Characterization of Human Prostatic Acid Phosphatase and Its Clinical Significance

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Summary Human prostatic acid phosphatase (EC: 3.1.3.2) belongs to the group of enzymes that can split simple phosphate esters, nucleotides, and phosphoamino acids in phosphoproteins including phosphotyrosine derivatives. The native phosphatase is a glycosylated homodimer with a molecular weight of 100 kDa. Specific inhibition and denaturation-renaturation studies showed that the catalytic activity of the enzyme depends on the formation of the dimer. The enzyme is androgen-regulated and is a prostate-specific protein; and its level is often elevated in the blood serum of prostate cancer patients. Prostatic acid phosphatase can be stabilized against various denaturation agents by cross-linking techniques and can be used in different biomedical and biotechnological processes.

Key Words: prostatic acid phosphatase, structure, clinical and biotechnological applications

Many biological processes in the cell involve the formation and cleavage of phosphate bonds. Enzymatic dephosphorylation being an opposing activity to phosphorylation reactions, is an important mechanism for different metabolic regulations, signal transduction, and cell proliferation.

Human prostatic acid phosphatase (hPAP) belongs to the group of enzymes that can split C-O-P and N-P bonds of simple esters, nucleotides, and phosphoamino acids in phosphoproteins including phosphotyrosine derivatives and displays a preference to catalyze aryl phosphates. The natural substrate for hPAP is unknown, and the discovery of the true biological function of the enzyme is a very important goal and requires further study. The androgen-regulated enzyme is a prostate-specific protein, and its level is often elevated in the serum of prostate cancer patients; therefore it has been used for many years as a clinical marker in the diagnosis of the above pathological process [1]. The results described in this paper shed further light on the structural assembly of the crystalline hPAP and

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provide more information on the relationship between its structure and catalytic activity. The practical use of the enzyme is also discussed.

Molecular properties of hPAP

Native hPAP is a glycoprotein with a molecular weight of approximately 100 kDa. Active hPAP consists of two identical subunits [2]. Upon isoelectric focusing, hPAP that is homogenous in ultracentrifugation pattern, elution profile of gel filtration, and immunological tests, has been resolved into multiple charged species with isoelectric points ranging from 4.5 to 5.3 with the major species at 5.0 [3]. Each of these charged species appears to have equivalent catalytic properties. Difference in degree of sialylation is one but probable not the only cause of charge heterogeneity, because several bands remain following neuraminidase digestion.

To define structure and basic organization of the hPAP molecule, we purified the enzyme by affinity chromatography [4] and crystallized it by using the standard hanging drop technique with a vapor diffusion setup with 30% PEG-1450, 50 mM glycine buffer at pH 10.0 [5]. Tablet-shaped crystals were grown, which measured $0.5 \times 0.5 \times 0.3$ mm and diffracted X-rays to about 3.0 Å resolution. The crystals were orthorhombic and belonged to space group $p2_12_12_1$ with $a = 126.3$ Å, $b = 207.9$ Å, and $c = 73.0$ Å. There were two dimers per asymmetric portion of the unit cell, which gave a Matthews' parameter [6] $V_m = 2.4$ Å³/Da. X-ray analysis data and all calculations were completed using CHAIN [7] and O [8] programs. The structure of the enzyme was periodically rebuilt including saccharide chains, additional amino acids, and solvent molecules [9]. The crystallographic model for two molecules of hPAP has 11,205 protein atoms, 519 carbohydrate atoms, 4 glycine residues at the active site, and 633 water molecules.

One molecule of native hPAP is a glycosylated homodimer in which the subunits are related by a 2-fold axis (Fig. 1). Each subunit contains two domains, the larger one is α/β type, composed of a central seven-stranded mixed β -sheet with α -helices on both sides. The second domain contains six α -helices and is formed by a long chain (residues 125–227) from the first domain and a loop between residues with no secondary structural elements.

The active site in hPAP is located in a large open cleft between the two domains. The intersubunit hydrogen bonds are between the side chain of Gln 33 and the main chain of His 67, between the side chain of Gln 40 and the main chain of Val 97, and between the side chain of His 112 and the side chain of Asp 76. Other interactions between the subunits are predominantly hydrophobic.

hPAP has three N-linked carbohydrate chains attached to Asn 62, Asn 188, and Asn 301 [10]. The oligosaccharide chains are close to the protein surface and are positioned between neighboring dimers, which stabilizes those conformations that result in favorable interactions. Conformational variability of oligosaccharide chains was observed as the function of different environment in the crystal lattice [9]. When the carbohydrate structures were superimposed from four protomers, considerable differences were visible, which resulted not only from interactions of

the parts of the carbohydrate chains, but also may have been affected by the disordered parts and not seen in the electron density.

To obtain more information about the specific surface features of the hPAP molecule, we used scanning electron microscopy (SEM) and atomic force microscopy (AFM). Fresh samples of the enzyme (1–5 μg protein/ml) in 0.01 M Tris-HCl buffer, pH 7.5, were deposited on cleaved mica; and specimens were coated with gold and imaged by secondary electrons in the SEM (LEO 435 Vpi, Leo, Cambridge, UK). Figure 2 shows a representative SEM image of hPAP. Besides single molecules of hPAP, we see aggregates composed of four, six or even more molecules, which probably result from the drying procedure used for the sample. The average size of the single molecules obtained from different SEM images is 30–50 nm.

Samples for AFM were prepared with the concentration of hPAP and buffer solution the same as in the case of SEM. The solution was deposited on freshly cleaved mica and left for 10 to 15 min before being rinsed 3 times with an excess of buffer, and then was dried at room temperature. AFM images were recorded with a Nanoscope E Microscope (Digital Instruments, Santa Barbara, CA). A typical high-resolution AFM image of hPAP molecules with the scan area of $1,000 \times 1,000$ nm is shown in Fig. 3. The image is similar to the results obtained by SEM. We can clearly see single molecules of hPAP of moderate asymmetry and aggregates composed of several molecules. The average size of molecules is 30–40 nm. Molecules have a height of about 3 nm. Compared with the dimensions obtained from the analysis of the structure of hPAP crystals, hPAP molecules adsorbed on mica appear broadened and flattened.

Dimeric structure and activity of hPAP

Because hPAP has a dimeric structure, it was interesting to learn if an association of two identical subunits is necessary for the catalytic activity of the enzyme. To answer the above question, Kuciel *et al.* [11, 12] undertook systematic studies on reversible denaturation of hPAP. In 6 M urea at pH 2.5, the enzyme is completely inactivated and dissociated into subunits. Tenfold dilution of the enzyme into 0.1 M phosphate buffer, pH 7.0, allowed hPAP to renature to about 90% of the initial activity. Based on the enzyme concentration dependence of the reactivation rate, it was established that reactivation of the phosphatase depends on the monomer association and that the subunit cannot be catalytically active. Spectral analysis (fluorometry, circular dichroism), fast size-exchange chromatography, and proteolytic tests of denatured and renatured hPAP led to the conclusion that in the folding pathway there exist several intermediates with very fast and slower steps of reconstitution of the catalytically active molecule [12]. The formation of the dimer leads also to a reduction of the accessible surface of the protomer from 15,200 to 13,300 $\text{\AA}^2$. This is likely the main factor responsible for the observed very tight binding between the subunits [2].

Oligomerization studies indicate that the dimer formation introduces struc-

Fig. 1. Diagrammatic drawing representing the structure of the hPAP dimer with oligosaccharide chains shown as black lines.

Fig. 2. Scanning electron microscopy image of hPAP dried on mica and coated with gold.

tural changes that propagate to the active site and formation of the catalytically active molecule. From site-directed mutagenesis of hPAP, the key role of Arg 79 in the formation of the catalytically active dimer was demonstrated [13]. Perhaps this residue plays a particular role in forming the phosphoenzyme intermediate during the enzymatic reaction pathway. In monomers, Arg 79 is probable located too far from the active center; and dimerization is thus necessary for its correct positioning.

Fig. 3. High-magnification AFM image of the surface of hPAP molecules deposited on freshly cleaved mica and recorded in contact "error signal" mode.

Calorimetric analysis of hPAP in the presence of L(+)-tartrate

The specificity of L(+)-tartrate as a potent competitive inhibitor of hPAP was investigated several years ago [14]. Tartrate, with an inhibition constant $K_i = 10^{-6}$ M, has a structure related to the transition state of hPAP in which the phosphorus atom of the substrate is pentacoordinated [15]. This property of tartrate, for instance, is routinely used in affinity chromatography for hPAP purification, where inhibitor molecule is immobilized through an amide linkage [4]. To characterize further the interaction between hPAP and L(+)-tartrate, we used differential scanning calorimetry, a technique well suited for a direct investigation of the interacting systems.

Experiments were performed using an adiabatic capillary scanning microcalorimeter DASM-4 (Academy of Sciences of Russia) with a cell volume of 0.4699 ml and a scanning rate of 1°C/min. Calorimetric measurements of the sample (1 mg of enzyme protein per ml of 0.01 M Tris-HCl buffer, pH 7.5) were recorded from 10–90°C at a heating rate 1°C/min. In the experiments with tartrate, the ligand was added to the enzyme solution in the same buffer to obtain the desired final concentration equal to 10^{-3} M. The T_m for a given transition was taken to be the point of maximum excess heat capacity. ΔH was calculated from the area under each calorimetric peak. The van't Hoff enthalpy change was calculated using the following equation [16]:

$$\Delta H_{\text{vH}} = \frac{4RT_m^2}{(T_e - T_o)/2},$$

where ΔH_{vH} is van't Hoff enthalpy; R , the gas constant; T_m , temperature of

maximum heat capacity; T_o , temperature of onset of the transition; and T_e , temperature at the end of the transition, all expressed in Kelvin degrees.

Typical DSC thermograms of hPAP recorded in the absence and presence of L(+)-tartrate are shown in Fig. 4. Transition peak temperatures are given in Table 1. Within the accuracy of our measurements, both samples showed an endothermic thermal transition. The value of T_m for hPAP amounted to 49.5°C; and in the presence of 0.001 M tartaric acid, the T_m value was 68.5°C. As can be seen in Table 1, DSC scanning of hPAP in the presence of competitive inhibitor showed a considerable increase in T_m value. Also, values for ΔH were larger in the presence of ligand, whereas values for ΔH_{vH} remained nearly the same. For both DSC

Fig. 4. DSC thermograms of hPAP without inhibitor (A) and in the presence of 1 mM L(+)-tartaric acid (B). Samples containing 1 mg protein in 1 ml of 0.01 M Tris-HCl buffer, pH 7.5, were measured from 10 to 90°C at a heating rate of 1°C/min.

Table 1. Summary of thermodynamic data for hPAP.

L(+)-Tartaric acid (M)	T_m (°C)	ΔH (kcal/mol)	ΔH_{vH} (kcal/mol)
0	49.5	895.6	114
0.001	68.5	1107.8	120

Fig. 5. Isothermal titration of the binding of L(+)-tartaric acid to hPAP at pH 7.5 and 25°C. Upper part of the picture represents injections of 1 mM tartaric acid solution in 0.01 M Tris-HCl buffer, pH 7.5, and the lower part, integrated calorimetric heats and best-fit curve to a simple single-site binding model.

curves, a ratio of the van't Hoff to calorimetric enthalpy ($\Delta H_{\text{vH}}/\Delta H$) was 0.10–0.13, which indicates a significant population of one or more intermediate states between the native and denaturated states [17]. A similar conclusion can be obtained from the work of Kuciel *et al.* [12] on reversible denaturation of hPAP.

In order to determine the enthalpy change for the binding of tartaric acid to hPAP, we carried out isothermal titration calorimetric measurements. A typical thermogram in Tris-HCl buffer, pH 7.4, at 25°C is shown in Fig. 5. To a solution of hPAP (1.2 mg protein/ml) were added 30 consecutive equivalent 5- μ l injections of tartaric acid solution. The negative sign of the measured heat indicates that the enthalpy change for each injection was negative and that the process of binding under the applied conditions was exothermic. The total heat calculated from the summation of the heats involved in each injection, i.e., ΔH , obtained at 25°C amounted to -6.49 kJ/mol, which corresponds to -1.55 kcal/mol and is in agreement with other similar systems [18].

Transcription of hPAP mRNA

As was observed earlier, hPAP is synthesized under steroid hormone regulation and is secreted by the epithelial cells into the seminal plasma. Most studies on the transcription of the PAP gene were carried out *in vivo* or on isolated cell lines cultured from cancer of the prostate gland or its metastases and brought inconsistent results [19, 20]. Therefore, we prepared an experimental model using tissue slices obtained from patients with diagnosed and histopathologically confirmed hyperplasia [21]. The tissue slices prepared under sterile conditions were incubat-

Fig. 6. Northern blot analysis of hPAP mRNA in total RNA isolated from six different specimens of hyperplastic human prostate gland. Agarose gel electrophoresis was carried out in the presence of 2.2 M formaldehyde in 20 mM MOPS buffer pH 7.0. I, total RNA stained with ethidium bromide; II, autoradiogram of mRNA hybridized to ³²P-labeled DNA probes [adapted from ref. 21].

ed for 1 to 5 h in RPMI 1640 medium with or without 5 α -dihydrotestosterone (DHT). Total RNA was then isolated, fractionated on an oligo-dT-cellulose column, and analyzed by agarose gel electrophoresis of gels containing 2.2 M formaldehyde. Hybridization with labeled DNA probes specific to hPAP was performed according to typical procedures, and nylon membranes soaked with scintillation fluid were counted in a scintillation spectrometer. Figure 6 indicates that the fraction of RNA specifically reacting with DNA probes was 3.3 ± 0.1 kb in size. Analysis of hPAP mRNA in samples of benign hyperplastic prostate slices, obtained from different donors, incubated in the presence of DHT showed a dose- and time-dependent increase in the mRNA level, which reached its highest value after about 5 h of incubation at a 5×10^{-9} M concentration (Fig. 7). As can be seen in the figure, the highest increase in the mRNA level with respect to the reference sample was usually 200–400% after 5 h-incubation of slices from different patients. On the other hand, estradiol at concentrations ranging from 10^{-9} – 10^{-8} M showed no effect on the expression of the hPAP gene; but at a 10^{-7} M concentration, transcription visibly increased. The above results suggest that DHT is necessary to sustain expression gene of hPAP in hypertrophic gland and that probably the ratio of androgens to estrogens might play important role in the transcriptional activity in prostate gland.

Fig. 7. Effect of DHT concentration (A) and time of incubation (B) on the level of hPAP mRNA. A, tissue slices were incubated for 3 h in RPMI 1640 medium containing different concentrations of DHT, and the mRNA level was then determined. B, tissue slices were incubated for different times in the same medium containing 10^{-10} M DHT. The results are mean values of three tissue samples obtained from different patients [adapted from ref. 21].

Practical use of hPAP

Human prostatic acid phosphatase, depending on its biological properties, can be utilized in different fields of practical application: in diagnosis of prostate cancer, for dephosphorylation of orthophosphate esters in different biotechnological processes, and in suppression of immune system cells.

Since 1936 hPAP levels in blood serum were routinely estimated to monitor both development and stage of prostate cancer [22]. For distinguishing phosphatase of prostate origin from extraprostatic types, various methods have been used, including radioimmunochemical tests.

On the account of possible cross reactivity between hPAP and phosphatases from other tissues, polyclonal immunoglobulins are not recommended for this purpose. Therefore, we developed a monoclonal antibody, termed Mab-14 [23], which is an IgG₁ immunoglobulin that does not react with the enzyme form leucocytes, kidney, liver, pancreas, spleen, and colon or with acid phosphatase from other tissues, but recognizes all isoenzymic forms of prostatic acid phosphatase. Mab-14 binds two molecules of hPAP with an apparent affinity constant of 2.4×10^{-8} M, and the interaction of the enzyme with antibody has no effect on the catalytic activity of the enzyme. The antibody reacts with native, denatured, and even partially degraded molecules of hPAP [24]. MAb-14 has been applied

Fig. 8. Immunohistochemical staining of hPAP in normal prostatic tissue (A), in carcinoma of the prostate (B), and in metastatic carcinoma of prostate gland in femoral bone (C). Indirect immunohistochemical technique with Mab-14 monoclonal antibody specific for hPAP was used [adapted from ref. 23].

to the immunohistochemical localization of hPAP distribution in normal human prostate gland, in adenocarcinoma of prostate, and its metastases to bone (Fig. 8).

Stable hPAP preparations can be used in continuous dephosphorylation of low- and high-molecular-weight substrates, when the enzyme is linked to a neutral carrier to make it insoluble. For this purpose we immobilized hPAP on Concanavalin A or on Sepharose obtaining active products resistant against various denaturation agents [25].

In the last decade studies have been initiated on the effect of hPAP on neutrophil and natural killer cell functions [26]. It was found that preincubation of natural killer cells with increasing amounts of hPAP, brought about a dose-dependent inhibition of their ability to kill the tumor target cells by about 85%. The secretion of large amounts of hPAP could effectively inhibit host anti-tumor immunological effector mechanism including both natural and acquired neutrophil immunity. It was also found that hPAP blocked superoxide production by neutrophils by about 50% [26].

The above observations led La Count *et al.* [27] to postulate that the inhibition of hPAP in the serum of prostate cancer patients should stop deterioration of their immune system and thus reduce the probability of metastasis of the tumor. Specific and potent inhibitors of hPAP could be used as an important component of therapy primarily for patients with prostate cancer, but probably also for patients with tumors of non-prostatic origin.

Most of this work was supported by grant No 501/P/18/L of the Polish Committee for Scientific Research. We would like to thank to Prof. A. and W. Zielenkiewicz from Institute of Physical Chemistry for help in calorimetric experiments and to Prof. J. Haber and his coworkers from the Institute of Catalysis and Physicochemistry of Surface, both of Polish Academy of Sciences, for atomic force microscopy.

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