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Dual coenzyme specificity of *Archaeoglobus fulgidus* HMG-CoA reductase

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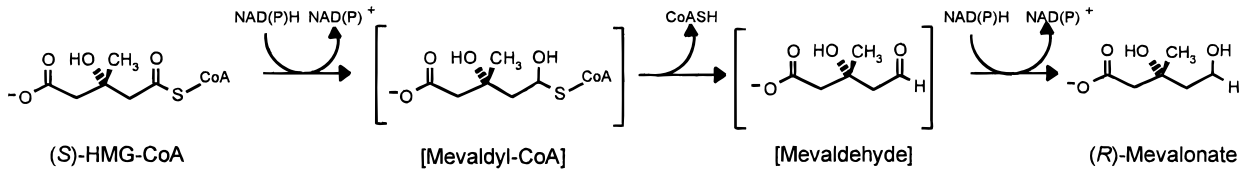
Abstract

Comparison of the inferred amino acid sequence of *orf* AF1736 of *Archaeoglobus fulgidus* to that of *Pseudomonas mevalonii* HMG-CoA reductase suggested that AF1736 might encode a Class II HMG-CoA reductase. Following polymerase chain reaction–based cloning of AF1736 from *A. fulgidus* genomic DNA and expression in *Escherichia coli*, the encoded enzyme was purified to apparent homogeneity and its enzymic properties were determined. Activity was optimal at 85 °C, ΔH_a was 54 kJ/mol, and the statin drug mevinolin inhibited competitively with HMG-CoA (K_i 180 μM). Protonated forms of His390 and Lys277, the apparent cognates of the active site histidine and lysine of the *P. mevalonii* enzyme, appear essential for activity. The mechanism proposed for catalysis of *P. mevalonii* HMG-CoA reductase thus appears valid for *A. fulgidus* HMG-CoA reductase. Unlike any other HMG-CoA reductase, the *A. fulgidus* enzyme exhibits dual coenzyme specificity. pH-activity profiles for all four reactions revealed that optimal activity using NADP(H) occurred at a pH from 1 to 3 units more acidic than that observed using NAD(H). Kinetic parameters were therefore determined for all substrates for all four catalyzed reactions using either NAD(H) or NADP(H). NADPH and NADH compete for occupancy of a common site. k_{cat}[NAD(H)]/k_{cat}[NADP(H)] varied from unity to under 70 for the four reactions, indicative of slight preference for NAD(H). The results indicate the importance of the protonated status of active site residues His390 and Lys277, shown by altered K_M and k_{cat} values, and indicate that NAD(H) and NADP(H) have comparable affinity for the same site.

Keywords: archaeal oxidoreductase; HMG-CoA reductase; mevaldehyde; mevalonate; redox coenzyme specificity; thermostable enzyme

Catalysis by HMG-CoA reductase (E.C. 1.1.1.34) of Reaction (1), the four-electron reductive deacylation of (*S*)-HMG-CoA to the isoprenoid precursor (*R*)-mevalonate, proceeds in three stages, the first and third of which are reductive. The putative intermediates mevaldyl-CoA and mevaldehyde remain enzyme-bound during the course of the reaction. HMG-CoA reductase also catalyzes Reactions (2) and (3), two 2-electron reactions of free mevaldehyde that appear to model the third stage and the reverse of the second and first stages of Reaction (1), respectively, and Reaction (4), the four-electron oxidative acylation of (*R*)-mevalonate to (*S*)-HMG-CoA, the reverse of Reaction (1) (Bochar et al., 1999a).



(1)

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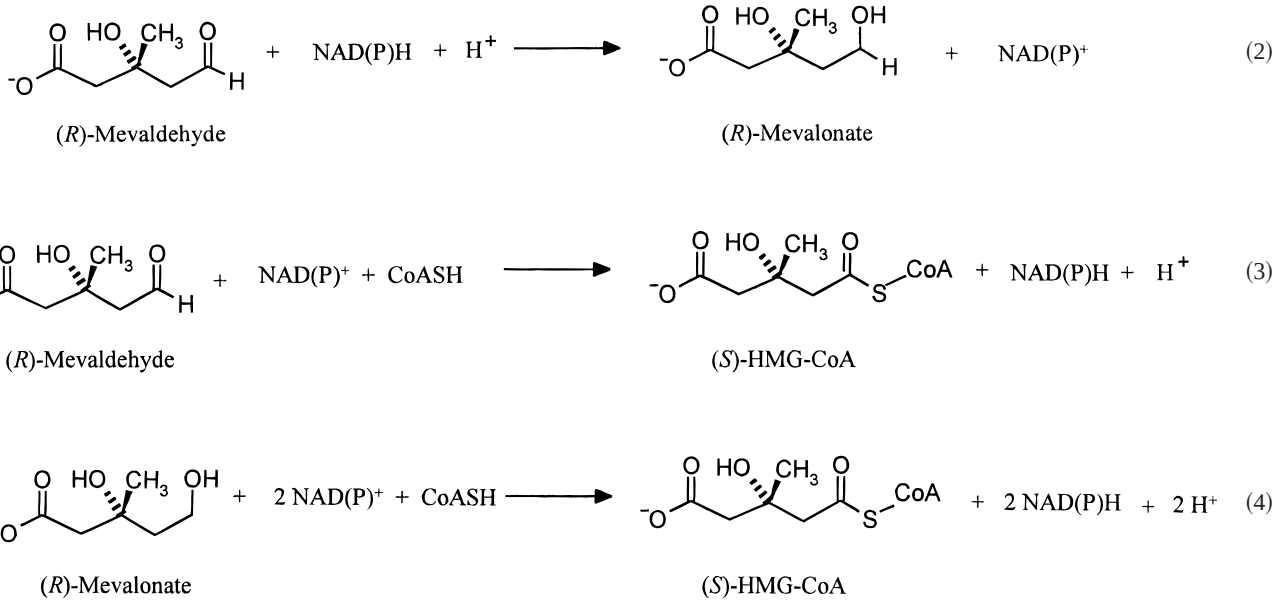
Abbreviations: CoASH, coenzyme A; HMG, 3-hydroxy-3-methylglutaryl; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The suffixes P and A refer to residues from the HMG-CoA reductases of *Pseudomonas mevalonii* and of *Archaeoglobus fulgidus*, respectively.



Genes that encode HMG-CoA reductase are ubiquitous in eukaryotes and archaea, and are present in some, but not all, eubacteria. Although prior investigations have established the probable functions of an active site glutamate, aspartate, histidine, and lysine that participate in a common mechanism of catalysis (Wang et al., 1990; Darnay et al., 1992; Darnay & Rodwell, 1993; Frimpong & Rodwell, 1994; Tabernero et al., 1998; Bochar et al., 1999c), only recently was it possible to infer from gene sequences that there are two structural classes of HMG-CoA reductase (Doolittle & Logsdon, 1998; Bochar et al., 1999b). Almost all prior research has addressed the class I enzymes of higher vertebrates that constitute the target for the inhibitory statin drugs employed to control cholesterol biosynthesis in human subjects. The class II enzymes are, however, of no less medical interest. Genes that appear to encode class II HMG-CoA reductases are present in several human pathogens, including *Borrelia burgdorferi*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (Bochar et al., 1999b), clinically important organisms that are rapidly becoming resistant to the armamentarium of available antibiotics.

The hyperthermophilic archaeon *Archaeoglobus fulgidus*, a strict anaerobe that inhabits hydrothermal environments and hot oil field-production waters, grows chemotrophically on various carbon sources or lithoautotrophically on hydrogen, thiosulfate, and carbon dioxide at temperatures as high as 95 °C (Steen et al., 1997). The sequence *orf* AF1736 of *A. fulgidus* (Klenk et al., 1997) suggested that it might encode a class II HMG-CoA reductase, the first example of an archaeal class II form of the enzyme (Bochar et al., 1999b). Both for this reason and because the only previously characterized class II HMG-CoA reductase is the biodegradative *Pseudomonas mevalonii* enzyme (Gill et al., 1985), we employed PCR to synthesize *orf* AF1736 DNA using *A. fulgidus* genomic DNA as a template. Following expression and purification to apparent homogeneity of *A. fulgidus* HMG-CoA reductase, characterization of its enzymic properties and kinetic parameters revealed its unique coenzyme specificity and implicated two ionizable active site residues as functional in a catalysis.

Results

Temperature profile for catalysis of Reaction (1)

Optimal activity was observed at ~85 °C (Fig. 1). An activation energy ΔH_a of ~54 kJ (13 kcal) per mole was calculated from the Arrhenius equation and the slope of a plot of $\log v$ vs. $1/T$ (Fig. 1, inset). For convenience, subsequent assays were conducted at 50 °C.

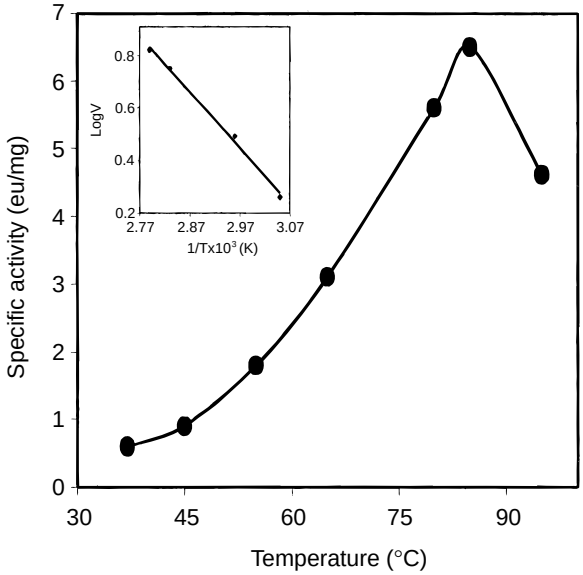


Fig. 1. Effect of temperature on activity. Catalysis of Reaction (1) using NADPH as coenzyme was conducted at the indicated temperatures under otherwise standard conditions. Insert: Arrhenius plot of activity from 35 to 90 °C.

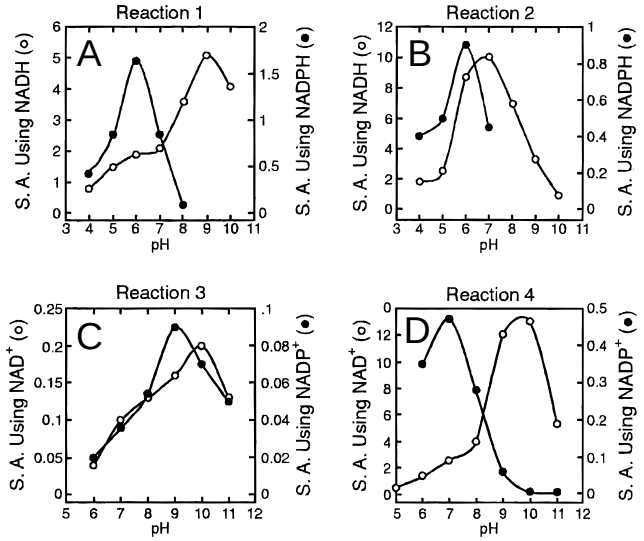


Fig. 2. Effect of hydrogen ion concentration on activity. The indicated reactions were assayed at the indicated pH values under otherwise standard conditions using either NADP(H) (●) or NAD(H) (○) as coenzyme. S.A. = specific activity. Assays were conducted in 100 mM KCl, 100 mM K_xPO_4, 100 mM Tris·HCl adjusted to the indicated pH values. **A:** Reaction (1), reductive deacylation of HMG-CoA to mevalonate. **B:** Reaction (2), reduction of mevaldehyde to mevalonate. **C:** Reaction (3), oxidative acylation of mevaldehyde to HMG-CoA. **D:** Reaction (4), oxidative acylation of mevalonate to HMG-CoA.

Coenzyme specificity and pH profiles for catalysis of all four reactions

The ability of *A. fulgidus* HMG-CoA reductase to use either NADPH or NADH as cosubstrate for catalysis of Reaction (1) at pH 7 was initially detected using crude enzyme preparations. Both coenzymes also function as a coenzyme for the catalysis of all four reactions at significant rates, but at different optimal pH values. Figure 2 illustrates the pH profiles for catalysis of Reactions (1) through (4) using either NADP(H) or NAD(H) as coenzyme. For all four reactions, activity was optimal at a more acidic pH using NADP(H). The differences were most pronounced for Reactions (1) and (4), for which the pH optima differ by about three pH units. Clearly, NAD(H) was not only an effective coenzyme, but based on specific activity values was preferred over NADP(H). This conclusion, however, compares activity at quite different hydrogen ion concentrations, and hence, for different ionic forms of the enzyme. To permit comparison of NAD(H) and NADP(H) using the same ionic form of the enzyme, activity was next assayed at the same pH with either coenzyme. For each reaction studied, higher specific activities still were obtained using NAD(H) (Table 1).

For catalysis of Reaction (1) using either NADPH or NADH, the principal determinant of k_{cat}/K_M as a function of hydrogen ion concentration was k_{cat} (Fig. 3). The variations in activity as a function of pH shown in Figure 2 thus do not appear to reflect an altered K_M for either NADPH or NADH. The pH profiles both of k_{cat} and of k_{cat}/K_M do, however, implicate two groups with pK_a values of about 6.5 and 9.5, whose deprotonation is accompanied by loss of activity.

Kinetic parameters

Figures 4–7 show double reciprocal plots for the determination of K_M values for all substrates for all four catalyzed reactions. Table 2 summarizes these data and includes a comparison to the K_M values for the class II HMG-CoA reductase of *P. mevalonii* and to the class I Syrian hamster enzyme. Apart from the K_M for NADH in Reaction (2) and for coenzyme A in Reaction (3), K_M values were generally higher for the *A. fulgidus* enzyme than for either hamster or *P. mevalonii* HMG-CoA reductase.

Competition between nucleotide coenzymes

We suspected that NADH and NADPH occupy the same site on the enzyme. If so, the two coenzymes should compete with one other. Reaction (2), the reduction of mevaldehyde to mevalonate, a reaction for which V_{max} using NADH is about 10 times that using NADPH, was selected for study to permit a clear distinction between catalysis using NADH or NADPH. The experiment employed a constant concentration of NADPH and variable concentrations of NADH. Under these conditions, NADPH indeed behaved as a classic competitive inhibitor (Fig. 8).

Inhibition by mevinolin

Statin drugs are competitive inhibitors of HMG-CoA reductase activity (Bochar et al., 1999a). As anticipated, catalysis of Reaction (1) was inhibited by the statin drug mevinolin. Inhibition was competitive with respect to HMG-CoA with a K_i of 0.18 mM (Fig. 9), a value similar to that for the class II HMG-CoA reductase of *P. mevalonii* rather than to the 5–15 nM K_i typical of a class I HMG-CoA reductase (Bischoff & Rodwell, 1996).

Discussion

It is essential for activity that two ionizable groups with pK_a values of about 6.5 and over 9 are in their protonated states. We propose

Table 1. *Activity of A. fulgidus HMG-CoA reductase using NAD(H) or NADP(H) as coenzyme*[a]

Reaction	pH	Specific activity (eu/mg) using NAD(H)	Specific activity (eu/mg) using NADP(H)
(1) HMG-CoA → mevalonate	7.0	2.1	0.36
(2) Mevaldehyde → mevalonate	7.0	6.7	0.25
(3) Mevaldehyde → HMG-CoA	8.0	0.13	0.055
(4) Mevalonate → HMG-CoA	8.0	6.3	0.27

[a] All assays employed the same enzyme preparation, the indicated pH, and were conducted on the same day.

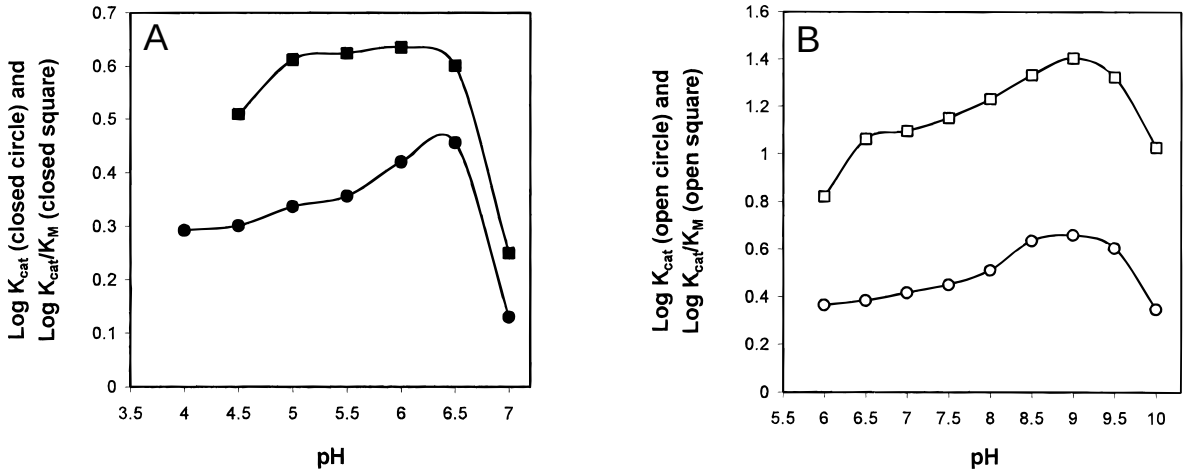


Fig. 3. Effect of hydrogen ion concentration on kinetic parameters for Reaction (1). Shown are the pH-dependent profiles of (**A**) $k_{cat(\mathrm{NADPH})}$ (●) and $k_{cat(\mathrm{NADPH})}/K_{M(\mathrm{NADPH})}$ (■), and (**B**) $k_{cat(\mathrm{NADH})}$ (○) and $k_{cat(\mathrm{NADH})}/K_{M(\mathrm{NADH})}$ (□). All assays employed saturating concentrations of HMG-CoA.

that these are $His390_A$ and $Lys277_A$, the apparent cognates of the active site residues $His381_P$ and $Lys267_P$ of *P. mevalonii* HMG-CoA reductase that have established functions in catalysis (Darnay et al., 1992; Bochar et al., 1999c). We further propose that the mechanism proposed for catalysis of *P. mevalonii* HMG-CoA reductase (Tabernero et al., 1998) is valid for *A. fulgidus* HMG-CoA reductase.

Despite wide differences in kingdom, ecological niche, and biologic function in their host organism, the HMG-CoA reductases of *A. fulgidus* and *P. mevalonii* share 61% amino acid identity. Although among characterized HMG-CoA reductases only these two enzymes use NAD(H), their abilities to discriminate against NADP(H) differ by almost five orders of magnitude. For catalysis of Reaction (1), $[k_{cat}/K_{M(\mathrm{NAD})}]/[k_{cat}/K_{M(\mathrm{NADP})}]$ is 6.3 for *A. fulgidus* HMG-CoA reductase, but is 5.5×10^5 for the *P. mevalonii* enzyme (Table 3). That both coenzymes bind *A. fulgidus* HMG-CoA reductase at the same site may be inferred from the ability of each coenzyme to act as a competitive inhibitor of the other during catalysis of Reaction (2). A preference for NAD(H) notwithstanding, *A. fulgidus* HMG-CoA reductase would appear to be a true biosynthetic enzyme. This may be inferred from its ability to use NADPH, the presence in archaeal membranes of mevalonate-derived isoprenoid lipids (de Rosa et al., 1986), and the existence of *A. fulgidus* DNA that encodes a putative mevalonate kinase. Other oxidoreductases that use either NADP(H) or NAD(H) include L-glutamate dehydrogenase (Maulik & Ghosh, 1986; Consalvi et al., 1991a, 1991b), isocitrate dehydrogenase (Danson & Wood, 1984; Leyland & Kelly, 1991), glucose 6-phosphate dehydrogenase (Ben-Bassat & Goldberg, 1980; Anderson & Anderson, 1995), 6-phosphogluconate dehydrogenase (Ben-Basset & Goldberg, 1980), aldose reductase (Neuhauser et al., 1997), biliverdin IXa reductase (Maines et al., 1996), and the liver alcohol

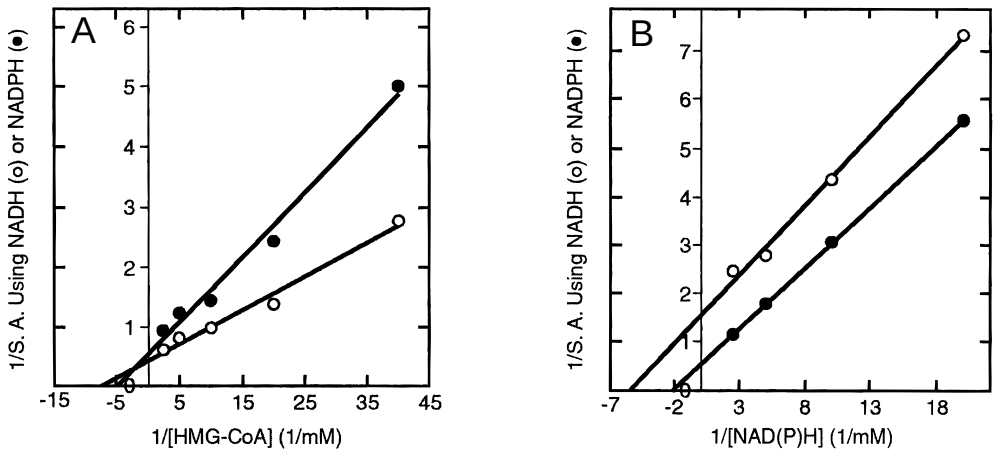


Fig. 4. Catalysis of Reaction (1), the reductive deacylation of HMG-CoA. Double-reciprocal plots for the effect of HMG-CoA and of coenzyme concentration. The NADPH- and NADH-dependent reactions were conducted at pH 6 and 9, respectively. **A:** HMG-CoA concentration was varied as shown using 0.5 mM NADPH (●) or 0.5 mM NADH (○). **B:** The concentration of NADPH (●) or NADH (○) was varied as shown using 2.0 mM HMG-CoA.

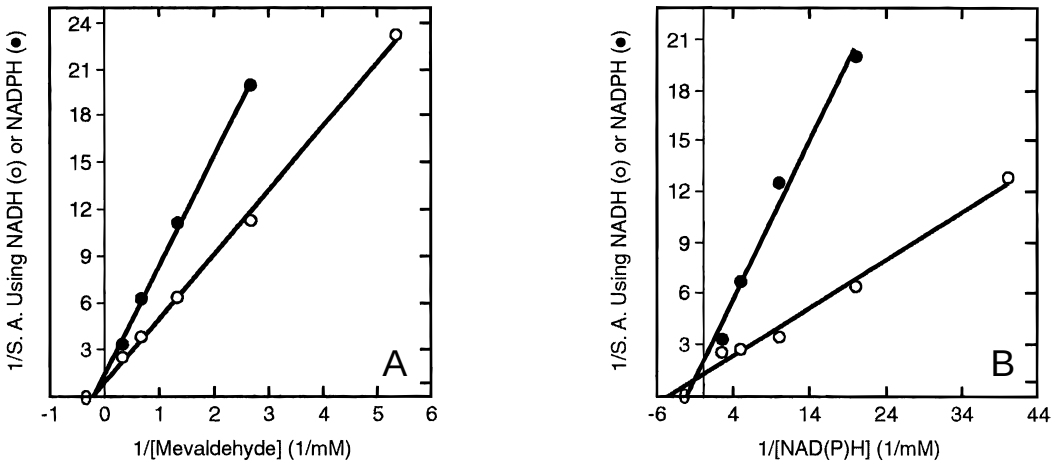


Fig. 5. Catalysis of Reaction (2), the reduction of mevaldehyde. Double-reciprocal plots for the effect of mevaldehyde and of coenzyme concentration. The NADPH- and NADH-dependent reactions were conducted at pH 6 and 7, respectively. **A:** Mevaldehyde concentration was varied as shown using 0.5 mM NADPH (●) or 0.5 mM NADH (○). Specific activity values using NADH were multiplied by 10 to allow representation on the same graph as NADPH. **B:** The concentration of NADPH (●) or NADH (○) was varied as shown at a fixed concentration of 120 mM mevaldehyde.

dehydrogenase of trout (Tsai et al., 1987) and pickerel (al-Kassim & Tsai, 1993). With the exception of fish liver alcohol dehydrogenase, coenzyme K_M values are an order of magnitude or more lower for NADP(H) than for NAD(H), suggesting a preference for NADPH. The HMG-CoA reductase of *A. fulgidus*, thus, is among a mere handful of oxidoreductases, and the only HMG-CoA reductase, for which the K_M is essentially the same for either coenzyme.

The ability to use both NAD(H) and NADP(H) was entirely unexpected. Crystal structures of *P. mevalonii* HMG-CoA reductase revealed that $Asp146_P$ interacts with the 2′-hydroxyl of the adenosyl ribose of NAD(H) (Lawrence et al., 1995; Tabernero et al., 1998), and mutagenic studies have established that $Asp146_P$ is indeed a major determinant of nucleotide coenzyme specificity (Friesen et al., 1996). Sequence alignments implicate $Asp162_F$ of *A. fulgidus* HMG-CoA reductase as the apparent cognate of $Asp146_P$, consistent with the ability of the *A. fulgidus* enzyme to use NAD(H), but suggesting that it should discriminate against NADP(H). Despite the 61% structural identity between the *P. mevalonii* and *A. fulgidus* enzymes, the ability of *A. fulgidus* HMG-CoA reductase to accommodate both forms of the coenzyme suggests, however, that significant differences in the coenzyme binding site may become apparent when the crystal structure of *A. fulgidus* HMG-CoA reductase is solved and compared to that of the *P. mevalonii* enzyme.

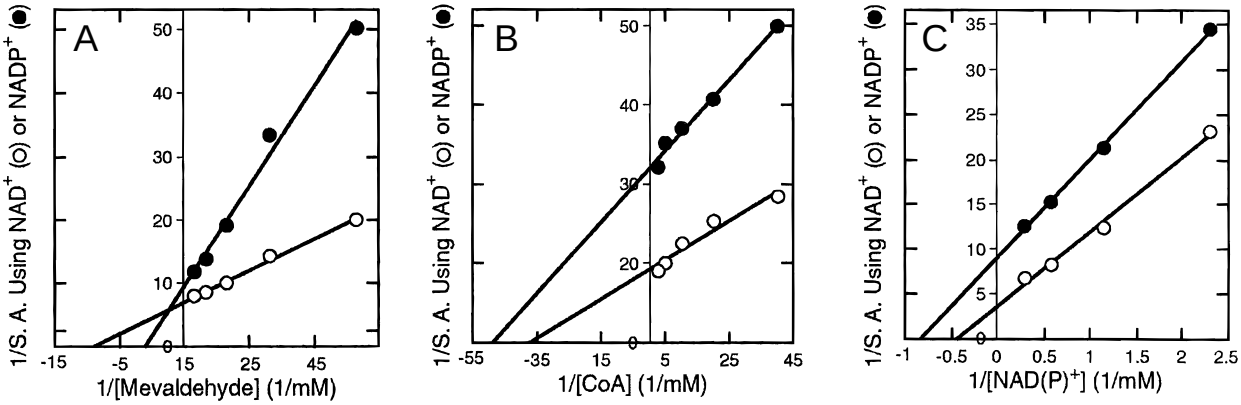


Fig. 6. Catalysis of Reaction (3), the oxidative acylation of mevaldehyde. Double-reciprocal plots for the effect of mevaldehyde, coenzyme A, and nicotinamide nucleotide coenzyme concentration. Reactions using $NADP^+$ or NAD^+ were conducted at pH 9 and 10, respectively. **A:** Mevaldehyde concentration was varied as shown using 0.5 mM coenzyme A and either 15 mM $NADP^+$ (●) or 15 mM NAD^+ (○). **B:** Coenzyme A concentration was varied as shown using 4.0 mM mevaldehyde and 15 mM $NADP^+$ (●) or 15 mM NAD^+ (○). **C:** The concentration of $NADP^+$ (●) or NAD^+ (○) was varied as shown using 4.0 mM mevaldehyde and 0.5 mM coenzyme A.

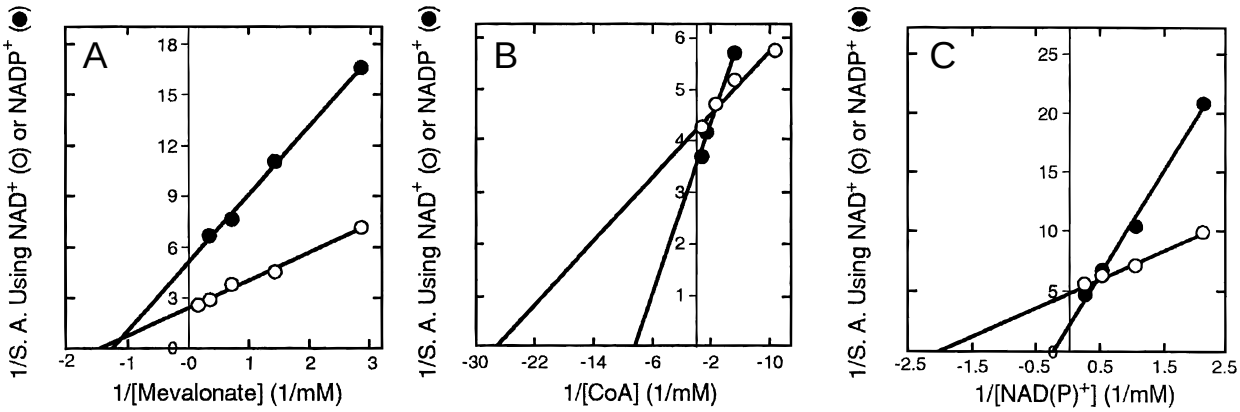


Fig. 7. Catalysis of Reaction (4), the oxidative acylation of mevalonate. Double-reciprocal plots for the effect of mevalonate, coenzyme A, and nicotinamide nucleotide coenzyme concentration. Reactions using $NADP^+$ or NAD^+ were conducted at pH 7 and 10, respectively. To permit representation for both NAD^+ and $NADP^+$ on the same graph, specific activity values using NAD^+ were multiplied by (**A**) 25 or (**B**, **C**) 50. **A:** Mevalonate concentration was varied as shown using 15 mM $NADP^+$ (●) or 15 mM NAD^+ (○), and coenzyme A (1.5 mM using $NADP^+$; 0.5 mM using NAD^+). **B:** Coenzyme A concentration was varied as shown using 15 mM $NADP^+$ (●) or 15 mM NAD^+ (○), and 6.0 mM mevalonate. **C:** $NADP^+$ (●) or NAD^+ (○) concentration was varied as shown using 6.0 mM mevalonate and coenzyme A (1.5 mM using $NADP^+$; 0.5 mM using NAD^+).

Materials and methods

Materials

Purchased reagents included $NADP^+$, NAD^+, NADPH, NADH, coenzyme A, (*R,S*)-HMG-CoA, mevalonolactone, mevaldic acid precursor, phenylmethylsulfonyl fluoride, and DEAE-Sepharose FF (Sigma, St. Louis, Missouri); T4 DNA ligase (Promega, Madison, Wisconsin); restriction enzymes (New England Biolabs, Beverly, Massachusetts, or Promega); Vent DNA polymerase (New England Biolabs); Qiaex gel extraction kit, Qiaprep Spin plasmid Miniprep Kit, Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany); and pre-stained low-range protein standards (Bio-Rad, Hercules, California). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. Buffer A contained 10% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride in 20 mM K_xPO_4, pH 7.3.

Table 2. *Comparison of K_M values for A. fulgidus, P. mevalonii, and Syrian hamster HMG-CoA reductase*[a]

Reactions and substrates	*A. fulgidus* K_M (μM)	*P. mevalonii* K_M (μM)	Hamster K_M (μM)
(1) HMG-CoA → mevalonate			
HMG-CoA 175 20 20			
NADPH	500		80
NADH	160	80	
(2) Mevaldehyde → mevalonate			
Mevaldehyde	5,000	8,000	1,600
NADPH	540		160
NADH	200	1,800	
(3) Mevaldehyde → HMG-CoA			
Mevaldehyde	400	80	90
CoASH	20	110	50
$NADP^+$	1,100		600
NAD^+	2,100	120	
(4) Mevalonate → HMG-CoA			
Mevalonate	620	260	20
CoASH	30/120[b]	60	10
$NADP^+$	1,700		510
NAD^+	500	300	

[a] Data for the HMG-CoA reductase of *A. fulgidus* is from Figures 4–7.
[b] 30 μM using NAD^+, 120 μM using $NADP^+$.

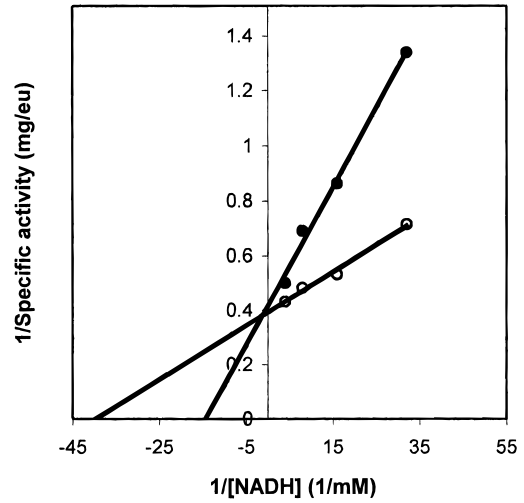


Fig. 8. Competition between NADH and NADPH for catalysis of Reaction (2), the reduction of mevaldehyde to mevalonate. Assays at 50 °C were conducted in 100 mM KCl, 100 mM K_xPO_4, pH 7.0, and employed 4.0 mM mevaldehyde and the indicated concentrations of NADH in the absence (○) or presence (●) of 0.25 mM NADPH. Reactions were initiated by adding NADH.

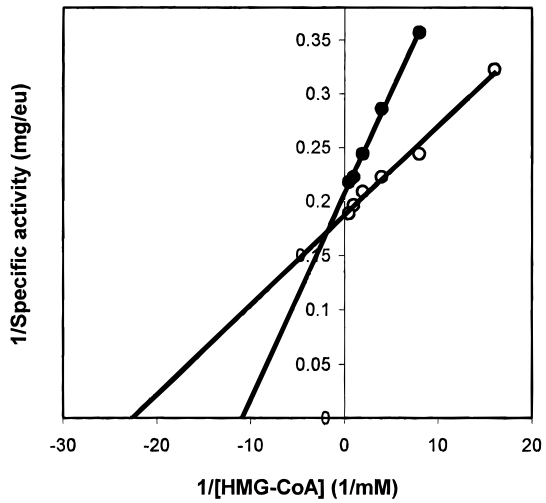


Fig. 9. Inhibition by mevinolin. Double-reciprocal plot for inhibition of Reaction (1), the reductive deacylation of HMG-CoA to mevalonate, using NADH as coenzyme. Analyses were conducted at pH 9.0 and 50 °C at the indicated concentrations of HMG-CoA in the presence of 0 μM (○) or 260 μM (●) potassium mevinolinate. All reactions were initiated by adding NADH.

Genomic DNA, bacteria, and culture media

A. fulgidus genomic DNA was a generous gift of Nils-Kåre Birkeland of the Department of Microbiology, University of Bergen, Norway. Plasmid pDC952, which encodes $tRNA^{AGG,AGA}$ that recognizes the AGG and AGA arginine codons (Andrews et al., 1996), was a generous gift from James R. Walker (Department of Microbiology, University of Texas, Austin, Texas). Plasmid pET-21b and *Escherichia coli* BL21(DE3) cells were from Novagen (Madison, Wisconsin) and *E. coli* DH5α cells were from Gibco BRL (Gaithersburg, Maryland). Media for growth of *E. coli* included LB (Sambrook et al., 1989) and $LB_{amp/cm}$ (LB plus 75 μg/mL ampicillin, 150 μg/mL chloramphenicol). DNA sequencing was conducted at the Purdue University DNA Sequencing Center.

Assays of HMG-CoA reductase activities

Spectrophotometric assays of HMG-CoA reductase activity employed a Hewlett-Packard model 8452 diode array spectrophotometer whose cell compartment was maintained at 50 °C during measurements at 340 nm of the appearance or disappearance of NAD(P)H. Assays were conducted in a final volume of 200 μL. Standard assay conditions for each reaction studied appear below.

Reaction (1), reductive deacylation of HMG-CoA to mevalonate

Using NADPH as coenzyme: 0.5 mM NADPH, 2.0 mM (*R,S*)-HMG-CoA, 100 mM KCl and 100 mM K_xPO_4, pH 6.0. Using NADH as coenzyme: 0.5 mM NADH, 2.0 mM (*R,S*)-HMG-CoA, 100 mM KCl, and 100 mM Tris·HCl, pH 9.0.

Reaction (2), reduction of mevaldehyde to mevalonate

Using NADPH as coenzyme: 0.5 mM NADPH, 20 mM (*R,S*)-mevaldehyde, 100 mM KCl, and 100 mM K_xPO_4, pH 6.0. Using NADH as coenzyme: 0.5 mM NADH, 20 mM (*R,S*)-mevaldehyde, 100 mM KCl, and 100 mM K_xPO_4, pH 7.0.

Reaction (3), oxidative acylation of mevaldehyde to HMG-CoA

Using $NADP^+$ as coenzyme: 15 mM $NADP^+$, 0.5 mM coenzyme A, 4.0 mM (*R,S*)-mevaldehyde, 100 mM KCl, 100 mM Tris·HCl, pH 9.0. Using NAD^+ as the coenzyme: 15 mM NAD^+, 0.5 mM coenzyme A, 4.0 mM (*R,S*)-mevaldehyde, 100 mM KCl, and 100 mM Tris·HCl, pH 10.

Reaction (4), oxidative acylation of mevalonate to HMG-CoA

Using $NADP^+$ as the coenzyme: 15 mM $NADP^+$, 1.5 mM coenzyme A, 6.0 mM (*R,S*)-mevalonate, 100 mM KCl, and 100 mM K_xPO_4, pH 7.0. Using NAD^+ as the coenzyme: 15 mM NAD^+, 0.5 mM coenzyme A, 6.0 mM (*R,S*)-mevalonate, and 100 mM Tris·HCl, pH 10.

Unless otherwise stated, all reactions were initiated by adding HMG-CoA, mevaldehyde, or mevalonate. For all assays, one enzyme unit (eu) represents the turnover, in 1 min, of 1 μmol of nicotinamide nucleotide coenzyme. This corresponds to the turnover of 1 μmol of mevaldehyde or to 0.5 μmol of HMG-CoA or mevalonate. Reported data represent mean values for at least triplicate determinations.

Cloning of A. fulgidus orf AF1736

The synthetic oligonucleotides AGGGGAAACA*T*ATGCAGGTTC TTAGACTCG (encodes a new *Nde*I site) and TTAAGTGAG*C*TC AGCTTCTTAACCTC (encodes a new *Sac*I site) were employed

Table 3. *Catalytic efficiency using NAD(H) or NADP(H) as coenzyme*[a]

	K_M[NAD(H)] (mM)	K_M[NADP(H)] (mM)	k_{cat}[NAD(H)] (s^{-1})	k_{cat}[NADP(H)] (s^{-1})	$\frac{k_{cat}/K_M[\text{NAD(H)}]}{k_{cat}/K_M[\text{NADP(H)}]}$	$\frac{k_{cat}/K_M[\text{NADP(H)}]}{k_{cat}/K_M[\text{NAD(H)}]}$
Reaction (1)	0.16	0.50	2.0	1.0	6.3	0.16
Reaction (2)	0.20	0.54	5.0	0.40	34	0.03
Reaction (3)	2.1	1.1	0.10	0.05	1.0	0.95
Reaction (4)	0.50	1.7	5.0	0.25	68	0.01

[a] k_{cat} is defined as V_{max} divided by the molar concentration of active sites, which based on analogy to the *P. mevalonii* enzyme (Lawrence et al., 1995; Tabernero et al., 1998) is assumed to be two per dimer.

Table 4. *Summary of purification of Archaeoglobus fulgidus HMG-CoA reductase*[a]

Fraction	Activity (eu)[b]	Protein (mg)	Specific activity (eu/mg)	Enrichment (fold)	Recovery of enzyme units (%)
Cytosol[c]		1,090			
Heat	45	95	0.50	(1.0)	(100)
DEAE-Sepharose	32	23	1.4	2.8	70

[a] The data are for the purification of enzyme from 4 L of culture.
[b] For catalysis of Reaction (1) using NADPH as a coenzyme.
[c] Precipitation at 50 °C hindered accurate measurement of activity in unheated cytosol.

as the forward and reverse primers for PCR amplification of *orf* AF1736. The resulting PCR product was digested with *Nde*I and *Sac*I and ligated into expression vector pET-21b that had been digested with the same enzymes, forming pET21b(AF,R2). A portion was digested with *Xba*I and *Hind*III, cloned into appropriately digested pUC19, and sequenced to verify that the cloned fragment was structurally correct.

Expression of pET21b(AF,R2) and pDC952

Of the 29 arginine codons of AF1736, 26 (90%) are AGA or AGG, and codons 17 and 18 form a tandem AGA-AGA pair. *E. coli* strain BL21(DE3) cells were therefore cotransformed with pET-21b(AF,R2) and with pDC952, which encodes $tRNA^{AGG,AGA}$, transferred to $LB_{amp/cm}$ medium and grown, with shaking, at 37 °C. At a cell density of about 0.8 at 600 nm (90 Klett units), isopropyl-thiogalactoside, final concentration 0.5 mM, was added, and growth was continued to a cell density of about 340 Klett units. Cells were harvested by centrifugation (4,000 × *g*, 15 min, 4 °C) and either disrupted immediately or stored at −20 °C for subsequent use.

Purification of A. fulgidus HMG-CoA reductase

Packed cells from 4 L of culture suspended in 120 mL of chilled buffer A were ruptured by passage twice through a French pressure cell. Following centrifugation of the cell lysate (105,000 × *g*, 60 min, 4 °C), the supernatant liquid was retained as the cytosol fraction. Significant expressed protein remained in the high-speed

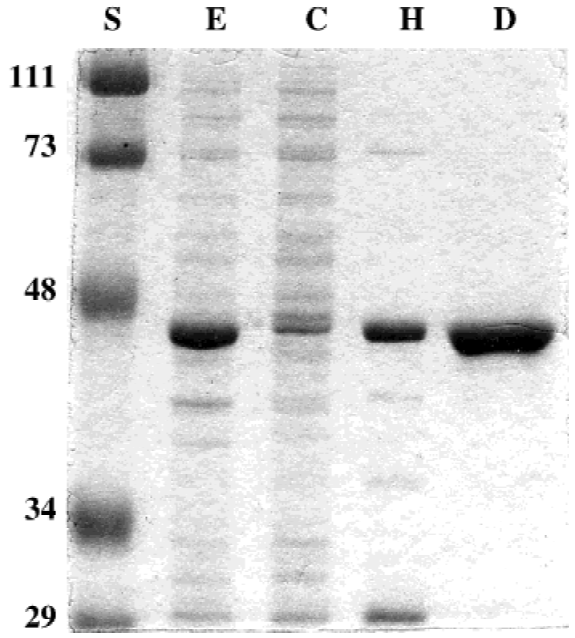


Fig. 10. Analysis of fractions by SDS-PAGE. Approximately 5 μg portions of the indicated fractions were subjected to SDS-PAGE using a 12% resolving gel and a 4% stacking gel. The gel was stained with Coomassie blue. Lanes contained pre-stained protein standards of the indicated molecular mass in kDa (S), crude extract, which includes both the soluble and insoluble fraction, (E), cytosol (C), heat fraction (H), and DEAE-Sepharose fraction (D).

pellet. The cytosol fraction was divided into four equal portions, then incubated at 75 °C for 25 min, chilled, and centrifuged (12,000 × *g*, 10 min, 4 °C). The supernatant liquid was retained. The precipitate was suspended in four volumes of chilled buffer A and centrifuged as above. Both supernatant liquids were then combined to give the heat fraction. Subsequent operations were at room temperature. The heat fraction was applied to a 3.0 × 15 cm (106 mL bed volume) column of DEAE-Sepharose FF equilibrated with buffer A. The column was washed with 350 mL of buffer A, then eluted with 350 mL of 150 mM KCl in buffer A. To each milliliter of the combined active fractions 1.86 mL of an $(NH_4)SO_4$ solution saturated at room temperature, pH 7.0, were added. Following centrifugation (23,000 × *g*, 20 min, 4 °C), the precipitate was dissolved in a minimal volume of buffer A to give the DEAE-Sepharose fraction. Table 4 summarizes the progress of the purification, which yielded an essentially homogeneous product (Fig. 10).

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