyanide and $H_2O_2$, also resulted in a loss of activity. The oxidation with ferricyanide was found to be reversible, with 60% of the original activity restored by reduction with sodium dithionite. Enzyme treated with $H_2O_2$ could not be reactivated at all by addition of sodium dithionite.

Kinetic studies

Steady-state kinetic measurements were carried out on both 3-methylcatechol 2,3-dioxygenase (from E. coli) and catechol 2,3-dioxygenase (from P. putida A.T.C.C. 23973) using the following substrates: catechol, 3-methylcatechol and 4-methylcatechol. Typical saturation behaviour was observed in all cases, allowing determination of $K_m$ and relative $k_{cat}$ values (Table 3). The relative $k_{cat}$ values for the 3-methylcatechol 2,3-dioxygenase as isolated from E. coli are identical (within experimental error) with those for the same enzyme from P. putida UCC2 [6]. The dependence of the reaction rate on pH for 3-methylcatechol 2,3-dioxygenase (under saturating conditions) gave a bell-shaped curve (Fig. 2). The $pK_a$ values from this curve were found to be 6.9±0.1.

Table 3. Kinetic properties of 3-methylcatechol 2,3-dioxygenase and catechol 2,3-dioxygenase

All values were determined from steady-state kinetic measurements at 25 ± 0.1 °C, in 0.20 M-phosphate buffer, pH 7.00. For the purposes of comparison all the $k_{cat}$ values have been normalized to a value of 100 for catechol as substrate.
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but with $PK_a$ values of $6.2 \pm 0.1$ and $8.5 \pm 0.2$ and a pH optimum of 7.0. The possibility that the lower $PK_a$ value had no effect on the enzymic activity. The fact that activity is restored following re-reduction indicates that Fe(II) is essential for enzyme function, with the oxidized Fe(III) state being inactive. The irreversible loss of activity following treatment of the enzyme with $H_2O_2$ suggests that this oxidant has effects other than a simple one-electron oxidation of the Fe(II) centre.

There are marked differences between the kinetic properties of 3-methylcatechol 2,3-dioxygenase and catechol 2,3-dioxygenase (Table 3). For example, there are differences in the substrate specificity as deduced from the relative rates of cleavage for the two enzymes. This can be partly explained by considering the catabolic pathway in which 4-methylcatechol 2,3-dioxygenase functions. The 3-methylcatechol 2,3-dioxygenase originates from \textit{P. putida} UCC2, which can utilize \textit{m}- or \textit{p}-toluidine as the sole carbon and nitrogen source. Oxidative deamination of toluidine leads to methyl-substituted catechols (as opposed to catechol) as ring-cleavage substrates [5]. Thus the enzyme might be expected to cleave a methyl-substituted catechol in preference to catechol itself. Another marked difference between the two enzymes is the fact that there is a 10-fold difference in substrate $K_m$ values (Table 3), with values for 3-methylcatechol 2,3-dioxygenase in the region of $0.3 \mu M$ and for catechol 2,3-dioxygenase around $3 \mu M$. This difference in $K_m$ values corresponds to a difference in binding energy of around 5–6 kJ mol$^{-1}$. The $K_m$ values for the three substrates with 3-methylcatechol 2,3-dioxygenase are surprisingly low, in fact a $K_m$ of $0.3 \mu M$ is lower than any other published $K_m$ value for a catechol dioxygenase, whether of the extradiol or intradiol type (see table of values in [3]). This low $K_m$ value implies a significant difference in the nature of the active site in 3-methylcatechol 2,3-dioxygenase compared to that of catechol 2,3-dioxygenase.

The dependence of 3-methylcatechol 2,3-dioxygenase enzyme activity on pH gave a bell-shaped curve (Fig. 2) with $pK_a$ values of around 6.9 and 8.7 and a pH optimum of 7.5. The two hydroxyl groups of catechol show $K_m$ values which are too high to account for these values, and we therefore ascribe them to protein-based protonations/deprotonations. The lower $pK_a$ value of 6.9 might be due to an active site base, perhaps histidine. This possibility was investigated by the attempted modification of such a histidine with diethylpyrocarbonate. Treatment of the enzyme with diethylpyrocarbonate, however, had no discernable effect on activity. This failure to inactivate the enzyme with diethylpyrocarbonate does not in itself negate the role of an active site base, it may simply reflect the inaccessibility of such a residue to the modifying agent. Alternatively this $pK_a$ might be the result of the protonation of some other active site residue such as a ligand to the iron.

From our studies on 3-methylcatechol 2,3-dioxygenase we drew the following conclusions. (i) The enzyme is a tetramer of identical subunits. (ii) The $N$-terminal amino acid sequence (35 residues) of the enzyme shows around 50% identity with published sequences of catechol 2,3-dioxygenases. (iii) Fe(II) is an essential cofactor of the enzyme. (iv) The $K_m$ for catechol is the lowest seen for EDTA and restored on incubation with ferrous ions, clearly demonstrating that activity is dependent on the presence of iron and also that metal removal and replacement is fairly easily achieved. Oxidation of the Fe(II) cofactor by ferricyanide also results in a loss of enzymic activity. The fact that activity is restored following re-reduction indicates that Fe(II) is essential for enzyme function, with the oxidized Fe(III) state being inactive. The irreversible loss of activity following treatment of the enzyme with $H_2O_2$ suggests that this oxidant has effects other than a simple one-electron oxidation of the Fe(II) centre.

D\textsc{iscussion}

We have described a purification procedure for a 3-methylcatechol 2,3-dioxygenase from \textit{E. coli} cells containing pNMN24 (N. C. McClure, unpublished work). The isolation procedure allows purification of the enzyme to > 90\% homogeneity (Table 1) within 4–5 days. After purification the enzyme has a native molecular mass of 120000 ± 20000, and consists of a single subunit type ($M_r$ 33500 ± 2000), consistent with the enzyme existing as a tetramer of identical subunits. This is similar to the catechol 2,3-dioxygenase from \textit{P. putida}, which has a molecular mass of 140000 and consists of four identical subunits [3]. It is therefore likely that these enzymes have similar quaternary structures.

The $N$-terminal amino acid sequence of the 3-methylcatechol 2,3-dioxygenase (Fig. 1) shows around 50\% identity to the published sequences of two catechol 2,3-dioxygenases [12,13], indicating that, although the enzymes may have similar quaternary structures, there are marked differences in primary structure. Both of the catechol 2,3-dioxygenase sequences begin with Met-Asn-Lys [12,13]; these three residues are absent from the 3-methylcatechol 2,3-dioxygenase expressed in \textit{E. coli}. This may be due to posttranslational modification of the enzyme during extraction from \textit{E. coli}. The DNA sequence encoding the 3-methylcatechol 2,3-dioxygenase is currently being determined (N. C. McClure, personal communication); this will eventually allow the deduction of the entire amino acid sequence and hence confirm the presence or absence of these three residues.

Enzyme activity can be removed by treatment with pH 7.5, which corresponds to the pH optimum.

and 8.7 ± 0.1 and the pH optimum was around 7.5. Similarly, catechol 2,3-dioxygenase gave a bell-shaped curve, but with $PK_a$ values of $6.2 \pm 0.1$ and $8.5 \pm 0.2$ and a pH optimum of 7.0. The possibility that the lower $PK_a$ value might be due to an active-site histidine was investigated by attempted modification of this residue with diethylpyrocarbonate. This would be expected to inactivate the enzyme by removing the ability of such a histidine to act as an active-site base. However, treatment of the enzyme with diethylpyrocarbonate (even in large excess, in which control experiments with bovine serum albumin showed complete modification of all accessible histidines) had no effect on the enzymic activity.

The kinetic determinations at each pH value were determined under saturating conditions. The $k_{cat}$ values were normalized to 100 for the value at pH 7.5, which corresponds to the pH optimum.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{pH-dependence of the catalytic activity of 3-methylcatechol 2,3-dioxygenase}
\end{figure}

The kinetic determinations at each pH value were determined under saturating conditions. The $k_{cat}$ values were normalized to 100 for the value at pH 7.5, which corresponds to the pH optimum.
any of the catechol dioxygenases to date. (v) The dependence of enzyme activity on pH follows a classic bell-shaped curve with a pH optimum of around 7.5.

We thank N. C. McClure for the gift of pNMN24 and the WELMET protein characterization facility of the University of Edinburgh for N-terminal sequencing. This work was aided by financial support from Unilever.

REFERENCES


Received 10 July 1989/29 September 1989; accepted 6 October 1989
APPENDIX IV : LIST OF LECTURE COURSES AND MEETINGS
ATTENDED

Lecture Courses Attended

1. "Business Management",
   Prof. S. Coke and colleagues, Business Studies Department, 1988.
2. "The Lanthanides and Actinides",
   Dr. S. Cradock, 1988.
3. "Introduction to X-Ray Structure Determination",
4. "Industrial Processes",
5. "Medicinal Chemistry",
   Prof. R. Baker and Dr. P. Leeson, Merck Sharp and Dohme Research Laboratories, 1989.
6. "Postgraduate Lectures",
7. "Medicinal Aspects of Inorganic Chemistry",
Meetings Attended

1. "Scribe Course",
2. "R.S.C. Dalton Scottish Division Meeting",
3. "University of Strathclyde Inorganic Chemistry
   Departmental Annual Conference",
4. "Fifth Ames Symposium",
   Edinburgh University, 1988.
5. "The Smith Kline and French Symposium on Chemical
   Approaches to Enzyme Catalysis and Structure",
6. "Scottish Protein Group 58th Meeting",
   St. Andrews University, 1989.
7. "SERC CRAC Graduate School",
   Brunel University, 1989.
8. "Inorganic Biochemistry Discussion Group Spring
   Meeting",
   Essex University, 1989.
9. "4th International Conference of Bioinorganic
   Chemistry",
   Massachusetts Institute of Technology, Boston, USA, 1989.
10. "10th International Symposium on Flavins and Flavoproteins",
Università Degli Studenti di Milano, Como, Italy, 1990.