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Conversion of hydroxyphenylpyruvate dioxygenases into hydroxymandelate synthases by directed evolution

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Abstract Hydroxymandelate synthase (HmaS) and hydroxyphenylpyruvate dioxygenase (HppD) are non-heme iron-dependent dioxygenases, which share a common substrate and first catalytic step. The catalytic pathways then diverge to yield hydroxymandelate for secondary metabolism, or homogentisate in tyrosine catabolism. To probe the differences between these related active sites that channel a common intermediate down alternative pathways, we attempted to interconvert their activities by directed evolution. HmaS activity was readily introduced to HppD by just two amino acid changes. A parallel attempt to engineer HppD activity in HmaS was unsuccessful, suggesting that homogentisate synthesis places greater chemical and steric demands on the active site.

Keywords: Enzyme mechanism; Dioxygenase; Protein engineering; Non-proteinogenic amino acids; Hydroxyphenylglycine; Non-ribosomal peptide synthesis

1. Introduction

Hydroxyphenylpyruvate dioxygenase (HppD) catalyses the first dedicated step of tyrosine catabolism, the oxidation of 4-hydroxyphenylpyruvate (HPP) to homogentisate (HGA), with incorporation of both atoms of molecular oxygen into the product and release of CO_2 (Fig. 1) [1]. This enzyme has been a subject of research over several decades, as mutations cause type III tyrosinaemia, an inherited disorder of tyrosine metabolism, and because homogentisate is a precursor of plastoquinone and tocopherols in plants. Inhibitors of HppD are clinically and commercially important for treatment of tyrosinaemias [2] and as herbicides [3].

The closest homologue to HppD, hydroxymandelic acid synthase (HmaS), is involved in the production of glycopeptide antibiotics in *Amycolatopsis orientalis* [4,5] and at least eight other homologues have since been found in microbes with clinically important secondary metabolites. Unusual non-proteinogenic amino acids are incorporated into non-ribosomal peptides, where they contribute to the impressive chemical and structural diversity of this family of natural products [6]. HmaS catalyses the first biosynthetic step of one such amino acid, 4-hydroxyphenylglycine, by reaction of molecular oxygen with HPP to yield 4-hydroxymandelic acid (HMA) (Fig. 1).

HppD and HmaS share high homology (20–35% amino acid identity), the same substrate and the same initial catalytic step. This presents a unique opportunity to study how a common reaction intermediate is partitioned along divergent pathways by closely related active sites.

In the crystal structure of HppD from *Pseudomonas fluorescens* the catalytic Fe^{II} is coordinated by two histidines and a glutamate [7]. A hydrophobic substrate binding pocket is observed, but the orientation of substrate binding is unknown. Recently the structures of HppDs from four other organisms have become available: *Zea mays*, *Arabidopsis thaliana*, *Streptomyces avermitilis* and *Rattus norvegicus* [8–10]. The structures of *Arabidopsis thaliana* and *S. avermitilis* HppD contain inhibitors in the hydrophobic cavity, which are coordinated to Fe^{II}. Using these structural data, and the increasing number of sequenced homologues, the aim of this study was to uncover the crucial differences between HmaS and HppD active sites by interconverting their activities. Since none of the crystal structures contain substrate or products, and there is currently no structure of any HmaS, a high throughput approach was chosen for the best chance of identifying mutants with interconverted activities.

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Abbreviations: HppD, 4-hydroxyphenylpyruvate dioxygenase; HppD_SA, 4-hydroxyphenylpyruvate dioxygenase *Streptomyces avermitilis*; HppD_PF, 4-hydroxyphenylpyruvate dioxygenase *Pseudomonas fluorescens*; HmaS, 4-hydroxymandelate synthase; HPP, 4-hydroxyphenylpyruvate; HGA, homogentisic acid; HMA, 4-hydroxymandelic acid

2. Materials and methods

2.1. Plasmids

The cloning of HmaS and HmaO from the antibiotic producer *Amycolatopsis orientalis* has already been described [4,15]. 4-Hydroxyphenylpyruvate dioxygenase *P. fluorescens* (HppD_PF) was amplified by PCR from *P. fluorescens* DNA, DSMZ strain 4358, and ligated in pET28a+. HppD_PF has 14 amino acid differences from the protein crystallised by Serre and coworkers [7]. The sequence has been deposited (accession number DQ364627). 4-Hydroxyphenylpyruvate dioxygenase *S. avermitilis* (HppD_SA) (accession number U11864) and HGD, a putative 1,2 homogentisate dioxygenase (accession number

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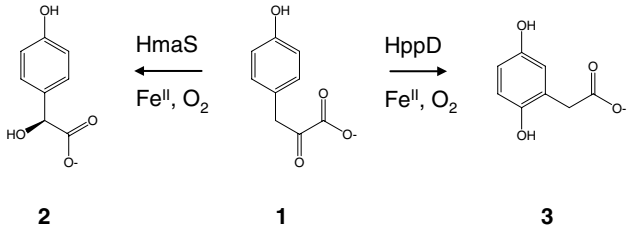

Fig. 1. Reactions of HmaS and HppD. HmaS catalyses the oxidation of 4-hydroxyphenylpyruvate (**1**) to (*S*)-4-hydroxymandelate (**2**), whereas HppD catalyses the oxidation of the same substrate to homogentisate (**3**).

AP005047, gi:29610176), were amplified by PCR from *S. avermitilis* genomic DNA and cloned in pET28a+. Primer sequences are available as supporting information.

Libraries were prepared by PCR, using oligonucleotides to encode diversified regions; a list of primers is available in the supporting information. Libraries were prepared in strain DH10B, the number of colonies was noted, then cells were scraped up to purify plasmid DNA. Libraries containing mutations at residue 335 plus one or both of 214 and 216 were prepared sequentially: residue 335 was diversified first, and this library was used as a template for PCR to introduce diversity at the other residue(s). Library quality was checked by sequencing four individual members from each library. Over 96% of library members contained full length genes with the expected diversified residues. The frequency of PCR-induced mutations was less than one per 4 kb.

2.2. Library screening in microplates

Libraries were transformed in *Escherichia coli* BL21-CodonPlus®-RP (Stratagene). Cultures were grown in 1 ml LB-tyrosine in Deepwell microplates (Fisher). LB-tyrosine was prepared from LB (1 litre) by the addition of 1 g tyrosine, dissolved in 20 ml 1 M HCl, followed by 100 ml 1 M disodium orthophosphate and 2 ml 30 mg/ml kanamycin sulphate. After 48 h growth at 30 °C, cells were harvested by centrifugation and 100 μl of the supernatant was transferred to a UV-transparent microplate (Grenier). The absorbance was measured at 280–440 nm, 10 nm intervals, using a Strectramax Plus spectrophotometer running Softmax Pro (Molecular Biosciences).

The phenotypes of the clones were classified as HmaS wild type-like ($A_{330} > 2.0$, $A_{430} < 0.1$, no visible pigmentation), HMA producer ($A_{330} > 0.3$, $A_{430} < 0.1$, no pigmentation), HppD wild type-like ($A_{330} > 2.0$, $A_{430} > 0.1$, dark pigmentation), HGA producer ($A_{330} > 0.3$, $A_{430} > 0.1$, brown pigmentation), or inactive ($A_{330} < 0.3$). Plasmids encoding genes of interest were prepared from the cell pellets by standard methods. To sample the range of amino substitutions that are tolerated by the enzymes, the genes of six wild type-like clones from each single-residue library were sequenced. Only one gene contained a PCR-induced mutation outside the active site: HmaS H106R T214V.

2.3. Overexpression and purification

All proteins were expressed in *E. coli* BL21-CodonPlus®-RP. The standard procedure was growth at 30 °C to mid-log phase followed by the addition of IPTG to 0.1 mM then 3 h expression. For HmaO, cells were grown at 37 °C to mid-log then grown at 40 °C for 6 h. Cells were lysed using lysozyme in phosphate buffer (100 mM sodium phosphate pH 8.0, 150 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme with Benzonase (Novagen)). Proteins were purified using nickel resin by standard methods, the concentration measured by the Bradford assay and the purity estimated by PAGE. Proteins were transferred to 50 mM sodium phosphate pH 7.5 for assay.

2.4. Measuring HMA and HGA production by coupled assay

HMA production was assayed spectrophotometrically by coupling to hydroxymandelate oxidase and measuring the absorbance of 4-hydroxybenzaldehyde at 330 nm. Reactions contained 20 nM–20 μM test protein and 2 μM hydroxymandelate oxidase in sodium phosphate buffer (50 mM, pH 8.0) plus 50 μM Fe^{II}, 0.5 mM ascorbic acid, 0.1 mg ml^{-1} catalase. Reactions were started by the addition of 1 mM HPP from a stock solution of 100 mM HPP in 500 mM sodium phosphate pH 8.0, and incubated at 37 °C. Reactions were determined to be tightly coupled as the rates were unchanged by increasing concentrations of hydroxymandelate oxidase. Rates quoted in Table 3 are mean values (standard error <10%) determined within the first 90 s. The extinction coefficient of 4-hydroxybenzoylformate was measured as 11 700 m^{-1} cm^{-1}.

HGA production was measured under the same conditions but using homogentisate dioxygenase as the coupling enzyme and measuring the absorption of 4-maleyl-acetoacetate at 318 nm (extinction coefficient 13 500 M^{-1} cm^{-1}).

3. Results

3.1. High-throughput activity assays

A microplate screen was developed to assay HppD and HmaS activities. Since *E. coli* lack a catabolic pathway for tyrosine, there is no endogenous production or degradation of HGA [11]. Expression of HppD in *E. coli* leads to the accumulation of HGA in the culture medium, where the spontaneous oxidation and polymerisation of HGA into ochronotic pigment provides a convenient assay [12]. Two HppDs were studied: HppD_PF from *P. fluorescens* and HppD_SA from *S. avermitilis*. Both are well expressed in *E. coli*, and the concentration of HGA in the culture supernatant reaches 0.3 g/l, whereas the detection limit for HGA is approximately 10 mg/l.

HmaS activity cannot be monitored by direct spectrophotometric assay because the absorption maxima of HPP and HMA are too similar. In cultures of *E. coli*, however, we observed slow conversion of HMA to 4-hydroxybenzaldehyde, absorption maxima at 270 and 330 nm. HMA is not an ordinary metabolite of *E. coli*, so it is likely that this conversion is catalysed by an oxidase with promiscuous substrate specificity. The product concentration in cultures overexpressing HmaS was estimated as 0.3 g l^{-1}, with a detection limit of approximately 10 mg l^{-1}. Simple analysis of culture supernatants thus enables detection of HmaS and HppD activities (Fig. 2).

3.2. Identification of crucial residues for HmaS activity

Initially five HmaS residues were chosen for mutagenesis: M199, T214, I216, I335 and Y339. These amino acids are conserved amongst all nine hydroxymandelate synthases, whereas alternative amino acids, L, P, N, F and F, are conserved in the corresponding positions in the HppD family. Furthermore, the analogous residues in HppD make up part of the active site cavity in the crystal structure of HppD_PF, and L199, P214,

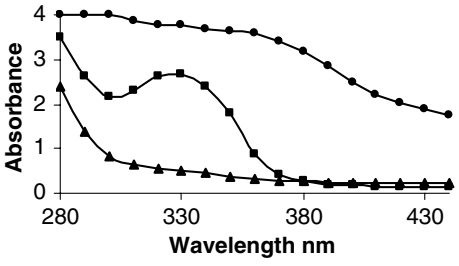

Fig. 2. High-throughput screen for HppD and HmaS activities in *E. coli*. Absorption spectra of the supernatants from *E. coli* expressing HmaS (squares), HppD (circles) or no recombinant protein (triangles).