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INITIAL STUDIES ON THE SUBSTRATE SPECIFICITY OF SOLUBLE RECOMBINANT 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE FROM RAT LIVER.

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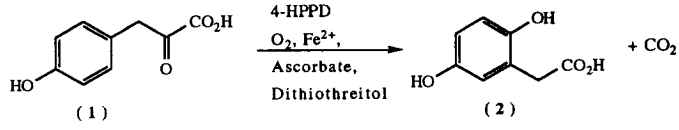
Abstract. The molecular cloning and expression of the cDNA encoding 4-hydroxyphenylpyruvate dioxygenase (rat F antigen) into *E.coli* and initial studies concerning the substrate specificity of the recombinant protein are described. Copyright © 1996 Elsevier Science Ltd

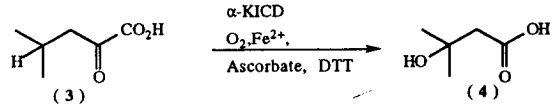
We recently reported[1] that the liver specific rat F antigen, a species variant of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) purified from rat liver displayed α-ketoisocaproate dioxygenase (α-KICD) activity - a result which has recently been confirmed independently[2]. This raised questions as to whether previous publications describing the purification of proteins possessing α-ketoisocaproate dioxygenase activity were in fact due specifically to α-KICD or to the purification of 4-HPPD which possesses α-KICD activity and thus whether a single enzyme, 4-HPPD, is responsible for the total observed cytosolic α-KICD activity. In an attempt to answer these questions we initiated efforts to clone and express the gene encoding 4-HPPD (rat F antigen) into *E. coli* and to then determine the substrate specificity of the recombinant protein. We now wish to report the preliminary results of these studies.

4-HPPD catalyses the oxidative decarboxylation and hydroxylation of 4-hydroxyphenylpyruvate **1** to homogentisate 2,5-dihydroxyphenylacetate[3] **2** (Scheme 1) and is an important enzyme in the metabolism of the aromatic amino acid, tyrosine.



Scheme 1

4-HPPD has been partially purified from pig[4], beef[5], dog[6], rat[7], human[3,8], and frog[9] liver and the co-factor and co-substrate requirements for this protein appear to be essentially the same as those of α-KICD[10-15], the protein believed to catalyse the conversion of α-ketoisocaproate **3** to β-hydroxyisovalerate **4** (Scheme 2).



Scheme 2

Screening of a λ ZAP II rat liver cDNA library (Stratagene, Sprague Dawley male rats, 6 months old) was carried out with the polymerase chain reaction (PCR)[16]. As the complete rat F antigen cDNA has never been isolated, primers used for screening were designed from the published incomplete sequence[17]. 60,000 plaque forming units were initially aliquoted from the library and divided into 20 pools each of approximately 3,000 units. Amplified pools were screened and positive pools with a band of correct molecular weight, as judged on agarose gel, were further subdivided into subpools each containing 200 plaque forming units. Re-

amplification, screening and subdivision were repeated to the level of a single plaque. A clone with an insert of 1.3Kb was selected for *in vivo* excision. Excision, plating and single stranded DNA rescue were performed according to the protocols provided by Stratagene[18]. Sequencing of the insert revealed that the cDNA was incomplete at the 5' end, however, comparison with 3 murine alleles[19] which had already been identified showed a > 95% homology. An intact rat F antigen gene with a complete 5' end has never been identified and in addition, attempted sequencing of wild type rat F antigen indicated that the N-terminus was blocked. After comparing the sequence of 4-HPPD from human, porcine and mouse sources, it was decided to add the two N-terminal amino acid residues from mouse 4-HPPD[19], namely Thr, Thr. Subsequently the cDNA was amplified using PCR with the addition of the two codons as well as a Nde I (5'-end) and a Bam H I (3'-end) restriction sites. The PCR product was isolated from agarose gel, enzyme digested and cloned into the expression vector (pMAT-4, donated by Mr D Hart) to be expressed as native enzyme.

Initially we screened the soluble recombinant 4-HPPD enzyme for its ability to convert 4-hydroxyphenylpyruvate **1** using the release of $^{14}CO_2$ from radiolabelled [1-^{14}C]-4-hydroxyphenylpyruvate as previously described[1]. Considerable levels of activity were detectable from a crude enzyme preparation which consisted solely of the soluble fraction from a cell lysate. No activity was detectable when an *E.coli* lysate without the plasmid was assayed in an identical manner to that described above. We next incubated [1-^{14}C]-α-ketoiso-caproate **3** and monitored conversion to β-hydroxyisovalerate **4** similarly by the release of $^{14}CO_2$ and again a high degree of enzyme activity was detected. Encouraged by these results we purified the protein to near homogeneity (as determined by SDS polyacrylamide gel electrophoresis) utilising a simple two column sequence - a S75 gel filtration step followed by mono-Q anion exchange chromatography - and repeated both assays as described above with similar results (see Table 1). No activity with either substrates **1**, or **3** was detected when they were incubated with co-factors in the absence of enzyme (see "control " entries in Table 1).

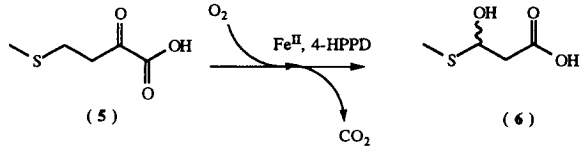
Enzyme Preparation	Protein Conc. $mgml^{-1}$	Substrate	Substrate Activity (cpm)	Incubation	Incubation Conditions *	$^{14}CO_2$ Produced (CPM)	Maximum Conversion %
Purified	1	[1-^{14}C]-4-HPP	1100	i	A	660	60
				ii		630	57
				control		128	-
		[1-^{14}C]-α-KIC	40,000	i	B	9000	23
				ii		5200	13
				control		105	-

* Incubation Conditions : A 27°C, 250rpm, 30min preincubation, 0.3mM substrate concentration
B 27°C, 250rpm, 30min preincubation, 1.2mM substrate concentration

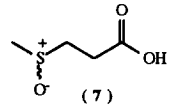
Table 1

These results indicate that recombinant 4-HPPD from rat liver is capable of converting both its natural substrate, 4-hydroxyphenylpyruvate **1** to homogentisate **2**, and α-ketoisocaproate **3** to β-hydroxyisovalerate **4**. Consequently it thus appears that previous references to an enzyme activity believed to be associated with α-ketoisocaproate dioxygenase were in fact due to 4-HPPD. We therefore suggest that any further reference to α-KICD be discontinued, primarily due to the much greater metabolic and catabolic importance of 4-HPPD.

Further studies regarding the substrate specificity of this interesting enzyme are underway in our laboratories and will be reported in due course. However, evidence is already present in the literature that the substrate specificity of 4-HPPD may be quite broad and this is worthy of comment here. Reports concerning α-KICD suggest that the ketoacid derived from methionine, β-thiomethyl,α-ketobutyrate **5**, is a substrate[15], it being converted to what was assumed to be the corresponding β-hydroxyacid **6** (Scheme 3) since the actual product was not identified. We find the proposal of **6** as the likely enzymic product unlikely in view of the known chemistry of the ketoacid dependant dioxygenases and suggest that a more likely product from this enzymic conversion would be the corresponding sulphoxide **7**.



Scheme 3



In the light of previous reports[15], we must now consider **5** to be a substrate for 4-HPPD, an inference which we are in the process of testing.

To conclude, it appears that apart from the well known involvement of 4-HPPD in normal aromatic amino acid metabolism, it also plays a significant role in the cytosolic catabolism of amino acids such as leucine, and possibly methionine and phenylalanine. The full significance of these findings remain to be determined and are currently under investigation in our laboratories.

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