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Purification and Some Properties of 4-Hydroxyphenylpyruvate Dioxygenase from *Pseudomonas* sp. P.J. 874†

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ABSTRACT: 4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) has been purified to apparent homogeneity from *Pseudomonas* sp. P.J. 874, induced to grow on tyrosine as the sole source of carbon. The enzyme protein was a tetramer of equally sized subunits, each with a mass of 36 kdaltons and the NH_2-terminal amino acid sequence Ala-Asp-Leu/Ile-Tyr-. It had a molecular mass of 150 kdaltons, a sedimentation coefficient in water at 20 °C of 7.6 S, and a Stokes radius of 4.9 nm. The isoelectric point was around pH 4.8. The enzyme protein showed a broad absorbance in the blue region of the visible spectrum. The purified enzyme protein contained variable amounts of iron, 0.6 to 1.3 mol/mol, and copper, 0.2 to 0.5 mol/mol. ^{59}Fe included in the culture medium followed enzyme activity through the purification. Inhibition experiments with metal chelators indicated an essential role for enzyme-bound Fe^{2+}. An optimized combination of ascorbate (50 mM), catalase (2.4 g/L), and iron (50 μM) activated the enzyme more than a combination of 2,6-dichlorophenolindophenol, glutathione, and catalase. The former system was also more effective than the latter in protecting the enzyme from inactivation at 37 °C. No stimulation by iron was observed when 2,6-dichlorophenolindophenol was the reductant. Reduced 2,6-dichlorophenolindophenol and ascorbate were hyperbolic uncompetitive activators when 4-hydroxyphenylpyruvate was the variable substrate. The enzyme showed a constant ratio of 4-hydroxyphenylpyruvate to phenylpyruvate activity during purification. One optimum around pH 7 was observed for both activities at 37 °C. The apparent Michaelis constant was 30 μM for 4-hydroxyphenylpyruvate and 0.52 mM for phenylpyruvate at pH 7.5 in the presence of 2,6-dichlorophenolindophenol (0.15 mM), glutathione (10 mM), and catalase (0.8 g/L). The corresponding values of the apparent maximal velocity were 900 and 20 nmol min^{-1} (mg of protein)$^{-1}$. The apparent Michaelis constant and maximal velocity were 0.15 mM for 4-hydroxyphenylpyruvate and 20 μmol min^{-1} (mg of protein)$^{-1}$, respectively, in the presence of the optimized combination of ascorbate, catalase, and iron. Substrate inhibition was observed at high concentrations of both 4-hydroxyphenylpyruvate and phenylpyruvate.

4-Hydroxyphenylpyruvate is enzymically converted to homogentisate by 4-hydroxyphenylpyruvate dioxygenase (4-hydroxyphenylpyruvate:oxygen oxidoreductase (hydroxylating, decarboxylating) (EC 1.13.11.27)). Hitherto, only the enzymes from human (Lindblad et al., 1971; Lindblad et al., 1977), chicken (Fellman et al., 1972a), and bovine (Nakai et al., 1975) liver have been obtained in highly purified forms. Both the human and the chicken liver enzymes have been resolved into multiple forms (Lindblad et al., 1972; Wada et al., 1975; Rundgren, 1977a). The proposed reaction mechanisms, which at present are seriously considered (Goodwin and Witkop, 1957; Lindblad et al., 1970; Hamilton, 1971), are closely related to those suggested for the 2-oxoglutarate-dependent oxygenases (Holme et al., 1968; Lindblad et al., 1969; Hamilton, 1971; for a review see Abbott and Udenfriend, 1974). In these mechanisms a transition-metal ion has been implicated for the activation of oxygen. Evidence for the presence of enzyme-bound copper or iron in 4-hydroxyphenylpyruvate dioxygenase has accumulated (Goodwin, 1972; Goodwin and Werner, 1973; Laskowska-Klita and Mochnacka, 1973; Wada et al., 1975; Lindblad et al., 1977). The enzymes from various sources have shown a requirement for catalase and a reductant, reduced 2,6-dichlorophenolindophenol being most effective (Goodwin, 1972; Laskowska-Klita and Mochnacka, 1973; Nakai et al., 1975; Lindblad et al., 1977). The stimulation by the naturally occurring reductant, ascorbate, is reported to decrease with purification (Zannoni, 1962). It has been reported that reductants prevent and reverse both the inactivation by storage and the inhibition by high concentrations of 4-hydroxyphenylpyruvate (Goodwin, 1972). This paper describes the purification of 4-hydroxyphenylpyruvate dioxygenase from a *Pseudomonas* strain, which has been isolated by enrichment culture (Midtvedt et al., unpub-

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lished results), and reports on some properties of the purified enzyme. The roles of catalase, reduced 2,6-dichlorophenolindophenol, and ascorbate are examined.

Experimental Procedures

Materials

Chemicals were obtained from the following sources: *N*-allyl-*N*,*N*-dimethylamine (25 g was treated with 5 drops of phenyl isothiocyanate at 40 °C under N_2 for 1 h and then distilled at 63 °C under N_2) from Eastman Kodak Co.; 1,2-dichloroethane (dried over K_2CO_3 and distilled at 84 °C) and ethyl acetate (dried over K_2CO_3 and distilled at 78 °C) from Fluka AG; iron wire (>99.999%) from Koch-Light Laboratories Ltd.; L-[1-^{14}C]tyrosine (~50 Ci/mol), L-[1-^{14}C]phenylalanine (~50 Ci/mol), and $^{59}FeSO_4$ as a solution in 0.05 M H_2SO_4 (1 Ci/L) from New England Nuclear Corp.; pyridine (100 g was refluxed with 1 g of phthalic anhydride for 2 h under N_2 and distilled at 117 °C under N_2) and phenyl isothiocyanate (distilled) of Sequanal grade from Pierce Chemical Company; L-[U-^{14}C]tyrosine (~500 Ci/mol) from The Radiochemical Centre; 4-hydroxyphenylpyruvic acid (purified and converted to the potassium salt as described by Lindblad, 1971) from Sigma Chemical Co.; chromatography papers no 1 (treated with 3 mM EDTA[1]) from Whatman Biochemicals Ltd. ^{14}C-Labeled phenylpyruvate was prepared from radioactive L-phenylalanine by the procedure used to obtain 4-hydroxyphenylpyruvate from L-tyrosine (Lindblad, 1971). Centrally deionized water was further purified by passage through the Super-Q System of Millipore (resistivity above 18 Mohm × cm).

In experiments with metal ions the glassware and dialysis tubings were washed in 0.5 M acetic acid containing 1 mM EDTA and then in Millipore-filtered water. Buffers were treated with Chelex-100 resin.

Methods

Cell Cultures. A strain of *Pseudomonas*[2] (sp. P.J. 874; Midtvedt et al., unpublished results) was induced to grow on a medium containing tyrosine as the single carbon source. The strain was maintained on blood-agar plates at 4 °C and transferred to new plates every month. Three transfers in the liquid medium of Jacoby (1964) preceded the final 10-L culture in conical flasks. The cultures were incubated at 30 °C with rotary agitation in air until a maximal absorbance at 535 nm of about 1.3 was reached, usually after 16 h. The bacteria were harvested by centrifugation at 8000*g* for 30 min and washed 4 times with 100 mL of 9 g/L NaCl solution per about 2.5 g of bacterial cell paste. The bacterial cell paste could be stored at −60 °C for several months.

Enzyme Assays. The activity of 4-hydroxyphenylpyruvate dioxygenase was measured by following the release of $^{14}CO_2$ from 4-hydroxy[1-^{14}C]phenylpyruvate or the formation of homogentisate from 4-hydroxy[U-^{14}C]phenylpyruvate as described by Lindblad (1971).

Assay 1. The standard reaction mixture, usually 0.25 mL, contained enzyme, 2,6-dichlorophenolindophenol (0.15 mM), glutathione (10 mM), bovine liver catalase (0.8 g/L), 4-hydroxy[1-^{14}C]phenylpyruvate (0.2 mM), and Tris-HCl at pH 7.5 (0.2 M). After 20 min in ice-water the reaction was started by placing the tubes in a water bath at 37 °C. The reaction time was 10 to 15 min at 37 °C. The enzyme activity with 25 μM phenylpyruvate as the substrate was determined by the same method. One unit (U) of enzyme activity is defined as the formation of 1 μmol of product per min under these conditions.

Assay 2. During the later part of the work the mixture of 2,6-dichlorophenolindophenol and glutathione was replaced by 50 mM ascorbate and 50 μM Fe^{2+}. The catalase concentration was increased to 2.4 g/L.

Protein. Protein was determined according to Lowry et al. (1951) with bovine serum albumin as the standard.

Gel Electrophoresis. Electrophoresis in 1% agarose gel was performed in 65 mM sodium phosphate buffer at pH 6.5 as described by Johansson (1972). Disc electrophoresis in 4% polyacrylamide gel was performed at pH 7.5 as described by Hedrick and Smith (1968). Only separating gel (0.6 × 10 cm) was used. Samples, 10 to 100 μL, in 3.4 M glycerol were applied after a 45-min prerun at 1 mA per tube. Electrophoresis was then performed at 2 mA per gel for about 2 h at 4 °C with the cathode at the top. Parallel nonstained gels were sliced in 5-mm sections. Each section was frozen at −60 °C for 2 h, eluted overnight at 4 °C with 500 μL of 0.2 M Tris-HCl buffer at pH 7.5, and analyzed for enzyme activity. The mobilities of proteins were calculated relative to that of bromophenol blue.

Experiments designed to reveal size and charge isomers were performed as described by Hedrick and Smith (1968), but without the spacer gel. Frictional ratios were calculated from plots of relative mobility vs. gel concentration (Parish and Marchalonis, 1970).

Disc electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was carried out as described by Weber and Osborn (1969). Samples of protein were denaturated in buffer containing sodium dodecyl sulfate (10 g/L) and 0.1 M 2-mercaptoethanol during 4 h at 37 °C or overnight at room temperature. Purified 4-hydroxyphenylpyruvate dioxygenase (4–20 μg) was run alone and in various combinations with marker proteins. After staining in Coomassie brilliant blue (2.5 g/L) the mobility was calculated relative to that of bromophenol blue.

Gel Filtration. The Stokes radius was determined by gel filtration (Laurent and Killander, 1964; Siegel and Monty, 1966) (for technical details see the Purification Procedure section, step 5). The elution volume (V_e) of blue dextran was used as void volume (V_0) and that of L-[1^{14}C]leucine was used as the sum of inner and void volumes ($V_i + V_0$). The total bed volume (V_t) was measured by volume.

Analytical Ultracentrifugation. The experiments were performed in a Beckman-Spinco Model E analytical ultracentrifuge equipped with electronic speed and rotor temperature control units and with schlieren and Rayleigh optic systems. Sedimentation velocity experiments were carried out in 0.2 M NaCl buffered with 10 mM potassium phosphate (pH 6.7), at 56 000 rpm. In a high-speed sedimentation equilibrium experiment according to Chervenka (1970) the protein was equilibrated with 0.1 M potassium phosphate buffer (pH 7.0), by filtration on Sephadex G-25 (medium), and diluted to a protein concentration of 0.6 g/L. The rotor speed was 12 000 rpm and the temperature 4 °C. The run was stopped after 21 h. The regression of ln fringe displacement on (radial distance)^2 was calculated by the least-squares method and used to calculate the weight-average molecular mass.

NH_2-Terminal Amino Acid Analysis. The phenyl isothiocyanate procedure of Iwanaga et al. (1969) was used. The phenylthiohydantoin derivatives of amino acids were identified

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DCIP, 2,6-dichlorophenolindophenol; GSH, glutathione; Tris, tris(hydroxymethyl)aminomethane.

[2] The bacterial strain used can be obtained from our laboratory.

by chromatography on precoated silica gel plates with fluorescense indicator (Jeppsson and Sjöquist, 1967) or on polyamide layers (Summers et al., 1973). Spectrophotometric determination of the phenylthiohydantoins was performed after paper chromatography as described by Sjöquist (1960).

Electrofocusing. Isoelectric focusing (pH range 3–10) was performed according to the method of Vesterberg and Svensson (1966) in a Model 8101 110-mL column (LKB-Produkter AB). Preparations of 4-hydroxyphenylpyruvate dioxygenase were exchanged for the light solution in the middle of the gradient. Electrofocusing was carried out at 300 V for 49 h at 4 °C.

Metal Studies. Copper was determined by spectrophotometry of the 2,2′-biquinoline-Cu^{+} complex (Felsenfeld, 1960; Broman et al., 1962) with $CuSO_4 \cdot 5H_2O$ as the standard. Iron was determined by spectrophotometry of the bathophenanthroline-Fe^{2+} complex (Doeg and Ziegler, 1962) with standards prepared from iron wire.

A mixture of $^{59}FeSO_4$ (1.5 mCi) and unlabeled $(NH_4)_2Fe(SO_4)_2$ was added to 2960 mL of growth medium to a final concentration of 2.6 μmol of iron/L and a specific radioactivity of 194 Ci/mol of iron. The distribution of radioactive iron was then followed through the purification of 4-hydroxyphenylpyruvate dioxygenase.

Results

Purification Procedure

All steps were performed at 4 °C unless otherwise stated. The enzyme activity was determined with assay 1.

Step 1: Cell-Free Extract. Bacterial cell paste was suspended in 10 mM potassium phosphate buffer at pH 6.7 (10 mL/g wet weight bacterial cell paste) and transferred into a Branson Rosette cell kept in ice-water. The bacterial cells were sonically disrupted for 5 × 3 min with a Branson S75 sonifier operating at 75 W and 20 kHz. The precipitate obtained by centrifugation at 100 000*g* for 60 min was discarded, since it contained less than 1% of the enzyme activity in the supernatant.

The stability of 4-hydroxyphenylpyruvate dioxygenase activity in cell-free extracts prepared in water was tested in 50 mM buffers at pH values between 3 and 9 after storage at 4, −20, and −60 °C for various times. At 4 °C the enzyme was most stable between pH 7.0 and 8.5; the activity decreased to about 80% after 1 day and to about 40% after 10 days. The enzyme activity was lost below pH 5. At −60 °C the enzyme was most stable in Tris-HCl buffer at pH 7.5; the activity decreased to about 40% after 120 days. The protecting effect of some compounds was tested when the enzyme was stored at 4 °C. No effect was observed with glutathione (2–42 mM), catalase (0.4–7 g/L), bovine serum albumin (0.7 g/L), 2-mercaptoethanol (0.1 M), glycine (0.1 M), or EDTA (0.3–3 mM).

Step 2: Streptomycin Precipitation. A 26% (w/v) solution of streptomycin in water (*a* μL), calculated from the formula $a = b0.75A_{260}$, was added dropwise to the clear supernatant from step 1 (*b* milliliters) during 30 min of continuous mechanical stirring. The solution was then stirred for an additional 15-min period and centrifuged at 18 000*g* for 30 min. The precipitate was discarded.

Step 3: DEAE-Cellulose Chromatography. A portion of the supernatant from step 2 (pH 6.4) was applied onto a DE52-cellulose column (1.6 × 31 cm), equilibrated with 10 mM potassium phosphate buffer at pH 6.7. The enzyme was eluted with a linear gradient formed from 500 mL of the equilibration buffer and 500 mL of that buffer, containing 0.4 M KCl. Fractions of 7.5-mL volume were collected at a flow rate of 50 mL/h. The peak of enzyme activity appeared at about 0.2 M concentration of KCl. The fractions with enzyme activity were pooled.

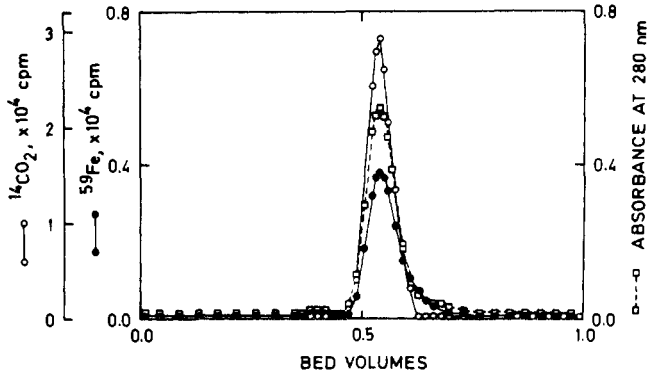

FIGURE 1: Sephadex G-200 filtration. A 2-mL sample from hydroxylapatite chromatography was put onto a column (1.5 × 86 cm) of Sephadex G-200 (see Purification Procedure). Portions of the 1.1-mL fractions were analyzed for enzyme activity (5 μL, O–O), ^{59}Fe (100 μL, ●–●), and absorbance at 280 nm (□---□).

Step 4: Hydroxylapatite Chromatography. A portion of the pooled enzyme fractions from step 3 was applied onto a hydroxylapatite column (5 × 24 cm), equilibrated with 10 mM potassium phosphate buffer at pH 6.7. The enzyme was eluted with a linear gradient formed from 2.5 L of the equilibration buffer and 2.5 L of 0.3 M potassium phosphate buffer at pH 6.7. Fractions of about 10 mL were collected at a flow rate of 200 mL/h. The enzyme activity appeared at about 35 mM potassium phosphate concentration. The fractions with enzyme activity were pooled and concentrated on a PM-10 Diaflo ultrafiltration membrane (Amicon Corp.). The enzyme solution was stored in portions at −60 °C.

Step 5: Sephadex G-200 Filtration. A column of Sephadex G-200 (1.5 × 86 cm) was prepared in 10 mM potassium phosphate buffer at pH 6.7, containing 0.2 M NaCl, and equilibrated at a flow rate of 6 mL/h. About 15-mg portions of the enzyme preparation obtained in step 4 in 2 mL of 0.3 M sucrose solution were layered on top of the column. Fractions of 1 or 2 mL were collected in preweighed polyethylene tubes. The elution volumes were determined by weight. The fractions with enzyme activity were pooled (see Figure 1) and concentrated by vacuum dialysis in a collodion bag (Model SM 13 200, Sartorius Membranfilter GmbH). The enzyme was stored in portions at −60 °C.

The purification procedure is summarized in Table I. The purification was followed by agarose gel electrophoresis at pH 6.5. Only one band, which contained more than 95% of the protein, was seen in the final step. The purified enzyme was stable for several months when stored at −60 °C in 0.2 M NaCl solution buffered with 10 mM potassium phosphate at pH 6.7.

Physicochemical Properties

Molecular Mass. Disc electrophoresis of the purified enzyme at pH 7.5 and 4 °C in 4% polyacrylamide gel showed one dominating band (Figure 2), which contained enzyme activity. The recovery of enzyme activity was about 20%. No size or charge isomers appeared at electrophoresis in different concentrations of polyacrylamide (4–12%) at pH 7.5 and 4 °C. The molecular mass was 166 kdaltons as determined from a

TABLE I: Purification of 4-Hydroxyphenylpyruvate Dioxygenase from *Pseudomonas* sp. P.J. 874.

Purification step	Vol (mL)	Total act. (U)	Total protein (mg)	Sp act. (U/mg)	4-HPP/PP[a]	Iron[c] nmol/mg	Iron[c] nmol/U	Sp radioact. of Fe[c] (Ci/mol)
1. Cell-free extract	97	140	1000	0.14	*b*	10.0	51.0	68
2. Streptomycin precipitation	97	110	1000	0.11	*b*	6.5	50.0	68
3. DE52-cellulose chromatography	100	72	110	0.66	1390	17.0	29.0	45
4. Hydroxylapatite chromatography	2.0	49	38	1.30	1460	9.1	5.0	47
5. Sephadex G-200 filtration	1.8	24	26	0.92	1520	4.1	6.4	43

[a] The ratio given is the activity obtained with 4-hydroxyphenylpyruvate (4-HPP), 0.2 mM, to that with phenylpyruvate (PP), 25 μM, as substrate for the enzyme. [b] The activity obtained with phenylpyruvate as substrate was too low to allow calculation of the ratio. [c] These data are taken from a small-scale purification, where radioactive iron (1.5 mCi of ^{59}Fe, specific radioactivity 194 Ci/mol of iron) was added to the culture medium.

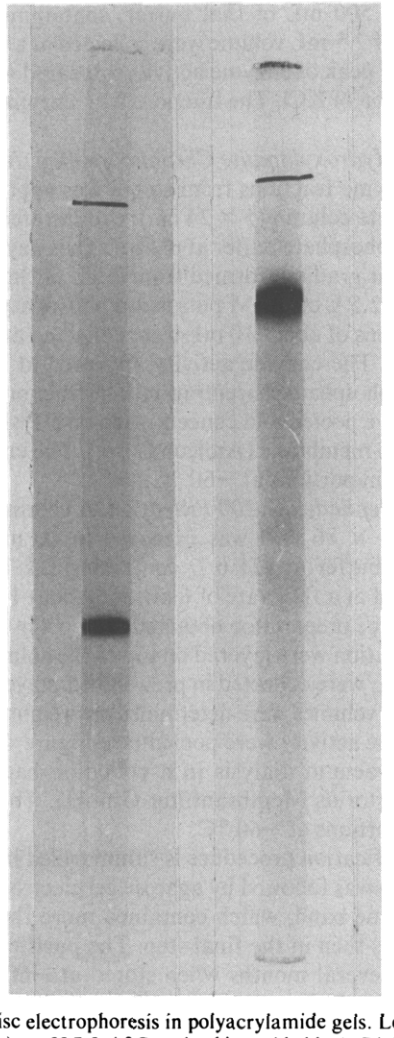

FIGURE 2: Disc electrophoresis in polyacrylamide gels. Left gel: Native enzyme (15 μg) at pH 7.5, 4 °C, stained in amido black. Right gel: Enzyme (20 μg) treated with sodium dodecyl sulfate (see Methods). The buffer (pH 7.0) contained sodium dodecyl sulfate (1 g/L) and 2-mercaptoethanol (14 mM). The gel was stained in Coomassie brilliant blue. The migration of bromophenol blue is indicated by steel wires at the top anodal parts of the gels.

plot of "frictional ratio" vs. molecular masses of bovine catalase, human transferrin, bovine serum albumin, and ovalbumin (for mass values see below).

The average distribution coefficients (K_{av}) of the enzyme and some marker proteins were determined by filtration on Sephadex G-200 columns (Laurent and Killander, 1964). The marker proteins used (for values of molecular mass and diffusion coefficient in water at 20 °C see Sober (1968) when no other reference is given) were: bovine liver catalase (Samejima and Yang, 1963), IgG (Rymo et al., 1972), rabbit muscle aldolase, human transferrin (Fish et al., 1969; Laurent and Killander, 1964), human serum albumin, bovine serum albumin, ovalbumin (Laurent and Killander, 1964), chymotrypsinogen A (Weber and Osborn, 1969; Laurent and Killander, 1964), and cytochrome *c* (Weber and Osborn, 1969; Laurent and Killander, 1964). The K_{av} value obtained with 4-hydroxyphenylpyruvate dioxygenase was 0.24 ± 0.11 (SD) (n = 8). Stokes radii of the marker proteins (calculated from their diffusion coefficients in water at 20 °C by the Stokes-Einsteins relation) were related to the K_{av} values obtained by the equation of Siegel and Monty (1966). The horizontal intercept in the plot of $(-\ln K_{av})^{1/2}$ vs. Stokes radius was set at 0.7 nm (Laurent and Killander, 1964). The Stokes radius of 4-hydroxyphenylpyruvate dioxygenase was 4.9 ± 0.1 nm (SD) (n = 8). A diffusion coefficient in water at 20 °C of 4.36×10^{-7} cm^2 s^{-1}, a molecular mass of 154 kdaltons, and a frictional ratio (f/f_0) of 1.39 (Siegel and Monty, 1966) were calculated from the Stokes radius and the sedimentation coefficient (see below) with the assumption of a partial specific volume of 0.725 mL/g of protein. The molecular mass of 4-hydroxyphenylpyruvate dioxygenase was determined to 172 ± 8 (SD) kdaltons (n = 8) from a plot of K_{av} vs. log molecular mass for the marker proteins.

The enzyme sedimented as a single symmetrical boundary at protein concentrations of 3.2 and 3.8 g/L and temperatures of 6 and 5 °C, respectively. Sedimentation coefficients in water at 20 °C of 7.58 and 7.64 S were obtained when a partial specific volume of 0.725 mL/g was assumed.

High-speed sedimentation equilibrium of the purified enzyme in 0.1 M potassium phosphate buffer at pH 7.0 gave a linear plot of ln fringe displacement vs. (radial distance)^2. A weight-average mass of 150 kdaltons was obtained from the slope (95% confidence interval, 147 to 154 kdaltons), when a partial specific volume of 0.725 mL/g was assumed. No dependence of point weight-average mass (Yphantis, 1964) on fringe displacement was noticed.

Subunit Size. One dominating band was observed when the purified enzyme was subjected to polyacrylamide gel electro-

TABLE II: NH_2-Terminal Residues of 4-Hydroxyphenylpyruvate Dioxygenase from *Pseudomonas* sp. P.J. 874.[a]

Step	Residue	mol/ 36 kg of protein[b]	Recovery (%) of residues from insulin
1	Ala	0.77	Phe-76 Gly-64
2	Asp	0.61	Val-84 Ile-78
3	Leu/Ile	0.48/0.49	Val-58 Asn-62
4	Tyr	0.36	Glu-67 Gln-26

[a] A sequential phenyl isothiocyanate procedure was applied to 2.6 mg of the purified enzyme as described under Methods. [b] The values are given per subunit mass of 36 kdaltons. They are not corrected for the recovery of residues from insulin.

phoresis in the presence of sodium dodecyl sulfate (Figure 2). The subunit mass was determined to be 36 kdaltons (n = 8, range 36–36.5 kdaltons) from a plot of relative mobility vs. log molecular or subunit masses of the marker proteins (human transferrin, bovine serum albumin, bovine liver catalase, ovalbumin, rabbit muscle aldolase, lactate dehydrogenase, and chymotrypsinogen A; for mass values see above and Weber and Osborn, 1969). The same minimal mass value was obtained with and without reduction of enzyme. In the latter case some faintly stained bands with mobilities corresponding to masses of about 110, 150, and 190 kdaltons became more apparent.

NH_2-Terminal Residues. Only a single amino acid residue was found in each of the first four steps when a sequential phenyl isothiocyanate procedure was applied on 2.6 mg of the purified enzyme (Table II). A polypeptide mass of 36 kdaltons could be calculated for 4-hydroxyphenylpyruvate dioxygenase.

Isoelectric Point. The enzyme showed a tendency to precipitate and to lose activity during electrofocusing. However, only one peak of 4-hydroxyphenylpyruvate dioxygenase activity appeared at about pH 4.8 when 1 mL of cell-free extract was electrofocused in a pH gradient between 3 and 10.

Light Absorption Spectrum. The ultraviolet spectrum of the purified enzyme, dissolved in 0.2 M NaCl buffered with 10 mM potassium phosphate at pH 6.7, showed a typical protein absorbance. The absorption coefficient, $A_{1cm}^{1\%}$, was 9.4 at 280 nm (mean value of five different preparations of purified enzyme with a range of 8.6–10.6). Highly concentrated preparations of purified enzyme were bluish and the visible spectrum showed a broad absorbance in the blue region. The absorption coefficient, $A_{1cm}^{1\%}$, at 595 nm was 0.19 for two different preparations of purified enzyme.

Metal Content. The iron contents in three different preparations of purified 4-hydroxyphenylpyruvate dioxygenase were 1.3, 0.83, and 0.62 mol/mol of enzyme. The copper contents of the last two preparations were 0.24 and 0.45 mol/mol of enzyme.

A summary of the distribution of ^{59}Fe and iron during a small-scale purification of the enzyme is given in Table I. Radioactive iron followed the enzyme activity through the chromatographic steps (cf. Figure 1). The ratio of iron content to enzyme activity was about the same in steps 4 and 5.

Catalytic Properties

Involvement of a Metal Ion. Fe^{2+} (10^{-6} to 10^{-3} M) and

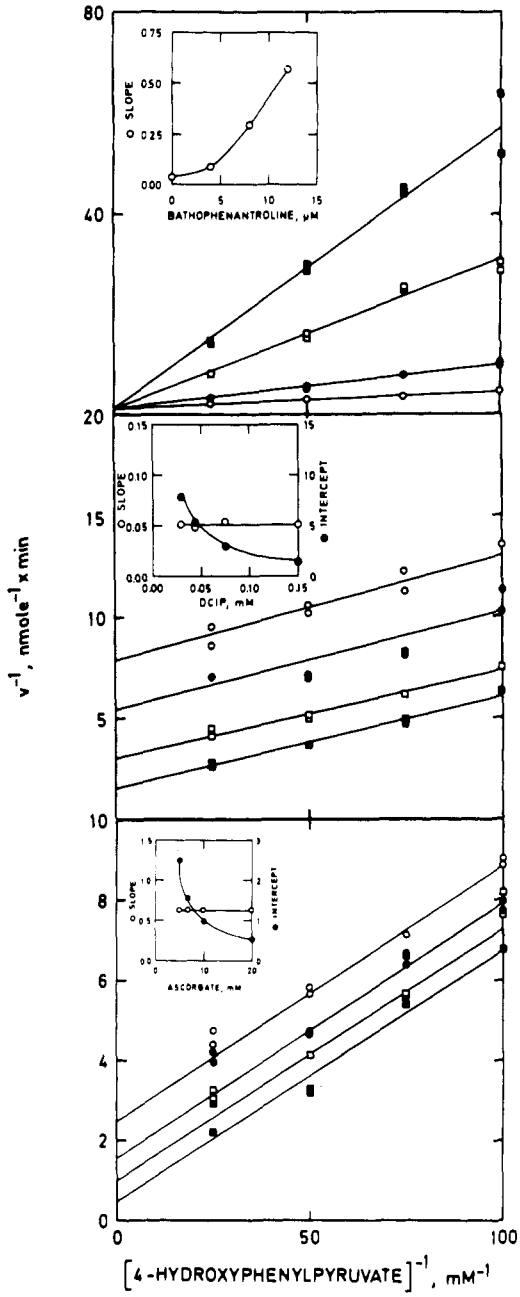

FIGURE 3: Double reciprocal plots with 4-hydroxyphenylpyruvate as the variable substrate and changing fixed concentrations of bathophenanthroline or reductants. Velocity was expressed as nanomoles of $^{14}CO_2$ evolved per minute per milliliter of reaction mixture. The insets show replots of slopes (○) and intercepts (●) vs. the concentrations of bathophenanthroline or of reductants. Upper panel: The concentrations of bathophenanthroline were 0 μM (○), 4 μM (●), 8 μM (□), and 12 μM (■). Assay 1 was used. Middle panel: The concentrations of 2,6-dichlorophenolindophenol were 0.030 mM (○), 0.045 mM (●), 0.075 mM (□), and 0.150 mM (■). Assay 1 was used. Lower panel: The concentrations of ascorbate were 5 mM (○), 6.7 mM (●), 10 mM (□), and 20 mM (■). Assay 2 was used.

Cu^{2+} (10^{-6} to 10^{-3} M) did not stimulate the enzyme activity in the presence of 2,6-dichlorophenolindophenol, glutathione, and catalase (see below). With the concentrations of these compounds used in assay 1 the enzyme activity was strongly inhibited by iron chelators, added to the incubation mixture at 4 °C about 15 min before the reaction was started by addition of 4-hydroxyphenylpyruvate. The concentration of the chelators giving 50% inhibition (I_{50}) was evaluated from plots of inhibition vs. log inhibitor concentration. I_{50} was 0.56 μM

TABLE III: Reactivation of Pseudomonad 4-Hydroxyphenylpyruvate Dioxygenase Inhibited by Bathophenanthroline with Fe^{2+}.

Sample	Rel act. (%) Before dialysis	After dialysis + Fe^{2+} 0	10^{-6} M	5×10^{-5} M	10^{-4} M	10^{-2} M	Loss of ^{59}Fe during dialysis
Control[a]	100	4	6		26		58
BP inhibited[b]	1	0.8	2		28		90
Control[a]	100	11	15	34	30	33	
BP inhibited[c]	2	8	10	27	30	26	

[a] Controls not inhibited with bathophenanthroline (BP) were run in parallel. [b] Purified ^{59}Fe-enzyme, 0.2 mg in 500 μL of Tris-HCl (pH 7.5), was inhibited with 1 mM bathophenanthroline during 15 min at 37 °C. The enzyme solution was then dialyzed at 4 °C against 100 vol of 0.2 M Tris-HCl (pH 7.5) containing 1 mM bathophenanthroline for about 4 h. The dialysis was then continued in the absence of bathophenanthroline for about 25 h with 4 to 5 changes of buffer. Assay 1 was used. The loss of iron was estimated by counting radioactivity of ^{59}Fe before and after dialysis. [c] Purified enzyme, 0.15 mg in 250 μL of 0.2 M Tris-HCl (pH 7.5), containing 60 mM ascorbate and 2.4 g/L catalase was inhibited with 5 mM bathophenanthroline during 15 min at 4 °C and then dialyzed against 50 mM Tris-HCl (pH 7.5), containing 10 mM ascorbate. Assay 2 was used.

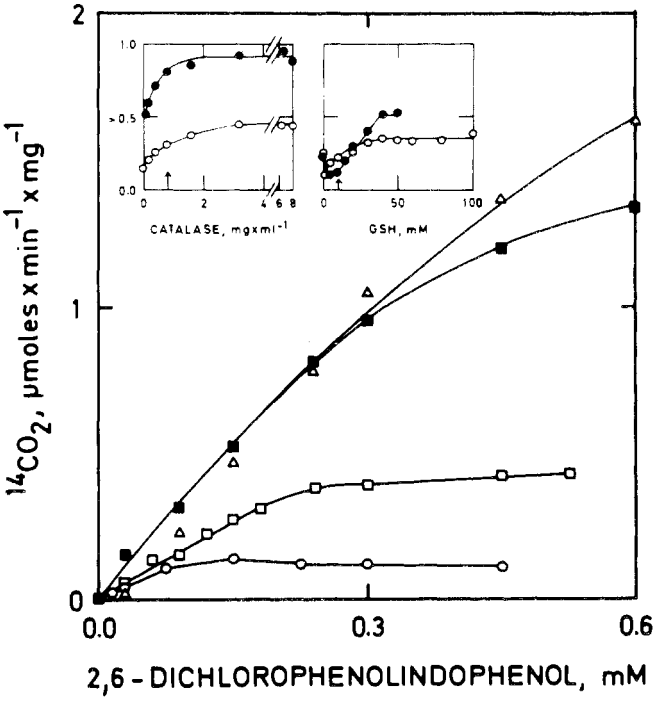

FIGURE 4: Effect of 2,6-dichlorophenolindophenol, glutathione, and catalase on the activity of the enzyme. Assay 1 was used when not otherwise stated. 2,6-Dichlorophenolindophenol was added alone (○) and in combination with: 50 mM glutathione and 1.2 g/L catalase at 25 μM 4-hydroxyphenylpyruvate concentration (□); 10 mM glutathione and 0.8 g/L catalase (■); 50 mM glutathione and 4 g/L catalase (△). Left inset: Catalase was added in combination with 0.15 mM 2,6-dichlorophenolindophenol (○), and with 0.15 mM 2,6-dichlorophenolindophenol plus 10 mM glutathione (●). Right inset: Glutathione was added in combination with 0.15 mM 2,6-dichlorophenolindophenol (○) and with 0.45 mM 2,6-dichlorophenolindophenol (●).

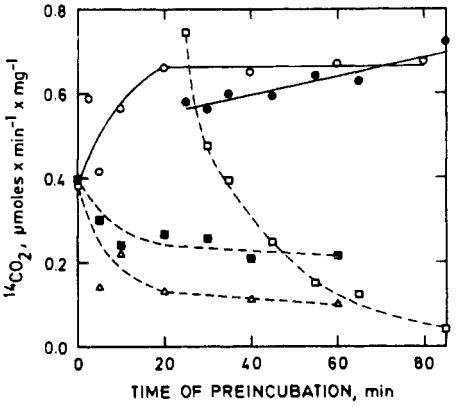

FIGURE 5: Effect of preincubation at 4 and 37 °C on the activity of the enzyme. The conditions of assay 1 were used unless otherwise stated: incubation of the reaction mixture except 4-hydroxyphenylpyruvate at 4 °C for various times (○—○); incubation of the reaction mixture except 4-hydroxyphenylpyruvate at 4 °C for 25 min followed by incubation at 37 °C for various times (□---□); incubation of the reaction mixture except 4-hydroxyphenylpyruvate at 4 °C for 25 min followed by addition of 4-hydroxyphenylpyruvate and further incubation at 4 °C for various times (●—●); incubation of enzyme in buffer at 37 °C for various times (■---■), followed by addition of 2,6-dichlorophenolindophenol, glutathione, and catalase and incubation at 4 °C for 30 min; incubation of enzyme and 4-hydroxyphenylpyruvate in buffer at 37 °C for various times (△---△), followed by addition of 2,6-dichlorophenolindophenol, glutathione, and catalase and incubation at 4 °C for 30 min.

for 8-hydroxyquinoline-5-sulfonic acid, 2.1 μM for sodium diethyldithiocarbamate, 7.1 μM for cupferron, 12 μM for bathophenanthroline, and 0.32 mM for 2,2′-bipyridyl. Weaker inhibition was obtained with chelators with low affinity for Fe^{2+}, e.g., tiron (I_{50} = 0.4 mM), bathocuproine (I_{50} = 3 mM), EDTA, and citrate (Sillén and Martell, 1971).

The inhibition by bathophenanthroline was not time dependent as determined during 60 min at 4 °C. The inhibition could be both prevented and reversed to about 80% by Fe^{2+} in concentrations above one-third of that of the inhibitor. Bathophenanthroline was a parabolic competitive inhibitor when 4-hydroxyphenylpyruvate was the variable substrate (Figure 3, upper panel).

Dialysis of the enzyme alone or in the presence of bathophenanthroline resulted in almost complete loss of activity. An undialyzed control lost about 40% of the initial activity. The same result was obtained when the dialysis was performed against Fe^{2+}-containing buffers (10^{-6} to 10^{-3} M Fe^{2+}) with or without 0.1 M 2-mercaptoethanol. The activity of dialyzed preparations could be restored to about 30% of the initial value by incubation with Fe^{2+} during 20 min at 4 °C in the presence of 2,6-dichlorophenolindophenol, glutathione, and catalase or in the presence of ascorbate and catalase in the concentrations used in assay 1 and assay 2, respectively (Table III). Attempts to reach higher activity by prolonged incubations in the presence of 2,6-dichlorophenolindophenol, glutathione, and catalase were unsuccessful. Dialysis of ^{59}Fe-labeled enzyme resulted in a loss of about 60% of the radioactivity. The loss increased to about 90% in the presence of bathophenanthroline.

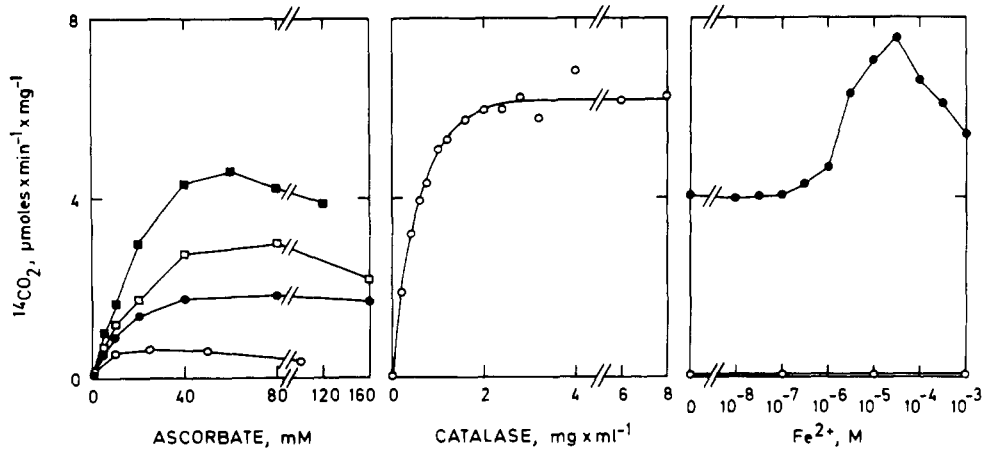

FIGURE 6: Effect of ascorbate, catalase, and Fe^{2+} on the activity of the enzyme. The compounds were added to a solution of the enzyme in 0.2 M Tris-HCl (pH 7.5), at 4 °C. 4-Hydroxyphenylpyruvate was added after 15 min to a final concentration of 0.2 mM and the reaction was started by placing the tubes in a water bath at 37 °C. Left panel: Ascorbate was added in combination with 1 mM Fe^{2+} (○), 0.8 g/L catalase (●), 4 g/L catalase (□), and with 1 mM Fe^{2+} plus 4 g/L catalase (■). Middle panel: Catalase was added in combination with 50 mM ascorbate and 50 μM Fe^{2+} (○). Right panel: Fe^{2+} was added alone (○) and in combination with 60 mM ascorbate plus 4 g/L catalase (●).

Stimulation by 2,6-Dichlorophenolindophenol, Glutathione, and Catalase. No activity was observed when the enzyme was incubated only with substrate. The results of a study of the stimulatory effect of 2,6-dichlorophenolindophenol, glutathione, and catalase are given in Figure 4. The specific activity was ~0.1 μmol min^{-1} (mg of protein)$^{-1}$ when the concentration of 2,6-dichlorophenolindophenol was above 0.15 mM and that of 4-hydroxyphenylpyruvate was 0.2 mM. Nonenzymic decarboxylation of the substrate rose from 1.5 to 15% when the concentration of 2,6-dichlorophenolindophenol was increased from 0 to 0.45 mM. Addition of 5 mM glutathione prevented this nonenzymic decarboxylation. Addition of catalase to the incubation mixture further increased the enzyme activity. Attempts were made to find an optimal combination of 2,6-dichlorophenolindophenol, glutathione, and catalase. At high concentrations of glutathione (50 mM) and catalase (4 g/L) no plateau in enzyme activity was reached, when the concentration of 2,6-dichlorophenolindophenol was increased to 0.6 mM. However, at a lower substrate concentration (25 μM) a plateau in specific activity was reached at 0.3 mM 2,6-dichlorophenolindophenol concentration. 2,6-Dichlorophenolindophenol was a hyperbolic uncompetitive activator when 4-hydroxyphenylpyruvate was the variable substrate (Figure 3, middle panel). With the combination of 2,6-dichlorophenolindophenol, glutathione, and catalase used in assay 1, 1 mM concentrations of Fe^{2+} and Cu^{2+} inhibited to 20 and 90%, respectively.

A nearly twofold increase in activity occurred when the enzyme was incubated during 20 min at 4 °C together with 2,6-dichlorophenolindophenol (0.15 mM), gluathione (10 mM), and catalase (0.8 g/L)(Figure 5). If substrate was then added no change in activity occurred during a following 60-min period of incubation at 4 °C. The enzyme activity was rapidly lost if the incubation of enzyme with 2,6-dichlorophenolindophenol, glutathione, and catalase was performed at 37 °C instead of at 4 °C. Incubation of the enzyme at 37 °C in the absence of 2,6-dichlorophenolindophenol, glutathione, and catalase resulted in about the same degree of inactivation both in the presence and in the absence of 4-hydroxyphenylpyruvate (Figure 5).

Stimulation by Ascorbate, Catalase, and Fe^{2+}. The results of experiments designed to find optimal concentrations of ascorbate, catalase, and iron are summarized in Figure 6. Ascorbate alone caused nonenzymic decarboxylation of the substrate and no stimulation of the enzymic reaction was noticed. In the presence of catalase (4 g/L) the specific activity was around 3 μmol min^{-1} (mg of protein)$^{-1}$. This could be further increased by the addition of Fe^{2+} in concentrations around 50 μM. Iron could not be replaced by 0.1 μM, 10 μM, or 1 mM concentrations of Ca^{2+}, Zn^{2+}, Ni^{2+}, Na^{+}, MoO_4^{2-}, or Cu^{2+}. Inhibition was observed at 1 mM concentrations of Ni^{2+} (20%), Zn^{2+} (53%), and Cu^{2+} (87%). Higher concentrations of Fe^{2+} (up to 1 mM) were not inhibitory as was the case with the activating system containing 2,6-dichlorophenolindophenol, glutathione, and catalase. Ascorbate was a hyperbolic uncompetitive activator when 4-hydroxyphenylpyruvate was the variable substrate (Figure 3, lower panel). Incubation of the enzyme during 40 min at 4 or 37 °C in the presence of ascorbate, catalase, and Fe^{2+} did not influence the activity.

Effect of H_2O_2. H_2O_2 (10 mM) decarboxylated the substrate to about 90% in the presence of 2,6-dichlorophenolindophenol and glutathione at the concentrations used in assay 1. At 1 mM or lower concentrations of H_2O_2 the nonenzymic decarboxylation was 1 to 2%, both in the presence and in the absence of catalase (0.8 g/L). H_2O_2 showed a weak inhibitory effect, about 30%, at 10 mM concentration in the presence of catalase and at 0.1 mM concentration in its absence.

Kinetics. Both under the conditions of assay 1 and assay 2 equal amounts of $^{14}CO_2$ and homogentisate were formed with 4-hydroxyphenylpyruvate as substrate for the purified enzyme. The release of $^{14}CO_2$ was maximal in Tris-HCl buffer between pH 7.0 and 7.5. During the conditions of assay 1 the apparent Michaelis constant was 30 μM and the maximal velocity 0.9 μmol min^{-1} (mg of protein)$^{-1}$ when determined from Lineweaver-Burk plots at low concentrations of 4-hydroxyphenylpyruvate. Concentrations of 4-hydroxyphenylpyruvate above 0.2 mM were inhibitory.

Phenylpyruvate could act as substrate for purified 4-hydroxyphenylpyruvate dioxygenase. The apparent Michaelis constant was 0.52 mM and the maximal velocity 20 nmol min^{-1} (mg of protein)$^{-1}$ when estimated from Lineweaver-Burk plots at low concentrations of phenylpyruvate. Concentrations of phenylpyruvate above about 0.5 mM were inhibitory and gave nonlinear time curves. At 25 μM concentration of phenylpyruvate the release of $^{14}CO_2$ was linear with time for

about 30 min and proportional to the amount of enzyme added until about 20% of the substrate had been consumed. The reaction had a broad pH optimum from pH 6.0 to 7.5. The elution profiles of the enzyme activities with phenylpyruvate and 4-hydroxyphenylpyruvate as substrates coincided in the chromatographic steps of the purification procedure. The ratio of 4-hydroxyphenylpyruvate to phenylpyruvate activity was constant in the later purification steps (Table I).

Under the conditions of assay 2 the reaction rate was constant for 30 min and proportional to the amount of protein until about 30% of the substrate had been consumed. The apparent Michaelis constant was 0.15 mM and the maximal velocity 20 μmol min^{-1} (mg of protein)$^{-1}$, respectively. Substrate inhibition was observed above 0.2 mM concentration of 4-hydroxyphenylpyruvate.

Discussion

The 4-hydroxyphenylpyruvate dioxygenase activity in *Pseudomonas* sp. P.J. 874 was isolated as a homogeneous protein as judged by several criteria of purity, i.e. congruent peaks of enzyme activity and protein concentration at gel filtration, one dominating band containing enzyme activity in different electrophoretic systems, a single boundary schlieren pattern, a linear slope at high-speed sedimentation equilibrium, and one NH_2-terminal residue per subunit mass. The minor bands observed at electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate had masses close to multiples of the minimal mass obtained. They were probably polymerization products, since they did not appear when a similar amount of protein was run in different concentrations of acrylamide at pH 7.5.

The molecular mass (150 kdaltons) obtained by sedimentation equilibrium and by the combined sedimentation velocity–gel filtration method showed a good agreement, whereas sieving methods resulted in a higher figure (170 kdaltons), which possibly indicates an asymmetric form of the protein. The molecular mass has been determined for 4-hydroxyphenylpyruvate dioxygenase prepared from livers of some vertebrates. We found 87 kdaltons for the human enzyme (Lindblad et al., 1977) and Wada and coworkers found 97 kdaltons for the chicken enzyme by sedimentation equilibrium (Wada et al., 1975). Estimations by gel filtration have resulted in values of 42 kdaltons for the pig enzyme (Goodwin, 1972), 150 kdaltons for the rabbit enzyme (Laskowska-Klita and Mochnacka, 1973), and 85 kdaltons for the frog enzyme (Laskowska-Klita, 1969).

The enzymes, so far studied in some detail, have been reported to be oligomers of 2 or 4 subunits. The human enzyme contains equally sized subunits of different charge and it appears to be a dimer (Lindblad et al., 1977; Rundgren, 1977a). The chicken enzyme also appears to be a dimer of subunits of equal size, but it has not been established if they differ in charge (Wada et al., 1975). Laskowska-Klita and Mochnacka (1973) reported that the rabbit enzyme dissociates into subunits on gel filtration and believe that the native enzyme contains four subunits. The bacterial enzyme dissociated into subunits with a mass of 36 kdaltons during denaturating conditions. The same value could be calculated from the NH_2-terminal amino acid analysis, if the yield of alanine in the first step was taken into account. The experimental data thus show that the bacterial enzyme is a tetramer composed of four subunits of equal size. The subunits of the human and chicken enzymes have a blocked NH_2-terminal residue, whereas in the bacterial enzyme there is a free NH_2-terminal alanine residue.

The human enzyme can be resolved into three major multiple forms, resulting from combination of two charge isomeric subunits (Rundgren, 1977a). Indications for the presence of multiple forms of the chicken enzyme have been presented by Wada et al. (1975), who suggested that they differ in degree of amidation. In both cases separation of the multiple forms was achieved by isoelectric focusing. The bacterial enzyme tended to precipitate at the isoelectric point and the technique could therefore not be used to reveal any multiple forms of the enzyme. At present there is no evidence that the subunits differ in charge.

So far, all reports on 4-hydroxyphenylpyruvate dioxygenase have indicated that a metal ion, either iron or copper, is involved in the enzymic reaction (Goodwin, 1972; Laskowska-Klita and Mochnacka, 1973; Goodwin and Werner, 1973; Wada et al., 1975; Lindblad et al., 1972, 1977). The fact that radioactive iron, added to the culture medium, followed the enzyme through the purification steps and the presence of iron in the final preparation indicates that the bacterial enzyme utilizes iron in the catalytic reaction. This is supported by the experiments with metal chelators, which indicate that iron rather than copper is involved. As for the human enzyme (Rundgren, 1977b) the iron chelator, bathophenanthroline, was a parabolic competitive inhibitor, which is consistent with the presence of a firmly enzyme-bound metal ion (Bardsley and Childs, 1974; Cleland, 1970). It was possible to remove most of the iron by dialysis with loss of the enzyme activity. However, the activity could only be partly restored by Fe^{2+} in the presence of a reductant and catalase. Possibly the loss of iron results in irreversible structural changes in the enzyme, but more extensive studies correlating enzyme activity with iron content are in progress.

The enzyme from all sources is stimulated by a reductant in the presence of catalase (Goodwin, 1972). Reduced 2,6-dichlorophenolindophenol or ascorbate has been the most widely used reductant. With the highly purified human and bovine enzymes, the two reducing systems have been found to be about equally effective (Nakai et al., 1975; Lindblad et al., 1977). With the bacterial enzyme no constant level in specific activity was reached with increasing amounts of reduced 2,6-dichlorophenolindophenol up to a concentration where solubility problems appeared. The highest specific activity obtained with a combination of 2,6-dichlorophenolindophenol, glutathione, and catalase was then about 20% of that reached by an optimized combination of Fe^{2+}, ascorbate, and catalase. The human enzyme had about 20% of maximal activity in the absence of a reducing system (Lindblad et al., 1977), whereas the bacterial enzyme as well as the bovine enzyme (Nakai et al., 1975) were inactive. Reductants were hyperbolic uncompetitive activators with respect to 4-hydroxyphenylpyruvate for the bacterial enzyme. We found mutual exclusive binding of a chelator with high specificity for divalent iron and 4-hydroxyphenylpyruvate to the reduced enzyme. The findings suggest that the reductant keeps the enzyme-bound metal reduced.

The Michaelis constant for 4-hydroxyphenylpyruvate was similar to that found for the mammalian enzymes (Goodwin, 1972; Fellman et al., 1972a,b; Raheja et al., 1973; Lindblad et al., 1977). Substrate inhibition appeared at about the same concentrations of 4-hydroxyphenylpyruvate as for the human enzyme (Lindblad et al., 1977). Phenylpyruvate was a substrate as shown for the enzyme from other sources, but the affinity for this substrate was about 10 times lower than that found with the mammalian enzymes (Taniguchi and Armstrong, 1963; Taniguchi et al., 1964; Fellman et al.,

1972a,b; Goodwin and Werner, 1972; Lindblad et al., 1977). As with the pig liver enzyme (Goodwin and Werner, 1972) substrate inhibition was also observed with phenylpyruvate, which was not the case for the human enzyme with up to 1 mM concentration of phenylpyruvate (Lindblad et al., 1977).

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