Properties of 5′-Nucleotidase from Avian Heart*

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ABSTRACT: 5′-Nucleotidase (EC 3.1.3.5) has been partially purified from pigeon ventricle. Unlike the enzyme from other vertebrate tissues which is predominantly bound to membranes, 5′-nucleotidase from pigeon heart was present entirely in the supernatant fluid following centrifugation of isotonic homogenates at 100,000*g* for 1 hr. The partially purified enzyme hydrolyzed nucleoside 5′-monophosphates, 5′-AMP being the preferred substrate. Other nucleotides were hydrolyzed at rates less than 10% that of 5′-AMP. The K_m for 5′-AMP was 12 mM. The enzyme required divalent cation for activity, Mg^{2+} being the most effective; the K_a for Mg^{2+} was 0.37 mM. Mn^{2+} also stimulated the enzyme (K_a = 55 μM) but maximal velocity with this cation was only 20% that with Mg^{2+}. Ca^{2+} inhibited the enzyme competitively with Mg^{2+}; the K_i for Ca^{2+} was 11 μM. The enzyme was inhibited by 2′-AMP and 3′-AMP in a manner competitive with substrate; the K_i for 2′-AMP was 2.1 mM and for 3′-AMP, 2.6 mM. When present at concentrations approximately equal to Mg^{2+}, ATP stimulated enzyme activity at low substrate concentrations (below 5 mM) and was slightly inhibitory at higher substrate concentrations. When present in excess of Mg^{2+}, ATP was strongly inhibitory at high substrate concentrations. 5′-Nucleotidase with similar solubility characteristics and substrate specificity was present in the hearts of two other avian species, the starling (*Sternus vulgaris*) and the domestic fowl.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been studied in a wide variety of vertebrate sources: bull semen (Levin and Bodansky, 1966), rat liver (Arsenis and Touster, 1968; Widnell and Unkeless, 1968; Fritzson, 1969), calf intestinal mucosa (Center and Behal, 1966), pig intestinal smooth muscle (Burger and Lowenstein, 1970), bovine pituitary (Lisowski, 1966), sheep brain (Ipata, 1968), rat cerebellum (Bosmann and Pike, 1971), and rat heart (Baer *et al.*, 1966; Edwards and Maguire, 1970; Sullivan and Alpers, 1971). The enzyme from these sources differs with respect to substrate specificity, substrate affinities, metal requirement, inhibitors, and stimulators (Drummond and Yamamoto, 1971). Although in most cases the intracellular location has not been defined, the enzyme appears to be predominantly membrane bound. Some evidence indicates that it exists largely in the plasma membrane of cells (Emmelot *et al.*, 1960; Emmelot and Bos, 1966); this has prompted the use of the enzyme as a plasma membrane marker (Bosmann and Martin, 1969; Benedetti and Emmelot, 1968). Considerable attention has been focused on the cardiac enzyme because of the possibility that adenosine functions as a physiological regulator of coronary blood flow (Berne, 1963). A sarcolemmal localization of 5′-nucleotidase is implicit in this hypothesis (Rubio and Berne, 1969). In accord with this the rat heart enzyme is predominantly membrane bound (Baer *et al.*, 1966). The enzyme from this source has broad substrate specificity and is strongly inhibited by ATP (Baer *et al.*, 1966; Edwards and Maguire, 1970) and by ADP (Sullivan and Alpers, 1971). In the course of studies designed to explore the properties and possible regulatory mechanisms of the enzyme in hearts of various species, we encountered a 5′-nucleotidase in avian heart with unique properties. In particular, it appeared to be exclusively soluble; it possessed narrow substrate specificity, and an unusually high K_m for 5′-AMP. This report describes these and other properties of the enzyme from pigeon ventricle.

Materials and Methods

Hydroxylapatite (suspension in 1 mM phosphate buffer, pH 6.8), 3′-AMP, 5′-AMP, 5′-dAMP, 5′-GMP, Na_2ATP, disodium D-ribose 5-phosphate, hypoxanthine, and rabbit muscle adenylic deaminase were purchased from Sigma Chemical Co. Adenine, adenosine, and 5′-CMP were obtained from Nutritional Biochemicals Corp.; 5′-UMP, 5′-IMP, inosine, and disodium glucose 6-phosphate from Calbiochem; 2′-AMP from Schwarz BioResearch; *p*-nitrophenyl phosphate from J. T. Baker Chemical Co.; and bis(*p*-nitrophenyl)phosphate from Aldrich Chemicals. Pigeon hearts (fresh frozen) were obtained from Pel-Freeze Biologicals (packed in Dry Ice) or from birds captured locally.

Assay of 5′-Nucleotidase. Enzyme activity was determined at 37° in a reaction mixture of 225 μl which contained 22 mM 5′-AMP, 10.7 mM magnesium chloride, 30 mM glycylglycine (pH 7.2), 20% by volume ethylene glycol, and the enzyme preparation to be tested. The incubation time was 5 or 10 min. The reaction was terminated by the addition of 1.0 ml of ice-cold 3% trichloroacetic acid. After centrifugation at 4° to remove denatured protein, inorganic phosphate was determined in a 0.5-ml aliquot by a modification of the Fiske and Subbarow method which utilized 0.25 N H_2SO_4 and a color development period of 30 min. Color intensity was measured in a Beckman DU spectrophotometer (light path, 1 cm). Reaction velocity was a linear function of time for at least 20 min and was proportional to enzyme concentration under all conditions used. Specific activity was defined as micromoles of substrate hydrolyzed per minute per milligram of protein.

Assay of Other Enzymes. Adenylate kinase was determined by the method of Colowick (1955); adenylic acid deaminase by the method of Lee (1963) and adenosine deaminase by the method of Kaplan (1955) except that the reaction was carried out in 0.1 M phosphate buffer (pH 7.5) containing 0.15 M KCl.

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TABLE I: Species and Tissue Distribution of 5′-Nucleotidase.

Tissue	Pigeon (*Columba livia*) ♂		Starling (*Sternus vulgaris*) ♀		Chicken (Leghorn Hens) ♀		Rat (Wistar) ♀	
	Total Act.[a]	Soluble[b] %	Total Act.[a]	Soluble[b] %	Total Act.[a]	Soluble[b] %	Total Act.[a]	Soluble[b] %
Heart	19.4	73.2	10.44	78.8	2.37	67.4	2.48	18.8
	18.7	64.9	9.93	84.6	2.47	61.4	2.47	15.5
Liver	1.47	42.9	0.58	0.0	1.90		3.35	14.2
	1.59	37.7	0.87	22.9	1.32		3.91	8.1
Kidney	2.55	69.2			1.55	59.2	4.43	5.3
	2.52	58.1			1.23	32.1	6.23	8.3
Muscle[c]	1.68	35.4	2.28	80.6	0.08		0.0	0.0
	1.36	44.1	3.57	78.4	0.08		0.12	0.0

[a] Activity expressed in micromoles per minute per gram wet weight. [b] Indicates the fraction of the activity that was recovered in the supernatant after centrifugation for 1 hr at 100,000*g*. Tissues were homogenized in 0.9% sodium chloride. [c] Breast muscle was used from pigeon, starling and chicken; hind leg muscle from rats. The values for each of two different experiments are given.

Protein was determined by the method of Lowry *et al.* (1951).

Results

Species and Tissue Distribution of 5′-Nucleotidase. The level of 5′-nucleotidase activity in saline homogenates of several tissues of three avian species and the rat is shown in Table I. Enzyme activity in pigeon and starling heart was considerably higher than in hearts of the chicken and rat. Activity in the other tissues examined was much lower than pigeon and starling ventricle.

Enzyme Solubility. In rat heart 85% of 5′-nucleotidase is bound to particulate material and can be solubilized with deoxycholate (Baer *et al.*, 1966). In contrast, the enzyme in pigeon ventricle appears to exist almost entirely in the soluble cytoplasm. Thus, when hearts were homogenized in 0.25 M sucrose–50 mM glycylglycine (pH 7.2) or in 0.28 M ethylene glycol–50 mM glycylglycine (pH 7.2), 67% of the activity was found in the supernatant fraction after centrifugation at 100,000*g* for 1 hr. Two subsequent washings of the pellet removed all but 2% of the remaining activity. This indicates that the enzyme was mostly in the soluble cytoplasm or that if it were bound to tissue membranes it must be readily released under isotonic extraction conditions. When several tissues of three avian species and the rat were homogenized in physiological saline (0.9% NaCl), activity present in 100,000*g* supernatant fractions varied widely in different tissues (Table I), and was considerably greater in hearts of each avian species than in the rat.

Preparation of Pigeon Ventricle 5′-Nucleotidase. Ventricular tissue from fresh or frozen pigeon hearts was washed with 0.9% sodium chloride and then homogenized in 10 volumes of this fluid at 4° in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was strained through gauze and centrifuged at 100,000*g* for 1 hr. The supernatant fluid was brought to 26% saturation with respect to ammonium sulfate by the slow addition of a saturated solution of this salt pH 6.8, while the mixture was stirred at 4°. After 5 min, precipitated protein was removed by centrifugation at 30,000*g*. The supernatant solution was brought to 39% ammonium sulfate in the above manner. After centrifugation the precipitate was dissolved in 10 mM glycylglycine (pH 7.2) containing 20% ethylene glycol (one-fifth original volume) and was then thoroughly dialyzed against this buffer. The dialyzed solution was treated with hydroxylapatite suspension using 5 mg of dry solids/mg of protein. After stirring for 10 min at 4°, the mixture was centrifuged, the clear fluid was removed and stored at −80°. The final preparation represented a 30-fold purification over the original saline homogenate, and an increase in specific activity from 0.21 to 6.6 in a typical preparation with a 30% yield.

Enzyme Stability and Effect of Ethylene Glycol. The enzyme was highly labile. Activity rapidly decreased in whole hearts stored at −80°. Thus, only 56% of the activity remained after 16 days and 34% after 22 days when tissue was stored at this temperature. Solutions of the semipurified enzyme, however, could be stored for 6 weeks at −80° without loss of activity. At elevated temperatures solutions of the enzyme were rapidly inactivated; at 50° (pH 7.2) 25% of the activity was destroyed in 30 sec and 58% in 1 min. The enzyme was also labile when stored at 4°; this complicated purification procedures. Stability could be improved by increasing the ionic strength and by the addition of ethylene glycol. This is illustrated in Figure 1A. Activity was rapidly lost when the enzyme was stored at 4° in 30 mM glycylglycine (pH 7.2); the addition of ethylene glycol to 20% by volume improved stability considerably (compare curves D and C). Stability was also improved by the addition of KCl (65 mM) (curve B) and when both KCl and ethylene glycol were present, the enzyme lost almost no activity in 24 hr (curve A). In addition to stabilizing the enzyme, both high ionic strength and ethylene glycol affected enzyme activity during the assay. Potassium chloride, when present in the assay mixture at concentrations up to 80 mM produced strong inhibition (Figure 1B, open circles). Sodium chloride produced similar inhibition. Ethylene glycol at a concentration of 20% by volume partially prevented KCl inhibition, (closed circles). Ethylene glycol at this concentration had a slight stimulatory effect on the activity (Figure 1B, inset); higher concentrations were inhibitory. The stimulatory effect of 20% ethylene glycol is also apparent in Figure 1B as evidenced by the difference in the activities at 0 M KCl. Inhibi-

TABLE II: Substrate Specificity of 5'-Nucleotidase.[a]

Substrate	Pigeon Ventricle	Starling Ventricle	Chicken Ventricle	Rat Ventricle	Starling Breast Muscle
5'-AMP	100.0	100.0	100.0	100.0	100.0
5'-CMP	6.8	6.0	7.6	33.7	45.9
5'-UMP	7.6	5.0	6.3	36.8	43.3
5'-GMP	7.8	5.7	7.4	35.5	48.0
5'-IMP	6.8	5.7	7.0	31.4	50.9
5'-dAMP	9.7	9.3	11.9	22.7	67.1
2'-AMP	0	0	0	1.8	0
3'-AMP	0	0	0	4.0	1.8
ATP	0	0	0	23.7	4.6
Glucose 6-phosphate	0	0	0	2.7	1.1
β-Glycerol phosphate	0	0	1.5	9.1	9.3

[a] All enzymes purified to the ammonium sulfate fraction as described for pigeon ventricle. Activities expressed relative to 5'-AMP and all substrates were tested at 22 mM.

tion by KCl was not due to denaturation because dilution restored full activity. Furthermore, when assayed with 45 mM KCl, velocity was a linear function of time of incubation. Glycylglycine up to a concentration of 45 mM had no inhibitory effect on enzyme activity. Routinely, ethylene glycol was used as the stabilizing agent; enzyme fractions were stored in 20% (v/v) solutions of this material in 10 mM glycylglycine (pH 7.2). Ethylene glycol was also present in the standard assay at a similar final concentration.

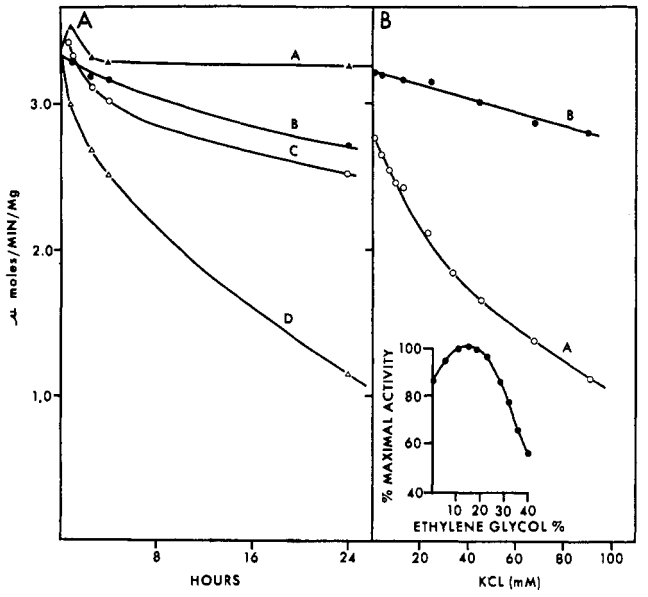

FIGURE 1: Effect of KCl and ethylene glycol on stability of 5'-nucleotidase at 4° and on the enzyme assay. Plate A: the purified enzyme was stored in four different buffers–30 mM glycylglycine–65 mM KCl–20% ethylene glycol, pH 7.2 (▲); 30 mM glycylglycine–65 mM KCl, pH 7.2 (●); 30 mM glycylglycine–20% ethylene glycol, pH 7.2 (○); 30 mM glycylglycine, pH 7.2 (Δ). Ten-microliter aliquots were assayed under standard conditions at the times indicated. Plate B: purified enzyme stored in 30 mM glycylglycine (pH 7.2) was assayed in the presence (●) and in the absence (○) of ethylene glycol 20% by volume. Otherwise standard assay conditions were used except that KCl was present at the concentrations indicated. Inset: enzyme (25 μg of protein, specific activity 3.25) stored in 30 mM glycylglycine (pH 7.2) was assayed under standard conditions except that the ethylene glycol concentration was varied.

Because of the considerable lability of the enzyme, efforts to purify it further were generally unsuccessful. Numerous attempts involving chromatography on DEAE-cellulose and CM-cellulose, adsorption on calcium phosphate gel, gel filtration on Sephadex G-200, solvent fractionation, and starch gel electrophoresis led to large losses in activity without purification. Even though the final purification was modest, certain enzymes which could complicate the assay were largely absent. When an amount of purified extract representing five times the 5'-nucleotidase usually present in the standard assay was examined for adenosine deaminase, adenylic acid deaminase, and adenylate kinase, only the latter could be de-

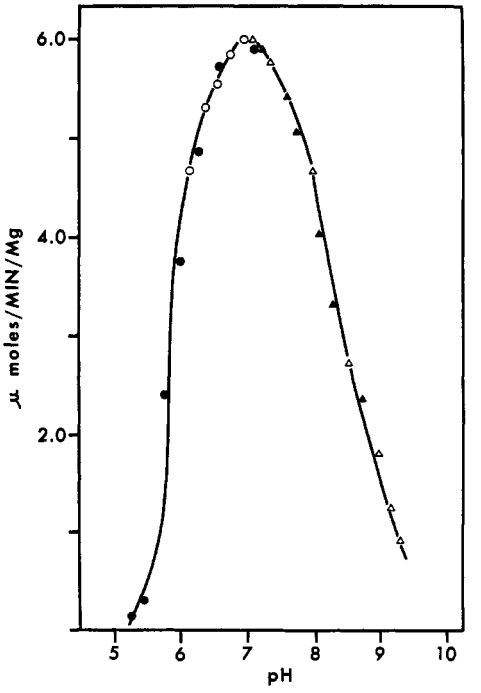

FIGURE 2: Effect of pH on reaction velocity. Standard assay conditions were used except the buffer was varied. Buffers used were (●) acetate, (○) maleate, (▲) glycylglycine, and (Δ) 2-amino-2-methyl-1,3-propanediol all at 33 mM. Incubation time 5 min; 18 μg of protein/assay.

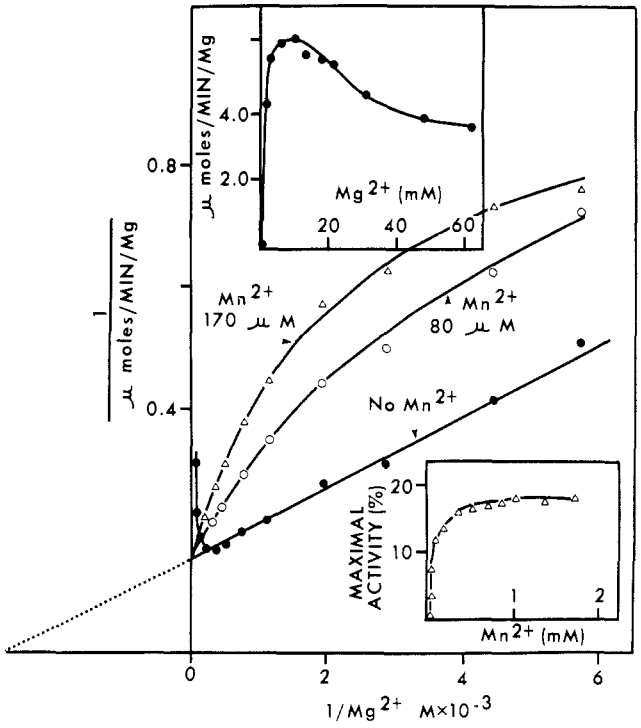

FIGURE 3: Effect of Mg^{2+} and Mn^{2+} on 5′-nucleotidase. Standard conditions (15 μg of protein) were used except that metal ion concentration was varied. (●) Mg^{2+} alone; (○) Mg^{2+} plus 80 μM Mn^{2+}; (△) Mg^{2+} plus 170 μM Mn^{2+}. Incubation time was 5 min, and 20 min when Mn^{2+} alone was present (bottom inset).

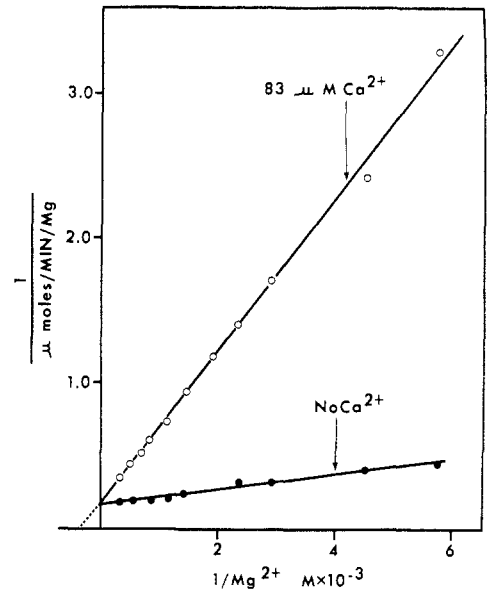

FIGURE 4: Inhibition of 5′-nucleotidase by Ca^{2+}. Standard assay conditions were used (15 μg of protein, 5-min incubation) except that Mg^{2+} was varied and Ca^{2+} was present as indicated. (●) No Ca^{2+}; (○) Ca^{2+} present at 83 μM.

tected. The activity of this enzyme in the hydroxylapatite-treated preparation was measured at 0.025 μmole/min per mg of protein, much lower than 5′-nucleotidase activity which measured 6.63 μmoles/min per mg.

Substrate Specificity. The enzyme from pigeon ventricle was specific for ribonucleoside 5′-phosphates with a distinct preference for 5′-AMP (Table II); 2′-AMP, 3′-AMP, and ATP were not attacked. The hydroxylapatite-treated fraction was also tested with D-ribose 5-phosphate, UDP-glucose, pyrophosphate, *p*-nitrophenyl phosphate each at 22 mM and bis(*p*-nitrophenyl) phosphate at 9 mM. No measurable release of inorganic phosphate occurred with any of these. It is apparent, Table II, that the enzyme from the other two avian species displayed a substrate specificity similar to that of the pigeon, but differed from that present in extracts of rat heart and starling breast muscle fractionated in an identical manner (ammonium sulfate precipitation of 100,000*g* supernatants). Some evidence indicated that the activity present in pigeon ventricle represents a single enzyme. Thus, the substrate specificity of the ammonium sulfate and hydroxylapatite-treated fractions was identical. When two preparations of the purified enzyme were heated at 51° so that 25 and 60%, respectively, of the original activity remained, the relative activities toward the substrates listed in Table II were unchanged. Furthermore, the hydrolysis of all substrates was absolutely dependent on divalent cation (see below).

Reaction Products. The quantitative liberation of inorganic phosphate as one product is implicit in the assay. Adenosine was identified as the other product by chromatography. Purified enzyme (27 μg of protein) was added to a doubled-reaction mixture and incubated for 10 min, followed by boiling for 3 min. Phosphate assay indicated that 1.33 μmoles of inorganic phosphate was produced. Aliquots of the reaction mixture were chromatographed in 1 M ammonium acetate–95% ethanol (15:35, v/v) for 20 hr. Except for unreacted substrate, adenosine was the only compound detectable under ultraviolet light. The spot was eluted with water, the solution was adjusted to 1.5 ml and the optical density was determined at 259 nm. The yield of adenosine was identical with the amount of inorganic phosphate expected in the aliquot. To further identify the product, material eluted from an identical chromatogram was rechromatographed using isobutyric acid–ammonium hydroxide–water (66:1:33, v/v). The R_F was identical with authentic adenosine.

pH Optimum. The pH dependency of enzyme activity is shown in Figure 2. Optimal activity occurred at pH 7. This compares with a value of 7.3 reported for the enzyme from sheep brain (Ipata, 1968) and 6.8 to 7.0 for the cerebellar enzyme (Bosmann and Pike, 1971), but differs from most other 5′-nucleotidases (Drummond and Yamamoto, 1971) including the recently described pig intestinal smooth muscle enzyme which has two pH optima, one at 5.5 and another at 7.0 (Burger and Lowenstein, 1970). Reaction velocity was not affected by buffer composition (Figure 2).

Divalent Cation Requirement. The pigeon ventricle enzyme was entirely devoid of activity in the absence of divalent cation. The enzyme functioned optimally at a Mg^{2+} concentration of 10 mM, Figure 3 (top inset). Higher concentrations were inhibitory. From double-reciprocal plots (Figure 3) a K_a for Mg^{2+} of 0.37 mM was calculated. Manganese was also capable of stimulating enzyme activity; however, maximal velocity in the presence of this cation was less than 20% that achieved with optimal Mg^{2+} (Figure 3, insert bottom right), although the affinity was significantly higher (K_a for Mn^{2+} was calculated to be 55 μM). Magnesium saturation of the enzyme was examined in the presence of two fixed concentrations of Mn^{2+} (80 and 170 μM). The double-reciprocal plots (Figure 3) indicate than Mn^{2+} behaved as a competitive inhibitor; the plots deviate from linearity reflecting the intrinsic stimulatory action of Mn^{2+} especially at low Mg^{2+} concentrations. Calcium had no stimulatory action on the enzyme, but interacted with

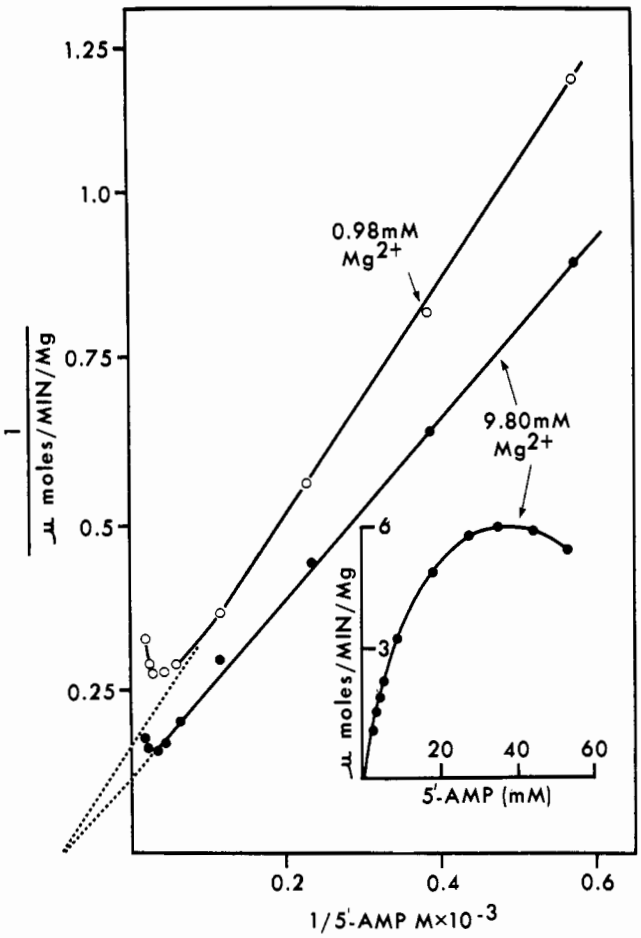

FIGURE 5: Effect of 5′-AMP concentration on reaction velocity. Standard assay conditions were used except that 5′-AMP concentration was varied and two fixed Mg^{2+} concentrations (●) 9.8 mM and (○) 0.98 mM were used. The incubation time was 10 min and 8 μg of protein was used.

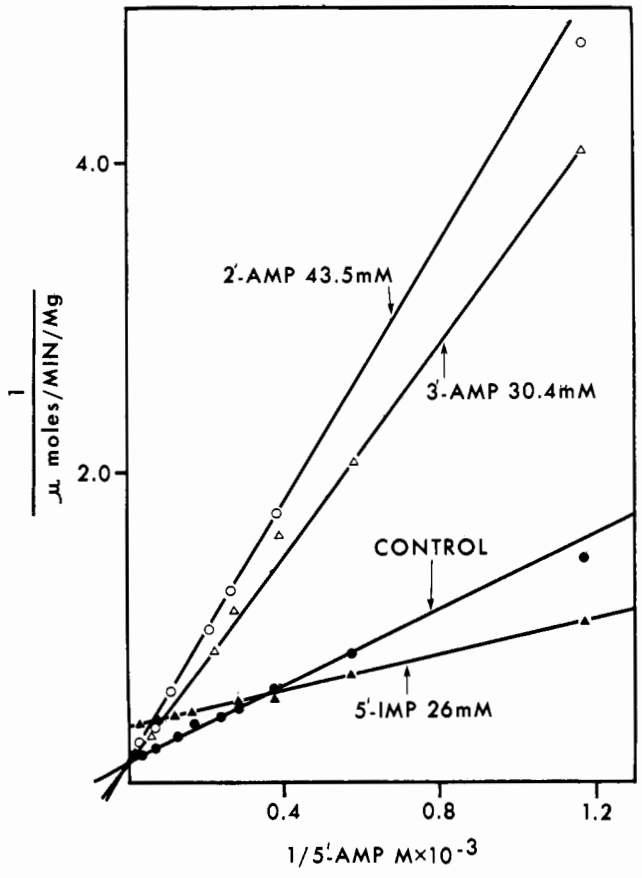

FIGURE 6: Action of nucleoside monophosphates on 5′-nucleotidase. Standard condition (15 μg of protein, incubation time 5 min) except that 5′-AMP concentration was varied, and other nucleotides were added. (●) No addition; (▲) 5′-IMP, 26 mM; (○) 3′-AMP, 30.4 mM; (△) 2′-AMP, 43.5 mM.

the metal binding site as a competitive inhibitor (Figure 4); the K_i for Ca^{2+} was calculated to be 11 μM.

Affinity for Substrate. The effect of 5′-AMP concentration on reaction velocity in the presence of two fixed Mg^{2+} concentrations is shown in Figure 5. Maximal velocities were achieved only at concentrations of this nucleotide between 30 and 40 mM. From the double-reciprocal plots the K_m for 5′-AMP was calculated to be 12 mM. This is in sharp contrast to the affinity constants for the enzyme from other vertebrate sources; 4 μM for the pig intestinal enzyme (Burger and Lowenstein, 1971), 10 μM for the membrane-bound enzyme from rat ventricle (Baer *et al.*, 1966), and 80 μM for the enzyme from rat cerebellum (Bosmann and Pike, 1971). From the data in Figure 5, it is also apparent that the affinity for substrate is likely not affected by Mg^{2+} concentration, the stimulatory action of this cation being to increase reaction velocity. The enzyme was inhibited by concentrations of 5′-AMP in excess of 40 mM and this also appeared to be independent of Mg^{2+} concentration.

Inhibition by Nucleotides. 3′-AMP and 2′-AMP inhibited the enzyme in a manner competitive with substrate (Figure 6); and were roughly equipotent; a K_i of 2.1 mM for 2′-AMP and 2.6 for the 3′ isomer was obtained. Adenosine, when tested at 8 mM, produced no inhibition. Thus the presence and position of the phosphate moiety seems critical for binding with the enzyme. The presence of 5′-nucleotides which are hydrolyzed slowly by the enzyme (Table II) also affected the hydrolysis of 5′-AMP. This is illustrated in Figure 6 for 5′-IMP (26 mM). At low 5′-AMP concentrations, 5′-IMP had an apparent stimulatory effect, possibly a reflection of its contribution as substrate at low 5′-AMP concentrations. Only at higher 5′-AMP concentrations was there an apparent weak inhibitory action of 5′-IMP. 5′-GMP behaved similarly.

ATP is a powerful inhibitor of 5′-nucleotidase from sheep brain (Ipata, 1968) and rat heart (Baer *et al.*, 1966), the K_i for the latter enzyme being 1.8 μM. ATP interacted with the pigeon heart enzyme in a complex manner. The effect of two fixed concentrations of ATP (8.6 and 13 mM) on 5′-AMP saturation kinetics in the presence of 10.4 mM Mg^{2+} is shown in Figure 7A. At substrate concentrations below 6 mM, ATP had a stimulatory effect, and a slight inhibitory effect at substrate concentrations above this. There appeared to be both a decreased V_{max} and decreased K_m for substrate with both concentrations of ATP. In Figure 7B, the effect of two fixed concentrations of ATP (2.7 and 8.9 mM) were examined on Mg^{2+} saturation kinetics, the substrate concentration being 22 mM. The highest concentration of ATP, which was always in excess of Mg^{2+} (the highest Mg^{2+} concentration present was 3 mM), produced inhibition in a manner competitive with Mg^{2+}. ATP at 2.7 mM also produced inhibition but the plot deviated from linearity at Mg^{2+} concentrations approaching this, indicating decreased inhibition when Mg^{2+}:ATP ratio approached unity. This would indicate that free ATP is the actual inhibitor. Indeed it was shown in a separate experiment

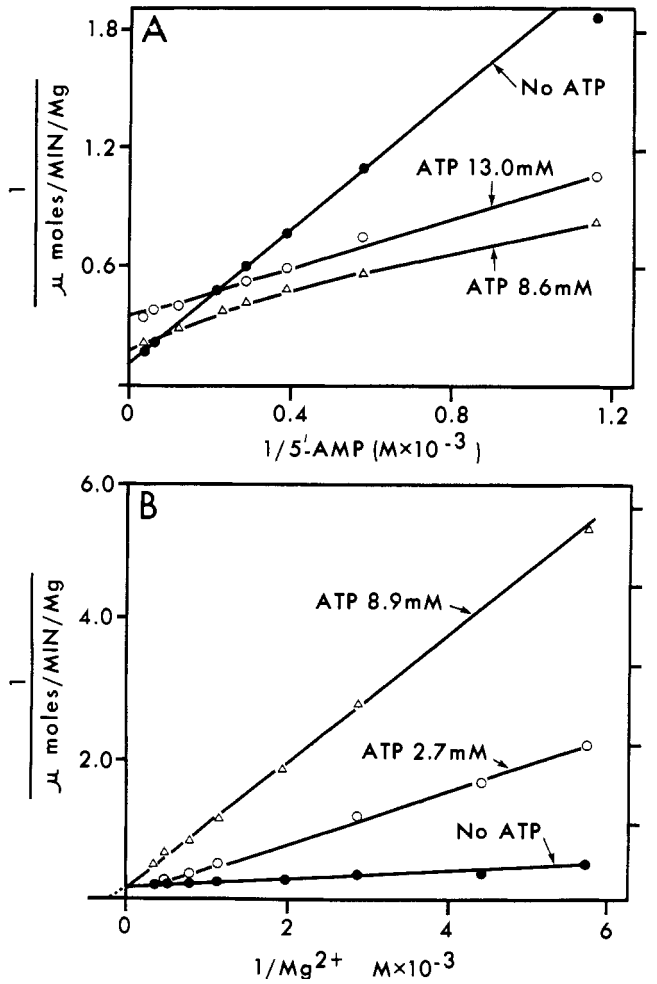

FIGURE 7: Action of ATP on 5'-nucleotidase activity. Plate A: 5'-AMP concentration was varied; Mg^{2+} concentration was 10.4 mM. ATP was present as indicated (●) no addition; (○) 13 mM ATP; (△) 8.6 mM ATP. Reaction time was 5 min. Plate B: Mg^{2+} concentration was varied and 5'-AMP concentration was 22 mM. ATP was present as indicated (●) no addition; (○) 2.7 mM ATP; (▲) 8.9 ATP.

that the stimulatory effect of Mg–ATP (Mg:ATP ratio kept constant) was essentially similar to that of Mg^{2+} alone (the apparent K_a for Mg–ATP was 0.43 mM compared with 0.37 mM for the cation alone).

Discussion

Pigeon ventricular 5'-nucleotidase differs in several respects from the enzyme from other vertebrate sources. It is largely soluble or at least it is present in the supernatant after centrifugation of isotonic homogenates at 100,000*g*. In other vertebrate sources the enzyme seems predominantly membrane bound. However, in most studies the exact intracellular origin of the activity is in doubt because of the extraction procedures used which have frequently employed detergent, high ionic strength, or low initial centrifugation velocities (see Drummond and Yamamoto, 1971). Even in those instances, particularly rat liver (Fritzson, 1969; Fritzson and Smith, 1971) where soluble activity is indicated, the pigeon enzyme is markedly different, especially with respect to substrate specificity. The pigeon ventricle enzyme is, of course, distinctly different from the rat heart enzyme previously studied in this laboratory (Baer *et al.*, 1966). Most (85%) of the activity in the latter tissue sediments at 100,000*g* and the enzyme possesses a broad substrate specificity. The small amount of activity present in the 100,000*g* supernatant fraction of rat heart, although not extensively studied also seems different from the pigeon enzyme at least with respect to substrate specificity (Table II). It may be of significance that the total 5'-nucleotidase activity of pigeon heart (expressed on a tissue wet weight basis) is considerably higher than that in a variety of other tissues and species (Table II) including rat heart. Activity in rat heart is much higher than that in hearts of several other mammalian species (Baer *et al.*, 1966). Even though the degree of purification achieved with the pigeon heart enzyme was modest, the final specific activity is equivalent to that obtained for the 110-fold purified enzyme from calf intestinal smooth muscle (Burger and Lowenstein, 1970) but somewhat less than the 855-fold purified rat cerebellar enzyme which was 25 μmoles/min per mg (Bosmann and Pike, 1971).

The pigeon heart enzyme is also unique in having a high K_m for substrate; it is at least two orders of magnitude greater than for other 5'-nucelotidases which have been examined (Drummond and Yamamoto, 1971). In fact, the K_m value of 12 mM for substrate is among the highest of a large number of enzymes for which such data have been compiled (Barman, 1969).

The cytoplasmic locus and the high K_m for substrate seem incompatible with a role in forming adenosine for autoregulation of coronary blood flow. It has been considered that adenosine formation occurs *via* a 5'-nucleotidase in plasma membrane structures which would ensure the delivery of the nucleoside into the interstitial fluid for availability at the precapillary resistance vessels (Rubio and Berne, 1969). Adenosine, formed in the soluble cytoplasm, would more likely be deaminated before it could leave the cell because of the presence of adenosine deaminase (Baer *et al.*, 1966).

It might be reasonable to suggest that 5'-nucleotidase in pigeon heart is adapted along with other enzymatic activities to conserve adenine nucleotides in a tissue which is capable of vigorous oxidative metabolism especially during flight. Thus the low affinity for substrate might allow significant concentrations of 5'-AMP to accumulate during vigorous activity, while still allowing adenosine formation for coronary autoregulation. Pigeon heart possesses high adenylate kinase activity, 85 μmoles/min per g wet weight (compared with 23 for rat heart) (K. Nakatsu and G. I. Drummond, unpublished), so an effective system for resynthesis of ATP is available. It is known that oxygen comsumption of birds is greatly increased during flight. For example, pigeons use 8.5 times as much oxygen during flight as at rest (Le Febvre, 1964) and the rate of energy utilization of gulls and budgerigars while flying may be 20 times that of the resting level (Tucker, 1969). These values are much greater than those achieved by mammals while exercising (Taylor *et al.*, 1970). One might expect that even greater energy demands would be placed on the myocardium of birds during flight. Under these circumstances, the conservation of adenine nucleotides and mechanisms for facile regeneration of ATP would constitute an important metabolic adaptation.

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Diagnostic Relationships in the Relaxation Spectrometry of Allosteric Enzymes*

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ABSTRACT: Because the relaxation spectra of multisubunit proteins showing cooperative effects are very complex, a systematic study of alternative binding patterns for a dimeric enzyme was made to determine diagnostic relationships between relaxation spectra and mechanism of ligand binding. These diagnostic relationships reveal patterns which can be generalized to larger systems. The relaxation rates show a range of concentration dependencies which may increase indefinitely, decrease to an asymptote, reach a maximum, etc. Different models for ligand binding will often predict relaxation spectra which are kinetically indistinguishable. A consideration of the experimental problems involved reveal that the shapes of the concentration dependence of relaxation rate curves can be used diagnostically, but that the mechanisms so deduced must be considered to be working hypotheses rather than final conclusions because of the assumptions required to solve the complex mathematics.

In understanding the nature of allosteric regulation in metabolic systems it is important to relate the structure of the protein to the kinetic phenomena associated with the enzyme. Various techniques are being employed to correlate kinetics with information about structure and function of enzymes, but one of the most important methods is the use of relaxation spectrometry, particularly the temperature-jump method. Binding data and steady-state kinetics can provide very important information, but many steps of an enzymatic reaction proceed at velocities which exceed those accessible to these techniques.

The application of the temperature-jump method to the study of very fast reactions in allosteric proteins has been pioneered by Eigen (1954) and his coworkers and applied to the specific protein yeast glyceraldehyde-3-phosphate dehydrogenase (Kirschner *et al.*, 1966). The details of the method are well documented in the classic treatise of Eigen and de Maeyer (1963), and reviews of the subject in varying degrees of mathematical complexity have been published (Czerlinski, 1967; Hammes, 1968, 1969; Faller, 1969; French and Hammes, 1969; Gutfreund, 1971).

To prove a mechanism it is not simply acceptable to show that the data are consistent with that mechanism, but one must also show that the data are incompatible with reasonable alternative mechanisms. Because kinetic derivations of relaxation spectrometry beyond the simplest systems are complex, most fast reaction papers have reported data consistent with one mechanism and occasionally discuss one alternative which does not fit the data. Practically never is the potential range of consistent mechanisms discussed, yet the scientist interested in protein properties wishes to know in a given situation what mechanisms have been "proved" or "ex-

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