STUDIES ON A POSSIBLE REACTION INTERMEDIATE OF p-HYDROXYPHENYLPYRUVATE DIOXYGENASE

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SUMMARY: A quinol has been proposed to be an intermediate of the p-hydroxyphenylpyruvate dioxygenase-catalyzed reaction. However, using a highly purified enzyme from bovine liver and chemically synthesized quinol, no significant formation of homogentisate, the product of the enzymic reaction, from the quinol was observed under any conditions tested. Furthermore, the quinol showed no appreciable inhibition on the enzymic activity. In light of these findings, the reaction mechanism of the enzyme is discussed.

p-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) catalyzes the conversion of p-hydroxyphenylpyruvate (I) to homogentisate (II), in which three types of reactions are involved, namely, hydroxylation of the benzene ring, oxidative decarboxylation, and migration of a side chain (1). A reaction mechanism for the enzyme in which a cyclic peroxide (III) and a quinol (IV) are involved as intermediates was originally proposed by Goodwin and Witkop (2) and further substantiated by Lindblad et al. with $^{18}O_2$ experiments (3) (Mechanism (a) in Scheme 1). Although this mechanism has been widely accepted (4), direct evidence to prove or disprove it has not been available. In fact, another mechanism ((b) in Scheme 1) involving

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a peracid (V) as an intermediate was proposed by Hamilton (5).

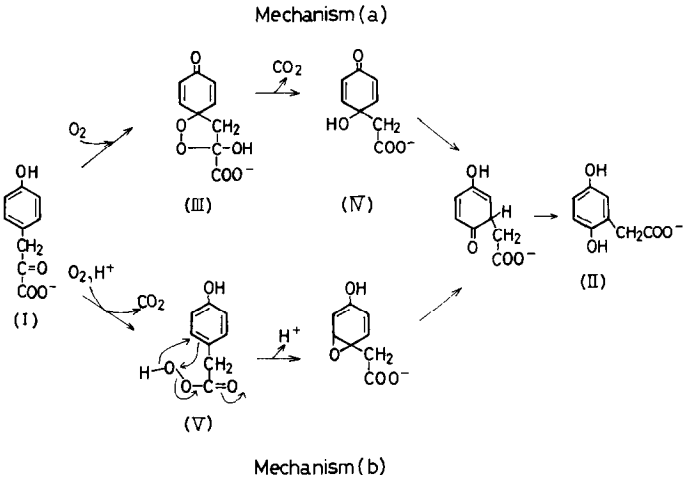
In previous experiments, the chemically synthesized quinol intermediate (IV), 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl) acetic acid, in the same way as the O-acetate (2), was shown to be converted nonenzymically to (II) in an alkaline solution by Saito et al. (6). In order to investigate whether or not the quinol is an intermediate of the enzymic reaction, compound (IV) was incubated with the highly purified enzyme under various conditions. This report describes the evidence against participation of a quinol as a free intermediate.

MATERIALS AND METHODS

p-Hydroxyphenylpyruvic acid (I) was obtained from Tokyo Kasei. DCIP[1/] was a product of Merck and urea of Schwarz-Mann. Homogentisic acid (II) and GSH were purchased from Sigma, and catalase from Boehringer. The quinol (IV) was synthesized from (I) with singlet oxygen as described previously (6). All other reagents, analytical grade, were purchased from commercial sources and used without further purification.

p-Hydroxyphenylpyruvate dioxygenase was purified from bovine liver acetone powder by essentially the same method as described by Lindblad et al. (7) with some modifications. The purification procedures included gel filtration with Bio-Gel A-0.5 m, chromatographies with SP-Sephadex C-50 and QAE-Sephadex A-50, and gel filtration with Sephadex G-200. All the purification was carried out at 4° in sodium acetate buffer, pH 5.6. An overall purification of about 19 fold was achieved with a yield of 22%. The purified enzyme thus obtained with a specific activity of 175 nmoles per

1/ Abbreviation: DCIP, 2,6-dichloroindophenol.



Scheme 1. Proposed reaction mechanisms for p-hydroxyphenylpyruvate dioxygenase.

min per mg of protein showed a major thick band with several faint bands on acrylamide gel electrophoresis.

The enzyme activity was assayed routinely by measuring the rate of formation of (II) by the method of Fellman et al. (8) with some modifications. The standard assay mixture contained in a final volume of 1.0 ml, 150 mM potassium phosphate buffer, pH 7.5, 100 µg of catalase, 200 µM the substrate (I), 10 µM DCIP, 1 mM GSH, and the enzyme. The reaction was started by the addition of the enzyme and the incubations were carried out at 37° for 15 min with gentle shaking. The reaction was terminated by the addition of 100 µl of 0.4 M L-cysteine and 100 µl of 6 M glycine-NaOH buffer, pH 10.6, and further incubated at 37° for 60 min. During this incubation, the homogentisate (II) formed was completely converted to 1,4-thiazine and the absorbance at 390 nm (ε = 5,520) was measured. Under these conditions, nonenzymic conversion of (IV) to (II) was not observed.

Compound (II) was also detected either by thin layer chromatography with a solvent system of ethyl acetate, acetic acid and ethanol (100:3:5, V/V/V) or by homogentisicase which specifically

Table I. Enzymic formation of homogentisate (II) from p-hydroxyphenylpyruvate (I) and quinol (IV)

Additions	Homogentisate formed (nmoles/15 min)	
	p-hydroxyphenylpyruvate	quinol
None	3.3	0.4
DCIP	6.0	0.0
GSH	12.3	0.0
Asc	47.6	0.0
GSH + Asc	61.4	0.0
DCIP + GSH	80.3	0.6
DCIP + Asc	72.1	0.0
DCIP + GSH + Asc	80.3	0.2

The reaction mixture contained in a final volume of 1.0 ml, 150 mM potassium phosphate buffer, pH 7.5, 100 µg of catalase, 200 µM either p-hydroxyphenylpyruvate (I) or the quinol (IV), and 30 µg of the enzyme. Where indicated, the following were added: 10 µM DCIP, 1 mM GSH, 1 mM ascorbic acid (Asc). Other conditions were the same as described under Materials and Methods. The values less than 1.0 are not significant with this assay method.

cleaves the benzene ring of (II) to form maleylacetoacetate having an absorption maximum at 320 nm.

RESULTS AND DISCUSSION

As shown in Table I, when (I) was used as the substrate, the enzyme showed a little activity in the absence of added cofactors. The activity was augmented by the addition of various cofactors

and maximum activity was obtained in the presence of DCIP in combination with GSH and/or ascorbic acid. On the other hand, when (IV) was used as the substrate, no significant formation of (II) was observed either in the absence or presence of cofactors. As reported previously (6), compound (IV) used in these experiments was nonenzymically converted to (II) in an almost stoichiometric amount at pH 12 at 24° within several hours.

Similar experiments were also carried out under various other conditions including increasing the amount of enzyme to 150 μg, increasing the quinol concentration to 1 m*M*, varying the pH over the range from 5.6 to 9.0, or adding urea (0.5 to 4.0 *M*) to the reaction mixture. The enzyme retained 100, 86, 62 and 10% of its original activity in the presence of 0.5, 1.0, 2.0 and 4.0 *M* urea, respectively, under the standard assay condition. However, in no case was the enzymic formation of (II) from (IV) observed. Furthermore, no significant inhibition was observed with 1 m*M* quinol (IV) under the standard assay conditions, even when the *p*-hydroxyphenylpyruvate (I) concentration was decreased to 20 μ*M*.

These results do not necessarily disprove Mechanism (a) for *p*-hydroxyphenylpyruvate dioxygenase but indicate that quinol may not be released from the ES-complex until the end product has been formed. Since Mechanism (a) requires the *para*-hydroxy group, the fact that phenylpyruvate (9, 10) and *p*-fluorophenylpyruvate (9) are slowly reacting substrates of the enzyme weakens the case for general applicability of Mechanism (a). Needless to say, however, these results neither prove nor exclude Mechanism (b). Further studies are required to clarify the reaction mechanism of the enzyme.

The quinol (IV) has also been postulated as an intermediate in the reaction catalyzed by *p*-hydroxyphenylacetate 1-hydroxylase,

an enzyme which catalyzes the conversion of p-hydroxyphenylacetate to homogentisate (II) in the presence of O_2 and a reducing agent (11). Since the para-hydroxy group in the substrate is apparently required for this enzymic reaction, quinol (IV) as an intermediate seems more probable. However, experiments with this hydroxylase have so far not been carried out.

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