The Metabolism of Nicotinic Acid

I. PURIFICATION AND PROPERTIES OF 2,5-DIHYDROXYPYRIDINE OXYGENASE FROM PSEU-
DOMONAS PUTIDA N-9*

(Received for publication, February 8, 1971)

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SUMMARY

Purification and crystallization of an enzyme which catalyzes the oxidation of 2,5-dihydroxypyridine, an intermediate in nicotinic acid catabolism, are described. The labile enzyme is stabilized by dithiothreitol. Activity lost by dialysis or purification procedures is restored by incubation with dithiothreitol and ferrous sulfate. The enzyme is inhibited by sulfhydryl reagents and iron-chelating agents.

The crystalline enzyme, on polyacrylamide electrophoresis with dithiothreitol, yields a single major band and a region of minor diffuse bands. In the absence of dithiothreitol the intensity of the minor bands is increased. On acrylamide gels containing sodium dodecyl sulfate, a single band with mobility corresponding to a molecular weight of 39,500 is obtained. A single region of enzyme activity corresponding to a molecular weight of 242,000 is obtained on sucrose gradients containing dithiothreitol. A portion of this activity is shifted to a region corresponding to lower molecular weight when dithiothreitol is omitted.

In this paper, we report the crystallization and further characterization of the 2,5-dihydroxypyridine-oxidizing enzyme. An accompanying paper (4) deals with the oxidation products of 2,5-dihydroxypyridine and with oxygen 18 experiments, which establish that the enzyme is an oxygenase. For convenience, we anticipate these data and will, in this paper, refer to the enzyme as 2,5-dihydroxypyridine oxygenase.

EXPERIMENTAL PROCEDURE

Materials and Methods

2,5-Dihydroxypyridine was synthesized as described by Behrman and Pitt (5). Nicotinic acid was obtained from Eastman Kodak Company, Rochester, New York. Dithiothreitol, Cellex-D, hydroxylapatite, 2-iodoacetamide, iodoacetic acid, α,α′-dipyridyl and p-chloromercuribenzoic acid were purchased from Calbiochem. N-Ethylmaleimide was obtained from Mann Research Laboratories. α-Phenanthroline was purchased from Matheson, Coleman and Bell. Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid, disodium salt) is the product of K and K Laboratories, Plainview, New York.

Enzyme Reactivation—Enzyme was routinely reactivated before assay by diluting an aliquot of the protein solution (0.05 to 0.20 ml) to 0.90 ml with 20 mM sodium phosphate, pH 7.5. To this, 0.05 ml of 200 mM dithiothreitol and 0.05 ml of 5.0 mM ferrous sulfate were added. After 15 min of incubation at room temperature, an aliquot (0.5 to 0.10 ml) of the reactivated enzyme solution was used for assay.

Enzyme Assay—Enzyme activity was determined by following the disappearance of 2,5-dihydroxypyridine (ε = 5200 M⁻¹ cm⁻¹) at 320 nm with a Cary recording spectrophotometer, model 15. The reaction was carried out at 25°C in a 3-ml quartz cuvette with a 1-cm light path and was initiated by the addition of substrate. The reaction mixture contained 0.50 μmole of 2,5 dihydroxypyridine, 50 μmoles of sodium phosphate, pH 8.0, and 0.05 to 0.10 ml of reactivated enzyme solution, in a total volume of 2.7 ml. One unit of activity is defined as the amount of enzyme which catalyzes the disappearance of 1.0 μmole of substrate in 1 min.

For testing substrate specificity, enzyme activity was followed by use of the Clarke Oxygen Electrode (Rank Brothers, Bottisham, Cambridge, England). The reaction mixture contained 40 μmoles of sodium phosphate, pH 8.0, and 0.05 to 0.10 ml of reactivated enzyme solution, in a total volume of 2.0 ml. The
reaction was started by adding 0.50 μmole of substrate through a small hole in the reaction chamber cover.

Protein was determined by the method of Lowry et al. (6).

**Acrylamide Gel Electrophoresis**—Analytical disc gel electrophoresis was done with 50 mM Tris-glycine buffer, pH 9.3, and 7.5% acrylamide-separating gel (7). Protein in a 20% sucrose solution was layered on the gels. Electrophoresis was for approximately 2 hours at 3 mA per tube.

The procedure of Weber and Osborn (8) was used to determine the molecular weight of the enzyme subunit. Gels and buffer contained 0.10% sodium dodecyl sulfate and electrophoresis was at 8 mA per gel.

**Sucrose Gradient Centrifugation**—Linear gradients from 5 to 20% sucrose (9) were made in a final volume of 12 ml of 50 mM sodium phosphate, pH 7.5. Centrifugation was for 16 hours at 286,000 × g. Fractions of 0.30 ml were collected. Each fraction was diluted to 1.0 ml, reactivated as described above, and assayed.

**Purification Procedure**

**Growth of Organism**—P. putida N-9, obtained from Professor R. Y. Stanier, was routinely grown in a 50-liter Pyrex carboy containing 40 liters of medium, under forced aeration at 30°C. A 1-liter culture incubated 18 hours in a 2-liter Erlenmeyer flask at 30°C on a rotary shaker at 250 rpm was the inoculum.

The growth medium contained 0.40% sodium nicotinate, 0.10% ammonium sulfate, 0.020% casein hydrolysate, 1.0% (v/v) "mineral base" (1) and 20 mM sodium phosphate, pH 7.0. Cells were grown for approximately 18 hours, reaching the stationary phase of growth, harvested with a refrigerated Sharples centrifuge, and stored at -40°C. This procedure yielded approximately 0.3 g of cells, wet weight, per liter of medium. All subsequent procedures were carried out at about 4°C and centrifugation was at 9000 rpm for 15 min, unless otherwise stated.

**Crude Extract**—Frozen cells (200 g) were suspended in 200 ml of 20 mM sodium phosphate, pH 7.5, containing 5.0 mM dithiothreitol. Approximately 0.1 mg of deoxyribonuclease was added to reduce viscosity, and the suspension was passed through a French pressure cell under 16,000 psi of pressure, followed by centrifugation to remove broken cells and debris.

**High Speed Centrifugation**—The crude cell free extract was centrifuged in the Beckman model L centrifuge at 80,000 × g for 2 hours to remove particulate matter.

**Ammonium Sulfate Fractionation**—The high speed supernatant solution was diluted to 1000 ml with 20 mM sodium phosphate, pH 7.5, and the dithiothreitol concentration was adjusted to 5.0 mM. Solid ammonium sulfate (265 g) was added with stirring, and the pH of the solution was adjusted to 7.5 with 5 N NaOH. After 1 hour of continued stirring, the precipitate was removed by centrifugation and discarded. To the supernatant solution, 125 g of solid ammonium sulfate were added and the pH was again adjusted to 7.5. After 1 hour, the precipitate was removed by centrifugation, resuspended in 100 ml of 20 mM sodium phosphate, pH 7.5, and dialyzed for 12 hours against three changes (4 liters each) of 10 mM sodium phosphate, pH 7.5.

**DEAE-cellulose Column Chromatography**—The DEAE-cellulose (Cellex-D, 0.77 meq per g) was prepared by suspending 100 g of the dry powder in 3 liters of 0.10 N NaOH containing 44 g of NaCl and 10 g of EDTA. After holding the slurry for 1 hour at room temperature, the DEAE-cellulose was collected by filtration and resuspended in 3 liters of 0.25 N NaOH. After 15 min, this material was filtered and resuspended in 3 liters of 0.10 N NaOH containing 44 g of NaCl. The DEAE-cellulose was then washed once with 3 liters of 2 mM NaCl, followed by four similar washes with 20 mM sodium phosphate, pH 7.5. Fine particles were removed by suspending the DEAE-cellulose in 3 liters of 20 mM sodium phosphate, pH 7.5, and decanting. This procedure was repeated four times. The DEAE-cellulose was then equilibrated with 0.10 mM sodium phosphate, pH 7.5, at 4°C. The column (4.5 × 35 cm) was packed under a pressure of 75 cm of H2O and washed with 4 liters of 0.10 mM sodium phosphate, pH 7.5.

The dialyzed ammonium sulfate fraction was applied, and the column was washed with 1200 ml of the equilibration buffer. The enzyme was eluted with a linear concentration gradient established between 700 ml of 0.10 mM sodium phosphate, pH 7.5, and 700 ml of 0.30 mM potassium phosphate, pH 7.5, both containing 5.0 mM dithiothreitol. The column was eluted at a flow rate of 120 ml per hour and 18-ml fractions were collected. Fractions with specific activity greater than 12, which emerged between 600- and 800-ml effluent volume, were pooled and dialyzed for 12 hours against three changes (4 liters each) of 20 mM sodium phosphate, pH 7.0.

**Hydroxyapatite Column Chromatography**—The dialyzed DEAE-cellulose fraction was applied to an hydroxyapatite column (2.0 × 15 cm) equilibrated with 20 mM sodium phosphate, pH 7.0. The column was washed with 200 ml of 60 mM sodium phosphate, pH 7.0, followed by elution of the enzyme with 90 mM sodium phosphate, pH 7.0, containing 5.0 mM dithiothreitol. Elution was at 75 ml per hour and 6-ml fractions were collected. Fractions with specific activity of at least 33 were pooled, placed in dialysis tubing, and concentrated to approximately 5 mg of protein per ml by covering the tubing with polyethylene glycol. This material was then dialyzed against 20 mM sodium phosphate, pH 7.5, for 12 hours.

**Crystallization**—Finely ground ammonium sulfate was added to the concentrated hydroxyapatite fraction until it became turbid and remained so for at least 5 min. The precipitate was removed by centrifugation. This procedure was repeated one or two more times until addition of ammonium sulfate produced a silver sheen. The solution was then dialyzed against 50 mM sodium phosphate, pH 7.5, for 6 hours, and then concentrated as described above to 5 to 10 mg of protein per ml. The solution was further concentrated by suspending the dialysis tubing in air for 24 hours. The crystals which formed (Fig. 1) were centrifuged, washed once with cold buffer, and dissolved in 50 mM sodium phosphate, pH 7.5, containing 5.0 mM dithiothreitol. Results of the enzyme purification procedure are presented in Table I.
subunits of lower molecular weight. When the crystalline enzyme was electrophoretically analyzed in the presence of 0.1% sodium dodecyl sulfate, a single protein band was obtained (Fig. 2E). The molecular weight of this polypeptide was approximately 39,500 (Fig. 3).

Centrifugation of the crystalline enzyme in a sucrose gradient containing dithiothreitol yielded a single region of enzyme activity corresponding to a molecular weight of 242,000 (Fig. 4A). When dithiothreitol was omitted, the peak corresponding to molecular weight 242,000 was reduced, and a broad region of enzyme activity corresponding to lower molecular weight was obtained (Fig. 4B). When Tris-glycine, pH 9.3, with no dithiothreitol was used, enzyme activity was observed only in the lower molecular weight region of the gradient (Fig. 4C).

Enzyme from the lower molecular weight region of the sucrose gradient (Fig. 4B) was concentrated and electrophoretically analyzed (Fig. 2C). Both major and minor bands were obtained, indicating reassociation of the low molecular weight material.

**Enzyme Reactivation**—Initial studies showed that enzyme activity was completely lost within 48 hours at 0°C. Treatments such as dialysis, Sephadex gel filtration, or any of the purification procedures listed in Table I resulted in marked loss of enzyme activity. Incubation of inactivated enzyme with the reducing agents, sodium borohydride, glutathione, ascorbate, or dithiothreitol, caused varying degrees of reactivation. Of these compounds, dithiothreitol was the most effective.

Behrman and Stanier (2) reported reactivation of their enzyme preparations by addition of ferrous ions to the assay mixture. Fig. 5 presents the results of incubating dialyzed high speed supernatant solution at 25°C with different concentrations of dithiothreitol or ferrous sulfate prior to assay.

Prior incubation of the dialyzed or partially purified enzyme with dithiothreitol and ferrous sulfate together is more effective than prior incubation with either compound alone (Fig. 6). The combination of dithiothreitol and ferrous sulfate in the prior incubation mixture had a stabilizing effect upon enzyme activity (Fig. 6). This made possible a reproducible assay if the enzyme was initially incubated at 25°C for 15 to 30 min immediately before testing. Optimal concentrations of these activating agents were found to be 10 mM dithiothreitol and 0.25 mM ferrous sulfate.

Although the dialyzed or partially purified enzyme could be

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**Table I**

| Step                  | Volume | Activity | Protein | Specific Activity | Recovery | Purification |
|-----------------------|--------|----------|---------|------------------|----------|--------------|
| Crude extract         |        | 320      | 61,440  | 17,640           | 100      | 1            |
| High speed centrifugation |      | 220      | 49,200  | 17,600           | 80       | 1.3          |
| Ammonium sulfate      | 120    | 38,712   | 5,810   | 6.7              | 63       | 3.2          |
| DEAE-cellulose        | 280    | 12,288   | 745     | 16.5             | 20       | 7.9          |
| Hydroxyapatite        | 22     | 3,070    | 85.3    | 36.0             | 5        | 17.1         |
| Crystallization       | 2.7    | 823      | 21.5    | 38.3             | 1.3      | 18.2         |
Fig. 3. Determination of the molecular weight of the 2,5-dihydroxypyridine oxygenase subunit. Gels and buffer contained 0.10% sodium dodecyl sulfate. Protein standards: phosphorylase A, mol wt 94,000; bovine serum albumin, mol wt 68,000; glutamate dehydrogenase, mol wt 53,000; ovalbumin, mol wt 43,000; pepsin, mol wt 35,000; trypsin, mol wt 23,300; β-lactoglobulin, mol wt 18,400; ribonuclease, mol wt 13,700. The mobility of 2,5-dihydroxypyridine oxygenase is indicated by the arrow. Each protein (20 to 50 μg) was applied to a separate gel.

Fig. 4. Sucrose gradient profiles of 2,5-dihydroxypyridine oxygenase activity. In each case, 0.57 mg of enzyme was applied to the gradient in a volume of 0.40 ml. A, gradient prepared in 50 mM sodium phosphate, pH 7.5, containing 5.0 mM dithiothreitol. Hemoglobin (–O–O), monitored at 410 nm, was used as the molecular weight standard. B, 50 mM sodium phosphate, pH 7.5, with no dithiothreitol. C, 50 mM Tris-glycine, pH 9.3, with no dithiothreitol.

reactivated to a considerable extent with either dithiothreitol or ferrous sulfate alone, the crystalline enzyme required both dithiothreitol and ferrous sulfate together for reactivation.

Stability—When the enzyme was stored at 0° in 20 mM sodium phosphate, pH 7.5, then reactivated and assayed, its half-life was approximately 2 to 3 days (Fig. 7). The presence of dithiothreitol in the buffer extended the half-life to about 2 weeks.

Addition of ferrous sulfate did not protect the enzyme, whereas dithiothreitol and ferrous sulfate together were detrimental on long term incubation at 0°. The enzyme retained 80% of its activity when stored for 3 months at −40°.

Absorption Spectrum—The crystalline enzyme yielded a single absorption peak at 280 nm. Absorption characteristic of heme or flavin could not be detected. The colorless appearance of
Fig. 7. Effect of dithiothreitol and ferrous sulfate on the stability of 2,5-dihydroxypyridine oxygenase. High speed supernatant solution dialyzed against 20 mM sodium phosphate, pH 7.5, was diluted with the same buffer to 2.0 mg protein per ml and stored at 0° with: 5.0 mM dithiothreitol; 0.25 mM ferrous sulfate; no stabilizing agents; 5.0 mM dithiothreitol and 0.25 mM ferrous sulfate. Aliquots (0.10 ml) were reactivated and assayed.

TABLE II
Activation of 2,5-dihydroxypyridine oxygenase by Fe²⁺ and Fe³⁺
Dialyzed DEAE-cellulose fraction was initially incubated in the standard reactivation mixture containing 0.25 mM FeSO₄, 0.25 mM FeCl₃, and 10 μM dithiothreitol, in the combinations indicated, and then assayed spectrophotometrically.

| Metal | Dithiothreitol | Units/ml |
|-------|----------------|----------|
| -     | -              | 0        |
| -     | +              | 3.4      |
| Fe²⁺  | -              | 21.1     |
| Fe²⁺  | +              | 67.0     |
| Fe³⁺  | -              | 0        |
| Fe³⁺  | +              | 30.7     |

the crystalline material indicated that it did not contain bound ferrie iron.

pH Optima—The purified enzyme, like the crude preparation tested by Behrman and Stanier (2) was most active when assayed at pH 8.0. The optimal pH for reactivation and that at which the enzyme was most stable was found to be pH 7.5. Maximal activity was obtained in sodium phosphate buffer (20 to 100 mM). Tris, Tris-glycine, and Tris-maleate were inhibitory.

Effects of Metal Ions—Behrman and Stanier (2) reported activation of the dialyzed enzyme solution with ferrous ions, but not by various other metal ions, when these were added directly to the assay mixture. This was reinvestigated by initially incubating the enzyme with 10 mM dithiothreitol and various metal ions (0.25 mM) for 15 min at 25°C. The enzyme was then assayed. The following metal salts were used: FeSO₄·7H₂O, Co(NO₃)₂·6H₂O, MgSO₄·7H₂O, BaCl₂·2H₂O, FeCl₃, CuCl₂·2H₂O, CuCl₂, MnSO₄·H₂O, ZnSO₄·7H₂O, CaCl₂·NiSO₄·6H₂O, AlCl₃·6H₂O, CdCl₂·2.5H₂O. Of these, only FeCl₃ and FeSO₄ were effective. Presumably, the Fe³⁺ is reduced to Fe²⁺ in the presence of dithiothreitol (Table II).

Substrate Specificity—The enzyme was tested, with the oxygen electrode, for its ability to catalyze the oxidation of the following compounds: 2,5-dihydroxypyridine, 2,3-dihydroxypyridine, 2,4-dihydroxypyridine, 2,6-dihydroxypyridine, 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine, dipicolinic acid, picolinic acid, nicotinic acid, 6-hydroxy nicotinic acid, pyridoxal, pyridoxamine hydrochloride, catechol, p-hydroxybenzoic acid. Only 2,5-dihydroxypyridine was oxidized, indicating a strict specificity of the enzyme.

Inhibitors—The enzyme was sensitive to the sulfhydryl reagents p-chloromercuribenzoate and N-ethylmaleimide when these compounds were added directly to the reaction mixture prior to adding the substrate (Table III). It was less sensitive to iodoacetamide and was not inhibited by iodoacetate. The enzyme was inhibited to some extent by the metal-chelating agents EDTA and KCN, and it was very sensitive to α,α'-dipyridyl and o-phenanthroline. The enzyme was also very sensitive to H₂O₂. K₃[Fe(CN)]₆ oxidized the substrate in the absence of enzyme.

DISCUSSION

The properties of the 2,5-dihydroxypyridine-oxidizing enzyme are similar to those of dioxygenases which catalyze the cleavage of the aromatic ring (10–13). It is unstable in the absence of dithiothreitol, sensitive to oxidizing agents, and is activated by ferrous ions and reducing agents. This enzyme does not contain heme or flavin and does not require an external reduced cofactor in stoichiometric amounts. The reaction does not proceed anaerobically in the presence of electron acceptors.

The rise and subsequent decrease in activity observed when the enzyme is incubated with ferrous sulfate suggests that the iron readily dissociates from the enzyme or is lost by auto-oxidation or formation of an insoluble product. Flamm and Cran-
dall (14) have shown that ascorbate maintains ferrous iron in a soluble form in phosphate buffer, pH 7.0. The marked stabilization of the enzyme activity upon prior incubation with ferrous ions and dithiothreitol together suggests that the dithiothreitol may be functioning in a similar fashion as ascorbate.

Unlike the crude preparations, which can be activated to an extent by either ferrous sulfate or dithiothreitol alone, the crystalline enzyme is reactivated only by prior incubation with iron and dithiothreitol together. This suggests that, besides maintaining the ferrous ions in solution, a second function of dithiothreitol may be to maintain an essential sulfhydryl group in the reduced form.

The 2,5-dihydroxypyridine-oxidizing enzyme appears most similar to the mammalian dioxygenases, homogentisate oxygenase (14) and 3-hydroxyanthranilate oxygenase (15-17) which contain loosely bound iron and are inhibited by metal-chelating agents. It differs from pyrocatechase (12) and protocatechuate 3,4-dioxygenase (18), which contain firmly bound ferric iron, giving a red color to the crystalline enzyme solution, and from tryptophan oxygenase, which contains iron in the form of heme (19).

The behavior of the 2,5-dihydroxypyridine-oxidizing enzyme on electrophoresis and in sucrose gradients indicates that it consists of an aggregate of six subunits of molecular weight 39,500. Whether the subunits are identical has not as yet been determined. Subunit material isolated on sucrose gradients could be reactivated by incubation with dithiothreitol and ferrous sulfate. Apparently, dissociation into subunits does not result in irreversible loss of activity. It is not known whether reaggregation, which most likely occurs during reactivation, is necessary for activity or whether the subunits themselves are enzymatically active.

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J. Biol. Chem. 1971, 246:3737-3742.

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