Steady State Kinetics of 4-Hydroxyphenylpyruvate Dioxygenase from Human Liver (III)*

MARIANNE RUNDGREN
From the Department of Clinical Chemistry, University of Gothenburg. Sahlgren’s Hospital. S-413 45 Gothenburg, Sweden

Kinetic experiments have been made with an apparently homogenous preparation of human liver 4-hydroxyphenyl- pyruvate dioxygenase Form 3 (4-hydroxyphenylpyruvate: oxygen oxidoreductase (hydroxylating, decarboxylating), EC 1.13.11.27) at 37°C in 0.2 M Tris/HCl, pH 7.5, by measuring the evolved carbon dioxide from the 1-14C-labeled substrate or the formation of homogentisate from the U-14C-labeled substrate. The effect of variations in the concentrations of substrates, products, and metal chelators on the velocity of the evolved carbon dioxide from the l-14C-labeled substrate or the formation of homogentisate from the U-14C-labeled substrate. A Theorell-Chance mechanism has not been excluded. A Theorell-Chance mechanism has not been excluded.

4-Hydroxyphenylpyruvate is enzymically converted to homogentisate by 4-hydroxyphenylpyruvate dioxygenase dioxygenase (4-hydroxyphenylpyruvate:oxygen oxidoreductase (hydroxylating, decarboxylating), EC 1.13.11.27). The reaction involves hydroxylation of the benzene ring, oxidative decarboxylation, and migration of the side chain. A one to one stoichiometric relation has been found between the disappearance of 4-hydroxyphenylpyruvate and O2 and the formation of homogentisate and CO2 (1-4). Thus far, reversibility of the reaction has not been demonstrated. Both atoms of molecular oxygen are incorporated into the product (5). Liver 4-hydroxyphenylpyruvate dioxygenase also catalyzes the conversion of phenylpyruvate to 2-hydroxyphenylacetate (4, 6-11). The reaction mechanism has been related to that of the 2-oxoglutarate coupled oxygenases (5, 12-14). However, both the peroxide mechanism of Lindblad et al. (5, 12) and the peracid mechanism of Hamilton (13) still lack experimental verification. No free intermediates have yet been identified in the reaction (10, 15). So far, no kinetic mechanism has been formulated for 4-hydroxyphenylpyruvate dioxygenase.

EXPERIMENTAL PROCEDURES

Materials

L-[U-14C]Tyrosine (~500 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks, England. L-[1-14C]Tyrosine (55 Ci/mmol) and L-[1-14C]Phenylalanine (~50 Ci/mmol) were obtained from New England Nuclear Chemicals, GmbH, Frankfurt (M), FDR. Glutathione, sodium 2,6-dichlorophenolindophenol, and sodium diethyldithiocarbamate were obtained from E. Merck A. G., Darmstadt, FDR. 4-Hydroxyphenylpyruvic acid, sodium phenylpyruvate, 2-hydroxyphenylacetic acid, homogentisic acid, 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), and L-amino acid oxidase from Crotalus adamanteus were obtained from Sigma Chemical Co., St. Louis, Mo. 4-Hydroxyphenylpyruvic acid was purified, converted to the potassium salt and determined by the modified enol-borate complex method of Gentz et al. (16). The dried salt was stored in a nitrogen-filled desiccator at -20°C. Bovine liver catalase was obtained as a crystalline suspension (80 g/liter) from C. P. Boehringer and Soehne, GmbH, Mannheim, FDR. Hyamine, 1 M, 10-X in methanol was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. O2/N2 mixtures were obtained from AGA, Lidingo, Sweden. Other reagents and solvents used were of analytical grade and obtained from commercial sources.

Methods

Enzyme—4-Hydroxyphenylpyruvate dioxygenase Form 3 was prepared from human liver by a purification procedure described elsewhere (11, 17, 18). The enzyme preparation used showed one band on a starch gel electrophoresis (pH 6.3), electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate, and isoelectric focusing in a polyacrylamide stabilized pH gradient between pH 6 to 8. In the latter case, the enzyme activity coincided with the protein band observed. At Sephadex G-200 column filtration, one peak with constant specific activity was obtained. A stock solution of 1.3 mg of enzyme protein/ml of 0.2 M Tris/HCl, pH 7.5, was kept in portions at -60°C. Immediately before each series of experiments, one portion was diluted with ice cold 0.2 M Tris/HCl, pH 7.5. The specific activity was ~1.1 µmol of product/min/mg of protein (see below).

Protein Estimations—Protein was determined with a modified Lowry procedure (19) with bovine albumin as the standard.

Enzyme Activity—The 14CO2 assay procedure of Lindblad (3) was generally used, except in some alternate product experiments where the formation of U-14C-labeled homogentisate was measured by the thin layer method of Lindblad (3). When phenylpyruvate was exchanged for 4-hydroxyphenylpyruvate as substrate the procedure was analogous. The standard reaction mixture contained: bovine catalase (0.6 g/liter), sodium 2,6-dichlorophenolindophenol (0.15 mM), glutathione (10 mM) (neutralized with equimolar amounts of sodium hydroxide), Tris/HCl (0.2 M) at pH 7.5, and carboxy-14C-labeled or U-14C-labeled 4-hydroxyphenylpyruvate (0.2 mM). The concentration of oxygen in the gaseous phase was that of air (21%). The incubation volume was 0.125 ml or a multiple of that. Polyethylene tubes were filled with all reaction components except water. The reaction was initiated by placing the tubes in a water bath at 37°C and allowed to proceed for 15 min with continuous shaking. The reaction was stopped by addition of an equal volume of 30 mM sodium diethyldithiocarbamate in 1 M sodium acetate buffer at pH 5.5. The evolved carbon dioxide was absorbed in 20 µl of Hyamine in a glass cup, suspended from a rubber stopper, during a 60-min shaking period at 37°C. The glass cup was then transferred to a scintillation vial and the radioactivity counted in a Tri-Carb (Pack-
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4-Hydroxyphenylpyruvate was the variable substrate and 4-hydroxyphenylpyruvate was the changing fixed substrate (Fig. 1). In both cases, the double reciprocal pattern was also obtained when oxygen was the variable substrate and the concentration of 4-hydroxyphenylpyruvate fixed at 21%. When the concentration of phenylpyruvate was increased, the degree of the same type of nonlinear inhibition was observed. When the experiment was repeated at 0.2 mM concentration of 4-hydroxyphenylpyruvate, the inhibitor lines of the double reciprocal plot showed increasing concave upward curvatures near the vertical axis. A lower degree of the same type of nonlinear inhibition was observed when the experiment was repeated at 0.2 mM concentration of 4-hydroxyphenylpyruvate. At low concentrations of oxygen, the inhibitor lines were now approximately parallel to the uninhibited line. Thus, the inhibitor lines showed a minimum and the type of inhibition could be classified as parabolic. (25).

Dead End Inhibition — Both bathophenanthroline and di-
and liquid scintillation spectrometer. In product inhibition experiments with bicarbonate, the amount of Hyamine was increased in order to be in excess of the amount of bicarbonate present in the reaction mixture. The maximal CO₂ binding capacity of Hyamine was 1 mol/mol. 1% to 300 μl of Hyamine did not cause any quenching at radioactivity determination. At high concentrations of NaHCO₃, the molarity of the sodium acetate buffer at pH 5.5 was doubled in order to lower the pH of the reaction mixture to about 5.7. A stock solution of 1 M NaHCO₃ at pH 7.5 was made in 0.4 M Tris/HCl, pH 6.8, and the final concentration was checked with a CO₂ titration method.

The reaction velocity has been expressed as nanomoles of product/min/ml of reaction mixture with the amount of enzyme indicated in the legends of the figures. An amount of enzyme was used that allowed less than about 25% of the organic substrate to be consumed. Velocity time curves were then linear for 30 min at 0.02 to 2 mM concentration of 4-hydroxyphenylpyruvate. A one to one stoichiometric relation between the formation of CO₂ and homogentisate was shown.

Variation in Oxygen Concentration — The rubber stopper had two tubes, one inlet tube for the different O₂/N₂ mixtures and one outlet tube for evacuation and equilibration at atmospheric pressure. The reaction tubes were filled with the incubation mixture when standing in ice water. The tubes were evacuated with a water pump for 1 min and were then equilibrated with the various gas mixtures for at least 15 min. Both tubes were then closed and incubation started as described above. The concentrations of oxygen given in the following are those in the gaseous phase.

Data Processing — The nomenclature and methods of Cleland were used to describe and elucidate the kinetic mechanism at steady state (20-23). Initial rates were obtained over a period of 15 min and double reciprocal plots were constructed. All velocity measurements were made at least in duplicate except in the experiments where the formation of homogentisate was measured. Agreement between two determinations is indicated by a single point in the figures. The experiments were repeated two to six times to confirm the observed patterns.

When the reciprocal plots were judged to be linear, the lines were calculated from the coefficients of the experimentally determined points to the general rate equation, v = VA/(Kᵣ + A), by the least squares method assuming equal variance for the velocities (24). The calculated values of slopes (V/K) and intercepts (1/V) of these lines were plotted versus the reciprocal of the changing fixed substrate concentration for initial velocity experiments and versus the inhibitor concentration in inhibition experiments. When the replots were linear, the numerical values of constants were obtained by iterative weighted fitting of the data to the corresponding rate equation (24). A linear bireactant initial velocity, linear competitive inhibition, and linear noncompetitive inhibition (24). All least squares fits were performed with the FORTRAN programs of Cleland (24), which provide values for the constants in a fitted equation and the standard errors of their estimates.

RESULTS

Initial Velocity Patterns — An intersecting pattern was obtained when 4-hydroxyphenylpyruvate was the variable and oxygen was the changing fixed substrate (Fig. 1). An intersecting pattern was also obtained when oxygen was the variable substrate and 4-hydroxyphenylpyruvate was the changing fixed substrate (Fig. 2). In both cases, the double reciprocal plots were linear over the concentration ranges used. Values of the kinetic constants derived from these experiments and the standard errors of their estimates are given in the legends to Fig. 1 and 2.

Substrate Inhibition — The reaction rate was maximal at concentrations of oxygen in the gaseous phase above 15 to 20%. Oxygen was not inhibitory at concentrations up to 100% in the gaseous phase. 4-Hydroxyphenylpyruvate was inhibitory at substrate to enzyme ratios above 0.5 mol/g of protein; lower ratios were used in all experiments (see legends to figures).

Alternate Substrate Inhibition — Studies with the substrate analogue phenylpyruvate gave linear intersecting initial velocity patterns. Homogentisate gave a linear noncompetitive inhibition when oxygen was the variable substrate and the concentration of phenylpyruvate was fixed at 0.2 mM. A linear competitive inhibition was obtained with unlabeled phenylpyruvate as the inhibitor, 4-hydroxyphenylpyruvate as the variable substrate and the concentration of oxygen fixed at 21% (Fig. 3, left).

Phenylpyruvate gave a nonlinear inhibition pattern when oxygen was the variable substrate and the concentration of phenylpyruvate was fixed at 0.2 mM (Fig. 3, right). When the concentration of phenylpyruvate was increased, the inhibitor lines of the double reciprocal plot showed increasing concave upward curvatures near the vertical axis. A lower degree of the same type of nonlinear inhibition was observed when the experiment was repeated at 0.2 mM concentration of 4-hydroxyphenylpyruvate. At low concentrations of oxygen, the inhibitor lines were now approximately parallel to the uninhibited line. Thus, the inhibitor lines showed a minimum and the type of inhibition could be classified as parabolic.
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**FIG. 3.** Double reciprocal plots with phenylpyruvate as alternate substrate inhibitor. Left, 4-hydroxyphenylpyruvate was the variable substrate and the concentration of oxygen was fixed at 21%. The concentrations of phenylpyruvate were: 0 (○), 10 μM (●), 20 μM (▲), and 40 μM (■). The amount of enzyme protein was 0.52 μg. Kinetic constants: $K_1 = 15 \pm 1 \mu M$, $K_2 = 20 \pm 1 \mu M$, and $V = 0.64 \pm 0.01 \text{ nmol min}^{-1}$. Right, oxygen was the variable substrate and the concentration of 4-hydroxyphenylpyruvate was fixed at 0.05 mM. The concentrations of phenylpyruvate were: 0 (○), 50 μM (●), 100 μM (▲), and 200 μM (△). The amount of enzyme protein was 1.1 μg.

**FIG. 4.** Dead end inhibition pattern with 4-hydroxyphenylpyruvate as the variable substrate and the concentration of oxygen fixed at 21%. The lines drawn in the double reciprocal plots were calculated from fits to the general rate equation (see "Methods"). The slopes and intercepts of these lines were used in replots. Upper left, bathophenanthroline was the inhibitor: 0 (○), 2 μM (●), 3 μM (▲), and 4 μM (■). The amount of enzyme protein was 0.43 μg. Upper right, replot of slopes and intercepts versus the concentration of bathophenanthroline. Lower left, diethyldithiocarbamate was the inhibitor: 0 (○), 0.5 μM (●), 0.75 μM (▲), 1.0 μM (■), and 1.5 μM (△). The amount of enzyme protein was 0.21 μg. Lower right, replot of slopes and intercepts versus the concentration of diethyldithiocarbamate.

**FIG. 5.** Dead end inhibition pattern with oxygen as the variable substrate and the concentration of 4-hydroxyphenylpyruvate fixed at 0.2 mM. The lines drawn in the double reciprocal plots were calculated from fits to the general rate equation (see "Methods"). The slopes and intercepts of these lines were used in replots. Upper left, bathophenanthroline was the inhibitor: 0 (○), 10 μM (●), 20 μM (▲), and 30 μM (■). The amount of enzyme protein was 3.1 μg. Upper right, replot of slopes and intercepts versus the concentration of bathophenanthroline. Lower left, diethyldithiocarbamate was the inhibitor: 0 (○), 2.5 μM (●), 5 μM (▲), and 10 μM (■). The amount of enzyme protein was 2.2 μg. Lower right, replot of slopes and intercepts versus the concentration of diethyldithiocarbamate.

Ethylidithiocarbamate gave competitive inhibitions with apparent parabolic slope replots when 4-hydroxyphenylpyruvate was the variable substrate and the concentration of oxygen was fixed at 21% (Fig. 4). Bathophenanthroline gave noncompetitive inhibition with apparently parabolic slope and intercept replots when oxygen was the variable substrate and the concentration of 4-hydroxyphenylpyruvate was fixed at 0.2 mM (Fig. 5, upper). The corresponding inhibition by diethyldithiocarbamate was also noncompetitive but the forms of the slope and intercept replots were more uncertain (Fig. 5, lower). When the two inhibitors were varied with the concentration of oxygen fixed at 21% and that of 4-hydroxyphenylpyruvate fixed at 0.2 mM, a parallel pattern was obtained when reciprocal velocities were plotted versus the concentrations of diethyldithiocarbamate at different levels of bathophenanthroline. This is the expected pattern for two competitive inhibitors with an infinite value of the interaction coefficient, indicating mutually exclusive binding to the enzyme (23).

**Product Inhibition** – A plot of reciprocal velocities versus 0 to 40 mM concentrations of homogentisate showed linear inhibition during standard assay condition (see "Methods"). Homogentisate gave linear competitive inhibition when 4-hydroxyphenylpyruvate was the variable substrate and the concentration of oxygen was fixed at 21% (Fig. 6, left). The mean value...
Fig. 6. Double reciprocal plots with 4-hydroxyphenylpyruvate as the variable substrate and homogentisate as the inhibitor at two concentrations of oxygen. Left, the concentration of oxygen was 21% and the concentrations of homogentisate were: 0 (○), 10 mM (●), 20 mM (□), 30 mM (■), and 40 mM (△). The amount of enzyme protein was 0.28 μg. Kinetic constants: $K_i = 1.1 \pm 0.1 \text{ mM}$; $K_m = 2.3 \pm 0.1 \text{ mM}$, and $V = 0.55 \pm 0.01 \text{ nmol min}^{-1}$. Right, the concentration of oxygen was 5.1% and the concentrations of homogentisate were: 0 (○), 10 mM (●), 20 mM (□), and 30 mM (■). The amount of enzyme protein was 0.74 μg. Kinetic constants: $K_e = 12 \pm 1 \text{ μM}$; $K_m = 3.8 \pm 0.4 \text{ mM}$, and $V = 0.70 \pm 0.02 \text{ nmol min}^{-1}$.

Fig. 7. Double reciprocal plot with oxygen as the variable substrate and homogentisate as the inhibitor: 0 (○), 15 mM (●), and 30 mM (□). The concentration of 4-hydroxyphenylpyruvate was 0.2 mM. The amount of enzyme protein was 5.2 μg. Kinetic constants: $K_i = 2.6 \pm 0.2$; $K_m = 30 \pm 3 \text{ mM}$; $K_m = 41 \pm 3.3 \text{ mM}$; $V = 0.7 \pm 0.1 \text{ nmol min}^{-1}$. Kinetic constants: $K_i = 2.6 \pm 0.2$; $K_m = 41 \pm 3.3 \text{ mM}$; $K_m = 41 \pm 3.3 \text{ mM}$; $V = 0.7 \pm 0.1 \text{ nmol min}^{-1}$.

Fig. 8. Double reciprocal plots with 4-hydroxyphenylpyruvate as the variable substrate and 2-hydroxyphenylacetate as alternate product inhibitor. The concentration of oxygen was 21%. The amount of enzyme protein was 0.61 μg. Left, the formation of CO$_2$ was measured. The concentrations of 2-hydroxyphenylacetate were: 0 (○), 10 mM (●), 20 mM (□), and 30 mM (■). Kinetic constants: $K_a = 15 \pm 1 \text{ μM}$; $K_m = 2.1 \pm 0.1 \text{ mM}$, and $V = 0.57 \pm 0.02 \text{ nmol min}^{-1}$. Right, the formation of homogentisate was measured. The concentrations of 2-hydroxyphenylacetate were: 0 (○), 10 mM (●), and 20 mM (□). Kinetic constants: $K_a = 41 \pm 5 \text{ μM}$; $K_m = 4.9 \pm 0.5 \text{ mM}$, and $V = 1.35 \pm 0.07 \text{ nmol min}^{-1}$.
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From the kinetic results presented, we propose an Ordered Bi Bi mechanism (20) for human liver 4-hydroxyphenylpyruvate dioxygenase Form 3, in which 4-hydroxyphenylpyruvate (A) binds to the enzyme prior to oxygen (B) and in which CO\textsubscript{2} (P) is released before homogentisate (Q) (Reaction 1).

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\begin{array}{c|c|c|c|c|c}
E & EA & (EAB-EPQ) & EQ & E \\
\end{array}
\]

**Reaction 1**

Several two substrate mechanisms had to be considered based on the results of initial velocity and product inhibition studies. Most of them were made unlikely by alternate substrate, dead end, and alternate product experiments.

The inhibition patterns obtained with unlabeled phenylpyruvate, via. linear competitive inhibition versus 4-hydroxyphenylpyruvate and parabolic nonlinear inhibition versus oxygen, are compatible with an ordered mechanism, in which 4-hydroxyphenylpyruvate is the first substrate (25).

With 4-hydroxyphenylpyruvate as the first substrate, the available product inhibition data conformed to those predicted for an ordered mechanism in which CO\textsubscript{2} and homogentisate correspond to P and Q. No evidence of dead end formation with oxygen as the variable substrate. Parabolic inhibition patterns are expected when two or more molecules of metal chelators bind to the enzyme in dead end fashion (26). Thus, the Cleland rules for dead end inhibition at steady state (22) could be used to verify an ordered addition of 4-hydroxyphenylpyruvate prior to oxygen.

The range of values obtained for the Michaelis constant for 4-hydroxyphenylpyruvate in the different experiments was 12 to 40 \(\mu\text{M}\). The dissociation and Michaelis constants for 4-hydroxyphenylpyruvate calculated from the inhibition constants for homogentisate (see legends to Figs. 6 and 7) (21) were 31 and 12 \(\mu\text{M}\), respectively. These values are similar to those obtained in initial velocity experiments as expected in the proposed mechanism. The values reported for the enzyme from other mammalian species have been of similar magnitude (20 to 60 \(\mu\text{M}\)) (8-10, 27). The enzyme from *P. aeruginosa* sp. P-3, 674 had a Michaelis constant of 30 \(\mu\text{M}\). The range of values obtained for the Michaelis constant for O\textsubscript{2} was lower (16 to 32 \(\mu\text{M}\) (28) than the value reported for the rat liver enzyme (100 \(\mu\text{M}\)) (9).

Isotopic exchange experiments would more decisively differentiate between possible two-substrate mechanisms, e.g. exclude a Theorell-Chance mechanism. However, since no inhibition has been observed with high concentrations of the proposed first product, CO\textsubscript{2}, and since the equilibrium of the reaction presumably is unfavorable (29), neither the reverse reaction nor any isotopic exchange between products and substrates is expected to occur.

The inhibitory effect of 4-hydroxyphenylpyruvate on the human liver enzyme Form 3 decreased when the amount of enzyme was increased. Inhibition of dog liver 4-hydroxyphenylpyruvate dioxygenase by high concentrations of 4-hydroxyphenylpyruvate has previously also been shown to increase with the substrate to enzyme concentration ratio and to be partially reversed by a variety of reductants (30, 31). It seems unlikely that the inhibitory effect of the substrate has influenced the results presented. Since noninhibitory substrate to enzyme ratios were used in all experiments designed to evaluate the kinetic mechanism. The substrate inhibition has been ascribed to accumulation of some inhibitory compound, e.g. \(H_2O_2\) formed during the reaction (30) or by nonenzymic interaction between the substrates (9), although experiments with the pure human enzyme have not provided evidence for the formation of \(H_2O_2\) (4). It has been suggested that tetrameric rabbit liver enzyme dissociates into less active dimers and inactive monomers upon oxidation of enzyme-bound iron (32), which would be in accordance with earlier suggestions that reductants stimulate \(Fe^{2+}\)-containing oxygenases by keeping iron in the reduced form (33). Further studies on the effect of substrate and reductants on the oligomeric structure of the enzyme are thus needed before any conclusions can be drawn from kinetic patterns at high concentrations of 4-hydroxyphenylpyruvate.

The mechanism proposed for human liver 4-hydroxyphenylpyruvate dioxygenase implies that oxygen only binds to the enzyme in the presence of the organic substrate. This has also been found to be the case for several other oxygenases, e.g. tryptophan 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, lysine 2-monooxygenase, and cytochrome P-450 (34). On the other hand, oxygen is reported to be the first substrate for steroid 4,5-dioxygenase (35) and half-reduced pseudomonomatropoxygenase 2,3-dioxygenase (36). Thymine 7-hydroxylase (EC 1.14.11.6) has recently been shown to have a mainly Ordered Bi Bi kinetic mechanism at steady state, in which 2-oxoglutarate binds to the enzyme before thymine and oxygen (37). Thus, the results presented provide further support for a relation between the reaction mechanism of 4-hydroxyphenylpyruvate dioxygenase and that of the 2-oxoglutarate coupled dioxygenases (5, 13). An Ordered Bi Bi mechanism (20) for 4-hydroxyphenylpyruvate dioxygenase does not discriminate between the peroxide mechanism of Lindblad et al. (5) and the peracid mechanism of Hamilton (13).

**REFERENCES**

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M Rundgren


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