Bovine Brain Purine-Nucleoside Phosphorylase Purification, Characterization, and Catalytic Mechanism†

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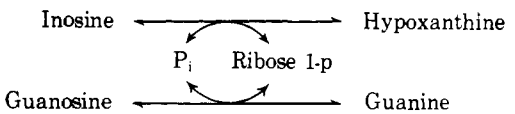
ABSTRACT: Bovine brain purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) was purified to homogeneity at a specific activity of 78 μmol min^{-1} mg of protein^{-1}. A molecular weight of 78 000–80 000 was calculated for the native enzyme by gel filtration on Sephadex. Gel electrophoresis in the presence of sodium dodecyl sulfate indicated subunits of molecular weight of 38 000. Chemical and kinetic studies strongly implicated histidine and cysteine as catalytic groups at the active site of the enzyme. The pK_a's determined for ionizable groups at the active site of the free enzyme were 5.8 and 8.2. Enzyme completely inactivated by *p*-chloromercuribenzoate was partially reactivated by adding an excess of 2-mercaptoethanol to the inactivated enzyme. A strong susceptibility to photooxidation in presence of methylene blue was observed. Photoinactivation was pH dependent, implicating histidine as the susceptible group at the active site. A rapid loss of catalytic activity upon incubation at 55 °C suggested heat lability. An activation energy of 9.6 kcal/mol was calculated. The nature of the catalytic mechanism of the enzyme was investigated, and initial velocity studies showed linear converging patterns of double-reciprocal plots of the data, consistent with a sequential catalytic mechanism. The product inhibition pattern was at variance with both the ordered Bi-Bi and random mechanisms. The observed competition between purine and nucleoside, and between inorganic orthophosphate and ribose 1-phosphate for this ordered mechanism, suggest a Theorell-Chance mechanism. Michaelis constants determined for substrates of the enzyme were 4.35×10^{-5} M for guanosine, 3.00×10^{-5} M for guanine, and 2.15×10^{-2} M for inorganic orthophosphate.

Purine nucleoside phosphorylase catalyzes the phosphorolysis and synthesis of nucleosides with almost equal efficiency. The following reaction scheme (Kim et al., 1968a) reflects the reversibility of the catalytic mechanism:



The enzyme has been isolated from various sources and a few of its properties have been examined in crude preparations of mammalian and avian organs and tissues, and in yeast and bacteria (Klein, 1935; Kalckar, 1947; Friedkin and Kalckar, 1950; Manson and Lampen, 1951; Heppel and Hilmoe, 1952; Korn and Buchanan, 1955; Abrams et al., 1965; Zimmerman et al., 1971; Murakami et al., 1971; Agarwal et al., 1975). Tsuboi and Hudson (1957) reported a 510-fold purification of the enzyme from human erythrocytes, and Kim et al., (1968a) reported the purification of the same enzyme to homogeneity and partial characterization. Within recent years the commercial enzyme from bovine spleen (Boehringer Ltd.) was determined homogeneous and partially characterized (Edwards et al., 1973). The enzyme from rabbit liver (Lewis and Glantz, 1976) and the enzyme from rabbit brain (Lewis, 1973) were also purified to homogeneity and partially characterized.

Major physical differences in the enzymes from the various sources have been reported. The heterogeneity of the human erythrocyte enzyme has been reported (Agarwal et al., 1973, 1975). The subunit structure of this enzyme and the commercial enzyme from bovine spleen has been elucidated, and three subunits of molecular weight 30 000 were reported for these enzymes (Agarwal et al., 1973). The crystalline commercial bovine spleen enzyme (Boehringer Ltd.), which was found to be homogeneous by electrophoresis on cellulose-acetate strips, was similarly resolved into three subunits of molecular weight 28 000 (Edwards et al., 1973). A monomeric species of the enzyme, isolated and purified to homogeneity from rabbit liver, showed a molecular weight of 45 000 ± (2000) by gel filtration and 38 000 by electrophoresis on polyacrylamide in presence of sodium dodecyl sulfate. This enzyme was homogeneous by isoelectric focusing (Lewis and Glantz, 1976). A similar preparation of the enzyme from rabbit brain, which was homogeneous by polyacrylamide disc gel electrophoresis, showed similar physical properties (Lewis, 1973).

This paper describes the purification to homogeneity, partial characterization, and catalytic mechanism of bovine brain purine-nucleoside phosphorylase. A molecular weight of 78 000–80 000 was determined by gel filtration of this enzyme on Sephadex G-200. A subunit molecular weight of 38 000 was determined by sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis.

An Ordered Bi-Bi mechanism was reported for human erythrocyte purine-nucleoside phosphorylase (Kim et al., 1968b). The product inhibition pattern for bovine brain purine-nucleoside phosphorylase suggests a Theorell-Chance mechanism.

Experimental Procedure

Materials. Frozen bovine brains were purchased from Pel Freez Biologicals (Rogers, Arkansas). DEAE[1]-cellulose (0.9 mequiv/g) and hydroxylapatite were purchased from Bio-Rad

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[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

(Richmond, Calif.). Nucleosides and purines, ribose 1-phosphate, and protein standards were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Phosphate buffers refer to mixtures of dibasic sodium phosphate and monobasic potassium phosphate. Citrate buffers were prepared from sodium citrate and citric acid solutions. All buffers, except buffers used for the enzyme assay, were made 10 mM with 2-mercaptoethanol just prior to use, unless otherwise stated.

Protein Determination. The protein was determined after the method of Zamenhof (1957).

Enzyme Assay. Method 1—Phosphorolysis of Nucleosides. Enzyme solutions of known protein concentrations were assayed in aliquots of 50 μl. The enzyme solution was added to a 3.0-ml cuvette, which was previously incubated at 40 °C in the thermostated cuvette compartment of the Beckman DU spectrophotometer. An aliquot of substrate (2.5 ml of purine-nucleoside, in 0.2 M phosphate buffer, pH 7.0) at 40 °C was added. After mixing, the disappearance of the substrate was recorded as ΔOD min^{-1} mg of protein^{-1} at the absorption maximum of the substrate. The results were converted to μmol of substrate hydrolyzed min^{-1} mg of protein^{-1} as shown below:

$$\left(\frac{\Delta\text{OD min}^{-1}\text{ mg of protein}^{-1}}{E_s - E_p}\right) \times \text{total volume of assay mixture in ml}$$

where E_s and E_p are the calculated extinctions of substrate (E_s) and products (E_p) at 1.0 μmol/ml, in 0.2 M phosphate buffer (pH 7.0), at the absorption maximum of the substrate only.

Method 2—Synthesis of Nucleosides. The ribosylation of purines in enzymatic synthesis of nucleosides was examined in 0.2 M citrate buffer (pH 7.0). Enzyme preparations were dialyzed against 0.1 M citrate buffer (pH 7.0) and 10 mM 2-mercaptoethanol prior to use, and an aliquot of 50 μl of known protein concentration was preincubated as before in a 3.0-ml cuvette. An aliquot of 50 μl (1.0 μmol) of ribose 1-phosphate solution was added, and followed by the addition of 2.5 ml (2.0×10^{-4} M) of purine substrate in 0.2 M citrate buffer (pH 7.0) at 40 °C. After mixing, the increase in absorbance of the product was recorded as ΔOD min^{-1} mg of protein^{-1}, at the absorption maximum of the product. The results were converted to μmol of nucleoside synthesized min^{-1} mg of protein^{-1}, as in the preceding example. In this case, $E_p - E_s$ was substituted for $E_s - E_p$.

The absorption maxima for some substrates of the enzyme in 0.2 M phosphate buffer (pH 7.0) are guanosine and deoxyguanosine (252 nm), inosine (249 nm), xanthosine (248 nm), and guanine (246 nm).

Active Site Studies. Initial velocity studies were made between pH 5.0 and 9.0, with guanosine as variable substrate in 0.2 M phosphate buffer (pH 7.0). The results were fitted to double-reciprocal plots (Lineweaver and Burk, 1934) for determination of Michaelis constants and maximal velocities for the enzyme. Plots of pK_M vs. pH and log V_{max} vs. pH, after the method of Dixon (1953), were utilized for the determination of ionizable groups at the active site of the free enzyme, and in the enzyme-substrate complex.

Inactivation of the Enzyme by Photooxidation. The high susceptibility of bovine brain purine-nucleoside phosphorylase to photoinactivation, in presence of methylene blue, allowed for simple apparatus to be utilized in the process. In this investigation, an adjustable desk lamp with reflector, fitted with a 100-W tungsten light bulb, served as the source of irradiation. The lamp reflected upwards through a tripod enclosed with white paper. At the upper end of the cone, a beaker containing crushed ice and water served as the receptacle for exposure of the sample to the emergent light rays, approximately 15 cm above the light source.

The buffer (0.05 M phosphate buffer, pH 7.0), with which the samples were prepared, was oxygenated by shaking vigorously with several volumes of air. Test samples consisting of 200 μg of homogeneous enzyme protein, in 1.0 ml of methylene blue solution (10 mg% in 0.05 M phosphate buffer, pH 7.0) were irradiated in a test tube (1.5 × 10.0 cm) with gentle agitation. Aliquots of 0.1 ml were removed every 30 s and immediately transferred to 0.4 ml of 0.2 M phosphate buffer (pH 7.0), containing 10 mM 2-mercaptoethanol, and stored in the dark for assay for residual activity. Samples that were stored in the dark in presence of 2-mercaptoethanol retained residual activity.

Light controls consisting of enzyme (200 μg of protein), in 0.05 M phosphate buffer (pH 7.0), were similarly irradiated and diluted in the dark prior to assay.

The dependence of photoinactivation of the enzyme on pH was examined between pH 6.0 and 9.0, at intervals of 0.5 pH units. Solutions of methylene blue were prepared by dilution in 0.05 M phosphate buffer at each of the above pHs. The method of irradiation and dilution were the same as before, except for a fixed period of 1-min irradiation for tests and controls. Aliquots were assayed immediately after dilution.

Purification of the Enzyme. All purification procedures were performed between 0 and 7 °C, and all buffers used in the purification process contained 10 mM 2-mercaptoethanol, unless otherwise specified.

Step 1—Homogenate Extract. Frozen bovine brains (1.69 kg) were homogenized in 4 l. of 0.2 M phosphate buffer (pH 7.5), containing 20 mM 2-mercaptoethanol, and centrifuged at 20 000*g* for 30 min at 0 °C. The supernatant (3820 ml containing 44.69 g of protein) was removed for fractionation with ammonium sulfate.

Step 2—Ammonium Sulfate Fractionation. The supernatant was fractionated in two steps (0–35% saturation then 35–70% saturation) near pH 7.0 with solid ammonium sulfate. Percent saturation was determined after Dawson et al. (1969). Enzyme activity was concentrated in the fraction which precipitated between 35 and 70% saturation. The precipitated protein was reconstituted in 0.2 M phosphate buffer (pH 7.5) containing 20 mM 2-mercaptoethanol and dialyzed overnight against running tap water near 7 °C. Precipitated proteins were removed by centrifugation, and the enzyme solution (600 ml containing 14.10 g of protein) was adjusted to pH 8.0 with dilute Tris base and made 10 mM with 2-mercaptoethanol.

Step 3—First DEAE-Cellulose Chromatography. The enzyme solution from the previous step was adsorbed on a column of DEAE-cellulose with bed dimensions of 4.8 × 30.0 cm, at a flow rate of 150 ml/h. The column was then washed with 2 (column bed) volumes of 0.05 M phosphate buffer (pH 8.0), followed by 2 volumes of the same buffer at pH 7.5. The flow rate during these washings was increased to 250 ml/h. The bound enzyme was then released by elution with 0.05 M citrate buffer (pH 6.5) and fractions of 25 ml were collected. Fractions with specific activity greater than twice the adsorbed protein were pooled and fractionated in two steps (0–45% saturation and 45–70% saturation) with solid ammonium sulfate near pH 7.0. Almost total enzyme activity was recovered in the reconstituted fraction, which precipitated between 45 and 70% saturation. The increase in total units in steps 2 and 3 may be

TABLE I: Summary of Purification Steps.

Step	Procedure	Vol. (ml)	Total Protein (mg)	Sp. Act. μmol min^{-1} mg of protein^{-1}	Total Units	Yield	Purification Factor
1	Homogenate extract	3820	44 690	0.018	804	100	1
2	$(NH_4)_2SO_4$ Fractionation	600	14 100	0.08	1,128	140	4
3	First DEAE-cellulose chromatography	38.5	1890	0.62	1,172	146	31
4	Second DEAE-cellulose chromatography	6.2	70	4.83	338	42.2	237
5	Chromatography on hydroxylapatite	4.5	5.1	43.20	220	36.1	2,117
6	Third DEAE-cellulose chromatography	2.0	2.95	73.50	217	27.0	3,603
7	Selective pption with saturated $(NH_4)_2SO_4$ soln	2.0	2.30	78.30	180	22.4	3,840

attributed to the elimination of inhibitors present in the crude homogenate.

Step 4—Second DEAE-Cellulose Chromatography. The dialyzed enzyme from the preceding step (38.5 ml containing 1.89 g of protein) was adsorbed on a column of DEAE-cellulose with bed dimensions of 2.8 × 20.0 cm. The column was washed as in step 3, and the flow rate adjusted to 100 ml/h. Enzyme activity was slowly released by elution with 0.025 M citrate buffer (pH 6.8), and fractions of 10.0 ml were collected. Fractions with activity greater than twice the adsorbed material were pooled and fractionated with ammonium sulfate (0–50% saturation and 50–70% saturation). The 50–70% precipitate was reconstituted in 5.0 ml of 0.05 M phosphate buffer (pH 7.5) at 4 °C, containing 5 mM 2-mercaptoethanol, and dialyzed against 3 l. of 0.01 M phosphate buffer (pH 7.5) at 4 °C.

Step 5—Chromatography on Hydroxylapatite. The dialyzed enzyme (6.2 ml containing 70 mg of protein) was adsorbed on a column of hydroxylapatite (1.5 × 10.0 cm) that was equilibrated against 0.01 M phosphate buffer (pH 7.5), containing 5 mM 2-mercaptoethanol. The column was washed with 3 (column) volumes of 0.04 M phosphate buffer (pH 7.5), and the enzyme was then eluted with 0.08 M phosphate buffer (pH 7.5). Fractions of 3.0 ml were collected, and fractions with activity greater than fourfold that of the adsorbed material were pooled and precipitated at 80% saturation with ammonium sulfate. The precipitated protein was reconstituted with buffer (pH 7.5) to 3.0 ml, and dialyzed against 1.5 l. of 0.01 M phosphate buffer (pH 7.5) at 4 °C, containing 5 mM 2-mercaptoethanol.

Step 6—Third DEAE-Cellulose Chromatography. The enzyme solution from the preceding step (4.5 ml containing 5.1 mg of protein) was adsorbed on a column of DEAE-cellulose (1.5 × 15.0 cm), and eluted in a manner identical to that of step 5. Fractions of 4.0 ml were collected, and fractions with specific activity greater than the adsorbed protein were pooled and precipitated at 80% saturation with solid ammonium sulfate near pH 7.0. At this stage of the purification of the enzyme, polyacrylamide disc gel electrophoresis showed a trace of protein contamination (see Figure 1A).

Step 7—Selective Precipitation with Ammonium Sulfate. The reconstituted enzyme (2.0 ml containing 2.95 mg of protein) was brought to a slight turbidity with saturated ammonium sulfate solution (pH 7.0). The resulting precipitate was removed by centrifugation. The supernatant was then brought to a definite turbidity with saturated ammonium sulfate solution and stored overnight at 4 °C. The precipitated enzyme was recovered by centrifugation and reconstituted to 2.0 ml (2.3 mg of protein) with 0.2 M phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and stored at 4 °C. A summary of the purification procedures is given in Table I.

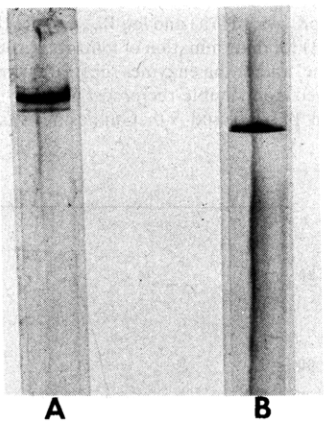


FIGURE 1: (A, B) photograph of polyacrylamide disc electrophoresis of enzyme during the final stages of purification. Pattern A was observed after purification step 6, and pattern B after step 7.

Results

Criteria of Purity of the Enzyme. Homogeneity of the enzyme was established by (1) polyacrylamide disc gel electrophoresis after the method of Davis (1964). Gels stained by Coomassie brilliant blue technique of Weber and Osborn (1969) showed a single homogeneous disk of protein (Figure 1B). (2) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) also showed a single disk of the homogeneous enzyme.

Molecular Weight of the Enzyme. The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-200 after the method of Andrews (1964). The calculated molecular weight of 78 000–80 000 agreed well with the documented values for the enzymes from bovine spleen (Agarwal et al., 1973; Edwards et al., 1973) and human erythrocytes (Kim et al., 1968a; Agarwal and Parks, 1969; Agarwal et al., 1973). The molecular weight calculated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) was 38 000, suggesting the possibility of two, probably identical subunits.

Active Site Studies. The pK_a's for ionized groups at the active site of the free enzyme were determined at pH 8.2 and 5.8, and strongly suggestive of the ionization of cysteine and histidine, respectively (Figure 2a). Ionized groups at the active site, in the enzyme–substrate complex, were observed near pH 7.7 and 5.3, suggesting a shift of 0.5 pH units in magnitude towards the acid region, as the groups became complexed with the substrate (Figure 2b). Another pK_a for either the free enzyme or the free substrate (Dixon, 1953), observed near pH 7.2–7.3 (Figure 2a), was attributed to the ionization of inorganic orthophosphate, a substrate, and the major component

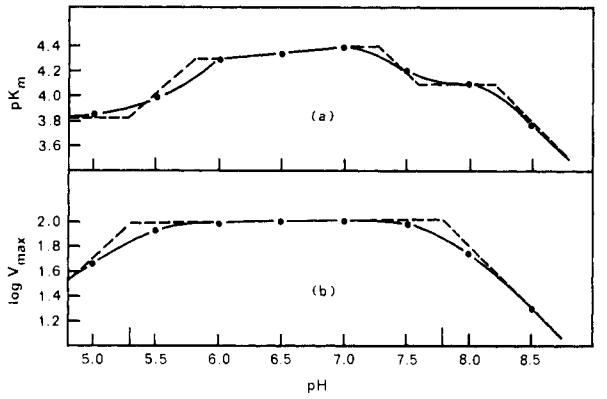


FIGURE 2: Determination of ionizable groups at the active site of the enzyme. Plots of pK_m vs. pH (a) and log V_{max} vs. pH (b) were constructed after Dixon (1953) for determination of ionizable groups at the active site of the free enzyme, and in the enzyme-substrate complex. K_m and V_{max} values were derived from double-reciprocal plots of initial velocity of the enzyme, between pH 5.0 and 9.0. Guanosine was the variable substrate.

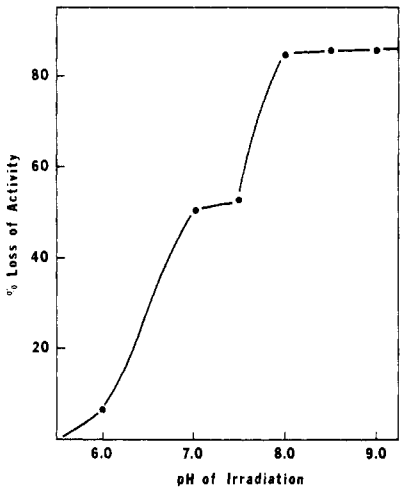


FIGURE 3: pH dependence of photoinactivation. A plot of percent loss of activity for 1 min of irradiation as a function of pH. Percent residual activity was determined as percent of light controls. Other conditions are specified at length under Experimental Procedure.

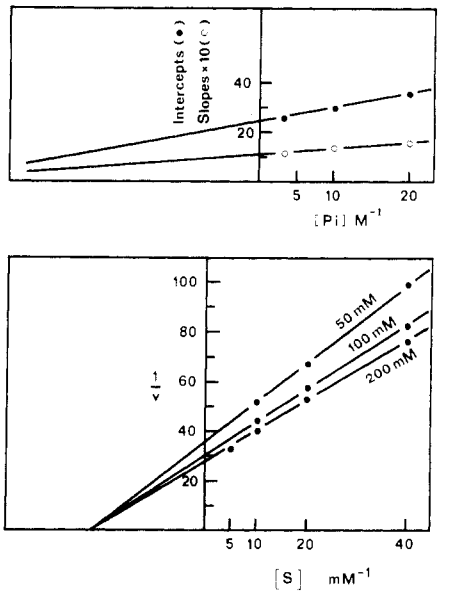


FIGURE 4: Plot of reciprocal of initial velocity vs. the reciprocal of concentrations of guanosine (mM) as variable substrate. Concentrations of inorganic orthophosphate were fixed at 200, 100, and 50 mM. Initial velocities are expressed as ΔOD/min at pH 7.0 and 40 °C. The amount of enzyme used in this experiment, and all subsequent experiments, was 0.4 μg of protein in 50 μl. Kinetic constants are listed in Table II.

TABLE II: Kinetic Constants for the Phosphorolytic Reaction of Bovine Brain Purine-Nucleoside Phosphorylase.[a]

Kinetic Constant	Value
K_a	4.35×10^{-5} M
K_b	2.15×10^{-2} M
K_{ia}	4.35×10^{-5} M
V_1	7.80×10^{-5} mol min^{-1} mg of protein^{-1}

[a] The constants K_a and K_b, limiting Michaelis constants for guanosine and inorganic orthophosphate, respectively, and V_1, the limiting maximal velocity of the enzyme for this reaction, were calculated from a replot of $1/V_{max}$ vs. $1/[S]$ (Florini and Vestling, 1957) for the data obtained from initial velocity studies.

of the assay mixture. The substrate guanosine is not ionized within the pH region of the investigation.

Reversible Inactivation by p-Chloromercuribenzoate. Almost complete inactivation of the enzyme was achieved within 15 min of incubation of the enzyme (1.0 μM) with an excess of *p*-chloromercuribenzoate (100 μM), in 0.2 M phosphate buffer (pH 7.0) at 40 °C. An approximate 10% of residual activity that persisted was abolished by increasing the concentration of the inhibitor to 300 μM. The completely inactivated enzyme regained 50% of its original activity upon treatment with an excess of 2-mercaptoethanol.

Photooxidation of the Enzyme. Photooxidation of the enzyme in presence of methylene blue caused a decline in catalytic activity, and complete inactivation was obtained within 3 min of irradiation of the enzyme. The susceptibility of the enzyme to photoinactivation was significantly enhanced as the pH was increased between pH 5.0 and 8.0. Photoinactivation was near maximal at pH 8.0, with 85% loss of catalytic activity for only 1 min of irradiation (Figure 3).

Stability and Activation Energy. The enzyme was stable when stored at 4 °C, but required protection of essential cysteine SH by 2-mercaptoethanol or dithiothreitol. Dilute solutions of the enzyme were irreversibly denatured by freezing. Rapid loss of catalytic activity was observed upon incubation at 55 °C. An activation energy of 9.6 kcal/mol was calculated between 20 and 50 °C with guanosine as substrate, in 0.2 M phosphate buffer (pH 7.0).

Catalytic Mechanism of the Enzyme. Initial velocity studies showed intersecting or sequential kinetics (Cleland, 1963a,b, 1970). When guanosine was varied at fixed concentrations of inorganic orthophosphate, the double-reciprocal plots for initial velocity intersected on the *x* coordinate with linear replots of slopes and intercepts (Figure 4). A similar pattern of primary and secondary reciprocal plots was also observed when inorganic orthophosphate was varied at fixed concentrations of guanosine. A sequential mechanism for the phosphorolytic reaction of the enzyme was indicated. Kinetic constants, calculated graphically after the method of Florini and Vestling (1957), are listed in Table II. Sequential mechanisms may be random, ordered Bi-Bi, or Theorell-Chance, a limiting case of the ordered mechanism (Cleland, 1963a,b, 1970). Product inhibition studies have been emphasized as diagnostic of the distinguishing features of the above mechanisms (Alberty, 1958; Fromm and Nelson, 1962; Cleland, 1963a,b), and have been largely utilized in recent years in identifying the specific

TABLE III: Product Inhibition Pattern and Kinetic Constants for the Reactions of Bovine Brain Purine-Nucleoside Phosphorylase.

Inhibitory Product	Variable Substrate	Fixed Substrate	Type of Inhibition	Inhibition Constants (M)
Guanine	Guanosine	P_i (200 mM)	Competitive	1.20×10^{-5}
Guanine	Guanosine	P_i (40 mM)	Competitive	1.45×10^{-5}
Hypoxanthine	Guanosine	P_i (200 mM)	Competitive	5.00×10^{-5}
Guanine	P_i	Guanosine (200 μM)	Noncompetitive	6.5×10^{-5}
Ribose 1-phosphate	P_i	Guanosine (200 μM)	Competitive	3.20×10^{-4}
Ribose 1-phosphate	Guanosine	P_i (20 mM)	Noncompetitive	3.60×10^{-4}
		Synthetic (Reverse) Reaction		
Guanosine	Guanine	Ribose 1-phosphate (400 μM)	Competitive	3.13×10^{-5}

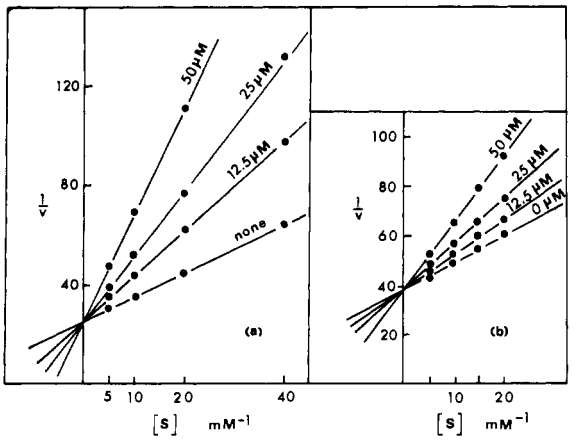


FIGURE 5: (a) Competitive inhibition of phosphorolysis by the product guanine. Guanosine was the variable substrate with inorganic orthophosphate fixed at 200 mM. The concentrations of guanine are shown on the graph. (b) Competitive inhibition of synthesis by the product guanosine. Guanine was the variable substrate with ribose 1-phosphate concentration fixed at 400 μM. Concentrations of guanosine are shown on the graph. Kinetic constants are listed in Table III.

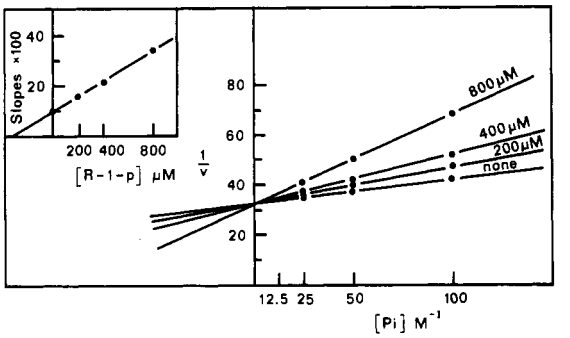


FIGURE 6: Competitive inhibition of phosphorolysis by ribose 1-phosphate. Inorganic orthophosphate was variable substrate. Guanosine was fixed at 200 μM. Concentrations of ribose 1-phosphate were 800, 400, 200, and 0 μM. Initial velocities were expressed as ΔOD/min. Kinetic constants are listed in Table III.

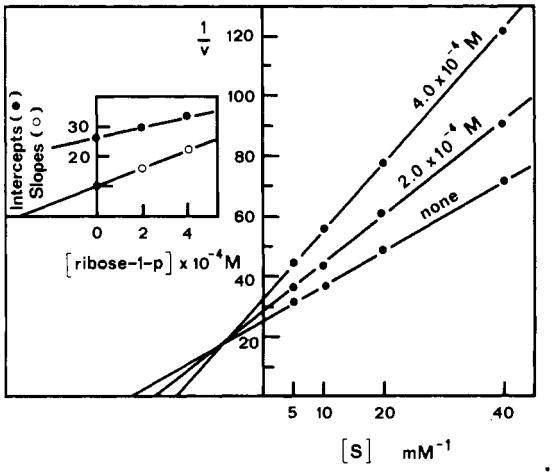
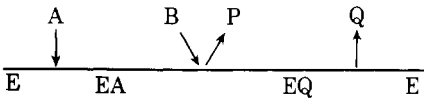


FIGURE 7: Noncompetitive inhibition of phosphorolysis by ribose 1-phosphate. Guanosine was variable substrate. Inorganic orthophosphate was fixed at 20 mM. Concentrations of ribose 1-phosphate were 400, 200, and 0 μM. Initial velocities were expressed as ΔOD/min at pH 7.0 and 40 °C. See Table III for kinetic constants.

catalytic mechanism of bisubstrate enzymes (Reynard et al., 1961; Zewe and Fromm, 1962; Wratten and Cleland, 1963, Morrison and James, 1965; Gulbinsky and Cleland, 1968; Kim et al., 1968b; Rudolph and Fromm, 1971; Danenberg and Cleland, 1975; Jamaluddin et al., 1975).

Product inhibition studies for the phosphorolytic reaction of bovine brain purine-nucleoside phosphorylase, as presented here, suggest that the predominant catalytic mechanism is that of the special case of an ordered Theorell–Chance mechanism (Theorell and Chance, 1951; Cleland, 1963a,b, 1970). The suggested mechanism:



is given in Cleland's terminology (Cleland, 1970), where E is the enzyme, A is substrate guanosine, B is substrate inorganic orthophosphate, P is product ribose 1-phosphate, and Q is product guanine (or purine). EA and EQ are the binary complexes postulated for the mechanism.

When guanosine was the variable substrate, at a fixed concentration of inorganic orthophosphate, both guanine (Figure 5a) and hypoxanthine were competitive product inhibitors for the phosphorolysis of guanosine. Mutual competitive inhibition between guanine and guanosine was demonstrated. In the reverse synthetic reaction of the enzyme, when guanine was varied at a fixed concentration of ribose 1-phosphate, guanosine was observed to competitively inhibit its own synthesis (Figure 5b). In a similar manner, when inorganic orthophosphate was varied at a fixed concentration of guanosine, ribose 1-phosphate was a competitive inhibitor for enzymatic phosphorolysis of guanosine (Figure 6). The reverse reaction was not investigated. When guanosine concentration was varied and inorganic orthophosphate concentration was fixed, ribose 1-phosphate was a noncompetitive inhibitor of phosphorolysis (Figure 7). Finally, guanine was observed to inhibit noncompetitively, when inorganic orthophosphate was varied at a fixed concentration of guanosine.

Given in Cleland's terminology (Cleland, 1970), Q gave competitive inhibition when A was the variable substrate, but noncompetitive inhibition when B was the variable substrate. Similarly, P gave competitive inhibition when B was varied, but noncompetitive inhibition when A was varied. The product inhibition pattern for this sequential mechanism was consistent with Theorell-Chance mechanism (Cleland, 1970). Product inhibition pattern and kinetic parameters are listed in Table III.

Discussion

Purine nucleoside phosphoroylase has been purified to homogeneity from human erythrocytes (Kim et al., 1968a), from rabbit liver (Lewis and Glantz, 1976) and from bovine brain (this investigation). The commercial enzyme from calf spleen (Boehringer Ltd.) was also determined homogeneous by electrophoresis on cellulose-acetate strips (Edwards et al., 1973). Despite possible differences in physical properties, these enzymes appear to be quite similar in chemical and kinetic properties. A pH-dependent photoinactivation of the enzyme suggested a high susceptibility of essential histidine to photooxidation (Weil et al., 1953; Weil and Seibler, 1954; Sluyterman, 1963; Westhead, 1965; Lewis and Glantz, 1976). The kinetically determined pK_a's for ionizable groups at the active site of the enzyme have implicated histidine and cysteine SH as catalytic groups. The same groups have been implicated as the catalytic species of the trimeric enzyme from human erythrocyte (Agarwal and Parks, Jr., 1969), and the monomeric enzyme from rabbit liver (Lewis and Glantz, 1976). The reactivation of enzyme inhibited by sulfhydryl reagents, as demonstrated in this investigation, has been reported for other purine-nucleoside phosphorylases (Kim et al., 1968a; Agarwal and Parks, 1971; Agarwal et al., 1975, Lewis and Glantz, 1976).

An ordered Bi-Bi catalytic mechanism has been reported for human erythrocyte purine-nucleoside phosphorylase (Kim et al., 1968b). The results of this investigation of the bovine brain enzyme were consistent with an ordered Theorell-Chance mechanism (Cleland 1963a,b; 1970). Primary and secondary reciprocal plots were linear, and competitive inhibition constants calculated for guanine from secondary plots were not significantly different for fixed concentrations of inorganic orthophosphate equal to $K_M \times 2$ and $K_M \times 10$, respectively. These observations are consistent with an ordered mechanism for the enzyme (Cleland, 1963b; Morrison and James, 1965). An obligatory order of substrate interaction with the enzyme is suggested by the experimental evidences. The nucleoside appears to be the first substrate to add to the enzyme, and the dissociation of the product guanine appears to be the rate-limiting step for the phosphorolytic reaction. The affinity of the enzyme for guanosine, as calculated kinetically, was 400-fold greater than its affinity for inorganic orthophosphate as substrates. Binding studies reported for the human erythrocyte enzyme (Kim et al., 1968b) indicated that while nucleosides and purines were strongly bound by the enzyme, inorganic orthophosphate and ribose 1-phosphate were only weakly associated.

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Mung Bean Nuclease I. Physical, Chemical, and Catalytic Properties†

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ABSTRACT: A simplified purification procedure for mung bean nuclease has been developed yielding a stable enzyme that is homogeneous in regards to shape and size. The nuclease is a glycoprotein consisting of 29% carbohydrate by weight. It has a molecular weight of 39 000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme contains 1 sulfhydryl group and 3 disulfide bonds per molecule. It has a high content (12.6 mol %) of aromatic residues. Approximately 70% of the enzyme molecules contain a peptide bond cleavage at a single region in the protein. The two polypeptides, 25 000 and 15 000 daltons, are covalently linked by a disulfide bond(s). Both the cleaved and intact forms of the enzyme are equally active in the hydrolysis of the phosphate ester linkages in either DNA, RNA, or adenosine 3′-monophosphate. The enzymatic activity of mung bean nuclease can be stabilized at pH 5 in the presence of 0.1 mM zinc acetate, 1.0 mM cysteine, and 0.001% Triton X-100. The enzyme can be inactivated and reactivated by the removal and readdition of Zn^{2+} or sulfhydryl compounds.

Mung bean nuclease is a member of a class of nucleases isolated from plants which possess a pronounced specificity towards nucleic acid substrates lacking ordered structure. Other so-called "single-strand specific" nucleases that are well characterized have been isolated from *Neurospora crassa* (Linn and Lehman, 1965), *Aspergillus oryzae* (S_1) (Ando, 1966; Sutton, 1971; Vogt, 1973; Rushizky et al., 1975), *Penicillium citrinum* (P_1) (Fujimoto et al., 1974a,c), and wheat seedlings (Hanson and Fairley, 1969; Kroeker et al., 1975). These enzymes are sugar unspecific endonucleases (*N. crassa* also possesses a single-strand specific exonuclease) which release 5′-phosphoryl-terminated products.

Mung bean nuclease, originally prepared by Sung and Laskowski (1962), and purified to homogeneity by Ardelt and Laskowski (1971), possesses DNase,[1] RNase, and ω-monophosphatase activities (Mikulski and Laskowski, 1970). The enzyme exhibits a preference for A↓pN and T↓pN linkages in denatured DNA (Sung and Laskowski, 1962). The enzyme was called "region specific" because a transient stage was observed in which native λDNA was cleaved at the A–T rich central region of the genome (Johnson and Laskowski, 1970). Mung bean nuclease has also been shown to release products enriched in A and T from early melting regions of native DNA (Kedzierski et al., 1973).

Due to the increasing importance of single-strand specific nucleases as probes of DNA structure and function, we have decided to simplify our method for preparation of mung bean nuclease and further characterize the physical, chemical, and catalytic properties of the enzyme.

Experimental Procedures

Materials. Calf thymus DNA was either prepared by the method of Kay et al. (1952) or purchased from Sigma (type V). Yeast RNA (type VI), adenosine deaminase (type I), and Triton X-100 were obtained from Sigma. Triton X-100 consists of mainly *p*-(1,1,3,3-tetramethyl)butylphenyl poly(oxyethylene glycol) with an average of 9.6 ethylene oxide units.

Ion-exchange celluloses used were Whatman DE-52 and CM-52. Frozen mung bean sprouts were supplied by R. J. Reynolds Foods, Inc., Jackson, Ohio.

General Methods. Analytical gel electrophoresis was performed on 5% acrylamide gels at pH 9.4 and run at 2 mA/gel (Kowalski and Laskowski, Jr., 1972). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 10% acrylamide gels according to the method of Weber and Osborn (1969). Gels stained with Coomassie blue were scanned at 550 nm using a Gilford Model 2400 spectrophotometer equipped with a linear transport. Gels were stained for carbohydrate using the periodic acid–Schiff reagent as described by Segrest and Jackson (1972). The content of sulfhydryl groups was determined according to the method of Habeeb (1972). Amino acid analysis was performed on a Beckman 121 M analyzer. The protein was oxidized with performic acid according to the procedure of Hirs (1956) and hydrolyzed for 22 h according to the procedure of Moore and Stein (1963).

Enzyme Assays. The standard mung bean nuclease assay employs acid precipitation of heat-denatured calf thymus DNA (Sung and Laskowski, Sr., 1962). One unit of mung bean

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[1] Abbreviations used are: 3′-AMP, adenosine 3′-monophosphate; DNase, deoxyribonuclease; RNase, ribonuclease; 3′-AMPase, adenosine 3′-monophosphatase; DEAE, diethylaminoethyl; CM, carboxymethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.