The C-terminal of rat 4-hydroxyphenylpyruvate dioxygenase is indispensable for enzyme activity

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Abstract We have cloned and overexpressed rat 4-hydroxyphenylpyruvate dioxygenase (4HPPD) in *Escherichia coli*. The soluble, active recombinant enzyme was shown to contain both 4HPPD and α-ketoisocaproate dioxygenase (αKICD) activity. However, upon truncation of the 14 amino acids at the C-terminus by site-directed mutagenesis, the resulting mutant enzyme (rat F antigen) exhibited complete loss of 4HPPD and αKICD activities. This finding suggests that the C-terminal extension domain plays an essential role in the catalytic activity of the enzyme.

Key words: 4-Hydroxyphenylpyruvate dioxygenase; α-Ketoisocaproate dioxygenase; Rat F antigen; C-terminal domain

1. Introduction

4-Hydroxyphenylpyruvate dioxygenase (4HPPD) is an enzyme involved in the metabolism of tyrosine, converting 4-hydroxyphenylpyruvate (4HPP) to homogentisate through decarboxylation and hydroxylation. The enzyme has been purified from porcine [1], human [2] and avian livers [3] as well as *Pseudomonas* sp. [4]. In mammalian species, the enzyme exists in dimeric form, whereas the *Pseudomonas* enzyme was shown to be a tetramer of 150 kDa.

4HPPD is a non-heme dioxygenase that requires Fe^{2+}, O_2, DTT and ascorbate for its activity. As an important enzyme in the degradation pathway of tyrosine, inhibition of 4HPPD has been used in the treatment of tyrosinaemia (type I) and liver cirrhosis [5]. It has also been demonstrated that 4HPPD is involved in the formation of melanin-like pigment in marine bacteria [6,7]. Most interestingly, we recently found that 4HPPD purified from rat liver is capable of catalysing the conversion of α-ketoisocaproate (αKIC) to β-hydroxyisovaleric acid [8].

In the liver, the cytosolic conversion of αKIC to β-hydroxyisovaleric acid was thought to be performed by another enzyme, α-ketoisocaproate dioxygenase (αKICD), a non-heme dioxygenase that also requires Fe^{2+}, O_2, DTT and ascorbate for activity. From our studies with the recombinant rat 4HPPD, we have confirmed that the activities of both 4HPPD and αKICD are due to a single enzyme [9].

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Abbreviations: DTT, dithiothreitol; 4HPP, 4-hydroxyphenylpyruvate; 4HPPD, 4-hydroxyphenylpyruvate dioxygenase; αKIC, α-ketoisocaproate; αKICD, α-ketoisocaproate dioxygenase; RFA, rat F antigen.

Database analysis and multiple alignment of amino acid sequences revealed that rat F antigen (RFA) exhibits a high degree of homology (>90%) with 4HPPD from a variety of species. RFA is a protein of about 43 kDa found predominantly in the hepatic cytoplasm of mammals. First identified in 1968, it has been extensively studied as a model of tolerance but to date, its function is still unknown [10–12]. One important difference between RFA and mammalian 4HPPD is the apparently shorter C-terminus of RFA, but despite this, it has been postulated that RFA is a species variant of 4HPPD or is in fact 4HPPD. Herein, we report that when active recombinant 4HPPD was converted to RFA by site-directed mutagenesis, the resulting mutant showed complete loss of activity.

2. Materials and methods

2.1. cDNA library screening

A λ ZAP II rat liver cDNA library (Sprague-Dawley, male, 6 months old from Stratagene) was screened for 4HPPD with the polymerase chain reaction (PCR), modified from the methods of Takumi and Lodish [13] as described previously [9]. Primers used for screening were designed according to the incomplete cDNA sequence of RFA [12]. Primers used were 5′GTGGTCAGTCATGTCATCAAGCAA3′ (forward) (169–192) and 5′ACAGAGAGTTGAAGTTACCTGCTCC3′ (reverse) (1102–1078). PCR reactions were carried out using *Taq* polymerase (Pharmacia) for 30 cycles: the first cycle was 94°C, 5 min, 55°C, 1 min, and 72°C, 1 min. The following 29 cycles were 94°C, 1 min, 55°C, 1 min and 72°C, 1 min.

60 000 plaque-forming units (pfu) were initially aliquoted from the library and divided into 20 pools, each of 3000 pfu. These pools were amplified and screened by PCR. Positive pools that showed a band of correct molecular weight as judged by agarose gel electrophoresis were further subdivided into subpools, each of 200 pfu. Reamplification, screening and subdivision were repeated to the level of a single plaque. A clone with an estimated insert of 1.3 kb was selected for in vivo excision. Single stranded DNA rescue was performed essentially according to the protocols provided by Stratagene (Stratagene, USA).

2.2. Subcloning into expression vector

Incomplete 4HPPD cDNA in pBluescript vector was amplified by PCR for the purpose of subcloning using Vent polymerase (New England Biolabs). The forward primer 5′GATCGACATATG**ACCACCTACTGGGACAAAGGA**CCAGAGCCTGAGAGAGGCCGGTTCCTC3′ and reverse primer 5′GATCGAGGATCCTCATTACATTCCAGACCTCACACCGTTGGTCTC3′ contained nucleotides introduced to give additional amino acids at 5′ end (bold). In addition, *Nde*I and *Bam*HI restriction sites (underlined) were added at the 5′ and 3′ ends for ease of subcloning. PCR was performed for 30 cycles and the DNA fragment was isolated and cloned into a *ptrc* vector (termed pHPD). Before expression, the recombinant plasmid was sequenced in both strands to confirm no unwanted mutation had been introduced by PCR.

2.3. Expression of 4HPPD

Clones in XL-1 Blue MRF′ were grown in 2TY broth supplemented with 34 μg/ml chloramphenicol at 27°C with shaking at 250 rpm until OD_{600} reached 1.5. Isopropyl-β-D-thiogalactopyranoside (IPTG)

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was added to a final concentration of 0.3 mM and the cultures were incubated for another 15 h. Cultures were centrifuged at 10 000 × *g* for 5 min. Cell pellets were resuspended in 50 mM Tris-HCl buffer, pH 8.0 with 1 mM EDTA. Cells lysis was carried out with sonication for 3 times (10 s) with a 15 s interval between repeats. Cell debris was removed by centrifugation at 10 000 × *g* for 25 min and the supernatants were collected for protein purification or enzyme assay.

2.4. Protein purification

4HPPD was precipitated from the above supernatant by adding ammonium sulfate (30% w/v) followed by stirring at 4°C for 1 h and centrifugation at 10 000 × *g* for 20 min. The pellet was redissolved in 50 mM Tris-HCl, pH 8.0 containing 1 mM EDTA (buffer A) before loading the resultant solution onto a DEAE Sepharose fast flow column pre-equilibrated with buffer A. After washing the column with 2 column volumes of buffer A, the absorbed proteins remaining on the column were eluted by running a linear gradient from 0 to 25% buffer B (1 M NaCl in buffer A). 4HPPD was typically eluted at 0.18 M NaCl.

2.5. Enzyme assay

αKICD activity was determined essentially according to the method described by Sabourin and Bieber [14]. A typical assay would consist of a final volume of 0.4 ml: 0.10 M Tris-maleate buffer, pH 6.0, 1 mM $FeSO_4$, 0.5 mM ascorbic acid, 1 mM DTT, 1 mM [1-^{14}C]αKIC (40 000 dpm/16.5 μl per assay), and 40 μl purified enzyme or crude lysates (protein concentration 2.0–2.5 mg/ml). The mixture was first preincubated for 30 min at 27°C without the substrate, αKIC. As negative controls, lysates of host cell without the plasmid, or Milli-Q water were used for incubation. [1-^{14}C]αKIC was added to initiate the reaction after 30 min and the $^{14}CO_2$ released was absorbed with 0.2 ml Hyamine solution (ICN Radiochemicals, Irvine, CA) in an Eppendorf overhanging the reaction mixture. After 1 h, 0.2 ml of 20% TFA was added to terminate the reaction and an additional 1 h was allowed for the absorption of $^{14}CO_2$. The Eppendorf containing the Hyamine solution was transferred to a scintillation vial, to which was then added 10 ml of Optiphase 'Safe' scintillation fluid (EG&G, UK) and the vial was counted for ^{14}C activity.

2.6. Site-directed mutagenesis

Site-directed mutagenesis was performed using a Pharmacia U.S.E. kit according to the manufacturer's instructions. The oligonucleotide used to generate C-terminal deletion mutant protein (rat F antigen) from pHPD was 5'CCAAGCTTCCCGGGATTCAGTGAGGTT-AACCCCGTAGGGCTTGCTCCTC3'. The mutant (termed pRFA) was subjected to sequencing in both orientations to confirm the introduction of the mutation at the site desired.

3. Results and discussion

3.1. Cloning of 4HPPD from rat

The 4HPPD fragment isolated from Sprague-Dawley cDNA revealed an open reading frame of 1155 nucleotides, lacking the 5′ end. The nucleotide sequence and deduced amino acids were shown to be almost identical with that of rat F antigen published by Gershwin et al. [12], with the exception of four base substitutions and a single base deletion. One base substitution is at position 26, where the cytosine (C) in RFA cDNA is replaced by an adenosine (A) (nucleotide position according to RFA sequence published by Gershwin et al. [12]). The second substitution is at position 28 (guanosine (G) instead of adenosine (A)), which changes the amino acid lysine to glutamic acid. The other two substitutions are both third base substitutions at positions 60 (thymidine (T) for cytosine (C)) and 729 (adenosine (A) for guanosine (G)), making no difference to the amino acids as coded for by the published RFA sequence. Nonetheless, the single base (thymidine) deletion at position 1138 eliminates the stop codon present in RFA cDNA. This enables a further 14 amino acids to be translated at the C-terminal (Fig. 1).

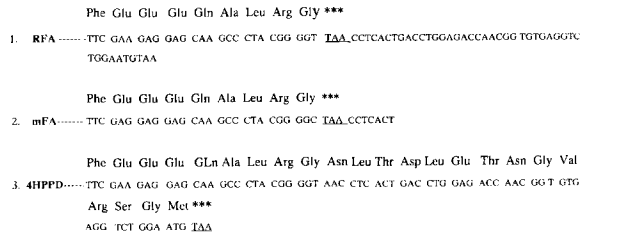

Fig. 1. Sequence alignment of the C-terminals of (1) rat F antigen (RFA), (2) mouse F antigen (mFA) and (3) 4-hydroxyphenylpyruvate dioxygenase (4HPPD) described here. In RFA, the thymidine (T) insertion at position 1138 introduced a termination codon (underlined). Consequently, the peptide lacks the final 14 amino acid residues compared with 4HPPD.

So far, a complete rat 4HPPD has never been isolated. Judging from the amino acid sequence of mouse 4HPPD [15] and RFA published [12], it was decided to overexpress the protein in *E. coli* with the N-terminus according to the cDNA sequence of RFA [12]. An additional 2 amino acids derived from mouse (namely Thr, Thr) were added downstream of the initiation codon.

3.2. Expression and purification of 4HPPD

When induced, SDS-PAGE analysis revealed an overexpressed protein of 45 kDa in cell lysates from clones containing the 4HPPD plasmid. This 45 kDa protein was not found in control host cell XL-1 Blue MRF′ (Fig. 2). The recombinant 4HPPD was purified to >90% purity after ammonium sulfate precipitation and DEAE chromatography, with substantial loss of activity (unpublished). The M_r of the purified recombinant protein measured by electrospray mass spectrometry was 45 085 ± 0.8 (calc. M_r 45,082). N-terminal sequencing confirmed that the first methionine had been cleaved off.

3.3. 4HPPD activity

As noticed by other groups cloning 4HPPD from *Streptomyces avermitilis* and *melA* gene from *Shewanella colwelliana*, *E. coli* harboring the plasmid containing the 4HPPD gene produced a characteristic brownish pigment after overnight

Table 1
Assays for αKICD and 4HPPD activity

	pHPD	pRFA	Host cell	Milli-Q water
αKICD assay: $^{14}CO_2$ released (cpm)	3571	204	191	213
4HPPD assay: pigment production	+++	–	–	NA

αKICD activity was measured by the release of $^{14}CO_2$ from α-[1-^{14}C]ketoisocaproate. The values above are the averages of 5 readings for pHPD and pRFA, and duplicate for negative controls. 4HPPD assay: +++, strong brownish pigment; –, no brownish pigment in culture. NA, not applicable.

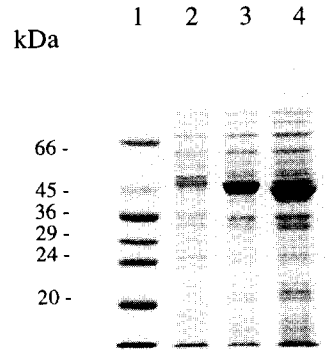

Fig. 2. Expression of 4HPPD and RFA in *E. coli*. Cells were induced and harvested as described in Section 2. Supernatants of soluble proteins were subjected to SDS-PAGE (12.5%) analysis and stained with Coomassie Blue. Lanes: 1, molecular weight markers; 2, host cell XL-1 Blue MRF'; 3, pHPD; 4, pRFA.

culture both on solid and liquid media [16,7]. The authors found that the intensity of this pigment could be increased by the addition of L-tyrosine. It is known that *E. coli* contains transaminases capable of converting L-tyrosine to 4-hydroxyphenylpyruvate (4HPP), the substrate of 4HPPD, which in turn, could be further catalysed by the recombinant 4HPPD to homogentisate (HGA). It was thus postulated that the brownish pigment produced was either ochronitic acid or a melanin-like substance, a product of the oxidation and polymerization of HGA. Thus, the presence of active 4HPPD in *E. coli* could be judged by the formation of this pigment. In our laboratory, we too noticed that the pHPD clone also produced similar brownish pigment, especially when grown in rich media. So far, the production of this pigment has been reported in 4HPPD clones of microorganisms like *Shewanella colwelliana* and *S. avermitilis*. In the mammalian enzyme, a direct link between 4HPPD and the production of this pigment has thus also been established.

pRFA showed the same level of protein expression and solubility profile as pHPD upon induction with IPTG. Nevertheless, no brownish pigment formation could be observed even after a prolonged growing period in L-tyrosine enriched medium (Table 1). The presence of RFA in this induced culture was confirmed by SDS-PAGE analysis. The data indicates that pRFA is inactive, i.e. incapable of converting 4HPP to HGA.

3.4. αKICD activity

We previously reported that native rat 4HPPD also showed αKICD activity [8]. In the recombinant clone, it was noted that *E. coli* lysates harboring the 4HPPD plasmid had shown high αKICD activity. Lysates of host cell, XL-1 Blue MRF' without plasmid were inactive. Since we found that purified 4HPPD from DEAE chromatography was less active and rather unstable in storage, lysates of pHPD and pRFA with the same protein concentrations were used for αKICD assay throughout this work. As shown in Table 1, 4HPPD is completely devoid of αKICD activity when mutated to RFA.

Rat F antigen was first identified in 1968 and has been studied extensively over the years because of its interesting properties as an alloantigen. When mice of responder strains are immunized with liver lysates from other strains, the antibody produced reacts against not only the immunogen, but also against its own allelic form of the protein. Early studies had concentrated on the protein per se. Thus cloning of rat F antigen was first reported by Gershwin et al. [12] in 1987 with the protein being overexpressed in *E. coli* as a fusion protein with β-galactosidase. However, no mass spectrometry or protease digestion has been reported on the recombinant protein to confirm the exact translation of the proposed cDNA.

Mouse F antigen, on the other hand, was isolated by Schofield et al. [17] using the PCR method, based on the rat cDNA sequence published earlier [12]. The primers were designed based on the 5′ and 3′ ends of rat F antigen. The mouse F antigen was amplified from first strand cDNA using *Taq* polymerase and the amplicon subcloned and sequenced. In mouse F antigen, it was postulated by Schofield et al. that the C-terminus was identical to that of rat. However, it should be noted that in the process of cloning, the same laboratory did observe that mouse F antigen had an apparent deletion of the first base of the stop codon [17]. Nonetheless, this was interpreted by the authors as a PCR artefact introduced by *Taq* polymerase.

The possible role of rat F antigen only came to light when 4HPPD from *Streptomyces*, human and porcine were cloned [16,18,19]. Sequence comparison showed that RFA bore a striking similarity to 4HPPD isolated from these organisms. In this paper, we have demonstrated that rat F antigen is inactive with respect to αKICD and 4HPPD activities. It is clear that the difference between rat 4HPPD and RFA cDNA lies solely at the termination codon. When compared with the cDNA of active 4HPPD in this work, the RFA cDNA sequence published by Gershwin et al. was shown to have an insertion of thymidine (T), which introduced a termination codon at position 1138, causing the protein to be shortened by 14 residues. 4HPPD is known to be a key enzyme in the catabolism of amino acid tyrosine and deficiency of this enzyme causes hypertyrosinaemia. On the other hand, evidence that RFA plays a role in regulating the catabolic function of 4HPPD or any other activity has not been found. We thus conclude that the previously isolated RFA cDNA could be a mutated 4HPPD gene which encodes for an incomplete enzyme, or could represent an artefact introduced during cloning and sequencing. Currently, we are investigating the rat genomic DNA in order to further our understanding of the relationship between RFA and 4HPPD.

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