# Bisphosphonates Are Potent Inhibitors of *Trypanosoma cruzi* Farnesyl Pyrophosphate Synthase\*

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carbocation transition state/reactive intermediate analogs of its substrates, the nitrogen-containing bisphosphonates currently in use in bone resorption therapy. The protein predicted from the nucleotide sequence of the gene has 362 amino acids and a molecular mass of 41.2 kDa. Several sequence motifs found in other FPPSs are present in TcFPPS. Heterologous expression of TcF-PPS in Escherichia coli produced a functional enzyme that was inhibited by the nitrogen-containing bisphosphonates alendronate, pamidronate, homorisedronate, and risedronate but was less sensitive to the non-nitrogen-containing bisphosphonate etidronate, which, unlike the nitrogen-containing bisphosphonates, does not affect parasite growth. The protein contains a unique 11-mer insertion located near the active site, together with other sequence differences that may facilitate the development of novel anti-Chagasic agents.

We report the cloning and sequencing of a gene encod-

ing the farnesyl pyrophosphate synthase of Trypano-

soma cruzi. The protein (T. cruzi farnesyl pyrophos-

phate synthase, TcFPPS) is an attractive target for drug

development, since the growth of T. cruzi is inhibited by

Infections caused by *Trypanosoma cruzi* (Chagas' disease or American trypanosomiasis) are responsible for heavy socioeconomic losses in most countries of Latin America (1). Therapy against Chagas' disease is unsatisfactory because of the toxicity of currently available drugs, together with the development of drug resistance (1). One possible solution to these problems is to find drugs active against *T. cruzi* that have already been developed for other uses in humans and have therefore been demonstrated to have low toxicity. An example of this approach is offered by the potential use of bisphosphonates, currently used in bone resorption therapy, as anti-Chagasic drugs (2, 3).

Bisphosphonates are pyrophosphate analogs in which the oxygen bridge between the two phosphorus atoms has been replaced by a carbon substituted with various side chains. Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget's disease, hypercalcemia caused by malignancy, tumor metastases in bone, and other ailments (4–7). Selective action in bone is based on binding of the bisphosphonate moiety to the bone mineral (4–7). The most potent class of drugs are the nitrogen-containing bisphosphonates, such as pamidronate, alendronate, and risedronate. Over the past several years, several groups have narrowed the site of action of these compounds to the mevalonate pathway (4, 8) and, more specifically, to inhibition of the enzyme farnesyl pyrophosphate synthase (FPPS)<sup>1</sup> (9–15). This inhibition results in a deficit in protein prenylation, and in mammals this deficit results in osteoclast apoptosis (16–18).

Nitrogen-containing bisphosphonates have also recently been shown to be active both in vitro and in vivo against T. cruzi without apparent toxicity to the host cells (2). For example, pamidronate and alendronate were active against the intracellular forms of the parasite (amastigotes) with IC<sub>50</sub> values of  $\sim$ 60 and 147  $\mu$ M, respectively (3). No toxicity to the host cells was detected except at high concentrations of bisphosphonates  $(>300 \ \mu M)$  (2). Pamidronate given intravenously to mice with an acute T. cruzi infection arrested the development of parasitemia during treatment (2). Since sterol biosynthesis should also be affected by FPPS inhibition, the effects on sterol biosynthesis of one of the more potent bisphosphonates, risedronate, were investigated in T. cruzi (3). The results were consistent with the idea that FPPS could be a principal target of the drug (3). It has also been postulated (2, 3) that the selective activity of nitrogen-containing bisphosphonates on *T*. cruzi could result from their preferential accumulation in the parasites due to the presence of a calcium- and pyrophosphaterich organelle named the acidocalcisome (19). This organelle would play the equivalent role of calcium hydroxyapatite in bone, to which bisphosphonates are known to bind with high affinity (4-7).

Although the presence of an FPPS activity in *T. cruzi* has been inferred (20), a thorough molecular characterization of the enzyme is essential for structure-function analysis and further development of potential chemotherapeutic agents. In the present study, we report the cloning, sequencing, and heterologous expression of a *T. cruzi* gene, designated *TcFPPS*, that encodes a functional FPPS. The *TcFPPS* sequence and enzymatic activity of the expressed protein indicate that it is an authentic member of the family of FPPSs, as found in other organisms (21). The recombinant enzyme was shown to be potently inhib-

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: FPPS, farnesyl pyrophosphate synthase; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; bp, base pair(s); kb, kilobase pair(s); Mops, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction.

ited by nitrogen-containing bisphosphonates, while a non-nitrogen-containing bisphosphonate (etidronate) was far less inhibitory, as found with the human enzyme (22). A threedimensional model of TcFPPS was constructed based on the x-ray structure of avian FPPS (23), and this has provided structural insights into several unique characteristics of the *T. cruzi* enzyme. These results are also of general interest, since they demonstrate, for the first time, the expression and specific inhibition by bisphosphonates of an FPPS enzyme in a human pathogen, opening up the way to the design and synthesis of even more potent and specific FPPS inhibitors.

### EXPERIMENTAL PROCEDURES

Materials-Newborn calf serum, Dulbecco's phosphate-buffered saline, protease inhibitor mixture, dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), and isopentenyl pyrophosphate (IPP) were purchased from Sigma. Restriction enzymes, T4 DNA ligase, Taq polymerase, the Klenow fragment of DNA polymerase, Trizol reagent, and 0.24-9.5-kb RNA ladder were from Life Technologies, Inc. The PolyATract mRNA isolation system was from Promega (Madison, WI). pCR2.1-TOPO cloning kit was from Invitrogen (Carlsbad, CA). Hybond-N nylon membrane and [α-<sup>32</sup>P]dCTP (3000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. [4-14C]IPP (57.5 mCi/mmol) was from PerkinElmer Life Sciences. The pET-28a<sup>+</sup> expression system, the His.Bind® kit, and the thrombin cleavage kit were from Novagen (Madison, WI). Oligonucleotides FPPS1 (5'-CGAGTG-GCACATCTG-3'), FPPS2 (5'-CCCGTACGACCTCCG-3'), ATG-FPPS (5'-GTCGCTGCTAGCATGGCGTCCATGGAGCGGTTT-3'), and TAA-FPPS (5'-CGGCCCAAGCTTACTTCTTACGCTTGTGGGTTTT-3') were synthesized at Sigma.

Culture Methods—T. cruzi amastigotes and trypomastigotes (Y strain) were obtained from the culture medium of  $L_6E_9$  myoblasts as described previously (24). T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose medium (25) supplemented with 10% newborn calf serum.

Isolation of the T. cruzi FPPS Gene and DNA Sequencing-The hybridization probe for screening a T. cruzi genomic library (26) was obtained by using the PCR technique. A fragment of 283 bp of the TcFPPS gene (TcFPPSf) was amplified using the oligonucleotides FPPS1 and FPPS2 derived from a T. cruzi expressed sequence tag (GenBank<sup>TM</sup> accession number AI046250) that showed pronounced similarity to FPPSs of other organisms. To amplify the TcFPPS gene, the PCR was performed with 35 cycles of 94 °C for 1 min for denaturation, 50 °C for 1 min for annealing, and 72 °C for 2 min for extension, using 1.5 units of Taq DNA polymerase with 500 ng of T. cruzi genomic DNA, 25 pmol of each of the two oligonucleotide primers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.2 mM each deoxynucleoside trisphosphate in a total volume of 50 µl using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). The genomic library constructed in  $\lambda$ GEM11 was replica-plated onto nylon membranes and screened as described previously (27). The isolated  $\lambda$ GEM11 DNA was digested with restriction endonucleases, and a 3.5-kb BamHI-HindIII fragment that hybridized to the PCR probe was ligated into pBSKS<sup>+</sup> and transformed into *Escherichia coli* DH5 $\alpha$ . The BamHI-HindIII insert was sequenced in a 373A DNA Automatic Sequencer (PerkinElmer Life Sciences). Appropriate primers were synthesized by using as a model both DNA strands from the coding region. The predicted amino acid sequence of TcFPPS was aligned with the sequences of other FPPSs using the Biology Workbench 3.2 utility (available on the World Wide Web at workbench.sdsc.edu).

Southern and Northern Blot Analyses-Total genomic DNA from T. cruzi epimastigotes was isolated by phenol extraction (26), digested with different restriction enzymes, separated on a 1% agarose gel, and transferred to nylon membranes. The blot was probed with a  $[\alpha^{-32}P]$ dCTP-labeled *TcFPPS*. After hybridization, the blot was washed three times in  $2 \times$  SSC, 0.1% SDS at 65 °C (SSC is 0.15 M NaCl, 0.015 M sodium citrate). For the Northern blot analysis, total RNA was isolated from amastigotes, trypomastigotes, and epimastigotes using Trizol reagent, according to the manufacturer's instructions. Polyadenylated RNA was obtained with the PolyATract mRNA isolation system. RNA samples were subjected to electrophoresis in 1% agarose gels containing 1× Mops buffer (20 mM Mops, 0.08 M sodium acetate, pH 7.0, 1 mM EDTA) and 6.29% (v/v) formaldehyde after boiling for 10 min in 50% (v/v) formamide,  $1 \times$  Mops buffer, and 5.9% (v/v) formaldehyde. The gels were transferred to a Hybond-N filter and hybridized with a probe containing the entire coding sequence of the TcFPPS gene obtained by PCR. All Southern and Northern blots were visualized by autoradiography. The *TcP0* (*T. cruzi* ribosomal protein 1) fragment used as a control in Northern blots was obtained as described before (24). Densitometric analyses of Northern blots were done with an ISI-1000 digital imaging system (Alpha Inotech Corp.). Comparison of levels of *TcFPPS* transcription between the different stages was performed by taking as a reference the densitometric values obtained with the *TcP0* transcripts and assuming a similar level of expression of this gene in all stages (28). Similar results were obtained when the densitometric values were compared by taking into account the amount of RNA added to each lane.

Expression and Purification of TcFPPS from E. coli—For expression in E. coli, the entire coding sequence of the TcFPPS gene was amplified using the PCR technique. Oligonucleotide primers for amplification of the FPPS coding region, ATG-FPPS and TAA-FPPS, were designed so that NheI and HindIII restriction sites were introduced at the 5'- and 3'-ends for convenient cloning in the expression vector  $pET-28a^+$  to give *pETcFPPS*, which was cloned and propagated in *E. coli* DH5 $\alpha$ . Doublestranded DNA sequencing was performed to confirm that the correct reading frame was used, with the polyhistidine tail placed in the Nterminal position. Subsequently, *pETcFPPS* was used to transform *E*. coli BL21(DE3). Bacterial clones were grown in LB medium containing 50  $\mu$ g/ml kanamycin. When induction was performed, bacterial cells transformed with *pETcFPPS* were first grown to an  $A_{600}$  of 0.6 at 37 °C, and then 1 mM isopropyl  $\beta$ -D-thiogalactoside was added. After 6 h of growth at 37 °C, cells were pelleted by centrifugation and resuspended in 4 ml of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9) buffer and sonicated. The soluble extract was applied to a nickel-chelated agarose affinity column that had been equilibrated with the same buffer. The protein was eluted from the column as described by the kit manufacturer (Novagen). The eluted fraction was transferred into a dialysis cassette (molecular weight 10,000) and dialyzed against 10 mM Hepes (pH 7.4). Extracts from E. coli expressing TcFPPS as a fusion protein possessed 40-fold higher activity than extracts prepared from control bacteria. Following affinity purification, the specific activity of TcFPPS was 80-fold higher than the activity in the extracts of the transformed *E. coli*, indicating that the gene encoded a functional FPPS activity. The polyhistidine tag was removed by thrombin cleavage as described by the kit manufacturer (Novagen). Proteins were determined by the method of Bradford (29) with bovine serum albumin as a standard and analyzed by SDS-polyacrylamide gel electrophoresis.

FPPS Assay and Product Analysis-The activity of the enzyme was determined by a radiometric assay based on that described before (30). Briefly, 100 µl of assay buffer (10 mM Hepes, pH 7.4, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 47 µM [4-14C]IPP (10 µCi/µmol)), and 55 µM DMAPP or GPP was prewarmed to 37 °C. The assay was initiated by the addition of 10-20 ng of recombinant protein. The assay was allowed to proceed for 30 min at 37 °C and was terminated by the addition of 10 µl of 6 м HCl. The reactions were made alkaline with 15  $\mu$ l of 6 M NaOH, diluted in 0.7 ml of water, and extracted with 1 ml of hexane. The hexane solution was washed with water and transferred to a scintillation vial for counting. One unit of enzyme activity was defined as the activity required to incorporate 1 nmol of [4-14C]IPP into [4-14C]FPP in 1 min. To identify the reaction products after the enzymatic reaction, the radioactive prenyl products in the mixture were hydrolyzed to the corresponding alcohols with alkaline phosphatase as described before (31). The mixture was extracted with hexane and concentrated after the addition of farnesol, geraniol, and geranylgeraniol, as carriers, and the reaction products were separated on silica gel 60 plates (Merck), using a mixture of benzene/acetone (9:1) as solvent. The positions of the prenyl alcohols were visualized by using iodine vapor. The radioactivity was visualized by autoradiography.

Bisphosphonates—Sodium salts of the bisphosphonate compounds were synthesized, purified, and characterized as described previously (3). The structures of etidronate, homorisedronate, pamidronate, alendronate, and risedronate are shown in Fig. 1. The purity of all samples was verified by microchemical analysis (H/C/N/P) and via <sup>13</sup>C, <sup>31</sup>P, and <sup>1</sup>H NMR spectroscopy, the <sup>1</sup>H NMR experiments being performed in triplicate using an internal maleic acid quantitation standard. Absolute compound purities as determined from these experiments were 97.9, 99.2, 98.1, and 98.8%, for homorisedronate, pamidronate, alendronate, and risedronate, respectively. Etidronate was obtained from Gador S.A. (Buenos Aires, Argentina).

Homology Modeling—The structural similarities between the TcF-PPS and the avian FPPS were examined by homology modeling the *T. cruzi* FPP synthase sequence onto the published structure of the avian FPPS in the presence of either GPP or FPP (23). The three-dimensional models of TcFPPS were constructed following the sequence alignment (Fig. 2) and the three-dimensional homology using the coordinates of



FIG. 1. Structure of GPP, FPP, and different bisphosphonates used in this work.

the avian x-ray crystal structure (Protein Data Bank code: 1UBW) (23). All steps of homology model building and refinement were performed within the Homology module of Insight II 2000 (Molecular Simulations Inc., San Diego, CA). The Search Loops algorithm was used to display a range of possible loop geometries for the unique 11-mer region in the TcFPPS sequence. The entire three-dimensional models were in general subjected to molecular mechanics energy minimization calculations using the CHARMM (Chemistry at Harvard Molecular Mechanics) force field with a convergence criterion of 0.01 kcal/mol, resulting in protein models that are conformationally reasonable.

*Electrostatic Potential Calculations*—We used *ab initio* Hartree-Fock methods (6-311G<sup>\*\*</sup> basis sets) to evaluate the molecular electrostatic potentials,  $\Phi(r)$ , and the charge density,  $\rho(r)$ , for etidronate and risedronate, basically as described previously (11). The results obtained are shown graphically in the docked ligand models described below (Fig. 9).

### RESULTS

Isolation of the TcFPPS Gene-A tBLASTn search of the data base from the Institute for Genome Research using the human FPPS sequence (P14324) indicated that the predicted amino acid sequence encoded by T. cruzi expressed sequence tag clone 18h5 (accession number AI046250) presented an aspartate-rich motif (region VI), which is one of the most conserved and characteristic motifs present in prenyltransferases. A PCR probe was obtained by amplification of the fragment of the putative T. cruzi FPPS gene, using oligonucleotide primers complementary to the expressed sequence tag and genomic T. cruzi DNA as template. The product of the amplification (283 bp) was ligated into vector pCR2.1TOPO for sequence analysis. A genomic DNA library constructed in  $\lambda$ GEM11 (26) was screened with the specific PCR probe. Thirty positive clones were obtained in the first screening of approximately  $3.0 \times 10^5$ plaque-forming units. After the third screening, one clone was selected for restriction analysis. The clone contained an insert that possessed the complete coding region of the FPPS gene. A

BamHI-HindIII fragment was identified that hybridized strongly with the PCR probe. This 3.5-kb segment was ligated into pBSKS<sup>+</sup>, resulting in pFPPS3.5, and its complete sequence was determined on both strands. Translation of the open reading frame of 1086 bp yielded a polypeptide of 362 amino acids with a predicted molecular mass of 41,180 Da. A BLAST search of the protein data base showed that the amino acid sequence from *T. cruzi* has 35–39% identity and 48–55% similarity with other representative (mammalian, plant, yeast) FPPSs (Fig. 2). The amino acid sequence from the *T. cruzi* enzyme was aligned with the sequences of avian, human, Arabidopsis thaliana, and Saccharomyces cerevisiae synthases as shown in Fig. 2. All the conserved residues involved in catalysis or binding (regions I–VII) identified in other FPPSs (21) are present in the *T. cruzi* enzyme.

Genomic Organization of the TcFPPS Gene—Southern blotting was performed with a PCR fragment that encompasses the entire coding region of TcFPPS (Fig. 3A). All restriction enzymes used, except NcoI, BglI, and NaeI, gave single, strong bands, which were distinct from one another. Observation of two bands with NcoI, BglI, and NaeI was due to the presence of unique restriction sites within the coding region of the gene, suggesting the presence of a single TcFPPS gene in the T. cruzi genome.

Analysis of FSPP Transcripts in Amastigote, Trypomastigote, and Epimastigote Stages of T. cruzi—In order to confirm the transcription of the TcFPPS gene, we performed Northern blot analysis using poly(A)<sup>+</sup> RNA from different forms of the parasite and the TcFPPS gene as a probe. The presence of a single TcFPPS transcript of ~1.85 kb after prolonged exposure was detected in each of the three life stages of T. cruzi (Fig. 3B). Analysis of the 1.85-kb band by densitometry indicated that the

T.cruzi	1	MASMERFLSVYDEVOAFLLDOLOSKYEIDPNRARYLRIMMDTTCLGGKYFRGMTVV
Avian	1	MHKFTGVNAKFOOPALRNLSPVVVEREREEFVGFFPOIVRDLTEDGIGHPEVGDAVA-RLKEVLOVNAPGGKCNRGLTVV
Human	1	MNG
A.thaliana	1	MADLKST FLDVYSVLKSDLLODP-SFEFTHESRO-WLERMLDYNVRGGKLNRGLSVV
S. cerevisiae	1	MASEKET PERFINUEPKI. VERINASI. LAYCMPKEACD. WYAHSI. NYNTPCCKI. NPCI. SVV
510010115140	-	
T.cruzi	57	NVARGELAVTOHDEATKER I LHDACVGGWMIEFLOAHYLVEDDIMDGSVMRRGKPCWYRFPGVTTOCAINDGIILKSWTO
Avian	80	AAVEELSCH COKDARS - LECALAVGWCIELFOAFFLVADDIMDOSLTPRGOLCWYKKEGVG LDAINDSFLLESSVY
Human	66	VAFPELVED - PRODADS - LOPAWTVCWCVFLLOAFFLVADDIMDSSLTPPCOTCWVORDCVC - LOPANDANLLFACIV
A thaliana	56	DSYMILING - ODLTEKE - TELSCALGWCIEWLOAVELUIDDIMDNSUTPROOPCWERKPKVG - MIAINDGILLENHIH
A. Chailana	61	DEVISION TO A CONTRACT TO A CONTRACT A CONTRACTACT A CONTRACTACTACT A CONTRACT A CONTRACTACTACTACTACTACTACTACTACTACTACTAC
S.CELEVISIAE	OT	DITALBRANT BUILDER IERVAI DER CIEDINGATED VAN BUILDER BUILARE GEGEN VERVERVERVERVERVERVERVERVERVERVERVERVERV
Teruzi	137	
Arrian	155	INKANIFALKEFILLENDIGER (AV DIRIK GOMI DI INKEDIGER AUF AV
Avian	141	
Human	141	RLLKLICKEQPIILNLIELFLQSSIQTEIGQTLDLLTAPQGNVDLVRFTEKRIKSIVKIKTAFISFILP
A.thaliana	131	RILKKHFREMPYYVDLVDLFNEVEFQTACGQMIDLITTFDGEKDLSKYSLQIHRRIVEYKTAYYSFYLP
S.cerevisiae	138	KLLKSHFRNEKYYIDITELFHEVTFQTELGQLMDLITAPEDKVDLSKFSLKKHSFIVTFKTAYYSFYLP
		*** IV V
T.cruzi	217	LVMGLFVSEAAASVEMNLVERVAHLIGEYFQVQDDVMDCFTPPEQLGKVGTDIEDAKCSWLAVTFLGKANAAQVAEFKAN
Avian	224	VAAAMYMVGIDSKEEHENAKAILLEMGEYFQIQDDYLDCFGDPALTGKVGTDIQDNKCSWLVVQCLQRVTPEQRQLLEDN
Human	210	IAAAMYMAGIDGEKEHANAKKILLEMGEFFQIQDDYLDLFGDPSVTGKIGTDIQDNKCSWLVVQCLQRATPEQYQILKEN
A.thaliana	200	VACALLMAGENLEN-HTDVKTVLVDMGIYFQVQDDYLDCFADPETLGKIGTDIEDFKCSWLVVKALERCSEEQTKILYEN
S.cerevisiae	207	VALAMYVAGITDEKDLKQARDVLIPLGEYFQIQDDYLDCFGTPEQIGKIGTDIQDNKCSWVINKALELASAEQRKTLDEN
		VI
T.cruzi	297	YGDKDFAKVAVVKRLYSEANLQADFAAYEAEVVREVESLIEQLKVKSPTFAESVAVVWEKTHKRKK
Avian	304	YGRKEPEKVAKVKELYEAVGMRAAFQQYEESSYRRLQ-ELIEKHSNR-LPKEIFLGLAQKIYKRQK
Human	290	YGOKEAEKVARVKALYEELDLPAVFLQYEEDSYSHIM - ALIEQYAAP - LPPAVFLGLARKIYKRRK
A.thaliana	279	YGKAEPSNVAKVKALYKELDLEGAFMEYEKESYEKLT - KLIEAHOSKAIQA - VLKSFLAKIYKROK
S.cerevisiae	287	YGKKDSVAEAKCKKIFNDLKIEOLYHEYEESIAKDLKAKISOVDESRGFKADVLTAFLNKVYKRŠK

FIG. 2. Comparison of the deduced amino acid sequence of *T. cruzi* with other FPPSs. The deduced amino acid sequence of *T. cruzi* FPPS (GenBank<sup>TM</sup> accession number AF312690) is compared with the sequences of the avian (P08836), human (P14324), *A. thaliana* FPPS isoform 2 (L46349), and *S. cerevisiae* (J05091) synthases. Similar residues are *shaded*. The seven conserved regions I–VII are *underlined*. The potential glycosomal targeting signal (*AHL*) is indicated by *asterisks above* the *alignment*.

FIG. 3. Southern and Northern blot analysis. A. Southern blot analysis. Total genomic DNA was digested with different endonucleases. The DNA fragments were separated in 1% (w/v) agarose, transferred to a nylon membrane, and hybridized with the FPPS coding sequence. B, Northern blot analysis of amastigotes (A), trypomastigotes (T), and epimastigotes (E) stages of T. cruzi. Approximately 3  $\mu$ g of poly(A)+ RNA were subjected to electrophoresis on the gel before transfer to nylon and hybridized with <sup>32</sup>P-labeled probe corresponding to the FPPS coding sequence. The membranes were stripped and reprobed with a <sup>32</sup>P-labeled PCR fragment of the TcP0 gene from T. cruzi as control.



TcFPPS gene was expressed at higher levels in trypomastigotes and epimastigotes than in amastigotes, while the amount of transcription of a ribosomal protein gene (TcP0) was at comparable levels in all three stages of the parasite.

Purification and Reaction Requirements of Recombinant Protein-TcFPPS was expressed in E. coli BL21(DE3) as a fusion protein with an N-terminal polyhistidine tail. Affinity chromatography on a nickel-chelated agarose column permitted a simple one-step protein purification. Enzyme purity was judged by using SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining (Fig. 4). The final protein preparation catalyzed the incorporation of [4-14C]IPP into hexane-extractable material when the allylic substrates, DMAPP and GPP, were used (data not shown). The product of the FPPS assay was analyzed by TLC, confirming that the purified protein shown in Fig. 4 catalyzed the synthesis of FPP. [4-14C]IPP incorporation into the organic solvent extractable material was linear with time for at least 60 min. The radioactive assay was performed in the presence of different concentrations of  $Mg^{2+}$  and  $Mn^{2+}$ , to determine their effect on the T. cruzi FPPS.  $Mg^{2+}$  and  $Mn^{2+}$ were added to the reaction mixture at concentrations between 0.5 and 20 mm. As shown in Table I, optimal levels of activity were obtained by the addition of  $Mg^{2+}$  (1–5 mM). However, the enzymatic activity was very low when the divalent cation was  $Mn^{2+}$ . The addition of 10 mM EDTA abolished FPPS activity. The *T. cruzi* enzyme activity was also assayed between pH 6 and 10.5 using a Tris-HCl (10 mM), Tris-glycine (10 mM) buffer. Optimum activity was observed between pH 8 and 9 (Fig. 5A). Since the polyhistidine tag could potentially affect the enzymatic activity of TcFPPS, in two experiments, the polyhistidine tag was removed by thrombin cleavage. However, this treatment resulted in an almost complete loss (99.74  $\pm$  0.07%) of the catalytic activity of the enzyme, presumably due either to misfolding or aggregation. Consequently, all activity and inhibition results reported were obtained with the His-tagged enzyme.

Kinetic Analysis—Standard procedures were used to determine kinetic parameters.  $K_m$  and  $V_{max}$  values were obtained by a nonlinear regression fit of the data to the Michaelis-Menten equation (SigmaPlot for Windows version 3.06). When the rate of FPP synthesis by the recombinant enzyme (10 ng) was measured in the presence of saturating IPP (47  $\mu$ M) and varying GPP concentration between 0.5 and 55  $\mu$ M, a  $K_m$  value of 7.48  $\pm$  1.25  $\mu$ M and a  $V_{max}$  of 214  $\pm$  12 units/mg were calculated (Fig. 5B). When the concentration of GPP was kept at 55  $\mu$ M and the IPP concentration was varied between 0.5 and 44  $\mu$ M, the  $K_m$ 



FIG. 4. **Purification of** *T. cruzi* **FPP synthase from** *E. coli*. A SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue. Lane 1, crude extract from pET-28a<sup>+</sup>-transformed cell; *lane 2*, crude extract of *E. coli* BL21(DE3)/pETcFPPS; *lane 3*, soluble fraction from extract of *E. coli* BL21(DE3)/pETcFPPS; *lane 4*, nickel column purified fraction.

### TABLE I

## Effect of divalent cations on FPPS from T. cruzi

FPPS activity was measured in the presence of the different concentrations of MgCl<sub>2</sub> or MnCl<sub>2</sub> indicated in a reaction medium containing 10 mM Hepes (pH 7.4), 2 mM dithiothreitol, 47  $\mu$ M [4<sup>-14</sup>C]IPP (10  $\mu$ Ci/ $\mu$ mol), 55  $\mu$ M GPP, and 10 ng of recombinant protein (final volume of 100  $\mu$ l). Reactions were incubated for 30 min at 37 °C, stopped by the addition of HCl, and made alkaline with NaOH. The radioactive prenyl product was extracted with hexane as described under "Experimental Procedures." No activity was detected in the absence of MgCl<sub>2</sub> or MnCl<sub>2</sub> and presence of 10 mM EDTA. Values shown are means ± S.D. of two experiments in duplicate.

Concentration	FPPS activity			
Concentration	$\mathrm{MgCl}_2$	$MnCl_2$		
тм	units/	units/mg		
0	$4.9\pm1.6$	$4.9\pm1.6$		
0.5	$189.9\pm21.1$	$17.9 \pm 11.0$		
1	$256.2\pm9.9$	$16.1\pm4.9$		
5	$279.6\pm6.7$	$6.0\pm2.8$		
10	$226.5\pm10.8$	$6.1\pm2.3$		
20	$202.2\pm10.3$	$5.2\pm2.4$		

value was 2.96  $\pm$  0.74  $\mu{\rm M}$  and the  $V_{\rm max}$  was 325  $\pm$  5 units/mg (Fig. 5C).

Inhibition by Bisphosphonates—Five bisphosphonates were tested for their ability to inhibit the *T. cruzi* enzyme. The  $K_i$  values for bisphosphonates were calculated using the Dixon equation (32), and the IC<sub>50</sub> values were obtained as described previously (3) (Table II). Bisphosphonates are known competitive inhibitors with respect to GPP of FPPSs having different origins (14). Three nitrogen-containing bisphosphonates used clinically, alendronate, pamidronate, and risedronate, inhibited the TcFPPS activity. Risedronate was significantly more potent than alendronate and pamidronate. The non-nitrogencontaining bisphosphonate analog homorisedronate had an intermediate activity (Table II).

Homology Modeling—Using homology modeling within the Homology module of Insight II 2000, we obtained the folded TcFPPS protein structure shown in Fig. 6B, which is to be compared with that of the avian enzyme whose structure has been reported previously (23, 33) (Fig. 6A). Both structures contain the substrate GPP. There is a 35% sequence identity and 48% sequence similarity between TcFPPS and the avian enzyme and a 35% sequence identity and a 50% sequence



FIG. 5. Effect of pH and substrate concentration on the FPP synthase activity. The FPP synthase activity was measured as described under "Experimental Procedures" over a range of pH between 6 and 10.5 using Tris-HCl buffer and Tris-glycine (A) and in the presence of different concentrations of GPP (B) or IPP (C). Insets in B and C represent the linear transformation, by double reciprocal plot, of each curve.

### TABLE II

### The effects of bisphosphonates on FPPS activity

The activity of the *T. cruzi* enzyme was assayed in the presence of bisphosphonates in mixtures containing 10 mM Hepes (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 47  $\mu$ M [4<sup>-14</sup>C]IPP (10  $\mu$ Ci/ $\mu$ mol), 18  $\mu$ M GPP, and 10 ng of protein in a final volume of 100  $\mu$ l. Reactions were incubated for 30 min at 37 °C, and the prenyl product was extracted and measured by liquid scintillation counting as described under "Experimental Procedures."

D'all a la sta	T. cruz	i FPPS	
Bisphosphonate	$K_i^{\ a}$	${\rm IC}_{50}{}^a$	Human FPPS IC <sub>50</sub>
	$\mu M$	$\mu M$	$\mu M$
Etidronate	$61.08 \pm 8.7$	$57.7 \pm 1.69$	
Homorisedronate	$8.17 \pm 1.36$	$9.07\pm0.7$	$2.93\pm0.58^b$
Alendronate	$1.04\pm0.18$	$0.77\pm0.0$	$0.05\pm 0.001^b; 0.46^c$
Pamidronate	$2.02\pm0.10$	$1.08\pm0.4$	$0.20 \pm 0.01^b; 0.5^c$
Risedronate	$0.032\pm0.002$	$0.037 \pm 0.003$	$0.01 \pm 0.002^b; 0.0039^c$

<sup>*a*</sup> Values are the means  $\pm$  S.D. of three independent experiments.

<sup>b</sup> From Ref. 22.

<sup>c</sup> From Ref. 14.

similarity with the human FPPS. The most obvious difference between the TcFPPS and all other sequences investigated is the addition of an 11-mer peptide sequence (between regions IV).



FIG. 6. *A*, x-ray crystallographic structure of avian FPPS (with GPP) (from Ref. 33). *B*, homology model of *T. cruzi* FPPS (with GPP). The conserved Asp-rich binding motifs of domain I and II are displayed in *yellow*. The 11-mer insert in the TcFPPS is shown in *gray*.

and V; Fig. 2), which, based on the alignment and homology modeling results shown in Fig. 6*B*, is located near the active site of the enzyme. Since the 11-mer contains two proline residues, any helical structure is unlikely in this region. We therefore generated a family of loop structures using the Search Loops algorithm in Insight II and a superposition of six energetically feasible structures is shown in Fig. 7. Interestingly, a number of these loops can readily penetrate the active site region of the protein, and it will clearly be of interest to see to what extent this loop affects enzyme activity.

### DISCUSSION

We report here that a gene, *TcFPPS*, encoding a functional FPPS, is present in the *T. cruz*i genome. Heterologous expression of *TcFPPS* in *E. coli* resulted in the production of a recombinant enzyme that was similar to other FPPSs with respect to its  $Mg^{2+}$  requirement, optimum pH, and sensitivity to bisphosphonates. This is the first report of a gene encoding a functional FPPS in a trypanosomatid.

Structural Aspects—As shown in the alignment in Fig. 2, there is considerable sequence similarity between different FPPSs, and this similarity extends to some 20 sequences we have aligned (data not shown). Detailed sequence comparisons between several known FPPSs have shown the presence of two highly conserved aspartate-rich domains having a DDXX(XX)D motif (where X can be any amino acid), which have been suggested to be involved in substrate binding through the formation of magnesium salt bridges between the pyrophosphate moieties of the isoprenoid substrates and the carboxyl groups of the aspartates (34). These two motifs reside within larger regions of homology, domain II and domain VI. Several site-directed mutagenesis experiments have shown that most of the conserved Asp and Arg residues in these two regions are crucial for catalytic function (35, 36).

An interesting feature of TcFPPS as compared with animal FPPSs is the change of two Phe residues to His<sup>93</sup> and Tyr<sup>94</sup>. An analysis of the x-ray structure of the avian enzyme, coupled with the information about conserved amino acids in the prenyltransferases so far cloned, has led to the idea that the aromatic rings of these two Phe residues, situated at the fifth and fourth amino acids before the first DDXXD motif in region II, are important for determining the ultimate length of the prenyl chains (33). The T. cruzi FPPS has His and Tyr residues at these positions, with the His<sup>93</sup> residue corresponding to Phe or Tyr in other organisms and the Tyr<sup>94</sup> corresponding to Phe (Fig. 2). In contrast, geranylgeranyl pyrophosphate and higher chain length synthases have smaller amino acid side chains in these positions, and it is this increase in size of the binding pocket that permits further prenyl chain growth. Based on molecular modeling (Fig. 8, A and B), our results suggest that



FIG. 7. Homology model of TcFPPS (with GPP) showing six possible 11-mer loop conformations (Search Loops algorithm, Insight II 2000).

at least the His<sup>93</sup> residue may play a similar role as Phe<sup>112</sup> in the avian enzyme, in regulating chain elongation, although it should be noted that mutation of the corresponding Phe to His in *Bacillus stearothermophilus* FPPS resulted in further chain elongation (35). This observation may be of interest from the perspective of designing novel parasite-specific inhibitors, since from the molecular modeling results, the TcFPPS His<sup>93</sup> residue is in apposition to the prenyl chain terminus and may provide an interaction site for binding to other, more highly functionalized bisphosphonates.

Another interesting feature of the sequence is that an SKLlike sequence (in positions 239–241) of the *T. cruzi* FPPS has previously been identified as a possible signal sequence for glycosomal import. Glycosomal targeting sequences are usually present at the C terminus of the protein; however, signals at an internal position have also been described (37). Since glycosomes in trypanosomatids are known to possess a phosphatepyruvate dikinase (38), it seems likely that pyrophosphate generated by the action of the TcFPPS could be utilized by the dikinase.

*Enzymatic Aspects*—The FPPS catalyzes the synthesis of FPP from IPP and DMAPP. This reaction is considered to be a rate-limiting step in isoprenoid biosynthesis, since it is the starting point of different branched pathways leading to the synthesis of key isoprenoid end products (39). As such, it represents an interesting drug target.





FIG. 8. Detail of avian FPPS (with FPP) (A) and TcFPPS (with FPP) (B) showing apposition of the prenyl chain terminus and Phe<sup>112</sup> or His<sup>93</sup>, respectively. The amino acid residues are shown as *stick structures* with carbon in *green*, nitrogen in *blue*, and oxygen in *red*. FPP is displayed in the *ball* and *stick representation* with carbon in *gray*, oxygen in *red*, and phosphorus in *orange*.

As shown in Table I and Fig. 5A, the protein we have expressed has optimum activity in the presence of 1–5 mM MgCl<sub>2</sub> and in the pH range 8-9, basically as found with the human enzyme (40). In addition, our results show that TcFPPS is potently inhibited by a number of bisphosphonates (Table II). The pattern of inhibition is that the  $K_i$  values decrease in the order etidronate to homorisedronate to pamidronate to alendronate to risedronate. Interestingly, the short chain non-nitrogen-containing bisphosphonate etidronate is in fact an inhibitor of the TcFPPS enzyme, although its  $K_i$  (or IC<sub>50</sub>) value is about 60  $\mu$ M (Table II). This is much larger than the  $K_m$ , implying that etidronate may act simply as an inert substrate rather than a transition state/reactive intermediate analog. The second generation bone resorption drugs pamidronate and alendronate have  $K_i$  (or IC<sub>50</sub>) values in the 1–2  $\mu$ M range (Table II), while the aromatic species risedronate has a  $K_i$  (IC<sub>50</sub>) of  $\sim$ 30 nm, making it a very potent inhibitor ( $K_i \ll K_m$ ) The activity of the methylene homolog of risedronate, homorisedronate, has a  $K_i$  (IC<sub>50</sub>) of about 8–9  $\mu$ M. In general, these  $K_i$  $(IC_{50})$  values are about a factor of 5 greater than those reported for a recombinant human enzyme (Table II), which may be related to the presence in TcFPPS of the 11-mer region (Figs. 2 and 5), although as shown in Table II, there is some variability in the reported  $IC_{50}$  values for the human enzyme, and small systematic differences between assays cannot be excluded.

The pattern of inhibition observed with the different bisphosphonates (Table II) is consistent with the proposal made previously that the more potent nitrogen-containing bisphosphonates act as aza-carbocation transition state/reactive intermediate analogs of the allylic substrates DMAPP/GPP (11). To illustrate this point further, we show in Fig. 9, *left* and *right*, space-filling models of risedronate and etidronate docked into the GPP binding site of the TcFPPS. Clearly, the much smaller methyl side chain in etidronate provides no electrostatic interaction with the protein (Fig. 9, *right*) and only a



FIG. 9. Docking of risedronate (*left*) and etidronate (*right*) into the active site of TcFPPS. The representations of the drug molecules are the electrostatic potentials  $\Phi(r)$  mapped onto the charge densities,  $\rho(r) = 0.05 \text{ ea}_o^3$ .

minimal hydrophobic interaction, while the 3-pyridyl substitution to form risedronate results in a much more favorable electrostatic interaction with the protein (Fig. 9, *left*) in addition to a much larger hydrophobic interaction. This enhanced hydrophobic interaction is most likely the reason that minodronate, containing a benzimidazole side chain, has an even smaller IC<sub>50</sub> than does risedronate in inhibiting a human FPPS (22).

It is also possible that some bisphosphonates may mimic the homoallylic (IPP) substrate. In order to address the question of the nature of the second (IPP) binding site, we extended our computer modeling studies to include not only the GPP substrate but also the bonding of the second, allylic pyrophosphate (*i.e.* isopentenyl pyrophosphate), which we find can be readily docked into the second, conserved DDXXD binding site in the active site of the TcFPPS (data not shown). While obtaining x-ray structures of e.g. FPPS + GPP + IPP might not be feasible, it seems likely that the tightly binding bisphosphonate inhibitors (such as risedronate) may substitute for GPP, leading to high resolution ternary complex structures, with IPP. This model of the FPPS active site also points to the possibility of developing larger inhibitors having four phosphonates (binding to both Asp-rich clusters) and does not exclude the possibility that some bisphosphonates we have investigated may already bind to the second Asp-rich region.

Removal of the polyhistidine tag of TcFFPS resulted in almost complete loss of activity. It has been suggested that polyhistidine tags may affect folding (41) or facilitate dimerization/ oligomerization (42) of some proteins. Polyhistidine tags have been used before for the purification of other FPPSs (43, 44), but no attempts to remove them were reported, and it is likely that structural studies will be required in order to distinguish between these possibilities. Likewise, detailed structure determinations are desirable in order to facilitate the design of new and more potent inhibitors.

Isoprenyl groups generated by FPPS and other enzymes in the isoprene pathway can be transferred to cysteine residues within carboxyl-terminal motifs present in several classes of proteins, including the family of GTP-binding proteins Ras, Rho, Rac, and Rab and the nuclear lamins, in a reaction catalyzed by at least three distinct cytoplasmic prenyl protein transferases (45). Post-translational modification of proteins with farnesyl or geranylgeranyl groups appears to be essential for the localization of these proteins to membranes and, consequently, for their biological function (45). Inhibition of protein prenylation by substrate inhibitors of prenyl protein transferases or by inhibitors of mevalonate or isopentenyl pyrophosphate synthesis (such as lovastatin, mevastatin, and phenylacetate) have a profound effect on cell morphology (46), cell

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replication (47, 48), and intracellular signal transduction (17) and can lead to the induction of apoptotic cell death (18, 50). It has been shown that apoptosis induced by bisphosphonates in J774 macrophages is associated with the inhibition of posttranslational prenylation of proteins such as Ras and that this effect can be reversed by the addition of components of the mevalonate pathway, such as FPP and geranylgeranyl pyrophosphate (51). Recent studies have indicated that protein prenylation also occurs in *T. cruzi* and other trypanosomatids, since the growth of T. cruzi intracellular forms is sensitive to protein farnesyl transferase inhibitors (20). Although it is not yet known whether treatment with nitrogen-containing bisphosphonates leads to apoptosis in T. cruzi, characteristics of apoptosis have been found to occur in D. discoideum (52) and in several trypanosomatids (53-55), and it has been reported that inhibition of protein prenylation in T. brucei using the statin compactin (an HMGCo-A reductase inhibitor) may lead to apoptosis (49, 56) or at least a process having many characteristics of apoptosis. Thus, the available evidence suggests that the nitrogen-containing bisphosphonates act in T. cruzi in much the same way that they do in bone resorption, by inhibiting FPPS. This results in disruption of protein prenvlation, and in T. cruzi there may also be additional effects due to disruption of sterol (ergosterol) biosynthesis, although, as noted previously, this effect alone would not be expected to result in cell death (3).

The results we have obtained to date suggest that the Ncontaining bisphosphonates and potentially related species such as imidodiphosphates may have potential for use in treating parasitic protozoan infections, by inhibition of protein prenylation via blocking the synthesis of FPP. Since millions of people have already been treated with bisphosphonates and since they have proven anti-Chagasic activity, bisphosphonate inhibitors of FPPS appear to constitute an attractive group of compounds to further develop as chemotherapeutic agents using structure-based drug design.

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#### REFERENCES

- 1. Docampo, R., and Schmuñis, G. A. (1997) Parasitol. Today 13, 129-130
- Urbina, J. A., Moreno, B., Vierkotter, S., Oldfield, E., Payares, G., Sanoja, C., Bailey, B. N., Yan, W., Scott, D. A., Moreno, S. N. J., and Docampo, R. (1999) J. Biol. Chem. 274, 33609-33615
- 3. Martin, M. B., Grimley, J. S., Lewis, J. C., Heath, H. T., III, Bailey, B. N., Kendrick, H., Yardley, V., Caldera, A., Lira, R., Urbina, J. A., Moreno, S. N. J., Docampo, R., Croft, S. L., and Oldfield, E. (2001) J. Med. Chem. 44, 909-916
- 4. Rogers, M. J., Watts, D. J., and Russell, R. G. G. (1997) Cancer (Suppl.) 80, 1652–1660
- 5. Rodan, G. A. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 375-388
- 6. Brown, D. L., and Robbins, R. (1999) J. Clin. Pharmacol. 39, 1–10
- 7. Rodan, G. A., and Martin, T. J. (2000) Science 289, 1508-1514
- 8. Amin, D., Cornell, S. A., Perrone, M. H., and Bilder, G. E. (1996) Drug. Res. 46, 759 - 762
- Overhand, M., Stuivenberg, H. R., Pieterman, E., Cohen, L. H., van Leeuwen, R. E. W., Valentijn, A. R. P. M., Overkleeft, H. S., van der Marel, G. A., and van Boom, J. H. (1998) Bioorg. Chem. 26, 269-282
- 10. Cromartie, T. H., Fisher, K. J., and Grossman, J. N. (1999) Pest. Biochem. Physiol. 63, 114–126
- 11. Martin, M. B., Arnold, W., Heath, H. T., Urbina, J. A., and Oldfield, E. (1999) Biochem. Biophys. Res. Commun. 263, 754-758
- 12. Van Beek, E., Peterman, E., Cohen, L., Löwik, C., and Papapoulos, S. (1999)

- Biochem. Biophys. Res. Commun. **264**, 108–111 13. Keller, R. K., and Fiesler, S. J. (1999) Biochem. Biophys. Res. Commun. **266**, 560 - 563
- 14. Bergstrom, J. D., Bostedor, R. G., Masarachia, P. J., Reszka, A. A., and Rodan G. (2000) Arch. Biochem. Biophys. 373, 231-241
- 15. Grove, J. E., Brown, R. J., and Watts, D. J. (2000) J. Bone Miner. Res. 15, 971-981
- Hughers, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R., and Boyce, B. F. (1995) J. Bone Miner. Res. 10, 1478 - 1487
- 17. Lerner, E. C., Qian, Y., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J., Der Cox, A. D., Hamilton, A. D, and Sebti, S. M. (1995) J. Biol. Chem. 270, 26802-26806
- 18. Danesi, R., Nardini, D., Basolo, F., del Tacca, M., Samid, D., and Myers, C. E. (1996) Mol. Pharmacol. 49, 972–979
- 19. Docampo, R., and Moreno, S. N. J. (1999) Parasitol. Today 15, 443-448
- Yokoyama, K., Trobridge, P., Buckner, F. S., Scholten, J., Stuart, K. D., Van Voorhis, W. C., and Gelb, M. H. (1998) Mol. Biochem. Parasitol. 94, 87–97
- 21. Koyama, T. (1999) Biosci. Biotechnol. Biochem. 63, 1671-1676
- 22. Dunford, J. E., Thompson, K., Coxon, F. P., Luckman, S. P., Hahn, F. M., Poulter, C. D., Ebetino, F. H., and Rogers, M. J. (2001) J. Pharmacol. Exp. Ther. 296, 235–242
   Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) Biochem-
- istry 33, 10871–10877
- 24. Furuya, T., Kashuba, C., Docampo, R., and Moreno, S. N. J. (2000) J. Biol. Chem. 275, 6428-6438
- 25. Bone, G. J., and Steinert, M. (1956) Nature 178, 308-309
- 26. Hill, J. E., Scott, D. A., Luo, S., and Docampo, R. (2000) Biochem. J. 351, 281 - 288
- 27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) (1987) Current Protocols in Molecular Biology, Chapter 6, John Wiley & Sons, Inc., New York
- 28. Skeiky, Y. A. W., Benson, D. R., Parsons, M., Elkon, K. B., and Reed, S. G. (1992) J. Exp. Med. 176, 201-211
- 29. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
- 30. Rilling, H. C. (1985) Methods Enzymol. 110, 145-152
- 31. Ogura, K., Nishino, T., Shinka, T., and Seto, S. (1985) Methods Enzymol. 110, 167 - 170
- Dixon, M. (1972) Biochem. J. 129, 197–202
  Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., Poulter, C. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 26, 15018–15023
- 34. Ashby, M. N., and Edwards, P. A. (1989) J. Biol. Chem. 264, 635-640 35. Koyama, T., Tajima, M., Sano, H., Doi, T., Koike-Takeshita, A., Obata, S.,
- Nishino, T., and Ogura, K. (1996) Biochemistry 35, 9533-9538 36. Song, L., and Poulter, C. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91,
- 3044 304837. Sommer, J. M., and Wang, C. C. (1994) Annu. Rev. Microbiol. 48, 105-138
- 38. Bringaud, F., Baltz, D., and Baltz, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7963-7968
- 39. Goldstein, J. L., and Brown, M. S. (1990) Nature 343, 425-430
- 40. Ding, V. D.-H., Sheares, B. T., Bergstrom, J. D., Ponpipom, M. M., Perez, L. B.,
- and Poulter, C. D. (1991) Biochem. J. 275, 61-65 41. Supattapone, S., Nguyen, H.-O. B., Muramoto, T., Cohen, F. E., Dearmond,
- S. J., Prusiner, S. B., and Scott, M. (2000) J. Virol. 74, 11928-11934
- Filutowicz, W. J. (1999) Acta Biochim. Pol. 46, 591–599
  Attucci, S., Aitken, S. M., Gulick, P. J., and Ibrahim, R. K. (1995) Arch. Biochem. Biophys. 321, 493–500
- 44. Gaffe, J., Bru, J.-P., Causse, M., Vidal, A., Stamitti-Berrt, L., Carde, J.-P., and Gallusci, P. (2000) Plant Physiol. 123, 1351-1362
- 45. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241-269
- 46. Pendergast, G. C., Davide, J. P., DeSolms, S. J., Giuliani, E. A., Graham, S. L.,
- Gibbs, J. B., Oliff, A., Kohl, N. E. (1994) Mol. Cell. Biol. 14, 4193–4202
  Quesney-Huneeus, V., Highes Wiley, M., and Siperstein, M. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5056–5060
- 48. Sinensky, M., and Logel, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3257-3261 Coppens, I.; Bastin, P.; Levade, T.; Courtoy, P. J. (1995) Mol. Biochem. 49. Parasitol. 69, 29-40
- 50. Perez-Sala, D., and Mollinedo, F. (1994) Biochem. Biophys. Res. Commun. 199, 1209 - 1215
- Luckman, S. P., Hughes, D. E., Coxon, F. P., Russell, R. G. R., and Rogers, M. J. (1998) J. Bone Miner. Res. 13, 581–589
- 52. Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D., and Golstein, P. J. (1994) J. Cell Sci. 107, 2691–2704
- 53. Ameisen, J. C., Idziorek, T., Billot-Mulot, O., Loyens, M., Tissier, J., Potentier, A., and Ouassi, A. (1995) Cell Death Differ. 2, 285-300
- 54. Moreira, M. E. C., Del Portillo, H. A., Milder, R. V., Balanco, J. M., Barcinski, M. A. (1996) J. Cell. Physiol. 167, 305–313 Welburn, S. C., Dale, C., Ellis, D., Beecroft, R., Pearson, T. W., and Maudlin,
- 55 I. (1996) Cell Death Differ. 3, 229-235
- 56. Welburn, S. C., Barcinski, M. A., Williams, G. T. (1997) Parasitol. Today 13, 22 - 26