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CRYSTALLIZATION AND SOME PROPERTIES OF PURINE NUCLEOSIDE PHOSPHORYLASE FROM CHICKEN LIVER

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Summary

Purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) from chicken liver has been purified about 650 fold and crystallized. The crystalline enzyme was cube shaped and showed a specific activity of 46 units per mg of protein. The homogeneity of the crystalline enzyme was shown by polyacrylamide gel-disc electrophoresis. The sedimentation coefficient ($s^{\circ}_{20,w}$) was 5.4 S. The crystalline enzyme was activated by the substrate inosine. The Hill coefficient was estimated to be 0.76, suggesting negative cooperativity with regard to the substrate inosine. The results of the kinetic analysis are consistent with the mechanism being a "rapid equilibrium random Bi-Bi reaction". The apparent equilibrium constant for phosphorolysis was 0.048.

Introduction

In previous papers, we have reported the developmental pattern of purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyl transferase, EC 2.4.2.1) in chicken liver, and differences in the inhibition by sulfate ion or ADP and in the stability at −20° C of the partially purified embryonic and adult enzyme [1—3]. The evidence showed that substrate activation occurred at high concentrations of inosine in both embryonic and chick liver enzymes [2,3].

Agarwal and Parks [4] have reported that crystalline human erythrocyte purine nucleoside phosphorylase displays the phenomenon of substrate activation at a high concentration of inosine, whereas crystalline bovine spleen enzyme does not display this phenomenon.

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For a comparison of the kinetic and physicochemical properties of the chicken liver with human erythrocyte and bovine spleen enzymes, it is important that the enzyme is further purified to a state of homogeneity.

The present report is concerned with studies on the crystallization of chicken liver purine nucleoside phosphorylase and some of its kinetic properties.

Materials and Methods

Materials

The following chemicals were used in this studies: Tris, hypoxanthine, ribose 1-phosphate and crystalline serum albumin from Sigma; inosine from Kohjin; protamine sulfate from Nutritional and Biochemical Corp.; acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylenediamine from Eastman; DEAE-cellulose and ECTEOLA-cellulose from Brown; Sephadex gel from Pharmacia; Whatman chromatography cellulose powder from Balston. All other reagents were of analytical grade. Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson [5]. Milk xanthine oxidase used in the following "Method I" was purified up to the $(NH_4)_2SO_4$ step by the method of Ball [6] and that used in "Method II" was purchased from Boehringer Mannheim.

Methods

The following two assay methods for purine nucleoside phosphorylase were used in this study. Method I and the definition of the enzyme activity were the same as described in the previous paper [1]. Method II was the xanthine oxidase method of Kalckar [7] in which the increase in the absorbance at 290 nm due to the formation of uric acid was measured. An Hitachi 124 spectrophotometer equipped with an Hitachi recorder was used. The reaction mixture contained the following components in a final volume of 3 ml; Tris/HCl buffer (pH 7.5), 340 μmol; xanthine oxidase, 400 units; the indicated amount of inosine, phosphate and inhibitors and an appropriate amount of purine nucleoside phosphorylase. The reaction was started by the addition of the enzyme in a cuvette of 1-cm light path at 37° C.

For the determination of the equilibrium constant of phosphorolysis, the forward and reverse reactions were assayed at 37° C. The reaction mixture for the forward reaction contained 1 or 1.5 mM of inosine and phosphate, and 100 mM of Tris/HCl buffer (pH 7.5) in a final volume of 1 ml. The reaction mixture for the reverse reaction contained 0.5 or 1 mM of hypoxanthine and ribose 1-phosphate and 100 mM of Tris/HCl buffer (pH 7.5) in a final volume of 1 ml. Both reactions were stopped by boiling the reaction mixture for 2 min at a given interval. Hypoxanthine in the reaction mixture was determined spectrophotometrically by the xanthine oxidase method.

Protein was determined by the method of Lowry et al. [8] using crystalline bovine serum albumin as the standard.

Electrophoresis of the crystalline enzyme was carried out on a polyacrylamide gel disc as described by Ornstein and Davis [9,10]. The degree of polymerization of the gel was 7.5%. The gel was stained for protein with 1% amido

black 10B/7% acetic acid solution for 1 h at room temperature and destained electrophoretically. For enzyme activity the gel was stained by the method of Mattson and Jensen as modified by Gardner and Kornberg [11].

Ultracentrifugal studies were conducted by using an Hitachi-1A analytical ultracentrifuge equipped with schlieren optics. Analyses were performed with various protein concentrations of the enzyme dissolved in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and dialyzed against the same buffer. Sedimentation velocity experiments were performed at 8° C with a rotor speed of 60 000 rev./min and double sector cells with RA 60H rotor.

Purification procedure

Purine nucleoside phosphorylase was purified and crystallized from 500 g of fresh chicken livers, which were obtained from a local meat market. The purification procedure from the homogenate to the second $(NH_4)_2SO_4$ fraction step, was adopted from the previous method [2], and the procedures were slightly modified as follows. The enzyme activities throughout the purification were assayed by Method I described above.

The second $(NH_4)_2SO_4$ fraction, desalted by Sephadex G-25 gel column equilibrated with 50 mM Tris/HCl buffer (pH 7.5), was applied to a DEAE-cellulose column (3 × 15 cm) equilibrated with the same buffer. After washing with 50 mM Tris/HCl buffer (pH 7.5) containing 0.05 M NaCl, the enzyme was eluted with the same buffer containing 0.4 M NaCl (first DEAE-cellulose fraction). The enzyme solution was concentrated to about 20% of the original volume by ultrafiltration. The first DEAE-cellulose fraction, diluted 10 times with 50 mM Tris/HCl buffer (pH 7.5), was added to DEAE-cellulose column (2 × 42 cm) equilibrated with 50 mM Tris/HCl buffer (pH 7.5). The column was developed with a linear gradient from 0 to 0.3 M NaCl in 1500 ml of 50 mM Tris/HCl buffer (pH 7.5). The enzyme emerged from the column when the NaCl concentration was about 0.15 M. The 10-ml fractions containing enzyme activities from the 60th to the 90th tubes were pooled (second DEAE-cellulose fraction). The second DEAE-cellulose fraction, concentrated by ultrafiltration, was dialyzed against 20 mM Tris/HCl buffer (pH 7.4) for 12 h and applied to an ECTEOLA-cellulose column (3 × 14 cm) and then eluted with the Tris/HCl buffer (ECTEOLA-cellulose fraction). The ECTEOLA-cellulose fraction was concentrated to about 10% of the original volume by ultrafiltration and dialyzed against 0.05 M potassium phosphate buffer (pH 6.5) for 12 h. The dialyzate was applied to a calcium phosphate gel-cellulose column (1.5 × 40 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 6.5), then washed with 0.05 M potassium phosphate buffer (pH 7.5) and developed with a linear gradient from 0.05 to 0.3 M potassium phosphate buffer (pH 7.5) in 600 ml. The 5-ml fractions containing enzyme activity (from 20th to 45th tubes) corresponding to about 0.15 M potassium phosphate were pooled. After the calcium phosphate gel-cellulose fraction had been concentrated up to about 1 ml by ultrafiltration, it was applied to a Sephadex G-200 column (2.6 × 40 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The enzyme was eluted between the 65th and 71st tubes of the 1.5-ml fractions. This fraction showed a major protein band with two minor bands on polyacrylamide gel disc

TABLE I

PURIFICATION OF PURINE NUCLEOSIDE PHOSPHORYLASE FROM CHICKEN LIVER

Activities were assayed by the Method I described in the text.

Fraction	Total volume (ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg protein)
1 Homogenate	2570	8130	46.0	0.0688
2 Supernatant at 10000 × *g*	1760	5820	21.2	0.156
3 Protamine sulfate	1975	4740	16.9	0.142
4 $(NH_4)_2SO_4$ (0.3—0.65 saturated)	292	3930	68.0	0.198
5 Calcium phosphate gel	357	3720	11.2	0.929
6 $(NH_4)_2SO_4$ (0.3—0.6 saturated)	109	2990	27.8	0.987
7 DEAE-cellulose column (I)	260	1690	2.39	2.72
8 DEAE-cellulose column (II)	328	1430	0.809	5.38
9 ECTEOLA-cellulose column	51.6	922	2.36	7.56
10 Calcium phosphate gel-cellulose column	135	734	0.169	32.2
11 Sephadex G-200 column	11.6	389	0.764	45.0

electrophoresis. A typical example of the purification procedure is summarized in Table I.

Results and Discussion

Crystallization

The enzyme from Sephadex G-200 fraction, which had a specific activity of 45 units per mg protein, was crystallized according to the method of Jacoby [12]. After the enzyme solution had been brought to the concentration of 70% saturation with saturated $(NH_4)_2SO_4$ solution in potassium phosphate buffer (pH 7.2) containing 5 mM 2-mercaptoethanol and 0.25 M sucrose, it was allowed to stand for 30 min at 4° C. The precipitate was collected by centrifugation at 18 000 × *g* for 20 min and extracted sequentially with 50, 48, 46, 45 and 44% saturation of $(NH_4)_2SO_4$ solution containing mercaptoethanol and sucrose. The supernatants were allowed to stand in a cold room at 4° C. After 4 days the enzyme precipitated as cubic crystals in the tubes extracted at 45 and 46% saturated $(NH_4)_2SO_4$ (Fig. 1). The enzyme from human erythrocytes and bovine spleen reported were crystallized in the form of needles or bundles of needles [4] and of flat plates [13], respectively. The specific activity of crystalline enzyme was 46 units per mg of protein.

Physicochemical properties

Some observations indicated the homogeneity of crystalline purine nucleoside phosphorylase from chicken livers. The crystalline enzyme revealed only a single major protein band on polyacrylamide gel-disc electrophoresis, which corresponded to the enzyme activity stained by the method described above. The sedimentation velocity pattern of the crystalline enzyme (10 mg/ml) showed only a single symmetrical peak (Fig. 2). The sedimentation coefficient was slightly affected by the enzyme concentration in the range from 2.5 to 10 mg per ml in 0.05 M potassium phosphate buffer (pH 7.5) containing

10 mM 2-mercaptoethanol. The sedimentation coefficient was calculated as 5.4 S by extrapolation to zero of the enzyme concentration.

Kinetic analysis

Kinetic analysis was carried out with crystalline enzyme by the assay method II described above. The previous paper reported a substrate activation at high concentrations of inosine with partially purified enzyme from embryonic and chicken liver [2,3]. With the crystalline enzyme, Lineweaver-Burk plots have also revealed a nonlinear and concave downward curve at high concentrations of inosine (Fig. 3). The slope of Hill plots was 0.76 suggesting a negative cooperativity in this enzyme reaction. The substrate activation at high concentrations of inosine has been reported with crystalline enzyme from human erythrocytes [4], partially purified enzyme from beef liver [14], chicken liver [2,3] and the hepatopancreas of *Helix pomatia* [15]. On the other hand, substrate activation was not seen at high concentrations of inorganic phosphate (Fig. 4). The following kinetic analyses were performed at low substrate con-

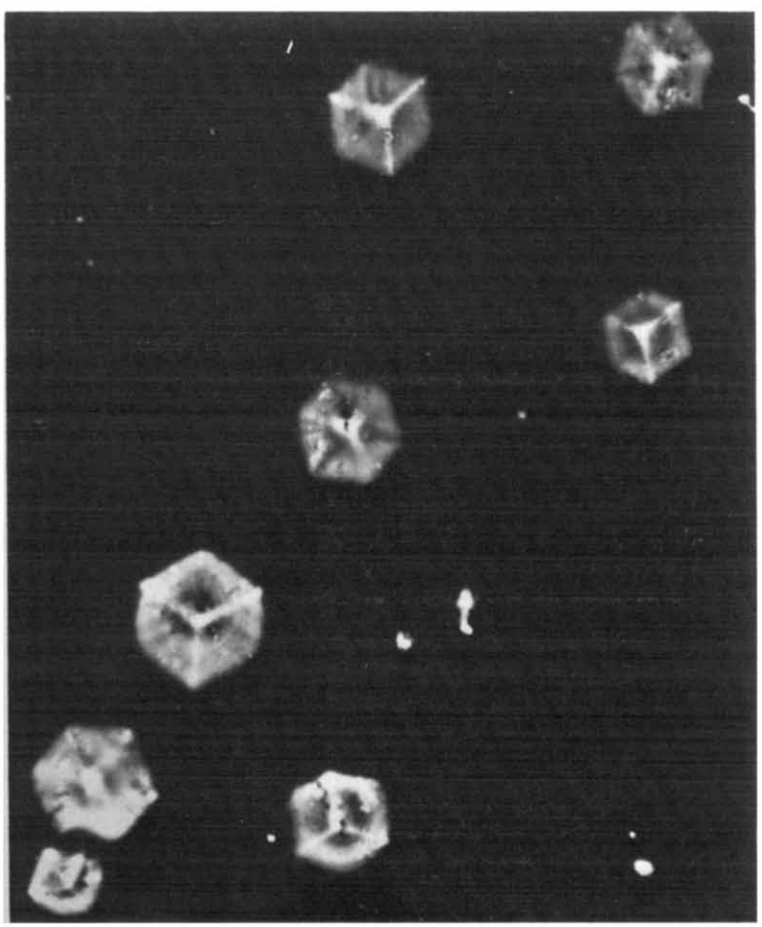

Fig. 1. Crystals of purine nucleoside phosphorylase from chicken liver in $(NH_4)_2SO_4$ solution. Phase contrast photomicrograph at 400-fold magnification.

centrations where the Lineweaver-Burk plots are linear. When inosine was the variable substrate with phosphate as a fixed substrate, the Lineweaver-Burk plots showed a crossing pattern in which the slope and the intercept changed. Similar patterns were obtained when phosphate was a variable substrate with inosine as a fixed substrate. From these results it was concluded, as reported by Kim et al. [16], that the predominant mechanism involved was a non ping-pong mechanism.

Product inhibition studies were also performed. The inhibition by the product, guanine or ribose 1-phosphate, was examined when inosine was the variable substrate below saturating concentration. Patterns of competitive inhibition were obtained in both cases as shown in Figs 5 and 6. When phosphate

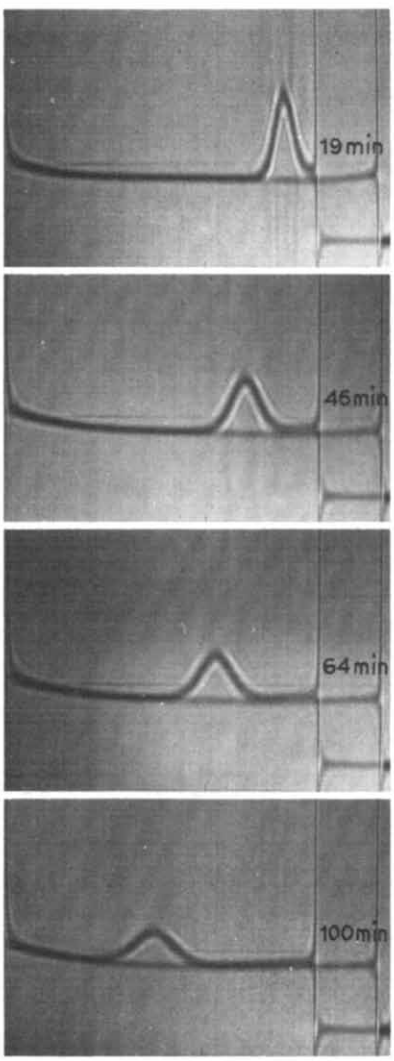

Fig. 2. Sedimentation pattern of crystalline purine nucleoside phosphorylase from chicken liver. The direction of centrifugation is from right to left. The photographs were taken with a phase-plate angle of 70° and at 19, 46, 64 and 100 min after reaching maximum speed. The other experimental conditions were described in the text.

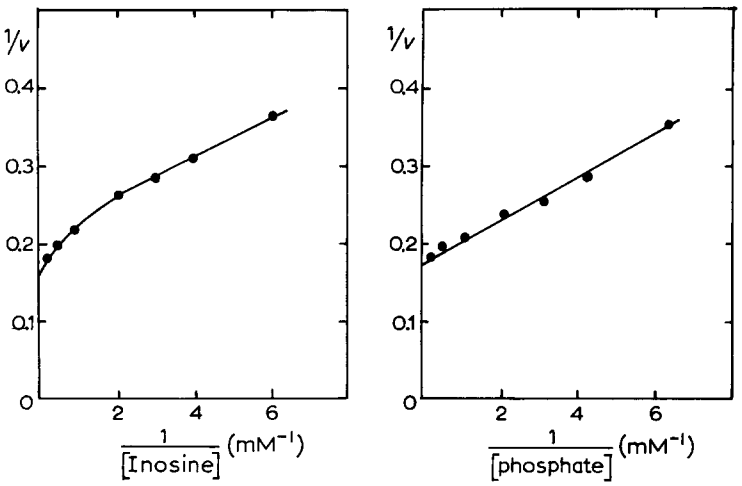

Fig. 3. The effect of concentration of inosine on the velocity of phosphorolysis. Plot of reciprocal of initial velocity with respect to reciprocal of inosine concentration. The reaction mixture contained 400 μmol of Tris/HCl buffer (pH 7.5), 60 μmol of phosphate buffer (pH 7.5), 400 units of xanthine oxidase, indicated amount of inosine and the enzyme preparation, in a final volume of 3 ml. Initial velocity was determined by Method II described in the text.

Fig. 4. The effect of concentration of phosphate on the velocity of phosphorolysis. Plot of reciprocal of initial velocity with respect to reciprocal of phosphate concentration. Conditions and methods were the same as in Fig. 3. The concentration of inosine was 18 μmol.

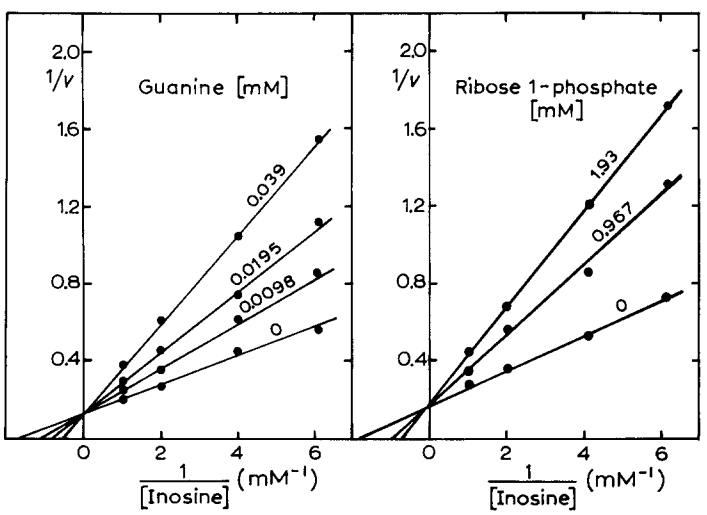

Fig. 5. Inhibition study with the alternative product guanine. Plot of reciprocal of initial velocity with respect to reciprocal of inosine concentration over a range of guanine concentrations. The reaction mixture contained, in addition to the indicated concentration of inosine and guanine, 340 μmol of Tris/HCl buffer (pH 7.5), 0.5 μmol of phosphate buffer (pH 7.5), 400 units of xanthine oxidase and the enzyme preparation in a final volume of 3 ml. Initial velocity was determined by Method II described in the text.

Fig. 6. Inhibition study with the product, ribose 1-phosphate. Plot of reciprocal of initial velocity with respect to reciprocal of inosine concentration over a range of ribose 1-phosphate concentrations. Conditions and methods were the same as in Fig. 5.

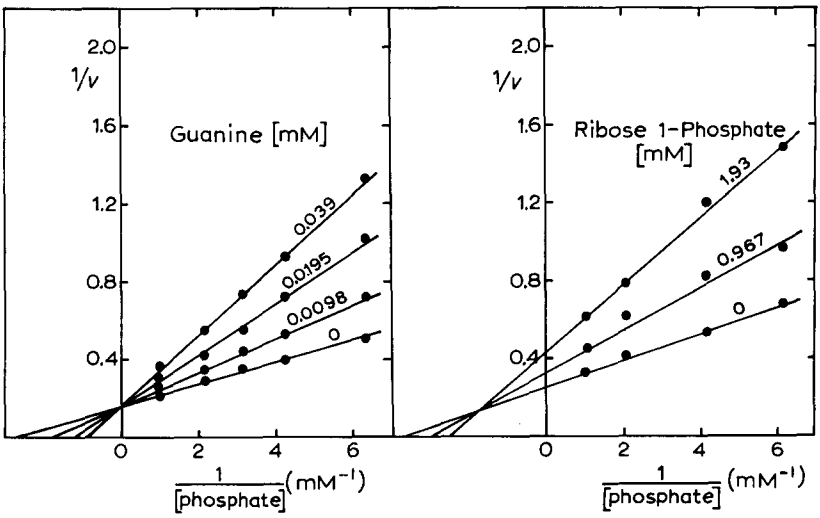

Fig. 7. Inhibition study with the alternative product guanine. Plot of reciprocal of initial velocity with respect to reciprocal of phosphate concentration over a range of guanine concentrations. The reaction mixture contained, in addition to the indicated concentration of phosphate and guanine, 340 μmol of Tris/HCl buffer (pH 7.5) 0.5 μmol of inosine, 400 units of xanthine oxidase and the enzyme preparation in a final volume of 3 ml. Initial velocity was determined by Method II described in the text.

Fig. 8. Inhibition study with the product, ribose 1-phosphate. Plot of reciprocal of initial velocity with respect to phosphate concentration over a range of ribose 1-phosphate concentrations. Conditions and methods were the same as in Fig. 7.

was the variable substrate with inosine at a fixed concentration below saturation, the inhibition experiments were done by using the products guanine or ribose 1-phosphate. Double reciprocal plots showed a competitive inhibition with guanine (Fig. 7) and noncompetitive inhibition with ribose 1-phosphate (Fig. 8), respectively. From the product inhibition studies, the reaction mechanism is assumed to be neither an "ordered Bi-Bi mechanism" nor a "random Bi-Bi mechanism", but a "rapid-equilibrium random Bi-Bi mechanism" as defined by Cleland [17]. The mechanism of erythrocyte enzyme has been reported as an "ordered Bi-Bi mechanism" [16,18,19].

Equilibrium constant

The apparent equilibrium constant of purine nucleoside phosphorylase from chicken liver was determined in order to compare it with those of the enzyme from ureotelic animals and others. These experiments were carried out according to the method described in the text. Starting with equimolar ratio of either inosine and phosphate or hypoxanthine and ribose 1-phosphate at pH 7.5, an equilibrium was reached at 70 min when 18% of inosine has been phosphorolyzed or 82% of hypoxanthine had been consumed to synthesize inosine. The apparent equilibrium constant for phosphorolysis was calculated to be 0.048. Equilibrium favors the reverse reaction, inosine synthesis, and its constant is approximately equal to the value of the enzymes from the ureotelic animals, fishes and microorganisms [20—25].

The other physical properties and the subunit structure of this enzyme will be discussed in the following papers.

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