Structural Basis for Bisphosphonate-Mediated Inhibition of Isoprenoid Biosynthesis.


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Abstract:

Farnesyl Pyrophosphate Synthetase synthesizes farnesyl pyrophosphate through successive condensations of isopentyl pyrophosphate with dimethylallyl pyrophosphate and geranyl pyrophosphate. N-containing bisphosphonate drugs used to treat osteoclast-mediated bone resorption and tumor-induced hypercalcemia are potent inhibitors of the enzyme. Here we present crystal structures of substrate and bisphosphonate complexes of FPPS. The structures reveal how enzyme conformational changes organize conserved active site residues to exploit metal-induced ionization and substrate positioning for catalysis. The structures further demonstrate how N-containing bisphosphonates mimic a carbocation intermediate to inhibit the enzyme. Together, these FPPS complexes provide a structural template for the design of novel inhibitors that may prove useful for the treatment of osteoporosis and other clinical indications including cancer.

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Introduction

Post-translational modification of C-terminal CAAX sequences by covalent attachment of isoprenyl chains is crucial for intracellular localization and proper function of small GTPases such as Ras, Rac, Rho, and CDC42\(^{(1,2)}\). The substrates for these modifications are the 15-carbon isoprenoid farnesyl pyrophosphate (FPP) or the 20-carbon isoprenoid geranyl-geranyl pyrophosphate (GGPP) synthesized by enzymes of the mevalonate pathway\(^{(3)}\) (Fig. 1A). A key branch point enzyme of the mevalonate pathway is Farnesyl Pyrophosphate Synthetase (FPPS), a ~30kda \(\text{Mg}^{2+}\)-dependent homodimeric enzyme that synthesizes (E,E)-FPP from isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP)\(^{(4,5)}\) (Fig. 1B).

Interest in understanding FPPS activity stems from the recent discovery that FPPS is the molecular target of N-containing bisphosphonates\(^{(6,7)}\). Bisphophonates are non-cleavable pyrophosphate (P-O-P) analogs in which the central oxygen is replaced by a carbon (P-C-P) with various side chains (Fig. 1C). Against parasitic organisms \(^{(8,9)}\) these agents have been shown in vitro to disrupt cell growth through FPPS inhibition. In people, bisphophonates are targeted to bone tissue\(^{(10)}\) where FPPS inhibition in bone-resorbing osteoclasts is a current therapeutic approach for treating postmenopausal osteoporosis\(^{(11,12)}\). Because of their bone targeting properties, bisphophonates have also found use as agents to treat tumor-induced hypercalcemia\(^{(13)}\), Paget’s disease\(^{(14)}\), and osteolytic metastases\(^{(15)}\).

Although structures of apo and ligand-bound avian FPPS have been solved\(^{(16,17)}\), the active sites are unassembled and do not provide substantial information concerning catalysis. Thus, to resolve the molecular basis of catalysis, and also to understand the structural features
governing bisphosphonate recognition, we determined the structures of unliganded *S. aureus* FPPS (FPPS-Sa), as well as two *E. coli* FPPS (FPPS-Ec) ternary complexes. These ternary complexes include a 2.4Å “substrate-bound” structure containing IPP and the noncleavable DMAPP analogue dimethylallyl S-thiolodiphosphate (DMSPP) (18), and a 1.95Å bisphosphonate-bound structure containing IPP and the osteoporosis drug 1-hydroxy-2-(3-pyridinyl)ethylidene bis-phosphonic acid (risedronate) (Fig 1C). Since the *E. coli* enzyme shares conserved active site residues with human FPPS(19) these complexes provide a structural framework for the development of novel FPPS inhibitors.

**Materials and Methods**

*Enzyme Expression and Purification* – Open reading frames encoding FPPS genes were amplified using the polymerase chain reaction from *E. coli* (JM109) and *S. aureus* (ATCC35556D) genomic DNA. The resulting PCR products were cloned into an *E. coli* expression vectors to give an open reading frame encoding FPPS-Ec fused to N-terminal six-histidine tag and FPPS-Sa fused to C-terminal six-histidine tag. To generate selenomethionine-labeled protein, the expression plasmid was transformed into the methionine auxotroph DL41(21). Recombinant FPPS’ were purified using ProBond Nickel-Chelating resin (Invitrogen; Carlsbad, CA) followed by diafiltration into 150 mM NaCl, 25 mM Tris pH 7.9, 0.25 mM TCEP.

*Crystallization and Structure Determination* - Crystals of selenomethionine-labeled *S. aureus* FPPS were grown using nanovolume crystallization techniques by mixing 50nL of protein solution with 50nL of reservoir(22). Substrate and inhibitor-bound crystals of *E. coli* FPPS were
Structures of FPPS Substrate and Inhibitor Ternary Complexes

obtained by pre-incubating enzyme (10mg/ml) with 2.5mM IPP, DMSPP and MgCl₂, or 2.5mM IPP, risedronate and MgCl₂, and then mixing 50nL of this solution with 50nL of reservoir. Crystals were harvested in reservoir solutions supplemented with 20% ethylene glycol, and flash frozen by direct immersion in liquid nitrogen. X-ray diffraction data from the S. aureus crystals were collected at the Advanced Light Source (ALS) Beam Line 5.0.2 using a wavelength of 0.978Å. X-ray diffraction data from the E. coli crystals were collected at ALS Beam Line 5.0.3 using a wavelength of 1.00Å. All data were integrated and scaled using HKL2000 (23). Crystals of the S. aureus enzyme belong to the space group P6₁22 and have two molecules in the asymmetric unit. Ligand-bound crystals of the E. coli enzyme belong to the space group P4₁22 and contain two molecules in the asymmetric unit. Data collection and refinement statistics are listed in table I. The structure of S. aureus FPPS was determined using SAD phases calculated with the program SHARP(24). The E. coli structures were determined by molecular replacement using AMoRe (25) with the refined S. aureus structure (~40% sequence identity) as a search model. Both structures were refined without non-crystallographic symmetry restraints in REFMAC and inspected, built, and rebuilt using Xfit(26). The atomic coordinates of the S. aureus (accession code – 1RTR) enzyme as well as the E. coli substrate (accession code – 1RQI), and risedronate (accession code – 1RQJ) complexes have been deposited with the Protein Data Bank.

RESULTS

S. aureus FPPS – The structure of FPPS-Sa confirms the conservation of the 3-layered all α-helical prenyl transferase fold and reveals regions of conformational flexibility important for
enzyme activity (Fig. 2A). The first α-helical layer is formed by helices 1 and 2 and is orthogonal to the two others. The second layer contains helices 3, 4, 6, and 11. The third layer is formed by helices 7, 8, 9, and 10. Helices 4 and 8 are located in the center of the protein core and contain the conserved Asp-rich motifs that mediate substrate binding and catalysis. As observed in avian FPPS (16,17), a complementary hydrophobic interface formed between helices 6 and 7 mediates enzyme dimerization (not shown).

*E. coli* FPPS IPP:DMSPP ternary complex – To determine the structural features governing FPPS substrate binding and catalysis we crystallized a trapped enzyme:substrate ternary complex containing IPP and the noncleavable DMAPP analogue DMSPP. FPPS undergoes significant substrate-induced active-site rearrangements that protect the centrally-located substrate binding cavity from bulk solvent and position active site residues in a catalytically competent conformation (Fig 2A). Compared to the unliganded *S. aureus* and avian structures(16,17), the *E. coli* ternary complexes reveal active-site conformational changes localized at the enzyme C-terminus and the α4-α5 and α9-α10 loops. Significantly, all three regions contain conserved residues that contact substrate and inhibitor diphosphate atoms. FPPS binds to the DMSPP diphosphate through a trinuclear Mg$^{2+}$ cluster that is ligated by three conserved aspartates located on opposite walls of the active-site cavity (Fig. 2B). All three Mg$^{2+}$ atoms are octahedrally coordinated by protein, pyrophosphate, and water oxygens (Fig 2B). The side chain of Asp244 from the second DDXXD motif, two diphosphate oxygen atoms, and three water molecules coordinate Mg1. The side chains of Asp105 and Asp111 from the first DDXXD motif, two diphosphate oxygens, and two water molecules complete the Mg2-binding site. Asp105 and Asp111, as well as a diphosphate oxygen and three water molecules coordinate the third active site Mg (Mg3). Significantly, the DMSPP diphosphate further contacts the side
chains of conserved Lys 202 as well as α4-α5 residue Arg116, and α8-α9 residue Lys258. These latter interactions likely play a key role in pyrophosphate release and substrate reloading (see below).

The hydrocarbon tail of DMSPP extends into a deep pocket that accommodates the growing isoprenyl product(16,17) (Fig 2B). The pocket extends to the dimer interface with residues from helix 4 (Tyr100, Ser101, His104, and Met110), helix 7 (Met 175 and Cys176), and helix 6 (Ile132, Leu133, Asp136) of the adjacent subunit forming the walls of the pocket. Hydrophobic interactions with Gln179 and Lys202 on one side and Ser101, Leu102, and Thr203 on the other, position the DMSPP hydrocarbon tail within van der Waals distance of the C1, C2, C3, and C4 atoms of IPP.

IPP binding is mediated by the enzyme’s C-terminus that organizes residues contacting the IPP pyrophosphate (Fig. 2B). A salt bridge between the C-terminal carboxylate and Lys66 positions its side chain Ne atom to directly interact with two nonbridging diphosphate oxygens. Arg318, which is also positioned by a C-terminal salt bridge, forms a water mediated interaction to a single diphosphate oxygen. Additional interactions to the IPP pyrophosphate include hydrogen bonds with Gly65 and His98, and salt bridges with Arg69 and Arg117. These enzyme:IPP pyrophosphate interactions anchor IPP in the FPPS active site such that its hydrocarbon tail is buttressed between conserved Phe240 and the hydrophobic tail of DMSPP (Fig 2B). Notably, these interactions position the nucleophilic C3-C5 double bond of IPP 3.2Å from the C1 atom of DMSPP (Fig 3A).
FPPS IPP:Risedronate ternary complex - The FPPS IPP:Risedronate complex reveals that bisphosphonates inhibit FPPS by binding to the trinuclear Mg\(^{2+}\) center in a manner identical to that of DMSPP (Fig 2C). The conformational changes, interactions with IPP, as well as the interactions to the inhibitors carbon-linked pyrophosphate, mimic those seen in the substrate complex. The pyridyl side chain of the bisphosphonate inhibitor binds to the same hydrophobic pocket that accommodates the C5-hydrocarbon tail of DMSPP. Similar stacking interactions to those described for DMSPP, as well as a hydrogen bond between the pyridyl nitrogen and conserved Thr203 position the inhibitor to make tight van der Walls contacts with IPP.

Discussion

Structure-Based Three-Metal-Ion Catalytic Mechanism – Biochemical studies have revealed that the reaction catalyzed by FPPS occurs by a three-step ionization-condensation-elimination mechanism(27) in which the double bond of IPP is stereospecifically alkylated by the C1 atom of the allylic substrate(28). The 2.4Å structure of the IPP:DMSPP ternary complex reveals the structural basis for this three-step mechanism.

The geometry of the FPPS trinuclear Mg\(^{2+}\) cluster is exquisitely tuned to initiate substrate ionization, with all three metal ions participating in catalysis. In the substrate complex Mg1 and Mg2 each form 6-membered ring chelate structures with two unesterified pyrophosphate
oxygen while Mg3 ligates a single unesterified oxygen of the linking pyrophosphate (Fig 2B). These interactions, as well as those to the IPP pyrophosphate, juxtapose the hydrophobic C5 isoprenoid tails in a conformation suitable for catalysis. Initial ionization of the allylic disphosphate triggered by the FPPS metal center would thus generate an allylic carbocation with its positive charge distributed over the C1, C2, and C3 atoms. As pyrophosphate is generated, the developing negative charge would be stabilized by interactions with the trinuclear metal center. Likewise, the positively charged allylic cation is stabilized by electrostatic interactions with the liberated pyrophosphate and also through conserved FPPS interactions. Notably, the main chain carbonyl oxygen of Lys202, and the side chain oxygens of Thr203 and Gln241, form a carbocation binding site by directing their negative dipoles toward the positive charge localized over the DMAPP C1, C2, and C3 atoms (Fig 3A,3B). Our trapped substrate complex suggests that this cation would be quickly condensed with the nucleophilic C3-C5 double bond of IPP which is only 3.2Å from the DMSPP C1 position (Fig 3A). Our structure further reveals that a non-metal ligated pyrophosphate oxygen is the catalytic base that deprotonates the condensed intermediate to generate the C5-extended isoprenoid reaction product. (Fig. 3A, 3B). In the ternary complex this catalytic oxygen is ligated by conserved Arg116 and Lys258 (Fig 3A) and, consistent with biochemical data(28,29), is correctly positioned to abstract the IPP C2 pro-R hydrogen. Notably, both Arg116 and Lys258 emanate from the conformationally variable α4-α5 and α9-α10 active-site loops that shield the reaction from bulk solvent. Pyrophosphate-mediated proton abstraction would weaken these interactions and could thus disrupt the structure of these loops to facilitate pyrophosphate release and IPP reloading.
Bisphosphonate-mediated FPPS inhibition – Nitrogen-containing bisphosphonates are proposed to inhibit FPPS by mimicking the carbocation intermediate formed after substrate ionization(30). The structure of the FPPS IPP:risedronate ternary complex presented here confirms this suggestion and reveals how key enzyme residues, as well as IPP participate in inhibitor binding. Risedronate binds to the FPPS allylic substrate binding pocket by using its carbon-linked pyrophosphate group to ligate the trinuclear metal center and by inserting its pyridyl group into the isoprenoid binding pocket. Significant enzyme affinity for nitrogen-containing bisphosphonates is thus achieved through interactions between the sp2 hybridized planar side chain that occupies the allylic carbocation binding site described above (Fig3A). Like DMSPP, the inhibitor makes significant interactions with the C2, C3, C4, and C5 atoms of IPP, and thus may be unable to bind tightly to the enzyme in its absence.

The recent discovery that bisphosphonates used to treat osteoporosis are potent FPPS inhibitors has stemmed much interest in understanding the structural features governing this inhibition. These FPPS ternary complexes illustrate how substrate-induced conformational changes facilitate catalysis and reveal how bisphosphonates target the closed, catalytically active conformation of the enzyme. The complexes presented here thus clarify the structural basis for bisphosphonate inhibition of isoprenoid biosynthesis and provide a structural template for designing novel molecules that could be efficacious in treating both microbial and parasitic infections and other human diseases.

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References


Figure Legends:

Fig. 1. Mevalonate Pathway, FPPS intermediates, and Bisphosphonate Structure. **A**, The mevalonate pathway consists of several enzymes that convert HMG-CoA to FPP. Inhibition of two key mevalonate pathway enzymes, HMG-CoA reductase by statins, and FPPS by bisphosphonates, is an effective strategy for treating several human diseases. **B**, FPPS synthesizes the C15 isoprenoid FPP by successively condensing IPP with DMAPP and GPP. In the DMAPP analogue DMSPP, the linking oxygen atom (shown) is replaced by sulfur. **C**, The carbon-linked phosphate groups of bisphosphonates structurally mimic the oxygen-linked phosphates of pyrophosphate. The bisphosphonate risedronate contains a standard carbon-linked diphosphate with a pyridyl side chain.

Fig. 2. Stereo Illustration of Substrate-Induced Conformational Changes and Substrate:Inhibitor Interactions. **A**, The open conformation of FPPS-Sa (green) is superimposed on the closed, catalytically active conformation of the FPPS-Ec ternary complex (gold). The DMSPP (yellow tubes) pyrophosphate is ligated to the three active site Mg2+ atoms (blue spheres) and is in close proximity to IPP (green tubes). **B**, FPPS substrate interactions highlighting polar, electrostatic, and hydrophobic interactions that maintain the two isoprenoid
substrates in a reactive conformation. For clarity, Asp111 is labeled as an asterisk (*). C, FPPS inhibitor interactions highlighting the structurally conserved binding modes of DMSPP and N-containing bisphosphonates. For clarity, labels for two Mg$^{2+}$ have been omitted and Asp 111 is labeled as an asterisk (*). Figures 2 and 3A were created with software from Advanced Visual Systems (AVS).

Fig. 3. Stereo Illustration of the FPPS carbocation binding site and structure-based three-metal ion mechanism for FPPS isoprenoid condensation. A, The side chain oxygen atoms of Thr203 and Gln241, and the main chain carbonyl of Lys202 are oriented with their negative dipoles directed towards the allylic carbocation binding site. The non-metal ligated pyrophosphate oxygen that serves as the catalytic base to remove the IPP C2 proR hydrogen, the reactive C3-C5 double bond of IPP and the C1 atom of DMSPP are illustrated. B, Substrate ionization by the trinuclear Mg$^{2+}$ center generates an allylic carbocation that alkylates that C5 atom of IPP. Double bond formation in the final product is mediated by a DMAPP pyrophosphate oxygen that serves as the catalytic base removing the IPP C2 pro-R hydrogen.
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Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis
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