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Minireview

# 4-Hydroxyphenylpyruvate dioxygenase

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**Abstract**

4-Hydroxyphenylpyruvate dioxygenase (HPPD) is an Fe(II)-dependent, non-heme oxygenase that catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate. This reaction involves decarboxylation, substituent migration and aromatic oxygenation in a single catalytic cycle. HPPD is a member of the α-keto acid dependent oxygenases that typically require an α-keto acid (almost exclusively α-ketoglutarate) and molecular oxygen to either oxygenate or oxidize a third molecule. As an exception in this class of enzymes HPPD has only two substrates, does not use α-ketoglutarate, and incorporates both atoms of dioxygen into the aromatic product, homogentisate. The tertiary structure of the enzyme would suggest that its mechanism converged with that of other α-keto acid enzymes from an extradiol dioxygenase progenitor.

The transformation catalyzed by HPPD has both agricultural and therapeutic significance. HPPD catalyzes the second step in the pathway for the catabolism of tyrosine, that is common to essentially all aerobic forms of life. In plants this pathway has an anabolic branch from homogentisate that forms essential isoprenoid redox cofactors such as plastoquinone and tocopherol. Naturally occurring multi-ketone molecules act as allelopathic agents by inhibiting HPPD and preventing the production of homogentisate and hence required redox cofactors. This has been the basis for the development of a range of very effective herbicides that are currently used commercially. In humans, deficiencies of specific enzymes of the tyrosine catabolism pathway give rise to a number of severe metabolic disorders. Interestingly, HPPD inhibitor/herbicide molecules act also as therapeutic agents for a number of debilitating and lethal inborn defects in tyrosine catabolism by preventing the accumulation of toxic metabolites.


*Keywords:* Dioxygenase; Oxygenase; Alpha-keto acid; Molecular Oxygen; Inhibitor; 4-Hydroxyphenylpyruvate; Activation; Review; Iron; Ferrous; Non-heme

In aerobic metabolism, the conversion of 4-hydroxyphenylpyruvate (HPP)[1] to 2,5-dihydroxyphenylacetate (homogentisate) is catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD). While this transformation is unique in nature and involves decarboxylation, aromatic hydroxylation, and substituent migration in a single catalytic cycle, the reaction is similar to those catalyzed by the α-keto acid dependent superfamily of oxygenase enzymes [1]. This conversion is the second step of a catabolic pathway that yields acetoacetate and fumarate from L-tyrosine (Scheme 1) [2]. While these ketogenic and glucogenic products have a direct energetic contribution, in higher organisms the pathway serves additional functions. In animals it is required to modulate blood tyrosine levels [3] and in plants the pathway is used for the anabolic production of essential cofactors such as plastoquinone and tocopherol from homogentisate [4]. This latter role for tyrosine catabolism has generated significant research by agrochemical interests who wish to design inhibitors/herbicides that uncouple photosynthesis by suppressing the production of the

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[1] *Abbreviations used:* HPP, 4-hydroxyphenylpyruvate; HPPD, 4-hydroxyphenylpyruvate dioxygenase; PDB, Protien Data Bank; HMS, hydroxy mandelate synthase; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexane dione, MCD, magnetic circular dichroism.

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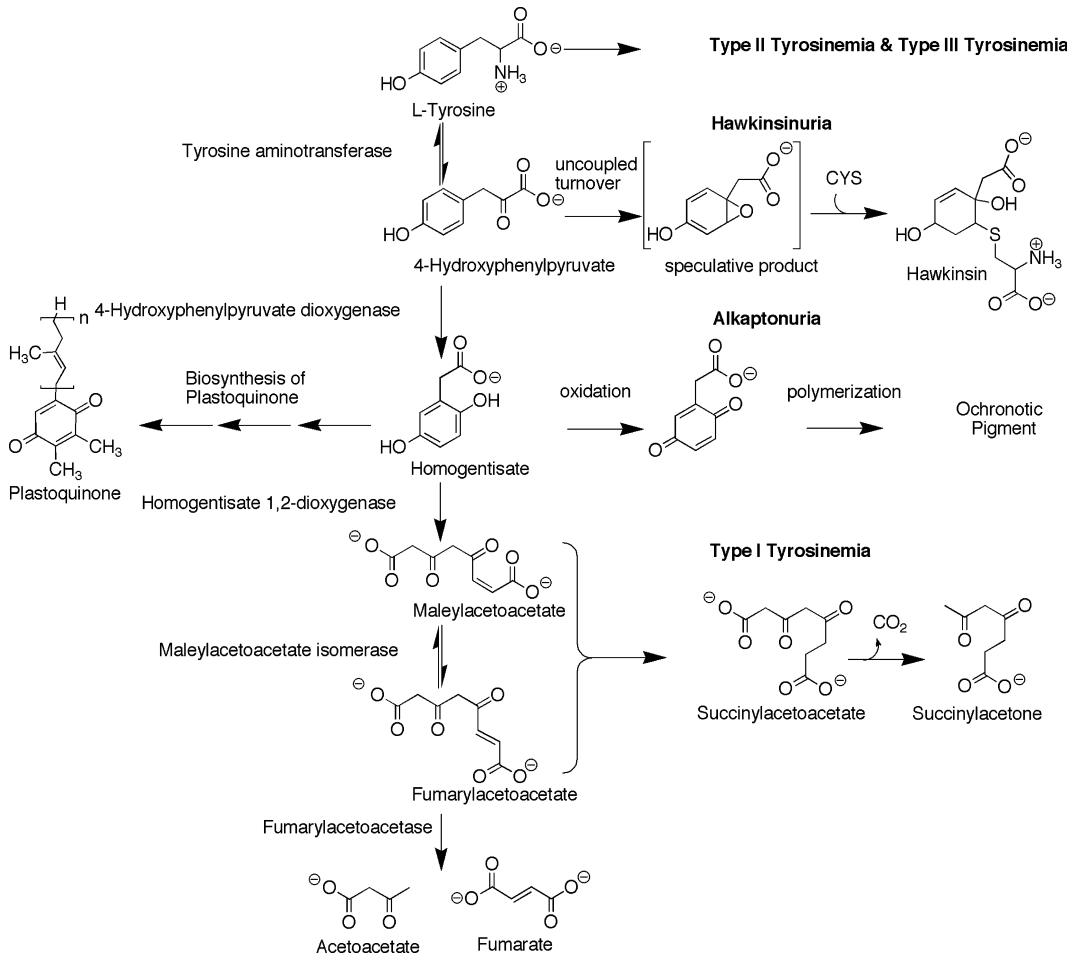


Scheme 1.

redox active lipophilic cofactors that link the two photosystems. Such investigations comprise the majority of the scientific literature for HPPD and have produced a number of commercially available herbicides. Interestingly, one of the early molecules developed as an herbicide is now used therapeutically; the specific inhibition of HPPD is currently used to alleviate the symptoms of two metabolic defects and could conceivably be applied to a third (vide supra). Considerably less research has been undertaken on the chemistry behind the quite extraordinary transformation that yields homogentisate from HPP.

In mammals, inborn defects in tyrosine catabolism give rise to disease states that range in severity from mild to lethal (right side of Scheme 1). A deficiency of the first tyrosine catabolism enzyme, tyrosine aminotransferase, produces type II tyrosinemia, a disease characterized by elevated levels of blood tyrosine that result in mild mental retardation at birth and corneal opacities later in life [5]. Type III tyrosinemia arises from a deficiency of active HPPD [6] and is largely indistinguishable from type II with regard to symptomology due to the reversibility of the preceding tyrosine aminotransferase reaction [7]. Hawkinsinuria is a result of a single mutation in the N-terminal region of the HPPD gene that brings about uncoupled turnover. The mutant enzyme releases an as yet unidentified intermediate/product that becomes covalently linked to thiols such as cysteine and glutathione and is excreted in large quantity in the urine [6]. The primary symptom of this disease is metabolic acidosis, which leads to a host of other deleterious symptoms including severe stunting. The oldest known inherited metabolic disorder is due to deficiency in active homogentisate 1,2-dioxygenase and is known as alkaptonuria [8]. Individuals who suffer from this deficiency accumulate large quantities of the homogentisate hydroquinone which readily oxidizes to the quinone. The reactive quinone can then polymerize to form a structurally uncharacterized caramel colored molecule known as the ochronotic pigment. While excretion of this pigment simply discolors urine without immediate adverse effects, it is the accumulation of it in cartilage and collagenous tissues that gives rise to the chronic debilitating symptoms of arthritic disability [9,10]. Deficiencies of the fourth tyrosine catabolism enzyme, maleylacetoacetate isomerase are not lethal or debilitating due to the propensity of maleylacetoacetate to form both succiny-

lacetoacetate and fumarylacetoacetate in the absence of the enzyme, both of which are substrates for the last enzyme in the pathway, fumarylacetoacetase [11,12]. However, a deficiency of fumarylacetoacetase causes the most severe of the tyrosine catabolism defects, type I tyrosinemia. Type I tyrosinemia patients suffer from liver dysfunction and renal proximal tubular failure and incur a high rate of mortality due to primary liver cancer [13,14]. Specific inhibition of HPPD thus has the capacity to alleviate the symptoms of hawkinsinuria, alkaptonuria and type I tyrosinemia [15,16] while inducing mild side effects whose symptomology in part mimicks those of types II and III tyrosinemia [3,17,18].

## HPPD structure

HPPD is found in all aerobic forms of life with the exception of some Gram positive bacteria [19]. The protein has a subunit mass of 40–50 kDa and typically associates to form tetramers and dimers in bacteria and eukaryotes, respectively [20–22]. The primary structure of the enzyme can be divided into a more variant N-terminus and a more conserved C-terminus. Plant sequences show the only significant variation in the latter half of the primary structure, exhibiting a 15 amino acid insertion close to the carboxy terminus [23,24]. The first X-ray crystal structure of HPPD published was from *Pseudomonas fluorescens* (Protein Data Bank, PDB ID = 1CJX) [25]. Since that time the structures of *Arabidopsis thaliana* (PDB ID = 1SP9 and 1TFZ) [23,24], *Zea mays* (PDB ID = 1SP8) [23], *Streptomyces avermitilis* (PDB ID = 1T47) [26] and Rat (PDB ID = 1SQI) [24] HPPD have been solved. These structures indicated that the N- and C-termini fold into discrete domains and that the active site is formed exclusively from residues of the latter (Fig. 1).While the function of the N-terminal half of the protein is not known, a single alanine to threonine mutation at position 33 in the human enzyme (corresponding to position 37 in Fig. 2) is the cause of hawkinsinuria (vide infra) [6]. The peptide fold of HPPD is unlike that of other α-keto acid dependent oxygenases that typically have a jellyroll fold motif (eight β-strands arranged in a barrel). HPPD has two topologically similar barrel-like domains that each contain a βαββββα module similar to the topology of a number of extradiol type dioxygenases [25–30] (Fig. 2).

Common to all non-heme Fe(II) dependent oxygenases is a 2-His-1-carboxylate facial triad that localizes the active site metal ion through direct coordination [31]. The α-keto acid dependent oxygenases can be categorized into three groups based on the spacing of these three ligands in the primary structure; the general spacing motif is $HX(D/E)X_{50-210}H$ [32]. HPPD cannot be placed into the α-keto acid enzyme categories having the motif, $HX_{\sim 80}HX_{\sim 80}E$, again more similar to that of the extradiol dioxygenase enzymes ($\underline{HX_{\sim 65}HX_{\sim 50}E}$) (Fig. 1). This, when considered with the overall similarity in peptide fold, suggests that HPPD mechanistic evolution converged with that of the α-keto acid dependent enzymes from an extradiol dioxygenase progenitor.

With the exception of a C-terminal helix, the active site metal ion of HPPD is surrounded by a bower of β-sheet structure (Fig. 2). Conserved active site amino acids extend toward the metal from this peptide, forming a sphere of inward pointing side chains. The binding pocket for the substrate is not currently known. Serre et al. [25] modeled the substrate in the active site and proposed a binding mode for HPP that was consistent with that observed for other α-keto acid dependent enzymes. This binding mode was reliant upon both bidentate coordination of the α-keto acid moiety with the active site metal ion and hydrogen bonding with conserved amide residues [25,30,33–38]. Recently, a different HPP binding position has been proposed which has proven consistent with the crystallographically observed binding position of a structurally related inhibitor in complex with HPPD [19,26] (vide supra). In this proposed binding pocket, the HPP α-keto acid moiety still makes bidentate contact with the active metal ion; however, the phenol is sandwiched between two conserved phenylalanine rings (Fig. 3).

## The reaction catalyzed

Scheme 2 depicts the reaction catalyzed by HPPD. In 1970 Lindblad et al. [39] demonstrated using $^{17}O_2$ that two atoms from dioxygen are incorporated in the formation of homogentisate, one at Cα of the pyruvate side chain and the other at C1 of the aromatic ring with respect to HPP. Early discontinuous assay methods by Rundgren were based on the displacement of labeled carboxylate from the pyruvate substituent for quantitation as $^{14}CO_2$ and thus established the occurrence of an oxidative decarboxylation in catalysis [40]. Rundgren showed that a tritium atom ortho to the pyruvate substituent is lost to solvent during catalysis, and thus proved that the 1,2-shift of the decarboxylated (now aceto) side chain was consistent with the NIH shift observed with P450 hydroxylases and pterin dependent oxygenase enzymes [41–47]. Using the assay methods described above, Rundgren also determined that the substrate binding mechanism of HPPD involved an ordered addition of substrates and an ordered release of products in which HPP was the first substrate to bind and $CO_2$ was the first product to dissociate [40].

## Substrate specificity

HPPD has been shown to have reasonably broad substrate specificity, recognizing a range of polar and

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MTQTTHHTPDTARQADPFPVKGMDAVVFAVGNAKQAAHYYSTAFGMQLVAYSGPENGSR 59
ETASYVLTNGSARFVLTSVIKPATPWGHFLADHVAEHGDGVVDLAIEVPDARAAHAYAI 118
EHGARSVAEPYELKDEHGTVVLAAIATYGKTRHTLVDRTGYDGPYLPGYVAAAPIVEPP 177
AHRTFQAIDHCVGNVELGRMNEWVGFYNKVMGFTNMKEFVGDDIATEYSALMSKVVADG 236
TLKVKFPINEPALAKKKSQIDEYLEFYGGAGVQHIALNTGDIVETVRTMRAAGVQFLDT 295
PDSYYDTLGEWVGDTRVPVDTLRELKILADRDEDGYLLQIFTKPVQDRPTVFFEIIERH 354
GSMGFGKGNFKALFEAIEREQEKRGNL 381
```

Fig. 1. The *Streptomyces avermitilis* primary structure and conservation when compared to that of Pseudomonas fluorescens, *Homo sapiens*, *Rattus norvegicus*, *A. thaliana*, *Daucus carota*, *Burkholderia fungorum*, and *Magnaporthe grisea*. Amino acids depicted in red are fully conserved while those in blue show similarity in all sequences. Amino acids represented by increased font size ligand the active site metal ion. Sequences underlined are within 10 Åof the active site metal ion.

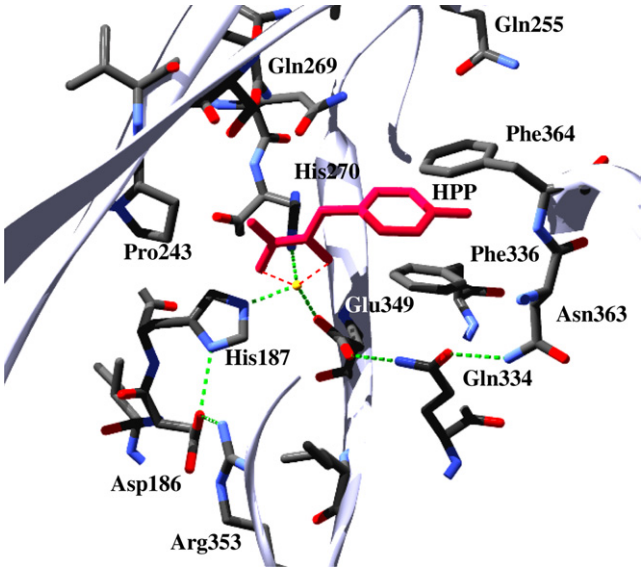
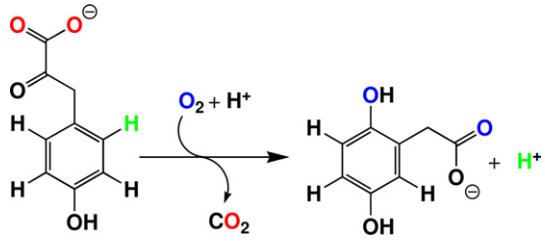



Fig. 3. A proposed binding site for HPP (depicted in red) in the active site of HPPD from *S. avermitilis*. The substrate was modeled into the cavity occupied by the inhibitor, NTBC, in this structure (see Fig. 5).




Scheme 2.

non-polar α-keto acids as substrate. Rat liver HPPD can decarboxylate and oxygenate α-ketoisocaproate ((4-methyl, α-keto)pentanoic acid) to *β*-hydroxyisovaleric acid ((3-hydroxy, 3-methyl)butyric acid) [48] and 2-oxo-5-thiahexanoic acid (the α-keto acid of methionine) to 4-thiapentanoic acid-4-oxide [49]. Reports for *Pseudomonas*, pig and human preparations of HPPD indicate phenylpyruvate as a substrate forming (2-hydroxyphenyl)acetic acid [22,50,51]. Interestingly, these latter observations seem at odds with the accumulation of phenylpyruvate in human subjects deficient in phenylalanine hydroxylase activity (phenylketonuria), as such activity by HPPD would deplete phenylpyruvate and alleviate the disease state [52]. Moreover, HPPD from *Streptomyces* has been shown to be entirely devoid of either decarboxylase or dioxygenase activity in the presence of phenylpyruvate, despite the apparent similar mode of association of this ligand with the active site metal ion to that of HPP [53].

While the requirement for the phenol of the substrate to stimulate the activity of HPPD appears to be species dependent, the enzyme will accept a number of molecules as substrates that have substituent additions to the aromatic ring and/or alterations to the pyruvate side chain. Pascal et al. showed that (2-fluoro)HPP was converted to (3-fluoro)homogentisate and that (4-hydroxyphenyl)-thiopyruvic acid produced (4-hydroxyphenyl)-sulfinylacetate as a consequence of the second

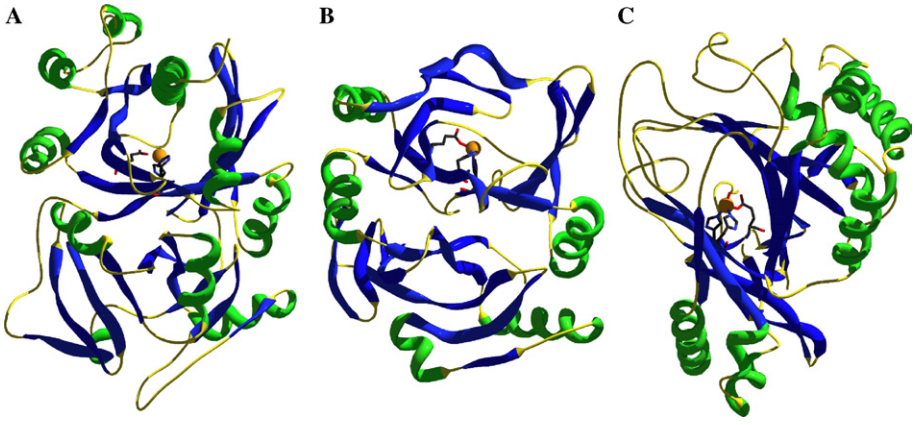
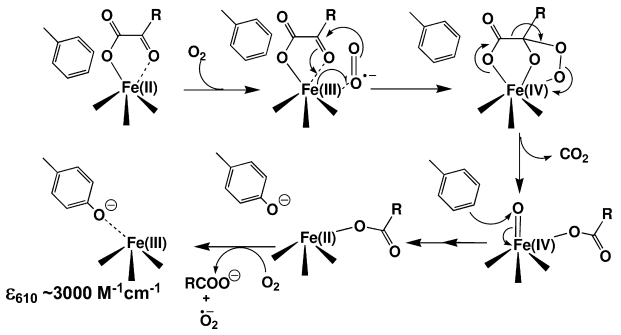



Fig. 2. Comparison of the tertiary structures of HPPD from *Pseudomonas fluorescens* (A), dihydroxybiphenyl dioxygenase from *Pseudomonas cepacia* (B), and clavaminate synthase from *Streptomyces clavuligerus* (C). For each structure the position of the active site is indicated by the position of the metal ion and its protein ligands.

oxygenation occurring on the electron rich sulfur atom of the thiopyruvic acid side chain [54]. In regard to this latter observation, HPPD has no tendency to oxygenate the benzylic carbon of HPP, however, the factors that dictate the position of hydroxylation appear to be relatively subtle. Hydroxymandelate synthase (HMS) has the same substrates and catalyzes a highly similar reaction to that of HPPD with the second atom of dioxygen being incorporated at the benzylic carbon and hence the product is the *para*-α-hydroxyaceto substituted phenol, hydroxymandelate, rather than homogentisate. HPPD and HMS show high sequence homology and based on the sequence differences between the two enzymes, Gunsior et al. were able to make a mutant form of HPPD that showed increased preference for hydroxylation of the benzylic carbon of HPP [19].




Scheme 3.

## Self hydroxylation

A number of forms of HPPD have a distinct purple or blue color when isolated in the presence of exogenous iron. This color is a consequence of a broad absorbance centered at around 600 nm that has an extinction coefficient of approximately 3000 $M^{-1}$ $cm^{-1}$ [50,55]. HPPD in this form is inactive, though the addition of reductants such as ascorbate slowly bleaches the absorbance and restores activity with a first order dependence on the concentration of the reductant. Interestingly, the rate of bleaching by the reductant was dramatically enhanced in the presence of the substrate, HPP [50]. Resonance Raman studies of the purple form of the enzyme indicated that the absorbance most probably arises from coordination of an active site Fe(III) atom by the oxygen atom of tyrosine in the phenolate form [56]. However, correlation of tertiary structure with available primary structures indicates that it is unlikely that any known HPPD has a tyrosine residue whose side chain is within contact distance to the active site metal ion. Moreover, when HPPD is isolated in the presence of an exogenous chelator, the apo-enzyme acquires no visible color in the presence of either Fe(II) or Fe(III) ions [53]. This raises the possibility of self-hydroxylation; the active site of HPPD has four fully conserved phenylalanines, all of which are sufficiently proximal to be hydroxylated and subsequently ligate to the metal ion [25,26]. Self-hydroxylation has been observed in other Fe(II) dependent oxygenases [57–59], in particular a number of other α-keto acid dependent oxygenases have shown this propensity, hydroxylating both active site tyrosine and tryptophan residues to yield visibly chromophoric complexes [60–62]. While the mechanism of self-hydroxylation has not been rigorously established, a plausible mechanism proposed by Liu et al. is that uncoupled turnover in the presence of an α-keto acid results in futile production of a high valency iron-oxo species that, in the absence of a suitable substrate recipient for oxygen, will attack surrounding amino acid residues (Scheme 3).

## HPP association and oxygen activation

Ordered substrate addition in which the organic substrate(s) associates with the free enzyme prior to dioxygen in productive catalysis has been proposed for a number of Fe(II)-dependent dioxygenases [63–66]. For α-keto acid dependent enzymes, a consensus is emerging as to the order of addition of substrates. Evidence indicates that for these enzymes, partially or fully ordered mechanisms are observed in which the α-keto acid is the first substrate to bind and $CO_2$ is the first product to dissociate [67–71]. The ordered addition of substrates observed by Rundgren for HPPD is thus fully consistent with what has been observed for other enzymes of this family [40]. Collectively these observations suggest that the association of the organic substrates within the active site heightens the reactivity of these enzymes with dioxygen. Structural and spectroscopic investigations have begun to elucidate the underlying processes that lead to this gated dioxygen activation phenomenon.

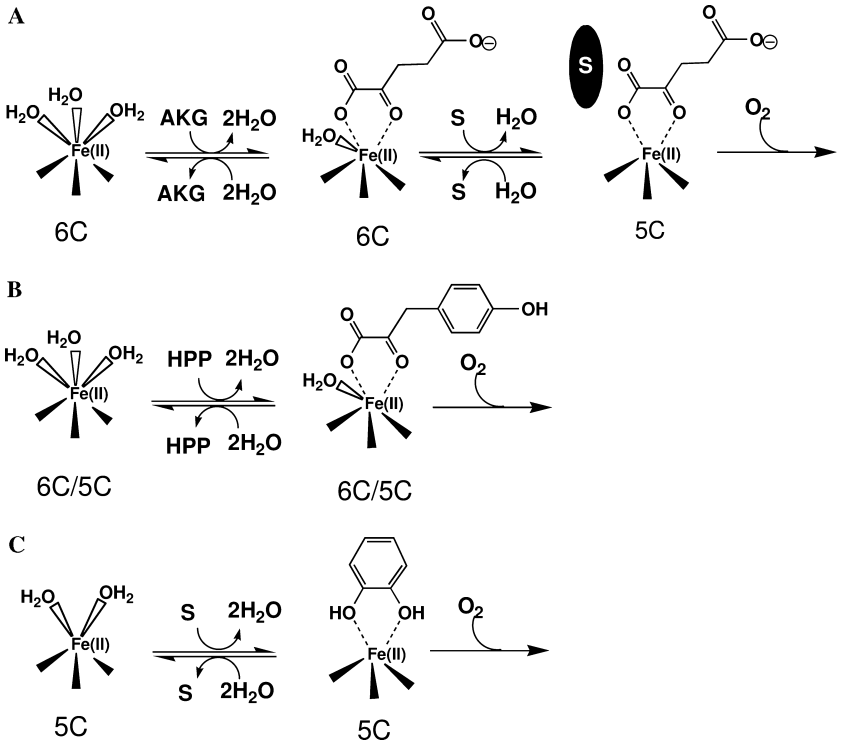
For a number of enzymes of the α-keto acid dependent family, α-ketoglutarate has been observed crystallographically to associate with the active site metal ion in a bidentate fashion through coordination to the 1-carboxylate and 2-keto oxygen atoms [33,34,37,38,72]. This association creates absorbance transitions that arise from metal to ligand charge transfer ($\varepsilon_{\sim 500\ nm} = 100$–$200\ M^{-1}\ cm^{-1}$). The absorbance shifts slightly to shorter wavelengths and the transitions become more resolved when the second organic substrate is added to this complex [73–75]. Charge transfer absorbance bands of this type are observed with a number of α-keto acids in complex with HPPD and strongly suggests that the coordination of HPP with the active site ferrous ion is also bidentate from the α-keto acid moiety. Interestingly, α-ketoglutarate, the cosubstrate and metal ion ligand for the majority of enzymes in the family, does not form a

bidentate association with the metal ion of HPPD [53]. The binding isotherms for α-keto acids with HPPD determined from an aerobic titration of this metal to ligand absorbance feature indicate that the phenol of HPP has a modest energetic contribution to binding. At 4 °C the dissociation constant for HPP is 30 μM, while that for phenylpyruvate is 4.4 kJ/mol weaker at 220 μM. Pyruvate binding energy is 10.5 kJ/mol weaker than HPP with a dissociation constant of ~3 mM.

Comparison of the resting and substrate bound near-IR magnetic circular dichroism (MCD) spectra of both an α-ketoglutarate dependent oxygenase and an extra-diol type oxygenase with that of HPPD, suggests that HPPD bridges these two classes of enzymes. Data show that for the α-ketoglutarate dependent oxygenases, coordination number is linked to dioxygen reactivity. The resting enzyme and the α-ketoglutarate complex are observed to be six coordinate and thus prevent the potentially futile reduction of molecular oxygen by blocking access to the metal ion. Only in the presence of both α-ketoglutarate and the second organic substrate, which does not ligand the metal ion (Scheme 4A), does the iron adopt a five coordinate, square pyramidal geometry [76,77]. On this basis, it would be inferred that in the presence of the single organic substrate, HPP, the HPPD active site iron atom would be five coordinate and poised to react with molecular oxygen. However, recent spectroscopic investigation of HPPD indicates that in both the resting and substrate bound states, the active site metal ion is a mixture of six and five coordinate species (9:1) [78] (Scheme 4B). The absence of a clear coordination number change in response to the binding of substrate is more similar to what has been observed using these methods for the extra-diol type of Fe(II) dependent non-heme oxygenases [79] and would imply that factors other than coordination number control the propensity of HPPD to reduce molecular oxygen (Scheme 4C).

The UV–Vis region of the MCD spectrum of α-keto acids in chiral environments includes a HOMO to LUMO ($n \rightarrow \pi^*$) transition. The energy of this transition is indicative of the degree of planarity of the α-keto acid moiety. In enzymes, this phenomenon has been studied most thoroughly for clavaminate synthase II. In the presence of saturating α-ketoglutarate, it is observed that this feature moves to significantly lower energy when coordinated to the active site metal ion, indicating that in the active site the α-keto acid moiety has coplanar oxygen atoms [76]. Intuitively, this conformation would maximize conjugation for the α-keto acid and assist delocalization of charge onto the metal ion (and ultimately to dioxygen). The energy of the $n \rightarrow \pi^*$ transition of the substrate α-keto acid moiety for the HPPD · Fe(II) · HPP complex has yet to be correlated with these data.

Although spectroscopic data indicate that α-keto acid dependent enzymes control their reactivity toward dioxygen, the factor of enhancement of the rate of reaction with molecular oxygen in the presence of substrates




Scheme 4.

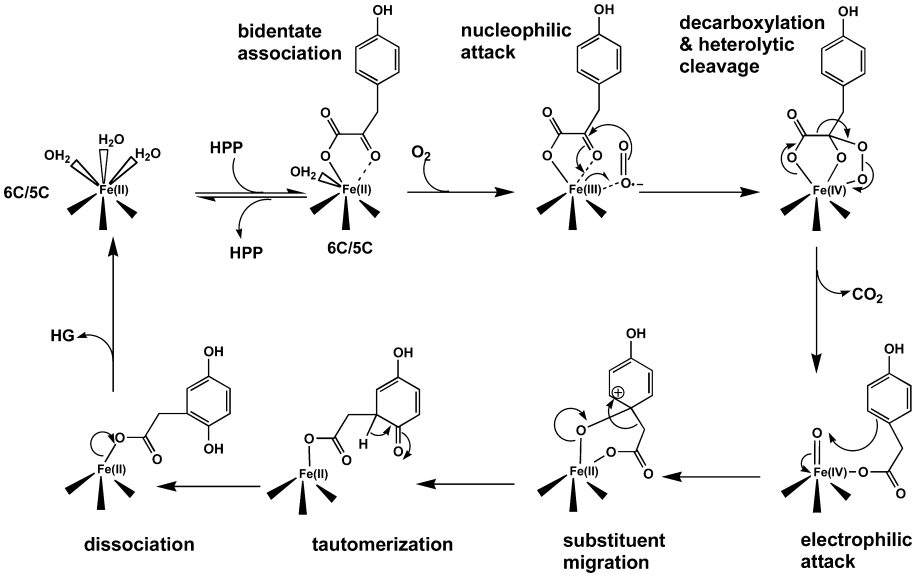
has generally not been demonstrated. To probe the oxygen reactivity of holo-HPPD in the presence and absence of HPP, the free holo-enzyme and the substrate in complex with the holo-enzyme have been reacted with molecular oxygen. The reaction of both the free enzyme and the substrate complex with molecular oxygen is second order. The substrate complex however exhibits a 3600-fold increase in the rate constant, clearly establishing the activating effector role for the α-keto acid substrate [53].

## Intermediates and mechanism

There can be little doubt that the characterization of catalytic intermediates is the current frontier of enzyme chemistry. However, not all enzymes will be amenable to this pursuit; only those enzymes that either have (or can be manipulated to have) significant accumulation of intermediate species, are appropriate to attempt to characterize intermediates from. For the α-keto acid dependent enzyme, taurine dioxygenase, a combination of spectrophotometric and freeze quench, rapid mixing methodologies revealed an Fe(IV) intermediate that accumulates prior to oxygenation of taurine [80–82]. This observation was a noteworthy advance in the study of α-keto acid dependent enzymes and entirely consistent with proposed mechanistic hypotheses [19,72,83]. Many unanswered questions remain, however, with regard to the chemical mechanism. Thorough pre-steady-state analyses of non-heme Fe(II) dependent oxygenases are required before the chemical identification of intermediates is definitive. Establishing the time frame in which intermediates accumulate and decay permits the determination of the fractional accumulation of each transient and in turn assists in the analysis of static physical data for trapped mixtures of intermediate states.

To date, the only published pre-steady-state evidence for HPPD was the observation of the accumulation of an intermediate at 490 nm that formed and decayed at catalytically relevant rates [53]. Recently, this investigation was expanded to a multi-wavelength absorbance and fluorescence pre-steady-state analysis that included the use of deuterated solvents and a ring deuterated substrate. These data indicated that two intermediates have significant fractional accumulation during a single catalytic turnover of HPPD from *S. avermiltilis*. The intermediates both have broad visible absorbance maxima at ~380 and 490 nm. The first intermediate has a significantly more intense absorbance at 380 nm. The final observed catalytic process exhibited kinetic sensitivity to deuterons derived from the solvent indicating that exchangeable protons are involved in general acid/base chemistry for this event. The penultimate phase in catalysis exhibits a kinetic isotope effect of two in the presence of 2,3,5,6-ring deuterated HPP indicating that processes of aromatic oxygenation are occurring during this phase. This assertion was supported by the observation that homogentisate is approximately 15 times more fluorescent than HPP and a significant increase in fluorescence occurs during this phase [84].

Scheme 5 depicts a tentative mechanistic hypothesis for HPPD compiled from a number of observations described in this review for HPPD and other mechanistically related enzymes. In Scheme 5, catalysis commences with the bidentate association of the substrate to form a complex that displays a mixture of five




Scheme 5.

and six coordinate geometry and whose overall coordination number is largely unchanged from the free enzyme [78]. This complex has heightened dioxygen reactivity [40,53] and in the presence of molecular oxygen delocalizes electron density from the planar α-keto acid moiety of the substrate to dioxygen [76], denuding the substrate of electrons and inducing a nucleophilic attack at the pyruvate Cα atom. This would be comprised of individual one-electron steps to satisfy the requirement for spin inversion in the two electron reduction of the dioxygen ground state triplet. Attack at the pyruvate Cα atom would yield an Fe(IV)-bridged peroxy species. Heterolytic cleavage of the peroxy oxygen bond would occur during decarboxylation to yield the highly electrophilic Fe(IV)-oxo intermediate [80,82] that can withdraw electrons from the aromatic ring inducing the formation of either an arenium cation as shown or alternatively a 1,2-epoxide (not shown) [19] that then presumably opens to the cation. The only direct evidence for an electrophilic species in catalysis is the hydroxylation of the electron-rich sulfur in (4-hydroxyphenyl)thio-pyruvate in preference to the aromatic ring C1[54]. The localization of the cation ortho to the now aceto substituent would direct the shift of the side chain to form a dienone that need only tautomerize to produce the homogentisate product.

## Inhibitors with specific reference to NTBC

Naturally occurring allelopathic diketone and triketone alkaloids are produced by a number of oil producing plants [85] and lichens [86]. These compounds are employed as agents to suppress the growth of surrounding plants [87]. The mode of action of this family of molecules is the specific inhibition of HPPD [24,88–90]. HPPD inhibition prevents the production of homogentisate, in turn preventing the synthesis of tocopherols and plastoquinones (Scheme 1) [4]. The inability to off load electrons from the photosystems results in a bleaching of the affected plants due to diminished chlorophyll levels. Synthetic efforts have developed many similar molecules that specifically inhibit HPPD for potential use as herbicides [91–95]. Several of these inhibitors, including mesotrione, sulcotrione and isofluxatole, are currently in use as selective broad leaf herbicides [96–98] (Fig. 4).

An interesting development from the synthetic work has been that two deleterious in born defects in mammalian tyrosine catabolism are treated effectively using a molecule whose structure is very similar the herbicides described above. NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) was one of the earliest triketones developed as an herbicide [99]. The pronounced improvement of a number of type I tyrosinemia patients treated with NTBC, led to its rapid adoption as a therapeutic agent [15]. The medical use of NTBC has been particularly successful and type I tyrosinemia patients now live essentially normal lives free of the lethal symptoms of the disease (vide infra) [100]. Recently, NTBC has also been used successfully to treat alkaptonuria patients [101] and, although it has not yet been reported, presumably hawkinsinuria treatment will soon follow (Scheme 1). Despite their importance to medicine and agriculture, how such inhibitors interacted with HPPD was, until recently, largely unclear. Conjecture over a number of important aspects of the interaction persisted including: the tautomeric state of the inhibitors, whether the inhibitors bound to the active site metal ion, the oxidation state of active site metal ion to which it binds and the meaning of numerous measured $IC_{50}$ values. Recently each of these characteristics of inhibitor interaction have been unambiguously established for NTBC acting upon HPPD from *S. avermitilis* [102].

As predominantly di- and triketone molecules, inhibitors of HPPD tend to undergo facile tautomerizations to form enols in aqueous solution (Scheme 6). Knowing

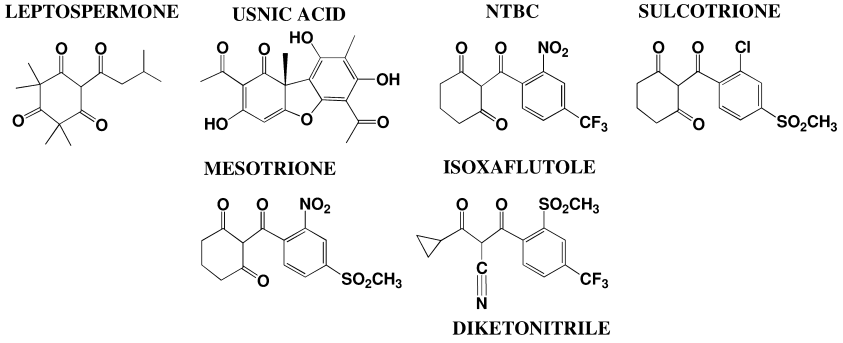
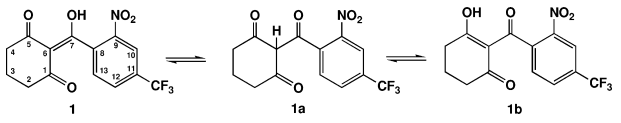



Fig. 4. Representative examples of naturally occurring and synthetic inhibitors of HPPD. Leptospermone is produced by oil producing plants such as the Australian bottle brush. Usnic acid is produced by lichens. NTBC is the drug administered as nitisinone for treatment of type 1 tyrosinemia and alkaptonuria by Swedish Orphan Pharmaceuticals, sulcotrione is the active ingredient in an herbicide by Essenchem, mesotrione is the active ingredient in the herbicide Callisto by syngenta, and isoxaflutole is from the herbicide Balance by Bayer that is active when converted in vivo to the diketonitrile form (as shown).



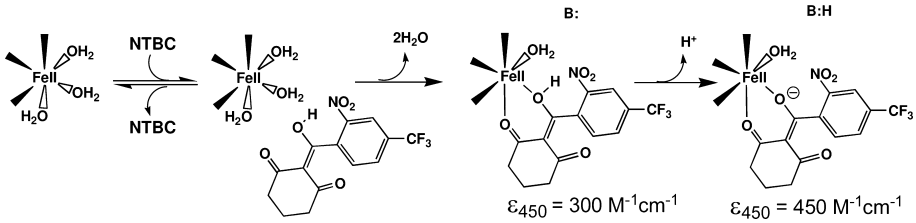

Scheme 6.

the dominant tautomer(s) of such molecules in aqueous conditions may be important to understanding the inhibitory mechanism and may even dictate selectivity. A number of studies have been concerned with a description of these equilibria. Calculations for a number of triketone molecules have suggested that the exocyclic (**1**) and endocyclic enols (**1b**) would dominate the aqueous forms [95]. This was consistent with X-ray crystallographic studies by Wu et al. that indicated an ambiguous structure in the solid state with bond lengths that would indicate both endocyclic and exocyclic enol character [103]. Nuclear magnetic resonance (NMR) studies performed in chloroform showed carbon atom resonances consistent with only the endocyclic enol. NMR studies presented by Kavana and Moran, performed with NTBC in aqueous solution at pH 7.0 show that there are two symmetrically inequivalent enol/keto carbon atoms, indicating that, at least for NTBC, it is the exocyclic enol that predominates under conditions more relevant to inhibitory processes [102].

Under aerobic conditions in the presence of reductants, HPPD cycles between the reduced Fe(II) and oxidized Fe(III) forms [53,104]. The action of HPPD inhibitors would be effective if they were to act upon the ferric or ferrous forms of HPPD. Prisbylla et al. have proposed that a triketone inhibitor has structural similarity to the substrate HPP and therefore will bind bidentate to the active ferrous form of the enzyme [90], a notion supported by the observation that a diketone moiety seems to be the minimum requirement for inhibition [86,93,96]. More recently, Garcia et al. showed using radiolabeled inhibitor that such a diketone inhibitor bound only to the ferrous form of the enzyme [91]. At odds with these assertions and based on the correlation between the binding strength of triketone inhibitors to Fe(III) in solution and their inhibitory strength, it was proposed by Wu et al. that the ferric form of HPPD is the relevant inhibited oxidation state [103]. Kavana and Moran observed that NTBC interacts only with the ferrous form of HPPD. A metal to ligand charge transfer at 450 nm is formed when NTBC is added to anaerobic HPPD . Fe(II). No spectroscopic changes in the UV–Vis or EPR spectra were seen upon mixing of NTBC and ferric HPPD, indicating that binding to the ferric form, if it occurs, does not involve the metal center [102].

The author has chosen not to conduct a comparison of published affinities for HPPD inhibitors, as it is now evident that many of these values may either not represent a measure of the binding affinity or are not directly comparable to one another. A general issue in determination of enzyme ligand interaction strength is the validity of the assumptions that precede measurement. For HPPD, the majority of such determinations were based on two assumptions, first that the binding of the inhibitor was readily reversible and second that it involved a single association and dissociation step. Furthermore, with the exception of the studies on carrot HPPD [91], all literature $K_i$ and $IC_{50}$ values were determined with partially purified or crude enzyme, leading to further fundamental problems with interpretation. The lack of experiments that accurately define dissociation rates for inhibitors make it impossible to differentiate between measuring the equilibrium of the free enzyme and the unbound inhibitor with the enzyme inhibitor complex and the measurement of one half of the enzyme concentration. In such experiments, slow dissociation of the inhibitor would dictate that the incubation time prior to measurement is the determining factor.

For NTBC, the binding mechanism is clearly not a simple reversible association. There are instead at least three steps before the inhibitor is bound to the enzyme. These are a relatively weak pre-equilibrium followed by two metal centered binding events, the last of which is ostensibly irreversible. The observation of a weak charge-transfer band for the HPPD · Fe(II) · NTBC complex provided an opportunity to define the binding mechanism by mixing the anaerobic ferrous enzyme with the anaerobic inhibitor in a stopped-flow spectrophotometer under pseudo-first order conditions. A hyperbolic dependence of the rate of the development of an initial charge-transfer band on NTBC concentration is consistent with an initial non-chromophoric binding event ($K_d = 1.2$ mM) and a subsequent association that produces metal to ligand charge-transfer transitions ($8\ s^{-1}$). This charge-transfer then intensifies during a final binding event ($0.76\ s^{-1}$) to give the irreversibly associated enzyme–inhibitor complex. A solvent kinetic isotope effect of 3 on the latter of these processes suggests that proton movement is involved in this step. Since the isotope effect coincided with the formation of the more intense charge-transfer absorbance, it has been tentatively ascribed to the formation of an irreversibly associated enolate bound bidentate to the active site Fe(II) atom (Scheme 7). The irreversibility of the latter binding step was proposed because the HPPD · Fe(II) · NTBC complex was observed to have no oxygen reactivity even over an extended time period [102]. Given that the oxygen reactivity of the HPPD holoenzyme was known ($39\ M^{-1}\ s^{-1}$) [53], it was possible to definitively state that oxidation of the HPPD · Fe(II) complex to form the colorless ferric form would occur concomitant with NTBC release. However, there are a number of factors that could influence reversibility; the irreversibility observed for HPPD from *S. avermitilis* in




Scheme 7.

association with NTBC may be species and/or inhibitor specific. Ellis et al. observed the recovery of 90% of HPPD activity from crude rat cytosol over the course of 10 h after inhibition with a related inhibitor, which implies slow dissociation from this form of the enzyme [105]. Moreover, the inhibitor mesotrione is used as an herbicide due to the observation that it is eliminated from the human relatively rapidly, suggesting a freely reversible complex with human HPPD [106].

The HPPD · Fe(II) · NTBC complex is unreactive toward molecular oxygen [102]. Since the di- and triketone inhibitors do not appear to interact significantly with the ferric form of the enzyme, the prevention of single-electron chemistry with dioxygen is a fundamental part of the inhibitory mechanism. The structure of NTBC in complex with HPPD from *S. avermitilis* has been solved. In this structure it is not steric considerations that prevent oxygen from reacting at the metal ion as the active site coordination sphere is predominantly five-coordinate, square pyramidal with unobstructed access to the sixth ligand site [26]. NTBC is observed to coordinate in a bidentate mode to the active site metal ion via its 5′ and 7′ oxygens (Fig. 5). There is a low occupancy/

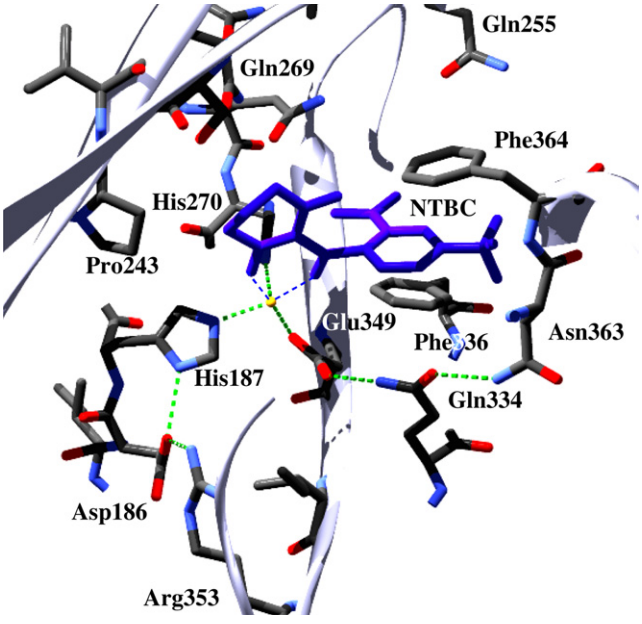



Fig. 5. The structure of the inhibitor NTBC (depicted in blue) bound in the active site of HPPD from *Streptomyces avermitilis*.

high temperature factor water molecule in the sixth coordination site of one of the two protomers of the asymmetric unit. The fold of the *S. avermitilis* enzyme is very similar to that of the previously solved *P. fluorescens* enzyme. A C-terminal helix however, is angled 40° out into solvent with respect to the earlier structure, as was observed in the unliganded plant enzyme [23]. Very recently, similar helix movements were also observed for the rat and *Arabidopsis* forms of HPPD in complex with inhibitory ligands [24]. This helix provides one of two phenylalanines that sandwich the phenyl ring of the inhibitor. This apparent $\pi$ stacking interaction together with association to the metal ion are the only clear energetically significant contacts with the active site surface. All other contacts are Van der Waals and occur almost exclusively with fully conserved active site residues. This latter observation would suggest that inhibitor selectivity is based more on bioavailability than direct affinity for HPPD from a specific organism. However, Yang et al. have shown that inhibitors can be designed that show increased affinity for plant over mammalian HPPD [24].

Both the exceedingly tight association of HPPD inhibitors, and the relatively slow onset of inhibition are consistent with such inhibitors acting as transition state analogs [102,107]. While such an assertion is largely speculative, in a comparison of Figs. 3 and 5 it is immediately evident that NTBC has some structural similarity to the substrate. The functional role of the phenylalanines that are observed to stack in a staggered conformation against the phenyl of the inhibitor may be to assist in oxygenation of the aromatic ring of the substrate. It is unlikely that such an interaction contributes significantly to binding the substrate as stacking aromatic rings in such a manner generally produce quite weak interactions [108]. However, donor acceptor $\pi$-cation stacking arrangements have considerable binding energies, consistent with stabilization of the proposed arenium cation of Scheme 5 [109,110].

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