Kinetic intermediates of unfolding of dimeric prostatic phosphatase*

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Kinetics of guanidine hydrochloride (GdnHCl)-induced unfolding of human prostatic acid phosphatase (hPAP), a homodimer of 50 kDa subunit molecular mass was investigated with enzyme activity measurements, capacity for binding an external hydrophobic probe, 1-anilinonaphtalene-8-sulfonate (ANS), accessibility of thiols to reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 2-(4'-maleimidylanilino)naphthalene-6-sulfonate (MIANS) and ability to bind Congo red dye. Kinetic analysis was performed to describe a possible mechanism of hPAP unfolding and dissociation that leads to generation of an inactive monomeric intermediate that resembles, in solution of 1.25 M GdnHCl pH 7.5, at 20°C, in equilibrium, a molten globule state. The reaction of hPAP inactivation in 1.25 M GdnHCl followed first order kinetics with the reaction rate constant 0.0715 ± 0.0024 min⁻¹. The rate constants of similar range were found for the pseudo-first-order reactions of ANS and Congo red binding: 0.0366 ± 0.0018 min⁻¹ and 0.0409 ± 0.0052 min⁻¹, respectively. Free thiol groups, inaccessible in the native protein, were gradually becoming, with the progress of unfolding, exposed for the reactions with DTNB and MIANS, with the pseudo-first-order reaction rate constants 0.327 ± 0.014 min⁻¹ and 0.216 ± 0.010 min⁻¹, respectively. The data indicated that in the course of hPAP denaturation exposure of thiol groups to reagents took place faster than the enzyme inactivation and exposure of the protein hydrophobic surface. This suggested the existence of a catalytically active, partially unfolded, but probably dimeric kinetic intermediate in the process of hPAP unfolding. On the other hand, the protein inactivation was accompanied by exposure of a hydrophobic, ANS-binding surface, and with an increased capacity to bind Congo red. Together with previous studies these results suggest that the stability of the catalytically active conformation of the enzyme depends mainly on the dimeric structure of the native hPAP.

**Keywords:** prostatic phosphatase, dimer, protein unfolding

**INTRODUCTION**

Human prostatic acid phosphatase (hPAP) is a dimeric glycoprotein composed of two identical polypeptide chains (Luchter-Wasyl & Ostrowski, 1974). In its three-dimensional structure, the catalytic center is located between the two domains of each subunit of the enzyme (Jakob et al., 2000). Studies on site-directed mutagenesis of rat prostatic acid phosphatase (Porvari et al., 1994), and also equilibrium unfolding, refolding and reassembly trials using denatured human prostatic acid phosphatase (Kuciel et al., 1990; 1996; Ostrowski et al., 1993; Kuciel & Mazurkiewicz, 1997; Wójciak et al., 2003) indicate that these enzymes are catalytically active as dimers. It is suggested that the main conformational change accompanying the association of the two subunits of human PAP is correct positioning of the loop with Arg79, which is thought to participate in catalytic center formation (Jakob et al., 2000) and is engaged in binding of the phosphatase inhibitor N-propyl-(+)-tartramate (LaCount et al., 1998).

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**Abbreviations:** ANS, 1-anilinonaphthalene-8-sulfonate; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GdnHCl, guanidine hydrochloride; hPAP, human prostatic acid phosphatase; MIANS, 2-(4'-maleimidylanilino)naphthalene-6-sulfonate; pNPP, p-nitrophenylphosphate; TNB, 2-nitro-5-thiobenzoic acid.
To understand the connection between folding and assembly of dimeric proteins with a catalytic function, it is important to track the relation between the local and global conformational changes that affect the protein’s biological activity. Investigation of guanidine hydrochloride-induced equilibrium unfolding of prostatic phosphatase suggests that hPAP dissociation and unfolding is a process proceeding through at least two stages: N₂ ↔ 2I ↔ 2U, where N₂ is the native, catalytically active, dimeric protein, I is the partially folded monomer devoid of phosphatase activity, and U is the unfolded monomer. Equilibrium experiments indicate that the most populous intermediate of hPAP in the 1.0–1.5 M range of guanidine hydrochloride concentrations is a monomer. In 1.25 M GdnHCl (pH 7.5), in equilibrium, the protein was completely devoid of catalytic activity and showed a high binding affinity for the hydrophobic probe ANS. The free thiol groups of the polypeptide were modified in reaction with MIANS, but their derivatives remained in the hydrophobic environment of the protein molecule (Wójciak et al., 2003). These features indicate that at this concentration of denaturant the population of monomeric equilibrium intermediates of hPAP resembles the molten globule state (Kuwajima, 1989; Ptitsyn, 1994).

The present study describes kinetic experiments in which prostatic phosphatase was denatured in 1.25 M GdnHCl (pH 7.5) at 20°C. In these conditions, the progress of inactivation and unfolding transitions can be followed on a reasonable time scale. The initial state of this process is the native form of the protein. The end point of the process might be described as an equilibrium with a high population of molten globules. As probes of local unfolding we employ measurements of the accessibility of free thiols to modifying reagents and the adsorption of Congo red dye to the loosened β-structured regions of the polypeptide. The protein’s catalytic activity and the exposure of its hydrophobic surfaces to solvent are considered to be global probes of the functional integrity of the biologically active protein.

MATERIALS AND METHODS

Materials. Ultrapure guanidinium hydrochloride (GdnHCl), sodium phosphate (NaH₂PO₄ and Na₂HPO₄), dimethylsulfoxide (DMSO), 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), 1-anilino-8-naphthalene-8-sulfonate (ANS) and p-nitrophenylphosphate (pNPP) were purchased from Sigma (St. Louis, MO, USA). 2-(4’-maleimidylanilino)naphthalene-6-sulfonate (MIANS) was obtained from Molecular Probes (Leiden, The Netherlands). Congo red was purchased from Aldrich Chemical Company. All other reagents were of analytical grade, obtained from POCh (Poland). Prostatic acid phosphatase (hPAP) was purified from human seminal plasma according to Van Etten and Saini (1978). The concentration of the purified phosphatase was determined using the absorption coefficient value ε₂₈₀ = 1.44 (Bobrzecka et al., 1968).

Stock solutions. GdnHCl (8 M) and ANS (2 mM) stock solutions were prepared in 0.1 M phosphate buffer (pH 7.5) with 1 mM EDTA. MIANS stock solution (5 mM) was prepared in DMSO.

Determination of enzyme activity. The catalytic activity of hPAP was determined at 20°C using 20 mM pNPP in 0.1 M acetate buffer (pH 5.0) as substrate. The enzyme solution (10 µl) was introduced into 1 ml of substrate solution, and 100 µl aliquots of the reaction mixture were withdrawn into 1 ml portions of 0.1 M NaOH at 30-s intervals for 3 min. To calculate the product concentration, the absorbance of alkaline samples was measured at 400 nm. The residual denaturant concentration did not inhibit the native enzyme, and no reactivation was observed during the activity measurements.

Kinetic and equilibrium measurements of the unfolding process. All unfolding experiments in GdnHCl followed the same protocol: 0.1 M phosphate buffer (pH 7.5), 8 M stock solution of GdnHCl and EDTA solution were mixed together to the desired final concentration of denaturant and 1 mM final concentration of EDTA. Then, 50 µl of stock protein solution (around 20 times the final protein concentration) was added. The final concentration of hPAP in each experiment described in this paper was 1 µM (100 µg/ml). All samples were incubated with the denaturant at 20°C. Each set of data was collected from at least five experiments.

Spectroscopic techniques. Absorption spectra were recorded with a Hitachi U200 spectrophotometer. Fluorescence spectra were registered with a Hitachi F4000 spectrofluorimeter.

Reaction of thiol groups with DTNB. The protein was added to 0.1 M phosphate buffer (pH 7.5) containing 1.25 GdnHCl, 1 mM EDTA and 200 µM DTNB. The final concentration of the protein was 1 µM. The reaction was monitored by measurement of absorption at 412 nm. The concentration of thiol groups was calculated using the absorption coefficient value ε₄₁₂ = 14150 M⁻¹cm⁻¹ (Riddles et al., 1979).

Labeling of thiol groups with MIANS. The protein was added to 0.1 M phosphate buffer (pH 7.5) containing 1.25 M GdnHCl, 1 mM EDTA and 10 µM MIANS. The final concentration of the protein was 1 µM. The reaction with thiol groups was monitored by measurement of fluorescence intensity at 420 nm. The excitation wavelength was 322 nm.
Determination of ANS binding. The protein was added to 0.1 M phosphate buffer (pH 7.5) containing 1.25 M GdnHCl, 1 mM EDTA and 100 µM ANS. The final concentration of the protein was 1 µM. The reaction progress was monitored by measurement of fluorescence intensity at 470 nm. The excitation wavelength was 400 nm.

Determination of Congo red binding. Several 1-ml samples containing 1 µM hPAP in 1.25 M GdnHCl (phosphate buffer pH 7.5) and 1 mM EDTA were prepared. At defined intervals from the beginning of denaturation, 100 µl aliquots of 250 µM Congo red dye were introduced into successive samples. After 1 min of incubation with the dye, a dense suspension of Sephadex G-25 (0.5 ml) preswollen in the denaturation buffer was added. The mixture was vortexed for 1 min and then the Sephadex particles were removed by centrifugation. The absorbance of the supernatants was measured at 485 nm.

Data analysis. Data analysis, graphs, statistics and calculations of standard errors of the determined values were performed by the SIMFIT program (Bardsley, 2005), with weighted least squares, using $R^2$ as a measure of goodness of fit.

Data collected in the course of first order or pseudo-first-order reactions were fitted using linear regression to formula:

$$\ln c = \ln a - kt \quad \text{or} \quad \ln \frac{a}{a-x} = kt$$

where $a$ was the concentration of substrate at the beginning of the reaction, the value for the native or unfolded protein; $c$ was the concentration of substrate at a given time of the reaction, being the value of the observable at time $t$, for example, concentration of the native protein measured through its catalytic activity; $x$ was the concentration of the product of the reaction at time $t$, in the reactions with DTNB, with MIANS or ANS and Congo red binding.

RESULTS

Kinetics of prostate phosphatase inactivation in 1.25 M GdnHCl (pH 7.5)

The enzyme inactivation reaction recorded in 1.25 M GdnHCl followed first-order kinetics (Fig. 1). The rate constant was determined to be 0.0715±0.0024 min⁻¹.

Reactivity of thiol groups with DTNB

Phosphatase unfolding in 1.25 M GdnHCl (pH 7.5) was followed by reaction of thiol groups with excess of DTNB. The absorbance of TNB at the end of the reaction corresponded to titration of two thiol groups per subunit. The rate constant for the pseudo-first-order reaction was determined to be 0.327±0.014 min⁻¹ (Fig. 2).

MIANS fluorescence

To monitor changes in the environment of hPAP thiols which were modified during the protein unfolding, reaction with excess of MIANS was performed and the spectra of derivatives were registered. The rate constant for the pseudo-first-order reaction was determined to be 0.216±0.010 min⁻¹ (Fig. 3).
Congo red dye is believed to form supramolecular complexes with relaxed β-structured regions of proteins (Piekarska et al., 1994; 1996). Previous experiments (Kuciel & Mazurkiewicz, 1997) have shown that Congo red binds irreversibly to partially unfolded forms of hPAP. Therefore we tried to follow the destabilization of the protein structure by estimating Congo red dye binding. At specified intervals, samples of the denatured protein were taken to determine their ability to bind the dye. Congo red binding to denaturing hPAP followed pseudo-first-order kinetics with a rate constant of 0.0409 ± 0.0052 min⁻¹ (Fig. 4).

Kinetics of ANS binding to denaturing hPAP

To study the exposure of hydrophobic surfaces during unfolding and dissociation transitions, hPAP was denatured in an excess of an external hydrophobic probe, ANS. Binding of ANS to native hPAP is very poor. Equilibrium binding of ANS in the 0.8–1.5 M range of GdnHCl solutions was enhanced 5–6 times, with a blue-shift of the emission spectra (Wójciak et al., 2003). Progress of the reaction of ANS binding to hPAP in 1.25 M GdnHCl followed pseudo-first-order kinetics with the reaction rate constant 0.0366 ± 0.0018 min⁻¹ (Fig. 5).

DISCUSSION

Human prostatic acid phosphatase is a large protein (100 kDa) formed by dimerization of identical polypeptide chains composed of 354 amino-acid residues each (Van Etten et al., 1991). In each subunit of the protein two domains can be distinguished: a large α/β domain formed of seven strands and of helical fragments; and a smaller α-helical domain. The active site of hPAP is located in a large open cleft between the two domains (Jakob et al., 2000). All amino-acid residues considered essential for catalytic activity, namely Arg11, His12, Arg15, Arg79, His257 and Asp258 (Ostanin et al., 1994, Lindquist et al., 1994; Ortlund et al., 2003), are found in the α/β domains. The intersubunit hydrogen bonds are formed through interaction of the side chains of the main chains of Gln33, Gln40, His67, Asp76, Val97, Trp106, Val111 and His112, located in the α/β domains. Other interactions between subunits are predominantly hydrophobic, and dimerization
results in reduction of the solvent-exposed surface of each subunit by 1900 Å² (Jakob et al., 2000). The analyses of the 3-D structure of hPAP that have been made so far suggest that the amino acids of the α/β domains participate in catalysis and also that they principally support the dimeric structure of the protein.

There are two free thiol groups in each subunit of hPAP, in the side chains of Cys183 and Cys281. The first one is situated in one of the helical fragments of the small α domain. Cys281 is found in a strand of the α/β domain. Free thiols of the cysteines are in minimal distances of 8 Å (Cys183) and 13 Å (Cys281) from the imidazole of His12, essential for catalytic activity. The thiol groups do not participate directly in catalysis. In the native form of this protein, the thiol groups of both cysteines are buried and not accessible to modifying reagents. Moreover, it seems that amino acids in the vicinity of these cysteines are not engaged in the interaction between the subunits. That is why these cysteines in the native protein can serve as local probes of the conformational variations around them. Free thiols of the hPAP equilibrium intermediate that dominates in 1.25 M GdnHCl (pH 7.5) react with MIANS. The high fluorescence of the MIANS derivatives indicates that the −SH groups of this intermediate are situated in a hydrophobic environment of a partially unfolded molecule (Wojcicka et al., 2003).

The goal of the kinetic experiments presented in this paper was to elucidate the sequence of events leading to the formation of the described equilibrium intermediate, and to distinguish the kinetic intermediates of the unfolding.

Reaction of the thiol groups with DTNB showed that both −SH groups per molten globular monomer are unmasked at the end of the reaction. The rate constant for the pseudo-first-order reaction is 0.327 ± 0.014 min⁻¹. After 10 min of 1 µM hPAP unfolding in 1.25 M GdnHCl, all free thiols present in the protein molecule were substituted. At the same time, around 60% of the catalytic activity of the protein was still preserved. Labeling with MIANS was carried out to characterize the environment of the thiols that became exposed. The strong fluorescence of the product indicated hydrophobic surroundings. The rate constant for the pseudo-first-order reaction of hPAP with MIANS was determined to be 0.216 ± 0.010 min⁻¹. This value is within the same range of magnitude as the rate constant of the reaction with DTNB. The difference between the two values may be explained by differences between the mechanisms of action of these two reagents and/or by various features of the products that serve as indicators of the reaction progress. In both cases, however, the end point of the reaction with thiols was reached much faster than complete inactivation of the enzyme.

The guanidine hydrochloride-induced decrease of catalytic activity of hPAP as a function of time followed first-order kinetics with a rate constant of 0.0715 ± 0.0024 min⁻¹. The rate constant for the pseudo-first-order reaction of Congo red binding (0.0409 ± 0.0052 min⁻¹) was in the same range. Congo red can generate polymolecular ligand–protein contacts with periodic motifs in the structure, especially with peptide chains of a destabilized β-sheet (Piekerska et al., 1994; 1996). During urea/acid-induced reversible dissociation and unfolding of hPAP, intermediates of the renaturing protein transiently bind Congo red molecules (Kuciel & Mazurkiewicz, 1997). The ability to bind Congo red that accompanies GdnHCl-induced phosphatase unfolding may be explained by loosening, in the course of the process, of β-sheet fragments present in the α/β domains.

Hydrophobic areas on the surface of the unfolding molecule were estimated using ANS as a probe. The emission of ANS fluorescence in the solution of native hPAP was low. The enhancement of ANS fluorescence during GdnHCl-induced unfolding of hPAP shows a rate constant of 0.0366 ± 0.0018 min⁻¹ in pseudo-first-order kinetics. The increase of ANS fluorescence might be partially attributable to unfolding of domains of each polypeptide, but mainly to the exposure, upon dissociation, of the monomer–monomer interface, which is rich in hydrophobic amino acids (Jakob et al., 2000).

The collected data show that the rate constants of GdnHCl-induced hPAP inactivation and the rate constants of the Congo red and ANS binding reactions are of the same order of magnitude, and are much lower than the rate constants of unfolding measured by the reactivity of free thiols. From these data the possible mechanism of hPAP unfolding and dissociation that leads to generation of molten globule species at 1.25 M GdnHCl (pH 7.5) can be proposed:

\[ N_2 \overset{fast}{\rightarrow} N_2^* \overset{slow}{\rightarrow} 2I \]

where \( N_2 \) is the native, fully active, dimeric protein; \( N_2^* \) is the partially active, probably dimeric molecule (a species with local alterations of structure around thiol groups that have become accessible but are still hidden in the hydrophobic environment); and \( I \) is an inactive monomer that resembles a molten globule state. Intermediate \( I \) exposes its hydrophobic areas to ANS solution. The destabilized β-sheet of the \( I \) intermediate is able to bind Congo red.

The conformation of regions with free thiol groups distant from the subunit interface is desta-
bibilized in a fast process. Much slower processes — the exposure of hydrophobic areas on the surface, and loosening of the β-sheet of α/β domains are followed by inactivation of the protein. Therefore we can assume that the interactions of the monomer–monomer interface are responsible for maintaining stable α/β domains and proper active site conformations. The kinetics of the described transitions support the conclusion from experiments on urea-induced-reversible denaturation (Kuciel et al., 1990; Ostrowski et al., 1993) and on equilibrium unfolding of hPAP in GdnHCl solutions (Wójciak et al., 2003), that monomeric forms of this protein cannot be active. Monomers of structurally similar rat prostate phosphatase (Schneider et al., 1993), created by site-directed mutagenesis, were also found to be completely inactive (Porvari et al., 1994). Details of the 3D structure were not determined, however, it was suggested that the overall folding of the dimerization mutants was abnormal. Even in the presence of inhibitor or substrate the engineered monomer was not induced to the form of an active enzyme.

The active center pocket of prostatic acid phosphatase is located far from the subunit interface. However, the positioning of Arg79, necessary for catalysis, probably requires movement of the Val77–Phe92 helix (Jakob et al., 2000). This might be accomplished by induced fitting during the association of partially folded subunits, a mechanism previously proposed for oligomerization of many enzymes composed of subunits, for example for dimeric triosephosphate isomerase (Schliebs et al., 1997), creatine kinase (Couthon et al., 1995; Ou et al., 2002), or for the formation of the active tetramer of pig kidney fructose 1,6-bisphosphatase (Reyes et al., 2003). Interaction between subunits seems to be a rather general mechanism by which oligomeric proteins gain their catalytic properties.

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REFERENCES

Bardsley WG (2005) SIMFIT a package for simulation, curve fitting, graph plotting and statistical analysis. University of Manchester, UK. http://www.simfit.man.ac.uk

Bobrzańska K, Ostrowski W, Rybarska J (1968) The effect of iodination on the activity and structure of acid phosphomonoesterase from human prostate. Acta Biochim Polon 15: 369–379.

Couthon F, Clottes E, Ebel Ch, Vial Ch (1995) Reversible dissociation and unfolding of dimeric creatine kinase isoenzyme MM in guanidine hydrochloride and urea. Eur J Biochem 234: 160–170.

Jakob CG, Lewiński K, Kuciel R, Ostrowski W, Lebioda L (2000) Crystal structure of human prostatic acid phosphatase. Prostate 42: 211–218.

Kuciel R, Mazurkiewicz A (1997) Molten globule as an intermediate on the human prostatic phosphatase folding pathway. Acta Biochim Polon 44: 645–658.

Kuciel R, Bakalova A, Mazurkiewicz A, Bilska A, Ostrowski W (1990) Is the subunit of prostatic phosphatase active? Reversible denaturation of prostatic acid phosphatase. Biochem Int 22: 329–334.

Kuciel R, Mazurkiewicz A, Ostrowski WS (1996) The folding intermediate of reversibly denatured human prostatic acid phosphatase. Int J Biol Macromol 18: 167–175.

Kuwajima K (1989) The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Proteins 6: 87–103.

LaCount MW, Handy G, Lebioda L (1998) Structural origins of 8(+)-tartrate inhibition of human prostatic acid phosphatase. J Biol Chem 273: 30406–30409.

Lindquist Y, Schneider G, Vilhko P (1994) Crystal structures of rat acid phosphatase complexed with the transition-state analog vanadate and molybdate. Implications for the reaction mechanism. Eur J Biochem 221: 139–142.

Luchter-Wasył E, Ostrowski WS (1974) Subunit structure of human prostatic acid phosphatase. Biochim Biophys Acta 365: 349–359.

Ortlund E, LaCount MW, Lebioda L (2003) Crystal structures of human prostatic acid phosphatase in complex with a phosphate ion and alpha-benzylaminobenzylphosphonic acid update the mechanistic picture and offer new insights into inhibitor design. Biochemistry 42: 383–389.

Ostanin K, Saeed A, Van Etten RL (1994) Heterologous expression of human prostatic acid phosphatase and site-directed mutagenesis of the enzyme active site. J Biol Chem 269: 8971–8978.

Ostrowski WS, Kuciel R, Tanaka F, Yagi K (1993) Fluorometric analysis of native, urea-denatured and refolded human prostatic acid phosphatase. Biochim Biophys Acta 1164: 319–326.

Ou W-B, Park Y-D, Zhou H-M (2002) Effect of osmolytes as folding aids on creatine kinase refolding pathway. Int J Biochem Cell Biol 34: 136–147.

Piekarśka B, Roterman I, Rybarska J, Konieczny L, Kaszuba J (1994) The melting of native domain structure in effector activation of IgG studied by using Congo red as a specific probe. J Physiol Pharmacol 45: 147–162.

Piekarśka B, Skowroniec M, Rybarska J, Stopa B, Roterman I, Konieczny L (1996), Congo red-stabilized intermediates in the lambda light chain transition from native to molten state. Biochimie 78: 183–189.

Porvari KS, Herrala AM, Kurkela RM, Taavitsainen PA, Lindquist Y, Schneider G, Vilhko PT (1994) Site-directed mutagenesis of prostatic acid phosphatase. Catalytically important aspartic acid 258, substrate specificity, and oligomerization. J Biol Chem 269: 139–142.

Porvari KS, Herrala AM, Kurkela RM, Taavitsainen PA, Lindquist Y, Schneider G, Vilhko PT (1994) Site-directed mutagenesis of prostatic acid phosphatase. Catalytically important aspartic acid 258, substrate specificity, and oligomerization. J Biol Chem 269: 139–142.

Ptitsyn OB (1994) Kinetic and equilibrium intermediates in protein folding. Protein Eng 7: 593–596.

Reyes AJ, Ludwig HC, Yanez AJ, Rodriguez PH, Slebe JC (2003) Nativelike intermediate on the unfolding pathway of pig kidney fructose-1,6-bisphosphatase. Biochemistry 42: 6956–6960.

Riddles PW, Blakeley RL, Zerner B (1979) Ellman’s reagent; 5,5’-dithiobis(2-nitrobenzoic acid) — a reexamination. Anal Biochem 94: 75–81.

Schliebs W, Thanki N, Jaenicke R, Wierenga RK (1997) A double mutation at the tip of the dimer interface loop

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of triosephosphate isomerase generates active monomers with reduced stability. *Biochemistry* 36: 9655–9662.

Schneider G, Lindquist Y, Vihko P (1993) Three-dimensional structure of rat acid phosphatase. *EMBO J* 12: 2609–2615.

Van Etten R, Saini MS (1978) Selective purification of tartrate-inhibitable acid phosphatases: rapid and efficient purification (to homogeneity) of human and canine prostatic acid phosphatases. *Clin Chem* 24: 1525–1530.

Van Etten RL, Davidson R, Stevis PE, MacArthur H, Moore LJ (1991) Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J Biol Chem* 266: 2313–2319.

Wójciak P, Mazurkiewicz A, Bakalova A, Kuciel R (2003) Equilibrium unfolding of dimeric human prostatic acid phosphatase involves an inactive monomeric intermediate. *Int J Biol Macromol* 32: 43–54.