Structural Basis for the Exceptional in vivo Efficacy of Bisphosphonate Drugs


Introduction

Bisphosphonates are potent inhibitors of osteoclastic activity that are now widely used for preventing the bone loss associated with osteoporosis,[1] Paget’s disease,[2] hypercalcemia[3] and metastatic bone disease.[4] At the biochemical level, there is now convincing evidence that the nitrogen-containing bisphosphonates (N-BPs, Figure 1a) inhibit the mevalonate pathway in osteoclasts and macrophages.[5,6] A key enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase (FPPS), is thought to be the main molecular target of N-BPs,[7–9] but the precise interaction between N-BPs and the human enzyme has remained elusive.

Human FPPS, a homodimeric enzyme of 41-kDa subunits, catalyzes the two-step synthesis of the C15 metabolite farnesyl pyrophosphate (FPP) from the C5 isoprenoids dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP, Figure 1b). FPP is required for the posttranslational prenylation of essential GTPase signalling proteins such as Ras and Rho[10] and is also a precursor for the synthesis of cholesterol, dolichol, and ubiquinone. Early biochemical studies have indicated that the FPPS reaction is Mg$^{2+}$- or Mn$^{2+}$-dependent and follows an ordered ternary-complex mechanism in which the allylic substrate binds first to the enzyme, then proceeds through the formation of an allylic carboxylation, leading to the formation of the next higher homologue of the allylic substrate with concomitant release of pyrophosphate (PPi).[11] Crystal structures of apo- and substrate-bound avian FPPS (42 kDa, 69% sequence identity to the human enzyme) have long served as a basis for the understanding of FPPS structure and mechanism.[12,13]

However, recent X-ray analyses of E. coli FPPS (32 kDa, 25% sequence identity) and S. aureus FPPS (32 kDa, 27% sequence identity) showed marked differences in enzyme conformation, the number of metal sites, the binding mode of the allylic substrate, and the role of the conserved DDXXD motifs.[14] The extent to which conclusions from the avian or E. coli FPPS structures can be transferred to human FPPS remains unclear.[15] Herein, we provide the first experimental structures of human FPPS and present a differential scanning calorimetry (DSC) analysis of the binding of a set of representative N-BPs. Our data form the basis for structure-guided design of optimized N-BPs with improved pharmacological properties.

To understand the structural basis for bisphosphonate therapy of bone diseases, we solved the crystal structures of human farnesyl pyrophosphate synthase (FPPS) in its unliganded state, in complex with the nitrogen-containing bisphosphonate (N-BP) drugs zoledronate, pamidronate, alendronate, and ibandronate, and in the ternary complex with zoledronate and the substrate isopentenyl pyrophosphate (IPP). By revealing three structural snapshots of the enzyme catalytic cycle, each associated with a distinct conformational state, and details about the interactions with N-BPs, these structures provide a novel understanding of the mechanism of FPPS catalysis and inhibition. In particular, the accumulating substrate, IPP, was found to bind to and stabilize the FPPS–N-BP complexes rather than to compete with and displace the N-BP inhibitor. Stabilization of the FPPS–N-BP complex through IPP binding is supported by differential scanning calorimetry analyses of a set of representative N-BPs. Among other factors such as high binding affinity for bone mineral, this particular mode of FPPS inhibition contributes to the exceptional in vivo efficacy of N-BP drugs. Moreover, our data form the basis for structure-guided design of optimized N-BPs with improved pharmacological properties.
Results and Discussion

Overall structure: open, partially closed, and fully closed conformations

In comparison with the smaller microbial enzymes, avian and human FPPS possess three short helices (α-1, α-2 and α-3) inserted between helices H and I. Unliganded human FPPS adopts an open conformation very similar to that originally observed with the avian enzyme. In marked contrast, the complexes with the N-BP inhibitors and the ternary complex with zoledronate and IPP display closed conformations, which resemble that observed with the E. coli FPPS complexes, although substantial differences in the orientation of the I and J helices are apparent. This conformational switch in the human enzyme can be approximated by a rigid body motion of the last 130 C-terminal residues (Figure 2a) and involves a two-step mechanism. First, upon occupation of the allylic binding site, the two DDXXD motifs come closer together, and two loops (H–I and D–E) that line the substrate binding site form a “gate”, which closes the entrance to the allylic binding site (“partially closed” conformation). This structural transition also requires a dramatic change in the orientation of the Tyr349 loop. The aliphatic amino group of pamidronate did not exhibit it a well-defined electron density, suggesting that it does not make strong interactions with the enzyme active site, or adopts more than one orientation in the complex. In contrast, the imidazole substituent of zoledronate is well-ordered and makes strong interactions with the enzyme active site, or participates in hydrogen bonding with Thr201 O.

Binary complexes with bisphosphonates

Alendronate, ibandronate, pamidronate, and zoledronate bind to the allylic substrate site and interact with both DDXXD motifs through a trinuclear metal centre (Figure 3). The binding of the bisphosphonate moiety is the same for all inhibitors. Three different divalent cations—Mg²⁺, Mn²⁺, and Zn²⁺—were investigated in the work reported herein. With all three cations, the geometry and coordination of the metal cluster is conserved and is reminiscent of the trinuclear co-catalytic centres observed in some phosphatases,[17] nucleases,[18,19] and phospholipases.[20] Each metal site shows octahedral coordination involving the bisphosphonate oxygen atoms, the side-chain carboxylate groups of Asp107 and Asp174 (first DDXXD motif) and water molecules. The conserved residues Lys200, Arg112, and Lys257 make direct salt-bridge interactions with the bisphosphonate oxygen moieties. Significantly, Lys257 is part of the mobile H–I loop. Bisphosphonate binding therefore stabilizes the closed conformation of FPPS by interacting with both DDXXD motifs and Lys257 of the H–I loop. The aliphatic amino group of pamidronate did not exhibit it a well-defined electron density, suggesting that it does not make strong interactions with the enzyme active site, or adopts more than one orientation in the complex. In contrast, the imidazole substituent of zoledronate is well-ordered and makes strong interactions with FPPS: the protonated heterocyclic nitrogen atom makes a bifurcated H-bond interaction with O₁ of Thr201 and Thr201 O₁, two conserved residues located at the kink of helix G which line the carbocation binding pocket (Figure 3). These interactions most likely contribute to the well-known preference for a nitrogen atom at the fourth position from the geminal carbon atom of the bisphosphonate moiety. The H-bonding interaction with O₁ of Thr201 is also observed in the alendronate complex, but not with Lys200 O. Pamidronate has its amino nitrogen atom in position 3 and hence cannot make any of these hydrogen bonds. Ibandronate also has its tertiary amino group in position 3 and does not form these hydrogen bonds; its methyl substituent makes van der Waals contacts with Thr201 O₁ and Lys200 O, where-
as its n-pentyl group is directed toward the FPPS dimer interface, filling a binding site thought to be normally occupied by the growing isoprenoid chain. The binding of the long ibandronate side chain is accompanied by conformational changes in the side chains of Phe99, Thr167, and Gln171. Notably, Phe99 (Phe113 in avian FPPS) has been shown to control product chain length.[13]

The higher potency and superior ligand efficiency (binding energy per ligand atom) of zoledronate[9] can probably be ascribed to the bifurcated H-bonding interaction described above and to the increased rigidity (lower entropic penalty) and bulkiness (additional van der Waals contacts) of the imidazole ring, in comparison with the flexible alkyl chains of other N-BPs. Furthermore, it has been proposed that N-BPs act as transition-state analogues, which mimic the putative carbocation intermediate formed during catalysis.[21] Structural overlays with the avian and *E. coli* FPPS complexes demonstrate that the substituted imidazole nitrogen atom of zoledronate indeed binds in the same region as the putative sp²-hybridized carbocation. Hence, the increased potency of zoledronate may also derive, in part, from the fact that its imidazole ring is a better transition-state mimetic than the primary or tertiary amino groups of alendronate, pamidronate, and ibandronate.[21]

The 1-OH group of N-BPs, together with the two phosphonate moieties, is responsible for the high affinity of these compounds to bone mineral,[22] thereby targeting these drugs to bone and increasing their efficacy for treating bone diseases. The X-ray crystal structures of the FPPS complexes presented herein suggest that the 1-OH group also contributes to FPPS recognition and binding through a water-mediated hydrogen bond to Gln240 Oₑ₁ and a direct polar contact with Asp243 Oᵈ₂.

### Ternary complex with zoledronate and IPP

The ternary complex with zoledronate and IPP locates the IPP binding site to a basic region adjacent to the allylic substrate site. IPP binding does not involve any of the DDXXD motifs and is therefore not metal dependent. Three conserved basic residues, Lys57, Arg60, and Arg113, interact directly with the pyrophosphate group of IPP, and three others, Arg112, Lys257, and Arg351, have their positively charged group within 5.0 Å of the pyrophosphate moiety. The hydrocarbon chain of IPP binds between the conserved Phe239 and the imidazole ring of zoledronate (Figure 4). Several of these residues—Phe239, Gln240, Lys257, and Arg351—are part of the secondary structural elements that are affected by the conformational switch of FPPS. Hence, the IPP binding site is fully formed only after FPPS is triggered into the partially closed conformation by binding of allylic substrate or N-BP.

### Calorimetric data for FPPS–N-BP complexes

Differential scanning calorimetry (DSC) was used to measure the thermal stability of FPPS–N-BP complexes. Parameters such as the melting temperature ($T_m$) of protein–ligand complexes can be used to compare the binding affinities of different ligands.[21] In our study, zoledronate ($T_m$ 80.6 °C, $\Delta T_m = +25.2$ °C)
was found to induce the largest increase in $T_m$, followed by
risedronate ($T_m = 76.1 { }^\circ C$, $\Delta T_m = 20.7 { }^\circ C$),
ibandronate ($T_m = 72.6 { }^\circ C$, $\Delta T_m = 17.2 { }^\circ C$), alendronate ($T_m = 68.9 { }^\circ C$, $\Delta T_m = 13.5 { }^\circ C$),
and pamidronate ($T_m = 64.5 { }^\circ C$, $\Delta T_m = 9.1 { }^\circ C$) as shown in Figure 5a. The ranking of N-BPs by
these calorimetric data is in full agreement with enzyme inhibi-
tion data using recombinant human FPPS.[9]

The addition of IPP leads to significant further stabilization of
FPPS–N-BP complexes (Figure 5b). The $T_m$ value of the
FPPS–zoledronate complex increased by 5.0 $^\circ C$ ($T_m = 85.6 { }^\circ C$)
upon addition of IPP. The same phenomenon was observed with
other N-BPs. Interestingly, stabiliza-
tion of the complex upon
binding of IPP is more pro-
nounced for weaker N-BPs such
as pamidronate ($T_m = 78.8 { }^\circ C$, $\Delta T_m = 14.3 { }^\circ C$), alendronate ($T_m = 83.2 { }^\circ C$, $\Delta T_m = 14.3 { }^\circ C$),
and ibandronate ($T_m = 82.9 { }^\circ C$, $\Delta T_m = 10.3 { }^\circ C$) in comparison with
stronger binders such as risedro-
nate ($T_m = 84.4 { }^\circ C$, $\Delta T_m = 8.3 { }^\circ C$)
and zoledronate ($T_m = 85.6 { }^\circ C$, $\Delta T_m = 5.0 { }^\circ C$). As a consequence, dif-
ferences among the various N-
BPs become relatively small in
the presence of IPP. Among all
the N-BPs studied, zoledronate had the largest stabilizing
effect on FPPS in presence or absence of IPP.

**Structural basis for the high in vivo efficacy of N-BPs**

Blockade of the FPPS enzymatic activity by zoledronate or
other N-BP drugs leads to the intracellular accumulation of the
IPP substrate.[8] Therefore, the ternary complex of human FPPS
with zoledronate and IPP most likely represents the inhibited
form of human FPPS in vivo. As shown above, IPP binds to and
further stabilizes the inhibited ternary complex in the fully
closed conformation, rather than competing with inhibitor, as
in the simple and most frequently observed case of competi-
tive inhibition with single-substrate enzymes (Figure 6). In the
fully closed conformation, competition of the N-BP inhibitor by
DMAPP/GPP is inefficient, as the N-BP is trapped inside the
enzyme, and the allylic substrate binding site is inaccessible
(Figure 7 and cover picture). Hence, in addition to other factors
such as efficient bone targeting, the high in vivo efficacy and
prolonged duration of action of some N-BPs results from the

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**Figure 3.** FPPS complexes with a) zoledronate/Mg$^{2+}$, b) pamidronate/Mn$^{2+}$, c) alendronate/Zn$^{2+}$, and d) ibandronate/Zn$^{2+}$. The metal ions are shown as large spheres. For ease of comparison, all complexes are shown in the same orientation. Black dashed lines show selected hydrogen bonds/electrostatic interactions. Yellow dashed lines show the coordination spheres of the metal ions. The position of the primary amino group of pamidronate was ill-defined in the electron density maps; in this case, one possible orientation is shown which would allow an H-bonding interaction with Tyr204. For ibandronate, the X-ray crystallographic data indicate that two alternative conformations related by nitrogen inversion are present in the bound state; both conformations are shown. Binding of the ibandronate side chain induces a conformational change of Phe99, Thr167, and Gln171.

**Figure 4.** Close-up view of the IPP binding interactions: structure of the ternary complex of human FPPS with IPP (yellow carbon atoms) and zoledronate (orange carbon atoms).
compulsory-order ternary-complex mechanism of FPPS and the noncompetitive mode of inhibition of N-BPs with respect to the second substrate.

Conclusions

Herein, we describe the first experimental structures of human FPPS.[24] Whereas structures of avian and S. aureus FPPS are available only in the open conformation, and of E. coli FPPS only in the fully closed conformation, our structures show human FPPS in an open, partially closed, and fully closed conformation and thus provide three snapshots of the enzyme catalytic cycle. One of our major findings is that the stabilization of the inhibitor complex through accumulation of substrate contributes to the high in vivo efficacy of the N-BP drugs.

N-BPs are highly charged hydrophilic compounds. Their pharmacokinetic profile is characterized by high affinity for mineralized bone and prolonged skeletal retention, low exposure in soft tissue, and low permeability across the cell membrane.[6,10] Although FPPS is present in all mammalian cells, the cells primarily targeted by N-BPs in vivo are endocytic or phagocytic cells in bone such as osteoclasts and macrophages, and to a lesser extent renal tubule epithelial cells, as bisphosphonates are excreted exclusively through the kidney. Overall, N-BPs have proven to be well-tolerated, highly efficacious drugs for the treatment of a variety of benign and malignant bone diseases characterized by elevated osteoclastic bone resorption.[1–4,10,25,26]

Although preclinical experiments have convincingly demonstrated antitumour properties of bisphosphonates (reviewed in reference [27]), the results of clinical studies reported to date have been inconsistent and controversial.[28] Likewise, alternative attempts to target the mevalonate pathway and disrupt protein prenylation with inhibitors of farnesyl transferase and geranylgeranyl transferase have also yielded disappointing results in clinical trials.[29] Therefore, it would be of great therapeutic interest to explore the antitumour potential of novel FPPS inhibitors. The currently available N-BPs are not optimal for this purpose for three reasons: first, they have low oral bioavailability (typically ≈1%); second, their pharmacokinetic properties do not permit efficacious drug concentrations to be reached in plasma and soft tissue; and third, these drugs do
not readily enter the cytosol of non-endocytic cells. Our new structural data for human FPPS could facilitate the design of novel inhibitors to overcome these shortcomings; they suggest new avenues toward the discovery of novel FPPS inhibitors such as bisubstrate analogues (such as N-BPs that extend into the IPP site) or allosteric inhibitors (such as compounds that block FPPS in the open state).

The potential therapeutic applications of such novel FPPS inhibitors could extend beyond the treatment of metastatic bone disease to encompass soft-tissue tumours and other diseases such as hypercholesteremia, in which targeting the mevalonate pathway has been shown to be effective. As FPPS is also an essential enzyme in protozoa, bisphosphonates have been proposed as chemotherapeutic agents for the treatment of trypanosomatid and apicomplexan protozoal infections such as Chagas’ disease and leishmaniasis.\(^{30,31}\) The structural data on bisphosphonate binding to trypanosomal FPPS\(^{32}\) combined with our new data on human FPPS could now facilitate the design of more potent and specific anti/protozoal drugs.

In conclusion, our work opens the possibility for structure-based design of novel FPPS inhibitors that may become a new class of therapeutics directed against a clinically validated target for the treatment of a wide range of diseases.

### Experimental Section

#### Cloning, expression, and purification:
Human FPPS (residues 6–353 with an N-terminal His tag followed by a PreScission protease cleavage site) was overexpressed in E. coli BL21 (DE3) Tuner cells (Novagen). The soluble fraction of His-tagged FPPS was purified from the cell lysate by anion-exchange (Q-Sepharose HP), hydrophobic (Phenyl Sepharose HP), and metal-chelation (HiTrap) chromatography. The N-terminal His tag was then removed with the PreScission protease, and the reaction products were separated by metal chelation chromatography (HiTrap). His-tag-free FPPS was further purified by size-exclusion chromatography (Superdex-200), concentrated by ultrafiltration to 16 mg/mL \(^{-1}\), and stored at \(-80^{\circ}\)C.

#### Crystallization and structure solution:
All crystals were grown by the vapor-diffusion method at 19\(^{\circ}\)C from either A) 1.2 mM Na/K phosphate (pH 4.7–5.3) with 25% (v/v) glycerol or B) 0.1 mM zinc acetate, 0.1 mM Na acetate, 12% PEG 4000 (pH 4.4). Apo crystals were prepared by mixing equal volumes of crystallization solution A and of a protein stock solution consisting of human FPPS (6-353, 16 mg/mL\(^{-1}\)) in 25 mM Tris-HCl (pH 8.0), 25 mM NaCl, and 2 mM DTT. The complexes with pamidronate, zoledronate, and IPP were prepared by co-crystallization at a decreased protein concentration (4.2 mg/mL\(^{-1}\) in the presence of 5 mM MgCl\(_2\) or MnCl\(_2\). Stock solutions of pamidronate (50 mM) and zoledronate (10 mM) were prepared in distilled water and added to the enzyme to a final concentration of 2.5 mM and 0.5 mM, respectively. A threefold molar excess of IPP (Sigma) was used. The zinc complexes with zoledronate, alendronate, and ibandronate were prepared by mixing equal volumes of crystallization solution B and a protein stock solu-

### Table 1. X-ray data collection and refinement statistics.

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<th>Zoledronate/Zn(^{2+})</th>
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tion consisting of human FPPS (6-353, 14 mg mL⁻¹) in 10 mM Tris (pH 7.4), 25 mM NaCl, 5 mM MgCl₂, and 1.0 mM bisphosphonate.

X-ray crystallographic data collection and structure determination: Crystalllographic data were collected at 95 K with a MARCCD detector and synchrotron radiation (Swiss Light Source, beam line XSO6A). The crystals were mounted in cryoloops and flash-frozen. For the zolendronate, alendronate, and ibandronate complexes with Zn²⁺, a cryoprotectant made of 20% glycerol and 80% well solution was used. Diffraction data were recorded as 1.0° oscillation images and processed with HKL[33] or XDS[34]. All crystals were in space group P4₁2,1 with one FPPS monomer per asymmetric unit. The structure of human FPPS (open form) was initially determined by molecular replacement with the program AmoRe[35] with avian FPPS (PDB code: 1FPS) as a search model. The structure was then refined with CnX[36] and O.[37] The structures of the FPPS complexes were determined by using an initial rigid-body refinement of the apo structure followed by full refinement with CnX and O. Data collection and refinement statistics are summarized in Table 1. All refined structures have been deposited into the Protein Data Bank, with entrycodes 2F7M (unliganded FPPS), 2F89 (pamidronate/Mn²⁺ complex), 2F8C (zoledronate/Mg²⁺ complex), 2F9K (zoledronate/Zn²⁺ complex), and 2F94 (ibandronate/Zn²⁺ complex).

Differential scanning calorimetry (DSC): DSC scans were obtained with a MicroCal VP-capDSC system (MicroCal LLC, Northampton, MA, USA) operated at a scan rate of 200 °C·h⁻¹. The final FPPS concentration was typically 30 μM in 25 mM Tris (pH 7.5), 25 mM NaCl and 5 mM MgCl₂. N-BPs and IPP (Echelon Inc., Salt Lake City, UT, USA) were added to the protein solution at a final concentration of 300 μM.

Note added in proof: After this manuscript was submitted, structural data for FPPS from T. cruzi were published (S. B. Gabelli, J. S. T. cruzi 2004 62, 80) which fully corroborate the findings presented herein.

Acknowledgement

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Keywords: bone metastasis · drug design · farnesyl pyrophosphate synthase · inhibitor · osteoporosis


[24] The structural coordinates of human FPPS complexes were recently deposited into the PDB by Kavanagh et al. Although no published manuscript is available, these structures are in agreement with the more comprehensive findings and conclusions described herein.