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# Effect of protein-modifying reagents on ecto-apyrase from rat brain

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**Abstract**

We have tested several chemical modifiers to investigate which amino acid residues, present in the primary structure of the ecto-apyrase, could be involved in catalysis. Synaptosomes from cerebral cortex of rats were prepared and the ATP diphosphohydrolase activity was assayed in absence or the presence of the modifiers. Percentages of residual activity for ATPase and ADPase obtained when the following reagents were tested, are respectively: phenylglyoxal (an arginine group modifier) 17 and 30%; Woodward's reagent (a carboxylic group modifier) 33 and 23%; Koshland's reagent (a tryptophan group modifier) 10 and 12%; maleic anhidride (an amino group modifier) 11 and 25% and carbodiimide reagent (a carboxylic group modifier) 56 and 72%. Otherwise, PMSF, a seryl protein modifier and DTNB, a SH-group modifier did not affect either ATPase or ADPase activity. Inhibitions observed after treatment with phenylglyoxal and Woodward's reagent were significantly prevented when the synaptosomal fraction was preincubated with ATP and ADP, indicating that the arginine and the side chain of glutamate or aspartate (carboxyl groups) participate in the structure of the active site. This interpretation was confirmed by using GTP and GDP, two other apyrase substrates. Phenylglyoxal and Woodward's reagent also inhibited the GTPase and GDPase activities and this inhibition was prevented by preincubation with these substrates. 

*Keywords:* ATP diphosphohydrolase; Apyrase; ATPDase; ATPase–ADPase; Amino acid modifiers

## 1. Introduction

ATP diphosphohydrolase (apyrase, ATPDase, EC 3.6.1.5) is an enzyme that catalyses the conversion of nucleoside di- and triphosphates to monophosphate and inorganic phosphate in the

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presence of $Ca^{2+}$ or $Mg^{2+}$. It is insensitive to known inhibitors of various ATPase (P-type, F-type and V-type) and has an optimum pH of about 8.0. Apyrase activity has been well established in plant and animal tissues, including mammalian [21,24]. In previous reports we have described an ATP diphosphohydrolase in synaptosomal fraction from the central [1] and peripheral [23] nervous systems.

One of the biochemical role proposed for this enzyme is a participation in an 'enzyme chain' together with a 5′-nucleotidase for the complete hydrolysis of ATP to adenosine in the synaptic cleft during neurotransmission [1,12,14]. These two enzymes have a dual function controlling the availability of ligands (ATP, ADP, AMP and adenosine) for either nucleotide or nucleoside receptors. By the hydrolysis of the nucleotide, they might be involved in controlling activation of receptor [37]. There was a controversy about the enzymes that can be involved in extracellular hydrolysis of ATP considering the existence of an ecto-ATPase described by others [10,20,28]. However, it has been recently demonstrated that both, the ecto-ATPase, that has a very high preference for ATP over ADP, and the ATP diphosphohydrolase, that hydrolyses ATP and ADP equally well, are coexpressed in rat brain [12].

We have shown that the enzyme ATP diphosphohydrolase is firmly associated with the synaptic plasma membrane [3]. This can be explained by the presence of two transmembrane domains at the N- and C-termini [11] as it was recently confirmed by the amino acid sequence analysis [12,34]. Recently it was shown [33], that the marked sensitivity of the enzyme to different detergents as observed by us [3] is due to the dissociation of the tetrameric structure of the protein. This feature of the enzyme makes the solubilisation and purification process difficult [2].

Recently, considerable progress has been made in the study of the molecular structure of ATP diphosphohydrolase of several sources [9,12,25,34]. Wang et al. [34] isolated rat and mouse brain ecto-apyrase cDNAs and suggested that there might be only a single copy of the ecto-apyrase (APY/CD39) gene. In an other report, with potato tubers apyrase, four well-conserved regions that were defined as "apyrase conserved regions" were identified [9]. Moreover, it was shown that ATP diphosphohydrolase is a highly glycosylated protein with six potential N-linked glycosylation sites [7,9,11,12,15,31,32,34].

Besides all these advancements, little is known about the participation of amino acid residues in the enzymatic activity of apyrase. In the present work, we address this question by studying the effects of protein-modifying reagents on ecto-apyrase from synaptosomal preparations from rat brain. Based on the results, we intend to provide evidence for participation of specific amino acid residues in the catalytic activity of the enzyme and to explore similarities and differences between apyrases from different sources.

## 2. Materials and methods

### 2.1. Materials

The carbodiimide (EDAC) and Koshland's reagent were kindly provided by Dr. Maria Antonieta Valenzuela (University of Chile). Nucleotides (ATP, ADP, GTP, GDP), HEPES, Trizma Base, EDTA, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K), phenylglyoxal, 2,5-furandione (maleic anhydride) and Percoll were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Percoll was routinely filtered through millipore AP15 pre-filters to remove aggregated, incompletely coated particles. Sucrose was obtained from Merck (Darmsdadt, Germany). All other reagents were of analytical grade.

### 2.2. Methods

#### 2.2.1. Subcellular fractionation

Adult Wistar female rats (age, 70–90 days, weight 240–280 g) were housed in controlled conditions with a 12 h light/dark cycle at constant temperature ($23 \pm 2^{\circ}C$) and food and water were available ad libitum. The animals were killed by decapitation, their cortex was removed and gently homogenised in 10 volumes of an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5, with a

Table 1
Conditions for the chemical modification on the synaptosomal ecto-apyrase activity

| | Modifier | | | | | |
|---|---|---|---|---|---|---|
| | Phenylglyoxal | Woodward's reagent | Koshland's reagent | | Maleic anhydride | Carbodiimide (EDAC) |
| Amino acid residue | Arginine | Carboxylic group | Tryptophan | | Amino group | Carboxylic group |
| Buffer | sodium bicarbonate 0.1 M pH 8.0 | MES 0.05 M pH 6.0 | sodium acetate 0.08 M pH 5.0 | | Tris–HCl 0.05 mM pH 8.0 | glycine methyl ester |
| Temperature (°C) | 30 | 0 | 30 | | 30 | 0 |
| Time (min) | 30 | 5 | 5 | | 10 | 5 |
| Concentration (mM) | 10 | 5 | 50 | | 50 | 20 |
| Solvent | water | water | 10% acetone | | water | water |

motor-driven Teflon–glass homogeniser. The synaptosomes were isolated as described by Ref. [18]. Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed by centrifuging at $15{,}000 \times g$ for 20 min with the same ice-cold medium to remove Percoll. The synaptosomes pellets were resuspended at a final protein concentration of approximately 0.9 mg/ml. The material was freshly prepared and maintained at 0–4°C throughout the experiments.

### 2.2.2. Chemical modification of amino acid residues

The synaptosomal fractions were preincubated with modifiers at determined conditions (Table 1). The samples of modified enzyme were assayed for ATP diphosphohydrolase activity as described below. Controls (100% of activity) to each modifier tested were run without addition of the modifier. In the case of Koshland's a control was made with buffer plus 10% acetone, since this reagent was prepared in this solvent (Table 1). In addition to the modifiers shown in Table 1 we have tested 1 mM bis-dithionitrobenzoic acid (DTNB, -SH group modifier) and 0.1 mM phenylmethylsulphonyl fluoride (PMSF, seryl protein modifier). These modifiers were preincubated with synaptosomal fractions for 10 min.

### 2.2.3. Enzyme protection by the substrate

To evaluate the protection against the modifiers by the substrates the following protocol was used: the synaptosomes were preincubated with substrate (ATP, ADP, GTP or GDP) at final concentration of 12 mM in presence of buffers (as described in Table 1). This first preincubation was made at 30°C for 10 min (except for carbodiimide where the temperature was 0°C) followed by a second preincubation in the presence of the modifiers described above. The synaptosomal fractions were then assayed for ATP diphosphohydrolase activity as described below. Controls

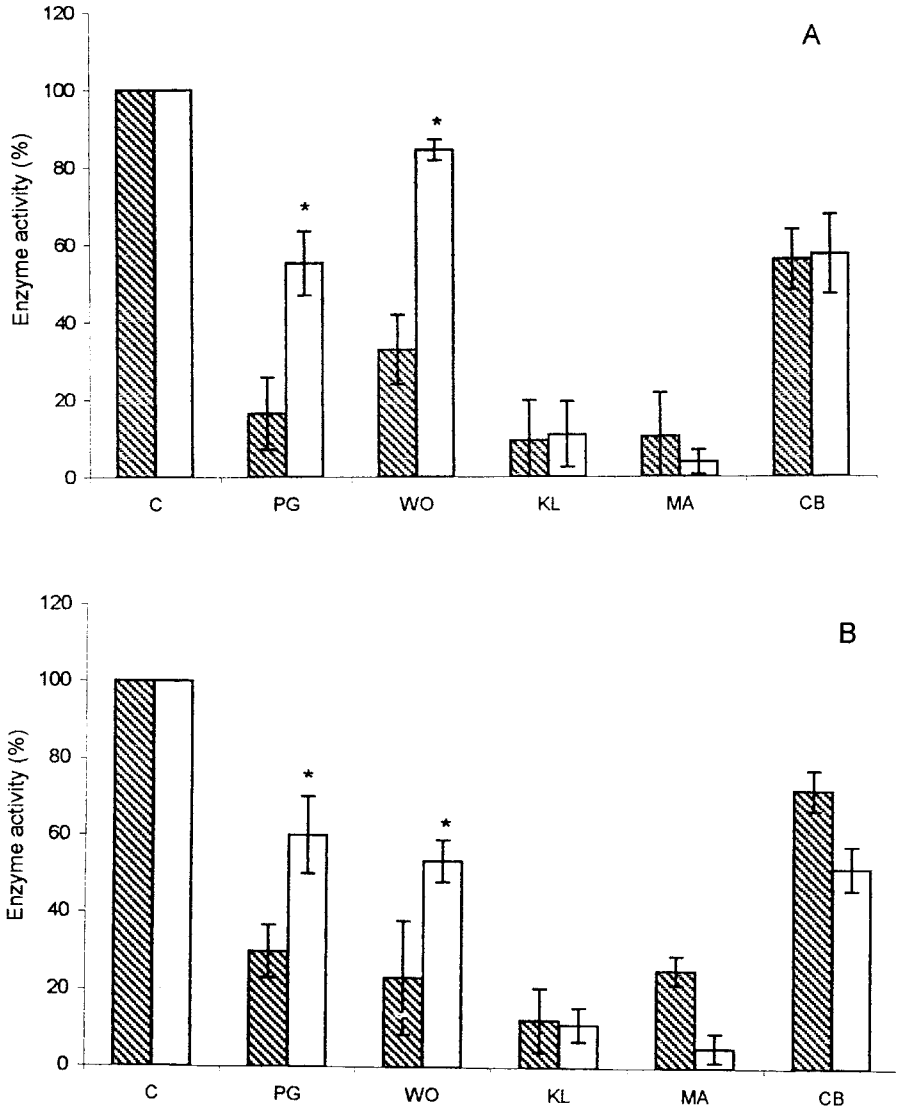


Fig. 1. Effect of modifiers on ATPase and ADPase activities of the ATP diphosphohydrolase from rat cerebral cortex synaptosomes. Synaptosomal preparations were submitted to modification (▨) and protection with substrate (□) for ATPase (A) and ADPase (B) activities measured as described in Materials and methods. The modification and protection time, temperature and reagent concentration used are listed in Table 1. The symbols represent: C (control), PG (phenylglyoxal), WO (Woodward's reagent), KL (Koshland's reagent), MA (maleic anhydride) and CB (carbodiimide). Bars represent the means ± S.D. of at least four different experiments. The control represent 100% of the activity (no modifier). The percentages of residual activity in the presence of modifiers were compared with the percentages of residual activity using substrates as protectors prior the incubation with the modifiers (for details see Materials and methods). Data were analysed by Student's *t*-test. Significantly different at $^{*}P < 0.01$.

(100% of activity) to each substrate and each modifier tested were run without modifier in the same conditions described above to chemical modification.

*2.2.4. ATP diphosphohydrolase assay*

Samples of enzyme were assayed for ATP diphosphohydrolase activity as described by Barrastini et al. [1], at 37°C for 20 min, in a med-

ium containing 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µl. The reaction was initiated by the addition of substrate (ATP, ADP, GTP or GDP) to a final concentration of 1.0 mM. In protocols to evaluate the protective effect of substrate the reaction was started by the addition of $CaCl_2$, since the nucleotides were already present. The reaction was stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 µl samples were taken for the assay of released inorganic phosphate (Pi) by the method of Chan et al. [6]. Controls with addition of the enzymatic preparations (or by the addition of $CaCl_2$ in the substrate protection protocol) after addition of trichloroacetic acid, were used to correct for nonenzymatic hydrolysis of the substrates. All samples were run in triplicate. Incubation times and protein concentrations were chosen in order to ensure linearity of reaction rate. Specific activity is expressed as nmol Pi released/min/mg of protein, at 37°C.

*2.2.5. Protein determination*

Protein was measured by the Comassie Blue method according to Bradford [4], using bovine serum albumin as standard.

*2.2.6. Statistical analysis*

The data obtained for the enzyme activities are presented as mean ± S.D. of at least four different experiments. The statistical analysis used in experiments was Student's *t*-test. *P* values of $<0.05$ or $<0.01$ were considered to represent significant differences, as indicated in the figures.

## 3. Results

A common strategy for identifying the amino acid residues essential for the biological function of a protein is to treat the protein with specific reagents [8,16,22,29]. This investigation provides clues about amino acids involved in catalysis.

PMSF at 0.1 mM (a seryl protein inhibitor) and 1 mM DTNB (a -SH group modifier) did not affect ATPase and ADPase activities (results not shown) suggesting that aliphatic hydroxyl and sulfhydryl groups are not involved in the enzymatic activity.

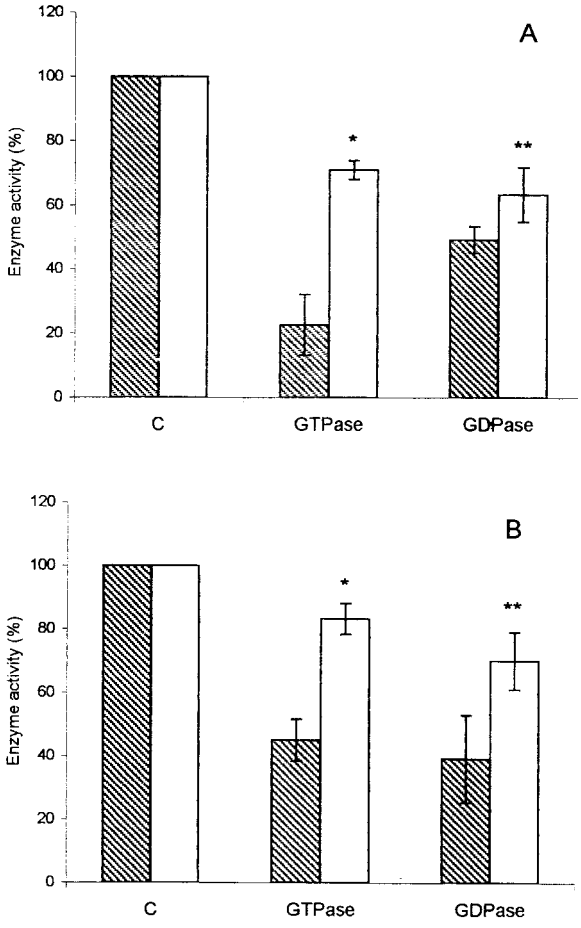


Fig. 2. Effect of modifiers phenylglyoxal and Woodward's reagent on GDPase and GTPase activities of the ATP diphosphohydrolase from rat cerebral cortex synaptosomes. Synaptosomal preparations were submitted to modification (▨) and protection with substrate (□) for GTPase and GDPase activities with phenylglyoxal (A) and Woodward's reagent (B), measured as described in Materials and methods. The modification time, temperature and reagent concentration used are listed in Table 1. The symbol C represents control. Bars represent the means ± S.D. of at least four different experiments. The control represent 100% of the activity (no modifier). The percentages of residual activity in the presence of modifiers were compared with the percentages of residual activity using substrates as protectors prior the incubation with the modifiers (for details see Materials and methods. Data were analysed by Student's *t*-test. Significantly different at $^{*}P < 0.01$ or $^{**}P < 0.05$.

Exposure to phenylglyoxal (arginine group modifier), Woodward's reagent (carboxylic group modifier), Koshland's reagent (a tryptophan group modifier), maleic anhydride (amino groups modifier) and carbodiimide (carboxylic modifier) was also carried out. The pattern of inactivation promoted by these different amino acid reagents on the enzyme activity is shown in Fig. 1. A strong inhibition on ATPase and ADPase activities was observed when the synaptosomal fraction was preincubated with phenylglyoxal, Woodward's reagent, Koshland's reagent and maleic anhydride in the conditions presented in Table 1. On the other hand, the treatment with carbodiimide caused a smaller inactivation on both activities. To determine if the inactivation observed could be avoided by the protection with substrates ATP and ADP, we used the protocol described in Material and methods. Fig. 1 shows beyond the effects of the modifiers alone, the comparison of these effects with the effects observed when we used preincubation with substrates. The results presented in Fig. 1 show that the inactivation promoted by phenylglyoxal and Woodward's reagent was significantly prevented when the synaptosomal fraction was preincubated with ATP (Fig. 1A) and ADP (Fig. 1B), suggesting that arginine and carboxylic groups are involved in the catalytic site of the enzyme. The participation of these groups in the catalytic site was confirmed by using two other substrates for the enzyme ATP diphosphohydrolase (GTP and GDP). The reagents phenylglyoxal and Woodward's reagent inhibited significantly GTPase and GDPase activities of the enzyme. In a similar way, for ATPase and ADPase activities, this inactivation was also prevented by preincubation with GTP and GDP, respectively (Fig. 2).

## 4. Discussion

Mammalian ecto-apyrases are integral membrane proteins with two transmembrane domains, with small cytoplasmic $NH_2$- and COOH-terminal segments, and a large extracellular domain with enzymatic activity. Moreover, several conserved regions were identified in different apyrases, suggesting that these enzymes are an evolutionary related family of proteins [5,9,11,12,19,25,32,34].

One important characteristic of membrane-bound apyrase is the marked sensitivity to different detergents which have made the solubilisation and purification of this enzyme difficult [2,13,17,30]. Loss of enzyme activity by detergent addition previously described by us [2], could be now explained by the dissociation of ecto-apyrase tetrameric to monomeric form [35].

The molecular structure of the enzyme, now available in the literature, prompted us to investigate which amino acid could be involved in the enzymatic activity of the ecto-apyrase from rat brain, by using different reagents specific for different amino acid side chains.

Our results indicate involvement of arginine, tryptophan, amino and carboxylic groups in the enzyme activity of the ATP diphosphohydrolase considering that phenylglyoxal, Koshland's reagent, maleic anhydride and Woodward's reagent promoted near 70–90% of inactivation for ATPase and ADPase activities (Fig. 1). Surprisingly, carbodiimide (a carboxylic modifier) caused a smaller but significant inhibition for both ATPase and ADPase activities. The difference in the results observed with carbodiimide when compared to obtained with Woodward's reagent (both carboxylic group modifiers) could be explained by different reactivities between different reagents and the carboxylic groups of the enzyme.

An important problem in interpreting the results of chemical modification is the possible side reactions. With some reagents, several different groups may be modified. Lack of specificity limits the usefulness of many reagents, but it is possible to limit the specificity by using adequate conditions. The reactivity of a group in a protein with a particular substance depends on the environment which includes reagent concentration, pH and temperature. For example, the specificity of Koshland's reagent to tryptophan residues is maintained at acidic pH. In fact, variation in reactivity as a function of pH is a convenient way to control the course of many modifications reactions [16]. The conditions used here to test

the chemical modifiers (Table 1) were chosen from literature in order to avoid these side reactions.

To determine if some of the amino acids affected by the modifiers are involved in the active site of the enzyme, we have tested the protection to the inactivation by using substrates before the treatment of the enzyme with the modifiers. The inactivation produced by Koshland's reagent and maleic anhydride indicates the involvement of tryptophan and amino groups in the catalysis, but probably not at the catalytic site, since the inactivation was not prevented by preincubation with both substrates of the enzyme, ATP and ADP. Actually, the involvement of tryptophan in the catalysis has recently been demonstrated by site-directed mutagenesis of a human brain ecto-apyrase [27]. Mutation of tryptophan 187 to alanine produces an ecto-apyrase without nucleotidase activity. In contrast, mutation of tryptophan 459 to alanine resulted in an ecto-apyrase with enhanced NTPase, but diminished NDPase activity. The participation of this amino acid residue in the maintenance of the tertiary structure and not at the catalytic site was suggested, being in agreement with results presented here.

On the other hand, the loss of activity observed after the treatment with phenylglyoxal and Woodward's reagent was significantly prevented when the synaptosomal fraction was preincubated with the substrates. This result suggests that arginine and carboxylic residues are important for the catalytic activity, participating directly or indirectly in the substrate hydrolysis. Similar behaviour was described for apyrase from the heart sarcolemmal fraction [8] and from renal microvillar membranes [22]. As we can see in Fig. 2, phenylglyoxal and Woodward's reagent inhibited significantly the hydrolysis of GTP and GDP, two other substrates of the enzyme and this inactivation was also prevented by the preincubation with the substrates. More recently, a chemical modification with phenylglyoxal has been used to demonstrate that arginine is located in the ATP biding site of the $Ca^{2+}$-transporting ATPase of rabbit skeletal muscle sarcoplasmic reticulum [36]. This observation may suggest that arginine can effectively be involved in the substrate-binding and in the catalysis of ATP hydrolysing enzymes.

It is important to note that the protection of inactivation by substrates does not prove definitively that the modified residue(s) lie in the active site of the enzymes. Changes in the conformational structure of the protein promoted by chemical modification could be responsible for the inactivation of the enzyme. Some topologically remote groups from the active centre can also interact with modifiers and thereby influence the accessibility of the active site to the substrate. However, when modification of a group affects the enzymatic activity usually this group is considered essential to that activity [16].

The primary structure of ecto-apyrase from different tissues recently identified revealed the existence of four highly conserved regions defined as 'apyrase conserved regions' [9]. The sequence analysis shows the presence of arginine and carboxylic groups in these four regions. Site-directed mutagenesis analysis showed that aspartic residues (Asp 62 and 219) present in the apyrase conserved regions (ACR) I and IV are essential for apyrase activity, indicating the importance of carboxilic groups in the hydrolysis of nucleotides by the enzyme [26]. Likewise, we show here that carboxylic groups involved in the hydrolysis of substrates probably are present in the catalytic centre.

Our results also show the participation of arginine and amino groups in the nucleotide hydrolysis. Site-directed mutagenesis studies should be done to confirm the participation of these amino acids residues in ecto-apyrase activity.

In conclusion, with the present work we intend to contribute to the elucidation of amino acids involved in the nucleotide hydrolysis by ecto-apyrase from synaptosomal fraction from rat brain.

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