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ISOLATION AND PROPERTIES OF CRYSTALLINE QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG KIDNEY *

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Summary

Crystalline quinolinate phosphoribosyltransferase (nicotinatenucleotide: pyrophosphate phosphoribosyltransferase (carboxylating), EC 2.4.2.19) was isolated from hog kidney and compared with the same enzyme prepared from hog liver. The enzyme preparation was homogeneous as shown by polyacrylamide gel electrophoresis and ultracentrifugation analysis. The enzyme had a molecular weight of 220 000 and the subunit 35 000. The physicochemical properties of the enzyme were: sedimentation coefficient ($s^0_{20,w}$), $7.75 \cdot 10^{-13}$ s; difussion coefficient ($D^0_{20,w}$), $5.04 \cdot 10^{-7}$ cm^2/s; Stokes' radius, 62.05 Å, frictional ratio (f/f_0), 1.62; and isoelectric point (pI), 4.5. The enzyme was stable at 37°C for 30 min between pH 4.5 and 9.5. Enzyme activity was inhibited by various carboxylic acids; however, this inhibition was reversed by raising the Mg^{2+} concentration. Optimum pH was 5.5, and no detectable amounts of Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} and Ca^{2+} were found by atomic absorption spectrophotometry. The enzyme was found to contain sugar. Mg^{2+} was completely replaceable by Mn^{2+}. The reaction mechanism of this enzyme was suggested to be of the 'ping-pong' type. K_m values of quinolinic acid and 5-phosphoribosyl 1-pyrophosphate were $4 \cdot 10^{-5}$ and $1.4 \cdot 10^{-4}$ M, respectively.

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Introduction

NAD is known to be synthesized from tryptophan in animals. In the tryptophan-NAD pathway, the first niacin-active product is nicotinic acid mononucleotide which is synthesized from quinolinic acid and 5-phosphoribosyl 1-pyrophosphate (*P*-Rib-*PP*) in the presence of Mg^{2+} [1]. The enzyme catalyzing reaction is called quinolinate phosphoribosyltransferase (nicotinatenucleotide:pyrophosphate phosphoribosyltransferase (carboxylating), EC 2.4.2.19). The enzyme is located in liver and kidney [2]. The hog liver enzyme was first crystallized in our laboratory [3], and its properties, characteristics and active site have been reported [4—7]. Bovine liver enzymes have been highly purified and their properties have been reported [8,9]. Kidney enzyme, however, has not been purified. Quinolinate phosphoribosyltransferase activity is 3-fold higher in hog kidney than in liver [2]. In order to compare it with the liver enzyme, quinolinate phosphoribosyltransferase was purified from hog kidney.

This paper describes the crystallization, homogeneity and properties of hog kidney quinolinate phosphoribosyltransferase.

Materials and Methods

Materials. Fresh hog kidney was obtained from a slaughterhouse in Kyoto and stored at −20°C until use. [2,3,7,8-^{14}C]Quinolinic acid (0.23 Ci/mol) was purchased from Daiichi Pure Chemicals Co., Tokyo. *P*-Rib-*PP* (tetrasodium salt) was purchased from Sigma Chemical Company. β-Nicotinic acid mononucleotide was obtained from Kyowa Hakko Co., Tokyo, and Sephacryl S-200 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals. Ampholine was obtained from LKB. Other reagents were purchased from Nakarai Chemicals Ltd., Kyoto.

Standard assay conditions. Quinolinate phosphoribosyltransferase activity was assayed by counting the radioactivity of $^{14}CO_2$ stoichiometrically released from *P*-Rib-*PP*-dependent decarboxylation of [2,3,7,8-^{14}C]quinolinic acid [2]. The radioactivity of $^{14}CO_2$ equals the amount of nicotinic acid mononucleotide formed.

The reaction tube (15 × 70 mm) and a counting vial in which a Whatman No. 3MM filter paper (15 × 77 mm) soaked in 0.2 ml 25% β-phenylethylamine dissolved in methanol was placed, were connected vertically by a thick rubber tube. The reaction mixture contained in a total volume of 0.5 ml: 20 mM sodium acetate/acetic acid buffer (pH 5.5), 0.306 mM [2,3,7,8-^{14}C]quinolinic acid (0.23 Ci/mol), 1 mM *P*-Rib-*PP*, 4 mM $MgCl_2$, and an appropriate amount of the enzyme. The reaction was initiated by adding the enzyme and stopped by adding 0.8 ml 4% perchloric acid through the rubber tube by a syringe after incubating the reaction mixture at 37°C for 30 min. The $^{14}CO_2$ evolved by shaking the acidified mixture at 37°C for 60 min was completely trapped by β-phenylethylamine on the filter paper in the counting vial. Then 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-[2-(5-phenyloxazoyl)]benzene per l toluene) was added to the counting vial, and the radioactivity was determined by Packard Tri-Carb liquid scintillation spectro-

photometer Model 2425. One unit of enzyme activity is expressed as 1 nmol $^{14}CO_2$ evolved (equals 1 nmol nicotinic acid mononucleotide formed) per h at 37°C.

Protein concentration. Protein concentration was determined by the method of Lowry et al. [10] with bovine serum albumin as standard. After purification was completed protein concentration was determined spectrophotometrically using $E^{1\%}_{1\,cm} = 8.23$ at 280 nm at pH 7.0.

Ultracentrifugation analysis [11]. Ultracentrifugation analysis was carried out with a Hitachi ultracentrifuge model UCA-1A. Sedimentation velocity experiments were conducted in double-sector cell. Sedimentation constants were obtained at a rotor speed of 40 370 rev./min at 20°C in 0.05 M potassium phosphate buffer (pH 7.0), containing 0.1 M KCl. Diffusion coefficient was determined in the same buffer, using a capillary type synthetic boundary cell at a rotor speed of 8270 rev./min at 20°C.

High speed sedimentation equilibrium was performed using an interference cell with saphire windows. Interference photos were taken after centrifugation at 12 290 rev./min for 14 h.

Isolation and crystallization of quinolinate phosphoribosyltransferase

All steps were performed at 0—4°C.

Step 1. Frozen hog kidney (750 g) was cut and homogenized in a Waring blendor in 5 volumes 0.05 M potassium phosphate buffer (pH 7.0), containing 0.01 M 2-mercaptoethanol (standard buffer). Connective tissues were removed by straining through two layers of gauze.

Step 2. The crude extract was adjusted to pH 5.0 by adding 1 M acetic acid under continuous stirring. After standing for 3 h the solution was centrifuged at 10 000 × *g* for 10 min and the precipitate was discarded. The supernatant solution was adjusted to pH 7.0 by adding 10% ammonia.

Step 3. $(NH_4)_2SO_4$ was added to 40% saturation. The pH of the solution was maintained at 7.0 by addition of 10% ammonia. After standing overnight the resulting precipitate was collected. The precipitated protein was dissolved in the standard buffer and the solution dialyzed for 24 h against the same buffer. Insoluble materials were discarded.

Step 4. The dialyzed solution was loaded onto a DEAE-Sephadex A-50 column (2.5 × 50 cm) equilibrated with the standard buffer. The column was washed with 2 l standard buffer. Then the enzyme was eluted with a linear gradient of 0.05—0.5 M potassium phosphate buffer (pH 7.0), containing 0.01 M 2-mercaptoethanol (total volume 2 l). Enzyme activity appeared between 0.96—1.34 l of the eluate. Active fractions were pooled and $(NH_4)_2SO_4$ was added to 60% saturation. The precipitated protein was collected by centrifugation and dissolved in the standard buffer.

Step 5. The protein solution was dialyzed overnight against standard buffer, containing 30% $(NH_4)_2SO_4$. The resulting precipitated protein was collected and dissolved in a small amount of standard buffer.

Step 6. In order to crystallize the enzyme, the solution was dialyzed for 24 h against standard buffer containing 20% $(NH_4)_2SO_4$. Fine crystals were formed and collected.

Step 7. The crystalline enzyme was dissolved in 0.05 M Tris-HCl buffer (pH

8.5) to obtain a protein concentration of approx. 20 mg/ml. This solution was dialyzed for 1 week at room temperature against 0.05 M Tris-HCl buffer (pH 8.5), containing 0.13 M sodium citrate, in a micro dialysis cell [12]. Large enzyme crystals were formed. They were collected and used in the following experiments.

Results

Purification

A summary of the purification is presented in Table I. In regard to purification of quinolinate phosphoribosyltransferase from hog kidney, pH 5.0 treatment and DEAE-Sephadex column chromatography were very effective. Without the addition of 2-mercaptoethanol to the extract buffer, enzyme activity abruptly decreased in the ammonium sulfate fractionation step. The lost activity was recovered immediately upon addition of 2-mercaptoethanol to the buffer. The yield was raised about 3-fold when a *P*-Rib-*PP*-degrading enzyme in the crude extract of hog kidney was removed by the $(NH_4)_2SO_4$ fractionation step.

Enzyme homogeneity

The enzyme sedimented as a single, symmetrical, sharp band throughout the cell on ultracentrifugation at 40 370 rev./min in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 M KCl. The sedimentation coefficient depended on the protein concentration and a value for $s^0_{20,w}$ of $7.75 \cdot 10^{-13}$ s was calculated. The enzyme appeared in a single band (Fig. 1A and B) on polyacrylamide disc gel electrophoresis at pH 9.4 by the method of Davis [13] and at pH 8.0 by the method of Williams and Reisfeld [14]. These results show that crystalline quinolinate phosphoribosyltransferase from hog kidney is homogeneous.

Amino acid composition and molecular weight determination

The amino acid composition of the enzyme is shown in Table II. The partial

TABLE I

SUMMARY OF THE PURIFICATION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG KIDNEY

One unit equals 1 nmol of nicotinic acid mononucleotide formed per h at 37°C under standard assay conditions.

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Crude extract	76 000	50 920	0.67	100
pH 5.0 treatment	24 350	58 683	2.41	115
$(NH_4)_2SO_4$ precipitate (0—40% sat.)	4 521	139 337	30.82	273
DEAE-Sephadex	101	102 616	1016	201
$(NH_4)_2SO_4$ precipitation (0—30% sat.)	48.7	88 877	1825	174
Crystallization	13.0	66 248	5096	130
Recrystallization	11.0	60 654	5514	119

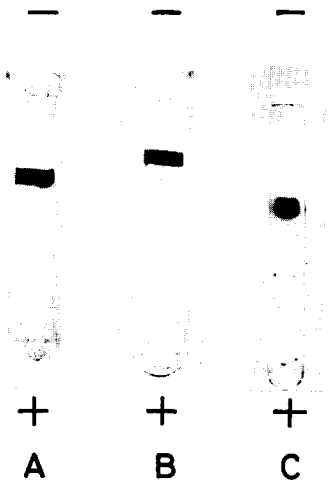

Fig. 1. Disc electrophoretic profiles of crystalline quinolinate phosphoribosyltransferase from hog kidney. A and B. Polyacrylamide gel electrophoresis was performed according to the methods of Davis (A) [13], and Williams and Reisfeld (B) [14]. Enzyme protein (50 μg) was electrophoresed at 2 mA per column for 80 min in a cold room. Protein was stained by Coomasie Brilliant Blue G-250. C. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [15]. The denatured protein (40 μg) was electrophoresed at 8 mA per column for 5.5 h at room temperature and stained by Coomasie brilliant blue G-250

TABLE II

AMINO ACID COMPOSITION OF QUINOLINATE PHOSPHORIBOSYLTRANSFERASE FROM HOG KIDNEY

Amino acid analysis was carried out with a Hitachi amino acid analyzer, model KLA-5. The enzyme protein was hydrolyzed at 110°C for 22 h in 6 M HCl. Half-cystine and methionine contents were determined after performic acid oxidation. Tryptophan content was determined spectrophotometrically [24]. Threonine and serine values were corrected for degradation during hydrolysis: thr, 5%; ser, 10% [25].

Amino acid	Residues per mol enzyme
Lys	52
His	31
Ammonia	150
Arg	63
Asp	106
Thr	84
Ser	70
Glu	159
Pro	104
Gly	164
Ala	264
Cys 1/2	31
Val	158
Met	21
Ile	17
Leu	187
Tyr	37
Phe	57
Trp	25

specific volume was calculated to be 0.74 ml/g [23].

The apparent molecular weight of the enzyme was determined by Sephacryl S-200 gel filtration. From the plot of the distribution coefficient (K_d) against the logarithm of the molecular weight of each standard protein the molecular weight of the enzyme was estimated to be about 220 000 using the following equation:

$$K_d = \frac{V_e - V_0}{(V_t - V_g) - V_0}$$

where V_e is the elution volume, V_0 the void volume, and $(V_t - V_g)$ the gel volume. The same molecular weight (220 600) was also obtained by high sedimentation equilibrium centrifugation. The diffusion coefficient of the enzyme was independent of the protein concentration and $D^0_{20,w} = 5.04 \cdot 10^{-7}$ cm^2/s, was obtained. Stokes' radius was calculated as 62.05 Å from the molecular weight and sedimentation coefficient. The frictional ratio f/f_0, was 1.62 based on the values for Stokes' radius and molecular weight. The molecular weight of the subunit was estimated by SDS-polyacrylamide gel electrophoresis [15], and it appeared in a single band (Fig. 1C). A logarithm of the molecular weight of standard proteins against the distance of migration gave a straight line. The molecular weight was estimated to be 35 000. These results indicate that hog kidney quinolinate phosphoribosyltransferase is composed of six subunits similar in size.

Isoelectric point

The isoelectric point (p*I*) of the enzyme was determined to be 4.5 by isoelectric focusing using Ampholine with a pH range of 4—7 [16].

Enzyme stability and optimum pH

Enzyme activity did not decrease even when it was kept at room temperature for 3 days in standard buffer. When the enzyme was kept in crystalline condition, it was found to be completely stable for at least 2 years at 0—4°C. The enzyme was kept at various pH values (3.0—10.5) for 30 min at 37°C; it denatured abruptly between pH 3.5 and 3.0, and was, however, stable at alkaline pH and it was completely stable between pH 4.5 and 9.5.

Maximum enzyme activity was attained at pH 5.5.

Metal ion requirement

The enzymic formation of nicotinic acid mononucleotide from quinolinic acid and *P*-Rib-*PP* showed an absolute requirement for divalent cations such as Mg^{2+}, Mn^{2+}, Fe^{2+} and Cd^{2+}. Mg^{2+} was the most effective one of the various divalent cations added at 1 mM to the reaction mixture.

Mg^{2+} requirement rose according to the increase in concentration of sodium acetate/acetic acid buffer (pH 5.5). The optimum concentrations of Mg^{2+} were 4, 6 and 10 mM for 20, 40 and 100 mM sodium acetate/acetic acid buffer (pH 5.5), respectively. The effects of various carboxylic acids were investigated (Table III). All carboxylic acids except for Leu, Asn and Gln inhibited this reaction, but inhibition was recovered by raising the Mg^{2+} concentration.

The most effective metal ion in quinolinate phosphoribosyltransferase-

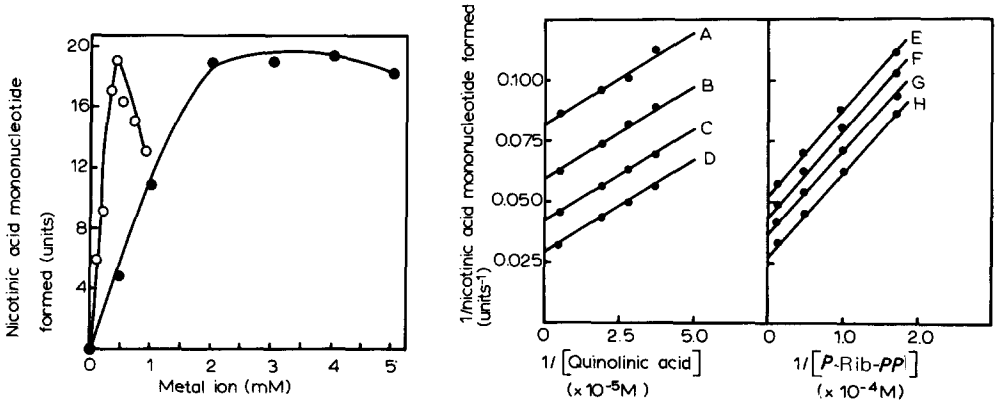

Fig. 2. Effect of metal ion concentration on quinolinate phosphoribosyltransferase activity. Enzyme activity was determined using standard assay conditions except for $MgCl_2$ and $MnCl_2$ concentrations and is expressed as units of nicotinic acid mononucleotide formed per h. ○, $MnCl_2$; ●, $MgCl_2$.

Fig. 3. Double reciprocal plots of initial velocity against substrate concentration. Standard assay conditions were varied as follows. *P*-Rib-*PP* concentrations were, $6 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ and $6 \cdot 10^{-4}$ M for curves A, B, C and D, respectively. Quinolinic acid concentrations were, $2.70 \cdot 10^{-5}$, $3.63 \cdot 10^{-5}$, $5.45 \cdot 10^{-5}$ and $1.82 \cdot 10^{-4}$ M for curves E, F, G and H, respectively. Each point on the curves represents the average of three independent reactions. Enzyme activity is expressed as units of nicotinic acid mononucleotide formed per h.

catalyzing reaction is Mg^{2+}. Mn^{2+} was partially effective in *Alcaligenes* sp. [17] and 'Shiitake' mushroom [18] enzymes, even when various concentrations were used. Mn^{2+} could completely replace Mg^{2+} in the kidney enzyme (Fig. 2). Similar results were obtained with the liver enzyme [19].

TABLE III

EFFECT OF CARBOXYLIC ACIDS ON QUINOLINATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

The standard assay for quinolinate phosphoribosyltransferase was used except for the addition of the indicated carboxylic acids.

Addition (1 mM)	Relative activity (%)
None	100
Maleic acid	58
Succinic acid	54
Fumaric acid	33
L-Malic acid	33
Citric acid	11
Lactic acid	71
Acetic acid	74
Formic acid	46
α-Ketoglutaric acid	45
Oxaloacetic acid	41
Glutamic acid	59
Glutamine	102
Aspartic acid	63
Asparagine	107
Leucine	105

Metal and sugar content

The metal content of the enzyme was analyzed with a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer equipped with a GA-2 graphite atomizer. The results indicated that the contents of Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} and Ca^{2+} were insignificant as in the case of hog liver enzyme [19].

Hog kidney enzyme was found to contain 1% mannose as determined by the orcinol/sulfuric acid method [20]. This content was the same in the liver enzyme [19].

Kinetics. Double reciprocal plots of the initial velocity vs. the second substrate are presented in Fig. 3. The plots were linear and parallel. It is very likely that the reaction mechanism of the enzyme from hog kidney may be a 'ping-pong' type. The same mechanism is found in hog liver enzyme [19]. The K_m values of quinolinic acid and *P*-Rib-*PP* were calculated to be $4 \cdot 10^{-5}$ and $1.4 \cdot 10^{-4}$ M, respectively from the seondary plots of Fig. 3.

Discussion

Large crystals of quinolinate phosphoribosyltransferase were isolated from hog kidney in a high yield by a straightforward procedure. Physicochemical and enzymic properties of the hog kidney enzyme were almost the same as those of the hog liver enzyme [4,5], but the two enzymes could clearly be detected as two bands by polyacrylamide gel electrophoresis. The final specific activity of the hog kidney enzyme was 1.8-fold higher than that of the liver enzyme.

Compared with quinolinate phosphoribosyltransferases from hog kidney and *Alcaligenes eutrophus* subsp. *quinolinicus* IAM 12305, which can utilize quinolinic acid as a sole carbon and nitrogen source [21], physicochemical properties [17] were substantially the same, but enzymic properties such as requirement of metal ion [17], optimum pH [17] and effect of carboxylic acid [22] were quite different.

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