Partial Purification and Properties of Purine Nucleoside Phosphorylase from Rabbit Erythrocytes

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1. The partial purification of purine nucleoside phosphorylase from rabbit erythrocytes is described. 2. Analytical and preparative isoelectric focusing gave a pI value for the enzyme of 4.65. 3. Gel-chromatography and sucrose-density-gradient-centrifugation techniques gave estimates of the molecular weight in the range 75 000–83 000. 4. Lineweaver–Burk plots of kinetic data were non-linear at high inosine concentrations. Extrapolation of the linear part of such plots yielded a K_m value for inosine of about $70 \mu\text{M}$ for the rabbit erythrocyte and liver enzymes. 5. A Hill interaction coefficient of 0.75 was obtained, suggesting negative co-operativity with respect to the binding of inosine. 6. Treatment of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) caused partial inactivation, and subsequent Lineweaver–Burk plots with inosine as substrate displayed complete linearity, with an increase in K_m value for inosine to $200 \mu\text{M}$. 7. Starch-gel electrophoresis did not reveal the presence of secondary isoenzymes; all tissue extracts examined gave electrophoretic patterns similar to those obtained with the partially purified enzyme from erythrocytes. 8. Results of hybridization studies with nucleoside phosphorylase from human foetal liver suggest that the rabbit enzyme is also a trimer.

Purine nucleoside phosphorylase (purine nucleoside-orthophosphate ribosyltransferase, EC 2.4.2.1) has been studied in a variety of mammalian tissues, including rat erythrocytes and bovine spleen (Agarwal *et al.*, 1975), and, in particular, human erythrocytes (Kim *et al.*, 1968*a,b*; Edwards *et al.*, 1971; Turner *et al.*, 1971; Agarwal *et al.*, 1975). Most of the enzymes studied share common properties, such as molecular weight and subunit structure, although differences in electrophoretic and kinetic properties, especially with regard to the phenomenon of substrate activation, have been demonstrated (Agarwal *et al.*, 1975). Evidence has been presented by Lewis & Glantz (1976) for the existence of a monomeric species of nucleoside phosphorylase in rabbit liver, with a molecular weight near 40 000. In contrast with this, we have obtained evidence that indicates that the enzyme from a variety of rabbit tissues, including the liver, has a trimeric subunit structure with a total molecular weight near 80 000.

In the present work the enzyme has been partially purified from rabbit erythrocytes, and preliminary studies on kinetic and electrophoretic properties are reported.

Experimental

Materials

Chemicals. Inosine and xanthine oxidase were obtained from BDH, Poole, Dorset, U.K. Phenazine methosulphate was a product of Ralph N. Emanuel, Wembley, Middx., U.K., and MTT tetrazolium salt

was from Wessex Biochemicals, Bournemouth, Hants., U.K. Hydrolysed starch was obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada. Sephadex (CM-, DEAE- and G-200) was obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. Ampholytes were supplied by LKB Instruments, South Croydon, Surrey, U.K.

Buffers. All pH measurements were done at room temperature (19°C). Anionic buffers were prepared by adding NaOH to the appropriate acid or base salt to give the required pH and molarity of anion. Similarly, unless otherwise stated, cationic buffers were prepared from the free base and HCl.

Methods

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis was carried out as described by Edwards *et al.* (1971).

Analytical isoelectric focusing. The pI value of the partially purified enzyme from rabbit erythrocytes was determined by isoelectric focusing in 5% (w/v) polyacrylamide gels containing LKB ampholytes. Gels were prepared in a mould ($0.1 \text{ cm} \times 16 \text{ cm} \times 20 \text{ cm}$) as described by Vesterberg (1972). Focusing was carried out for 24 h on a water-cooled plate with the current controlled to give a maximum power of 3 W.

Both starch and isoelectric-focusing gels were stained for enzyme activity with an agar overlay as described by Edwards *et al.* (1971).

Preparative isoelectric focusing. Electrofocusing was carried out in a sucrose gradient with 1% (w/v) Ampholine in the pH range 4–6 by using an LKB

focusing column (110 ml). A sucrose gradient, 0–64% (w/v), was prepared by the method described in the LKB 8101 Ampholine Electrofocusing Equipment Instruction Manual (LKB-Producter AB, S-161 25 Bromma 1, Sweden). The sample (2 ml) was a stroma-free rabbit haemolysate from which the haemoglobin had been removed by treatment with solid CM-Sephadex as described below. Electro-focusing was carried out at 4°C with an applied voltage of 500 V. After 24 h, fractions (1 ml) were collected and nucleoside phosphorylase activity and pH were determined for each fraction.

Determination of molecular weight. Approximate molecular weights were determined by gel filtration through Sephadex G-200 (Andrews, 1965) and by sucrose-gradient centrifugation (Martin & Ames, 1961). Sephadex G-200 equilibrated in 100 mM-Tris/acetate buffer, pH 7.5, was packed into a column (3 cm × 45 cm) and eluted with the same buffer at 4°C by using an operating pressure of 17 cm and a flow rate of 15 ml/h. The following proteins were used as standards: ovalbumin (mol.wt. 45 000); bovine serum albumin (mol.wt. 67 000); creatine kinase (mol.wt. 82 000); lactate dehydrogenase (mol.wt. 140 000); aldolase (mol.wt. 158 000). Sucrose-gradient centrifugation was carried out essentially as described by Martin & Ames (1961). Gradients were layered by hand by using 10, 15, 20, 25 and 30% (w/v) solutions of sucrose in 50 mM-Tris/HCl, pH 7.5. Solutions (1.2 ml) were carefully layered on to the surface of the preceding denser layer in cellulose acetate MSE centrifuge tubes, which were then left for approx. 3 h to equilibrate at 4°C. Just before centrifugation, the sample (0.1 ml) was carefully layered on to the surface of the gradient. When crude stroma-free lysate samples were used it was necessary to dilute the sample with the buffer so that the density of the sample did not exceed that of the uppermost sucrose layer. The following proteins were used as standards: carbonic anhydrase (mol.wt. 30 000); ovalbumin (mol.wt. 45 000); lactate dehydrogenase (mol.wt. 140 000). Centrifugation was done in an MSE Super-speed 75 ultracentrifuge precooled to 4°C, by using a swing-out titanium rotor (3 × 6.5 ml) at 50 000 rev./min for 16 h. After centrifugation approx. 16 fractions (eight drops per fraction) were collected from the top of each tube by using an Isco density-gradient fractionator (model 183). Fractions were analysed for nucleoside phosphorylase activity and protein as described below. From the relative migration of nucleoside phosphorylase and the standards in the gradient an estimate of the molecular weight was obtained.

Lactate dehydrogenase activity. This was measured in the direction lactate to pyruvate by the method of Cohen *et al.* (1964).

Carbonic anhydrase activity. The method described by Rickli *et al.* (1964) was used.

Purine nucleoside phosphorylase activity. This was measured spectrophotometrically by using xanthine oxidase in the coupled assay method of Kalckar (1947). Assays were carried out at room temperature (19°C) in a double-beam Unicam SP.1800 spectrophotometer connected to a Unicam AR 25 linear recorder, by using quartz cuvettes of 1 cm path-length. The standard reaction mixture contained 2 mM-inosine in 50 mM-sodium phosphate buffer, pH 7.5, and 0.06 units ($\mu\text{mol/min}$) of xanthine oxidase in a final volume of 3 ml. Reaction rate, as measured by the increase in A_{293} due to the formation of uric acid, was proportional to enzyme concentration provided that the absorbance change did not exceed 0.04/min. Protein concentration was measured as described by Brownson & Spencer (1972). One enzyme unit is expressed as the amount of enzyme that catalyses the phosphorolysis of 1 μmol of inosine/min, corresponding to an increase in A_{293} of 4.17/min, under the conditions described. Specific activity is expressed as units/mg of protein.

Hybridization experiments. These were performed by the procedures of Edwards *et al.* (1971), and only a brief outline is given here. Crude extracts of human foetal liver (age 16 weeks) and partially purified nucleoside phosphorylase from rabbit erythrocytes were used in these experiments. The liver extracts were prepared by homogenizing small pieces (0.3 g) of tissue in about 2 ml of 50 mM-sodium phosphate buffer, pH 6.5, containing 10 mM-mercaptoethanol, and removing cell debris by centrifugation at 18 000 rev./min for 20 min. Partially purified rabbit enzyme (specific activity 15 units/mg) in 50 mM-phosphate buffer, pH 6.5, was diluted to give a concentration of 1.0 unit/ml. Equal volumes (0.2 ml) of liver extract and enzyme solution were mixed, solid NaCl was added to give a final concentration of 2 M and the solution was frozen at –20°C. Subsequently the mixture was thawed and refrozen twice daily for 5 days and finally dialysed overnight against water containing 50 mM-mercaptoethanol. A similar mixture, but with NaCl omitted, and controls consisting of separate samples of liver extract and rabbit enzyme both with and without NaCl were subjected to the same treatment. After dialysis samples were analysed by starch-gel electrophoresis in the pH 6.5 buffer system of Edwards *et al.* (1971). The human foetal enzyme was chosen for these experiments, as previous results (Edwards *et al.*, 1973) had indicated that this enzyme was a trimer with three identical subunits and since its electrophoretic mobility at pH 6.5 was sufficiently different from that of the rabbit enzyme to allow the detection of possible hybrid forms.

Partial purification of purine nucleoside phosphorylase from rabbit erythrocytes. All purification procedures were carried out at 4°C. Erythrocytes from New Zealand White rabbits were separated and washed as described by Brownson & Spencer (1972).

Cells were lysed with an equal volume of water containing 5 mM-mercaptoethanol and cell debris was removed by centrifuging at 18000 rev./min for 50 min. The supernatant remaining was used in all experiments involving crude haemolysates and for further purification steps. All solutions contained 5 mM-mercaptoethanol. The large difference between the pI values of nucleoside phosphorylase and haemoglobin facilitated removal of the latter with CM-Sephadex by using the batchwise procedure described by Osborne & Spencer (1973). The resulting filtrate was adjusted to 60% saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (35.4 g/100 ml of haemoglobin-free solution) and the solution was left for about 1 h. The precipitate was then collected by centrifugation at 6000 rev./min for 30 min and redissolved in 50 mM-imidazole/HCl buffer, pH 6.0 (10 ml). The enzyme solution was dialysed for 24 h against the same buffer, concentrated by pressure dialysis on an Amicon Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) with a UM-10 filter and the enzyme concentrate (8 ml) applied to a column (2.4 cm $\times$ 40 cm) of DEAE-Sephadex (A50) equilibrated against 50 mM-imidazole/HCl, pH 6.0. The column was eluted with a linear gradient (200–400 mM) of NaCl in the same buffer, at a flow rate of 25 ml/h. Fractions (4 ml) were collected and analysed for nucleoside phosphorylase activity and protein. The Cl^- concentration was estimated by titration with AgNO_3 . Those fractions containing nucleoside phosphorylase activity were combined, concentrated by pressure dialysis (to 5 ml) and dialysed for 24 h against frequent changes of 50 mM-Tris/HCl, pH 7.5. The dialysed solution was

applied to a column (3 cm $\times$ 35 cm) of Sephadex G-150 equilibrated with 50 mM-Tris/HCl, pH 7.5, that was then eluted with the same buffer at a flow rate of 30 ml/h. Fractions (4 ml) were collected and analysed for nucleoside phosphorylase activity and protein. Fractions containing the enzyme were pooled and concentrated by pressure dialysis.

Results

Purification

Fig. 1 shows the elution profile obtained when a crude haemoglobin-free lysate was chromatographed on DEAE-Sephadex. Most of the protein impurities were eluted by 200–250 mM-NaCl (not shown in Fig. 1), but a concentration of 300 mM was required to elute the enzyme. The major protein contaminant remaining at this stage was removed by gel filtration through Sephadex G-150 (Fig. 2). A typical purification scheme is shown in Table 1. The variation of the data in Table 1 did not exceed $\pm 10\%$ for triplicate experiments.

A final specific activity of about 20 units/mg was achieved. Kim *et al.* (1968a) reported a value of 80 units/mg for a crystalline preparation of the enzyme from human erythrocytes, and the crystalline bovine spleen enzyme, which is available commercially (from Boehringer, Mannheim, Germany), has a specific activity of 22 units/mg.

Molecular weight

Crude haemolysates and partially purified enzyme gave values for the molecular weight in the range

Fig. 1. Chromatography of a haemoglobin-free rabbit erythrocyte lysate on DEAE-Sephadex. Experimental conditions are described in the text. ●, Nucleoside phosphorylase activity; ○, protein (A_{280}); ▲, [NaCl].

83000–85000 for triplicate determinations. Similar estimates were obtained for liver and brain homogenates from rabbits. Estimates of molecular weight by sucrose-gradient centrifugation were in the region 75000–78000. Both approaches gave values close to that of 80000 reported by Kim *et al.* (1968a) for the human enzyme. No evidence was found for the existence of a form with mol.wt. 46000, reported by Lewis & Glantz (1976), in the rabbit liver or in any of the tissue extracts examined.

Tissue distribution

Nucleoside phosphorylase activity was found in crude homogenates of the following rabbit tissues: liver, spleen, kidney, erythrocyte, brain and bone marrow. Preliminary experiments using starch-gel electrophoresis indicated the presence of a single active species of the enzyme in extracts of all these tissues (Fig. 3). The buffer system used was that devised by Edwards *et al.* (1971) to give maximum resolution of the multiple molecular forms of the

Fig. 2. Chromatography on Sephadex G-150 of the pooled and pressure-dialysed active fractions from the DEAE-Sephadex column (Fig. 1)

Experimental conditions are described in the text.
●, Nucleoside phosphorylase activity; ○, protein (A_{280}).

enzyme found in human erythrocyte lysates. When human lysates were run in parallel with rabbit tissue extracts, patterns of well-resolved zones of activity were observed similar to those described by the above authors (Fig. 3).

The additional zone of activity consistently observed in liver extracts may be protein-bound xanthine or hypoxanthine, both of which are intermediates in the linked enzyme-staining procedure used to detect nucleoside phosphorylase activity. This component was also visible when the substrate, inosine, was omitted from the usual staining mixture; its electrophoretic mobility is consistent with protein-bound rather than free xanthine or hypoxanthine, and this is confirmed by gel-filtration studies, which indicate a molecular weight of approx. 10000 for this component.

Isoelectric focusing

Preliminary experiments using Ampholine mixtures covering the pH range 3–10 on analytical plates consistently gave a pI value of about 4.6 for the enzyme. When partially purified enzyme was examined in a preparative column, by using a pH range of 4–6, a value of 4.65 was obtained (Fig. 4).

Hybridization experiments

Analysis of mixtures of human and rabbit nucleoside phosphorylase by starch-gel electrophoresis suggest that hybridization has occurred (Fig. 5). Sample 5 corresponds to a mixture of human foetal liver extract and partially purified rabbit erythrocyte enzyme; after the freezing and thawing procedure described in the Experimental section, four distinct bands of enzyme activity were obtained on subsequent electrophoresis. A comparison of the bands obtained from sample 5 with control samples shows that the band nearest to the anode corresponds to the rabbit erythrocyte enzyme, whereas the band nearest the cathode corresponds to the human foetal liver

Table 1. Purification of nucleoside phosphorylase from rabbit erythrocytes
For details see the text.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification factor	Recovery (%)
Stroma-free haemolysate (50ml)	50.0	2860	0.0175	1	100
Supernatant liquid remaining after CM-Sephadex treatment	47.5	136	0.350	20	95
Combined fractions containing nucleoside phosphorylase activity from DEAE-Sephadex column	25.0	7.1	3.500	200	50
Combined fractions containing nucleoside phosphorylase activity from Sephadex G-150 column	17.5	0.8	21.550	1225	35

Fig. 3. Starch-gel electrophoresis of rabbit tissue extracts stained for nucleoside phosphorylase activity. Extracts were prepared by homogenizing the tissue (1.5g) in about 3ml of water containing 5mM-mercaptoethanol, and removing cell debris by centrifugation at 18000rev./min for 20min. The conditions of electrophoresis were as described by Edwards *et al.* (1971) for human erythrocyte nucleoside phosphorylase.

Fig. 4. Preparative isoelectric focusing of rabbit erythrocyte nucleoside phosphorylase. Experimental details are described in the text. ●, Nucleoside phosphorylase activity; ○, pH.

enzyme. The two bands with intermediate electrophoretic mobilities appear to represent hybrids of human and rabbit nucleoside phosphorylase. Hence the four bands from sample 5 may be considered as corresponding to R_3 , R_2H , RH_2 and H_3 in order of decreasing anodic mobility, where R represents a single rabbit enzyme subunit and H the analogous human enzyme subunit.

The pattern of bands obtained in sample 5 indicates that NaCl is not necessary to induce formation of human/rabbit hybrid forms of the enzyme: by contrast Edwards *et al.* (1971) found that NaCl was apparently necessary to induce hybrid formation between the human and mouse enzymes. Since the

Fig. 5. Starch-gel electrophoresis of samples from the hybridization experiments after staining for nucleoside phosphorylase activity. Samples 1 and 2 correspond to a human foetal liver homogenate. Samples 3 and 4 correspond to a partially purified preparation of rabbit erythrocyte nucleoside phosphorylase. Samples 5 and 6 correspond to a mixture of the human and rabbit preparation. Sample 7 corresponds to a stroma-free rabbit haemolysate. Samples 2, 4 and 6 were made 2M in NaCl before the freezing and thawing procedure described in the Experimental section. For details see the text.

human foetal enzyme was used by Edwards *et al.* (1971) and in the present work, this apparent difference may reflect an increased tendency for the rabbit enzyme to dissociate into subunits compared with the mouse enzyme. However, it is not necessary to assume that dissociation into subunits is a prerequisite for hybrid formation, since it is possible that a mechanism involving intermediate complexes and subunit exchange may be involved.

Kinetic studies

When kinetic data for nucleoside phosphorylase from a variety of sources are plotted by the method of Lineweaver & Burk (1934), non-linear plots are obtained (Turner *et al.*, 1971; Agarwal *et al.*, 1975). In the present work, by using partially purified enzyme from rabbit erythrocytes, we have also obtained non-linear double-reciprocal plots from measurements of initial rates of phosphorolysis of inosine over a wide range of inosine concentrations at a fixed concentration of the second substrate, phosphate (Fig. 6). The kinetics were unaffected on addition of further xanthine oxidase. Results similar to those shown in Fig. 6 were obtained by using crude liver extracts and haemolysates: extrapolation of the linear part of such plots gave a K_m value for inosine of about 70 μM , which is similar to the range of values

Fig. 6. Effect of concentration of inosine on the initial velocity of the reaction catalysed by rabbit erythrocyte nucleoside phosphorylase

The reaction mixture contained 50mM-sodium phosphate buffer, pH 7.5, 0.06 EC unit of xanthine oxidase, 5 μ l of partially purified rabbit erythrocyte nucleoside phosphorylase (concentration 48 μ g/ml and specific activity 20 units/mg) and various amounts of inosine in a final volume of 3 ml. Conditions for the measurement of reaction rates were as described in the text, except that assays were performed at 40°C. The inset shows the results obtained by using a crude rabbit liver extract under identical conditions. Duplicate determinations were performed for each concentration of inosine; where single points are shown, duplicate results were identical.

Fig. 7. Effect of 5,5'-dithiobis-(2-nitrobenzoic acid) on the activity of rabbit erythrocyte nucleoside phosphorylase. Partially purified rabbit erythrocyte enzyme (concentration 37 μ g/ml and specific activity 18 units/mg) in 50mM-sodium phosphate buffer, pH 7.5, was incubated with 2mM-5,5'-dithiobis-(2-nitrobenzoic acid) at room temperature (19°C). Samples were removed after various time intervals and immediately assayed for nucleoside phosphorylase activity. The insert shows the double-reciprocal plot obtained for the enzyme activity remaining after treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) for 2h. Conditions were as described for Fig. 6.

(20–50 μM) reported by Turner *et al.* (1971) for the multiple forms of the enzyme in human erythrocyte lysates. Lewis & Glantz (1976) did not observe this type of phenomenon, but found that the purified rabbit liver enzyme gave double-reciprocal plots which curved upwards at high substrate concentrations.

Concave-downward plots like those in Fig. 6 are often formally described as examples of substrate activation at high concentrations, without any detailed explanation for the mechanism of such activation. These descriptions assume that velocities measured at low substrate concentrations represent uncomplicated Michaelis–Menten-type kinetics that become modified as substrate concentrations are increased (Kim *et al.*, 1968*b*). There seems to be no obvious reason for accepting this assumption in preference to the alternative interpretation that the observed changes in reaction velocity could be due to negative co-operativity of substrate binding. Some support for this suggestion is provided when the kinetic data are plotted in the form of a Hill-type plot; an interaction coefficient of 0.75 may be indicative of negative co-operative binding of inosine by the enzyme. Experiments with 5,5'-dithiobis-(2-nitrobenzoic acid) may also be relevant in this context; like the human enzyme, rabbit nucleoside phosphorylase is partly inactivated, in a time-dependent manner, on incubation with this reagent (Fig. 7). The enzymic activity remaining after this treatment gave perfectly linear double-reciprocal plots of reaction velocity versus inosine concentration, with a K_m value for inosine of 200 μM . It may be significant that this value for the K_m is close to that obtained by extrapolation of the downward-curving part of the plots shown in Fig. 6.

Discussion

Rabbit liver nucleoside phosphorylase has been studied in some detail (Lewis & Glantz, 1976), but only limited data are available for the erythrocyte enzyme (Duhm, 1974). Many of the properties reported here for the erythrocyte enzyme contrast with those reported by Lewis & Glantz (1976) for the rabbit liver enzyme. The molecular-weight and hybridization studies reported here indicate that the enzyme is a trimer with a total molecular weight close to 80000; no evidence was found for a form of the enzyme of molecular weight close to 40000 in the liver, or in any of the other tissues examined. Lewis & Glantz (1976) determined molecular weights by means of Sephadex gel filtration and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. It is possible that these authors could have obtained an erroneous value for the molecular weight by both methods if their enzyme preparation

contained a major protein contaminant, particularly since column eluates were apparently monitored by estimating protein concentration rather than by activity measurements.

As indicated in the section on kinetics the data here are also at variance with those of Lewis & Glantz (1976). However, our results are similar to those reported for the enzyme from several species, indeed the concave-downward double-reciprocal plots observed in the present work appear to be characteristic of the enzymes from human (Kim *et al.*, 1968*b*), rat (Agarwal *et al.*, 1975), pig and guinea-pig erythrocytes (Duhm *et al.*, 1974). A possible explanation of this phenomenon is that these preparations contain two or more isoenzyme forms having significantly different kinetic properties. For the human erythrocyte enzyme, however, this explanation appears unlikely since Agarwal *et al.* (1975) found that each of the six isoenzyme forms, isolated by preparative isoelectric focusing, displayed similar kinetic behaviour to the unfractionated starting material. Further, our present data indicate that most of the nucleoside phosphorylase activity in rabbit erythrocytes is represented by a single molecular form of the enzyme. It has been suggested that the curved double-reciprocal plots observed with this enzyme could be the result of co-operative binding interactions between subunits (Agarwal *et al.*, 1975; Kim *et al.*, 1968*a*); the possibility that the co-operativity may be negative does not appear to have been proposed. The preliminary results reported here for the rabbit enzyme are consistent with a model, similar to that proposed by Conway & Koshland (1968), in which the binding of inosine to one subunit induces changes in the other subunits so that their affinity for inosine is decreased.

The possible existence of an equilibrium between the trimeric form of the rabbit erythrocyte enzyme and its constituent subunits cannot be ruled out as a cause of the observed kinetic behaviour; if the degree of dissociation of the trimer varies with substrate concentration and if dimers and/or monomers are enzymically active but with significantly different kinetic properties, then kinetics of the type observed could result. In the present work we have not examined this possibility in any detail. However, gel-filtration experiments have not revealed any differences in the apparent molecular weight of the enzyme when it was passed through columns of Sephadex G-200 in the absence or in the presence of either or both substrates.

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