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Substrate specificity of *p*-hydroxyphenylpyruvate hydroxylase

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SUMMARY

Evidence is presented that a single enzyme is responsible for hydroxylation of three naturally occurring aromatic keto acids; *p*-hydroxyphenylpyruvate, phenylpyruvate and 3,4-dihydroxyphenylpyruvate.

The origin of large amounts of *o*-hydroxyphenylacetic acid in the urine of patients afflicted with phenylketonuria has been suggested to arise largely if not totally from the hydroxylation of phenylpyruvate. Partially purified liver fractions have been obtained which will convert phenylpyruvate to *o*-hydroxyphenylacetate and *p*-hydroxyphenylpyruvate to homogentisate[1,2]. This observation has been the main support for the conclusion that a single enzyme, *p*-hydroxyphenylpyruvate hydroxylase [*p*-hydroxyphenylpyruvate, ascorbate O_2 oxidoreductase (hydroxylating), EC 1.14.2.2] is responsible for the hydroxylation for the aromatic keto acids.

The advent of 3,4-dihydroxy-L-phenylalanine therapy for the treatment of Parkinsonism has led to a renewed interest in the metabolic products of this aromatic amino acid. 3,4-Dihydroxy-L-phenylalanine has been shown to undergo transamination; the product of this enzymatic reaction is 3,4-dihydroxyphenylpyruvate[3]. Thus the metabolic fate of this keto acid was of some interest in relation to the enzymatic hydroxylation of the other aromatic keto acids.

Recent success in the purification of *p*-hydroxyphenylpyruvate hydroxylase[4] and newer methods for the assay of enzyme activities have allowed us to investigate this question of enzyme substrate specificity. The results are described in this brief communication.

Enzyme activity was determined by a radiochemical assay described earlier[5].

The purification of the enzyme was carried out by the method of Lindblad *et al.*[4] using chicken liver as a source of enzyme.

The enzyme fraction was activated with an equal volume of a solution containing 2.5 mM dichlorophenolindophenol and 52 mM glutathione. The enzyme solution was added to a Warburg vessel containing 100 mM potassium phosphate (pH 7.3) and 1000 units of beef liver catalase (EC 1.11.1.6) in the main compartment of the flask. 1 μmole *carboxy*-^{14}C-labeled substrate was tipped in and the flask incubated for 10 min at 37 °C. The reaction was stopped by the addition of 0.3 ml of 1 M H_2SO_4 and the CO_2 collected on a small filter disc containing 0.025 ml of 1 M hyamine hydroxide. The $^{14}CO_2$ released was determined with a liquid scintillation counter.

[*carboxy*-^{14}C] Phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and 3,4-dihydroxyphenylpyruvic acid were prepared from the corresponding *carboxy*-^{14}C-labeled amino acids using snake venom L-amino acid oxidase (EC 1.4.3.2) and catalase[6].

Table I includes enzyme activity data for the purified chicken liver *p*-hydroxyphenylpyruvate hydroxylase fractions with three different substrates. The ratios of the activities did not change as the purification proceeded and the final pure enzyme exhibited essentially this same ratio of activities.

TABLE I

HYDROXYLASE ACTIVITY OF PURIFIED FRACTIONS USING VARIOUS SUBSTRATES

Enzyme Fraction *	*Hydroxylase activity (μmoles/mg protein per h)*			*Ratios of activity*		
	(a) p-Hydroxyphenylpyruvate	*(b) Phenylpyruvate*	*(c) 3,4-Dihydroxyphenylpyruvate*	*a/b*	*a/c*	*c/b*
Step I - Ammonium sulfate fractionation	1.86	0.04	0.20	46.6	9.1	5.1
Step II - After SP-Sephadex C-50	7.50	0.18	0.66	41.6	11.3	3.7
Step III - After hydroxylapatite	15.90	0.31	1.62	51.0	9.8	5.2
Step IV - After QAE-Sephadex A-50	27.90	0.66	3.45	42.3	8.1	5.2

* Chicken liver enzyme fractionation was carried out as described by Lindblad *et al.*[4]. Enzyme purity criteria of final fraction after Step IV is detailed in results.

This final enzyme fraction behaved as a single protein using two criteria of purity: (1) The enzyme exhibited a single protein band by disc gel electrophoresis at pH 8.9, pH 7.0 and pH 4.5. (2) The enzyme appeared to be composed of a major single protomer in sodium dodecylsulfate disc gel electrophoresis, having an estimated molecular weight of 35 000.

A second line of evidence supports the conclusion that a single enzyme is responsible for hydroxylation of the substrate *p*-hydroxyphenylpyruvate, phenylpyruvate, and 3,4-dihydroxyphenylpyruvate. The competitive inhibition of one

substrate by the other two is shown in Fig. 1. Phenylpyruvate and 3,4-dihydroxyphenyl pyruvate behaved as competitive inhibitors of *p*-hydroxyphenylpyruvate. Similar data was obtained using [*carboxy*-^{14}C] phenylpyruvate as the substrate and the other two keto acids as inhibitors.

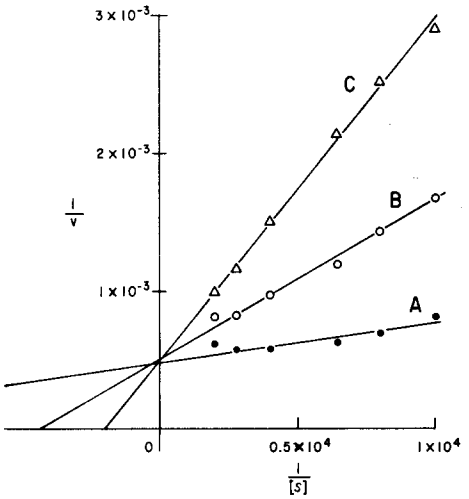

Fig.1. Competitive inhibition of hydroxylase activity by various substrates. The Lineweaver–Burk plot was determined using *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvate as substrate (A). The effect of simultaneous addition of non-radioactive $1 \cdot 10^{-3}$ M phenylpyruvate in (B) and $1 \cdot 10^{-5}$ M 3,4-dihydroxyphenylpyruvate in (C) is shown. Similar data is obtained using [*carboxy*-^{14}C] phenylpyruvate as substrate and *p*-hydroxylphenylpyruvate and 3,4-dihydroxyphenylpyruvate as inhibitors.

The data presented is consistent with the view that a single enzyme is responsible for the hydroxylation of three biologically occurring aromatic keto acids. An extension of this conclusion is the observation of elevated amounts of keto acids in two human diseases of aromatic amino acid metabolism. In tyrosinemia increased urinary excretion of phenylpyruvate is observed along with expected increase in *p*-hydroxyphenylpyruvate[7,8], and in phenylketonuria elevated urinary excretion of *p*-hydroxyphenylpyruvate has been reported[9]. Presumably, the elevation of one keto acid as a result of the inborn error of metabolism, results in the elevation of the other keto acid by competitively occupying their common hydroxylase enzyme.

The behavior of 3,4-dihydroxyphenylpyruvate as a substrate for the enzyme is of some interest in the metabolism of 3,4-dihydroxy-L-phenylalanine. Since 3,4-dihydroxy-L-phenylalanine has been established as a substrate for tyrosine aminotransferase (EC 2.6.1.5)[3] it is likely that significant amounts of 3,4-dihydroxyphenylpyruvate occur in Parkinson patients treated with 3,4-dihydroxy-L-phenylalanine. The product of the hydroxylase action on 3,4-dihydroxyphenylpyruvate is 2,4,5-trihydroxyphenylacetic acid. This latter substance is related to the metabolism of 6-hydroxy-

dopamine in that it is the expected product from the action of monoamine oxidase on the amine.

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