ATPase–ADPase ACTIVITIES OF RAT PLACENTAL TISSUE

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Abstract—1. Calcium-stimulated ATPase–ADPase activities were studied in a microsomal fraction of rat placental tissue.

2. The kinetic characteristics correspond to those of ATP-diphosphohydrolase, also known as apyrase (E.C. 3.6.1.5).

3. These characteristics include the lack of specificity towards nucleoside di- and triphosphates, activation by Ca^{2+}, Mg^{2+} or Mn^{2+}, insensitivity to specific inhibitors of some ATPase and absence of an effect of sulphydryl reagents.

4. Chemical modification of tyrosine, tryptophan, arginine and carboxylic residues decreases both ATPase and ADPase activities.

5. The substrate analogue, 5′-(β,γ-methylene)triphosphate, protected both enzyme activities against all the modifying amino acid reagents tested.

6. Placental fractions (homogenate and microsomes) inhibit ADP-dependent platelet aggregation.

7. The solubilized microsomal enzyme has a molecular mass of 67 kDa by size-exclusion chromatography; the pI is 9.36.

8. A differential effect is observed on the activation produced by Concanavalin A on microsomal and solubilized fractions when treated in the presence and absence of α-methylmannoside.

INTRODUCTION

Human placenta possesses an ADPase activity that inhibits platelet aggregation, because of its ability to remove ADP (Hutton *et al.*, 1980a,b; O'Brien *et al.*, 1987; Barradas *et al.*, 1990). O'Brien *et al.* (1987) compared the ADPase activity in placentas from normal patients with those in placentas of subjects with pregnancy-induced hypertension, patients giving birth to neonates with intrauterine growth retardation (IUGR) and women after natural abortion. This last group had placentas with unusually low activities of this enzyme (about half). The ADPase activity in samples from gestations with IUGR demonstrated a trend towards lower values, although this difference did not reach statistical significance.

The aim of the present work was to ascertain whether or not the diphosphohydrolytic activity in rat placenta corresponded to apyrase or ATP-diphosphohydrolase (E.C. 3.6.1.5). Apyrase that hydrolyses ATP and ADP has been described in microsomes from several animal preparations such as rat liver (Wattiaux-De Coninck and Wattiaux, 1969; Knowles *et al.*, 1983), pig pancreas (LeBel *et al.*, 1980), tumours (Knowles *et al.*, 1983), bovine aortic epithelial tissue (Miura *et al.*, 1987) and synaptosomes (Schadeck *et al.*, 1989). Our group has recently reported that apyrase is also found in microsomal fractions of rat uterus, salivary gland and mammary gland (Valenzuela *et al.*, 1989).

Lüthje *et al.* (1988) have proposed that the ATP- and ADP-hydrolysing activities in red cells can be ascribed to apyrase. Both these activities are associated with the external surface of the plasma membrane and could be related to the ectoenzymic pathway of nucleotide degradation. Pearson *et al.* (1980) proposed that the ectonucleotidase systems (ATPase, ADPase and 5′-nucleotidase) could be of importance in the regulation of neurotransmission, blood platelet function and vasodilatation.

Shami and Radde (1971) have described placental nucleotidase activities and characterized a calcium-stimulated ATPase from guinea pig. They attributed to the enzyme a direct function in the active transport of calcium. Since this report, this activity has been identified in human placenta where it has also been associated with a functional role in transport (Miller and Berndt, 1973). Biochemical characterization has, however, only recently been reported (Treinen and Kulkarni, 1986; Tuan and Kushner, 1987; Kelley *et al.*, 1990). The latter group have shown the presence of two components of Ca^{2+}-stimulated ATP hydrolysis in human basal plasma membranes. One of these components is ATP-specific, requires Mg^{2+} and Ca^{2+}, is inhibited by sulphydryl reagents and has a molecular mass of about 150 kDa—characteristics similar to those reported for the calcium transport system. The second component, a Ca^{2+}- or Mg^{2+}-dependent nucleotide phosphatase activity, cannot be involved in calcium transport because it has a low selectivity for nucleotide substrates, is inhibited by GTP (Kelley and Smith, 1987), has a greater activity

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Abbreviations used: MES, 2(*N*-morpholino) ethane sulphonic acid; TES, *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid; Tricine, *N*-Tris (hydroxymethyl) methyl glycine.

than does ATP-dependent Ca^{2+}-transport and is not inhibited by sulphydryl reagents. These data lead us to propose that this second component with both ATPase and ADPase activities may be apyrase. Similarly, the low substrate specificity of the Ca^{2+}-stimulated ATPase described by Shami and Radde (1971) is more reminiscent of apyrase.

Here we present evidence that the placental Ca^{2+}- and Mg^{2+}-stimulated ATPase activity is similar to that of apyrase. We suggest that the ADPase activity is relevant to the inhibition of platelet aggregation found in placenta (Hutton *et al.*, 1980a,b; O'Brien *et al.*, 1987; Barradas *et al.*, 1990).

MATERIALS AND METHODS

Subcellular fractionation

Placentas from Sprague–Dawley rats (19 days pregnant) were minced and homogenized at 4°C with a Potter–Elvehjem homogenizer (glass pestle) in 4 volumes of 0.25 M sucrose, 5 mM $MgCl_2$, 25 mM KCl, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM Tris–HCl, pH 7.5. PMSF was dissolved in dimethylsulphoxide and added to the buffer just before use. All subsequent procedures were at 4°C. The homogenate was filtered through nylon and centrifuged at 1000 *g* for 10 min; the supernatant was centrifuged at 12,000 *g* for 20 min. The second supernatant was centrifuged at 100,000 *g* for 1 hr. The three pellets were washed with the extraction buffer. The pellets were suspended with a Potter–Elvehjem homogenizer (Teflon pestle) in the same homogenization buffer without PMSF.

Enzyme assays

Apyrase activity was measured with ATP (ATPase activity) or ADP (ADPase activity) as substrate at a final concentration of 2 mM in the presence of 5 mM $CaCl_2$ in 100 mM Tris–HCl, pH 8.0; inorganic phosphate released was determined (Fiske and SubbaRow, 1925). Phosphate was measured according to Ernster *et al.* (1950) for the determination of kinetic parameters and also of the isoelectric point, because of the interference by the ampholines with the older method. A unit of activity (U) is defined as 1 μmole of pI liberated per min at 30°C. The effect of pH on apyrase activity was measured using 100 mM acetate, MES, TES or Tricine-glycine.

The 5′-nucleotidase, glucose-6-phosphatase, glutamate dehydrogenase and lactate dehydrogenase were measured as described by Beaufay *et al.* (1974), Trams and Lauter (1974), Leighton *et al.* (1968) and Bergmeyer *et al.* (1974), respectively.

Protein determination

Protein was determinated by the Lowry method using bovine serum albumin as standard (Lowry *et al.*, 1951).

RNA detection

RNA was detected qualitatively in agarose gels with 1% ethidium bromide.

Microsomal enzyme solubilization

Octylglucoside (2 mg per 1 mg protein) was added to microsomal fractions. No incubation was necessary. Solubilized proteins were separated by centrifugation at 100,000 *g* for 1 hr and the supernatants were collected.

Molecular mass and pI of the solubilized microsomal fraction

The molecular mass was estimated by gel filtration (Andrews, 1964) with a calibrated Sephacryl S-200 column. The column was equilibrated with 0.3 M NaCl in 50 mM Tris–HCl, pH 8.0. Aggregation was prevented by further addition of octylglucoside to the sample (final 4 mg per 1 mg protein) before gel filtration.

The isoelectric point was determined by isoelectrofocusing according to Sánchez *et al.* (1980) in the presence of 2 M urea.

After measurement of the pH gradient, the gel was cut into 2 mm fractions and the protein eluted with 100 mM Tris–HCl, pH 8.0, containing 5 mM $CaCl_2$. After 6 hr, samples were removed for assay.

Platelet aggregation

Platelet aggregation was monitored in an aggregometer PAP-4 (Bio-Data Corp.) using citrate-treated platelet-rich plasma according to Born (1962). A final concentration of 4 μM of ADP was added to induce aggregation. The decrease in light scattering was followed and expressed as a percentage of that seen in controls in which tissue samples were replaced by homogenization buffer or by 0.15 M NaCl.

Modification of amino acid residues

Modifications were performed as described by Means and Feeney (1964).

All reactions were performed at 20°C and were stopped by dilution 20-fold in 100 mM NaCl; a portion of the treated sample was then taken for assay. Blank determinations without specific reagents were performed in parallel. Reactions with *bis*-dithionitrobenzoic acid and 2-hydroxy-5-nitrobenzyl bromide were stopped after 10 min and the others after 1 hr. The reaction conditions employed in each case were 10 mM *bis*-dithionitrobenzoic acid in 100 mM Tris–HCl, pH 8.0; 20 mM tetranitromethane in 100 mM Tris–HCl, pH 8.0; 20 mM phenylglyoxal in 100 mM Na-bicarbonate, pH 8.0; 20 mM 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide in 1.2 M glycine methyl ester; 20 mM 2-hydroxy-5-nitrobenzyl bromide in 100 mM acetate, pH 4.0.

Effect of Concanavalin A

Samples were incubated for 30 min at 30°C with Concanavalin A (1 mg/ml) before assay. Concanavalin A was replaced by water in the control. α-Methylmannoside was added to a final concentration of 50 mM.

RESULTS

The ATPase–ADPase activities of the different subcellular fractions are summarized in Table 1. The highest specific activity was associated with membrane fractions; the activity detected in the soluble

Table 1. Subcellular distribution of ATPase–ADPase activities. Assay conditions are described in Materials and Methods. Each determination was performed in duplicate

Fraction	ATPase %	ATPase U/mg protein	ADPase %	ADPase U/mg protein	ATPase / ADPase
Homogenate	100.0	0.080	100.0	0.03	2.7
1000 *g* pellet	12.9	0.15	11.0	0.10	1.5
12,000 *g* pellet	15.7	0.34	13.9	0.2	1.7
100,000 *g* pellet	25.5	0.56	18.3	0.50	1.1
Final supernatant	13.5	0.06	4.6	0.02	3.0

Table 2. Substrate specificity of microsomal fraction. Assay conditions are described in Materials and Methods. All experiments were run at least in duplicate

Substrate (2 mM)	Percentage of enzymic activity
ATP	100.0
GTP	69.3
UTP	78.9
ADP	90.0
IDP	63.9
CDP	42.8
PPi	11.1
AMP	28.6
G6P	13.9
*p*NPP*	13.2

**p*NPP: *p*-nitrophenylphosphate.

fraction had a very low specific activity. The characterization of these diphosphohydrolytic activities was done on the microscomal fraction (100,000 *g*). This membrane fraction showed activity of 5′-nucleotidase and glucose-6-phosphatase (respectively markers for plasma membrane and endoplasmic reticulum fractions). The presence of RNA indicated that at least some of the endoplasmic reticulum was "rough".

Characterization of microsomal ATPase–ADPase activities

Substrate specificity. The relative enzymatic activities are shown in Table 2. The major phosphorolytic activities present in this fraction are directed towards nucleoside di- and triphosphates, with the phosphomonoesterase activity being rather low. Activity with 5′-AMP and on other phosphomonoesters may be due to the 5′-nucleotidase and general unspecific phosphatases in the fraction.

Bivalent metal activation. The activities of both ATPase and ADPase are strongly inhibited by 10 mM EDTA (to less than 10% ADPase and to 0.5% ATPase). Ca^{2+} is the most effective activator of the metal ions tested (Table 3).

Optimum pH. Both activities (ATPase and ADPase) have an optimum pH value of 8.0 in the presence of Ca^{2+}.

Determination of K_m. The Hanes–Woolf plots were linear, giving no indication of co-operativity or substrate inhibition in the experimental conditions used. In the presence of 5 mM Ca^{2+} the K_m for ATP is 50 μM and for ADP 33 μM.

Effect of inhibitors. The insensitivity to oligomycin and insignificant effect of ouabain indicate that the contributions to ATPase activity by mitochondrial ATPase and (Na^+, K^+)-ATPase were negligible (Table 4). The adenylate kinase inhibitor, adenylyl (3′, 5′)-adenosine pentaphosphate, Ap5A (Feldaus *et al.*, 1976) also has very little effect on ADPase activity. These results indicate that the activity is that of a true ADPase and is not the result of a coupled action of adenylate kinase and an ATPase.

Table 3. Metal ion activating effect. Assay conditions are described in Materials and Methods. All experiments were run at least in duplicate

Metal ion (5 mM)	Relative activity (%) ATPase	ADPase
Ca^{2+}	100	100
Mg^{2+}	92	76
Mn^{2+}	54	46
Zn^{2+}	13	34
Co^{2+}	25	33

Table 4. Effect of inhibitors on microsomal fraction. Assay conditions are described in Materials and Methods. All experiments were run at least in duplicate

Inhibitors	Concentration	Inhibition (%) ATPase	ADPase
Ouabain	5 mM	5.5	0
Oligomycin	0.9 mg/ml	0.5	0
Ap5A	0.5 mM	2.8	0.7
o-Vanadate	1 mM	19.6	21.7
Azide	10 mM	25.0	36.4
DES	1 mM	29.7	35.6
Fluoride	10 mM	40.2	56.8
DCCD	1 mM	59.2	59.7

Other inhibitors such as azide, *o*-vanadate, diethylestilbestrol (DES), fluoride and dicyclohexylcarbodiimide (DCCD), all of which are less specific (Serrano, 1973), produced parallel effects on the enzymatic activities (Table 4).

Solubilized enzyme. The recovery of both ATPase and ADPase activities after solubilization with octylglucoside was 38%. No activity was detected in the pellet remaining after centrifugation.

Molecular mass. Both enzyme activities eluted from Sephacryl S-200 with similar molecular masses of 67 kDa.

Isoelectric point. To avoid protein aggregation isoelectrofocusing was performed in the presence of 2 M urea. Both ATPase and ADPase activities exhibited the same pI of 9.36, supporting the suggestion that ATP-diphosphohydrolase is present in placental tissue.

Inhibition of ADP-induced platelet aggregation. Figure 1 shows the effect of the homogenate and the microsomal fraction on platelet aggregation induced by ADP. Both fractions caused an inhibition of the ADP response proportional to their ADPase specific activity.

Participation of amino acid residues in enzyme reaction. The participation of thiol groups has been excluded by the insensitivity of apyrase to *bis*-dithionitrobenzoic acid (Table 5). This lack of effect

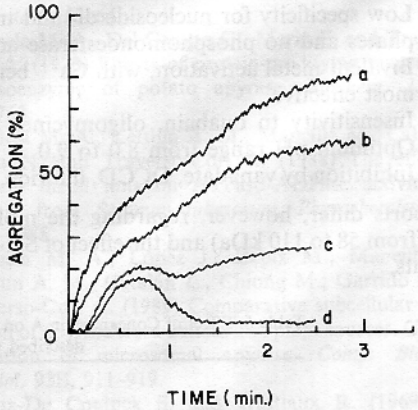

Fig. 1. Effects of homogenate and microsomal fraction on platelet aggregation induced by ADP. (a) Aggregation control in response to 4 μM ADP. (b) Addition of 58 μl of homogenate. (c) Addition of 10 μl of microsomal fraction. (d) Addition of 20 μl of microsomal fraction.

Table 5. Effect of modifying reagents on ATPase–ADPase activities. Preincubation and assay conditions are described in Materials and Methods. All experiments were run at least in duplicate

Reagent	ATPase		ADPase	
	No addition	AMP-PCP* (10 mM)	No addition	AMP-PCP (10 mM)
Tetranitromethane (20 mM; 60 min)	85	24	81	30
2-Hydroxy-5-nitrobenzyl bromide (20 mM; 10 min)	96	57	96	32
Water-soluble carbodiimide (20 mM; 60 min)	79	29	65	50
Phenylglyoxal (20 mM; 60 min)	100	21	100	51
bis-Dithionitrobenzoic acid (1 mM; 10 min)	0	—	0	—

*AMP-PCP: 5′-(β,γ-methylene)triphosphate.
Microsomal fraction was treated with the protein reagents either with or without the substrate analogue (AMP-PCP). Enzyme activity in the absence of modifying reagents was taken as 100%.

of thiol groups makes the tetranitromethane reaction specific for tyrosine groups.

The effect of the same specific amino acid modifiers and the protection by 5′-(β-γ-methylene)triphosphate suggest the participation of aromatic amino acids (tyrosine and tryptophane), carboxylic residues and some basic groups such as arginine in both ATPase and ADPase activities. These results are shown in Table 5.

Effect of Concanavalin A. This plant lectin causes an appreciable activation of ATPase and ADPase activities present in the placental microsomal fraction (Table 6). It is noteworthy that the effect is greater with the solubilized enzyme. Table 6 also shows the ability of α-methylmannoside to prevent the effect only with the solubilized enzyme. Carraway *et al.* (1975) have also reported a specific activation of Mg-ATPase of mammary gland by Concanavalin A.

DISCUSSION

In the present work we have shown that rat placental ATPase–ADPase activities are membrane bound and are probably associated with a single protein species.

Mammalian apyrases have the following general properties.

(a) Low specificity for nucleoside di- and triphosphates and no phosphomonoesterase activity.
(b) Bivalent metal activation, with Ca^{2+} being the most effective.
(c) Insensitivity to ouabain, oligomycine, Ap5A.
(d) Optimum pH range from 8.0 to 9.0
(e) Inhibition by vanadate, DCCD, fluoride, azide.

Reports differ, however, regarding the molecular mass (from 58 to 110 kDa) and the effect of SH-group reagents.

We found that the enzyme in the placental microsomal fraction has properties similar to the above and suggest that it is an apyrase. We have purified to homogeneity and further characterized potato ATP-diphosphohydrolase. This apyrase has kinetic properties similar to those of the mammalian enzyme but differs in the molecular mass; the plant enzyme is also mainly in the soluble fraction (Valenzuela *et al.*, 1973, 1988, 1989; Kettlun *et al.*, 1982). The similar response of both rat and potato enzyme to chemical agents modifying amino acids suggests that there may be a degree of homology between the two.

The ADPase activity of this placental apyrase could be important in regulating the ADP available for platelet aggregation. Hutton *et al.* (1980a,b) proposed that the ADPase activity was a property of alkaline phosphatase, which is very abundant in this tissue; our studies indicate that this is unlikely. Barradas and his group have suggested that the enzyme could play a role in protecting from thrombosis (Barradas *et al.*, 1990, see references therein). They found that the ADPase activity in umbilical cord plasma was significantly greater than that in maternal blood samples. They suggested that this enzyme activity arises from the placental tissues. It is interesting that this group also found that the elevated concentrations of plasma non-esterified fatty acids decreased ADPase activity in placenta, possibly by detaching the loosely bound enzyme from the endothelium. This may be of relevance since the concentrations of fatty acids are elevated in maternal plasma during delivery. These authors point out that the elevated ADPase activity in human placenta may also compensate for the limited amounts of prostacyclin, the major vascular prostaglandin, a vasodilator and a very potent inhibitor of platelet aggregation.

Table 6. Effect of Concanavalin A on microsomal and solubilized enzyme. Assay conditions are described under Materials and Methods

Addition	Microsomal enzyme		Solubilized enzyme	
	ATPase	ADPase	ATPase	ADPase
	% of activity			
None	100	100	100	100
Concanavalin A (1 mg/ml)	142	155	222	267
Concanavalin A (1 mg/ml) +α-methylmannoside (50 mM)	132	163	122	115

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