Characterization and regulation of *Leishmania major* 3-hydroxy-3-methyl-glutaryl-CoA reductase

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In eukaryotes the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase catalyses the synthesis of mevalonic acid, a common precursor to all isoprenoid compounds. Here we report the isolation and overexpression of the gene coding for HMG-CoA reductase from *Leishmania major*. The protein from *Leishmania* lacks the membrane domain characteristic of eukaryotic cells but exhibits sequence similarity with eukaryotic reductases. Highly purified protein was achieved by ammonium sulphate precipitation followed by chromatography on hydroxyapatite. Kinetic parameters were determined for the protozoan reductase, obtaining K_m values for the overall reaction of 40.3 ± 5.8 μM for (*R,S*)-HMG-CoA and 81.4 ± 5.3 μM for NADPH; V_{max} was 33.55 ± 1.8 units·mg^{-1}. Gel-filtration experiments suggested an apparent molecular mass of 184 kDa with subunits of 46 kDa. Finally, in order to achieve a better understanding of the role of this enzyme in trypanosomatids, the effect of possible regulators of isoprenoid biosynthesis in cultured promastigote cells was studied. Neither mevalonic acid nor serum sterols appear to modulate enzyme activity whereas incubation with lovastatin results in significant increases in the amount of reductase protein. Western- and Northern-blot analyses indicate that this activation is apparently performed via post-transcriptional control.

Key words: ketoconazole, lovastatin, mevalonic acid, sterol, trypanosomatid.

INTRODUCTION

Leishmania species are protozoan parasites that cause the leishmaniases, diseases for which there is a need of improved chemotherapy. These flagellated parasites contain sterols of fungal type such as ergosterol [1,2], which differs from cholesterol, the predominant mammalian sterol, by the presence of 24-methyl group and Δ^7 and Δ^{22} bonds. Cholesterol can be taken up in large amounts by *Leishmania* promastigotes from the medium, but cannot be further metabolized or synthesized *de novo* from exogenous mevalonic acid [3]. Sterol biosynthesis inhibitors are effective anti-proliferative agents that have been used in the treatment of diseases produced by fungi and yeast [4,5]. *Leishmania* is also highly sensitive to this class of agents and the combined administration of lovastatin, a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, and 14α-demethylase inhibitors such as miconazole or ketoconazole present synergistic effects when inhibiting growth of the parasite *in vitro* [6]. Hence, the combined use of sterol biosynthesis inhibitors that act at different steps of the pathway appears to offer a promising approach for an effective treatment of leishmaniasis.

In mammalian cells, cholesterol is either obtained by receptor-mediated uptake of plasma low-density lipoprotein (LDL) or by synthesis from acetyl-CoA through the sterol biosynthetic pathway. Each cell must balance the external and internal sources of cholesterol so as to ensure the biosynthesis of molecules produced by the isoprenoid pathway while avoiding sterol overload. Cholesterol homoeostasis is achieved through feedback regulation of at least two enzymes of sterol biosynthesis, HMG-CoA synthase and HMG-CoA reductase, as well as by means of control of LDL receptors. The key rate-limiting enzyme, HMG-CoA reductase, catalyses the conversion of HMG-CoA into mevalonate, which is required for synthesis of a large family of compounds. These include cholesterol and isoprenoids such as haem A, ubiquinone, dolichol, isopentenyl tRNA, steroid hormones and other isoprenoid groups, which are covalently linked to growth-regulating proteins and oncogenic products [7].

HMG-CoA reductase is a soluble protein in prokaryotes [8–10] and in the parasitic protozoan *Trypanosoma cruzi* [11], while in all other eukaryotic organisms characterized the enzyme presents a variable N-terminal membrane domain and its subcellular location appears to be preferentially the endoplasmic reticulum [12]. This domain is necessary and sufficient for the regulated degradation of HMG-CoA reductase. Little is known about the nature and regulation of this enzyme in protozoa, while it has been studied extensively in mammalian cells. This regulation, which is mediated by sterols and non-sterol mevalonate-derived metabolites, is complex and occurs at many levels, including transcription, translation, protein degradation and protein phosphorylation [7].

In the current studies, we describe the isolation and characterization of HMG-CoA reductase from *Leishmania major* and also demonstrate that in this protozoan the enzyme lacks the membrane-domain characteristic of eukaryotic reductases. We examine the modulation of the soluble enzyme in promastigote forms of the parasite, showing that activity is not subject to regulation by mevalonic acid or medium sterols.

EXPERIMENTAL

Materials

Restriction enzymes, T4 DNA ligase, *Taq* polymerase and the Klenow fragment of DNA polymerase were from Boehringer Mannheim. [α-^{32}P]ATP was from ICN Pharmaceuticals (Irvine, CA, U.S.A.). BSA, dithiothreitol (DTT), D,L-mevalonic acid lactone, ketoconazole and sodium phosphate were from Sigma.

Abbreviations used: CHEF, contour-clamped homogeneous electric field; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; *lmhmgr* gene, *Leishmania major* HMG-CoA reductase gene; LDL, low-density lipoprotein; DTT, dithiothreitol.

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The nucleotide sequence data reported here appear in the GenBank nucleotide sequence database under the accession number AAF155593.

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Leupeptin was purchased from Boehringer Mannheim. Lovastatin was supplied by Merck Sharp and Dohme. The pET expression system was from Stratagene. Oligonucleotides HMG1 (5′-ATGGGNATGAAYATG-3′), HMG2 (5′-GTTBGCBGCV-TGBGCGTT-3′), HMG3 (5′-TCTGCGCTTCTCTGCCTTTA-3′) and HMG4 (5′-TTACGGAGTCGGAGGCTT-3′) were synthesized in a Millipore ConSep LC500DNA synthesizer at the Analytical Services of the Instituto de Parasitología y Biomedicina 'López-Neyra', Granada, Spain.

Cell culture

L. major promastigotes were grown in tissue-culture flasks at 28 °C in filter-sterilized 199 medium supplemented with either 10% heat-inactivated fetal calf serum (Gibco) or 10% lipid-poor serum, which was prepared by the method of Kirsten and Watson [13]. The cultures were initiated at a cell density of 5×10^5 cells$\cdot$ml^{-1}, and the drugs were added at a cell density of 1×10^6 cells$\cdot$ml^{-1}. Cell densities were determined by counting with a Coulter® Z1 counter. Lovastatin and D,L-mevalonic acid lactone were added from stock solutions in ethanol, the lactone rings of the drugs were hydrolysed by a 30-min incubation in 0.2 M NaOH at 37 °C, followed by neutralization with 0.2 M HCl. Ketoconazole was added as an aqueous solution titrated to pH 2.4 with HCl.

Isolation of the *L. major* HMG-CoA reductase (*lmhmgr*) gene and DNA sequencing

The hybridization probes for screenings were obtained by the PCR technique. A fragment of 303 bp of the *lmhmgr* gene was amplified, using the degenerate oligonucleotides HMG1 and HMG2 derived from highly conserved sequences of the catalytic domain, and used as hybridization probe for screening of a cDNA library. A PCR fragment of the *lmhmgr* gene was further obtained using the oligonucleotides HMG3 and HMG4, and used as a probe to screen the library of genomic DNA. The PCR reaction mixture (50 μl) contained 25 pmol of each of the two oligonucleotide primers, 500 ng of *L. major* genomic DNA, dNTPs each at 400 μM, 50 mM KCl, 10 mM Tris/HCl (pH 8) and 1.5 mM $MgCl_2$. Amplification was initiated with 1.5 units of *Taq* polymerase. PCR parameters were 35 thermal cycles consisting of a 1-min denaturation at 94 °C followed by a 1-min annealing period at 45 °C and a 2-min extension at 72 °C. The genomic library constructed in λEMBL3 was replica plated on to nitrocellulose and screened as described in [14].

The isolated λEMBL3 DNA was digested with restriction endonucleases and a 2.4-kb *Bam*HI–*Xho*I fragment that hybridized to the PCR probe was ligated into pBSKS$^-$ and transformed into XL1-Blue *Escherichia coli*. The *Bam*HI–*Xho*I insert was sequenced in an Applied Biosystems 373 DNA sequencer.

Southern- and Northern-blot analyses

Total genomic DNA from *L. major* was isolated from *Leishmania* by phenol extraction [15], digested with different restriction enzymes using the conditions recommended by the supplier. Endonuclease-cleaved DNA was electrophoresed on 0.8% (w/v) agarose gels. The isolation of the poly(A)$^+$ RNA was accomplished with the QuickPrep® *Micro* mRNA purification kit (Pharmacia Biotech). mRNA samples were subjected to electrophoresis on 1% (w/v) 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 the samples had been boiled for 10 min in 50% (v/v) formamide/1 × Mops buffer/5.9% (v/v) formaldehyde. A 0.24–9.5-kb RNA ladder (Gibco) was used as a standard. The gels were transferred to a Hybond-N filter and hybridized with the probe containing the entire coding sequence of the *lmhmgr* gene obtained by PCR. All Southern and Northern blots were visualized by autoradiography. The β-tubulin gene from *L. major* was used for normalization of hybridization signals on Northern blots and the relative intensities of the bands were quantified by scanning densitometry.

Low-melting-point agarose blocks were prepared as described in [16]. Chromosomes were separated on a 1% agarose gel in 0.5 M Tris/borate/EDTA and 0.5 mg$\cdot$ml^{-1} ethidium bromide using a CHEF (contour-clamped homogeneous electric field) electrophoresis system (Pharmacia) as described previously [17]. The resulting gel was transferred to a Hybond-N (Amersham) nylon filter and subjected to Southern-blot analysis [18] with the PCR-amplified *lmhmgr* gene as a probe.

Construction of expression system

For expression in *E. coli* the entire coding sequence was amplified by the PCR technique. Oligonucleotide primers for amplification of the HMG-CoA reductase coding sequence were designed so that *Nde*I and *Bam*HI restriction sites were introduced at the 5′ and 3′ ends for convenient cloning in the expression vector pET-11c to give pETLMHMG, which was cloned and propagated originally in XL1B. Double-stranded DNA sequencing was performed to confirm the correct sequence after amplification. Subsequently, pETLMHMG was used to transform the *E. coli* expression host BL21(DE3). Bacterial clones were grown in Luria–Bertani medium containing 50 mg$\cdot$ml^{-1} ampicillin. When induction was performed, bacterial cells transformed with pETLMHMG were first grown to an absorbance of 0.6 at 37 °C and then 1 mM isopropyl β-D-thiogalactoside was added; cultures were then grown for 6 h at 37 °C, cells were collected by centrifugation and, when not used immediately, frozen at −80 °C.

Purification of *L. major* HMG-CoA reductase overexpressed in *E. coli*

A frozen pellet from 1 l of *E. coli* BL21(DE3)/pETLMHMG cells overexpressing *L. major* HMG-CoA reductase was thawed at room temperature and resuspended in 20 ml of buffer A [100 mM sucrose/20 mM Na_2HPO_4, pH 6.75/10 mM DTT/20 μg$\cdot$ml^{-1} leupeptin/10% (v/v) glycerol]. Cells were ruptured by sonication and centrifuged at 12000 ***g*** for 30 min at 4 °C. The supernatant was brought to 30% saturation with $(NH_4)_2SO_4$, maintained on ice, shaken occasionally for 20 min, and then centrifuged at 12000 ***g*** for 30 min. The precipitate was discarded and the supernatant liquid was brought to 50% saturation with $(NH_4)_2SO_4$. The resulting precipitate was dissolved in 5 ml of buffer A and stored overnight at −80 °C. The thawed ammonium sulphate fraction was applied to a column (1 cm × 20 cm; 9 ml) of hydroxyapatite Bio-Gel® HTP Gel (Bio-Rad) in buffer A. The column was washed with 25 ml of buffer A and the enzyme was eluted with a 300-ml gradient of 0–1.5 M NaCl in buffer A (3-ml fractions). Active fractions were combined and brought to 50% saturation with $(NH_4)_2SO_4$. The precipitate was desalted and stored at −80 °C until use. Protein was determined by the method of Bradford [19] with BSA as a standard. In gel-filtration experiments, a Superdex™ 200 HR 10/30 column attached to ÄKTA purifier 10 FPLC equipment (Pharmacia Biotech) was used.

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Measurement of HMG-CoA reductase activity

The activity of the enzyme was determined either by a radiometric assay as described previously [11] or by a spectrophotometric assay based on that described by Frimpong et al. [20]. Assays were performed in a final volume of 200 μl and contained 150 mM $NaPO_4$, pH 6.75, 75 mM NaCl, 10 mM DTT, 1 mM EDTA, 270 μM (*R*,*S*)-HMG-CoA, 270 μM NADPH and 125 ng of recombinant enzyme. The kinetic parameters were determined in experiments where the non-varied substrate was kept at 270 μM. NADH oxidation was assessed using two different cofactor concentrations (350 and 700 μM) and two different enzyme concentrations (625 and 2500 $ng \cdot ml^{-1}$). The analysis of HMG-CoA reductase activity in *L. major* extracts was made using the radiometric assay. *Leishmania* promastigote cells were grown to exponential phase (16×10^6 $cells \cdot ml^{-1}$), collected by centrifugation, washed with PBS and resuspended in buffer A with protease inhibitors. Cells were ruptured by sonication, the resulting extract was clarified by centrifugation (12000 ***g***, 15 min, 4 °C) and used as an enzyme source for determination of reductase activity. When the intracellular distribution of the enzyme was analysed, the 12000 ***g*** supernatant was centrifuged further at 105000 ***g*** for 60 min at 4 °C. The pellet was resuspended in buffer A with protease inhibitors, and both the pellet and the 105000 ***g*** supernatant were used for determination of HMG-CoA reductase activity. Enzyme activity (1 unit) was defined as the amount of enzyme that converts 1 nmol of [^{14}C]HMG-CoA into [^{14}C]mevalonate/min per mg of protein.

All activity measurements for the purification scheme and characterization of the enzyme were performed using the spectrophotometric assay at 340 nm in a Hewlett-Packard model 8452A diode array spectrophotometer. HMG-CoA reductase activity (1 unit) was defined as the amount of enzyme that converts 1 μmol of NADPH into $NADP^+$ in 1 min/mg of protein.

Western-blot analysis

Polyclonal anti-*L. major* HMG-CoA reductase antibody was generated by immunizing rabbits with the purified protein. Promastigotes were lysed by sonication, and cell supernatants were prepared by centrifugation at 12000 ***g*** for 15 min. Protein (30 μg) was subjected to electrophoresis through an SDS-polyacrylamide (12 %) gel and blotted on to Immobilon-P membranes (Millipore) at 25 V for 30 min, using a Semi-Dry Transfer Cell (Bio-Rad). Western-blot analysis of HMG-CoA reductase was performed with a 1:20000 dilution of the indicated antiserum, using anti-rabbit-IgG-conjugated alkaline phosphatase as the secondary antibody. Bound antibody was visualized with chromogenic substrates [14].

RESULTS

Isolation of the *lmhmgr* gene

The PCR probe was obtained by amplification of a fragment of the *lmhmgr* gene using as primers degenerate nucleotides complementary to highly conserved regions contained within the soluble catalytic domain, and genomic *L. major* DNA as template. A PCR product of the expected size (303 bp) was identified that showed pronounced similarity to HMG-CoA reductases. A cDNA library constructed in λZAP Express was screened with the specific PCR probe (303 bp). One clone was selected that contained a 2.3-kb insert, although the 5′ end of this cDNA was truncated and only 729 bp of the 3′ end of the *lmhmgr* gene were present. The complete coding sequence was obtained from a library of *L. major* genomic DNA constructed in λEMBL3 and screened with a PCR probe (691 bp) obtained from the cDNA sequence. Two positive plaques were carried through a tertiary screening, and one clone was selected for restriction mapping and further characterization. The clone contained a 13.5-kb *Bam*HI–*Bam*HI insert that possessed the complete coding region of the HMG-CoA reductase gene. A *Bam*HI–*Xho*I restriction fragment was identified that hybridized strongly with the PCR probe. This 2.395-kb segment was ligated into $pBSKS^-$ to give pHMG2.4 and sequenced on both strands by using universal and synthetic primers. The complete coding sequence (1302 bp), together with 500 and 593 bases of the 5′ and 3′ flanking regions, respectively, was established. Prediction of the initiation codon was based on the comparison of the *L. major* sequence with the *T. cruzi* HMG-CoA reductase sequence and the identification of non-coding regions by codon-usage studies. The nucleic acid sequence is highly conserved between these protozoan parasites (62 % identity). The open reading frame presented a codon usage that correlated closely with that determined for other *L. major* genes. Translation of the open reading frame of 1302 bp yielded a polypeptide of 434 residues with an estimated molecular mass of 45953 Da. The amino acid sequence was aligned with the sequences of *T. cruzi*, human, yeast, *Arabidopsis thaliana*, *Haloferax volcanii* and *Pseudomonas mevalonii* (Figure 1). The *lmhmgr* gene encoded for a protein similar to *T. cruzi* HMG-CoA reductase (80 % similarity), that lacks the membrane N-terminal domain characteristic of other eukaryotic enzymes.

Northern-blot analysis and genomic organization of the HMG-CoA reductase gene

A Northern blot of poly$(A)^+$ RNA extracted from the promastigote form of the parasite was performed by using the *lmhmgr* gene as a probe. The analysis indicated a transcript size of 4.4 kb. A prolonged exposure of the autoradiogram did not reveal any additional bands.

Genomic DNA from *L. major* cells was digested with different endonucleases, blotted and hybridized with the HMG-CoA reductase gene probe. Digestions with enzymes that cut at sites not contained within the coding region of the HMG-CoA reductase gene gave single bands, whereas two bands were obtained with enzymes with unique restriction sites in the coding region, therefore suggesting that the gene is of single copy.

The chromosomes of *Leishmania* range in size between 200 and 2000 kb [21] and can be resolved by CHEF electrophoresis. The *Leishmania* HMG-CoA reductase probe was used to hybridize a filter replicate of chromosomes of *L. major* under conditions that favoured the separation of larger chromosomal bands. As shown in Figure 2, HMG-CoA reductase sequences were located on a single chromosome of 1.4 Mb.

Purification and kinetic analysis

Table 1 summarizes the results of a typical purification. The enzyme was purified approx. 6-fold and represented 28 % overall recovery of activity. The HMG-CoA reductase preparation finally obtained had a specific activity of 32.9 $units \cdot mg^{-1}$. Enzyme purity was judged homogeneous by SDS/PAGE (Figure 3). The single band present on the gel had an apparent molecular mass of 46000 Da, a value in agreement with the DNA-derived protein sequence of HMG-CoA reductase. The molecular mass of the native purified protein was estimated, by exclusion chromatography, to be 184585 Da, a value consistent with a tetrameric subunit organization. Optimal activity of *Leishmania* HMG-CoA reductase was observed in the range of pH 5.7–7. K_m values for the overall reaction were 40.3 ± 5.8 μM for (*R*,*S*)-HMG-CoA

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Lmajor  ------------------------------------------------------------  0
Tcruzi  ------------------------------------------------------------  0
Human   DSVEEETGINRERKVEVIKPLVAETDTPNRATFVVGNSSLLDTSSVLVTQEPEIELPREP  442
Yeast   VQQKSAT----------IEFSETRSMPASSGLETPVTAKDIIISEEIQNNECVYALSSQD  591
Arath   ALIASFIYLLGFFGIDFVQSFISRASGDAW-----------DLADTIDDDDHRLVTCSPP  148
Hvolc   ------------------------------------------------------------  0
Pmev    ------------------------------------------------------------  0

Lmajor  --MRRSLLLACSAAKGESWASMSDTEIMKQVENKKIAFHGLEQALAPDYDRAIAIRREIV  58
Tcruzi  -MFRRAILLGCSAAKT-PWSECSNAQLVDAVKSRKISFYGLEQALEPDYRRAIEVRREVV  58
Human   RPNEECLQILGNAEKGAKF--LSDAEIIQLVNAKHIPAYKLETLMETH-ERGVSIRRQLL  499
Yeast   EPIRPLSNLVELMEK-EQLKNMNNTEVSNLVVNGKLPLYSLEKKLE-DTTRAVLVRRKAL  649
Arath   TPIVSVAKLPNPEPIVTESLPEEDEEIVKSVIDGVIPSYSLESRLG-DCKRAASIRREAL  207
Hvolc   --------------------MTDAASLADRVREGDLRLHELEAHADA--DTAAEARRLLV  38
Pmev    --------------------------------------MSLDSRLPAFRNLSPAARLDHI  22

Lmajor  KKKICPSPAATH---PLERVPYKNYDWSSVVGQSCENILGYVPVPVGLAGPLLLDGKEVA  115
Tcruzi  SEIASQQPEAKKKQSALHTIPFENYDWNKVVGQSCENILGYVPVPVGLAGPLLLDGKEVA  118
Human   SKKLSEP-------SSLQYLPYRDYNYSLVMGQNCENIIGYVPIPLGVAGPLLIDGKEYP  552
Yeast   STLAESPILVS------EKLPFRNYDYDRVFGACCENVIGYMPIPVGVAGPLCLDEKEFQ  703
Arath   QRVTGR---------SIEGLPLDGFDYESILGACCENVIGYMPIPVGVIGPLIIDGTSYH  88
Hvolc   ESQSGAS---------LDAVGNYGFPA-EAAESAIENMVGSIQVPMGVAGPVSVDGGSVA  258
Pmev    GQLLGLSHDDVSLLANAGALPMD------IANQCCEMPVGYIQIPVGIAGPLLLDGYEYS  76

Lmajor  LPMATTEGALVASAHRGARAINLSGGCRTAVLKEGMTRAPVVEVNSFDEAITVIKF--CE  173
Tcruzi  IPMATTEGALVASTHRGARAITRSGGCKTLLLGEGMTRAPVVELPSLEEAGRLHKY--CN  176
Human   VPMATTEGCLVASTNRGCRAIGLGGGASSRVTADGMTRGPVVRLPRACDSAEVKAWLETS  612
Yeast   IPMATTEGCLVASAMRGCKAINAGGGATTVLLKDGMTRGPVVRFPTLIRSGACKIWLDSE  763
Arath   VPMATTEGCLVASTNRGCKAMFISGGATSTVSKDGMTRAPVVRFASARRASELKFFLENP  318
Hvolc   LPLATTEGALLASVNRGCSVINSAGGATARVLKSGMTRAPVFRVADVAEAEALVSWTRDN  118
Pmev    VPLVVEEPSIVAAASYMAK-LARANGGFTTSSSAPLMHAQVQIVGIQDPLNARLSLLRRK  135

Lmajor  ----ERFDVLR---EAFESTTRFGKLLSIKCAMAGRQVHLRFSAFTGDAMGMNMITKGCD  226
Tcruzi  ----ENFLSLK---EAFESTTQYGKLNSLKCSLAGRKAYLRFRATTGDAMGMNMITKGVD  229
Human   ----EGFAVIK---EAFDSTSRFARLQKLHTCIAGRNLYIRFQSRSGDAMGMNMISKGTE  665
Yeast   ----EGQNSIK---KAFNSTSRFARLQHIQTTLAGDLLFMRFRTTTGDAMGMNMISKGVE  816
Arath   ----ENFDTLA---VVFNRSSRFARLQSVKCTIAGKNAYVRFCCSTGDAMGMNMVSKGVQ  371
Hvolc   FALKEAA-------EAAEETTNHGELLDVTPYVVGNSVYLRFRYDTKDAMGMNMATIATE  181
Pmev    DEIIELANRKDQLLNSLGGGCRDIEVHTFADTPRGPMLVAHLIVDVRDAMGANTVNTMAE  195

Lmajor  KALQVLQQHIP--SVRVLTLSGNFCTDKKPSALNWVEGRGKSVVAEAVIKRD-VVESVLK  283
Tcruzi  KALSVLQQHFP--SMEILALSGNYCTDKKPSAVNWIDGRGKSVVAEATLLAD-VVEDTLK  286
Human   KALSKLHEYFP--EMQILAVSGNYCTDKKPAAINWIEGRGKSVVCEAVIPAK-VVREVLK  722
Yeast   YSLKQMVEEYGWEDMEVVSVSGNYCTDKKPAAINWIEGRGKSVVAEATIPGD-VVKSVLK  875
Arath   NVLEYLTDDFP--DMDVIGISGNFCSDKKPAAVNWIEGRGKSVVCEAVIRGE-IVNKVLK  428
Hvolc   AVCGVVEAETAA---SLVALSGNLCSDKKPAAINAVEGRGRSVTADVRIPRE-VVEERLH  257
Pmev    -AVAPLMEAITGGQVRLRILS-NLADLRLARAQVRITPQQ---LETAEFSGEAVIEGILD  250

Lmajor  CTVDSVVSLNVTKNLRGSALAGSIGGFNAHAANIVAALYIATGQD--------PAQVVES  335
Tcruzi  CTVDSLVSLNIDKNLVGSAMAGSVGGFNAQAANAVAAIFIATGQD--------PAQVVES  338
Human   TTTEAMIEVNINKNLVGSAMAGSIGGYNAHAANIVTAIYIACGQD--------AAQNVGS  774
Yeast   SDVSALVELNISKNLVGSAMAGSVGGFNAHASNLVTALFLALGQD--------PAQNVES  927
Arath   TSVAALVELNMLKNLAGSAVAGSLGGFNAHAMNIVSAVFIATGQD--------PAQNVES  480
Hvolc   TTPERGRELNTRKNLVGSA-KAASLGFNAHVANVVAAMFLATGQD--------EAQVVEG  308
Pmev    AYAFAAVD-------------PYRAATHNKGIMNGIDPLIVATGNDWRAVEAGAHAYACRS  298

Lmajor  ATCMTTVDKAG---EDLVISLMMPSIEVGAVGGGTGLSS-QRAMLELMGCAGSNKEDPGA  391
Tcruzi  SMCITTMSKVG---NDLLISVTMPSIEVGVVGGGTGLAA-QRGCLELIGCGGPSKESPGT  394
Human   SNCITLMEASGPTNEDLYISCTMPSIEIGTVGGGTNLLP-QQACLQMLGVQGACKDNPGE  833
Yeast   SNCITLMKEVD---GDLRISVSMPSIEVGTIGGGTVLEP-QGAMLDLLGVRGPHPTEPGA  983
Arath   SQCITMMEAIN-DGKDIHISVTMPSIEVGTVGGGTQLAS-QSACLNLLGVKGASTESPGM  538
Hvolc   ANAITTAEVQD---GDLYVSVSIASLEVGTVGGGTKLPT-QSEGLDILGVSGG-GDPAGS  363
Pmev    GHYGSLTTWEKDNNGHLVGTLEMP-MPVGLVGGATKTHPLAQLSLRILGVKT--------  349

Lmajor  HSRQIARVVAGAVICGELSLLSGLAAGHLLSAHMKLNRKPPTP  434
Tcruzi  NAQLLSRVVAAGVLSAELSLMSGLAAGHLLSSHMRLNRKKK  435
Human   NARQLARIVCGTVMAGELSLMAALAAGHLVKSHMIHNRSKINLQDLQGACTKKTA  888
Yeast   NARQLARIIACAVLAGELSLCSALAAGHLVQSHMTHNRKTNKANELPQPSNKGPPCKTSA  1043
Arath   NARRLATIVAGAVLAGELSLMSAIAAGQLVRGHMKYNRSSRDISGATTTTTTTT  592
Hvolc   NADALAECIAVGSLAGELSLLSALASRHLSSAHAELGR  401
Pmev    -AQALAEIAVAVGLAQNLGAMRALATEGIQRGHMALHARNIAVVAGARGDEVDWVARQLV  408
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Figure 1 Comparison of the deduced amino acid sequence of *L. major* with other reductases

The deduced amino acid sequence of *L. major* HMG-CoA reductase is compared with the sequences of the *Trypanosoma cruzi* (Tcruzi, complete sequence), human (residues 383–888), yeast (yeast HMG-CoA reductase isoenzyme 2, residues 542–1043), *Arabidopsis thaliana* (Arath, residues 100–592), *Haloferax volcanii* (Hvolc, complete sequence) and *Pseudomonas mevalonii* (Pmev, residues 1–408) reductases. Solid boxes indicate regions of amino acid sequence identity.

and 81.4 ± 5.3 μM for NADPH, and the V_{max} was 33.55 ± 1.8 units·mg^{-1}. No activity was detectable when NADH was used instead of NADPH, even when high concentrations of both cofactor and enzyme were used. Lovastatin was a competitive inhibitor with respect to HMG-CoA with a K_i of 1.83 ± 0.005 nM.

Intracellular distribution of HMG-CoA reductase

The *L. major* promastigote extract was fractionated by differential centrifugation as described in the Experimental section. All of the fractions were assayed for HMG-CoA reductase activity. As

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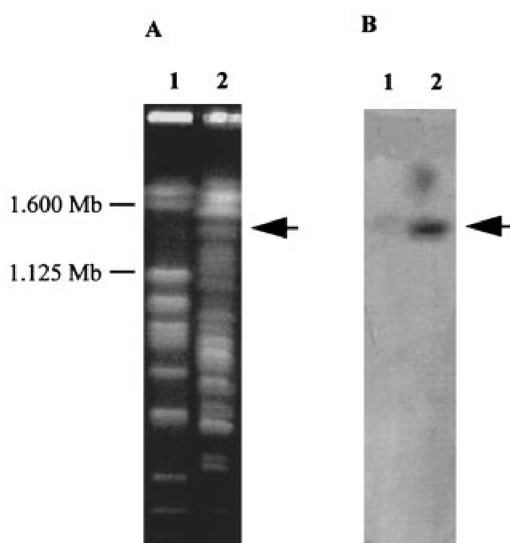

Figure 2 Chromosomal localization of the *lmhmgr* gene

(**A**) Ethidium bromide-stained gel. Lane 1, *Saccharomyces cerevisiae* as molecular-mass marker chromosomes; lane 2, *L. major* chromosomes. (**B**) Autoradiograph of a nylon transfer of the gel shown in (**A**) probed with the ^{32}P-labelled *lmhmgr* probe.

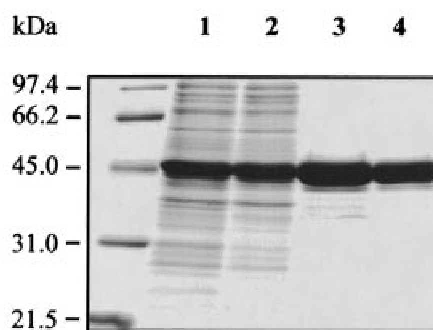

Figure 3 Purification of *L. major* HMG-CoA reductase

An SDS/polyacrylamide gel was stained with Coomassie Brilliant Blue. Lane 1, soluble extract BL21(DE3)/pETLMHMG; lane 2, ammonium sulphate 30–50% precipitate; lane 3, hydroxyapatite fraction; lane 4, ammonium sulphate 50% precipitate.

Table 2 Distribution of HMG-CoA reductase activity in *L. major* extracts

S12 refers to the whole supernatant obtained after sonication and centrifugation at 12000 ***g*** for 15 min, S105 and P105 refer to the supernatant and pellet, respectively, obtained after centrifugation of S12 at 105000 ***g*** for 60 min. Values are the means of four determinations. S.D. values did not exceed 8% of the mean value.

Fraction	Specific activity (nmol · min^{-1} · mg^{-1})	Total activity (nmol · min^{-1})
S12	8.87	77.22
S105	11.73	59.23
P105	1.82	5.12

shown in Table 2, *L. major* reductase specific activity in 105000 ***g*** supernatant was 6.5-fold that obtained in the pellet or 'membrane' fraction. When the total activity recovered in each fraction was calculated, 92% of the total enzyme activity was located in the supernatant. Similar results were found with *T. cruzi* [11], in contrast with what has been observed in other eukaryotic cells.

Modulation of HMG-CoA reductase activity by mevalonate-derived products

The regulation of HMG-CoA reductase in mammalian cells has been revealed through the use of potent reductase competitive inhibitors, such as lovastatin and compactin. In cultured cells, they block the synthesis of mevalonate and trigger adaptive reactions that result in increases in reductase protein within a few hours. The increase in enzyme can be reversed by incubating compactin-treated cells with mevalonate. Large amounts of mevalonate suppress reductase activity by nearly 99% [22,23].

To study the modulation of HMG-CoA reductase in *Leishmania*, we incubated *L. major* promastigotes with lovastatin, at concentrations near the EC_{50} of the inhibitor, and/or mevalonate. As shown in Table 3, the activity of HMG-CoA reductase was increased about 2- and 3-fold in promastigotes treated for 10 h with 40 and 80 μM lovastatin respectively. In the presence of both mevalonate and lovastatin, the activity was comparable with that of control cells. The changes in HMG-CoA reductase activity were associated with changes in the amount of reductase protein, as determined by immunoblot analysis (results not shown). Protein levels were increased 2–3-fold in cells grown in the presence of 40 and 80 μM lovastatin.

We determined whether HMG-CoA reductase was regulated by exogenous mevalonate, a modulator of the mammalian enzyme. When *L. major* promastigotes were grown with different concentrations of mevalonate for 12 h, reductase activity was not suppressed significantly (Table 3). Similar results were seen when the cells were treated with mevalonate for different times of incubation.

To examine further whether activation of the HMG-CoA reductase by lovastatin and reversal by mevalonate were associated with levels of reductase mRNA, *Leishmania* promastigotes

Table 1 Purification summary of recombinant HMG-CoA reductase

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units · mg^{-1})	Enrichment (-fold)	Yield (%)
Soluble extract	830.0	147.84	5.61	1.0	100
Ammonium sulphate (30–50%)	554.11	55.86	9.92	1.76	66.7
Hydroxyapatite	408.5	25.15	16.24	2.89	49.2
Ammonium sulphate (50%)	233.54	7.1	32.9	5.86	28.1

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Table 3 Regulation of HMG-CoA reductase activity in *Leishmania* by lovastatin and mevalonate

A series of cultures were incubated with lovastatin and/or mevalonate for 10–12 h. Cell suspensions were harvested, washed and incubated for 1 h with fresh medium to minimize the possibility of interference by residual lovastatin or mevalonate with reductase activity. Values are the means of four determinations. S.D. values did not exceed 8% of the mean value. The experiment was repeated twice with similar results.

Addition	HMG-CoA reductase activity (nmol · min^{-1} · mg^{-1})
Lovastatin (μM)	
0	4.79
40	8.18
80	12.68
80 + Mevalonate (10 mM)	4.43
Mevalonate (mM)	
0	4.03
5	3.86
10	3.26
20	3.1

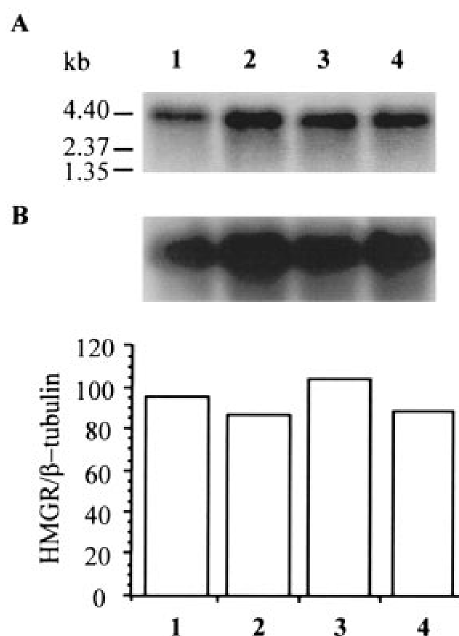

Figure 4 Analysis of HMG-CoA reductase mRNA in *Leishmania* treated with lovastatin (80 μM) and/or mevalonate (10 mM)

Northern-blot analysis of the *Leishmania* HMG-CoA reductase (**A**) and the β-tubulin (**B**) transcripts with ^{32}P-labelled specific probes. Approx. 7 μg of poly(A)$^{+}$ RNA were subjected to electrophoresis on the gel before transfer to nylon. Lanes 1, control cells; lanes 2, *L. major* grown in presence of lovastatin and mevalonate; lanes 3 and 4, cells grown in presence of lovastatin and mevalonate, respectively. The graph shows the ratio between the density of the bands corresponding to the HMG-CoA reductase and the β-tubulin transcripts. Control cells were set at a ratio of 100%. HMGR, HMG-CoA reductase.

were grown in the presence of lovastatin and/or mevalonate and poly(A)$^{+}$ RNA was prepared for hybridization analysis. As shown in Figure 4, no variations were seen in the levels of a specific HMG-CoA reductase transcript.

Table 4 Modulation of HMG-CoA reductase activity in *Leishmania* by availability of exogenous sterols and ketoconazole

To analyse the influence of lipoproteins on *Leishmania* reductase activity, promastigote forms were grown in medium containing 10% complete serum. After washing, cells were incubated in medium supplemented with 10% complete serum or 10% lipoprotein-poor serum for 24 h. Cells were treated with ketoconazole for 24 h in medium containing 10% complete serum. Values are the means of four determinations. S.D. values did not exceed 8% of the mean value.

Addition	HMG-CoA reductase activity (nmol · min^{-1} · mg^{-1})
Complete serum	6.44
Lipoprotein-poor serum	6.18
Ketoconazole (μM)	
0	8.81
0.5	10.93
1.0	13.38
1.5	13.75

Regulation of HMG-CoA reductase activity by availability of exogenous and endogenous sterols

In the absence of LDL, mammalian cells maintain high reductase activity whereas, when LDL is present, HMG-CoA reductase activity declines by more than 90%, and cells produce only the small amounts of mevalonate needed for non-sterol end-products [24]. To analyse the effect of serum sterols on reductase activity, log-phase promastigotes were harvested, washed and incubated for 24 h in medium supplemented with complete or lipoprotein-poor serum. As shown in Table 4, no differences were observed in cells grown in the presence of lipoprotein-poor serum. Similar results were obtained when promastigotes were adapted to grow in deficient medium. Consequently, serum sterol does not appear to be a regulator of the *Leishmania* enzyme, as occurs in mammalian cells.

Finally, we exploited ketoconazole as a tool to study the regulation of HMG-CoA reductase. This drug blocks ergosterol biosynthesis at the level of the cytochrome P450-dependent 14α-demethylation of lanosterol. In promastigote forms treated with different concentrations of ketoconazole for 24 h, reductase activity was increased by nearly 55% (Table 4). Similar effects were seen when cells were incubated for different time periods with ketoconazole (1 μM). The activation of the enzyme was associated with a parallel change in the amount of HMG-CoA reductase protein, as determined by Western-blot analysis (results not shown).

DISCUSSION

In the current paper we describe the isolation and characterization of the *lmhmgr* gene. The deduced amino acid sequence of the *Leishmania* enzyme was compared with other HMG-CoA reductases. Alignment analysis and hydrophobicity plots show clearly that the enzyme lacks the membrane N-terminal domain characteristic of eukaryotic enzymes. The shorter polypeptide encoded by *lmhmgr* exhibits pronounced similarity (80%) to the *T. cruzi* protein and all of the conserved residues involved in catalysis or binding identified in other HMG-CoA reductases are present in the *L. major* enzyme. The absence of a membrane-spanning domain and the existence of protease-sensitive reductase activity in the 105000 ***g*** supernatant firmly support that *L. major* HMG-CoA reductase is a soluble enzyme. The indications of a soluble reductase in *Leishmania* add to the observation that *T. cruzi* also lacks a membrane-spanning domain

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and that activity is located in the cellular soluble fraction [11] and suggest that this feature is a common characteristic of the Trypanosomatidae family. Recently, Concepción et al. have reported that the *T. cruzi* enzyme is soluble and that activity is associated mainly with the glycosomal subcellular fraction [25]. However, HMG-CoA reductase from *T. brucei*, another trypanosomatid, has been described as microsomal [26], which would contrast with what we have observed in two closely related protozoa. HMG-CoA reductase appears to be of single copy in both *T. cruzi* and *Leishmania* and Northern blots suggest the existence of only one transcript species. Therefore the existence of membrane-bound isoforms is highly improbable. More likely, the microsomal activity is unique to *T. brucei* or was an artifact, since in this organism reductase activity was two orders of magnitude lower than the values obtained in the present study. The gene coding for HMG-CoA reductase from *T. brucei* has not yet been characterized. The availability of recombinant enzyme will allow for more detailed cellular studies that would aid in establishing the definite location of the enzyme in *Leishmania*.

The purification procedure allowed for the production of highly purified protein available for structural and enzymological studies. Enzyme was relatively stable as an ammonium sulphate precipitate and could be stored as such for several weeks at −80 °C. The availability of highly stable homogeneous protein will allow for both structural and functional studies. In this sense, kinetic properties of protozoan reductase seem to resemble those of the mammalian enzyme (52% identity with the human enzyme); however, the lack of a membrane domain is unique to this kind of organism. The kinetic characterization of *L. major* HMG-CoA reductase showed that the K_m for NADPH was similar to those previously reported for other NADPH-dependent reductases. However, the K_m for HMG-CoA was one order of magnitude higher than the value described for the human enzyme [27] and the affinity for lovastatin was therefore 20000 times higher than its affinity for the natural substrate HMG-CoA.

When the molecular mass was estimated by gel-filtration experiments the value obtained was 184585 Da, suggesting that *L. major* HMG-CoA reductase is a tetramer, similar to what has been described in the case of the radish seedlings enzyme [28] and the recently described catalytic portion of the human enzyme [29].

We have analysed some of the mechanisms involved in the regulation of *Leishmania* reductase. HMG-CoA reductase activity was clearly increased in promastigotes incubated with lovastatin. Inhibition of growth rate and activation of enzyme activity mediated by lovastatin was reverted by mevalonate, suggesting that exogenous mevalonate is capable of satisfying the cell requirements for non-sterol, mevalonate-derived substances that are vital for diverse cellular functions. The lovastatin-mediated increase in HMG-CoA reductase activity was associated with a increase in the amount of protein, as determined by Western-blot analysis, although no variations were determined in the levels of reductase mRNA. In *Leishmania*, control of gene expression is generally achieved through post-transcriptional regulation [30]. Our data indicate that the increase in activity is attributable solely to an increase in the amount of enzyme without parallel changes in mRNA levels, which would agree with what has been observed previously for the control of expression of other genes in this organism.

The lack of a membrane-spanning domain might account for absence of mevalonate-mediated suppression of HMG-CoA reductase activity. Studies on the mammalian enzyme have shown that the membrane domain is essential for sterol- and non-sterol-mediated degradation [31,32]. Apparently, *Leishmania* HMG-CoA reductase is a very stable protein and is not subjected to degradation regulatory mechanisms. Coppens et al. [26] have reported in *T. brucei* that HMG-CoA reductase activity is decreased 2-fold in cells incubated with mevalonate. However, the present study performed measuring soluble enzyme activity shows that the *Leishmania* enzyme is not down-regulated by the addition of exogenous mevalonate.

Serum sterols have no effect upon *L. major* HMG-CoA reductase. A plausible explanation is that *Leishmania* lacks the ability to synthesize cholesterol *de novo* from mevalonate [3], thus this sterol is not a physiological regulator of the enzyme. Moreover, when ergosterol biosynthesis was inhibited by ketoconazole, HMG-CoA reductase activity was increased. Previous studies in *Leishmania* demonstrated that this drug causes accumulation of abnormal sterols with a 14α-methyl group [3]. Since ketoconazole is a cytochrome P450 inhibitor, the possibility exists that the drug prevents suppression of reductase activity from *L. major* by inhibiting a cytochrome P450-dependent formation of endogenous oxysterols. Alternatively, the activation of the enzyme could be caused by inhibition of the synthesis of end-products of the sterol metabolism, such as ergosterol. The ketoconazole-mediated increase in HMG-CoA reductase activity was associated again with an increase in the amount of protein.

In summary, *Leishmania* HMG-CoA reductase is up-regulated when sterol synthesis is inhibited by drug pressure and this activation is apparently performed via post-transcriptional control. The lack of sensitivity to mevalonate and sterols is consistent with the absence of a membrane domain and may be a consequence of unique biological properties of the isoprenoid biosynthetic pathway in protozoa. Trypanosomatids are early-branching eukaryotic cells and their cell organization differs considerably from that of mammalian cells. Specific features present in trypanosomatids but absent from their hosts may be exploitable in providing targets for rational drug design. Studies are currently underway to determine the exact intracellular localization of reductase as well as the characteristics of other enzymes involved in this metabolic route.

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