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Note

The interaction of hydroxymandelate synthase with the 4-hydroxyphenylpyruvate dioxygenase inhibitor: NTBC ☆

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Dedicated to Edward Solomon

Abstract

Hydroxymandelate synthase (HMS) catalyzes the committed step in the formation of *para*-hydroxyphenylglycine, a recurrent substructure of polycyclic non-ribosomal peptide antibiotics such as vancomycin. HMS uses the same substrates as 4-hydroxyphenylpyruvate dioxygenase (HPPD), 4-hydroxyphenylpyruvate (HPP) and O_2, and also conducts a dioxygenation reaction. The difference between the two lies in the insertion of the second oxygen atom, HMS directing this atom onto the benzylic carbon of the substrate while HPPD hydroxylates the aromatic C1 carbon. We have shown that HMS will bind NTBC, a herbicide/therapeutic whose mode of action is based on the inhibition of HPPD. This occurs despite residue differences at the active site of HMS from those known to contact the inhibitor in HPPD. Moreover, the minimal kinetic mechanism for association of NTBC to HMS differs only slightly from that observed with HPPD. The primary difference is that three charge-transfer species are observed to accumulate during association. The first reversible complex forms with a weak dissociation constant of 520 μM, the subsequent two charge-transfer complexes form with rate constants of 2.7 s^{-1} and 0.67 s^{-1}. As was the case for HPPD, the final complex has the most intense charge-transfer, is not observed to dissociate, and is unreactive towards dioxygen.

Keywords: Hydroxymandelate; Hydroxyphenylpyruvate; Dioxygenase; Dioxygen; Ferrous; Non-heme

1. Introduction

Hydroxymandelate synthase (HMS) catalyzes an α-keto acid dependent dioxygenase reaction, forming 4-hydroxymandelate (HMA) from 4-hydroxyphenylpyruvate (HPP) in what appears to be an analogous reaction to that catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD). HPPD forms homogentisate (2,5-dihydroxyphenylacetic acid, HG) by hydroxylation of the aromatic ring, inducing a 1,2-migration, while HMS hydroxylates instead the benzylic carbon (Scheme 1). HPPD has been studied quite extensively as it is a target of di- and triketone molecules that act as herbicides and therapeutics [1]. In plants these molecules prevent the synthesis of HG, which is precursor to the essential plant cofactors, plastoquinone and tocopherol [2–4]. In mammals, the inhibition of HPPD can be used to completely alleviate the debilitating and/or lethal symptoms of specific inborn defects in tyrosine catabolism [5–8].

HMS is a relatively recent discovery [9,10] that functions in a small number of bacteria to assist in the production of *para*-hydroxyphenylglycine, a recurrent substructure of non-ribosomal peptide antibiotics such as vancomycin

Abbreviations: HMS, hydroxymandelate synthase; HPPD, (4-hydroxyphenyl)pyruvate dioxygenase; HMA, hydroxymandelate; HPP, (4-hydroxyphenyl)pyruvate; PHPG, *para*-hydroxyphenylglycine; HEPES, (*N*-(2-hydroxyethyl) piperizine-*N*′-(2-ethane sulfonic acid)); NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione.

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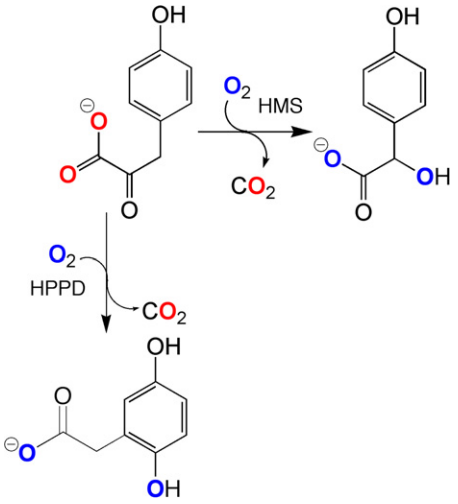

Scheme 1.

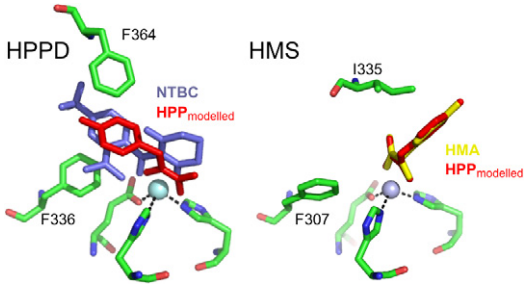

Fig. 1. Comparison of the ligand binding sites in HPPD and HMS. The HPPD figure is derived from the crystal structure of the HPPD · Fe(II) · NTBC complex (PDB ID 1T47). NTBC is depicted in blue, while the predicted binding of HPP based on this structure is shown in red. The HMS portion of the figure depicts the HMSCo(II)HMA complex (PDB ID 2R5V). HMA is depicted in yellow and the predicted binding of HPP based on this structure is shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and chloroeremomycin [11]. HMS has the same fold as HPPD [12]. The enzyme's tertiary structure can be divided into two domains, an N-terminal domain and a C-terminal domain that are topologically very similar. This fold was first observed for a number of extradiol type dioxygenases [13] and suggests that HMS and HPPD evolved from an extradiol dioxygenase precursor. The C-terminal domain is the catalytic portion of the structure and HMS and HPPD have significant identity and similarity in this domain. There are however, 24 residues in the C-terminus that are conserved only in the HMS primary structures. These are candidate residues critical to the delivery of the second oxygen atom. Recent mutagenesis studies have attempted to switch the catalytic specificity of HPPD to form HMA based largely on sequence comparison and structural insight. The conversion of a phenylalanine conserved only in HPPD structures (F364 in Fig. 1) to valine or isoleucine, the latter of which is conserved in HMS, resulted in the formation of HMA in a fraction of total turnover [14,15]. This phenylalanine residue had also been implicated as vital to catalysis in HPPD as it was one of two phenylalanine residues observed to sandwich the aromatic ring of the inhibitor 2-[2-nitro-4-(triflouromethyl)benzoyl]-1,3-cyclohexanedione (NTBC) that has a qualitative similarity to the structure of the substrate, HPP [16] (Fig. 1). If the substrate phenol were stacked similarly, these phenyl rings could play a role in stabilizing a cationic ring species during electrophilic aromatic hydroxylation. Moreover, the complex of HPPD · Fe(II) · NTBC is ostensibly non-dissociable [17] even though the inhibitor makes no hydrogen bonding or ionic interactions with the enzyme. The only significant energetic contact observed is the bidentate contact made by the 5′ and 7′ oxygens of NTBC with the active site metal ion. It has been surmised recently that this contact alone does not account for the stability of the complex [18]. This has lead to the conclusion that the phenylalanines that stack with the relatively electron deficient aromatic ring of the inhibitor may contribute significant energy to the HPPD · Fe(II) · NTBC complex [16,18]. On this basis we would therefore minimally predict that HMS would at least show diminished affinity for known HPPD inhibitors. In an added complication to this issue, the crystal structure of HMS derived from crystals grown in the presence of HPP indicates that although the density for the ligand is inconclusively assigned to either the substrate or product (HMA) it clearly adopts an entirely different orientation and conformation to that predicted by the HPPD inhibitor complex structures [12] (Fig. 1). In this research note, we investigate whether HMS is subject to inhibition by NTBC, the paradigm example of molecules designed to inhibit HPPD.

2. Experimental

2.1. Materials

HPP was purchased from Sigma–Aldrich Co. NTBC was a gift from Swedish Orphan Pharmaceuticals. The sodium salt of HEPES buffer was purchased from ACROS.

HMS from *Amycolatopsis orientalis* was purified and concentrated according to previously published methods [19].

2.2. Extinction coefficients

The extinction coefficient of apo-AoHMS was calculated to be 20 525 M^{-1} cm^{-1} at 280 nm by the method of Pace [20]. The molar extinction coefficient of NTBC was 20 550 M^{-1} cm^{-1} at 257 nm as previously reported [17]. The molar extinction coefficient of HPP was 3 400 M^{-1} cm^{-1} at 276 nm at pH 7.0 [19].

2.3. Stopped-flow measurements of inhibitor binding

The binding of NTBC was monitored under anaerobic conditions by stopped-flow spectrophotometry observing the accumulation of charge-transfer absorbance [17]. Anaerobic HMS · Fe(II) complex (48.3 μM final) was