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Homotropic allostery of nucleotidase activity of human prostatic acid phosphatase

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ABSTRACT

The steady-state kinetics of hydrolysis of four purine ribonucleotides: 3′-AMP, 5′-AMP, 5′-GMP and 5′-IMP catalysed by human prostatic acid phosphatase (PAP; EC 3.1.3.2) *in vitro* was examined in this study. It has been shown for the first time that nucleotidase activity of PAP exhibits positive cooperativity, or homotropic allostery, in binding the purine ribonucleotides. Therefore, these substrates are homotropic positive effectors, or homotropic allosteric activators, of PAP-catalysed reaction. Enzyme-substrate saturation curves described by Hill equation are sigmoidal and the values of Hill cooperativity coefficient h are higher than 1. The affinity of PAP to substrates, the degree of cooperativity and the efficiency all depend on the chemical nature of the ribonucleotide and they increase in the following order: 5′-AMP < 5′-IMP < 5′-GMP < 3′-AMP, but the enzyme reactivity remains almost equal. Therefore, 3′-AMP is the most efficient PAP substrate while 5′-AMP is the least efficient one of the four ribonucleotides studied. The noncovalent, homoallosteric regulation of the nucleotidase activity of PAP *in vitro* discovered in this study may be physiologically important *in vivo*.

1. Introduction

Human prostatic acid phosphatase (PAP), a glycoprotein synthesized by the epithelia of the prostate gland and secreted at high concentration into the seminal fluid, is a non-specific histidine-dependent phosphatase (EC 3.1.3.2) hydrolyzing various phosphomonoesters, including phosphoproteins, phospholipids and nucleotides *in vitro* [1–6]. PAP being a phosphoprotein phosphatase dephosphorylates proteins at phosphoserine and phosphothreonine [7], as well as at phosphotyrosine residues [5,6,8]. Moreover PAP as a phospholipid phosphatase dephosphorylates inositol 1,4,5-trisphosphate (IP_3) [9], lysophosphatidic acid (LPA) [10] and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [6]. Furthermore, PAP exhibits phosphotransferase [1] and protease activities [11]. Despite over 80 years of research, the physiological function of the seminal fluid PAP in the fertilization process remains unknown. The so-far suggested biological role of PAP seemed to be maturation of semenogelins by dephosphorylation [8] and by proteolysis [11] or dephosphorylation of phospholipids [6,9,10].

PAP belongs to the homotropic allosteric enzyme family since positive cooperativity in binding aromatic phosphomonoesters was evidenced in our previous study [12–15]. The kinetics of the PAP-catalyzed hydrolysis obeys Hill equation [16], but not the Michaelis-Menten one as it was believed before [1]. These substrates are thus the allosteric activators (homotropic positive effectors) of the reaction. The enzyme-substrate saturation curves are sigmoidal and the values of the Hill cooperativity coefficient are in the range 1–4. The affinity of PAP to aromatic phosphomonoesters and the degree of cooperativity exhibited by the enzyme both depend on the chemical character of the substrate molecule, at constant enzyme concentration [12,15]. Moreover, the affinity of PAP to each of the aromatic phosphomonoesters increases, but the degree of cooperativity decreases when the enzyme is diluted. Furthermore, the degree of PAP cooperativity also goes up when the water-pool radius of reverse micelles increases [13,15].

The changes of quaternary and tertiary PAP structures accompanying the process of enzyme dilution were observed by high speed

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air-fuge ultracentrifugation and tryptophan fluorescence measurements performed over a broad enzyme concentration in native conditions in the absence of any effector (substrate) [14,15]. Active dimers of PAP are formed at higher protein concentration, but active monomers—at lower enzyme concentration. Dilution causes the shift of the equilibrium of protein's oligomeric forms to lower oligomers, according to Ostwald's dilution law [17]. Therefore, PAP exists in ligand-induced concentration-dependent association-dissociation equilibria of catalytically active oligomeric species (monomer-dimer-tetramer) [12–15]. These processes offer a reasonable explanation for the allosteric behaviour of PAP, observed in kinetic studies. Concentration-dependent regulation of proteins' biological activity is a well-known phenomenon observed [16,18–20].

Moreover, we have evidenced for the first time that the monomeric PAP subunit obtained by dissociation of active dimeric PAP at low enzyme concentration is catalytically active [14,15]. Thereby, the process of dimerization is not necessary for the formation of the active dimeric enzyme molecule from the inactive subunits as it was wrongly concluded in the renaturation studies of Kuciel et al. [2,21].

The 2′-, 3′- and 5′- nucleotidase catalytic activities of seminal fluid PAP have been known for long time [22–27]. Various purine and pirymidine nucleoside monophosphates (with ribose, deoxyribose, arabinose and glucose), their derivatives, oligonucleotides, polynucleotides and RNA are known substrates of PAP. The dephosphorylation rate of the monophosphonucleotides was depended on the type of sugar, on the type of nitrogen base as well as on the position of the phosphate moiety [23,25]. This rate was higher for 3′-ribonuclotides than for 5′- and 2′- isomers. Unfortunately the Michaelis-Menten equation, but not the general Hill equation [16], was used for calculations in the studies on PAP's nucleotidase activity performed until now [23–26].

The aim of the present study was to establish if the nucleotidase activity of the seminal fluid PAP is regulated by homotropic allostery or by positive cooperativity in substrate binding, as the acid phosphatase activity of this enzyme [12–15] and as the activity of several nucleotidases [28–31]. Therefore, the steady-state kinetics of hydrolysis of four purine ribonucleotides: 3′-AMP, 5′-AMP, 5′-GMP and 5′-IMP catalysed by PAP was examined.

2. Experimental

2.1. Materials

Sodium salts of adenosine 5′-monophosphate (5′-AMP) and adenosine 3′-monophosphate (3′-AMP), disodium salts of guanosine 5′-monophosphate hydrate (5′-GMP) and inosine 5′-monophosphate octahydrate (5′-IMP), crystalline oxalate salt of Malachite Green for microscopy, ammonium molybdate tetrahydrate, and Triton X100 were purchased from Sigma Chemical Co. Sodium acetate, acetic acid, and Tris (tris(hydroxymethyl) aminomethane) came from Fluka. All the other chemicals were of the highest purity available and were used without further purification.

2.2. Enzyme preparation

Homogenous human prostatic acid phosphatase was purified from human seminal plasma, using the affinity method of van Etten and Saini [32]. The molar concentration of phosphatase was determined by the absorbance measurement at 280 nm ($A^{1\%}_{280} = 14.4$), assuming a molecular mass of 100 kDa [12].

2.3. Hydrolysis of purine nucleotides catalysed by human prostatic acid phosphatase

The incubation mixture of 200 μl contained purine nucleotide solution of up to 2.5 mM concentration in 0.1 M acetate buffer, pH 5.0. Nucleotidase assay was performed in a discontinuous way. Enzymatic reaction was started by adding 50 μl of 242 nM PAP solution (in 10 mM Tris-HCl buffer of pH 7.4, containing 100 mM NaCl) at 20 °C; thus the enzyme concentration in the incubation mixture was 48.4 nM. In order to determine the concentration of the produced inorganic phosphate by using modification of Lanzetta procedure [26,33], a sample of the incubation mixture (50 μl) was taken every 15 s for 1 min and added to 950 μl of the Malachite Green reagent (0.323 mM Malachite Green oxalate salt, 1.72 mM ammonium molybdate sodium salt ($(NH_4)_2MoO_4$) and 0.05% v/v Triton X100, dissolved in 0.7 M hydrochloric acid). After incubation (15 min at room temperature) a 23 μl sample of 38% sodium citrate solution was added. Absorbance measurements of the coloured product were performed at 650 nm wavelength on a double-beam Varian Cary 300 Bio UV–vis Spectrophotometer. In the blank experiment the enzyme was not added. The value of the absorbance coefficient of the coloured reaction product at 650 nm was determined to be $5.17 \pm 0.11\ mM^{-1}\ cm^{-1}$. The calibration curve was linear in the absorbance range 0.05–2.20 (phosphate concentration range 0.02–0.4 mM). The kinetic curve was plotted on the basis of five experimental points for each incubation mixture.

2.4. Initial rate calculation

The initial reaction rate (v_o) was calculated from the tangent of the kinetic curve of the first-order reaction at t = 0, by using Excel software. The initial reaction rate was estimated from triplicate measurement over a substrate concentration range up to 2.5 mM.

2.5. Calculation of the kinetic parameters for PAP-catalysed hydrolysis of purine nucleotides

In order to determine the steady-state reaction constants ($K_{0.5}$, k_{cat} and h) for each substrate, the Hill equation was fitted to the experimental data with EZ-Fit (Perrella Scientific, Inc.; http://www.jlc.net/~fperrell) [34] or Sigma Plot (Jandel Scientific) software:

$$v_0 = \frac{k_{cat}[E]_0([S]_0)^h}{(K_{0.5})^h + ([S]_0)^h} \quad (1)$$

where v_o is the initial reaction rate, $[E]_o$ is the overall molar concentrations of enzyme, $[S]_o$ is the initial concentrations of substrate, h is the Hill cooperativity coefficient, k_{cat} is the catalytic constant (turnover number) and $K_{0.5}$ is the half saturation constant.

3. Results and discussion

3.1. Kinetics of PAP- catalysed hydrolysis of purine nucleotides

This paper presents the results on steady-state kinetics of hydrolysis of four purine ribonucleotides: 3′-AMP, 5′-AMP, 5′-GMP and 5′-IMP catalysed by the prostatic acid phosphatase (PAP) isolated from human seminal fluid in 0.1 M acetate buffer at pH 5.0, at enzyme concentration of 48.4 nM (Fig. 1, Table 1). The experiments were conducted in order to characterise the type of kinetics reaction: hyperbolic or sigmoidal and to establish if the nucleotidase activity of PAP is regulated by homotropic allostery. The Hill Eq. (1), the general one [16], instead of the Michaelis-Menten one used previously [23–26], was applied to fit the experimental data and to calculate the kinetic constants of the PAP-catalysed reaction.