Characterization of ATP-Diphosphohydrolase from Rat Mammary Gland

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ATP-diphosphohydrolase (or apyrase) hydrolyses nucleoside di- and triphosphates in the presence of millimolar concentration of divalent cations. It is insensitive towards sulfhydryl and aliphatic hydroxyl-selective reagents and to specific inhibitors of ATPases. We present further evidence that ATPase and ADPase activities present in rat mammary gland correspond to apyrase. Two kinetic approaches have been employed, competition plot and chemical modification with group-selective reagents. The M_r of these activities was determined by ^{60}Co radiation–inactivation. The kinetic approaches employed, competition plot (which discriminate whether competitive reactions occur at the same site) and chemical modification, point to the presence of a single protein which hydrolyses ATP and ADP. The similar M_r values of ATPase and ADPase activities also support this proposal. ATPase and ADPase activities of mammary gland show a similar sensitivity or insensitivity towards several chemical modifiers. These results suggest that this enzyme is ATP-diphosphohydrolase, also known as apyrase. The results obtained are compared with the ones obtained by us and other authors with the enzyme isolated from other sources.

Keywords: Apyrose ATP-diphosphohydrolase Mammary gland

INTRODUCTION

ATP-diphosphohydrolase (EC 3.6.1.5), also known as apyrase, hydrolyzes nucleoside di- and triphosphates to monophosphate esters and inorganic phosphate in the presence of a divalent cation. ATP-diphosphohydrolases from various sources share the following common features: (i) a broad nucleotide specificity, (ii) dependence on millimolar concentrations of Ca^{2+} or Mg^{2+}, (iii) insensitivity towards sulfhydryl or aliphatic hydroxyl-selective reagents and to certain specific inhibitors of ATPases (such as ouabain and oligomycin) (Kettlun *et al.*, 1982, 1992, 1994; Sarkis *et al.*, 1986; Lüthje *et al.*, 1988; Valenzuela *et al.*, 1989; Pieber *et al.*, 1991; Yagi *et al.*, 1991, 1992; Frassetto *et al.*, 1993; Picher *et al.*, 1993). The M_r of the mammalian native enzyme (always membrane-bound) depends on the tissue, and ranges from 65 to 190 kDa (Pieber *et al.*, 1991; Côté *et al.*, 1991; Yagi *et al.*, 1992; Kettlun *et al.*, 1994). Studies with protein-modifying reagents on potato, rat and human placental apyrases suggest the involvement of Arg and His residues in the catalytic mechanism (Valenzuela *et al.*, 1973; Kettlun *et al.*, 1982, 1992, 1994; Pieber *et al.*, 1991).

In this present paper we have characterized apyrase from rat mammary gland using both kinetic and chemical methods, and compared this enzyme with similar activities from other sources.

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MATERIALS AND METHODS

Animals

Adult Sprague–Dawley rats (weight 250–300 g) were housed under controlled conditions of photoperiod (12L : 12D) and temperature (22°C) and were fed with rat chow pellets *ad libitum*.

Microsomal fraction

The microsomal fraction was prepared as previously described in Valenzuela *et al.* (1989).

Enzyme assays

Apyrase activity was assayed by measuring Pi liberation from ATP or ADP (final concentration 2 mM) in the presence of 5 mM $CaCl_2$ in 100 mM Tris–HCl, pH 8.0. According to the sensitivity required, Pi was determined either by Fiske and SubbaRow (1925) or Ernster *et al.* (1950).

For optimum pH determination, Tris was replaced as follows: 0.1 M MES pH 5.5–6.5; 0.1 M TES pH 6.5–8.5 and 0.1 M glycine pH 8.5–10.0.

Protein determinations

Protein were determined according to Lowry *et al.* (1951) with bovine serum albumin as standard.

The competition plot

The competition plot described by Chevillard *et al.* (1993) consisted in choosing ADPo and ATPo concentrations that give the same rate. A series of mixtures of both substrates was then prepared at concentrations ADP = pADPo and ATP = (1-p)ATPo, respectively. p values ranged from 1 to 0. The total rates determined for each mixture were plotted against p. The same rate was obtained with the following two pairs: 22 μM ADP-35 μM ATP and 40 μM ADP-56 μM ATP.

Chemical modification of amino acid residues

Aliquots of microsomal membrane suspensions were incubated as follows: 1 mM DTNB in 50 mM Tris–HCl, pH 8.0 for 15 min at 30°C; 0.1 mM methyl methanethiosulfonate (MMTS) in 50 mM Tris–HCl, pH 7.0 and 8.0 for 60 min at 20°C; 20 mM EDAC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] in 0.83 M glycine methyl ester, pH 4.0 for 15 min at 30°C; 30 mM phenylglyoxal in 100 mM $NaHCO_3$ for 15 min at 30°C; 20 mM diethylpyrocarbonate (DEP) in 70 mM Na–acetate, pH 4.0 for 15 min at 0°C; 50 mM tetranitromethane (TNM) in 50 mM Tris–HCl, pH 8.0 for 60 min at 0°C; 20 mM 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) in 20 mM Na–acetate, pH 4.0 for 10 min at 0°C; 16 mM maleic anhydride in 50 mM Tricine, pH 9.0 for 15 min at 0°C. In protection experiments the concentration of AMP-PcP was 10 mM.

At the end of the reaction, ATPase–ADPase activities were determined using the standard assay. Controls contained the appropriate solvent without the specific reagent.

Determination of relative molecular mass

The M_r values of the microsomal ATPase–ADPase activities were determined by the ^{60}Co radiation–inactivation method (Kempner and Schlegel, 1979). This method is based on the fact that the susceptibility of a given protein to irreversible inactivation by irradiation is related to its molecular size. Freeze-dried microsomal samples placed in vacuum-sealed ampoules were irradiated at the Comisión Chilena de Energía Nuclear in a ^{60}Co irradiator Naratom 3500, with an average rate dose of 417.3 Gy/hr. Non-irradiated control samples were kept under the same conditions. True apyrase activity was measured because neither 0.1 mg/ml oligomycin nor 0.5 mM Ap_5A [adenylyl (3′-5′)-adenosine pentaphosphate] affected ATPase and ADPase activities. These inhibitors were tested to discard any interference by mitochondrial ATPase or adenylate kinase. The logarithms of the residual activity is plotted as a function of the dose; the slope of this function was determined by linear regression analysis. The inverse of this value corresponded to the 37% dose; these data were used empirically to calculate the M_r (Kempner and Schlegel, 1979).

RESULTS

Kinetic studies

The competition plot can discriminate whether competitive reactions occur at the same active site, or in multiple sites. The horizontal straight lines presented in Fig. 1 indicates that ATPase and ADPase activities compete for the same site (Chevillard *et al.*, 1993).

Both ATPase and ADPase activities of the microsomal fraction were maximal at pH 8.0 with half maximal activity at pH values of 6.8

the difference of 4% is within the standard deviation of the individual determinations.

DISCUSSION

The work presented here provides kinetic and physicochemical evidence that the di- and triphosphonucleotidase activities reside in a single enzyme. The competition plot which showed independence of p when the proportion of the nucleotides (ATP and ADP) was changed is consistent with hydrolysis of both substrates occurring at the same active site (Chevillard *et al.*, 1993).

The analogous reactivity and insensitivity pattern towards several group-selective reagents of the nucleoside di- and triphosphatase activities also supports the hypothesis that they share a common active site. These chemical modification studies on rat mammary gland apyrase show similarities with the *Solanum tuberosum* enzyme (Valenzuela *et al.*, 1973; Kettlun *et al.*, 1982; Kettlun *et al.*, 1992). These preliminary studies on the active site suggest the participation of His, Arg, Tyr and Trp. Histidine could participate as a general base catalyst and arginine may have the typical pyrophosphate-neutralizing role, facilitating nucleophilic attack on the phosphorus atom (Cotton *et al.*, 1973). The aromatic amino acids might be involved in nucleotide binding. There are some differences, however between the rat and human placental ATP-diphosphohydrolases. Carboxyl groups seem to be essential for activity of the rat enzyme (Pieber *et al.*, 1991). Whereas, for human apyrase, no substrate protection was observed when the aromatic residues were modified (Kettlun *et al.*, 1994).

Additional evidence that the same catalytic site is responsible for the hydrolysis of ATP and ADP comes from the similarity of the M_r values obtained in the radiation–inactivation experiments. The radiation–inactivation technique has been widely used for the M_r determination of enzymes bound to native membranes (Kempner and Schlegel, 1979). We employed this technique because the hydrolytic activities of the solubilized microsomal fraction of mammary gland eluted in the void volume of a Sephacryl S-300 gel column (even in the presence of 0.5 M urea and 1% detergent). The ^{60}Co radiation–inactivation results indicate that this is the smallest unit required to carry out the enzyme function. The data, around 70 kDa for both activities under native conditions, are close to the values reported for the ATP-diphosphohydrolase from rat and human placenta (Pieber *et al.*, 1991; Kettlun *et al.*, 1994), human umbilical vessels (Yagi *et al.*, 1992) and bovine lungs (Picher *et al.*, 1993). These results contrast with those for the apyrase from pig pancreas and bovine aorta, where the functional units have M_r values of 132 and 189 kDa, respectively (Côté *et al.*, 1991).

In summary, the constant rate independent of p obtained from the competition plot experiments indicates that the ATP and ADP hydrolysis occur at the same active site. Similar effects of several chemical modifiers on ATPase and ADPase activities, together with the very close inactivation by ^{60}Co of both activities, agree with the existence of a single enzyme with nucleoside di- and triphosphatase activities, which is the distinctive characteristic of ATP-diphosphohydrolase.

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