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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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 mevaloni, 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. HMG-CoA reductase purified from S. solfataricus HMG-CoA reductase was purified in 40% yield to a specific activity of 17.5 μU 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 μM) and NADPH (23 μM) 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 archaeabacteria (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. 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 mevaloni 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 thermophilic 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 some of 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...
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, Thermo Scientific aquatic DNA polymerase, and T4 DNA ligase, NEB, Promega, or Bethesda Research Laboratories; isopropylthiogalactoside, Riedel-de Haën, U.S. Biochemicals; silica gel thin-layer chromatography sheets, Kodak; and expression vector pET-21b, Novagen. (S)-HMG-CoA, (R)-HMG-CoA (3), and homologous catalytic domain Syrian hamster HMG-CoA reductase (20) were prepared as previously described. Polyclonal antibodies specific for the Syrian hamster HMG-CoA reductase were a gift from Joe Papiz 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 (DSM 1617) was cultured according to the method of Grogan (25). E. coli DH5α was grown in 2× YT medium (38) supplemented with 50 μg of ampicillin per ml when required. E. coli BL21(DE3) was grown on LB medium (Luria–Bertani medium) (38) plus 75 μg 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 T4 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 pBluescript was sequenced by dideoxy chain-termination method (39) using T7 DNA polymerase. Both DNA strands were sequenced in their entirety using 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/T/C/G/A/T/C/G/ATGGG/G/A/C/T/G/ATGA/C/A/G/T-G-3'), which encodes the conserved DAMGMMN 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 GC-biased M13I recognition site A/CCCGT, which occurs at a relatively low frequency in the A/T-rich S. solfataricus genome, provided a cloning landmark.

Integration of hmgA into the expression vectors pET-7.7 and pET-21b. PCR amplification of hmgA employed a Pfu DNA polymerase, S. solfataricus genomic DNA as the template, and the oligonucleotide primers HMG-F (5'-G C/T/G/A/C/T/G/ATGGG/G/A/C/T/G/ATGA/C/A/G/T-G-3') and HMG-R (5'-G/C/T/G/A/C/T/G/AAATGGGGAATGC/CCAGAAACCTTCTTCA-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 pET-7.7 (44) and pET-21b to produce the expression vectors pET-7.7(Sol)HMG and pET-21b(Sol)HMG, respectively. The presence in these expression vectors of hmgA was confirmed by Sanger dideoxy nucleotide 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 pET-7.7(Sol)HMG in E. coli BL21(DE3) cells grown at 37°C on LB medium, with shaking at 300 rpm, to a density of 80 Klett units (red filter), and maintained at 30°C for 20 h. Following centrifugation to remove denatured protein, the supernatant liquid was retained as the heat fraction.

Sulfobolus solfataricus HMG-CoA reductase. E. coli BL21(DE3) cells containing pET-21b(Sol)HMG were grown on LB medium, with shaking and vigorous aeration. When the culture had a density of 200 Klett units (red filter), the cells were harvested by passage twice through a French pressure cell. The cell lysate was centrifuged at 105,000 × g for 30 min, and the supernatant liquid was maintained at 70°C for 10 min. Following centrifugation to remove denatured protein, the supernatant liquid was retained as the heat fraction.

Sulfobolus solfataricus HMG-CoA reductase was applied to a column (1.5 by 60 cm) of SP Sepharose FF in buffer A linked in series to a column (1.5 by 60 cm) of Q Sepharose FF in buffer B. Following elution with 100 ml of buffer A, material that flowed through both columns was combined to give the ion fraction.

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 archaean H. volcanii (4, 31). The derived amino acid sequence of hmgA contained 250 M(6,220 M) proteins, similar in length to the 404-residue HMG-CoA reductase of H. volcanii (4, 31). The derived amino acid sequence of hmgA contained 250 M(6,220 M) proteins, similar in length to the 404-residue HMG-CoA reductase of H. volcanii (4, 31).
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.
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. A downstream open reading frame appears to encode serine hydroxymethyltransferase.

Evidence for expression. Portions of high-speed supernatant liquid and of the heat fraction from cells expressing pT-7(Sol) HMG-CoA reductase 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]mevalonate. Incubations contained the following in a final volume of 28 ml: 100 mM (R,S)-[3-14C]HMG-CoA (specific activity, 58 mCi/mmol), 1.1 mM NADPH, 1.5 nCi of (R,S)-[5-3H]mevalonate, 5 mM dithiothreitol, 100 mM KCl, 100 mM K$_2$PO$_4$, 100 mM Tris, and 100 mM glycine (pH 5.5). Reactions were initiated by adding 2.0 ml of H$_2$O, 1.7 mg of the heat fraction of *S. solfataricus* HMG-CoA reductase, or 1.2 mg 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 μl 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 $^1$4C and $^3$H in a Beckman model LS1801 scintillation spectrometer. Shown is the fraction of the total $^1$H and $^1$4C recovered at the indicated median $R_f$ values.

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

<table>
<thead>
<tr>
<th>Source of HMG-CoA reductase gene</th>
<th>Identity with $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. solfataricus</td>
<td>0.807</td>
</tr>
<tr>
<td>H. volcanii</td>
<td>1.093</td>
</tr>
<tr>
<td>C. griseus</td>
<td>0.410</td>
</tr>
<tr>
<td>D. discoideum</td>
<td>0.434</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>0.437</td>
</tr>
<tr>
<td>P. mevalonii</td>
<td>0.178</td>
</tr>
</tbody>
</table>

$^a$ 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.
lonate and with [14C]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 ΔΗ 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 assumed (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...
mevalonate and cross-reacted with antibodies raised against rat liver HMG-CoA reductase. The \textit{S. solfataricus} HMG-CoA reductase was purified in an approximately 40% yield to a specific activity of 17.5 \( \mu \text{U per mg} \) 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.

\textit{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 \textit{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 \textit{S. solfataricus} glyceraldehyde 3-phosphate dehydrogenase (37).

\textit{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 \textit{H. volcanicii} (4), and the optimal pH of 6.1 for the Syrian hamster enzyme (11).

While HMG-CoA reductase serves essential anabolic roles in both archaebacteria and eukaryotes, the only form of the enzyme whose crystal structure has been solved is that of the biodegradative HMG-CoA reductase of \textit{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.

**ACKNOWLEDGMENTS**

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The enzymology data are from the Ph.D. thesis of D.A.B. We thank our colleagues Dan Gilchrist, Dong-Yul Kim, Kenneth Li, Ronald Somerville, and Yunhui Yang for helpful suggestions.

**REFERENCES**


**TABLE 3. Comparison of the kinetic parameters of \textit{S. solfataricus} HMG-CoA reductase with those of other biosynthetic HMG-CoA reductases**

<table>
<thead>
<tr>
<th>Source of HMG-CoA reductase</th>
<th>( V_{\text{max}} ) (( \mu \text{U/mg} ))</th>
<th>( K_m ) (( \mu \text{M} )) for:</th>
<th>( K_m ) (( \mu \text{M} )) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. solfataricus} ( a )</td>
<td>16.7</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>\textit{H. volcanicii}</td>
<td>34</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>Syrian hamster</td>
<td>37</td>
<td>35</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\( a \) Kinetic parameters determined at 37°C are from reference 4 for \textit{H. volcanicii} and reference 21 for Syrian hamster.

\( a \) For the \textit{S. solfataricus} enzyme, assays were conducted at 50°C.

**FIG. 6.** Effect of temperature on activity and stability of \textit{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.