Studies on non-haem ferrous-dependent oxygenases and oxidases


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Biological reactions involving dioxygen are commonly mediated via initial complexation of dioxygen to iron. In the cases of the iron–porphyrin and iron–sulphur enzyme families, extensive experimental and theoretical studies have been carried out over the last 30 years. Although important aspects of the catalytic chemistry of these enzymes remain uncertain, for example the precise nature of dioxygen activation and the structure of the oxidizing species, detailed mechanistic proposals have been made. In contrast, the reactivity of many other biological iron centres remains relatively unexplored. In the last decade it has become clear that there is a superfamily of non-haem iron proteins, which includes both oxygen-transport proteins, oxygenases and oxidases. In parallel with porphyrin-utilizing proteins, the non-haem family contains members with a requirement for a single iron cofactor and those with a requirement for two iron cofactors. A polynuclear iron protein is also known in the form of the iron-storage protein ferritin (for a review see [1]).

As studies on iron-dependent proteins progress, it is unavoidable that they are categorized according to the presence or absence of porphyrin cofactor, the oxidation state of the iron and the number of iron atoms. Such classifications are useful for highlighting differences between the different types of enzyme, but may also hide similarities. Recent mechanistic and spectroscopic studies are beginning to provide detailed insights into the structures and mechanisms of the non-haem iron-dependent enzymes, which complement those carried out on the porphyrin-utilizing enzymes. Comparison of data from the two families may suggest why nature has evolved superficially very different metal co-ordination platforms for dealing with the problems associated with directing the oxidizing potential of dioxygen.

We have been studying iron-dependent oxidases and oxygenases that have no cofactor other than ferrous iron. The majority of members of this subfamily identified have a requirement for a 2-oxo acid as a cosubstrate, normally 2-oxoglutarate (Figure 1a) (for a review see [2]). The 2-oxoglutarate-dependent oxygenases are involved in a wide range of metabolic processes, including collagen biosynthesis (proline-4-hydroxylase [3]), antibiotic biosynthesis (e.g. clavaminic acid synthase, CAS [4,6]) and plant ‘hormone’ biosynthesis (e.g. gibberellic C-20 oxidase [7]). Kinetic studies on proline-4-hydroxylase and lysyl hydroxylase have led to proposals of an ordered sequential mechanism, in which Fe²⁺, 2-oxoglutarate and dioxygen bind sequentially to the enzymes [3]. Reaction then occurs to generate CO₂, succinate and an enzyme-bound [ferryl(Fe(IV)=O)] species, which effects substrate oxidation via a ‘rebound’ mechanism [1,3,8]. These mechanistic proposals are supported by kinetic and substrate analogue experiments on the 2-oxoglutarate-dependent oxygenase, deacetoxy/deacetylcephalosporin C synthase [9–11], and by analogy with proposals for IPNS [11–13].

Comparison of the sequences of many 2-oxoglutarate-dependent oxygenases suggests a common evolutionary origin [2,14]. However, some 2-oxo acid-dependent oxygenases (e.g. CAS [15], proline-4-hydroxylase [16], p-hydroxyphenylpyruvate dioxygenase [17,18]) apparently do not display any sequence similarity to each other or any other members of the family, resulting in proposals of convergent evolution to a closely related function [2,15]. This proposal is supported by spectroscopic analyses and chemical modification experiments, which have indicated that two to three histidine residues act as iron ligands in all the non-haem ferrous-dependent oxygenases studied. For example,

Abbreviations used: CAS, clavaminic acid synthase; IPNS, isopenicillin N-synthase; ACC oxidase, 1-aminoacyclopropane-1-carboxylic acid oxidase.

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CAS and proline-4-hydroxylase [16] (and IPNS [19] and ACC oxidase [20]), which do not show any significant sequence similarity to each other, each apparently use the side chains of histidine residues as iron ligands.

Figure 1

Reactions catalysed by non-haem ferrous-dependent oxygenases and oxidases

(a) Stoichiometry of a 2-oxoglutarate-dependent oxygenase hydroxylation reaction. A typically less than stoichiometric amount of oxygen from dioxygen is incorporated into the product alcohol (see [3–5] for examples). (b) The isopenicillin N-synthase (IPNS)-catalysed conversion of L-δ-(α-aminoadipoyl)-L-cysteine-α-valine (ACV) to isopenicillin N. LAA = L-δ-(α-aminoadipoyl). (c) The 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase catalysed conversion of ACC into ethylene. (d) The 'partial' reaction of 2-oxoglutarate-dependent oxygenases in which reduction of dioxygen is coupled to ascorbate and 2-oxoglutarate turnover. (e) Proposed 'partial' reaction of 2-oxoglutarate-dependent oxygenases in which reduction of dioxygen is coupled to turnover of two molecules of 2-oxoglutarate. •, 18O; 16O/18O; O, 16O.
similarities to each other and to the sequence-related subfamily of 2-oxoglutarate-dependent oxygenases [2,14,21], but the intradiol dioxygenases do not display any overall sequence similarity to any other non-haem ferrous-dependent enzymes [22].

Crystallographic studies on IPNS [23] and the intradiol dioxygenases [24,25] have provided insights into the iron co-ordination chemistry and should provide a platform for detailed mechanistic studies. The crystal structure of *Aspergillus nidulans* IPNS complexed to manganese, substituted for iron, has revealed a single metal at the active site with an approximately octahedral metal co-ordination, utilizing the side chains of four amino acid residues, Asp-214, His-216, His-270 and Gln-330, and two water molecules [23]. In comparison, crystal structures of the intradiol dioxygenases [24,25] have revealed a square pyramidal geometry utilizing three protein-bound ligands (two histidines and a glutamate) and two water molecules. Impressions of similarities between the co-ordination chemistry of IPNS and the aromatic intradiol dioxygenases are enhanced by the proposal (based on mechanistic suggestions and sequence analyses) that the side chain of Gln-330 is displaced from the iron during the catalytic cycle by the thiol of the tripeptide substrate, L-δ-(α-aminoadipoyl)-L-cysteine-D-valine, during the catalytic cycle [23].

Interpretation of extensive substrate analogues, kinetic and spectroscopic studies on IPNS in the light of its crystal structure is allowing a detailed mechanism to be established [13,23]. In contrast, the ACC oxidase reaction mechanism is less clear, despite the apparent structural similarity to IPNS and remains a considerable challenge.

ACC oxidase catalyses the final step in the biosynthesis of the plant 'hormone' (or signalling molecule) ethylene. Instead of using 2-oxoglutarate, ascorbate acts as a cosubstrate being apparently stoichiometrically oxidized to dehydroascorbate [25]. Uniquely amongst the family of known ferrous-dependent oxidases and oxygenases, ACC oxidase also requires CO₂ or bicarbonate as an activator [26,27]. Furthermore, although catalytic inactivation of the ferrous-dependent oxygenases has been previously reported, purified ACC oxidase is particularly labile with a typical half-life of less than 20 min under optimal catalytic conditions [28]. Iron-ascorbate-mediated inactivation of a number of enzymes has been reported (e.g. see [29]), but in none of the previous studies was the enzyme iron dependent nor was ascorbate used as a substrate. In the case of the 2-oxo acid-dependent oxygenases, ascorbate is not required for catalysis but is commonly added to incubation mixtures to maximize the lifetime of the activity. However, in some cases the addition of ascorbate may be inhibitory (e.g. CAS) or has little effect [16]. In the case of proline-4-hydroxylase, stoichiometric conversion of ascorbate with 2-oxoglutarate has been observed in the absence of a peptide substrate (the 'partial reaction', Figure 1d) [30]. However, even in the absence of ascorbate (and dithiothreitol) proline-4-hydroxylase still catalyses turnover of 2-oxoglutarate to succinate, albeit at a reduced rate. A possible explanation for this is that a second molecule of 2-oxoglutarate effects reduction of the ferryl intermediate, i.e. two molecules of 2-oxoglutarate are consumed for each dioxygen molecule (Figure 1e).

Inactivation of ACC oxidase is not due to product inhibition by dehydroascorbate or cyanide, and, in the absence of ACC, a distinct ascorbate-dependent inactivation is observed. This inactivation can be partially prevented by the addition of catalase, implying the involvement of H₂O₂ [28]. Further studies on the inactivation and kinetics of the ACC oxidase reaction have demonstrated that its catalytic instability results from several different processes. Unfolding of the catalytically active conformation to a less active conformation may occur in the absence of iron or substrates. However, more rapid inactivation is observed in the presence of iron, dioxygen and ascorbate. This type of oxidative inactivation is only partially protected against by the addition of catalase, implying at least two processes, only one of which involves H₂O₂ present in solution. The H₂O₂ may be generated in solution by Fenton-type reactions or may 'leak' from the ACC oxidase active site. The oxidative damage to the enzyme that is not catalase 'protectable' results in partial proteolysis of ACC oxidase, the fragmentation pattern of which depends on the presence of ACC even in the absence of bicarbonate or CO₂. One cleavage site was located between Leu-186 and Phe-187, which maps close to the predicted active site of ACC oxidase based on the structure of IPNS. The peptide sequence immediately after the cleavage point, FQDD, is similar to a cleavage sequence, FNDD, reported for 'malic' enzyme after fragmentation with iron and ascorbate [29], but the cleavage point in 'malic' enzyme occurs...
immediately after this sequence, not before. It is possible that the polar side chain residues contained within the 'cleavage' sequences provide ligands for iron-binding sites, e.g. in ACC oxidase there is a second iron-binding site, which is involved in the fragmentation process.

The role of bicarbonate or CO$_2$ in the ethylene-forming reaction of ACC oxidase is not known. The rate of enzyme inactivation in the presence of ACC, iron and ascorbate was slowed down dramatically on inclusion of bicarbonate, but bicarbonate has very little effect on the rate of iron-ascorbate-mediated inactivation in the absence of ACC. It is likely that the protective effect of bicarbonate against inactivation under catalytic conditions is directly related to its role as a catalytic activator. Together with supporting kinetic and inhibitor studies, the inactivation work has led to a working hypothesis for the catalytic cycle of ACC oxidase. By analogy with proposals for the Z-oxo acid-dependent dioxygenases and IPNS, it is proposed that ascorbate and dioxygen bind and react with an ACC oxidase-Fe(II) complex (A, Figure 2) to form an iron(II)-linked peroxide intermediate (B, Figure 2) concomitant with production of dehydroascorbate. ACC may then bind to the peroxide intermediate to give complex C (Figure 2). Bicarbonate or CO$_2$ then binds causing formation of a ferryl intermediate (D, Figure 2), which oxidizes ACC to ethylene and cyanoformic acid, completing the catalytic cycle. Clearly these proposals require further experimental testing and may also need to accommodate the possibility of more than one iron-binding site in ACC oxidase.

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Structural studies on the catalytic component of benzene dioxygenase from Pseudomonas putida

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Introduction

Pseudomonas putida ML2 utilizes benzene as its sole source of carbon and energy. Benzene is first oxidized to cis-1,2-dihydroxycyclohexa-3,5-diene through the addition of both atoms of dioxygen to the aromatic nucleus [1,2]. This reaction is catalysed by the multicomponent enzyme, benzene dioxygenase (EC 1.14.12.3). The individual components of the enzyme have been purified [1,3]. A flavoprotein, reductaseRED, accepts electrons from NADH and transfers them to a small iron-sulphur protein, ferredoxinRED [4,5]. The latter component then reduces a terminal oxygenase, ISPRED, which is a large iron–sulphur protein composed of two disimilar subunits in an α2β2 configuration [6]. The reduced terminal oxygenase catalyses the dihydroxylation of benzene. The genes encoding the enzyme, bedA (reductase), bedB (ferredoxin) and bedC1C2 (terminal oxygenase α- and β-subunits respectively), have been cloned and sequenced. From the deduced amino acid sequence, motifs have been identified for the ligation of the redox centres [7].

The ISPRED contains two types of redox centre, namely catalytic non-haem iron and iron–sulphur clusters. The latter have been demonstrated, by cluster-extrusion experiments [8] and by Mössbauer spectroscopy [9], to be two [2Fe-2S] clusters. In addition, the latter showed that in the oxidized form the two iron atoms within each centre are high-spin ferric but with considerable inequivalence. ISPRED gives an EPR signal in the reduced state with rhombic symmetry and the g factors $g_z = 2.018$, $g_r = 1.917$, $g_\theta = 1.917$. 