Structural Origins of L(+)-Tartrate Inhibition of Human Prostatic Acid Phosphatase*

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Acid phosphatase activity in the blood serum is usually separated into tartrate-resistant and tartrate-refractory, which is reported as the prostatic acid phosphatase level. Human prostatic acid phosphatase crystals soaked in N-propyl-L-tartramate were used to collect x-ray diffraction data to 2.9 Å resolution under cryogenic conditions. Positive difference electron density, corresponding to the inhibitor, was found. The quality of the electron density maps clearly shows the orientation of the carboxylate and N-propyl-substituted amide groups. The hydroxyl group attached to C3 forms the C3 hydroxyl group on the d(-)-stereoisomer of tartrate, which does not significantly inhibit prostatic acid phosphatase, does not form strong hydrogen bonds with Arg-79 or His-257. The structure of human prostatic acid phosphatase, noncovalently bound in N-propyl-L-tartramate, is used to develop inhibitors with higher specificity and potency than L(+)-tartrate.

Human prostatic acid phosphatase (PAP)† has been of significant medical interest ever since tests screening for serum PAP levels were successfully used to diagnose and stage prostate cancer (1). Recently, the primary diagnostic protocol for detecting prostate cancer has shifted from evaluating serum PAP levels to utilizing the prostate specific antigen test. However, accurately detecting serum PAP levels is still of considerable interest because of its effectiveness in staging metastatic prostate cancer and evaluating the progress of chemotherapy in prostate cancer patients (2).

PAP, which is produced by the prostate gland, is found in the seminal fluid at concentrations near 1 mg/ml (3). The enzyme is categorized as an acid phosphatase, because its optimum pH range is between 4 and 7. PAP belongs to the family of high molecular weight phosphatases. Mature PAP is active as a glycosylated homodimer with $M_r = -100,000$. The enzyme is capable of hydrolyzing a wide spectrum of substrates including alkyl, aryl, and acyl orthophosphate monoesters and phosphorylated proteins (4). The natural substrate for PAP is uncertain, thus, the discovery of the specific biological function is of great interest and awaits further investigations.

The catalytic mechanism has been intensely studied and it was concluded that the enzyme should be classified as a histidine phosphatase (5). The crucial intermediate is phosphoryamide, namely phosphohistidine. The rate-limiting step is the breakdown of this covalent phosphoenzyme intermediate through addition of a nucleophilic water molecule to phosphoamidate with concomitant elimination of inorganic phosphate, via a $S_2$ mechanism, to form a noncovalent binary enzyme-inorganic phosphate complex. Extensive studies of chemically modified enzyme and a series of site-directed mutants allowed Van Etten and co-workers (5) to propose a sound description of the catalytic process.

L(+)-Tartrate is a fairly good inhibitor of PAP ($K_I = 2.9 \times 10^{-3}$ M at pH 5.0). It is specific for acid phosphatases; in addition to PAP, it also inhibits homologous lysosomal acid phosphatases and acid phosphatases isolated from such tissues as liver, bone, and kidney, which are usually not present in the blood serum (6). The inhibition is a stereospecific property because d(-)-tartrate and mesotartrate are only very poor inhibitors. On the other hand, other α-hydroxy carboxylic acids have been tested, and it was concluded that the inhibitor must possess an α-hydroxyl group in the d-configuration. The β-carboxyl must be part of a carboxyl group or be attached to a carboxyl or hydroxyl group (7). The specificity of L(+)-tartrate inhibition was further investigated by Lindqvist et al. (8) who determined the crystal structure of rat PAP with noncovalently bound L(+)-tartrate. Unfortunately, these studies were based on data at 3 Å resolution and yielded only poor density for the ligand. In addition, a wrong stereoisomer of the tartrate model was used.

Nontoxic and specific inhibitors of PAP should be a valuable tool in the search for its natural substrate(s). Structure-based development of inhibitors can only proceed by first elucidating the accurate molecular structure of human-PAP and its complexes with lead inhibitors. We report here the three-dimensional structure of human PAP complexed with N-propyl-L-tartramate (NPT), which is a mono-n-propylamide derivative of L-tartrate, at 2.9 Å resolution.

MATERIALS AND METHODS

Crystallization and Data Collection—Human prostatic acid phosphatase was purified from semen using tartrate affinity chromatography as described by Van Etten and Saini (9). NPT was synthesized from L(+)-tartrate according to procedures previously described by Van Etten and Saini (9). Crystals of native human PAP were grown from solutions containing 30% PEG 1500, 7% PEG 1000, 6% PEG 400, 100 mM KCl, and 100 mM glycine at pH 10.0. Typical native crystals grew to 0.2 × 0.15 × 0.15 mm in size. The complex with NPT was prepared by soaking a native human PAP crystal in artificial mother liquor contain-
with phases based on the refined structure of native human PAP solved at 3.1 Å resolution. Visual examination of the maps and model building were done with the interactive graphics program CHAIN (8). NPT was manually fitted into the appropriate Fo − Fc electron density initially contoured at a 2.3 σ level. The agreement between the NPT model and the initial difference density was excellent (Fig. 1). The complex was refined using simulated annealing, positional (conventional least squares) and temperature factor refinement with noncrystallographic symmetry restraints imposed on the four PAP subunits present in the crystal. The crystals were orthorhombic, space group P212121, with unit cell dimensions a = 119.9 Å, b = 202.7 Å, and c = 71.1 Å and contained two homodimers per asymmetric subunit.

Data for the binary complex were collected by flash freezing the crystal at 123 K in a stream of nitrogen generated by a Molecular Structure Corp. low temperature X-Stream attachment. A Rigaku rotating anode source at 50 kV and 100 mA with mirror optics and an R-Axis IV area detector at a distance of 150 mm were used to collect data as 1° oscillation frames over 180° range yielding a redundancy factor of 5 and an Rmerge = 13.8%. A total of 224,797 reflections with I > 2σ(I) was measured and reduced to 39,548 unique reflections using the HKL suite of programs (10). The data set is 98.5% complete to 2.9 Å resolution and contains 60% reflections with I > 2σ(I) in the 2.98–2.8 Å resolution shell.

Molecular Modeling—The coordinates for NPT were generated in SYBYL using the interactive model building protocol Sketch Molecule (11). The resultant structure was then subjected to 100 cycles of energy minimization using the Powell method and a gradient termination. Torsion angles of NPT for atoms O1-C1-C2-C3 (D5 = 134°, D4 = 125°, D3 = 122°), C1-C2-C3-C4 (D5 = 122°, D4 = 135°, D3 = 118°), and C2-C3-C4-O4 (D5 = 178°, D4 = 178°, D3 = 178°) were measured and reduced to 39,548 unique reflections using the HKL suite of programs (10). The data set is 98.5% complete to 2.9 Å resolution and contains 60% reflections with I > 2σ(I) in the 2.98–2.8 Å resolution shell.

Map Interpretation and Refinement—Initial difference electron density maps for the binary complex were calculated with X-PLOR (online) with phases based on the refined structure of native human PAP solved at 3.1 Å resolution. Visual examination of the maps and model building were done with the interactive graphics program CHAIN (11). The resultant structure was then subjected to 100 cycles of energy minimization using the Powell method and a gradient termination. Torsion angles of NPT for atoms O1-C1-C2-C3 (D5 = 134°, D4 = 125°, D3 = 122°), C1-C2-C3-C4 (D5 = 122°, D4 = 135°, D3 = 118°), and C2-C3-C4-O4 (D5 = 178°, D4 = 178°, D3 = 178°) were measured and reduced to 39,548 unique reflections using the HKL suite of programs (10). The data set is 98.5% complete to 2.9 Å resolution and contains 60% reflections with I > 2σ(I) in the 2.98–2.8 Å resolution shell.

RESULTS AND DISCUSSION

Inhibitor Binding in PAP—Initial difference Fourier maps of the binary complex, which was phased with the nonliganded native structure, revealed interpretable predictive electron density within the active site for three of the four monomers present in the asymmetric subunit. NPT was manually positioned within the observed density as a rigid body for subunits A, B, and D. The asymmetric nature of the electron density, because of the mono-n-propyl moiety, facilitated the correct positioning of the tartrate pharmacophore.

The t-tartrate PAP binary complex reported by Lindqvist et al. (8) suffered from incorrect modeling of tartrate, due in part to the symmetrical nature of tartrate and ambiguous electron density. Also, their tartrate model (Protein Data Base accession code: 1rpa) corresponds to D-tartrate (2S, 3S) and does not accurately portray the structure of the used inhibitor (Fig. 2). D-Tartrate does not inhibit PAP although its enantiomer, L-tartrate (2R, 3R), is a moderate (Ki = 10–5 M) and specific PAP inhibitor (9). Torsion angle measurements for the final tartrate model reported by Lindqvist et al. (8) were 147° (D = 82°), −83° (D = 91°), and 18° (D = 224°) for τ1, τ2, and τ3, respectively, with D indicating angular deviation from average small molecule values calculated for L-tartrate. Our final tartrate model in subunit A has torsion angles of −125° (D = 65°), 178° (D = 85°), and −134° (D = 16°) corresponding to τ1, τ2, and τ3, respectively. Average torsion angle values and sample standard deviations (s) for all three NPT molecules included in the final model are τ1 = −123° (s = 3°), τ2 = 178° (s = 0°), and τ3 = −135° (s = 3°). Although the torsion angles of NPT deviate minimally outside the σ range calculated for small molecule data on L-tartrate, both NPT and L-tartrate have a similar conformational profile. In both cases, τ1 and τ2 are mobile whereas τ3 remains conformationally rigid. This suggests that the hydroxyl groups attached to C2 and C3 in NPT and L-tartrate remain relatively rigid, whereas the carboxylate groups adjust to optimize intermolecular interactions.

The major difference between modeled D-tartrate and observed L-tartrate within the active site stems from the position of the C3 hydroxyl group. In L-tartrate, the C3-OH group points away from His-257 (distance = 4.57 Å) and lies underneath and parallel to the plane of the guanidino group of Arg-79 (Fig. 2). Thus, the C3-OH group of D-tartrate is not in a geometrically favorable position to form strong hydrogen bonds with either Arg-79 or His-257. In contrast, the C3-OH group of L-tartrate have a similar conformational profile. In both cases, τ1 and τ2 are mobile whereas τ3 remains conformationally rigid. This suggests that the hydroxyl groups attached to C2 and C3 in NPT and L-tartrate remain relatively rigid, whereas the carboxylate groups adjust to optimize intermolecular interactions.

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bond with the amide oxygen (O41) of NPT, however, the distance between Tyr-123’s hydroxyl group and the O41 atom of NPT is 4.16 Å, thus denying a strong hydrogen bond. A bridging water molecule could afford an indirect hydrogen bonding interaction between Tyr-123 and NPT, however, we did not see electron density to support this hypothesis.

The N-propyl moiety from the amide portion of NPT is in a trans geometry typical of trans-peptide bonds observed in proteins. This geometry extends the propyl group of NPT into a hydrophobic region formed by Trp-174, Phe-171, Ser-175, and Ile-18. The additional noncovalent interactions most likely account for the small decrease in $K_i$ for NPT ($K_i = 2.5 \times 10^{-5}$ M) versus that of L-tartrate ($K_i = 2.9 \times 10^{-5}$ M) (9). The NPT-PAP binary complex provides a good lead inhibitor enzyme model to initiate structure-based inhibitor design.

**Overall Structure and Tartrate Inhibition**—The overall topology of human PAP is consistent with rat PAP with the active site located at the C-terminal end of the $\beta$-sheet in the $\alpha/\beta$ domain (17). Furthermore, human PAP does not experience a significant structural change upon binding the inhibitor. Superposition of the $\alpha$-carbons from a single subunit of native human PAP, vanadate-rat-PAP and tartrate-rat-PAP complexes onto the $\alpha$-carbons of human-PAP-NPT complex yielded root mean square deviations of 0.384 Å, 0.786 Å, and 0.714 Å, respectively. Most of the structural differences in the active site occurred among arginine and histidine residues and involved positional shifts of the side groups to accommodate optimal hydrogen bonding to the inhibitor.

The $\alpha/\beta$ domain of human PAP has a similar fold compared with the $\alpha/\beta$ domains found in Saccharomyces cerevisiae (yeast) phosphoglycerate mutase (SPM) (Protein Data Bank access code: 4pgm) and the recombinant C-terminal (bisphosphatase activity) domain of rat liver fructose-2,6-bisphosphatase (RFB) (Protein Data Bank access code: 1fbt) (18, 19). Superposition of $\alpha/\beta$ domains of SPM and RFB onto the $\alpha/\beta$ domain of human PAP, utilizing 123 and 121 equivalence points (CA atoms), respectively, yielded corresponding root mean square deviations of 2.85 Å and 3.14 Å.

The sequence motif of RHG$\times$R$\times$P, which is conserved in rat and human prostatic acid phosphatases and in human lysosomal, rat liver lysosomal, yeast, and Escherichia coli acid phosphatases (20), has similar conformation among PAP (RHG-DRSP), SPM (RHGESEL), and RFB (RHGQSEW) (Fig. 3). However, the sequence corresponding to the $\times$XRXP portion in acid phosphatases differs from those present in glycolytic phosphatases. Arg-15 of PAP, corresponding to the second arginine in RHG$\times$R$\times$P, is in a position to donate a single hydrogen bond to the carboxylate group of L-tartrate in PAP. However, SPM and RFB do not have the second arginine found in the motif and instead have a serine (Ser-11). Modeling shows that this serine is too far from L-tartrate and in either case, Ser-11 of SPM and RFB most likely does not hydrogen bond to L-tartrate.

Moreover, the conserved proline residue in the sequence motif RHGXRXP has a significant impact on the conformational differences observed between PAP, SPM, and RFB. The absence of proline (Pro-17 (PAP), Leu-13 (RFB), and Trp-13 (SPM)) in phosphoglycerate and fructose-2,6-bisphosphatase leads to the formation of a short $\alpha$-helix-coil loop structure. The loop narrows the active site and functions to impart specificity for small molecule phosphate esters such as phosphoglycerate and fructose-2,6-bisphosphate (18). Conversely, Pro-17 in PAP does not allow for the formation an $\alpha$-helix-coil
loop structure to close in on the active site. Instead, the large random coil loop structure, which is structurally conserved in rat PAP, extends downward into the α-domain causing the active site to be open and accessible to large substrates such as phosphorylated proteins.

In addition to the sequence motif RHGXRXP, residues Arg-79, His-257, and Asp-258 are involved in binding L-tartrate. Residues corresponding to Arg-79 and His-257 in PAP are conserved in RFB (Arg-57 and His-142) and SPM (Arg-59 and His-181). However, Asp-258, which is conserved in the known acid phosphatases, is not conserved in the SPM or RFB. Asp-258 in PAP participates in two hydrogen bonds to L-tartrate. One of the interactions originates from the main-chain and involves the amide nitrogen of Asp-258 donating a hydrogen bond to the C1 carboxylate group of L-tartrate (Asp-258-N...NPT-O2 distance 3.07 Å). The second involves the side-chain carboxylic acid oxygen of Asp-258 and the C2 hydroxyl group of L-tartrate (Asp-258-OD2...NPT-O2 distance 2.83 Å). The residues, which would correspond to Asp-258 in SPM and RFB, are Gly-182 and Gln-143, respectively. The main chain hydrogen bond to L-tartrate would be conserved because of the similar folds of SPM and RFB, however, the side chain of Gly-182 in SPM is not capable of forming a hydrogen bond and the side chain of Gln-143 in RFB, which is one carbon unit longer than Asp-258, would most likely be out of position. Thus, a strong binding of L-tartrate to SPM and RFB is unlikely, even though the phosphate binding site of these enzymes is structurally similar to PAP. Therefore, tartrate resistance is not only a function of differing topology and active site structure, but also is dependent upon the residues within structurally similar active sites.

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