

3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase of *Sulfolobus solfataricus*: DNA Sequence, Phylogeny, Expression in *Escherichia coli* of the *hmgA* Gene, and Purification and Kinetic Characterization of the Gene Product†

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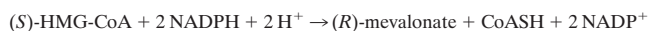
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The gene (*hmgA*) for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) from the thermophilic archaeon *Sulfolobus solfataricus* P2 was cloned and sequenced. *S. solfataricus* HMG-CoA reductase exhibited a high degree of sequence identity (47%) to the HMG-CoA reductase of the halophilic archaeon *Haloferax volcanii*. Phylogenetic analyses of HMG-CoA reductase protein sequences suggested that the two archaeal genes are distant homologs of eukaryotic genes. The only known bacterial HMG-CoA reductase, a strictly biodegradative enzyme from *Pseudomonas mevalonii*, is highly diverged from archaeal and eukaryotic HMG-CoA reductases. The *S. solfataricus* *hmgA* gene encodes a true biosynthetic HMG-CoA reductase. Expression of *hmgA* in *Escherichia coli* generated a protein that both converted HMG-CoA to mevalonate and cross-reacted with antibodies raised against rat liver HMG-CoA reductase. *S. solfataricus* HMG-CoA reductase was purified in 40% yield to a specific activity of 17.5 μ U per mg at 50°C by a sequence of steps that included heat treatment, ion-exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography. The final product was homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The substrate was (S)- not (R)-HMG-CoA; the reductant was NADPH not NADH. The K_m values for HMG-CoA (17 μ M) and NADPH (23 μ M) were similar in magnitude to those of other biosynthetic HMG-CoA reductases. Unlike other HMG-CoA reductases, the enzyme was stable at 90°C and was optimally active at pH 5.5 and 85°C.

Among prokaryotes there are two evolutionarily distinguishable groups, the *Bacteria*, or eubacteria, and the *Archaea*, or archaeobacteria (47, 48). The *Archaea* comprise two highly divergent subdivisions or kingdoms, the *Euryarchaeota* (includes methanogenic, halophilic, and some thermophilic *Archaea*) and the *Crenarchaeota* (includes extreme thermophiles and thermoacidophiles) (46). The members of the *Archaea* share important biochemical features with eukaryotes. These include similar transcription factors (33, 34, 36) and a pathway of isoprenoid biogenesis that involves biosynthesis of mevalonate by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34).

HMG-CoA reductase employs two equivalents of NADPH to reductively deacylate the thioester group of HMG-CoA to the primary alcohol of mevalonate:



In eukaryotes, this reaction is rate limiting for the biosynthesis of isoprenoids. Consequently, the sensitivity of HMG-CoA reductase to inhibition by drugs such as Lovastatin has been extensively exploited to treat hypercholesterolemias in human subjects (16). While numerous eukaryotic HMG-CoA

reductases have been studied, no true biosynthetic HMG-CoA reductase has been detected in bacteria. An NAD⁺-dependent, biodegradative HMG-CoA reductase from *Pseudomonas mevalonii* has, however, been characterized (2, 23), and its three-dimensional structure has been solved (32).

In members of the *Archaea*, HMG-CoA reductase serves a key biosynthetic role. Archaeal membranes are rich in ether-linked isoprenoid membrane lipids (13), whose phytanyl groups are derived from mevalonate (29). The first archaeal HMG-CoA reductase gene to be cloned was that of *Haloferax volcanii*, a representative of the kingdom *Euryarchaeota* (30, 31). The *H. volcanii* gene has been expressed in *Escherichia coli*, and its encoded HMG-CoA reductase has been purified to homogeneity and characterized (4, 5).

We report here the cloning and sequencing of *hmgA* from the thermoacidophile *Sulfolobus solfataricus* P2. This gene encodes the HMG-CoA reductase from a typical species of *Crenarchaeota*. The evolutionary relationship of the encoded enzyme to other HMG-CoA reductases has been analyzed. Following expression of *hmgA* in *E. coli*, the HMG-CoA reductase of *S. solfataricus* was purified to homogeneity and its enzymic properties were characterized. This thermostable HMG-CoA reductase offers potential for solution by X-ray crystallography of the first three-dimensional structure of a true biosynthetic HMG-CoA reductase.

MATERIALS AND METHODS

Chemicals. Purchased materials included the following: NADPH, NADH, (R,S)-HMG-CoA, phenylmethylsulfonyl fluoride, anti-rabbit immunoglobulin G-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, nitro

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blue tetrazolium chloride, and reactive red-120 agarose, Sigma; (*R,S*)-[3-¹⁴C] HMG-CoA and [γ -³²P]ATP, Amersham; [5-³H]mevalonolactone, New England Nuclear; restriction enzymes, *Thermus aquaticus* DNA polymerase, and T4 DNA ligase, NEB, Promega, or Bethesda Research Laboratories; isopropylthiogalactoside and T7 polymerase, U.S. Biochemicals; polynucleotide kinase and vector pIBI131, International Biotechnologies, Inc.; prestained low-range protein standards, Bio-Rad; GeneScreen Plus, Dupont; ProBlott polyvinylidene difluoride membranes, Applied Biosystems; butyl Sepharose 4 FF, SP Sepharose FF, Q Sepharose FF, and G-25 Sephadex, Pharmacia; silica gel thin-layer chromatography sheets, Kodak; and expression vector pET-21b, Novagen. (*S*)-HMG-CoA, (*R*)-HMG-CoA (3), and homogeneous catalytic domain Syrian hamster HMG-CoA reductase (20) were prepared as previously described. Polyclonal antibodies specific for rat liver HMG-CoA reductase were a gift from Joe Papiez and David Gibson, Department of Biochemistry, Indiana University School of Medicine, Indianapolis. Additional purchased materials were from previously listed sources (3, 4).

Conditions for growth of archaeal and bacterial strains. *S. solfataricus* P2 (DSM 1617) was cultured according to the method of Grogan (25). *E. coli* DH5 α was grown in 2 \times YT medium (38) supplemented with 50 μ g of ampicillin per ml when required. *E. coli* BL21(DE3) was grown on LB_{amp} medium (Luria-Bertani medium [38] plus 75 μ g of ampicillin per ml).

DNA manipulations. Standard protocols (38) were used for most DNA manipulations. Restriction enzymes and T4 ligase were used as recommended by the vendor. Synthetic oligonucleotides were labeled with [γ -³²P]ATP using polynucleotide kinase (22). DNA immobilized to GeneScreen Plus was hybridized to ³²P-labelled DNA probes under conditions recommended by the manufacturer. Double-stranded DNA cloned into the plasmid vector pIBI131 was sequenced by the dideoxy chain-termination method (39) using T7 DNA polymerase. Both DNA strands were sequenced in their entirety with a combination of internal oligonucleotide primers and ordered sets of deletions (27).

Cloning and sequencing of the *S. solfataricus* HMG-CoA reductase gene. Following digestion of *S. solfataricus* P2 genomic DNA with several restriction enzymes, the resulting DNA fragments were separated on 0.7% agarose gels and transferred to nylon membranes. The degenerate oligonucleotide HMG3 (5'-G A[C/T]GCG[G/A/T/C]ATGGG[G/A/C/T]ATGAA[C/T]ATG-3'), which encodes the conserved DAMGMNM motif characteristic of all HMG-CoA reductases, was used as a hybridization probe. An approximately 3.7-kb *EcoRI-MluI* fragment was chosen for cloning. The G/C-biased *MluI* recognition site A/CGCGT, which occurs at a relatively low frequency in the A/T-rich *S. solfataricus* genome, provided a cloning landmark.

In preparation for cloning, *EcoRI*-digested genomic DNA was applied to a 10 to 40% sucrose gradient and centrifuged at 35,000 rpm for 12 h in a Beckman SW40 swinging bucket rotor (38). Portions (300 μ l each) were removed, resolved on agarose gels, immobilized on nylon filters, and reprobed with HMG3. A strong hybridization signal was obtained for *EcoRI* fragments approximately 14 kb in size. These DNA fragments were digested with *MluI*, inserted into *EcoRI-MluI*-cut pIBI131, and transformed into *E. coli*. Transformed colonies were transferred to membrane filters and reprobed with HMG3. Several positive clones contained a 3.7-kb *EcoRI-MluI* fragment, of which a 1,885-bp section was sequenced.

Evolutionary analysis. HMG-CoA reductase sequences were obtained from public databases. DNA sequences were complete except for those of the fungi *Gibberella fujikuroi* and *Phycomyces blakesleeana* (10). Phylogenetic analyses used the complete sequences of the archaeobacteria *S. solfataricus* and *H. volcanii* and the bacterium *P. mevalonii*, as well as the sequences of the catalytic domains of eukaryotic HMG-CoA reductases. Amino acid sequences aligned by the program MULTALIN (9) were then edited by eye to better align certain consensus motifs. Maximum-parsimony analysis employed the program PAUP 3.1.1 (43). The confidence limits of branch points were estimated by 500 random bootstrap replications. Neighbor-joining distance analysis was performed with the PHYLIP 3.57 software package (17). Pairwise distances were estimated by the program PROTDIST with the "Dayoff" option invoked. The programs SEQBOOT, NEIGHBOR, and CONSENSE were used to derive neighbor-joining trees in which the confidence limits of branch points were estimated by 300 random bootstrap replications.

Integration of *hmgA* into the expression vectors pT7-7 and pET-21b. PCR amplification of *hmgA* employed *P. furiosus* DNA polymerase, *S. solfataricus* genomic DNA as the template, and the oligonucleotide primers HMG-F (5'-G CTGACATATGAAAATTGATGAA-3') and HMG-R (5'-CCTTAGGAATTC CAAGAACTTTTCTA-3'). HMG-F anneals at the 5'-end of *hmgA* and encodes an *NdeI* site (underlined) at the start codon of *hmgA* (boldfaced). HMG-R anneals downstream of the 3' end of *hmgA* and encodes an *EcoRI* site (underlined). The PCR product was digested with *NdeI* and *EcoRI* and subcloned into pT7-7 (44) and pET-21b to produce the expression vectors pT7-7(Sol)HMGR and pET-21b(Sol)HMGR, respectively. The presence in these expression vectors of *hmgA* was confirmed by Sanger dideoxynucleotide sequencing (39) at the Purdue Department of Biochemistry DNA Sequencing Facility.

Expression of *S. solfataricus* HMG-CoA reductase in *E. coli*. *hmgA* was initially expressed from pT7-7(Sol)HMGR in *E. coli* BL21(DE3) cells grown at 37°C on LB_{amp} medium, with shaking at 300 rpm, to a density of 80 Klett units (red filter). Isopropylthiogalactoside was then added to a concentration of 0.5 mM. When the culture had attained a density of 200 Klett units, the cells were harvested by

centrifugation. Cells were suspended in 10% (vol/vol) glycerol and 1.0 mM phenylmethylsulfonyl fluoride in buffer A (1.0 mM ethylenediaminetetraacetic acid in 20 mM K₂PO₄ [pH 7.0]) and ruptured by passage twice through a French pressure cell. The cell lysate was centrifuged (541,000 \times g, 8 min, 4°C), and precipitated debris was discarded. The supernatant liquid was heated to and maintained at 70°C for 10 min. Following centrifugation to remove denatured protein, the supernatant liquid was retained as the heat fraction.

Purification of *S. solfataricus* HMG-CoA reductase. *E. coli* BL21(DE3) cells containing pET-21b(Sol)HMGR were grown at 37°C on 30 liters of LB_{amp} medium, with stirring and vigorous aeration. When the culture had attained a density of 320 Klett units (red filter), the cells were concentrated by passage through a Pellicon cassette system tangential flow filtration device equipped with a 0.45- μ m-pore-diameter nitrocellulose Millipore filter and then harvested by centrifugation. Cells from 10 liters of culture expressing *S. solfataricus* HMG-CoA reductase from pET-21b(Sol)HMGR were suspended in 10% (vol/vol) glycerol and 1.0 mM phenylmethylsulfonyl fluoride in buffer A and ruptured by passage twice through a French pressure cell. The cell lysate was centrifuged (105,000 \times g, 60 min, 4°C). Portions (40 ml each) of the supernatant liquid were maintained at 80°C for 25 min, placed on ice, and centrifuged to remove denatured protein. The precipitates were washed with 4 volumes of buffer A. Following centrifugation, the initial supernatant liquid and wash were combined to give the heat fraction. The heat fraction was applied to a column (1.5 by 6.0 cm) of SP Sepharose FF in buffer A linked in series to a column (1.5 by 6.0 cm) of Q Sepharose FF in buffer A. Following elution with 100 ml of buffer A, material that flowed through both columns was combined to give the ion fraction. The ion fraction was adjusted to 0.82 M in (NH₄)₂SO₄ by adding 0.25 ml of saturated (NH₄)₂SO₄ solution (saturated at room temperature [pH 7.0 when diluted 1:20]) per ml. Precipitated protein was removed by centrifugation and discarded. The supernatant liquid was applied to a column (2.5 by 12 cm) of butyl Sepharose 4 FF in 0.82 M (NH₄)₂SO₄ in buffer A. The column was then washed with 200 ml of 0.82 M (NH₄)₂SO₄ in buffer A. Bound proteins were then eluted with a decreasing gradient of 0.82 to 0 M (NH₄)₂SO₄ in buffer A (400 ml total). Active fractions were combined and adjusted to 65% saturation in (NH₄)₂SO₄ by adding 1.86 ml of saturated (NH₄)₂SO₄ solution per ml. Precipitated protein was recovered by centrifugation and suspended in buffer A to give the butyl fraction. To remove (NH₄)₂SO₄, the butyl fraction was passed through a column (2.5 by 19 cm) of Sephadex G-25 equilibrated in buffer A. The desalted butyl fraction was applied to a column (1.5 by 12 cm) of reactive red-120 agarose equilibrated in buffer A. The column was washed with 150 ml of buffer A and then was eluted with a gradient of 0 to 1.5 M KCl in buffer A (400 ml total). Active fractions were combined and adjusted to 65% saturation in (NH₄)₂SO₄. Following centrifugation, the pellet was suspended in 10% glycerol in buffer A to give the red fraction.

Spectrophotometric assay of HMG-CoA reductase activity. The HMG-CoA-dependent oxidation of NADPH was monitored in a Hewlett-Packard model 8452A diode array spectrophotometer equipped with a cell holder adjusted to the desired assay temperature. Because of the instability of NADPH at pH 5.5 and elevated temperatures, we used an initial concentration of NADPH too great to be measured accurately at 340 nm ($\epsilon_{340} = 6,220 \text{ M}^{-1}\text{cm}^{-1}$). Disappearance of NADPH was therefore monitored at 366 nm ($\epsilon_{366} = 3,300 \text{ M}^{-1}\text{cm}^{-1}$). Standard assays (final volume, 200 μ l) contained 250 μ M (*R,S*)-HMG-CoA and 500 μ M NADPH in 100 mM potassium acetate–100 mM potassium 2-[*N*-morpholino]ethanesulfonic acid (pH 5.5). Reactions, which unless otherwise noted were conducted at 50°C, were initiated by adding HMG-CoA. Protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as the standard. One microunit of enzyme is defined as the amount that catalyzes the oxidation of 1 μ mol of NADPH per min.

Nucleotide sequence accession number. The GenBank accession number for the *hmgA* gene of *S. solfataricus* is U95360.

RESULTS

***hmgA* appears to encode an HMG-CoA reductase.** Sequencing of 1,885 bp of the 3.7-kb *EcoRI-MluI* subclone and comparison of this information to that in sequence databases showed that it contained the entire *S. solfataricus* HMG-CoA reductase gene, *hmgA*. *hmgA* encodes a 410-residue protein, similar in length to the 404-residue HMG-CoA reductase of the halophilic archaeon *H. volcanii* (4, 31). The derived amino acid sequence of *hmgA* showed significant sequence similarity to *H. volcanii* HMG-CoA reductase and to the catalytic domains of the eukaryotic enzymes (Fig. 1). Three residues, nested in conserved sequences, have been previously implicated in catalysis by the HMG-CoA reductases of *H. volcanii* (5), Syrian hamster (11, 21), and *P. mevalonii* (12, 45). These residues were also present in the derived amino acid sequence of *hmgA*. The sequences of the two archaeal HMG-CoA reductases were marginally more similar (47% identity) to each

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SS      1      MKIDEVVEKLVKGEISFHEVDNLLA--NAAMVARR---LALEKIVGVGLPSIGS
HV      MTDAASLADRVREGDLRLHELEAHADA--DTAAEARR---LLVESQSGASLDAVGN
H      ...FLSDAEIIQLVNAKHIPAYKLETLMET--HERGVSI RRQLLSTKLEPFPSSLQYLPY
DD      ...QKEHQRALHAQAVVAAA EKAATS GEDP--SSIQPVVPT--SNLDFEG-SLTNLPV
AT      ...PEEDEEIVKSVIDGVIPSYSLESRLGD--CKRAASIRRE--ALQRTVGRSIEGLPL
PM      MSLDSRLPAFRNLS PAARLDHIGQLLGLSHDDVSLLAN

                [ I ]                                [ II ]
SS      51      TVIDYSEIKNKNAENVIGAIQIPLGIVGPIRVNGDYAKGDFYVPMATTEGALIASVNRGI
HV      YGFP-AEAAESAIENVMGSIQVPMGVAGPVSVDGGSVAGEKYLPPLATTEGALLASVNRGC
H      RDYNYSLVMGACCENVIGYMPIPVGVAGPLCLDGK----EYQVPMATTEGCLVASTNRGC
DD      DHFDYTKVLGACCENVIGYIPIPVGVAGPILLDGK----LVSIPMATTEGCLVASTHRGA
AT      DGFDYESILGQCCEMPVGYIQIPVGIAGPLLLDGY----EYSVPMATTEGCLVASTNRGC
PM      AGALPMDIANGMIENVIGTFELPYAVASNFQINGR----DVLVPLVVEEPSIVAAASYMA

SS      111     KAVTLSGGVRAKVLKDEMTRAPVFKFDSIEQIPNFLKFIE--ENLEKIR-----NIAN
HV      SVINSAGGATARVLKSGMTRAPVFRVADVAEAEALVSWTR--DNFAALK-----EAAE
H      RAIGLGGGASRVLADGMTRGPVVRVLRPRACD SAEVKAWLETPEGFAVIK-----DAFD
DD      KAITKSGGAKTVLLQSGMTRAPVCRLPSSIRAGELKQWIENQENFYQVA-----SAFN
AT      KAMFISGGATSTVLKDG MTRAPVVRFASARRASELKFLENPENFDTLA-----VVFN
PM      K-LARANGGFTTSSSAPLMHAQVQIVGIQDPLNARLSLLRRKDEI IELANRKDQLLSNLG

                [ III ]
SS      162     STSHHGKLSITPFVLGNNVWLRFSFETGDDAMGMNMVTIAVEKVCFEIEENFPSADCLAV
HV      ETTNHGELLDVT PYVVGNSVYLRFYDTRDAMGMNMAT IATEAVCGVVEAE-TAASLVAL
H      STSRFARLQKLHVTMAGRNLYIRFQSKTDAMGMNMISKGTEKALVKLQEFFPEMQILAV
DD      STSRFARLKSIKVVIAGRLVYLRFKSSTDAMGMNMVSKGVEKALEVITEYFPEMEVLSL
AT      RSSRFARLQSVKCTIAGKNAYVRFCCSTDAMGMNMVSKGVQNVLEYLTD DFPDMDVIGI
PM      GGCRDIEVHTFADTPRGFMLVAHLIVDVRDAMGANTVNTMAEAVAPLMEAITGGQVRLRI

SS      222     SGNMCSDDKKQTNVNSLFGRGKTVLAEALIKKDVIRNIIHSNAQLIHDINLRKNWLGTARA
HV      SGNLCSDDKKPAAINAVEGRGRSVTADVRIPREVVEERLHTTPERGRELNTRKNLVGSAKA
H      SGNYCTDKKPAAVNWIEGRGKTVVCEAVI PARVVREVLKTTTEAMIDVNNKNLVGSAMA
DD      SGNVCTDKKPSINWLEGRGKSVVAEAVISGDIVRDVLKTTVEALVSLNIDKNLIGSAMA
AT      SGNFCSDDKKPAAVNWIEGRGKSVVCEAVIRGEIVNKVLKTSVAALVELNMLKLAGSAVA
PM      LSNLADLRLARAQVRITPQQ--LETAEFSGEAVIEGILDAYAFAAVD-----P

SS      282     GSLSQFN AHFANIVTAIFIATGQD-----VAQIVES-SSGYTWTEV---RGEDLYISVT
HV      ASLG-FNAHVANVVAAMFLATRQD-----EAQVVEG-ANAITAEV---QDGDLYVSVS
H      GSIGGYNAHAANIVTAIYIACGQD-----AAQNVGS-SNCITLMEASGPTNEDLYISCT
DD      GSIGGFNAHASNIVTALYIATGQD-----PAQNVES-SNCITLMESIN-GGKDLYISVT
AT      GSLGGFNAHASNIVSAVFIATGQD-----PAQNVES-SQCITMMEAIN-DGKDIHISVT
PM      YRAATHNKGIMNGIDPLIVATGNDWRAVEAGAHAYACRSGHYGSLLTWEKDNNGHLVGLT

                [ IV ]
SS      332     LPSLEVGTVGGGTRLPTQKE-ALSIMGVYGSNPPG SNAKKLAEIIASTVLSGELNLLA
HV      IASLEVGTVGGGTKLPTQSE-GLDILGVSGGG-DPAGSNADALAECIAVGS LAGELSLLS
H      MPSIEIGTVGGGTNLLPQQA-CLQMLGVQGACKDNPGENARQLARIVCGTVMAGELSLMA
DD      MPSIEVTVGGGTHLPAQSA-CLDLLKIRGANLERPGANSEQLARVVAAAVLSGELSLMS
AT      MPSIEVTVGGGQLASQSA-CLNLLGVKGASTESPGMNARRLATIVAGAVLAGELSLMS
PM      EMPMPVGLVGGATKTHPLAQLSLRILGVKT-----AQA LAEIAVAVGLAQN LGAMR

SS      390     ALSNKELGKAHAKLGRAMKV
HV      ALASRHLSSAHAELGR
H      ALAAGHLVRSHMVHNRSKIN      [+ 13]
DD      ALAAGHLVRSHLKHNRKTEA      [+ 18]
AT      AIAAGQLVRSHMKYNRSSRD      [+ 12]
PM      ALATEGIQRGHMALHARNIA      [+ 37]

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FIG. 1. Amino acid sequences of selected HMG-CoA reductases. Alignments generated by the Pileup program of the Wisconsin package (14) are for sequences for the HMG-CoA reductases from *S. solfataricus* (SS), *H. volcanii* (HV), and *P. mevalonii* (PM) and for the catalytic domains of the enzymes from Syrian hamster (H), *Arabidopsis thaliana* (AT), and *Dictyostelium discoideum* (DD). Bracketed Arabic numerals indicate that sequences extend for the indicated number of residues beyond the last residue shown. Residues underlined and in boldface are those that have been implicated by mutagenesis and kinetic analyses as functioning in catalysis by *P. mevalonii* (12, 45), hamster (11, 21), and *H. volcanii* HMG-CoA reductase (5). Additional highlighted sequences are those that based on the crystal structure of the *P. mevalonii* enzyme (32), have been proposed to form the binding site for (S)-HMG-CoA ([I] and [II]) and for reduced and oxidized NAD(P) ([III] and [IV], respectively). Numbers refer to residues of the *S. solfataricus* enzyme.

TABLE 1. Pairwise comparisons of the encoded amino acid sequences of selected HMG-CoA reductase genes

Source of HMG-CoA reductase gene	Identity with ^a :					
	<i>S. solfataricus</i>	<i>H. volcanii</i>	<i>Cricetulus griseus</i>	<i>D. discoideum</i>	<i>A. thaliana</i>	<i>P. mevalonii</i>
<i>S. solfataricus</i>		0.807	1.093	0.938	0.942	2.951
<i>H. volcanii</i>	0.469		1.098	1.032	1.049	2.781
<i>C. griseus</i>	0.410	0.410		0.571	0.588	2.466
<i>D. discoideum</i>	0.434	0.420	0.596		0.533	2.536
<i>A. thaliana</i>	0.437	0.422	0.584	0.607		2.566
<i>P. mevalonii</i>	0.178	0.195	0.193	0.184	0.189	

^a Tabulated above the diagonal are estimated numbers of amino acid substitutions per site, calculated with the "Dayhoff 120" option in the program PROTDIST of the PHYLIP 3.57 package (17). Shown below the diagonal are the fractions of identical amino acids.

other than to the eukaryotic sequences (41 to 44% identity) (Table 1). In contrast, both archaeal HMG-CoA reductases shared less than 20% amino acid identity with the only known bacterial homolog, that from *P. mevalonii*. Figure 2 illustrates the results of phylogenetic analyses of available HMG-CoA reductase sequences by maximum-parsimony and neighbor-joining analyses.

Also sequenced were 328 bp upstream and 325 bp downstream of *hmgA*. The sequence TTTATA, present in an archaeal promoter for the 16S rRNA gene (26), lay 61 to 66 bp upstream of the AUG initiation codon. The putative Shine-Dalgarno motif AGCUGA began 9 bp upstream of the AUG initiation codon.

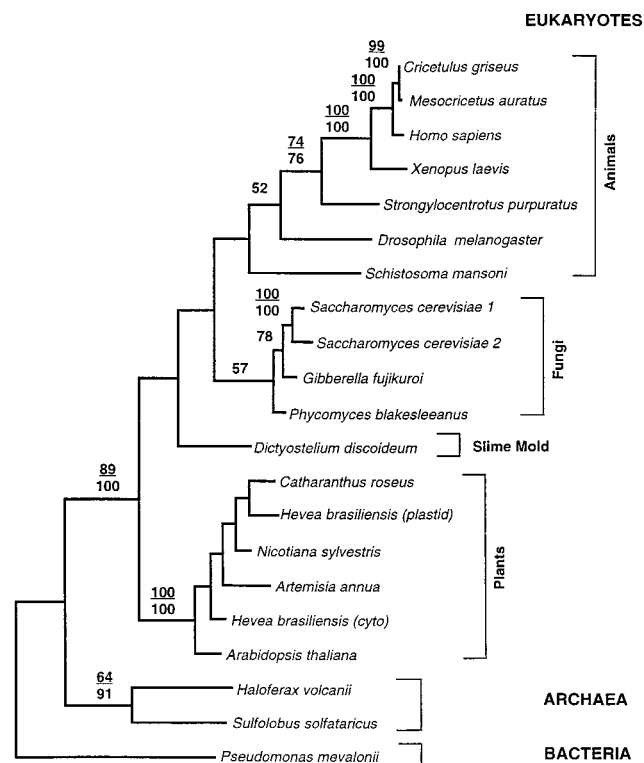


FIG. 2. Minimal-length maximum-parsimony tree of HMG-CoA reductase protein sequences. The single shortest tree was 1,770 steps long. Top numbers or single numbers at branch points represent the percent occurrence of nodes in 500 bootstrap replications of the maximum-parsimony analysis. Bottom numbers show the percent occurrence of the same nodes in 300 bootstrap-replicated neighbor-joining trees based on pairwise estimates of the number of amino acid changes per site. Only bootstrap occurrences greater than 50% in either analysis are shown.

A downstream open reading frame appears to encode serine hydroxymethyltransferase. Inspection revealed no significant open reading frame upstream of *hmgA*. However, a second transcriptional promoter sequence, TTTAAA, and a second open reading frame were located 14 and 43 bp downstream of the termination codon of *hmgA*, respectively. The sequenced 282 bp of the open reading frame appeared to encode a portion of serine hydroxymethyltransferase (glycine hydroxymethyltransferase [EC 2.1.2.1]) (42). The serine hydroxymethyltransferase genes of eukaryotes and bacteria encode 414 to 483 residue proteins. BLASTX scores (1) suggested that *S. solfataricus* serine hydroxymethyltransferase was most similar to the bacterial enzymes.

Evidence for expression. Portions of high-speed supernatant liquid and of the heat fraction from cells expressing pT-7(Sol) HMGR were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting with antibodies directed against rat liver HMG-CoA reductase revealed a reactive band of approximately the predicted molecular weight. The heat fraction also converted [¹⁴C]HMG-CoA to a ¹⁴C-labeled compound whose chromatographic mobility coincided both with that of an internal standard of [³H]meva-

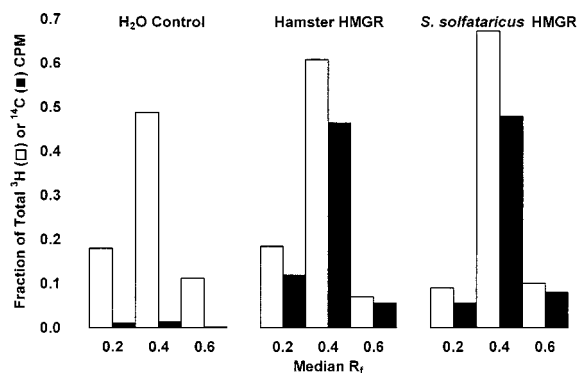


FIG. 3. Mobility of the ¹⁴C-product formed corresponds to that of authentic [³H]mevalonate. Incubations contained the following in a final volume of 28 μ l: 100 μ M (*R,S*)-[3-¹⁴C]HMG-CoA (specific activity, 58 μ Ci/ μ mol), 1.1 mM NADPH, 1.5 nCi of (*R,S*)-[5-³H]mevalonate, 5 mM dithiothreitol, 100 mM KCl, 100 mM K₂PO₄, 100 mM Tris, and 100 mM glycine (pH 5.5). Reactions were initiated by adding 2.0 μ l of H₂O, 1.7 μ g of the heat fraction of *S. solfataricus* HMG-CoA reductase, or 1.2 μ g of purified hamster HMG-CoA reductase. Incubations were for 3 min at 37°C (hamster enzyme) or at 80°C (water control and *S. solfataricus* enzyme). Following termination of incubations by addition of 8 μ l of 6 N HCl, samples were subjected to silica gel thin-layer chromatography in toluene-acetone (1:1) (40). Strips (2 cm wide) were then cut out and counted for total ¹⁴C and ³H in a Beckman model LS1801 scintillation spectrometer. Shown is the fraction of the total ³H and ¹⁴C recovered at the indicated median R_f values.

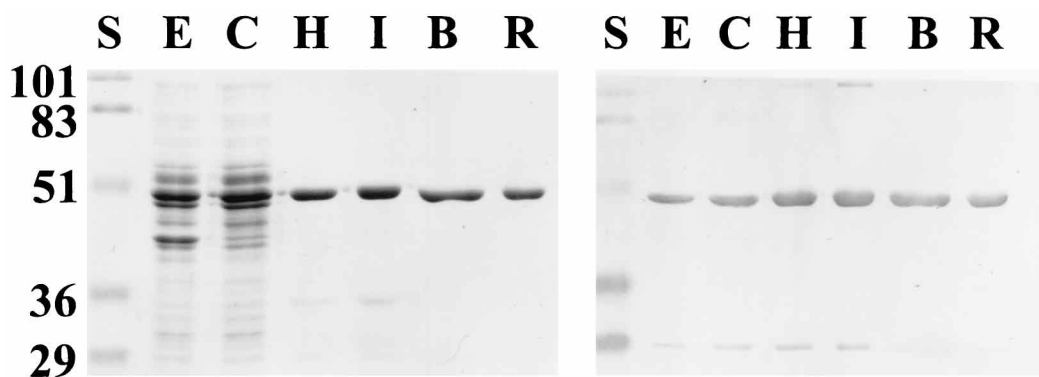


FIG. 4. SDS-PAGE and Western blotting of fractions from the purification of *S. solfataricus* HMG-CoA reductase. (Left) The gel was stained with Coomassie blue. Lanes contained prestained protein standards (S) of the indicated molecular mass in kilodaltons, crude extract (E), cytosol (C), heat fraction (H), ion fraction (I), butyl fraction (B), and red fraction (R). (Right) Western blotting of an unstained duplicate of the preceding gel employed rabbit polyclonal antibodies raised against rat liver HMG-CoA reductase as the primary antibody and anti-rabbit immunoglobulin G alkaline phosphatase conjugate as the secondary antibody. The blot was developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride (38).

lonate and with [^{14}C]mevalonate formed simultaneously by purified Syrian hamster HMG-CoA reductase (Fig. 3).

Purification yields homogeneous *S. solfataricus* HMG-CoA reductase. Figure 4 and Table 2 summarize the results of a typical purification. A portion of the red fraction was subjected to SDS-PAGE, transferred to a ProBlott polyvinylidene difluoride membrane, and submitted for N-terminal sequencing (six cycles) at the Purdue Laboratory for Macromolecular Structure. The sequence Met Lys Ile Asp Glu Val Val of the major band (Fig. 4) corresponded to the first six residues of the translated sequence of *hmgA*.

Kinetic parameters. Optimal activity of the red fraction for the reductive deacylation of HMG-CoA was observed at approximately pH 5.5 (Fig. 5). The substrates were (*S*)-HMG-CoA and NADPH. No activity was detectable when NADH was substituted for NADPH or when (*R*)-HMG-CoA replaced (*S*)-HMG-CoA. K_m values for the overall reaction, calculated from double-reciprocal plots, were 23 μM for NADPH and 17 μM for HMG-CoA, and V_{\max} was 16.7 μU per mg at 50°C.

Temperature profile for activity and for stability. Optimal activity of the red fraction was observed at approximately 85°C. The activation energy ΔH for the reaction was approximately 47 kJ (11 kcal) per mol (Fig. 6, left). The half-life of the red fraction ranged from 150 h at 40°C to 3.2 h at 90°C (Fig. 6, right).

DISCUSSION

Although less suitable as a phylogenetic reporter than rRNAs (46), elongation factors (28), ATPase subunits (24), RNA polymerases (35), or aminoacyl-tRNA synthetases (7), the HMG-CoA reductase tree supports the majority view, that of an *Archaea*-eukaryote clade, if a midpoint rooting is as-

TABLE 2. Summary of a typical purification of *S. solfataricus* HMG-CoA reductase^a

Fraction	Activity (μU)	Amt of protein (mg)	Sp act ($\mu\text{U}/\text{mg}$)	Enrichment (fold)	Yield (%)
Cytosol	1,330	3,700	0.36	1.0	100
Heat	870	440	2.0	6	65
Ion	850	290	3.0	8	64
Butyl	690	110	6.2	17	52
Red	500	29	17.5	49	38

^a The data are for the purification of the enzyme from 10 liters of culture.

sumed (15). The gene for HMG-CoA reductase was also found in *Methanococcus jannaschii*, an archaeon of the kingdom *Euryarchaeota* whose complete genome sequence was published while this paper was in preparation (8). However, a particular concern is the lack of bacterial sequences other than that of *P. mevalonii* (2), a γ -purple bacterium (46). No HMG-CoA reductase gene is present in the whole genome sequence of *Haemophilus influenzae* (18), also a γ -purple bacterium, or in that of the gram-positive bacterium *Mycoplasma genitalium* (19). (Note that the putative attribution of sequence MG085 of *M. genitalium* as HMG-CoA reductase [19] probably is incorrect, since the translated sequence lacks the DAMG, ENVIG, and GTVGG signature sequences and the catalytic Glu, Asp, or His is not readily apparent.) The HMG-CoA reductase gene thus has either been selectively lost from several bacterial lineages or has been recently acquired by *Pseudomonas* through either horizontal gene transfer or convergent evolution (41) and represents a unique adaptation for the utilization of mevalonate as a carbon source (23).

Biochemical evidence supports the sequence-based inference that the *hmgA* gene product is a true biosynthetic HMG-CoA reductase. The expression of *hmgA* in *E. coli* was accompanied by synthesis of a protein that converted HMG-CoA to

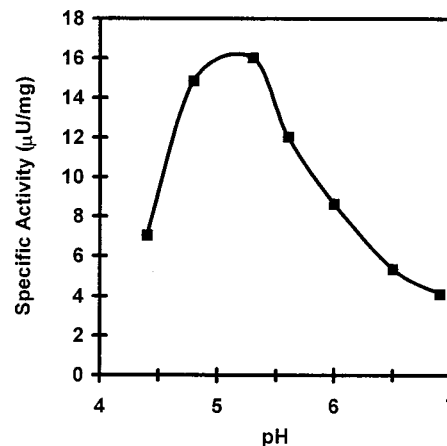


FIG. 5. Effect of pH on the activity of purified *S. solfataricus* HMG-CoA reductase. Assays of HMG-CoA reductase activity of the red fraction were conducted at the indicated pH, but under otherwise standard conditions.

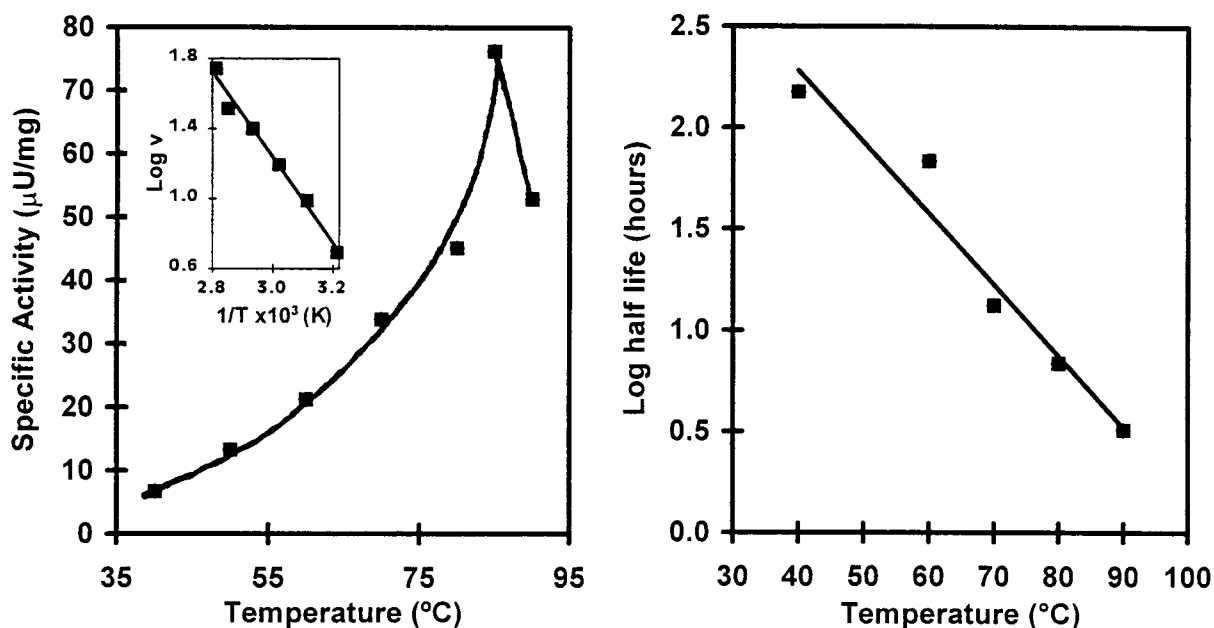


FIG. 6. Effect of temperature on activity and stability of *S. solfataricus* HMG-CoA reductase. (Left) Assays of HMG-CoA reductase activity of the red fraction were conducted at the indicated temperatures under otherwise standard conditions. The data appeared to be linear over the range of 40 to 85°C. The inset shows an Arrhenius plot of the data over this range. (Right) Portions of concentrated red fraction (2.8 mg/ml) were maintained at the indicated temperatures for 4 to 80 h. Following a 1:10 dilution into 10% glycerol in buffer A at 50°C, residual activity was determined at 50°C under standard assay conditions. Shown is the log of the half-life at the indicated temperatures.

mevalonate and cross-reacted with antibodies raised against rat liver HMG-CoA reductase. The *S. solfataricus* HMG-CoA reductase was purified in an approximately 40% yield to a specific activity of 17.5 μ U per mg at 50°C by successive heat treatment and chromatography on ion-exchange, hydrophobic interaction, and affinity chromatographic supports. The final product, the red fraction, appeared to be homogeneous as judged by SDS-PAGE.

S. solfataricus HMG-CoA reductase exhibits a substrate specificity that is typical for biosynthetic HMG-CoA reductases. The substrate is (*S*)- not (*R*)-HMG-CoA, and the reductant is NADPH not NADH. In addition, the K_m values for HMG-CoA and NADPH parallel those for characterized biosynthetic HMG-CoA reductases (Table 3). Significant differences from other HMG-CoA reductases include high thermal stability and different optimal temperatures and optimal pHs for activity. As might be anticipated for an enzyme from a thermophile, concentrated solutions of *S. solfataricus* HMG-CoA reductase are stable for several hours at temperatures as high as 90°C, and the activity of dilute solutions is optimal at 85°C. While no activation energies have been reported for

other soluble HMG-CoA reductases, an activation energy of 47 kJ per mol is similar to the 55 kJ per mol for *S. solfataricus* glyceraldehyde 3-phosphate dehydrogenase (37). *S. solfataricus* HMG-CoA reductase is optimally active at pH 5.5, a pH significantly below the pH 7.3 optimum of the only other characterized archaeal HMG-CoA reductase, that of the halophile *H. volcanii* (4), and the optimal pH of 6.1 for the Syrian hamster enzyme (11).

While HMG-CoA reductase serves essential anabolic roles in both archaeobacteria and eukaryotes, the only form of the enzyme whose crystal structure has been solved is that of the biodegradative HMG-CoA reductase of *P. mevalonii* (32). Since the purified catalytic domain of the Syrian hamster enzyme (20) lacks long-term stability, a purified thermostable HMG-CoA reductase offers significant potential for solution by X-ray crystallography of the three-dimensional structure of a true biosynthetic HMG-CoA reductase.

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TABLE 3. Comparison of the kinetic parameters of *S. solfataricus* HMG-CoA reductase with those of other biosynthetic HMG-CoA reductases^a

Source of HMG-CoA reductase	V_{max} (μ U/mg)	K_m (μ M) for:	
		NADPH	HMG-CoA
<i>S. solfataricus</i> ^b	16.7	23	17
<i>H. volcanii</i>	34	66	60
Syrian hamster	37	35	4.3

^a Kinetic parameters determined at 37°C are from reference 4 for *H. volcanii* and reference 21 for Syrian hamster.

^b For the *S. solfataricus* enzyme, assays were conducted at 50°C.

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