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STUDIES ON A Ca^{2+}-DEPENDENT NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE IN RAT LIVER PLASMA MEMBRANES

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Summary

A membrane-bound Ca^{2+}-dependent nucleoside triphosphate pyrophosphohydrolase was solubilized in deoxycholate, separated from inorganic pyrophosphatase, and partially characterized. The K_m for a variety of substrates was determined. At 10^{-4} M free Ca^{2+} (pH 8.0) the K_m values for ATP and GTP were 0.32 and 2.2 μM, respectively. With ATP as substrate, Mg^{2+}, Sr^{2+}, and Ba^{2+} could only replace Ca^{2+} to a limited degree. Both purine and pyrimidine nucleoside triphosphates were hydrolyzed yielding PP_i and mononucleotides and similarly AMP was formed from adenosine-($\beta\gamma$-methylene)triphosphate. UDPglucose was hydrolyzed at the pyrophosphate bond. Tripolyphosphate and phosphoribosyl-1-pyrophosphate (*P*-rib-*PP*) were not hydrolyzed.

Substrate competition experiments showed that GTP inhibited pyrophosphohydrolysis of ATP competitively. However, UDPglucase and adenosine-($\beta\gamma$-methylene)triphosphate inhibited ATP pyrophosphohydrolysis in a non-linear manner. Adenosine-($\beta\gamma$-methylene)triphosphate inhibited pyrophosphohydrolysis of UDPglucose non-competitively, whereas UDPglucose inhibition of adenosine-($\beta\gamma$-methylene) triphosphate pyrophosphohydrolysis was competitive.

The molecular weight of ATP pyrophosphohydrolase was estimated at 120 000 and the p*I* at 5.1

Pyrophosphohydrolysis of adenosine-($\beta\gamma$-methylene)triphosphate was studied in a number of rat organs. Nearly all activity could be sedimented at

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Abbreviations: p(NH)ppA, adenosine-($\beta\gamma$-imido)triphosphate; p(CH_2)ppA, adenosine-($\beta\gamma$-methylene)-triphosphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; *P*-rib-*PP*, phosphoribosyl-1-pyrophosphate.

50 000 X g. Very high activities were found in liver, kidney and small intestine, whereas low activities were found in brain and blood.

Introduction

A membrane-bound ATP pyrophosphohydrolase (EC 3.6.1.8) was first demonstrated in rat liver plasma membranes by Lieberman et al. [1]. This observation has since been confirmed by several authors [2—4]. Nucleotide pyrophosphatase (EC 3.6.1.9) has also been studied in rat liver and shown to be present in the plasma membrane [5,6]. Nucleotide pyrophosphatase has been partially purified from rat liver [7,8] and purification to apparent homogeneity has been reported [9,10]. From the purification patterns and substrate competition experiments it was concluded that an enzyme with a broad specificity is able to hydrolyze a variety of dinucleotides and sugar nucleotides at their pyrophosphate bond. It was also concluded, that phosphodiesterase I activity (EC 3.1.4.1) was due to this enzyme. All purified nucleotide pyrophosphatase preparations contained ATP pyrophosphohydrolase activity. However, substrate competition experiments involving ATP did not show competitive inhibition. Thus, it is not certain whether ATP pyrophosphohydrolase activity was due to the same enzyme as nucleotide pyrophosphatase.

Johnson and Welden [11] demonstrated hydrolysis of p(NH)ppA to PNP and AMP by rat liver plasma membranes and suggested this reaction was due to nucleotide pyrophosphatase. Pyrophosphohydrolysis of ATP, p(NH)ppA, and $p(CH_2)$ppA has been shown to be dependent on low free concentrations of Ca^{2+} and it was suggested that all three reactions were caused by the same enzyme [12].

In the present investigation ATP pyrophosphohydrolysis by a rat liver plasma membrane enzyme is further characterized and the tissue distribution of pyrophosphohydrolysis of $p(CH_2)$ppA studied. This substrate was chosen since it is not known to be hydrolyzed by enzymes other than ATP pyrophosphohydrolase and/or nucleotide pyrophosphatase.

Materials and Methods

Materials

[8-^{3}H]Adenosine-5′-($\beta\gamma$-methylene)triphosphate (ammonium salt; specific radioactivity 23 Ci/mmol), [2-^{3}H]AMP (ammonium salt; specific radioactivity 5 Ci/mmol), [5-^{3}H]UTP (ammonium salt; specific radioactivity 30 Ci/mmol), 5′-[γ-^{32}P]ATP (triethylammonium salt; specific radioactivity 3000 Ci/mmol), and 5′-[γ-^{32}P]GTP (ammonium salt; specific radioactivity 20 Ci/mmol) were from the Radiochemical Centre, Amersham, Bucks., U.K. ATP, GTP, AMP, 5-phosphorylribose-1-pyrophosphate, adenosine, TES, lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), β-galactosidase (EC 3.2.1.23) and bovine serum albumin were from Sigma Chemical Company (St. Louis, MO, U.S.A.). $p(CH_2)$ppA, inosine, hypoxanthine, ATP, UDPglucose, inorganic pyrophosphatase (EC 3.6.1.1), phosphoglucomutase (EC 2.7.5.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and UDPglucose pyrophosphorylase (EC

2.7.7.9) were from Boehringer, Mannheim, F.R.G. Nitrilo-triacetic acid, Triton X-100 and polyethyleneimine-cellulose thin layer sheets (product No. 5579) were from Merck, Darmstadt, F.R.G. Tripolyphosphate, *N,N,N',N'*-tetramethylenediamine (TEMED), acrylamide, bis(*N,N'*-methylene bisacrylamide) were from B.D.H., Poole, U.K. Ampholine (pH range 3—10) was from Pharmacia, Sweden. 3-deoxy-ATP was a generous gift from Professor Hans Klenow, Department of Biochemistry B, The Panum Institute. Ion-exchange solution for the Ca^{2+} electrode was a generous gift from Professor W. Simon, Swiss Federal Institute of Technology, Zürich. All other reagents were of analytical grade.

Methods

Rat liver plasma membranes were prepared as described by House et al. [4]. ATP pyrophosphohydrolase was solubilized and separated from inorganic pyrophosphatase as previously described [12].

The following media were used for enzyme analysis: (A) 10 mM nitrilo-triacetic acid, 20 mM TES, pH 8.0, (B) 10 mM nitrilo-triacetic acid, 1 mM EGTA, 20 mM TES, pH 8.0, and (C) 5 mM EDTA, 5 mM EGTA, 20 mM TES, pH 8.0.

To these media substrate and divalent metal ions were added. Free concentrations of divalent metal ions were calculated using a computer program described earlier [13]. In these calculations formation of Ca_2EDTA is also taken into account. This explains why low free Ca^{2+} concentrations can be obtained in medium C with a total Ca^{2+} concentration higher than the concentrations of chelators.

Different values for the association constants between divalent metal ions and ATP have been published [14]. Using one set of constants a discrepancy was found between electrode measurements and calculated values [15]. Consequently, the validity of calculated free Ca^{2+} concentrations were checked with a Ca^{2+}-electrode [16] when Ca^{2+} was the only divalent metal ion, and low concentrations of ATP or other substrates were chosen.

All enzyme assays were performed in duplicate.

Assay of ATP pyrophosphohydrolase using p(CH_2)ppA as substrate

The assay was performed essentially as previously described [12] by measuring [^{3}H]AMP production from [^{3}H]p(CH_2)ppA. Modifications were introduced by addition of IMP, inosine and hypoxanthine to the carrier mixture to ensure full recovery of counts using crude tissues as enzyme. Furthermore separation of substrate from products was obtained by thin-layer chromatography on polyethyleneimine-cellulose thin layer sheets by elution for 7 cm in 0.3 M sodium formiate buffer, pH 3.4.

Assay of nucleotide pyrophosphatase and nucleoside triphosphate pyrophosphohydrolase using [^{3}H]UDPGlc or [^{3}H]UTP as substrates

These assays were performed as described above for measurement of p(CH_2)ppA pyrophosphohydrolase with the following exceptions: [^{3}H]-UDPGlc or [^{3}H]UTP replaced [^{3}H]p(CH_2)ppA and the carrier mixture consisted of 2 mM each of UDPGlc, UDP, UMP and uridine. Thin-layer chromatographic separation of nucleotides on polyethyleneimine-cellulose thin-layer

sheets was performed as described by Randerath and Randerath [17]. In all experiments no activity was found in the UDP spot, indicating that the enzyme preparation used did not contain UTPase activity.

Assay of nucleoside triphosphate pyrophosphohydrolase using either (γ-^{32}P)-labelled nucleotides (ATP or GTP) or unlabelled trinucleotides (CTP, UTP, or 3-deoxy-ATP) as substrates

$^{32}PP_i$ formation from (γ-^{32}P)-labelled substrates was measured as described previously [12] with the modification that the reaction was stopped by addition of 1.3 M perchloric acid (final concentration) followed by neutralization with 1.3 M KOH.

Assays using unlabelled substrates were performed in a similar manner by direct measurement of PP_i production [13].

Assay of 5'-nucleotidase

This assay was performed by measurement of [^{3}H]AMP to [^{3}H]adenosine [18]. The following modifications were introduced: substrate concentration was 1 mM, assay temperature was 37°C, and carrier mixture contained AMP, adenosine, IMP, inosine and hypoxanthine.

Protein was assayed by the method of Lowry et al. [19] with some modifications [20].

Tissue distribution of p(CH_2)ppA pyrophosphohydrolysis

For each experiment one rat was killed by a blow on the head. A number of organs (see Table III) were immediately removed and placed on ice. Part of each organ was minced with a pair of scissors in 5 mM Tris (pH 7.4) containing 250 mM sucrose. The mince was homogenized for 30 s in a Virtis homogenizer (model 45) at setting 40. The homogenate was filtered through 2 layers of cheese cloth, and centrifuged at 50 000 $\times g$ for 1 h. Supernatants and pellets (resuspended in 20 mM TES, pH 8.0) were stored at −20°C until use.

p(CH_2)ppA pyrophosphohydrolase analyses were assayed in medium A (see above) containing 5 μM [^{3}H]p(CH_2)ppA and 9.37 mM $CaCl_2$ (free Ca^{2+} concentration 10^{-4} M).

Polyacrylamide gel electrophoresis

Polyacrylamide gels of concentrations from 5 to 11% were prepared as described by Dewald et al. [21] except that the gels were photopolymerized using riboflavin at a concentration of 2 mg/100 ml.

For electrophoresis 25 μl of liver plasma membrane (approx. 100 μg protein) were solubilized for 15 min at room temperature by addition of 2.5 μl 10% Triton X-100. 5 μl 60% sucrose were added and the mixture centrifuged at 100 000 $\times g$ for 60 min at 4°C. 5 μl 0.004% bromophenol blue were added to the supernatant (always containing more than 90% of p(CH_2)ppA pyrophosphohydrolase activity), which was then applied on top of a gel (5 × 100 mm). Electrophoresis was run at 3 mA per gel until the dye reached the bottom of the gel.

Gels were stained for ATP pyrophosphohydrolase by incubating in 20 mM TES (pH 8.5), 20 mM $CaCl_2$ and 0.2 mM ATP (modified from Wang and Morris

[22]). The PP_i formed appeared as a white calcium precipitate after at least 15 min.

Alkaline phosphatase activity was detected as described by Braatz and McIntire [23] and 5'-nucleotidase as described for ATPase by Vilhardt and Hope [24] except that AMP was used instead of ATP.

Isoelectric focusing in polyacrylamide gels was performed as described by Holtlund and Kristensen [25]. The position of ATP pyrophosphohydrolase was found as described above.

Results and Discussion

Except for the tissue distribution and electrophoresis experiments, enzyme analyses were carried out using deoxycholate-solubilized liver plasma membrane preparation described in the Methods.

Activation of ATP pyrophosphohydrolase by Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+}

In Table I activation of ATP pyrophosphohydrolase by Ca^{2+} is compared with activation by Mg^{2+}, Sr^{2+} and Ba^{2+}. Medium B, which contained 10 mM nitrilo-triacetic acid was chosen for these experiments to ensure well defined levels of free Ca^{2+} [26]. Since very small total concentrations of Ca^{2+} are required in a pure nitrilo-triacetic acid system to obtain free Ca^{2+} concentrations below 10^{-7} M, 1 mM EGTA was in addition included in medium B. This compound binds endogenous Ca^{2+} which in the pure nitrilo-triacetic acid system would contribute significantly to the total Ca^{2+} concentration calcu-

TABLE I

ACTIVATION OF ATP PYROPHOSPHOHYDROLASE ACTIVITY BY Ca^{2+}, Sr^{2+} AND Ba^{2+}

Pyrophosphohydrolysis of ATP was measured using partially purified enzyme preparation from rat liver plasma membranes, solubilized in deoxycholate and free of inorganic pyrophosphatase. Enzyme activity was estimated by following $^{32}P_i$ formation from 5 μM [γ-^{32}P]ATP in a medium containing 10 mM nitrilo-triacetic acid and 1 mM EGTA at pH 8.0. Assays were performed in duplicate at 30 and 60 min. Divalent metal ions were added to the incubation medium to obtain the required free concentration. Enzyme activity is expressed relative to activity with 10^{-5} M free Ca^{2+} which was set to 1. Mean ±S.E.. *n* is the number of experiments.

Ion	Total (mM)	Free (M)	Enzyme activity relative to activity at 10^{-5} M free Ca^{2+}	*n*
none	0	0	0.063 ± 0.03	5
Ca^{2+}	1.09	10^{-7}	0.45 ± 0.06	5
Ca^{2+}	6.43	10^{-5}	1.0 ± 0.1	5
Ca^{2+}	11.90	10^{-3}	2.1 ± 0.2	5
Mg^{2+}	0.0113	10^{-7}	0.10	2
Mg^{2+}	1.02	10^{-5}	0.23	2
Mg^{2+}	10.90	10^{-3}	1.1	2
Sr^{2+}	0.146	10^{-7}	0.14	2
Sr^{2+}	1.33	10^{-5}	0.30	2
Sr^{2+}	10.10	10^{-5}	0.99	1
Ba^{2+}	0.0716	10^{-}	0.16	2
Ba^{2+}	1.14	10^{-}	0.27	2

lated to be added. Free Ca^{2+} concentrations were checked with a Ca^{2+} electrode (see Methods). Table I shows that the other divalent cation can only replace Ca^{2+} to a limited degree. It was calculated [12] that 0.5 mM total Ca^{2+} would be necessary to allow 10^{-7} M free Ca^{2+} in the medium containing 10^{-3} M free Sr^{2+}. It is therefore concluded that the activity obtained with Mg^{2+}, Sr^{2+}, and Ba^{2+} are not due to displacement of endogenous Ca^{2+} from the chelators since such heavy contamination is very unlikely.

Enzyme activity is increased by a factor of 2 when free Ca^{2+} is raised from 10^{-7} to 10^{-5} M. This result was also found using medium A (nitrilo-triacetic acid) containing 5 μM ATP in three experiments. In a previous publication [12] a factor of 10 increase was reported using medium C (EGTA, EDTA) containing 2.5 mM ATP. Probably, the calculated free Ca^{2+} concentration of 10^{-7} M in medium C containing 2.5 mM ATP was different from the actual value due to underestimation of ATP affinity to Ca^{2+} [14].

In agreement with our results Decker and Bischoff [9] found inactivation of phosphodiesterase I and UDPGlc pyrophosphatase by EDTA and partial reactivation by Mg^{2+} or Ca^{2+}. Similarly Evans et al. [10] found inactivation of phosphodiesterase I by EDTA and a subsequent 120% reactivation by the addition of 10 mM Ca^{2+}.

Kinetic experiments

Double reciprocal plots for the pyrophosphohydrolysis of ATP in medium C containing 11.9 mM $CaCl_2$ (free Ca^{2+}, $5.5 \cdot 10^{-4}$ M) were linear. From four experiments the K_m was estimated as 0.32 μM (see Table II). In a medium containing 10^{-5} M free Ca^{2+}, 10^{-3} M free Mg^{2+}, the K_m was somewhat higher at 0.94 μM (Table II). The K_m for pyrophosphohydrolysis of GTP was also very low (2.22 μM), whereas the K_m values for UTP, p(CH_2)ppA and UDPGlc were higher (Table II).

A value of 780 μM for K_m using ATP as substrate has been reported [9]. In an attempt to verify this, three experiments were performed with ATP concentrations up to 5 mM. However, the reaction rates were constant with ATP concentrations above 5 μM. The K_m for UDPGlc in the interval from 95 to 590 μM has been measured [5,8,9] as compared with our value of 11 μM (Table II). Johnson and Welden [11] found the K_m for p(NH)ppA at 19 μM, which is close to our value for p(CH_2)ppA at 21 μM in Table II.

There are at least two explanations for these discrepancies. First, a pH ranging from 7.5 to 11 was used in the various investigations. It has been shown that the K_m for pyrophosphohydrolysis of p(NH)ppA [11], UDPGlc, and coenzyme A [5] is dependent on pH. Second, careful control of free Ca^{2+} was not ensured in previous investigations of these enzyme activities. Endogenous Ca^{2+} may, under such circumstances, be important for enzyme activity. At higher substrate concentrations, chelation of endogenous Ca^{2+} by the substrate will then inhibit the enzyme activity and cause overestimation of the K_m.

Pyrophosphohydrolysis of CTP and 3-deoxy-ATP was compared to pyrophosphohydrolysis of ATP in medium C containing 11.9 mM $CaCl_2$ (free Ca^{2+}, $5.5 \cdot 10^{-4}$ M) and a substrate concentration of $5 \cdot 10^{-5}$ M. In two separate experiments pyrophosphohydrolysis of CTP and 3-deoxy-ATP was 76 and 68% of the value for ATP at similar concentration. In the same medium 5-phospho-

TABLE II

K_m AND V FOR PYROPHOSPHOHYDROLYSIS OF A VARIETY OF SUBSTRATES USING A PARTIALLY PURIFIED ATP PYROPHOSPHOHYDROLASE FROM RAT LIVER PLASMA MEMBRANES AS ENZYME

The analyses were performed in final volume of 20 μl incubation medium containing 5 mM EDTA, 5 mM EGTA, and 20 mM TES, pH 8.0 (medium C). $CaCl_2$ and $MgCl_2$ were added to obtain the indicated free concentrations. 0.01—0.1 μg enzyme preparation was added to the incubation medium, and incubation was carried out for 5—20 min. Pyrophosphohydrolysis of trinucleotides was estimated by incorporating released PP_i into [^{3}H]UTP. When the trinucleotides were labelled with ^{32}p at the γ-phosphate (ATP and GTP) the resulting [^{3}H,^{32}p]UTP was separated from the substrate. Analysis using [^{3}H]p(CH_2)ppA or [^{3}H]UDPGlc as substrate was estimated from appearance of ^{3}H-labelled mononucleotide. Initial velocities were estimated from mean of at least two measurements with substrate breakdown below 10%, and linearity with time was always observed. The kinetic constants were calculated as described by Wilkinson [29]. This method gives also the S.E.

Substrate	K_m (μM)	V (nmol/min per mg protein)
Medium C (total Ca^{2+}: 11.9 mM, free Ca^{2+}: $5.5 \cdot 10^{-4}$ M)		
ATP	0.16 ± 0.03	17.1 ± 1.5
	0.38 ± 0.15	9.14 ± 2.29
	0.46 ± 0.10	
	0.30 ± 0.09	
GTP	1.1 ± 0.4	163 ± 41
	2.2 ± 0.5	156 ± 28
Medium C (total Ca^{2+}: 6.83 mM, Mg^{2+}: 6.62 mM; free Ca^{2+}: 10^{-5} M, Mg^{2+}: 10^{-3} M)		
UTP	14.1 ± 0.7	583 ± 10
p(CH_2)ppA	21 ± 2	58 ± 2
	11 ± 2	96 ± 17
UDPGlc	11 ± 2	913 ± 56
ATP	0.94 ± 0.03	22 ± 0.4
	1.5 ± 0.7	34 ± 13

ribosyl-1-pyrophosphate and tripolyphosphate at concentrations of $5 \cdot 10^{-5}$ M did not release detectable PP_i.

Substrate competition experiments were performed in medium C containing 6.83 mM $CaCl_2$ and 6.62 mM $MgCl_2$ (10^{-5} M free Ca^{2+} and 10^{-3} M free Mg^{2+}). The results were as follows:

1. GTP competitively inhibited pyrophosphohydrolysis of ATP with a K_i close to the K_m (K_i = 1.1 μM, mean of two experiments) calculated as described by Dixon and Webb [27].

2. Inhibition of pyrophosphohydrolysis of $5 \cdot 10^{-7}$ M ATP by UDPGlc and p(CH_2)ppA was estimated in two sets of experiments. At a concentration of $5 \cdot 10^{-6}$ M, UDPGlc inhibited ATP pyrophosphohydrolysis by 10%, while p(CH_2)ppA caused 17% inhibition. At a concentration of $5 \cdot 10^{-5}$ M, UDPGlc caused 60% and p(CH_2)ppA 64% inhibition. Attempts to further classify the inhibition failed, since double reciprocal plots with ATP concentrations from $5 \cdot 10^{-8}$ to $5 \cdot 10^{-7}$ M in the presence of UDPGlc and p(CH_2)ppA were not linear.

3. UDPGlc inhibited p(CH_2)ppA pyrophosphohydrolysis non-competitively, whereas p(CH_2)ppA inhibited UDPGlc pyrophosphohydrolysis competitively

(K_i = 34 μM, mean of two experiments). Johnson and Welden [11] similarly found non-competitive inhibition of p(NH)ppA pyrophosphohydrolysis by UDPglc.

Substrate competition experiments have previously been performed using enzyme preparations from rat liver [5,7—10]. Competitive inhibition was found between a variety of dinucleotides and sugar nucleotides including *p*-nitrophenyl thymidine-5′-phosphate. The inhibition patterns when ATP was the inhibiting agent, is less simple. Thus, non-competitive inhibition by ATP on UDPglc pyrophosphohydrolysis has been found [7], and the same result has been reported for UDPglucuronic acid [6]. Competitive inhibition of *p*-nitrophenyl thymidine-5′-phosphate hydrolysis by adenosine-($\alpha\beta$-methylene)-5′-triphosphate and competitive inhibition of p(NH)ppA hydrolysis by ATP have also been reported [9,11].

Polyacrylamide gel electrophoresis

When a 5% gel was stained for ATP pyrophosphohydrolase a sharp band appeared with R_F of 0.25. Staining for alkaline phosphatase showed a band with R_F of 0.35, while staining for 5′-nucleotidase showed a band which had only just entered the gel. In disagreement with the suggestion of Johnson and Welden [11] we suggest that these three activities represent separate enzymes. In agreement with our results purified nucleotide pyrophosphatase [6,8—10] contained very little 5′-nucleotidase and alkaline phosphatase.

In one experiment the band stained for ATP pyrophosphohydrolase was cut out, homogenized in 20 mM TES, pH 8.0, and left for 24 h at 4°C. The polyacrylamide was run down at 10 000 × *g* for 30 min. The supernatant was assayed for ATP pyrophosphohydrolase, p(CH_2)ppA pyrophosphohydrolase and UDPglc-pyrophosphatase. Substrate concentration was 2.5 μM in medium C containing 6.83 mM $CaCl_2$ and 6.62 mM $MgCl_2$ (10^{-5} M free Ca^{2+} and 10^{-3} M free Mg^{2+}). The ratio of the three enzyme activities did not differ significantly from activities in the deoxycholate solubilized preparation.

In one experiment, polyacrylamide gels ranging from 5 to 11% were prepared. Electrophoresis was run with the enzyme preparation, as well as with lactic acid dehydrogenase, pyruvate kinase, β-galactosidase and bovine serum albumin. Using the latter four proteins as standards, the molecular weight was estimated to be 120 000 by the method of Rodbard and Crambach [28]. This value is close to 130 000 as found for phosphodiesterase I in plasma membranes from rat liver [10].

Isoelectric focusing was performed in 5% polyacrylamide gels. The gels were stained for ATP pyrophosphohydrolase and p*I* was estimated to be 5.1 ± 0.1 (S.E.M., n = 3). In a single experiment isoelectric focusing was performed in a G-75 gel (Pharmacia, Sweden) using a LKB Multiphor (LKB Instruments, Sweden). The fraction containing the peak of p(CH_2)ppA pyrophosphohydrolase activity was analysed for ATP pyrophosphohydrolase, p(CH_2)ppA pyrophosphohydrolase and UDPglc pyrophosphatase as above. Again no relative enrichment was found.

Tissue distribution of p(CH_2)ppA pyrophosphohydrolase

Table III shows a tissue distribution of this activity in the rat. Tissues were

TABLE III

DISTRIBUTION OF $p(CH_2)$ ppA PYROPHOSPHOHYDROLASE ACTIVITY IN RAT TISSUES

Rat organs were homogenized and centrifuged at 50 000 × *g* for 1 h. Pellets and supernatants were analysed for their ability to hydrolyse $p(CH_2)$ppA to AMP and PCP at 10^{-4} M free Ca^{2+}. Pellets were also analysed for 5′-nucleotidase. *n* is the number of experiments.

Organ	*n*	Pellet		Supernatant
		$p(CH_2)$ppA pyrophosphohydrolase (μmol/h per mg protein × 10^3 ± S.E.M.)	5′-Nucleotidase (μmol/h per mg protein ± S.E.M.)	$p(CH_2)$ppA pyrophosphohydrolase (μmol/h per mg protein × 10^3 ± S.E.M.)
Forebrain	3	2.1 ± 1	1.2 ± 0.2	0.77 ± 0.8
Hindbrain	2	2.6 ±	1.8	0.56
Salivary gland	2	149 ±	2.9	9.3
Thyroid gland	2	50 ±	0.92	2.4
Thymus	3	9.2 ± 3	1.7 ± 0.6	1.5 ± 1
Heart	3	22 ± 3	1.1 ± 0.1	1.2 ± 0.2
Lung	3	28 ± 5	1.6 ± 0.3	0.62 ± 0.3
Liver	3	105 ± 23	2.7 ± 0.5	0.69 ± 0.1
Spleen	3	14 ± 2	2.2 ± 0.7	1.0 ± 0.8
Pancreas	2	19 ± 1	0.89 ± 0.2	n.d.
Ventricle	3	37 ± 1	0.68 ± 0.1	1.7 ± 0.6
Small intestine	3	180 ± 65	8.3 ± 1	1.3 ± 0.7
Large intestine	3	45 ± 24	1.9 ± 1	2.1 ± 0.7
Kidney	3	232 ± 55	4.3 ± 1	0.69 ± 0.5
Abdominal fat	3	96 ± 32	1.6 ± 0.9	7.1 ± 4.8
Leg muscle	3	30 ± 3	1.6 ± 0.9	0.37 ± 0.3
Uterus	3	75 ± 22	1.7 ± 0.6	n.d.
Whole blood	2	0.75	0.018	0.37

separated into high speed pellets and supernatants. The activities are presented relative to protein concentration. In all cases $p(CH_2)$ppA pyrophosphohydrolase activity was much higher in the pellet than in the supernatant. The activities relative to protein concentration in the various pellets differ greatly. To obtain an alternative reference with some relevance to membranes, 5′-nucleotidase activity was also measured. The pellet activities of $p(CH_2)$ppA pyrophosphohydrolase also differ very much when compared to this reference.

The tissue distribution of $p(CH_2)$ppA pyrophosphohydrolysis shows that this activity is due to either one or several membrane-bound enzymes. Values from different tissues should be compared with caution, since various organs are not homogenized with equal efficiency and inactivation during preparation may also be organ-dependent. However, it seems clear that brain and blood have much lower activities than found elsewhere, whereas very high activities are found in liver, kidney and small intestine. In small intestine this activity has been shown to be present in the brush border vesicles (Torp-

Pedersen, C., Flodgaard, H., Sjöström, H. and Norén, O., unpublished data) and isolation from this source is in progress.

Concluding remarks

The present study supports the theory that ATP pyrophosphohydrolase activity and nucleotidepyrophosphatase activity are due to one single enzyme since these two activities could not be separated by polyacrylamide gel electrophoresis or by isoelectric focusing. The kinetic experiments, however, indicate that ATP and GTP act as substrates for the enzyme with different properties compared to UDPglc and p(CH_2)ppA. The unusual low K_m values found for these two substrates and the complex inhibition patterns observed in the presence of other substrates indicate the presence of more than one active site/enzyme or a complex reaction mechanism.

It is confirmed that ATP pyrophosphohydrolase requires a low concentration of free Ca^{2+}. It can therefore be concluded that during investigation of either ATP pyrophosphohydrolase or nucleotide pyrophosphatase careful control of free concentrations of divalent cations is necessary.

It has been shown, that p(CH_2)ppA pyrophosphohydrolase is widely distributed in tissues from the rat, and the location is in membranes. This may be a hint for the function of the enzyme.

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References

1 Lieberman, I., Lansing, A.I. and Lynch, W.E. (1967) J. Biol. Chem. 242, 736—739
2 Ray, T.K., Tomasi, V. and Marinetti, G.V. (1970) Biochim. Biophys. Acta 211, 20—30
3 Franklin, J.E. and Trams, E.G. (1971) Biochim. Biophys. Acta 230, 105—116
4 House, P.D.R., Poulis, P. and Weidemann, M.J. (1972) Eur. J. Biochem. 24, 429—437
5 Skidmore, J. and Trams, E.G. (1970) Biochim. Biophys. Acta 219, 93—103
6 Touster, O., Aronson, Jr. N.N., Dulaney, J.T. and Henrickson, H. (1970) J. Cell Biol. 47, 604—618
7 Schliselfeld, L.H., Van Eys, J. and Touster, O. (1965) J. Biol. Chem. 240, 811—818
8 Bachorik, P.S. and Dietrich, L.S. (1972) J. Biol. Chem. 247, 5071—5078
9 Decker, K. and Bischoff, E. (1972) FEBS Lett. 21, 95—98
10 Evans, W.H., Hood, D.O. and Gurd, J.W. (1973) Biochem. J. 135, 819—826
11 Johnson, R.A. and Welden, J. (1977) Arch. Biochem. Biophys. 183, 216—227
12 Flodgaard, H. and Torp-Pedersen, C. (1978) Biochem. J. 171, 817—820
13 Flodgaard, H. and Fleron, P. (1974) J. Biol. Chem. 249, 3465—3474
14 Mohan, M.S. and Rechnitz, G.A. (1972) J. Am. Chem. Soc. 94, 1714—1716
15 Kim, Y.S. and Padilla, G.M. (1978) Anal. Biochem. 89, 521—528
16 Oehme, M., Kessler, M. and Simon, W. (1976) Chimia 30, 204—206
17 Randerath, E. and Randerath, K. (1964) J. Chromatogr. 16, 126—129
18 Torp-Pedersen, C., Treiman, M. and Thorn, N.A. (1979) J. Neurochem. 32, 1085—1091
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Lous, P., Plum, C.M. and Schou, M. (1956) Nord. Med. 55, 693—695
21 Dewald, B., Dulaney, J.T. and Touster, O. (1974) Methods Enzymol. 32, 82
22 Wang, J.K. and Morris, A.J. (1974) Arch. Biochem. Biophys. 161, 118—124

23 Braatz, J.A. and McIntire, K.R. (1977) Prep. Biochem. 7, 495—509
24 Vilhardt, H. and Hope, D.B. (1975) Biochem. J. 143, 181—190
25 Holtlund, J. and Kristensen, T. (1978) Anal. Biochem. 87, 425—432
26 Reed, K.C. and Bygrave, F.L. (1975) Anal. Biochem. 67, 44—54
27 Dixon, M. and Webb, E.C. (1964) Enzymes, 2nd edn., p. 327—331, Academic Press, New York
28 Rodbard, D. and Crambach, A. (1971) Anal. Biochem. 40, 95—113
29 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332