Chinese Hamster Purine-Nucleoside Phosphorylase: Purification, Structural, and Catalytic Properties†

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ABSTRACT: Purine-nucleoside phosphorylase (EC 2.4.2.1; purine-nucleoside:orthophosphate ribosyltransferase) was purified to apparent homogeneity from Chinese hamster liver and kidneys and from V79 tissue culture cells. The enzymes from both sources appear to have identical structural and catalytic properties. A simple rapid radioisotope assay capable of detecting 0.1 nmol of product for both directions of the purine-nucleoside phosphorylase reaction is described using Bio-Rad Cu^{2+} Chelex in Pasteur pipet columns. At 37 °C, the purified enzyme converts 60 μmol of guanine to guanosine per min per mg of protein. Electrophoresis in sodium dodecyl sulfate-polyacrylamide gels indicates that the enzyme is composed of identical subunits of 30 000 molecular weight. The native enzyme behaves as a mixture of dimers of 68 000 molecular weight and trimers of 89 000 molecular weight during Sephadex G-100 chromatography. Sucrose gradient centrifugation indicates that the enzyme has a sedimentation coefficient of 5.4 S, which corresponds to a molecular weight of 94 000 and suggests a trimer structure. The enzyme displays Michaelis-Menten kinetics with apparent Michaelis constants of 20 μM for both hypoxanthine and guanine, 35 μM for guanosine, 50 μM for inosine, and 200 μM for both ribose 1-phosphate and phosphate. During isoelectrofocusing, the enzyme forms a single major band at a p*I* of 5.25.

Purine-nucleoside phosphorylase (EC 2.4.2.1; purine-nucleoside:orthophosphate ribosyltransferase) in eucaryotes catalyzes the reversible conversion between the purine bases, hypoxanthine and guanine, and their corresponding nucleosides, inosine and guanosine:

$$\text{(hypoxanthine or guanine)} + \text{ribose-1-P}^{1} \underset{\text{"breakdown"}}{\overset{\text{"synthesis"}}{\rightleftharpoons}} \text{(inosine or guanosine)} + P_i$$

The literature on this enzyme has been reviewed by Friedkin and Kalckar (1961) and by Parks and Agarwal (1972). The equilibrium constant for the purine-nucleoside phosphorylase reaction strongly favors nucleoside synthesis (Friedkin and Kalckar, 1961; Parks and Agarwal, 1972):

$$K_{eq} = \frac{[\text{purine}][\text{ribose-1-P}]}{[\text{nucleoside}][P_i]} = 0.018$$

Since there are no known eucaryotic nucleoside kinases capable of converting inosine or guanosine to their corresponding nucleotides, IMP or GMP, it is generally assumed that purine-nucleoside phosphorylase functions in vivo in the direction of nucleoside breakdown. The enzyme hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) synthesizes IMP or GMP from the corresponding purine bases, hypoxanthine or guanine. Thus, purine-nucleoside phosphorylase in concert with hypoxanthine-guanine phosphoribosyltransferase could recycle nucleosides formed during nucleic acid breakdown, into nucleotides for nucleic acid synthesis. Both enzymes are found in high concentrations in erythrocytes, liver, and kidneys.

Although the eucaryotic enzymes involved in the interconversion of purine bases, nucleosides, and nucleotides have been studied in detail, little is known about their regulatory role. Deficiencies in hypoxanthine-guanine phosphoribosyltransferase produce the physiological and neurological symptoms of the Lesch-Nyhan syndrome (Lesch and Nyhan, 1964), although tissue culture cells survive without the enzyme. The high concentrations of purine-nucleoside phosphorylase and hypoxanthine-guanine phosphoribosyltransferase in erythrocytes suggest that these cells may transport nucleotide precursors around the body. There appears to be a linkage between nucleoside metabolism and the immune system. The absence of adenosine deaminase, the enzyme that converts adenosine into inosine, results in combined immunological deficiency (Hirschhorn, 1975). Recently, a patient with severe defective T-cell immunity was shown to be lacking purine-nucleoside phosphorylase (Giblett et al., 1975). Purine analogues are frequently used in cancer chemotherapy, because they are toxic to rapidly growing cells utilizing purine salvage pathways. A better understanding of purine metabolism might suggest improved methods of treatment.

The study of purine metabolism in tissue culture cells offers two major advantages over that in animals or organs. First, it is easier to alter the concentration of components outside the cells. Second, by examining mutants selected in tissue culture, one may be able to elucidate the in vivo role of purine enzymes. We have chosen to work with Chinese hamster cells because they offer many advantages over other cell lines for genetic and biochemical studies (Puck, 1972). In a previous paper, we described the purification and properties of Chinese hamster hypoxanthine-guanine phosphoribosyltransferase (Olsen and Milman, 1974). In this paper, we describe the purification and properties of purine-nucleoside phosphorylase from Chinese hamster V79 tissue culture cells and from liver and kidneys. In addition, we describe direct, sensitive, and rapid radioisotope

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[1] Abbreviations used: P and P_i, phosphate and inorganic phosphate, respectively; EDTA, ethylenediaminetetraacetic acid; tricine, *N*-tris(hydroxymethyl)methylglycine; tris, tris(hydroxymethyl)aminomethane.

assays for both directions of the purine-nucleoside phosphorylase reaction.

Experimental Procedure

Enzyme Assays. Cu^{2+} Chelex Columns. Both the nucleoside synthesis assay and the nucleoside phosphorylase assay for purine-nucleoside phosphorylase utilize Cu^{2+} Chelex prepared by a modification of the procedure described by Goldstein (1967). Typically, 250 g of Chelex 100 (Bio-Rad) is allowed to stand overnight in 2 l. of 0.5 M $CuCl_2$, then washed with 10 l. of H_2O and 1 l. of 1 N NH_4OH, and stored in 1 N NH_4OH. This preparation is used for the nucleoside synthesis assay. For the nucleoside phosphorylase assay, it is essential that the free Cu^{2+} ions be removed with 0.1 M EDTA to decrease nucleoside leakage through the columns. Generally, 250 g of Cu^{2+} Chelex is rinsed with 2 l. of 0.1 M EDTA, then with 4 l. of H_2O, and stored in H_2O. The Cu^{2+} Chelex is loaded into Pasteur pipet columns (3.5 × 0.5 cm) containing a glass-fiber plug. We usually use Cu^{2+} Chelex columns only once. They can be regenerated, first by washing with 25 ml of 5 N NH_4OH, and then with either 12 ml of 1 N NH_4OH for the nucleoside synthesis assay or with 25 ml of H_2O for the nucleoside phosphorylase assay.

Nucleoside Synthesis Assay. The formation of guanosine from guanine and ribose 1-phosphate is measured in a radioisotope assay. A standard assay mixture contains: 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 2 mM dithiothreitol, 1.15 mM ribose 1-phosphate (Sigma, dicyclohexylammonium salt), 80 μM guanine, [8-^{14}C]guanine (Amersham/Searle, 55 mCi/mmol) to give 20 000 to 200 000 cpm per assay, and an enzyme sample in a final volume of 100 μl. Bovine serum albumin is added to the partially purified enzyme fractions where indicated to improve the stability of the enzyme. A reaction mixture is incubated for 15 min at 37 °C, and the incubation is terminated by adding 150 μl of 1 N NH_4OH. A 200-μl aliquot is removed and applied to a Cu^{2+} Chelex column equilibrated in 1 N NH_4OH. Nucleoside is eluted with 1.5 ml of 1 N NH_4OH and counted in 10 ml of scintillation fluid (9.1 g of 2-5-diphenyloxazole, 0.61 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 2140 ml of toluene, and 1250 ml of Triton X-100) in a Packard Tri-Carb scintillation counter with a counting efficiency of 80%.

The formation of inosine is measured in an analogous manner by replacing guanine with hypoxanthine and [8-^{14}C]guanine with [8-^{14}C]hypoxanthine (Schwarz, 53.7 mCi/mmol). Background cpm in the absence of enzyme of less than 0.5% of the initial cpm in the assay were obtained by purifying the ^{14}C-labeled purine bases from the suppliers prior to use. ^{14}C-labeled purine base was adsorbed to a standard Cu^{2+} Chelex column in 1 N NH_4OH, washed with 1 N NH_4OH, eluted with 5 N NH_4OH, and lyophilized.

Nucleoside Phosphorylase Assay. The conversion of guanosine to guanine and ribose 1-phosphate is measured by the formation of [^{14}C]ribose 1-phosphate from [U-^{14}C]guanosine. A standard assay mixture contains 50 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 10 mM potassium phosphate (pH 7.8), 75 μM guanosine, [U-^{14}C]guanosine (Amersham/Searle, 464 mCi/mmol, or ICN, 220 mCi/mmol) to give 20 000 to 60 000 cpm per assay, and an enzyme sample in a total volume of 100 μl. Bovine serum albumin is added to partially purified enzyme fractions where indicated to improve the stability of the enzyme. A reaction mixture is incubated for 15 min at 37 °C, and the incubation is terminated by adding 150 μl of H_2O and immediately placing the mixture in an 80 °C water bath for 10 min. A 200-μl aliquot of the cooled mixture is then applied to a Cu^{2+} Chelex column equilibrated in H_2O. The [^{14}C]ribose 1-phosphate produced during the reaction is eluted with 1.5 ml of H_2O and counted in 10 ml of scintillation fluid as described above. The unreacted guanosine and guanine produced during the enzyme reaction remain bound to the Cu^{2+} Chelex column. Rate calculations are based on the assumption that 50% of the radioactivity in [U-^{14}C]guanosine is in the ribose 1-phosphate moiety.

The formation of ribose 1-phosphate during the enzyme-mediated phosphorylation of inosine is measured in an analogous manner by replacing quanosine with inosine and [U-^{14}C]guanosine with [U-^{14}C]inosine (Amersham/Searle, 505 mCi/mmol). Background cpm in the absence of enzyme of less than 1% of the initial cpm in the assay was obtained using the ^{14}C-labeled nucleoside from the supplier without further purification.

Purification of Chinese Hamster Purine-Nucleoside Phosphorylase. All procedures were performed at 0–5 °C unless otherwise specified. All enzyme fractions were stored in a liquid-nitrogen freezer. Enzyme activity was measured by the formation of guanosine from guanine and ribose 1-phosphate in the nucleoside synthesis assay described above. Protein concentrations were determined by the Lowry method with crystalline bovine serum albumin as a standard unless otherwise indicated.

V79 Tissue Culture Cell Enzyme. Crude Extract. Chinese hamster V79 cells (Ford and Yerganian, 1958) were grown in suspension to a density of 0.5 to 1 × 10^6 per ml in Joklik's minimum essential media (Gibco) supplemented with nonessential amino acids (Gibco), 1 mM sodium pyruvate, 50 mM Tricine (pH 7.6), and 5% fetal bovine serum (Irvine Scientific). The cells were harvested by centrifugation and resuspended in 2 volumes of enzyme buffer (20 mM Tris-HCl (pH 7.8), 6 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol) containing 120 mM KCl. The cells were lysed by freezing and stored at −70 °C. The lysate from 25 ml of packed cells was thawed and centrifuged at 20 000*g* for 20 min and the supernatant was saved. The pellet was resuspended in an additional 10 ml of the same buffer and centrifuged as described above. The two supernatants were combined to form the crude extract.

High-Speed Supernatant Fraction. The crude extract (76 ml) was centrifuged for 2.5 h at 60 000*g* in a Spinco 40 rotor. The supernatant formed the high-speed supernatant fraction.

DEAE-Sephadex Fraction. To 73 ml of high-speed supernatant fraction was added 18.25 ml of enzyme buffer containing 20 mM KCl to adjust the KCl concentration to 100 mM. The adjusted enzyme fraction (90 ml) was applied to a column (0.9 × 20 cm) containing DEAE-Sephadex A-50 equilibrated in enzyme buffer containing 100 mM KCl. The column was washed with 40 ml of the same buffer, and then the enzyme was eluted in a 400-ml, 100–270 mM KCl linear gradient in enzyme buffer. Fractions of 4 ml were collected and assayed for enzyme activity. Fractions containing peak enzyme activity (70–93) were pooled and concentrated to 1.9 ml using an Amicon ultrafiltration cell with a PM-10 membrane.

G-100 Sephadex Fraction. DEAE-Sephadex fraction enzyme (1.6 ml) was mixed with 0.15 ml of glycerol and applied to a column (1.5 × 90 cm) containing G-100 Sephadex equilibrated in enzyme buffer containing 100 mM KCl. The column was developed with the same buffer, and 2-ml fractions were collected and assayed for enzyme activity. Fractions containing enzyme activity (31–38) were pooled and concentrated to 1.4 ml using an Amicon ultrafiltration cell with a PM-10 mem-

TABLE I: Purification of Chinese Hamster V79 Tissue Culture Cell Purine Nucleoside Phosphorylase.

Fraction	Protein Concn (mg/ml)	Spec Act. (units/mg)[a]	Cumulative Recovery (%)	Purification (fold)
Crude extract	21.4	0.14	100	1
High-speed supernatant	12.6	0.16	79	1.1
DEAE-Sephadex	19.5	1.59	26	11
G-100 Sephadex	3.8	11.8[b]	33	84
Isoelectric focusing	0.08[c]	60.0[b]	13	430

[a] One unit is defined as the amount of enzyme which catalyzes the formation of 1 μmol of guanosine per min at 37 °C in the nucleoside synthesis assay. [b] Assay includes 1 mg per ml of bovine serum albumin. [c] Protein concentration determined from intensity of enzyme band on sodium dodecyl sulfate-polyacrylamide gel in comparison with protein standards.

brane. The G-100 Sephadex fraction was used for the K_m determinations.

Isoelectric Focusing Fraction. A 110-ml, 0–46% linear sucrose gradient with 1% pH 5 to 7 ampholytes and 2 mM dithiothreitol was prepared manually in an LKB Model 8180 ampholine column following the procedure in the LKB manual. G-100 Sephadex fraction enzyme (0.5 ml) was applied to the middle of the gradient, replacing the "light" solution in that region. The electrofocusing was conducted for 5 days at 3–5 °C. During that time, the voltage was increased from 300 to 800 V. One-milliliter fractions were collected, and the pH and enzyme activity were measured. Fractions containing peak enzyme activity (72–73) were pooled.

Chinese Hamster Liver and Kidney Enzyme. The livers and kidneys (29.4 g) from 12 adult male Chinese hamsters were homogenized in 30 ml of enzyme buffer. The homogenate was centrifuged for 20 min at 20 000*g* and the supernatant was saved. The pellet was homogenized with an additional 30 ml of enzyme buffer and centrifuged as above. The two supernatant fractions were combined to form the crude extract (2.6 g of protein). The specific activity of the liver and kidney crude extract was 0.1 unit/mg as compared with 0.14 unit/mg for the V79 tissue culture cells crude extract. Additional purification steps were analogous to those described for the purification from V79 tissue culture cells. The specific activity of the G-100 Sephadex fraction used for K_m determinations was 11.1 units/mg as compared with 11.8 units/mg for the G-100 Sephadex fraction purified from V79 tissue culture cells.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in vertical gel slabs (0.8 mm thick) as previously described (Olsen and Milman, 1974). Standards of chymotrypsinogen, and carbonic anhydrase were purchased from Worthington. Ovalbumin, ribonuclease A, bovine serum albumin, bovine hemoglobin, and glucose-6-P dehydrogenase (*Leuconostoc mesenteroides*) were obtained from Sigma. Hexokinase and lactate dehydrogenase were purchased from Boehringer Mannheim.

Results

Product formation in both the nucleoside synthesis and nucleoside phosphorylase assays is directly proportional to enzyme concentration and a linear function of time for at least 1 h. The products eluted from the Cu^{2+} Chelex columns were characterized by thin-layer chromatography as described by Randerath (1961). In all cases, the nucleosides synthesized cochromatographed with the nucleoside of the purine base used as substrate, and the ^{14}C-labeled "ribose 1-phosphate fraction" cochromatographed with a ribose 1-phosphate standard (data not shown).

Table I summarizes the purification of purine-nucleoside

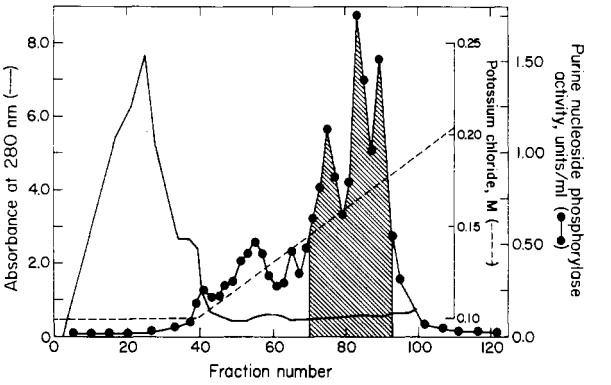

FIGURE 1: DEAE-Sephadex chromatography of Chinese hamster purine-nucleoside phosphorylase. Enzyme activity (●—●) was measured by the nucleoside synthesis assay. The fractions indicated by cross-hatching were pooled. The dashed line indicates the KCl gradient. The protein content is indicated by absorbance at 280 nm (—). Other details are described in Experimental Procedure.

phosphorylase from approximately 25 g of Chinese hamster V79 tissue culture cells. A crude extract was prepared by freezing the cells, and cellular debris was removed by centrifugation. Microsomes and additional cell debris were removed by high-speed centrifugation, and the supernatant was adsorbed to a DEAE-Sephadex column. While the majority of the protein passed through the column, the purine-nucleoside phosphorylase activity was retained, and then eluted by a linear KCl gradient in an irregular peak from 0.14 to 0.18 M KCl as shown in Figure 1. No additional activity was removed by higher salt concentrations. Only the most active fractions were pooled resulting in a recovery for this step of 33%. This value may be artificially low since the recovery increased after the G-100 Sephadex step. The concentrated DEAE-Sephadex fraction was applied to a Sephadex G-100 column and the enzyme activity eluted as a single peak separated from the majority of the protein as shown in Figure 2. In the final purification step, isoelectric focusing, purine-nucleoside phosphorylase was located in a single major peak corresponding to an isoelectric pH of 5.25 as shown in Figure 3. A minor peak at a lower isoelectric point may be due to a modification of a labile amino acid (Turner et al., 1971; and our unpublished observations) during purification or storage. The purification of purine-nucleoside phosphorylase from Chinese hamster liver and kidneys was analogous to that from the tissue culture cells, and the purified enzyme also focused at an isoelectric pH of 5.25.

A sodium dodecyl sulfate-polyacrylamide gel of the electrofocusing enzyme fraction (Figure 4) showed a single protein band. Enzyme activity correlated with the intensity of this band

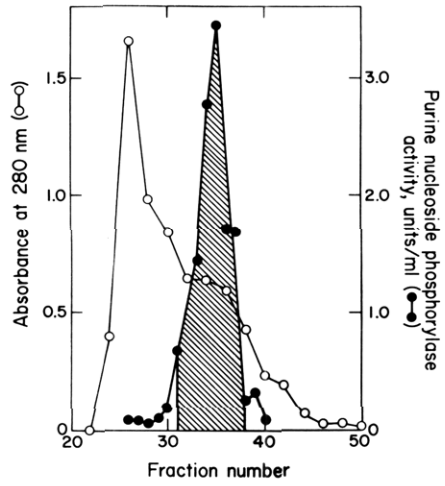

FIGURE 2: G-100 Sephadex chromatography of Chinese hamster purine-nucleoside phosphorylase. Enzyme activity (●—●) was measured by the nucleoside synthesis assay. The fractions indicated by cross-hatching were pooled. The protein content is indicated by absorbance at 280 nm (o—o). Other details are described in Experimental Procedure.

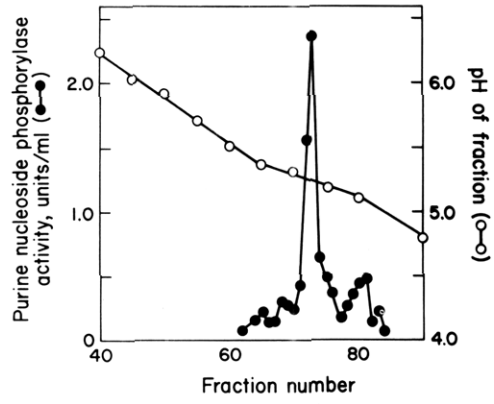

FIGURE 3: Isoelectric focusing of Chinese hamster purine-nucleoside phosphorylase. Isoelectric focusing was performed as described in Experimental Procedure. Enzyme activity (●—●) was measured by the nucleoside synthesis assay. The pH of the fractions is indicated by the open circles (o—o). Other details are described in Experimental Procedure.

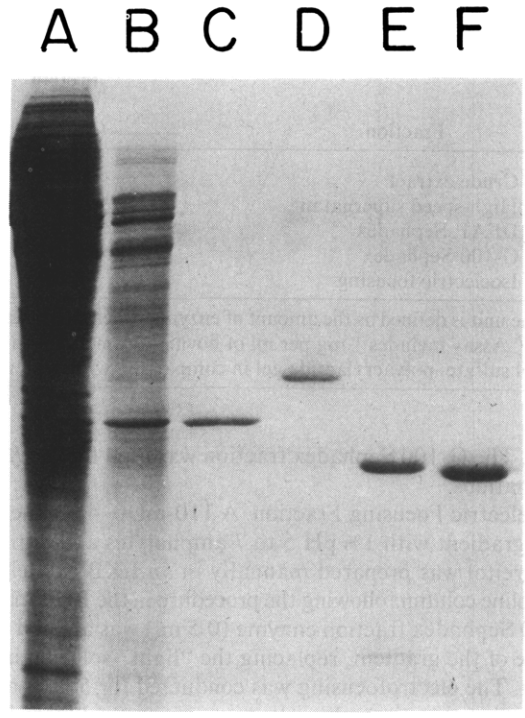

FIGURE 4: Sodium dodecyl sulfate—polyacrylamide gel electrophoresis of Chinese hamster purine-nucleoside phosphorylase enzyme fractions. Electrophoresis was performed as described in Experimental Procedure. The samples are: (A) DEAE-Sephadex fraction enzyme, 60 μg; (B) G-100 Sephadex fraction enzyme, 7.6 μg; (C) isoelectric focusing fraction enzyme, 1.6 μg; (D) lactate dehydrogenase, 1 μg; (E) chymotrypsinogen, 3 μg; (F) human hypoxanthine-guanine phosphoribosyltransferase, 3 μg.

observed in fractions from the isoelectric focusing column run on sodium dodecyl sulfate–polyacrylamide gels (data not shown), strongly suggesting that this band is the purine-nucleoside phosphorylase subunit. Figure 4 shows that the purine-nucleoside phosphorylase band is a major component of the G-100 fraction and is distinguishable in the DEAE-Sephadex fraction. The mobility of purine-nucleoside phosphorylase in sodium dodecyl sulfate–polyacrylamide gels is slightly less than carbonic anhydrase (29 000 molecular weight). The molecular weight of purine-nucleoside phosphorylase is 30 000 determined from a linear plot of the log of the molecular weight vs. the mobilities of standard proteins run in the same sodium dodecyl sulfate–polyacrylamide slab gel. The proteins, their molecular weights, and relative mobilities are: lactate dehydrogenase (36 000, 3.84), purine-nucleoside phosphorylase (30 000, 4.36), carbonic anhydrase (29 000, 4.41), chymotrypsinogen (25 700, 4.81), and RNase (13 700, 7.01).

The native molecular weight of purine-nucleoside phosphorylase was determined by gel filtration. The elution profile of the enzyme on an analytical Sephadex G-100 column (Figure 5) showed a major peak centered at a molecular weight of 68 000 and a shoulder centered at a molecular weight of 89 000. The same skewed pattern has been consistently obtained in repeated experiments. These data suggest that, during Sephadex G-100 chromatography, purine-nucleoside phosphorylase may dissociate from a trimer (molecular weight = 3 × 30 000) to a dimer (molecular weight = 2 × 30 000). Fraction 59 from the high-molecular-weight side of the purine-nucleoside phosphorylase peak and fraction 67 from the low-molecular-weight side of the peak were separately applied to the same Sephadex G-100 column. The elution profiles of enzyme activity for both fractions were essentially identical with that of the initial enzyme sample, suggesting that dimers and trimers of purine-nucleoside phosphorylase are in equilibrium.

The native molecular weight of purine-nucleoside phosphorylase was also determined by sucrose gradient centrifugation. In sucrose gradient centrifugation (Figure 6), purine-nucleoside phosphorylase migrated between glucose-6-phosphate dehydrogenase and hemoglobin with a sedimentation coefficient of 5.6 S. The molecular weight of 5.6S protein calculated relative to glucose-6-phosphate dehydrogenase and hemoglobin assuming proportionality to $(s_{20,w})^{3/2}$ (Martin and Ames, 1961) is 94 000, implying that the enzyme is a trimer (molecular weight = 3 × 30 000).

Purine-nucleoside phosphorylase displays Michaelis–Menten kinetics for ribose 1-phosphate, purines, nucleosides, and phosphate when one substrate is in limiting amounts and the other is in excess. Double-reciprocal Lineweaver–Burk plots of $(\text{velocity})^{-1}$ vs. $(\text{substrate})^{-1}$ for hypoxanthine, guanine, inosine, and guanosine are shown in Figure 7A, and for ribose 1-phosphate and phosphate in Figure 7B. The velocities

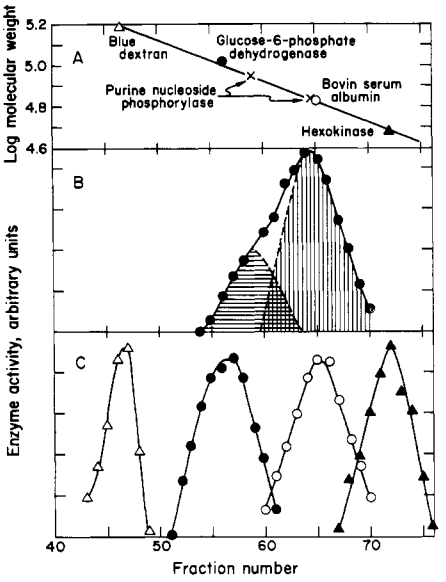

FIGURE 5: Native molecular weight of Chinese hamster purine-nucleoside phosphorylase determined by gel filtration on Sephadex G-100. A 0.68-ml sample containing 60 μg of the G-100 fraction of purine-nucleoside phosphorylase, 20 μg of glucose-6-P dehydrogenase from *Leuconostoc mesenteroides,* 3.6 mg of bovine serum albumin, and 250 μg of yeast hexokinase was applied to a column (1.5 × 90 cm) containing Sephadex G-100 equilibrated in enzyme buffer. Fractions of 1.0 ml were collected. In panel B, purine-nucleoside phosphorylase was located by the nucleoside synthesis assay (●—●). A separation of the peak into its probable components is indicated by the dashed lines and cross-hatching. In panel C, glucose-6-P dehydrogenase (●---●) and hexokinase (▲—▲) were located as described by Olsen and Milman (1974). The void volume of the column was determined with blue dextran (△—△), located by the absorbance at 660 nm. The molecular weights of the standards are: bovine serum albumin, 68 000; glucose-6-P dehydrogenase, 103 700; yeast hexokinase, 48 000 at pH above neutral; blue dextran, 150 000 apparent molecular weight on G-100 Sephadex. In panel A, the log of the molecular weight is plotted as a function of the elution volume.

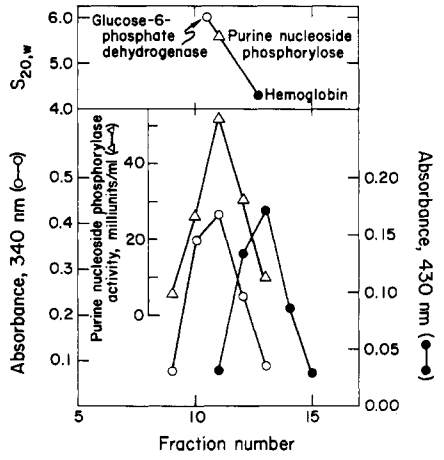

FIGURE 6: Sedimentation coefficient determination of Chinese hamster purine-nucleoside phosphorylase. A 110-μl sample containing 10 μg of glucose-6-P dehydrogenase, 200 μg of bovine hemoglobin, and 30 milliunits of G-100 Sephadex fraction purine-nucleoside phosphorylase in enzyme buffer was applied to a 5.2-ml, 6 to 20% linear sucrose gradient. After centrifugation for 13 h at 4 °C in a Spinco L-2 ultracentrifuge at 45 000 rpm in a SW 60.1 swinging bucket rotor, 17 fractions of 0.31 ml were collected and assayed for purine-nucleoside phosphorylase activity (△—△) by the nucleoside synthesis assay. Glucose-6-P dehydrogenase (○—○) has a molecular weight of 103 700 and a sedimentation coefficient of 6.0 S and was located by an assay measuring the increase in absorbance at 340 nm as described by Olsen and Milman (1974). Hemoglobin (●—●) has a molecular weight of 64 000 and a sedimentation coefficient of 4.3 S and was located by diluting 100-μl samples with 300 μl of enzyme buffer and measuring the absorbance at 430 nm.

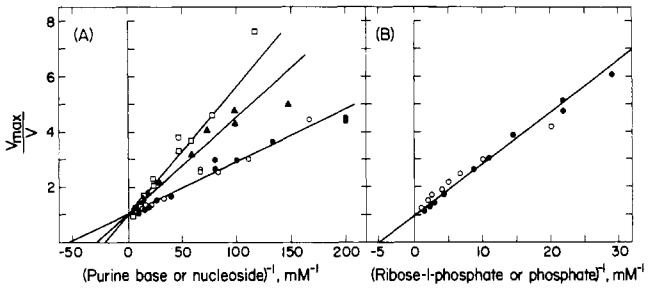

FIGURE 7: Determination of kinetic constants for Chinese hamster purine-nucleoside phosphorylase. The V79 cell G-100 fraction enzyme was used in the illustrated studies, but identical results were obtained from the liver and kidneys G-100 fraction enzyme. All assays contained 0.1 mg/ml bovine serum albumin and other modifications as described below. Velocity (V) is the amount of nucleoside or ribose 1-phosphate formed per minute, and V_{max} is determined from the ordinate intercept of the Lineweaver-Burk plot of V^{-1} vs. (substrate)$^{-1}$. The amount of enzyme used in each study is expressed in units of activity in the nucleoside synthesis assay with guanine as substrate. (A) Guanine (●—●) and hypoxanthine (○—○) K_m data were determined with 76 μunits of enzyme in the nucleoside synthesis assay, except that the concentration of ribose 1-phosphate was 1.75 mM. V_{max} was 76 pmol/min for guanine and 52 pmol/min for hypoxanthine. Guanosine (▲—▲) and inosine (□—□) K_m data were determined in the nucleoside phosphorylase assay with 151 and 227 μunits of enzyme, respectively. V_{max} was 64 pmol/min for guanosine and 119 pmol/min for inosine. (B) Ribose 1-phosphate (○—○) K_m data were determined with 222 μunits of enzyme in the nucleoside synthesis assay with 125 μM guanine as substrate, and the V_{max} was 222 pmol/min. Phosphate (●—●) K_m data were determined with 222 μunits of enzyme in the nucleoside phosphorylase assay with 276 μM inosine as substrate, and the V_{max} was 131 pmol/min. The ratio of the specific activity of the enzyme for other substrates relative to that for guanine is 0.68 for hypoxanthine, 0.58 for inosine, and 0.42 for guanosine.

are expressed as V_{max}/V so that the data for nucleoside synthesis and for nucleoside phosphorolysis can be plotted on the same scale. The ratio of the specific activity of the enzyme for other substrates relative to that for guanine is 0.68 for hypoxanthine, 0.58 for inosine, and 0.42 for guanosine. The apparent K_m values are 20 μM for both hypoxanthine and guanine, 35 μM for guanosine, 50 μM for inosine, and 200 μM for both ribose 1-phosphate and phosphate. Identical results were obtained from the G-100 fraction of the liver and kidneys enzyme and from the V79 cell enzyme.

Discussion

The radioisotope assays we have developed to measure the purine-nucleoside phosphorylase reaction in both directions are extremely sensitive and have the added advantage of measuring a direct product of the reaction. We can easily detect 1 μunit of enzyme activity in our standard assays. The assay sensitivity is 100- to 1000-fold greater than the coupled xanthine oxidase spectrophotometric assay of Kalckar (1947) used by most investigators. The high sensitivity enables 100-fold dilution of crude extracts in an assay, thus minimizing the effect of other components in an extract and extending the linearity of product formation with time.

Purine-nucleoside phosphorylase from Chinese hamster liver and kidneys appears identical with that in V79 tissue culture cells. Based on the specific activities of the crude extracts, the enzyme represents 0.2% of the soluble protein in the liver and kidneys or in V79 tissue culture cells. An extract from 10^7 cultured Chinese hamster cells contains approximately 1 mg of soluble protein. Based on a native molecular weight of 90 000, each cell contains approximately 3×10^5 enzyme molecules of purine-nucleoside phosphorylase. We have previously shown that Chinese hamster cells also contain ap-

proximately 3×10^5 enzyme molecules of hypoxanthine-guanine phosphoribosyltransferase, which is sufficient to totally satisfy a cell's requirement for nucleotides (Olsen and Milman, 1974). Since the turnover number for the phosphorylase activity of purine-nucleoside phosphorylase is approximately twofold higher than for hypoxanthine-guanine phosphoribosyltransferase, a cell using both enzymes could supply its total needs for nucleotides from nucleosides.

The structural and catalytic properties of Chinese hamster nucleoside phosphorylase are quite similar to those of the enzyme isolated from other eucaryotes. The purified Chinese hamster enzyme specific activity in the synthesis direction is 60 units/mg, compared with specific activities of 30, 45, and 96 units/mg observed for the purified chicken (Murakami and Tsushima, 1975) and bovine and human (Agarwal and Parks, 1969) enzymes, respectively. Chinese hamster purine-nucleoside phosphorylase has a single p*I* of 5.25, which is lower than that of the bovine enzyme with a p*I* of 5.4, or the human erythrocyte enzyme which has multiple p*I*'s between 5.85 and 6.25 (Agarwal et al., 1975). The Chinese hamster enzyme sedimentation coefficient of 5.4 S determined by sucrose gradient centrifugation is close to the value of 5.6 S determined for the chicken enzyme by analytical ultracentrifugation (Murakami and Tsushima, 1975). The Chinese hamster subunit molecular weight of 30 000 is identical with that reported by Agarwal et al. (1973) and Edwards et al. (1973). Our evidence for a trimer enzyme structure is in agreement with substrate binding studies (Agarwal and Parks, 1969), genetic analysis, and electrophoretic and sedimentation studies of the human erythrocyte and bovine spleen enzymes (Edwards et al., 1971, 1973). It is interesting that the *Bacillus cereus* enzyme which is composed of 24 000 molecular weight subunits has a native molecular weight of 80 000 to 95 000 in the presence of phosphate but dissociates to 47 000 in the absence of phosphate (Gilpin and Sandoff, 1971). This dissociation may be similar to that observed for the Chinese hamster enzyme during Sephadex G-100 chromatography. The *Escherichia coli* and *Salmonella typhimurium* enzymes also are composed of 25 000 molecular weight subunits, but they associate to form hexamers (Jensen and Nygaard, 1975).

The apparent Michaelis constants for Chinese hamster purine-nucleoside phosphorylase are close to the lowest values observed for the enzyme from other eucaryotes (Parks and Agarwal, 1972). Lineweaver–Burk plots of $(\text{velocity})^{-1}$ vs. $(\text{substrate})^{-1}$ show good fits with straight lines. The apparent Michaelis constants for Chinese hamster purine-nucleoside phosphorylase are 20–50 μM for both purine bases and nucleosides, and 200 μM for both ribose 1-phosphate and phosphate. A simple interpretation is that the enzyme contains two sites: one site which has an affinity for the purine base either free or as part of a nucleoside, and a second site which has an affinity for phosphate, either free or as part of ribose 1-phosphate. One cannot tell from the kinetic data the in vivo direction of the purine-nucleoside phosphorylase reaction. However, in the synthetic direction purine-nucleoside phosphorylase would have to compete for purine base with the enzyme hypoxanthine-guanine phosphoribosyltransferase which has a 20–40-fold lower K_m (Olsen and Milman, 1974). Although the equilibrium constant for the purine-nucleoside phosphorylase reaction favors nucleoside synthesis, the V_{max} we observe for phosphorolysis of guanosine is only one-half that for synthesis, and the V_{max} for phosphorolysis of inosine is approximately the same as that for synthesis. Thus, it is not unlikely that in vivo the enzyme catalyzes predominantly nucleoside breakdown.

We have tried to alter the level of purine-nucleoside phosphorylase activity in Chinese hamster V79 tissue culture cells. The specific activity of the enzyme in a crude cell extract is not changed if the cells are grown in the presence or absence of hypoxanthine, or in the presence of aminopterin which blocks de novo synthesis of purines (data not shown). We have also attempted to select for cells lacking purine-nucleoside phosphorylase activity. Szybalski (Szybalski et al., 1962) initially demonstrated that cells lacking hypoxanthine-guanine phosphoribosyltransferase activity could be selected by growing cells in the presence of purine analogues (e.g., 6-thioguanine), and revertants could be selected by growing cells in the presence of an inhibitor of de novo purine synthesis (e.g., aminopterin in "HAT media" also containing hypoxanthine, thymidine, and glycine). We reasoned that mutants in purine-nucleoside phosphorylase could be selected by growing cells in "HAT media" containing a purine nucleoside analogue (e.g., 6-thioguanosine). The nucleoside analogue should have no effect on cells, unless it were first converted to the purine base and then to the nucleotide. The presence of aminopterin in the media should ensure that cells maintain hypoxanthine-guanine phosphoribosyltransferase in order to synthesize nucleotides. Therefore, surviving cells ought to have a deficiency in purine-nucleoside phosphorylase or in nucleoside transport. We have selected cells resistant to 6-thioguanosine in "HAT media," but they all retained normal purine nucleoside phosphorylase activities in cell extracts. Further studies are required to determine if these cells are deficient in nucleoside transport, and if mutants in purine-nucleoside phosphorylase can be obtained.

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Heart Phosphofructokinase: Allosteric Kinetics with Fructose 6-Sulfate†

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ABSTRACT: The allosteric regulation of heart phosphofructokinase was studied at pH 6.9 with an alternative substrate, fructose 6-sulfate. The alternative substrate allowed kinetic studies to be carried out at high enzyme concentrations (0.1 mg/ml) where the effect of allosteric ligands on enzyme physical structure has been studied. A K_m for ATP binding (8-10 μM) in the presence of saturating AMP concentrations was found which agreed well with the value obtained at pH 8.2. ATP inhibitory effects closely followed saturation of its substrate site. Hill plots for ATP inhibition gave an interaction coefficient of 3.5, indicating cooperativity between at least four enzyme subunits. Neither AMP nor fructose 6-sulfate affected the cooperativity between the ATP inhibitory sites but only increased the inhibitory threshold. As the ATP concentration was increased from suboptimal to inhibitory levels, interaction coefficients for AMP and fructose 6-sulfate changed from 1 to 2. Increasing citrate concentration resulted in an increase in the interaction coefficient for fructose 6-sulfate to a value of 1.9. Citrate inhibition was synergistic with ATP inhibition with an interaction coefficient of 2. The data indicate that allosteric kinetics of the enzyme can be shown at high enzyme concentrations with the alternative substrate. ATP inhibition appears to involve interaction between at least four subunits, while citrate, AMP, and fructose 6-sulfate interact minimally with two subunits.

The important role played by phosphofructokinase in regulating glycolysis is reflected in its complex allosteric regulation (cf. reviews by Mansour (1972) and by Lardy and Bloxham (1973)). The enzyme is inhibited by increasing concentrations of ATP, the phosphoryl donor substrate. The kinetic effect of ATP inhibition is manifested by a lowered affinity and an increased cooperative response to the second substrate, fructose 6-phosphate. The allosteric inhibitor, citrate, displays a kinetic effect similar to that of ATP, and its inhibitory action is dependent on the concentration of ATP. Allosteric inhibition is counteracted by the products of the phosphofructokinase reaction, ADP and fructose-1,6-P_2,[1] as well as by the allosteric effectors AMP and cyclic AMP. The kinetic effect of these activators is opposite to that of ATP and citrate. The enzyme is also activated by divalent anions HPO_4^{2-} and SO_4^{2-} as well as by monovalent cations K^+ and NH_4^+.

Physical studies of phosphofructokinase have demonstrated that the enzyme exists in a variety of polymeric forms which undergo transformations in response to enzyme concentration, metabolic effectors, temperature alteration, and pH changes. Information on enzyme structure and ligand binding properties carried out at high enzyme concentration (mg/ml) is often correlated with kinetic studies utilizing enzyme concentrations at μg/ml levels. Since the degree of polymerization of certain regulatory enzymes affects their kinetic behavior and is dependent on their concentration, it is possible that the kinetic data obtained on highly diluted enzyme may not apply to the enzyme in concentrated form.

We reported that fructose 6-sulfate can be used as an alternative substrate for the enzyme (Martensen and Mansour, 1976b). The use of this substrate for studies on phosphofructokinase allosteric regulation seemed to offer several advantages. The reduced catalytic efficiency of greater than 30-fold and the decreased binding affinity by 100-fold allowed activity measurements at enzyme concentrations two orders of magnitude greater than that possible with the native substrate. The effect of enzyme concentration on allosteric regulation could therefore be investigated.

Experimental Procedure

Materials. Fructose 6-sulfate was synthesized by sulfurylation of fructose. The 6-sulfuryl ester was isolated and purified by a procedure which was reported before (Martensen and Mansour, 1976b). Nucleotides, fructose-6-P, fructose-1,6-P_2, and dithiothreitol were purchased from Sigma Chemical Co. Pyruvic kinase, lactic dehydrogenase, both from rabbit skeletal muscle, and phosphoenolpyruvate were purchased from Boehringer Mannheim Corporation.

Methods: Preparation and Assay of Phosphofructokinase. Sheep heart enzyme was purified and crystallized by the procedure described previously (Mansour et al., 1966; Lorenson and Mansour, 1969). The low catalytic activity of phosphofructokinase with the alternative substrate allowed the use of high concentrations of the enzyme to study its allosteric properties at pH 6.9. All assays described herein contained

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[1] Abbreviations used: fructose-6-P, fructose 6-phosphate; fructose-1,6-P_2, fructose 1,6-diphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide.