**Purification and Properties of Avian Liver p-Hydroxyphenylpyruvate Hydroxylase**

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**SUMMARY**

Avian liver p-hydroxyphenylpyruvate hydroxylase (EC 1.13.11.27) was purified to a 1000-fold increase in specific activity over crude supernatant, utilizing a substrate analogue, o-hydroxyphenylpyruvate, to stabilize the enzyme.

The preparation was homogeneous with respect to sedimentation with a sedimentation velocity (s_{20,w}) of 5.3 S. The molecular weight of the enzyme was determined to be 97,000 ± 5,000 by sedimentation equilibrium, and the molecular weight of the subunits was determined to be 49,000 ± 3,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis revealed heterogeneity of the purified enzyme. The multiple molecular forms were separable by isoelectric focusing, and their isoelectric points ranged from pH 6.8 to 6.0. The amino acid compositions and tryptic peptide maps of the three forms isolated by isoelectric focusing were very similar. The forms of the enzyme had the same relative activity toward p-hydroxyphenylpyruvate and phenylpyruvate. Conditions which are known to accelerate nonenzymic deamidation of proteins caused interconversion of the multiple molecular forms.

Iron was the only transition metal found to be associated with the purified enzyme at significant levels. The amount of enzyme-bound iron present in equilibrium-dialyzed samples was equivalent to 1 atom of iron per enzyme subunit. Purification of the enzyme activity correlated with the purification of the enzyme-bound iron.

An EPR scan of the purified enzyme gave a signal at g = 4.33, which is characteristic of ferric iron in a rhombic ligand field.

The early studies on p-hydroxyphenylpyruvate hydroxylase by La Du and Zannoni (2) revealed that ascorbate was required for the oxidation of p-hydroxyphenylpyruvate. This requirement for ascorbate was nonspecific, being fulfilled by nonstoichiometric amounts of reduced 2,6-dichlorophenolindophenol as well as several other reducing agents including hydroquinone, isoscorbate, reduced coenzyme Q_{10}, and a variety of hydroquinoidal compounds (2-4). Subsequently, Goswami (5) had shown that p-hydroxyphenylpyruvate hydroxylase can be reversibly inactivated by oxidation and can be reactivated by reducing agents. The reversible inactivation of the enzyme may reflect changes in the oxidation state of a transition metal prosthetic group. The existence of a metal prosthetic group had been suspected since certain metal chelators, α,α' dipyridyl, sodium azide, and diethylthiocarbamate inhibited the enzyme (2, 6). The inhibition of the enzyme by diethylthiocarbamate, a strong copper chelator, suggested the participation of copper as the prosthetic group. La Du and Zannoni (2) noted that copper-deficient dogs were also deficient in liver p-hydroxyphenylpyruvate hydroxylase; however, the low enzyme level did not increase with in vitro addition of copper. Evidence supporting ferrous iron as the metal prosthetic group has been presented by Goswami and Knox (7) and Goswami (5). Ferrous iron was reportedly removed from crude rat liver p-hydroxyphenylpyruvate hydroxylase by dialysis against o-phenanthroline and exhaustive dialysis against water, which resulted in a loss of activity. Contradictory evidence was obtained by Taniguchi and Armstrong (8), who noted that most of the enzyme activity was returned by the removal of the metal chelator by passing the enzyme through a bed of Sephadex G-50. Our own investigation of this question indicated that the enzyme activity was not significantly reduced by dialysis against chelators. Furthermore, o-phenanthroline and diethylthiocarbamate both inhibited the enzyme through a strictly competitive mechanism, as evidenced by enzyme kinetics studies (9, 10). Thus, if a metal prosthetic group is required for p-hydroxyphenylpyruvate hydroxylase activity, it must be tightly associated to the enzyme protein.

The purification of the avian liver enzyme was undertaken with the purpose of analyzing the purified enzyme for associated transition metals. Up until recently, this enzyme has resisted purification and exhibited extreme instability. We wish to report a successful purification of avian liver p-hydroxyphenylpyruvate hydroxylase utilizing a substrate analogue, o-hydroxyphenylpyruvate, to stabilize the enzyme during purification. Some of the chemical and physical properties of this highly active, purified enzyme preparation are described, including the transition metal content of the enzyme.

**EXPERIMENTAL PROCEDURE**

**Materials**

p-Hydroxyphenyl[1-^{14}C]pyruvate (9.3 μCi/mmol) and phenyl-[1-^{14}C]pyruvate (18 μCi/mmol) were prepared enzymatically from

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1 The name suggested for p-hydroxyphenylpyruvate hydroxylase by the 1972 Commission on Biochemical Nomenclature is 4-hydroxyphenylpyruvate dioxygenase (1).
was performed to determine the degree of homogeneity of the purified enzyme with respect to molecular size. The column was equilibrated with 100 mM NaCl, 50 mM potassium phosphate, pH 7.5, and 0.4 mM o-hydroxyphenylpyruvate. An examination of the sedimentation velocity of the purified enzyme was carried out with the Beckman E analytical ultracentrifuge equipped with schlieren optics. An 8.7-mg/ml sample of enzyme was dialyzed against several changes of 100 mM NaCl, 0.4 mM o-hydroxyphenylpyruvate at 4°C for 24 hours before the sedimentation velocity experiment was conducted at 20°C. Polyacrylamide gel electrophoresis was conducted at pH 8.0 according to Laemmli (15). Approximately 40 μg of protein were applied per gel in 40% sucrose to the top of the stacking gel, and gels were usually stained with Amido black. When gels were assayed for enzyme activity, the gels were prerun for 15 min with 0.4 mM o-hydroxyphenylpyruvate included in the top buffer. Enzyme samples were then added, and electrophoresis was carried out at 4°C with a current of 4 mA/tube for 3 hours. The gels were rapidly frozen by rolling them on a block of Dry Ice, and the frozen gels were then sliced into 1 mm sections, which were incubated in assay medium for 10 min to elute the enzyme before assay.

**Methods**

**Purification of Asian Liver p-Hydroxyphenylpyruvate Hydroxylase**—Lindblad et al. (12) have recently reported a purification of human liver p-hydroxyphenylpyruvate hydroxylase. We purified chicken liver enzyme following their procedure with the exception of the addition of 0.4 mM o-hydroxyphenylpyruvate to all buffers employed in the purification. This procedure entails preparation of chicken liver acetone powder, extraction of the acetone powder, ammonium sulfate fractionation, sulfoaryl (SP)-Sephadex (cation exchange chromatography), hydroxylapatite chromatography, and Ampholine ampholytes (anion exchange chromatography).

**Radiochemical Assay System for p-Hydroxyphenylpyruvate Hydroxylase**—The radiochemical enzyme assay system previously described by Fellman et al. (13) was used throughout the purification. This assay system is based on the evolution of 14CO2 from carboxyl-14C-labeled substrate incubated with the enzyme. The 14CO2 evolved from the reaction was collected on a hyamine-soaked paper wick suspended from the rubber stopper of the reaction vessel, and the evolved gas from the reaction was collected on the hyamine wick. After a 15-min postreaction incubation period, the hyamine wick was tipped in to stop the reaction and release the 14CO2 for collection on the hyamine wick. 14CO2 was counted in 19 ml of Buhler's solution by scintillation spectrometry in the Packard Tri-Carb.

**Examination of Enzyme Purity—** Gel filtration in Sephadex G-100

1 Dansyl-Cl is the trivial name for 5-dimethylaminonaphthalene-1-sulfonyl chloride.

2 Buhler's solution consists of 16 g of Omnifluor and 2 liters of toluene and 2 liters of absolute ethanol.

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This was calculated from values published in the *International Critical Tables of Numerical Data, Physics, Chemistry and Technology* (19).
enzyme was treated with trypsin, reacted with dansyl-Cl, and the dansylpeptides mapped according to the procedure of Zanetta et al. (23).

Analyses for Associated Transition Metals—p-Hydroxyphenylpyruvate hydroxylase was analyzed for associated transition metals by neutron activation analysis. The purified fraction of enzyme eluted from QAE-Sephadex, and a fraction of lower purity, a fraction eluted from SP-Sephadex, were dialyzed for 24 hours against two changes of 60 mM Tris-HCl, pH 8.0, at 4°C in a nitrogen-gassed chamber. Both of the samples, designated QAE (3.9 ml, 1 mg of protein/ml, 17 units/mg of protein), and SP (2.75 ml, 2.6 mg of protein/ml, 17 units/mg of protein), were irradiated along with separate aliquots of their dialysates in the Triga Mark 1 reactor at Reed College (Portland, Oregon). On the basis of the γ emission spectrum, it was possible to semiquantitatively determine the transition metal content of each sample. The copper and iron content of the enzyme was quantitated by atomic absorption spectroscopy. The Varian-Technicon AA-5 atomic absorption spectrophotometer was employed for the metal analyses utilizing an acetylene flame for atomization of samples. The procedure used for sample preparation was that used for the analysis of iron and copper in blood serum (24, 25), since the atomic absorption analysis of the samples from the activation analysis studies required the separation of metals from precipitated protein. One milliliter of sample, 1 ml of 10% trichloroacetic acid, and 0.25 ml of 0.05% diethylidithiocarbamate and 0.05% o-phenanthroline were stirred together for 20 min at room temperature. The precipitated protein was centrifuged and washed with 0.5 ml of 10% trichloroacetic acid. The spectrophotometer was calibrated with standard solutions of CuSO4·5H2O and Fe(NH4)2(SO4)2·6H2O from 5 to 15 μM.

An EPR spectrum was run on the purified enzyme. The enzyme was concentrated to 6.0 mg/ml in 10 mM Tris-HCl, pH 8.1, 0.4 mM o-hydroxyphenylpyruvate, 100 mM KCl by vacuum dialysis. The dialyzed enzyme (0.3 ml) was carefully frozen in a sample tube, and the EPR spectrum was scanned with the Varian V-4502 EPR spectrometer.

RESULTS AND DISCUSSION

The study of p-hydroxyphenylpyruvate hydroxylase has been hindered by the inability to obtain highly purified enzyme preparations which retain their enzymatic activity. Lindblad et al. (12) purified the enzyme to a high degree of purity, a single band in polyacrylamide gel electrophoresis; yet, the increase in the specific activity was no better than 100-fold, an achievement previously reported by Edwards et al. (26) and Zannoni and La Du (27). Testing various methods of enzyme stabilization, we found that storage under nitrogen gas in the presence of a substrate analogue, o-hydroxyphenylpyruvate, significantly increased the stability of the enzyme. Furthermore, inclusion of 0.4 mM o-hydroxyphenylpyruvate in the buffer systems of the purification process resulted in increased yields and a 16-fold increase in the specific activity of the final preparation (Table I). p-Hydroxyphenylpyruvate was also found to be a required constituent of buffers used in electrophoretic procedures which depend upon the preservation of enzyme activity to mark the position of the enzyme. Without the inclusion of the substrate analogue, electrophoretic procedures such as polyacrylamide gel electrophoresis and isoelectric focusing resulted in proteins which were distributed in the same patterns but without activity. Our final preparation of avian liver p-hydroxyphenylpyruvate hydroxylase, the QAE fraction, was found to be homogeneous with respect to molecular weight and size. During gel filtration in Sephadex G-150, the enzyme activity eluted with the protein peak at a constant ratio, with no other protein peaks observed by optical density at 280 nm. During the sedimentation velocity experiment, a symmetrical boundary was observed to sediment at a velocity corresponding to an e0.1% value of 5.38 at 50,780 rpm. The equilibrium sedimentation data, a linear plot of log AY versus t2, also indicated a homogeneous solution with a molecular weight of 97,000 ± 5,000. The method of sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue revealed a single principal protomer with a molecular weight of 49,900 ± 3,000 and some minor contamination (Fig. 1).

A more serious challenge to the homogeneity of the enzyme preparation was raised when polyacrylamide gel electrophoresis without sodium dodecyl sulfate demonstrated heterogeneity, denoted by multiple, close banding of protein as stained gels. The multiple banding of purified enzyme was observed consistently, in several different enzyme preparations. Furthermore, preincubation of the enzyme with 1% β-mercaptoethanol and running the polyacrylamide gel electrophoresis with 0.1% β-mercaptoethanol in the upper buffer did not alter the multiple banding. Since each
of these bands was demonstrated to possess enzyme activity by the elution of the activity from sliced gels and correlation of the peaks of activity with a scan of a stained gel from the same run, Fig. 2, the microheterogeneity was that of p-hydroxyphenylpyruvate hydroxylase and not that of contaminants. It was also noted that, during the purification of the enzyme, QAE-Sephadex chromatography resolved the enzyme activity into multiple peaks, an observation that had been previously puzzling. These peaks may now be interpreted as representing different molecular forms of the enzyme.

This observation of the microheterogeneity of avian liver p-hydroxyphenylpyruvate hydroxylase was confirmed by the separation of the purified enzyme into at least four active forms by isoelectric focusing. The isoelectric points of the four detectable forms were: I, 6.0; II, 6.2; III, 6.4; and small amounts of IV, 6.8 (Fig. 3). Since the ratio of p-hydroxyphenylpyruvate/phenylpyruvate activities was the same (approximately 45) for the four forms of the enzyme, the four forms of the p-hydroxyphenylpyruvate hydroxylase appear to be the same with respect to their substrate specificity. The fractions containing I, II, and III were separately pooled and dialyzed for 30 hours against three changes of 50 mM Tris-HCl, pH 7.6, to remove the ampholytes and buffer glycine for further structural analyses.

**Table II**

| Amino Acid   | µMol/mg Protein | Mols/49,000 g Protein | Ratios of Forms II/I, III/I |
|--------------|-----------------|----------------------|-----------------------------|
| Lysine       | 0.630           | 30.8                 | 0.94                         |
| Histidine    | 0.285           | 14.0                 | 1.13                         |
| Amide-N      | 1.201           | 38.9                 | 1.26                         |
| Arginine     | 0.392           | 10.3                 | 1.09                         |
| Aspartic acid| 0.890           | 43.6                 | 1.01                         |
| Threonine    | 0.458           | 22.4                 | 0.92                         |
| Serine       | 0.504           | 24.7                 | 0.95                         |
| Glutamic acid| 1.157           | 56.7                 | 0.80                         |
| Proline      | 0.375           | 18.8                 | 1.12                         |
| Glycine      | 0.663           | 32.5                 | 0.97                         |
| Alanine      | 0.489           | 26.8                 | 0.94                         |
| ½ Cystine    | 0.064           | 3.1                  | 0.76                         |
| Valine       | 0.627           | 30.7                 | 1.10                         |
| Methionine   | 0.159           | 7.8                  | 0.83                         |
| Isoleucine   | 0.370           | 18.1                 | 0.92                         |
| Leucine      | 0.743           | 36.4                 | 1.01                         |
| Tyrosine     | 0.352           | 17.3                 | 1.10                         |
| Phenylalanine| 0.492           | 24.0                 | 1.10                         |
| Cystine      | 0.102           | 4.9                  |                             |
| Tryptophan   | 0.012           | 0.5                  |                             |

Values estimated from 22-hour hydrolysis. Values extrapolated to zero time from 22, 48, and 72-hr hydrolyses in 3 M p-toluene sulfonic acid. 0.2% 3-(2-aminoethyl)-indole at 110° (28).

Values from 72-hour hydrolysis. Value from oxidation of cystine and cysteine to cysteic acid by performic acid and hydrolysis in 6 N HCl at 110° for 20 hours (29).

Values estimated from enhancement of p-chloromercuribenzoate absorption at 250 nm by thiol (30).

Forms I, II, and III were separated by isoelectric focusing.

The nature of the difference distinguishing each of the multiple molecular forms of p-hydroxyphenylpyruvate hydroxylase appeared to be that of charge, since this is the physical property by which electrophoretic and ion-exchange chromatographic methods separate proteins of the same molecular weight. The possibility of subunit polymerization through disulfide linkage was obviated by the observation that β-mercaptoethanol did not influence the microheterogeneity and by the homogeneity of the enzyme with respect to molecular weight. The possibility of grossly different primary structure of the multiple molecular forms of the enzyme was ruled out by the observed similarity of the amino acid compositions and dansyl-peptide maps of the three forms isolated by isoelectric focusing. The amino acid compositions of the Forms I, II, and III were determined by analysis of the 20-hour hydrolysates in 0 N HCl at 110°. The ratios, II/I and III/I, were approximately 1 for most of the amino acids, indicating very similar compositions (Table II). The tryptic dansylpeptide maps of Forms I and II were almost identical, except for three spots which did not match indicated by arrows to a, b, and c (Fig. 4). The dansylpeptide map of III was also similar to I and II in the pattern of the spots, but more than two unmatched spots were noted. NH2-terminal analysis by the method of Gros and Labouesse (31) failed to detect a free NH2-terminal amino acid for all three forms of the enzyme.

A total amino acid composition of the purified p-hydroxy-
Dansylpeptide maps of reduced, carboxymethylated \( p \)-hydroxyphenylpyruvate hydroxylase, Forms I, II, and III. The two-dimensional thin layer chromatography was run 60 min in Solvent 1 (methyl acetate/isopropyl alcohol/25\% ammonia (9/8/4, v/v/v)) and 140 min in Solvent 2 (isobutyl alcohol/acetic acid/water (15/4/2, v/v/v)) with a 10-min activation at 110° between runs. The peptide spots unique to the enzyme forms are indicated in the chromatograph as a, b, c, d, and e. Hatched areas indicate most intense spots, while dotted areas indicate barely visible spots.

The existence of multiple alleles for \( p \)-hydroxyphenylpyruvate hydroxylase was determined by hydrolysis of the enzyme with 3 M \( p \)-toluenesulfonic acid, 0.2\% 3-(2-aminoethyl)-indole at 110° for 24, 48, and 72 hours, and automatic amino acid analysis according to the method of Liu and Chang (28) (Table II). Hexosamines, which can be determined by this method, were not found in the enzyme hydrolysates. Since almost all glycoproteins contain at least 1 residuc of hexosamine (32), the possibility that the microheterogeneity of the enzyme was due to a heterogeneous carbohydrate moiety is unlikely.

Polyacrylamide gel electrophoresis was utilized to characterize enzyme incubated for varying lengths of time in 0.5 M potassium phosphate, pH 9.0. A progressive loss of protein from the bands of lower mobility and a relative increase of protein in the bands of higher mobility were observed in the stained gels (Fig. 5). The over-all loss of total protein seemed to be due to denaturation and precipitation at the high pH.

From the enzyme incubated for a total of 70 hours, the gels revealed the appearance of an additional protein band of higher mobility than those observed in the unincubated control. This new band was labeled \( f \) in Fig. 5. Preincubation of the treated enzyme with \( \beta \)-mercaptoethanol and inclusion of \( \beta \)-mercaptoethanol in buffers during electrophoresis did not alter these results. The conversion of lower to higher mobility corresponds to an increase in the net negative charge of the protein, neglecting possible changes in protein size or shape. Nonenzymic deamidation could account for the increase in negative charge and electrophoretic mobility. The increase in mobility could not be explained by the formation of intramolecular disulfide bridges since \( \beta \)-mercaptoethanol had no effect on the mobility of treated enzyme forms. Thus, the available evidence indicates nonenzymic deamidation as the probable cause of the multiple molecular forms of \( p \)-hydroxyphenylpyruvate hydroxylase; the significance of this phenomenon is unclear, since it may be occurring both in vivo and as a purification artifact.

Neutron activation analysis was performed by neutron activation analysis. Only copper was found to be present in significant amounts greater than the dialysate, and activation analysis proved inadequate for the detection of iron at the microgram level. Therefore, the copper and iron content of the enzyme sample was quantitated by atomic absorption spectroscopy. The results are presented in Table III, where they are expressed as the difference in metal content between the dialysate and enzyme samples. The amount of bound iron found in the enzyme fraction eluted from QAE-Sephadex was 12 times the amount of the bound copper found in the same fraction, and the iron to protein mole...
TABLE III

| Volume | Cu | Bound Cu | Total bound Cu | Total protein | Cu/protein | Specific activity |
|--------|----|----------|----------------|---------------|------------|------------------|
| QAE-fraction | 11.7 | 3.75 | 0.5 | 5.85 | 78 | 0.075 | 430 |
| QAE-dialysate | 3.25 | | | | | |
| SP-fraction | 5.25 | 3.75 | 0.75 | 3.94 | 91 | 0.043 | 20 |
| SP-dialysate | 3.00 | | | | | |

TABLE IV

| Volume | Fe | Bound Fe | Total bound Fe | Total protein | Fe/protein | Specific activity |
|--------|----|----------|----------------|---------------|------------|------------------|
| QAE-fraction | 3.5 | 0.9 | 3.15 | 67 | 0.047 | 230.0 |
| QAE-dialysate | 1.3 | | | | | |
| SP-fraction | 3.0 | 1.0 | 3.0 | 60 | 0.045 | 2.55 |

The ratio approached 1 atom of iron per enzyme subunit molecule. The increase in the iron to protein mole ratio, also, correlated well with the purification of the enzyme specific activity, going from a partially purified SP fraction to the QAE fraction. The copper to protein mole ratio, however, remained unchanged (Table III).

When special precautions were taken to eliminate nonspecifically bound metal ions from the enzyme samples, a decrease in the iron to protein mole ratio was noted with an accompanying decrease in the enzyme specific activity. The QAE fraction was dialyzed against buffer containing EDTA, and this QAE fraction and the SP fraction were run through separate beds of Sephadex G-25. These enzyme samples were then passed through Chelex 100 deionizing resin columns, and the samples were aspirated directly into the atomic absorption spectrophotometer.

The data presented in Table IV demonstrated that iron was again co-purified with the enzyme activity, even after the removal of nonspecifically bound metal ions. The 88-fold increase in the total bound iron of the QAE fraction over the SP fraction corresponded to the 90-fold increase in specific activity. The fact that a portion of iron was removed by the Chelex resin with a corresponding loss of specific activity could be explained by assuming denaturation of the enzyme and release of the tightly bound metal ion. An attempt at reactivation of Chelex-treated enzyme by the addition of ferrous ions was unsuccessful.

A confirmation of the atomic absorption data supporting the existence of an iron prosthetic group for p-hydroxyphenylpyruvate was obtained by EPR spectroscopy. A single strong signal was obtained in the g = 4 region for scans of the purified enzyme (Fig. 6). A precise g value for the signal was calculated from an

FIG. 6. EPR signal obtained from purified (QAE-fraction) p-hydroxyphenylpyruvate hydroxylase. Instrument settings: modulation amplitude, 3200; time constant, 1; gain, 100; field range, 1000 G; scan time, 5 min; klystron frequency (Kv), 9.143 Kmegacycles; p-hydroxyphenylpyruvate hydroxylase concentration, 6.6 mg/ml in 10 mM Tris-HCl (pH 8.0), 0.4 mM o-hydroxyphenylpyruvate, and 100 mM KCl.

Integration of the signal and was found to be 4.33. This signal is a common ferric iron EPR characteristic of high spin ferric iron in a rhombic ligand field (35). A similar signal has been observed for iron associated to transferrin, g = 4.14 (36) and for pyrocatechase which is an iron activated oxygenase, g = 4.2 (37). Ferric EDTA is a model system which also has been noted to give the g = 4.3 EPR. The EPR of p-hydroxyphenylpyruvate
hydroxylase is probably due to the ferrie form of the enzyme, since either the high or low spin configuration of ferrous iron would be expected to be diamagnetic giving no EPR. Activation of the enzyme with ascorbic acid or reduced dichlorophenolindophenol would result in reduction of the enzyme to the ferrous form which is, apparently, the active form of the enzyme; reduction of the iron would also lead to a loss of the EPR signal. Proof that iron plays a catalytic role in the enzymic reaction mechanism is yet to be accomplished, but the evidence for the specific association of iron to p-hydroxyphenylpyruvate hydroxylase is now directly and unambiguously demonstrated by its co-purification with enzyme activity and the failure of EDTA and Chelex 100 to remove it without destruction of enzyme activity.

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