5′-Nucleotidase from Rat Heart†

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ABSTRACT: 5′-Nucleotidase has been extracted from rat heart and purified to apparent homogeneity. The enzyme is a glycoprotein. Gel electrophoresis in the presence of sodium dodecyl sulfate indicates that the apparent molecular weight of the subunit is 74 000 at several different gel concentrations. Cross-linking of the native enzyme with dimethylpimelimidate followed by gel electrophoresis shows that the enzyme is a dimer. The enzyme hydrolyzes all nucleoside 5′-monophosphates tested. A comparison of V_{max}/K_m for 14 different substrates shows that AMP is the best substrate. The enzyme shows lowest K_m values for AMPS, AMP, isoAMP, GMP, and IMP. It shows no activity with nucleoside 2′- and 3′-monophosphates, sugar phosphates, and *p*-nitrophenyl phosphate, even when tested at high enzyme concentrations. The optimum activity of the enzyme occurs at pH 7.5 with AMP as substrate. Above this pH, buffer ions affect the activity in a complex manner, a second optimum being observed under some conditions. Magnesium ions activate the enzyme above pH 7.5 in the presence of some buffer ions but not of others. Magnesium ions show only a slight activation when the reaction is run in diethanolamine buffer, pH 9.5, at 30 °C; the activation in this buffer is considerably greater when the reaction is run at 37 °C. The enzyme is strongly inhibited by free ADP, maximum inhibition occurring below pH 6. The ADP inhibition is diminished as the pH is raised above 6, becoming negligible above pH 9. The enzyme is inhibited by EDTA. The inhibition is partially reversed when the EDTA is removed from the enzyme by gel filtration. This as well as other evidence indicates that the enzyme contains a tightly bound metal ion.

The 5′-nucleotidase of heart is largely confined to the cell membranes, in particular, the plasma membrane [reviewed by Burger & Lowenstein (1970)]. Experiments with perfused rat heart indicate that 5′-nucleotidase hydrolyzes externally added AMP with a concomitant transfer of adenosine across the cell membrane (Frick & Lowenstein, 1978). On the basis of these findings, it was proposed that 5′-nucleotidase acts as a translocase which converts an intracellular signal of energy deficiency in the form of AMP into an extracellular signal in the form of adenosine (Schultz & Lowenstein, 1978). Adenosine is a powerful vasodilator, and its release results in increased coronary blood flow, which brings more oxygen to the tissue (Berne & Rubio, 1979), thus helping to correct the energy deficiency.

This paper describes the extraction of the enzyme from rat heart membranes with the detergent Triton X-100. The major degree of purification is achieved in two steps involving affinity chromatography on columns of concanavalin A–Sepharose and ADP–Agarose, but several other steps are necessary to prepare the enzyme in pure form. The activity of the pure enzyme as a function of the hydrogen ion concentration is complex above pH 7.5, as previously shown for the relatively crude enzymes from bovine seminal plasma (Levin & Bodansky, 1966) and smooth muscle of small intestine (Burger & Lowenstein, 1970). A number of other properties of the enzyme are also presented.

Materials and Methods

Frozen rat hearts were obtained from Pel-Freeze Biologicals, Rogers, AR. CM-Sephadex, Sephadex G-200, and concanavalin A–Sepharose were obtained from Pharmacia, DE 23 was obtained from Whatman, and agarose–hexane–adenosine 5′-diphosphate, type 2 (agarose cross-linked to the 6-amino group of ADP), AMP, and εAMP[1] were obtained from P-L Biochemicals. Basic fuchsin and Malachite Green were purchased from Fisher Scientific Co., Lubrol WX was obtained from Supelco, and Triton X-100 was obtained from Bio-Rad. The solution of Lubrol WX was filtered through a Millipore filter before use. Methyl α-D-mannoside and cholic acid were purchased from Sigma. Cholic acid was decolorized with activated charcoal and recrystallized twice from 70% ethanol before use. Adenosine deaminase, ADP, and AMPS came from Boehringer Mannheim. IsoAMP was a gift from Dr. D. C. Cole. Dimethylpimelimidate dihydrochloride was obtained from Pierce. [^{32}P]AMP was obtained from Amersham. YM30 ultrafiltration membranes were purchased from Amicon. All other chemicals were of reagent grade or of the highest quality available.

Three assays were used for 5′-nucleotidase. The enzyme solution was diluted to an appropriate concentration with assay buffer containing 50 mM sodium cholate. One unit of activity is equivalent to 1 μmol of product formed per min at 30 °C.

Assay 1. 5′-Nucleotidase was assayed using AMP as substrate by converting the adenosine formed to inosine with an excess of adenosine deaminase (Burger & Lowenstein, 1970). Unless otherwise indicated, the reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1.67 mM sodium cholate, 0.1 mM AMP, 1.0 μg of adenosine deaminase, and 5′-nucleotidase in a final volume of 3.0 mL. The reaction was started by adding AMP and was run at 30 °C. The change in absorbance was measured at 262.5 nm in a Zeiss PMQ II spectrophotometer. When scale expansion was necessary, the instrument used was a Perkin-Elmer double-beam spectrophotometer (Model 557) set to read absorbance at 262.5 nm minus absorbance at 300 nm. Absorbance changes were converted to concentrations

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[1] Abbreviations used: isoAMP, 3-β-ribofuranosyladenine 5′-monophosphate (Leonard & Laursen, 1965); εAMP, 1,N^6-ethenoadenosine 5′-phosphate; AMPS, adenosine 5′-*O*-thiophosphate; $AMPNH_2$, adenosine 5′-phosphoramidate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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by using a $\Delta\epsilon_{262.5nm}$ of 8.8×10^3 M^{-1} at pH 7.0 (Tornheim & Lowenstein, 1972). A calibration curve of $\Delta\epsilon_{262.5nm}$ vs. pH was prepared to ensure use of the correct extinction coefficients at higher pH values.

Assay 2. Orthophosphate was determined by the method of Itaya & Ui (1966) with a slight modification. The reaction mixture was as for assay 1, except that adenosine deaminase was omitted, the final volume being 1.0 mL. The reaction was stopped by adding a mixture of 0.8 mL of 15 mM ammonium molybdate and 2 N HCl. To the resulting mixture was added 0.2 mL of 0.3% Malachite Green dissolved in 1 N HCl; this was followed immediately by 0.05 mL of 4% Lubrol WX. The absorbance at 645 nm was read after 30 min. The absorbance was affected by sodium cholate which was present in the reaction mixture. The highest absorbance was obtained when the assay mixture contained 0.5–1 mM sodium cholate. For example, ϵ_{645nm} was 1.07×10^5 M^{-1} with 0.625 mM sodium cholate and 0.77×10^5 M^{-1} without sodium cholate. The color was stable for at least 1 h.

Assay 3. This was similar to assay 1, except that adenosine deaminase was omitted and ^{32}P-labeled AMP was used. The liberated orthophosphate was extracted by the method of Sanui (1974), and the organic phase was assayed for radioactivity.

Protein was determined by the modified Lowry method (Peterson, 1977) using crystalline bovine serum albumin as standard.

Analytical disc gel electrophoresis was performed according to the method of Davis (1964) using 5% polyacrylamide gel which contained 0.1% Triton X-100. Detergent in the enzyme solution was changed from cholate to Triton X-100 by adding Triton X-100 to a final concentration of 0.1% and then dialyzing the enzyme solution against 100 volumes of a mixture containing 50 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100 for 18 h with one change of the mixture. Enzyme activity in the gel was detected by the method of Allen & Hyncik (1963). The gel was incubated in a mixture which contained 1 mM AMP, 50 mM Tris-HCl, pH 7.5, and 15 mM $CaCl_2$ at 37 °C until a visible line or lines of calcium phosphate precipitate appeared.

Sodium dodecyl sulfate–gel electrophoresis was carried out as described by Laemmli & Favre (1973). Protein samples were denatured in a mixture containing 1.25% $NaDodSO_4$, 1.25% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8, at 100 °C for 3 min and then dialyzed against 100 volumes of a mixture containing 0.1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8, for 24 h with one change of the mixture. When necessary, the dialyzed sample was lyophilized and dissolved in a small amount of denaturing buffer. Molecular weight standards employed were chymotrypsinogen A (25 700), ovalbumin (43 000), bovine serum albumin (68 000), and phosphorylase *a* (92 500). Gels were stained for protein or carbohydrate by the method of Fairbanks et al. (1971).

Native enzyme was cross-linked according to the method of Davis & Kaplan (1972). The reaction mixture contained 5 or 12 μg of purified 5′-nucleotidase, 20 mM dimethylpimelimidate dihydrochloride, 50 mM sodium cholate, and 0.155 M triethanolamine hydrochloride, pH 8.5, in a final volume of 0.2 mL. After 1 h of incubation at room temperature, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 20%. The precipitated protein was recovered by centrifugation and washed first with a mixture of 1 volume of concentrated HCl and 200 volumes of acetone and then with acetone. Prior to gel electrophoresis, the precipitate was dissolved in a small amount of a mixture containing 1.25% $NaDodSO_4$, 1.25% 2-mercaptoethanol, 12.5% glycerol, and 0.125 M Tris-HCl, pH 6.8, and heated at 100 °C for 3 min.

Results

Purification of 5′-Nucleotidase from Rat Heart. Unless otherwise indicated, all steps were performed at 0–4 °C starting with 100 hearts.

Step 1: Extraction of 5′-Nucleotidase from Membrane Fraction. Rat hearts were forced through a Fisher mincer (a miniature meat grinder) and homogenized in 4 volumes of 10 mM Tris-HCl buffer, pH 7.5, with a Polytron PT35 (Brinkmann Instruments, Westbury, NY) at half-maximum speed for 30 s, followed by a waiting period of 30 s. The homogenization was repeated twice. The homogenate was centrifuged at 77000*g* for 60 min. The precipitate was resuspended in the same volume of homogenizing buffer and centrifuged as before. The resulting precipitate was resuspended in 4 volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 1% Triton X-100. The suspension was stirred at 37 °C for 30 min and was then centrifuged at 77000*g* for 30 min. The supernatant was saved, and the precipitate was extracted as before. The supernatants were combined.

Step 2: Heat and pH 5.4 Treatment. A 100-mL portion of the supernatant from step 1 was made 1 mM with respect to ADP and was heated in a water bath at 70 °C with continuous stirring until the temperature was 60 °C. This took about 2.5 min. The supernatant was held at 60 °C for 30 s and was then cooled rapidly on ice. The pH of the mixture was adjusted to 5.4 with 2 N acetic acid, and the mixture was centrifuged at 8000*g* for 20 min.

Step 3: CM-Sephadex Chromatography. The supernatant from step 2 was applied to a CM-Sephadex C-50 column (3.5 × 24 cm) equilibrated with 20 mM Tris–acetate buffer, pH 5.4, containing 0.1% Triton X-100 at a flow rate of 40 mL/h. The column was then washed with 400 mL of the starting buffer followed by 100 mM Tris–acetate, pH 6.5. About 15% of enzyme activity was eluted by the time the pH of the eluate had changed from 5.4 to 6.2. The CM-Sephadex was then transferred to a beaker, and the rest of the 5′-nucleotidase activity was eluted by stirring the slurry with a mixture of 200 mL of 100 mM Tris–acetate, pH 6.5, and 0.1% Triton X-100 for 2 h. The CM-Sephadex suspension was filtered on a Büchner funnel. Extraction of the CM-Sephadex slurry followed by filtration was repeated twice, and the eluates were combined. The pH of the filtrate was adjusted to 7.5 with 1 M Tris, and the solution was concentrated to about 200 mL by ultrafiltration using a YM30 membrane. $MgCl_2$, $MnCl_2$, and $CaCl_2$ were added to the concentrated solution so that the concentration of each was 1 mM.

Step 4: Concanavalin A–Sepharose Affinity Chromatography. The solution from step 3 was applied at a flow rate of 15 mL/h to a concanavalin A–Sepharose column (1 × 20 cm) equilibrated with a mixture of 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The column was washed with a mixture of 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 50 mM sodium cholate until the absorbance at 280 nm of the efluent ceased to change (300–400 mL). Enzyme activity was then eluted at a flow rate of 15 mL/h with the same mixture containing 0.3 M methyl α-mannoside. Fractions of 15 mL were collected. Fractions containing the highest enzyme activity were pooled and concentrated to about 30 mL by ultrafiltration using a YM30 membrane. The concentrated solution was dialyzed against 1000 mL of a mixture containing 50 mM Tris-HCl,

Table I: Purification of 5′-Nucleotidase from Rat Heart[a]

stage	total protein (mg)	total act. (units)	sp act. [units/(mg of protein)]	yield (%)
homogenate	19 800	221	0.011	100
77000*g* precipitate	11 900	196	0.017	89
Triton X-100 extract	2 590	224	0.087	101
heat and pH 5.4 treatment	460	179	0.389	81
CM-Sephadex	246	78	0.319	35
concanavalin A–Sepharose	22.4	94	4.2	43
DEAE-cellulose	13.3	73	5.5	33
ADP–agarose	0.25	59	236	27

[a] The preparation started with 100 hearts. Assay 1 was used to measure activity.

pH 7.5, and 0.05% Triton X-100 for 7 h, with one change of the mixture.

Step 5: *DEAE-cellulose Column Chromatography*. The dialyzed solution from step 4 was applied to a DEAE-cellulose column (1 × 20 cm) equilibrated with a mixture of 50 mM Tris-HCl, pH 7.5, and 0.05% Triton X-100. Most of the enzyme activity passed through the column. The active fractions were concentrated to about 6 mL as described before and dialyzed against 50 mL of a mixture containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 50 mM sodium cholate for 18 h with one change of the mixture.

Step 6: *ADP–Agarose Affinity Chromatography*. The dialyzed solution from step 5 was applied to an ADP–agarose column (1.1 × 5 cm) equilibrated with a mixture containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 50 mM sodium cholate, and the column was washed with the same buffer. 5′-Nucleotidase was eluted with the same mixture plus 0.5 mM AMP and 5 mM $MgCl_2$ at a flow rate of 5 mL/h. Fractions of 3 mL were collected. Fractions containing the highest activity of 5′-nucleotidase were concentrated first by ultrafiltration and then in a dialyzing tube surrounded with dry Sephadex G-200 powder until the volume was about 2 mL. The concentrated solution was dialyzed against 100 mL of a mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM sodium cholate, and 1 M NaCl. It was stored at 4 °C.

Results of the purification procedure are shown in Table I. The average specific activity from four preparations ± SD was 240 ± 18 units/mg.

Comments on the Purification Procedure. The CM-Sephadex step leads to a considerable loss of activity; however, this step is necessary for obtaining enzyme of the highest purity as judged by gel electrophoresis. Part of the activity lost during chromatography on CM-Sephadex is routinely recovered during the concanavalin A–Sepharose step which involves elution in the presence of Mn^{2+}, Mg^{2+}, and Ca^{2+} ions. When the eluate from the CM-Sephadex column was incubated with the same mixture of metal ions, no increase in activity was observed. This indicates that the concanavalin–Sephadex column removes an inhibitor or modifies the activity in some other way. In contrast to ADP–agarose, the enzyme did not bind tightly to ATP–agarose.

Gel filtration on Sephadex-200 (column bed 2.2 × 40 cm) may be employed after the DEAE-cellulose step instead of the CM-Sephadex step. The Sephadex-200 column is equilibrated with a mixture containing 50 mM Tris-HCl buffer, pH 7.5, 50 mM sodium cholate, and 0.1 M NaCl, and elution is carried out with the same mixture. The enzyme thus obtained has the same specific activity as the enzyme prepared by the standard procedure shown in Table I, but several faint protein bands are observed on gel electrophoresis which are absent when the enzyme is prepared by the standard procedure.

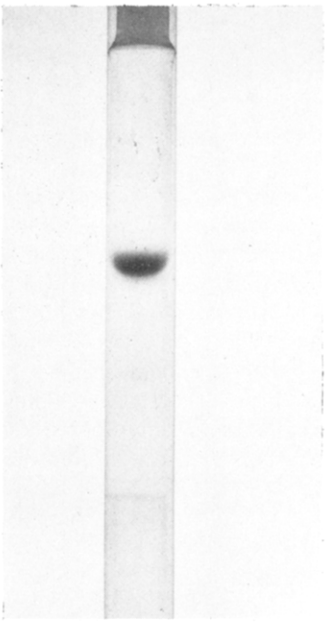

FIGURE 1: Electrophoresis of 5 μg of 5′-nucleotidase from rat heart on sodium dodecyl sulfate–7.5% polyacrylamide gel columns 5 × 70 mm. The gel was stained with Coomassie blue.

Purity and Molecular Weight of 5′-Nucleotidase. The purified preparation gave one major band and two very faint minor bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by staining with Coomassie blue (Figure 1). Attempts to remove these very faint bands by further purification using Sephadex G-200, hydroxylapatite, and ATP–agarose were unsuccessful. However, only a single band was detected on polyacrylamide gel electrophoresis without sodium dodecyl sulfate in the presence of Triton X-100 followed by staining with Coomassie blue; this band coincided with a single band of 5′-nucleotidase activity detected on a separate gel by precipitation of calcium phosphate as described under Materials and Methods (not shown). The apparent molecular weight of the polypeptide was 74 000; a constant value for the molecular weight was obtained with gels containing between 5% and 12.5% acrylamide.

Conventional methods for determining the molecular weight of the native enzyme, such as gel filtration and sucrose density gradient centrifugation, gave the apparent molecular weight of the enzyme–detergent complex. A bifunctional cross-linking reagent, dimethylpimelimidate, was employed to overcome this difficulty. When enzyme and cross-linking reagent were incubated for 1 h at room temperature, 50% of the original activity remained, and one major protein band corresponding to an apparent molecular weight of 147 000 was observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This compares with an apparent molecular weight of 74 000 obtained with the untreated enzyme (Figure 2). Staining of the polyacrylamide gel with periodic acid–Schiff reagent (Segrest & Jackson, 1972) revealed single bands for the cross-linked and untreated enzyme which ran in precisely the same positions as the bands stained for protein. These results constitute evidence that 5′-nucleotidase is composed of two subunits which are probably identical and that the enzyme is a glycoprotein.

Effect of Detergent. The purified enzyme showed no significant loss of activity when stored in 50 mM Tris-HCl, pH 7.5, and 0.03% Triton X-100 or 50 mM sodium cholate at 4 °C for 45 days; with the same buffer, it lost 40% of its activity in 0.005% Triton X-100, 50% in 10 mM sodium cholate, and

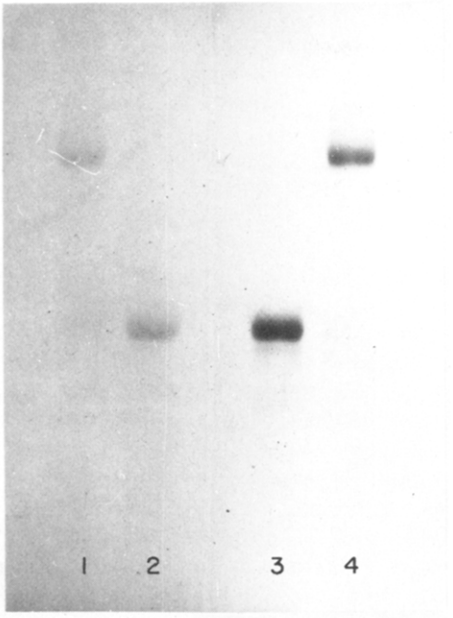

FIGURE 2: Cross-linking of 5′-nucleotidase with dimethylpimelimidate. The procedure for cross-linking the enzyme is described under Materials and Methods. Electrophoresis was performed on a sodium dodecyl sulfate–7% polyacrylamide slab gel. Lanes 1 and 2 contained 12 μg of enzyme and were stained for carbohydrate. Lanes 3 and 4 contained 5 μg of enzyme and were stained for protein. Lanes 2 and 3 contained untreated enzyme, and lanes 1 and 4 contained cross-linked enzyme. Similar experiments with molecular weight markers showed that the untreated and cross-linked enzyme yielded proteins with molecular weights of 74 000 and 148 000, respectively. This experiment was performed by Dr. Ming-kun Yu.

70% in 0.5 mM sodium cholate in 45 days.

The initial reaction velocity is linear for at least 1 h in reaction mixtures which contain >1 mM sodium cholate; in a reaction mixture containing 3.3 μM sodium cholate, the reaction velocity begins to decrease after 10 min. Sodium cholate slightly stimulates enzyme activity. With 22–52 mM sodium cholate in the assay mixture, the enzyme shows 25% greater activity than with 1.7 mM sodium cholate.

Effect of pH and Mg^{2+} on Activity. 5′-Nucleotidase shows optimum activity in Tris-HCl buffer at pH 7.5 when assayed with 100 μM AMP (Figure 3). A somewhat higher activity is observed in Mes–Tris buffer as reported previously for 5′-nucleotidase from smooth muscle of small intestine (Burger & Lowenstein, 1970). In the absence of Mg^{2+} ions, glycine–NaOH buffer yields a lower activity than Tris-HCl buffer. Glycine–NaOH buffer but not bicarbonate–NaOH buffer yields a second optimum at pH 9.5. The activity of 5′-nucleotidase in glycine–NaOH buffer is approximately doubled by 10 mM Mg^{2+} ions. The activity of the enzyme in bicarbonate–NaOH buffer is increased by Mg^{2+} ions at pH values greater than 9, and in the presence of this buffer, Mg^{2+} ions yield a second optimum at pH 9.3. In Tris-HCl buffer, the enzyme activity is inhibited slightly by Mg^{2+} ions at pH >7, and no second pH optimum is observed in the standard assay at 30 °C. However, when the assay is conducted at 37 °C, 10 mM $MgCl_2$ activates the enzyme, and a second optimum is observed (not shown in Figure 3). These observations are consistent with and extend results obtained with 5′-nucleotidase from bull seminal plasma (Bodansky & Schwartz, 1963; Levin & Bodansky, 1966) and mouse liver plasma membrane (Evans & Gurd, 1973).

When the activity of 5′-nucleotidase is measured in diethanolamine hydrochloride buffer, pH 9.5, at 30 °C, it is stimulated 20% by 6 mM $MgCl_2$. However, the same concentration of $MgCl_2$ increases its activity by 70% at 37 °C (Figure 4). The same effect is observed irrespective of whether $MgCl_2$ is added before or after the AMP. At concentrations higher than 10 mM, $MgCl_2$ inhibits the enzyme. The K_m for AMP in Tris-HCl, pH 9.5, and in glycine–NaOH, pH 9.5, was the same in the presence and absence of 10 mM

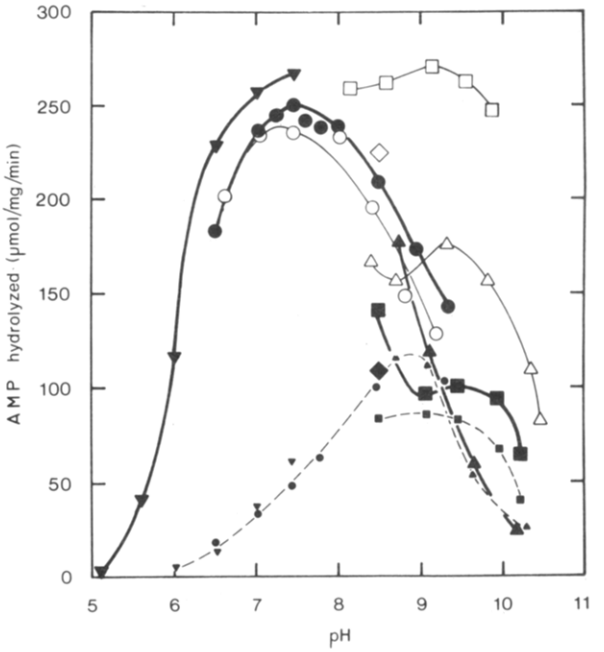

FIGURE 3: Effect of pH on activity with 100 μM AMP in the absence (solid symbols) and presence (open symbols) of 10 mM $MgCl_2$. Large symbols and solid lines are used for data obtained in the absence of ADP; small symbols and broken lines are used for data obtained in the presence of 17 μM ADP. The reactions were run in the following 50 mM buffers: (▼) Mes–Tris; (●, ○) Tris-HCl; (♦, ◊) Tricine–NaOH; (▲, △) bicarbonate–NaOH; (■, □) glycine–NaOH. The pH values were measured at the end of the reaction. Assay 1 was used in the absence of $MgCl_2$. Assay 2 was used in the presence of $MgCl_2$ because adenosine deaminase, which is used in assay 1, is significantly inhibited by $MgCl_2$ at pH values greater than 8. The reactions were run at 30 °C.

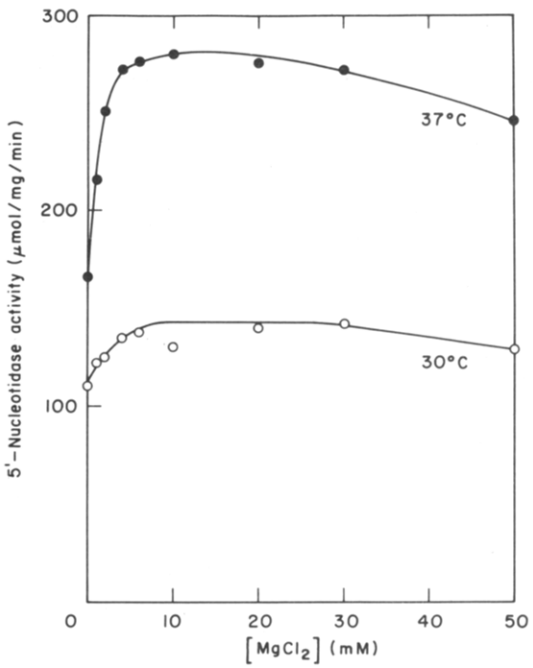

FIGURE 4: Effect of $MgCl_2$ on 5′-nucleotidase activity at 30 and 37 °C. The reaction mixture contained 50 mM diethanolamine hydrochloride, pH 9.5, 1.7 mM sodium cholate, 100 μM AMP, and $MgCl_2$ as indicated. Assay 2 was used.

Table II: Substrate Specificity of Rat Heart 5′-Nucleotidase[a]

compound tested	relative V_{max} (%)	K_m (μM)	relative V_{max}/K_m
AMP	100	5.6	18
6-chloropurine ribonucleoside 5′-monophosphate	50	5.3	9.4
GMP	54	6.4	8.4
IMP	55	8.0	6.9
ϵ-AMP	45	6.5	6.9
UMP	93	14	6.6
iso-AMP	24	7.0	3.4
AMPS	6	2.0	3.0
CMP	87	35	2.5
dTMP	19	14	1.4
dAMP	20	77	0.3
dGMP	12	48	0.3
$AMPNH_2$	6	57	0.2
dCMP	28	290	0.1
adenylosuccinate	1	103	0.01
2′,3′-AMP[c]	0		
2′,3′-CMP[c]	0		
ribose 5-phosphate	0		
glucose 6-phosphate	0		
p-nitrophenyl phosphate	0		

[a] Assay 2 was used for all substrates except AMPS and $AMPNH_2$ for which assay 1 was used. Purified enzyme (5 ng) (1.09 milliunits), 100 μM substrate, 50 mM Tris-HCl, pH 7.5, and 1.7 mM sodium cholate were used for determination of relative velocities by using a 20-min incubation period. V_{max} relative to the V_{max} for AMP (249 units/mg) was then calculated from the K_m for each substrate. The substrate concentration ranges used for K_m determination were the following: 5–80 μM for AMP,[b] GMP, IMP, UMP, dTMP, and iso-AMP; 10–100 μM for CMP; 10–160 μM for dGMP; 20–200 μM for dAMP; 30–490 μM for dCMP; 40–400 μM for adenylosuccinate. The assay mixtures contained 50 mM Tris-HCl, pH 7.5, 1.7 mM sodium cholate, enzyme, and substrate. The amount of enzyme and the incubation period was adjusted to give less than 20% hydrolysis. A continuous spectrophotometric assay (Burger & Lowenstein, 1970) was used to determine the K_m for AMPS and $AMPNH_2$ with [substrate] ranges of 0.5–5 and 10–100 μM, respectively. All nucleotides yielded Michaelis-Menten kinetics in the concentration ranges used. [b] Because assay 2 has a sensitivity limit of about 5 μM substrate, the K_m for AMP was also determined by using the continuous spectrophotometric assay which permits the use of much lower AMP concentrations. The K_m value for AMP obtained with this assay was 5.3 ± 0.6 μM. [c] Mixed isomers. All other nucleotides tested were 5′-nucleotides.

$MgCl_2$. However, both V_{max} and K_m were increased in bicarbonate–NaOH, pH 9.5, upon addition of 10 mM $MgCl_2$, suggesting a different mechanism of activation in this buffer system. These experiments were performed by using assay 3. Different effects of Mg^{2+} ions at different temperatures were also reported for the enzyme from bull seminal plasma (Levin & Bodansky, 1966).

ADP inhibits enzyme activity more at a lower pH than at a higher pH. At pH 6, enzyme activity is almost completely inhibited by 17 μM ADP, whereas at pH values greater than 9, the inhibition is virtually abolished (Figure 3). These observations suggest that the inhibitory species is ADP^{2-}.

Substrate Specificity. The activity of 5′-nucleotidase toward a number of nucleoside monophosphates and other phosphate esters is shown in Table II. The enzyme shows the highest V_{max} and among the lowest K_m values with AMP. The K_m values for the purine ribonucleoside monophosphates AMP, GMP, IMP, ϵAMP, and 6-chloropurine ribonucleoside 5′-monophosphate are similar; K_m values for the purine deoxyribonucleoside monophosphates dAMP and dGMP are about 10 times higher. Ribonucleotides are hydrolyzed with a V_{max} that is 3–5 times greater than the V_{max} for the corresponding deoxyribonucleotides. The enzyme hydrolyzes isoAMP at 24% of the V_{max} for AMP, with a K_m similar to that for AMP. Among the substrates tested by us, 5′-nucleotidase shows the lowest K_m value for AMPS but with a V_{max} only 6% of that observed with AMP. Murray & Atkinson (1968) reported that 5′-nucleotidase from *Crotalus adamanteus* venom hydrolyzes AMPS with one-fiftieth of the maximum velocity and about two-thirds the K_m for AMP.

Nucleoside 2′- and 3′-phosphates, sugar phosphates, and *p*-nitrophenyl phosphate are not hydrolyzed at concentrations of 100 μM. No activity is observed at pH 7.5 toward 1 mM glucose 6-phosphate and 1 mM *p*-nitrophenyl phosphate by using 10 times more enzyme than in the assay with AMP. Moreover, *p*-nitrophenyl phosphate (1 mM) is not hydrolyzed in 50 mM acetate buffer, pH 5.0, or in 50 mM bicarbonate–NaOH buffer, pH 10.0, with or without 10 mM $MgCl_2$, by using 10 times more enzyme than in the assay with AMP. In the case of the 1 mM ribose 5-phosphate tested by us, 0.05% was hydrolyzed initially, as determined by liberation of orthophosphate; however, prolonged incubation resulted in no further release of orthophosphate, suggesting that the enzyme hydrolyzed a small amount of a contaminant in the ribose 5-phosphate.

Specificity regardless of concentration is best expressed by V_{max}/K_m. By this criterion, AMP is the best substrate, four different purine nucleotides and UMP are between one-half and one-third as efficacious, and the deoxyribonucleoside monophosphates are much poorer substrates. 6-Chloropurine ribonucleoside 5′-phosphate and ϵAMP are relatively good substrates, while adenylosuccinate is a very poor substrate, indicating that while a bulky substituent in the 6-position is tolerated, it must not be too big, or that it must not be negatively charged, or both.

Inhibition by EDTA and Reactivation by Divalent Cation. 5′-Nucleotidase is inhibited by EDTA. The inactivated enzyme can be reactivated almost completely by dialysis against divalent cations. Several divalent cations are able to restore enzyme activity, Zn^{2+} ions being the most effective (Table III, experiment 2). After reactivation of the enzyme by dialysis against Zn^{2+} ions, its activity remains high even after the excess Zn^{2+} ions are dialyzed away (Table III, experiment 2). Mg^{2+} or Mn^{2+} did not enhance the effects of Zn^{2+} ions, but note that a mixture of Mg^{2+} and Mn^{2+} ions was able to restore the activity of the enzyme partially.

These observations raised the question of whether EDTA removes a metal bound to the enzyme or whether it inhibits by chelating a bound metal without removing it. 5′-Nucleotidase was therefore incubated with 1.38 mM [^{14}C]EDTA for 9 h, at which point 5.4% of the original enzyme activity remained. The incubation mixture was then subjected to gel filtration through a Sephadex G-25 column under conditions which completely separated free EDTA from 5′-nucleotidase. Virtually no radioactivity remained associated with the enzyme (Figure 5), and 45% of the original activity was restored by the gel filtration. Attempts to restore the rest of the activity by incubating the enzyme with 1 mM $MgCl_2$ or 1 mM $ZnCl_2$ at 4 °C were unsuccessful. These results suggest that the inhibition was due to the chelation by EDTA of a metal on the enzyme and that about half of the metal was not removed under the conditions used by us. The irreversible part of the inhibition may have been caused by the actual removal of the metal followed by denaturation of the enzyme.

Discussion

The membrane fraction from rat heart obtained by us contains about 1 unit of 5′-nucleotidase per mg of membrane protein assayed at 30 °C. The specific activity of the pure

Table III: Inactivation of 5′-Nucleotidase by EDTA and Reactivation by Divalent Cations[a]

expt	1 mM salt present during dialysis after EDTA treatment	reactivation (%)
1	none	0
	$MgCl_2$	73
	$MnCl_2$	40
	$CoCl_2$	56
	$CaCl_2$	19
2	none	0
	$ZnCl_2$	82
	$ZnCl_2$ + $MgCl_2$	76
	$ZnCl_2$ + $MnCl_2$	76
	$ZnCl_2$ + $CoCl_2$	86
	$MgCl_2$ + $MnCl_2$	41

[a] Cholate in the stock solution of enzyme was changed to Triton X-100 by adding the latter to a final concentration of 0.05% and then dialyzing the enzyme solution against 50 volumes of mixture A containing 50 mM Tris-HCl, pH 7.5, and 0.05% Triton X-100 for 2 days with three changes of mixture A. EDTA was added to the enzyme solution to give a final concentration of 1 mM, and the mixture was incubated for 3 h at 0 °C in experiment 1 and for 8 h in experiment 2. The protein concentration was 0.39 μg/mL for experiment 1 and 0.48 μg/mL for experiment 2. The remaining activity was 5% in experiment 1 and 0.45% in experiment 2. After incubation with EDTA, the enzyme solutions were dialyzed against mixture A, which also contained 1 mM of the divalent metal chloride indicated, for 2 days with one change of mixture A containing metal ion. In experiment 1, the enzyme activity was determined by using assay 1 at the end of the dialysis with divalent metal chloride; in this case, the assay contained 33 μM divalent metal chloride, except in the control which lacked divalent metal. In experiment 2, dialysis of the enzyme against divalent metal chlorides was followed by dialysis of the enzyme with mixture A without divalent metal ion for 1 day with one change of mixture A.

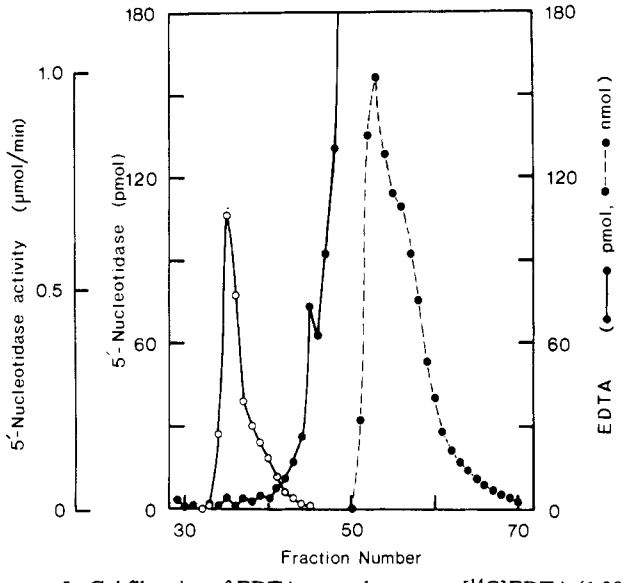

FIGURE 5: Gel filtration of EDTA-treated enzyme. [^{14}C]EDTA (1.38 μmol, 2.76 μCi/μmol) was added to 25.6 μg (346 pmol) of 5′-nucleotidase. The mixture, which had a volume of 1 mL, was incubated at 0 °C for 9 h. At this point, it showed 5.4% of the original 5′-nucleotidase activity. The mixture was applied to a column packed with Sephadex G-25 (2.2 × 40 cm) equilibrated with a mixture of Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.02% Triton X-100. The column was eluted with the same buffer at a flow rate of 9 mL/h, and fractions of 1.5 mL were collected. An aliquot of each fraction was assayed for 5′-nucleotidase activity (○) and for radioactivity (●).

enzyme is 236 units/mg of protein at 30 °C. Thus 5′-nucleotidase accounts for about 0.4% of the plasma membrane protein.

The purification procedure described here yields 2 μg of the pure enzyme per g of fresh heart. Purification of the membrane-bound enzyme has been reported from various sources with the following specific activities at 37 °C in units per milligram: smooth muscle of small intestine,[2] 13.8 (Burger & Lowenstein, 1970); rat cerebellum, 26 (Bosmann & Pike, 1971); mouse liver, 4.8 (Evans & Gurd, 1973); rat liver, 193 (Nakamura, 1976) and 221 (Slavik et al., 1977); pig lymphocytes, 41 (Dornand et al., 1978); rat heart,[3] 360 (this paper). The enzyme from pig lymphocyte membranes has been reported to be a glycoprotein. The enzyme was absorbed on *Lens culinaris* lectin coupled to Sepharose and eluted with methyl α-mannoside (Hayman & Crumpton, 1972). The enzyme from mouse liver has been partially purified and shown to be a dimer and a glycoprotein (Evans & Gurd, 1973). Antibodies against plasma membranes from mouse liver inhibit 5′-nucleotidase of plasma membranes prepared from mouse liver, rat liver, and pig lymphocytes (Gurd & Evans, 1974).

The enzyme is inhibited by EDTA, but the inhibition is largely reversed by dialysis of the enzyme against bivalent metal ions (Table III). When the EDTA is removed from the enzyme by gel filtration, much of the original activity is restored. In the case of the experiment shown in Figure 5, presumably half of the bound metal was not removed from the enzyme during the time the enzyme was incubated with EDTA. Incubation of the EDTA-treated, gel-filtered enzyme with various bivalent metal ions failed to restore the 55% of the activity that was lost, possibly because the enzyme was denatured to this extent by removal of a bound metal. Restoration of the lost activity may be possible under special conditions which remain to be elucidated. It was shown previously that the particulate 5′-nucleotidase from bovine brain is inactivated by acid and alkali treatments and that activity can be partially restored with zinc and other bivalent metal ions (Tanaka et al., 1973). On the other hand, it was reported that the membrane-bound enzyme of rat glioma cells is markedly inhibited by zinc ions and is activated somewhat by manganese and cobalt ions (Stefanovic et al., 1976).

The complex behavior of the enzyme in the presence of various buffer ions above pH 7.5 (Figure 3) suggests that some buffer ions, such as glycine and carbonate–bicarbonate, interact with the enzyme, possibly with a metal ion bound to the enzyme. Magnesium ions modify this behavior since they activate the enzyme above pH 8 in the presence of some but not of other buffer ions. Because magnesium ions interfere with the adenosine deaminase assay, we followed the 5′-nucleotidase reaction by measuring the orthophosphate liberated. We failed to observe an increase in activity when magnesium ions were added to 5′-nucleotidase assays containing Tris (Figure 3), perhaps because Tris acted as a phosphate acceptor. This possibility is raised because of the observation that in the case of alkaline phosphatase from *Escherichia coli*, Tris is 100 times more effective as a phosphate acceptor than water (Reid & Wilson, 1971). However, alkaline phosphatase from calf intestine transfers phosphate from *p*-nitrophenyl phosphate to Tris between pH 6.9 and 7.9, but not between pH 8 and 10 (Neumann et al., 1975). Alkaline phosphatases from *E. coli* (Reid & Wilson, 1971; Bosron et al., 1977) and mammalian sources (Fernley, 1971; Cathala et al., 1975) contain zinc and are activated by magnesium ions. The *E. coli* enzyme also contains magnesium (Bosron et al., 1977). Other types

[2] Corrected from a specific activity of 5.5 at 24 °C to 37 °C by using a temperature coefficient of 2.5 for the solubilized enzyme (Stanley & Luzio, 1978).

[3] Corrected from a specific activity of 240 at 30 °C to 37 °C by using a temperature coefficient of 1.5 for the solubilized enzyme (Stanley & Luzio, 1978).

of phosphatases, for example, fructose-1,6-bisphosphatase (Benkovic et al., 1978), also contain zinc and exhibit complex interactions with magnesium ions. Note also that nucleotide-metabolizing enzymes generally contain zinc.

Arrhenius plots for 5′-nucleotidase from rat liver show a change in slope, suggesting that a conformational change occurs in the enzyme at 30.4 °C (Stanley & Luzio, 1978). This change may be related to the change in sensitivity of the enzyme to Mg^{2+} ions when the temperature is raised from 30 to 37 °C (Figure 4). A possible explanation for the stimulation of 5′-nucleotidase activity by Mg^{2+} ions is that the AMP–Mg complex is the preferred substrate. However, the curve showing the effect of increasing the Mg^{2+} concentration at 37 °C (Figure 4) does not support this possibility, because at Mg^{2+} concentrations above 10 mM, the activity of the enzyme declines in a manner predicted by assuming that the substrate is free AMP, that AMP–Mg is not a substrate, and that the stability constant for AMP–Mg is 100.

The specificity studies reported in Table II suggest strongly that AMP is the normal substrate of 5′-nucleotidase from heart. This is consonant with its involvement in the control of vasodilation by the production of adenosine.

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