PURIFICATION AND CRYSTALLIZATION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG LIVER

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Summary: Quinolinate phosphoribosyltransferase (an intermediary enzyme in the *de novo* NAD biosynthetic pathway) was purified and crystallized for the first time from mammalian tissue. The crystalline preparation was certified to be homogeneous by ultracentrifugal analysis and polyacrylamide gel disc electrophoresis. The molecular weight of this enzyme protein was calculated as 172,000 with the sedimentation velocity method and as 173,000 using gel permeation chromatography on Sephadex G-200. The polypeptide chain molecular weight of this enzyme protein was calculated as 34,000 using the dodecyl sulfate-polyacrylamide gel electrophoresis and as 35,000 with the sedimentation equilibrium method. Quinolinate phosphoribosyltransferase from hog liver may consist of five identical subunits.

The *de novo* NAD biosynthetic pathways are known to be derived either from tryptophan or from aspartate-glycerol and related compounds. The tryptophan pathway has been found in *Neurospora* (1,2), mammalian tissues (3,4), *Xanthomonas pruni* (5) and aerobicaly grown yeast (6), while the aspartate-glycerol pathway has been found in plants (7), anaerobicaly grown yeast (6), and various bacteria including *Escherichia coli* (8,9), *Mycobacterium tuberculosis* (10-14), *Clostridium butylicum* (15-17) and *Mycobacterium bovis* (18). In all pathways, quinolinic acid is thought to be the common intermediate which is converted to NAD by three subsequent steps (19). Quinolinate phosphoribosyltransferase catalyzes the stoichiometric conversion of quinolinic acid to nicotinic acid mononucleotide and CO_2 in the presence of PRPP[1] and Mg^{++}. The enzyme has been crystallized from *Pseudomonad* (20). Highly purified preparations of this enzyme from beef liver (21,22) have been reported, but crystallization from mammals has never succeeded.

The present paper describes the purification, crystallization and some physicochemical properties of quinolinate phosphoribosyltransferase from hog liver.

Hog liver was obtained immediately after slaughter and was rapidly frozen and

1) 5-phosphoribosyl-1-pyrophosphate

maintained at -20°C until use. The substrate, 2,3,7,8-[^{14}C]quinolinic acid, was synthesized from aniline-U-[^{14}C] by Daiichi Pure Chemicals Co., Ltd., Tokyo (23). PRPP (tetrasodium salt, purity 90-95 %) was purchased from Sigma Chemical Company. Protein was determined using the method of Lowry *et al.*(24) with bovine serum albumin as standard. Ultracentrifugal studies were carried out at 20°C with a Hitachi Analytical Ultracentrifuge, Model UCA-1A equipped with schlieren optics or with a photoelectric scanner.

Assay Conditions Reaction mixtures contained potassium phosphate buffer (30 μmoles, pH 6.5); 2,3,7,8-[^{14}C]quinolinic acid (250 mμmoles, 50 mμCi); PRPP (100 mμmoles); magnesium sulfate (2.5 μmoles) and an appropriate amount of enzyme in a final volume of 0.5 ml. The reaction tube (15 x 70 mm) and counting vial, in which Whatman No.3MM filter paper (15 x 77 mm) soaked with 0.2 ml of 25 % β-phenylethylamine dissolved in methanol, were connected with a thick rubber tube (25). The reaction was initiated by adding a specified amount of enzyme, and incubating the whole at 37°C for 1 hr. The reaction was stopped by injecting 0.8 ml of 4 % perchloric acid with a syringe through a rubber tube. During shaking at 37°C for 90 min in a shaking incubator, the [^{14}C]CO_2 evolved was completely trapped by β-phenylethylamine on filter paper. Then 10 ml of scintillator (4 g of PPO[2] and 0.1 g of POPOP[3] per liter of toluene) was added to each counting vial. Radioactivity was determined with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 2425. One unit of enzyme activity is defined as the amount of enzyme which decarboxylates 1 mμmole of quinolinic acid per hour under the assay conditions.

Purification All manipulations were carried out in the cold (0-4°C). Hog liver (21 kg) was homogenized for 1 min with five volumes of 0.05 M potassium phosphate buffer at pH 6.0 in a blendor, and connective tissue was removed by passing the liver through a wire screen. The crude homogenate was adjusted to pH 5.5 by

2) 2,5-diphenyloxazole
3) 1,4-*bis*[2(5-phenyloxazolyl)]benzene

adding 1 N acetic acid with continuous stirring. After standing overnight in the cold, this solution was centrifuged at 10,000 x *g* for 15 min. The supernatant solution was adjusted to pH 7.0 by adding 10 % ammonia with continuous stirring. Solid ammonium sulfate was added to this acid-treated solution until 40 % saturation was attained. After stirring it for 40 min, the protein suspension was kept overnight in the cold. The resulting precipitate was collected by centrifugation at 10,000 x *g* for 20 min, then it was dissolved in 300 ml of 0.005 M potassium phosphate buffer, pH 7.0. The solution was dialyzed overnight against a total of 93 liters of the same buffer (3 x 31 liters). Insoluble materials were discarded by centrifugation at 10,000 x *g* for 30 min. The dialyzed solution was placed on a DEAE-cellulose column (9.5 x 43 cm) equilibrated with 0.005 M potassium phosphate buffer, pH 7.0. The column was washed with 15 liters of the same buffer, then the enzyme was eluted with a linear gradient consisting of a mixing chamber, containing 5 liters of the equilibration buffer fed by a reservoir of 5 liters of 0.3 M potassium phosphate buffer, pH 7.0. Fractions of 20 ml were collected. Enzyme activity appeared between 6.4 liters and 9.2 liters of the eluent. Active fractions were pooled and ammonium sulfate was added to 60 % saturation. The resulting precipitate was collected by centrifugation at 10,000 x *g* for 30 min. This was dissolved in 60 ml of 0.005 M potassium phosphate buffer, pH 7.0, and dialyzed for 45 hr against a total of 27 liters of the same buffer (3 x 9 liters). The dialysate was centrifuged at 10,000 x *g* for 15 min, and its supernatant solution was charged on a DEAE-cellulose column (2.5 x 36 cm) equilibrated with 0.005 M potassium phosphate buffer, pH 7.0. The column was washed with 660 ml of the same buffer, then the enzyme was eluted with a linear gradient consisting of a mixing chamber containing 1 liter of the equilibration buffer fed by a reservoir of 1 liter of 0.3 M potassium phosphate buffer, pH 7.0. Fractions of 10 ml were collected. Enzyme activity appeared between 1.05 liters and 1.65 liters of the eluent. Active fractions were pooled and ammonium sulfate was added to 60 % saturation. The resulting precipitate was collected by centrifugation at 10,000 x *g* for 15 min, and was dissolved in 30 ml of 0.05 M potassium phosphate buffer at

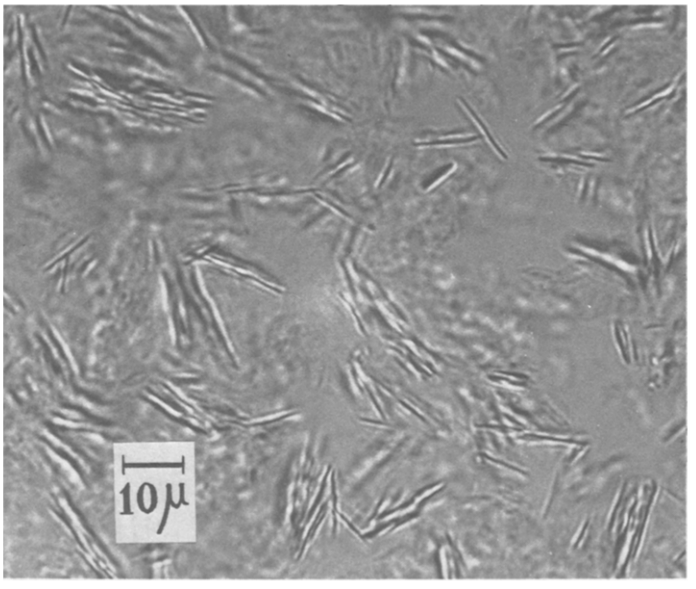

Fig. 1. Microphotograph of Crystalline Quinolinate Phosphoribosyltransferase from Hog Liver
(Microphotograph taken by a Nikon microscope model S-1P equipped with automatic exposure photomicrographic apparatus model AFM at 600 x.)

pH 7.0, then it was cooled at 0°C, after which fine crystals of the enzyme were gradually formed. Recrystallization was carried out by dissolving the crystals in 6 ml of 0.05 M potassium phosphate buffer at pH 7.0 and adding a small amount of ammonium sulfate. The solution was kept overnight at 0°C to crystallize the enzyme protein. The profile of recrystallized enzyme is shown in Fig. 1. A summary of the purification method is presented in Table 1.

Homogeneity Upon ultracentrifugation in 0.05 M potassium phosphate buffer, pH 7.0 at 60,000 rpm, the recrystallized enzyme sedimented as a single symmetrical sharp boundary throughout the cell. The sedimentation coefficient was independent of protein concentration. A value for $s^0_{20,w}$ of 8.00×10^{-13} sec was obtained.

On polyacrylamide gel disc electrophoresis at pH 8.9, according to the method of Davis (26), the recrystallized enzyme appeared in a single band (Fig. 2-A) and its relative mobility was calculated as 0.51 against the mobility of bromophenol blue. No minor band was ever observed.

Table 1. Summary of the Purification of Quinolinate Phosphoribosyltransferase from Hog Liver

Fraction	Total activity* [units x 10^{-6}]	Total protein [mg]	Specific activity [units/mg]	Purification [ratio]	Yield [%]
Crude homogenate	1.170	1961400	0.5966	1	100
Acid treatment	1.210	514300	2.352	3.9	103.4
Ammonium sulfate fraction	1.757	64300	27.33	45.8	150.1
1st. DEAE-cellulose eluate	1.142	4070	280.5	470.2	97.6
2nd. DEAE-cellulose eluate	0.7875	645	1221	2046.6	67.3
Crystallization	0.7381	240	3075	5154.2	63.1
Recrystallization	0.7161	231	3100	5196.1	61.2

*A unit of activity is defined as the amount of enzyme which decarboxylates 1 mμmole of quinolinic acid per hour at 37°C.

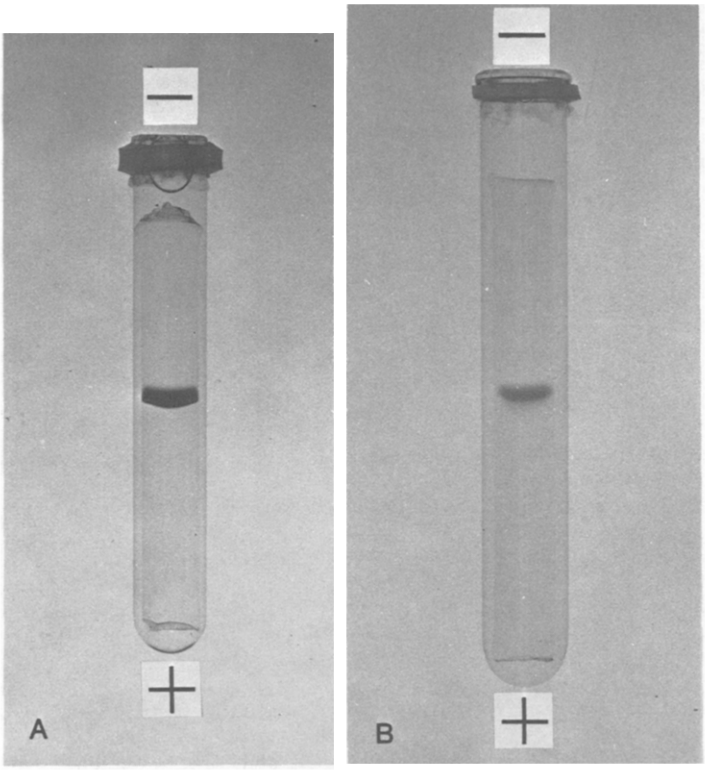

Fig. 2. Polyacrylamide Gel Disc Electrophoresis of Crystalline Quinolinate Phosphoribosyltransferase from Hog Liver without SDS (A) and with SDS (B)
A: Enzyme protein (60 μg) was electrophoresed at 3 mA per column for 90 min, using the method of Davis, in a cold room (4°C). Protein was stained by Amido Black 10B.
B: Enzyme protein was electrophoresed at 8 mA per column for 6 hr, using the method of Weber and Osborn, at room temperature. Protein was stained by Coomasie Brilliant Blue G-250.

Molecular Weight The apparent molecular weight was determined using gel permeation chromatography on a Sephadex G-200 column (1.5 x 85 cm) with eleven standard proteins, blue dextran and $[^3H]H_2O$. A distribution coefficient of 0.203 was obtained and an apparent molecular weight of 173,000 was estimated.

The diffusion coefficient was determined in 0.05 M potassium phosphate buffer, pH 7.0 at 8,270 rpm using a capillary type synthetic boundary cell. The diffusion coefficient was also independent of protein concentration and had a value of 4.33×10^{-7} $cm^2 \cdot sec^{-1}$. By amino acid analysis with a Hitachi Amino Acid Analyzer,

Model KLA-5 (tryptophan content was determined using the method of Goodwin and Morton (27)), the partial specific volume of this enzyme was estimated as 0.74 $ml \cdot g^{-1}$ (28). From the sedimentation and diffusion coefficients and from the partial specific volume, a molecular weight of 172,000 was calculated. Stokes' radius was calculated as 49.1 Å from the molecular weight and sedimentation coefficient (29). A frictional ratio, f/f_o, was 1.33 based on the values for Stokes' radius and molecular weight (29).

The molecular weight of the subunit was determined with SDS[4]-polyacrylamide gel disc electrophoresis and sedimentation equilibrium analysis in a guanidine hydrochloride solution. SDS-polyacrylamide gel disc electrophoresis was performed using the method of Weber and Osborn (30). This enzyme showed a single band on SDS-polyacrylamide gel as shown in Fig. 2-B. The molecular weight of its polypeptide chain was 33,500 in comparison to the relative mobility values of eight standard proteins.

The enzyme was dialyzed 9 days against 6 M guanidine hydrochloride dissolved in 0.05 M potassium phosphate buffer, pH 7.0. On sedimentation equilibrium analysis at 19,780 rpm with a photoelectric scanner, denatured protein (0.5 mg/ml) in the 6 M guanidine hydrochloride solution reached equilibrium within 17 hr; the molecular weight was calculated as 34,200. Sedimentation velocity analysis was performed in 5 M guanidine hydrochloride dissolved in 0.05 M potassium phosphate buffer, pH 7.0 at 40,370 rpm using a capillary type synthetic boundary cell. A single sharp boundary appeared and the sedimentation coefficient was dependent upon protein concentration. An $s^0_{20,w}$ of 3.20×10^{-13} sec was calculated for an infinitely low protein concentration. From the molecular weight of 34,200 and the sedimentation coefficient of 3.20×10^{-13} sec, a Stokes' radius for the polypeptide chain of 24.4 Å and a frictional ratio of 1.13 were calculated. This polypeptide chain completely lost the enzyme activity, an attempt to reactivate it by dialysis against 0.05 M potassium phosphate buffer at pH 7.0 was unsuccessful

The quinolinate phosphoribosyltransferase from hog liver may consist of five

4) sodium dodecyl sulfate

identical subunits, as deduced from the molecular weight of native enzyme (172,000), of the polypeptide chain (34,200), and from homogeneity of the polypeptide chain.

Studies of the properties and kinetics of the enzyme are in progress.

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