3-Hydroxy-3-methylglutaryl-coenzyme A reductase of *Haloferax volcanii*: Role of histidine 398 and attenuation of activity by introduction of negative charge at position 404

KENNETH M. BISCHOFF¹ AND VICTOR W. RODWELL

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

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Abstract

Mutant 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductases of the halophilic archaeon *Haloferax volcanii* were constructed to test the proposed mechanism that phosphorylation downregulates the activity of higher eukarya HMG-CoA reductases via charge–charge interaction with the active site histidine. To first verify the sequence-based inference that His 398 is the catalytic histidine of the *H. volcanii* enzyme, enzyme H398Q was constructed, purified, and assayed for catalysis of three reactions: [1] reductive deacylation of HMG-CoA, [2] reduction of mevaldehyde, and [3] oxidative acylation of mevaldehyde. Enzyme H398Q had low activity for catalysis of reaction [1] or [3], but readily catalyzed mevaldehyde reduction. By analogy to hamster HMG-CoA reductase, we conclude that His 398 is the active site histidine. Mutant forms of the 403-residue *H. volcanii* enzyme were constructed to model phosphorylation and infer whether attenuated activity involved interaction with His 398. Chimeric *H. volcanii*-hamster enzymes constructed in an effort to create an active, phosphorylatable chimeric enzyme were inactive or not phosphorylated. We therefore added Asp at position 404 to mimic the introduction of negative charge that would accompany phosphorylation. Enzyme 404D/H398Q was inactive for reaction [1] or [3], but catalyzed reaction [2] at 35% the wild-type rate. These observations are consistent with the model that attenuation of catalytic activity results from an ionic interaction between the imidazolium cation of His 398 and the carboxylate anion of Asp 404.

Keywords: attenuation of catalytic activity; HMG-CoA reductase; identification and role of the catalytic histidine

In eukarya, biosynthesis of the isoprenoid precursor mevalonate employs NADPH and the enzyme HMG-CoA reductase to reductively deacylate HMG-CoA:

This 4-electron oxidoreduction may be considered to proceed via two successive reductive stages in which mevaldehyde participates as an enzyme-bound intermediate:

Reprint requests to: Victor W. Rodwell, Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153; e-mail: rodwell@biochem.purdue.edu.

¹Present address: Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061.

While all attempts to trap free mevaldehyde during the course of the overall reaction have proved unsuccessful, HMG-CoA reductase catalyzes reactions [2] and [3] of free mevaldehyde, reactions that appear to parallel stage [1.2] and the reverse of stage [1.1] of the overall reaction, respectively:

Kinetic analysis of catalysis of reactions [2] and [3] by mutant forms of the Syrian hamster enzyme has identified His 865 as an active site residue whose function appears to be to protonate the CoAS⁻ thioanion formed at the conclusion of the first reductive stage, reaction [1.1]. Mutation of His 865 to glutamine blocks release of the thioanion, which results in severe inhibition of the second reductive stage of the overall reaction, reaction [1.2] (Frimpong & Rodwell, 1994).

Unlike true bacteria, for which the pathway of isoprenoid biosynthesis remains largely unknown, archaeobacteria utilize HMG-CoA reductase to form mevalonate, the precursor of archaeal ether-linked membrane phospholipids (DeRosa et al., 1986). The *hmgR* gene that encodes HMG-CoA reductase of the halophilic archaeon *Haloferax volcanii* (Lam & Doolittle, 1992) has been expressed in *Escherichia coli* and its encoded protein has been purified to homogeneity and characterized kinetically (Bischoff & Rodwell, 1996). The vast evolutionary distance that separates the archaea from the eukarya notwithstanding, *H. volcanii* HMG-CoA reductase shares significant properties with its eukaryotic counterparts. These include 42% identity and 61% similarity in amino acid sequence to the hamster enzyme (Lam & Doolittle, 1992) and similar kinetic parameters. Purified *H. volcanii* and hamster HMG-CoA reductases have comparable specific activities for catalysis of reaction [1], share specificity for (S)-HMG-CoA and for NADPH, and are

competitively inhibited by (R)-HMG-CoA and by the cholesterol-lowering drug Lovastatin (Bischoff & Rodwell, 1996).

The catalytic activity of the HMG-CoA reductases of higher eukarya has long been known to be controlled in a reversible manner by phosphorylation and dephosphorylation (Hunter & Rodwell, 1980; Kennelly & Rodwell, 1985). More recently, phosphorylation by AMP-activated protein kinase has been shown to involve a single serine (Clarke & Hardie, 1990; Sato et al., 1993; Omkumar et al., 1994; Dale et al., 1995). The regulatory serine, Ser 871 of the hamster enzyme, while it clearly plays no role in catalysis or substrate recognition (Omkumar et al., 1994), is located six residues from the active site histidine, His 865 (Darnay & Rodwell, 1993). This precise spatial relationship between the putative active site histidine and regulatory serine is strictly conserved in the HMG-CoA reductases of all higher eukarya (Fig. 1). Kinetic analysis of reactions [1-3] catalyzed by mutant forms of hamster HMG-CoA reductase led to a novel hypothesis to explain why phosphorylation downregulates activity: that the introduced phosphate interacts ionically with the active site histidine, blocking its ability to protonate the potentially inhibitory CoAS⁻, arresting its release from the active site and halting the overall reaction at the conclusion of the first reductive stage (Omkumar & Rodwell, 1994).

A striking feature of *H. volcanii* HMG-CoA reductase is that its N-terminal residue, Arg 403, occurs just five residues beyond

<i>Haloferax volcanii</i>	HLSSA H AELGR (+ 0)
Syrian hamster (<i>Mesocricetus auratus</i>)	HLVRS H MVHNR S KIN (+13)
Human (<i>Homo sapiens</i>)	HLVKS H MIHNR S KIN (+13)
Frog (<i>Xenopus laevis</i>)	HLVKS H NVHNR S KIN (+13)
Fruit fly (<i>Drosophila melanogaster</i>)	DLVKS H MRHNR S SIA (+19)
Potato-4 (<i>Solanum tuberosum</i>)	QLVKS H MKYNR S IKD (+ 6)
Rubber tree-1 (<i>Hevea brasiliensis</i>)	QLVKS H MKYNR S SKD (+ 6)
Tomato-1 (<i>Lycopersicon esculentum</i>)	QLVKS H MKYNR S IKD (+ 8)
Arabidopsis (<i>Arabidopsis thaliana</i>)	QLVRS H MKYNR S SRD (+13)

Fig. 1. Sequence alignments of C-terminal amino acids of selected HMG-CoA reductases. Sequences were obtained from the Genbank database. Alignments were produced by the Pileup program available on the Wisconsin package (Devereux et al., 1984). Shown in bold face are the conserved histidine, which for *H. volcanii* HMG-CoA reductase is His 398, and the regulatory serine of higher eukaryote HMG-CoA reductases. Numbers in parenthesis indicate the number of residues that follow the last listed amino acid.

His 398, the putative active site histidine, and one residue short of position 404, which based on sequence alignments would be the appropriate location for the absent regulatory serine (Fig. 1). The *H. volcanii* enzyme also lacks an appropriate phosphorylation motif for the AMP-activated protein kinase (Friesen & Rodwell, 1997). These properties, coupled with the structural and kinetic similarity to the hamster enzyme, make *H. volcanii* HMG-CoA reductase an attractive scaffold for testing the validity of the proposed mechanism by which phosphorylation down-regulates the activity of the HMG-CoA reductases of higher eukarya. Since substitution of Asp for the regulatory Ser of the hamster enzyme attenuated catalytic activity, mimicking the effects of phosphorylation (Omkumar & Rodwell, 1994), we tested the generality of the proposed mechanism by mutating, individually and in combination, His 398 of *H. volcanii* HMG-CoA reductase to glutamine and introducing an aspartyl residue at position 404. The effects on catalysis of reactions [1], [2], and [3] were then compared to comparable mutations of the hamster enzyme.

Results

Mutation of histidine 398 to glutamine

To establish that *H. volcanii* HMG-CoA reductase constitutes a valid model for catalysis by the HMG-CoA reductases of the higher eukarya, we first sought to verify the sequence alignment-based inference that His 398 is the essential active site histidine. Mutant enzyme H398Q was constructed, expressed in *Escherichia coli*, purified, and assayed for its ability to catalyze reactions [1], [2], and [3]. Enzyme H398Q, while it catalyzed reactions [1] and [3] at only 1.3% and <1.4% of wild-type activity, respectively, catalyzed reaction [2], at 24% the rate of the wild-type enzyme (Table 1). These observations closely parallel those for hamster mutant enzyme H865Q (Omkumar & Rodwell, 1994). We therefore conclude that His 398 is indeed the active site histidine of *H. volcanii* HMG-CoA reductase.

As for the hamster enzyme, the probable role of the active site histidine is protonation of the CoAS⁻ thioanion. This inference

Table 1. Kinetic parameters for catalysis of reactions [1], [2], and [3] by *Haloferax volcanii* wild-type and mutant enzymes

Enzyme	Reaction [1] ^a				Reaction [2] ^b				Reaction [3]	
			NADPH	HMG-CoA			NADPH	Mevaldehyde		
	V_{max}^c (μ U/mg)	V_{max} (%)	K_m (μ M)	K_m (μ M)	V_{max}^c (μ U/mg)	V_{max} (%)	K_m (μ M)	K_m (μ M)	V_{max}^c (μ U/mg)	V_{max} (%)
Wild-type	12.0	(100)	66	60	11	(100)	32	550	2.1	(100)
H398Q	0.15	1.3	177	200	2.6	24	100	3000	<0.03	<1.4
404N	9.2	77	72	77	12.3	112	31	4200	1.5	71
404D	0.017	0.1	160	2000	<0.03	<0.3	ND	ND	<0.05	<2.3
404D/H398Q	0.017	0.1	68	83	3.9	35	84	2100	<0.04	<0.19

^aData for catalysis of reaction [1] employed the spectrophotometric assay for wild-type enzyme and enzyme 404N. For enzymes H398Q, 404D, and 404D/H398Q, data were collected using the radioisotopic assay.

^bReaction [2] was assayed in the presence of 1 mM desthio-CoA. ND = not determined.

^c V_{max} values are given both as μ U/mg and relative to the value for the wild-type enzyme as 100%.

was supported by the observation that mutant enzyme H398Q catalyzed reaction [2] in the presence of desthio-CoA, but not in the presence of coenzyme A. These observations parallel those for the hamster enzyme (Frimpong & Rodwell, 1994). The inability of enzyme H398Q to catalyze the reduction of mevaldehyde in the presence of coenzyme A is best explained by loss of the ability to protonate and release the inhibitory CoAS⁻ thioanion. The requirement for desthio-CoA, a non-stoichiometric reactant in reaction [2], parallels the ability of coenzyme A or desthio-CoA to stimulate catalysis of reaction [2] by the wild-type rat (Sherban et al., 1985) and yeast (Veloso et al., 1981) enzymes. The non-thiol portion of coenzyme A thus may be essential for maintaining conformation of the active site. We therefore conclude that His 398 appears to perform the postulated role in catalysis.

Construction and properties of chimeric enzymes

We first attempted to engineer into *H. volcanii* HMG-CoA reductase a phosphorylation site recognized by the AMP-activated protein kinase that phosphorylates the hamster enzyme. Mutant genes encoded chimeric enzymes that contained the C-terminus of the hamster enzyme and a target serine at position 404 (Fig. 2). Following expression and purification, chimeric enzymes VH-1 and VH-2 were assayed for catalysis of reaction [1] and for the ability to serve as substrates for the AMP-activated protein kinase. Chimera VH-1 was active (specific activity 12 μ U/mg), but was not phosphorylated. Using synthetic peptides as models, Dale et al. (1995) have identified primary structural elements within five residues on the N-terminal side and four residues on the C-terminal side of the target serine, the "P-5 to P+4 region" essential for recognition by the AMP-activated protein kinase. Since chimera VH-1 lacks one such element, a basic residue at position P-3 or P-4, we next constructed chimeric enzyme VH-2. Although VH-2 contained the P-5 to P+4 region of the hamster enzyme, and hence, the known kinase recognition elements, it was not a substrate for the AMP-activated protein kinase. However, VH-2 was also inactive for reaction [1], and thus might have had a conformation in which Ser 404 was not accessible to the kinase. We therefore denatured VH-2 with urea prior to attempting its phosphorylation. While in a parallel control experiment urea-denatured hamster HMG-CoA was phosphorylated, VH-2 was not. In view of our failure to construct an active, phosphorylatable chimeric enzyme, we therefore examined the introduction of negative charge at position 404 by addition of an acidic residue.

Introduction of negative charge at the carboxy-terminus of *H. volcanii* HMG-CoA reductase

Substitution of Asp, but not of Asn, for the regulatory Ser of hamster HMG-CoA reductase mimics the effect of phosphoryla-

tion (Omkumar & Rodwell, 1994). We therefore inquired whether the introduction of Asp or of Asn at position 404 attenuated *H. volcanii* HMG-CoA reductase activity. As anticipated, mutant enzyme 404N catalyzed all three reactions at essentially wild-type rates (Table 1). By contrast, enzyme 404D failed to catalyze reaction [2] or [3] and was severely impaired for catalysis of reaction [1] (Table 1). While these observations appear to mimic the effect of phosphorylation, for enzyme 404D the K_m for HMG-CoA was elevated 30-fold and purification failed to yield a homogeneous product. Since inactivity could have resulted solely from an unfavorable change in conformation, we next asked whether the simultaneous replacement of His 398 by Gln restored the ability of enzyme 404D to catalyze reaction [2].

Catalysis by enzyme 404D/H398Q

Mutant enzyme 404D/H398Q, which contains both the H398Q and the 404D mutations, was constructed to decide whether Asp 404 lowered activity via interaction with His 398 or merely as a consequence of an unfavorable conformational change. As anticipated in the absence of the active site histidine, enzyme 404D/H398Q was severely impaired for catalysis of reactions [1] and [3] (Table 1). By contrast, enzyme 404D/H398Q catalyzed reaction [2] at 35% of the wild-type rate and had essentially wild-type K_m values for HMG-CoA and NADPH in reaction [1] and for mevaldehyde and NADPH in reaction [2] (Table 1). These observations parallel those for hamster mutant enzyme H865Q/S871D (Omkumar & Rodwell, 1994). Had the low activity of enzyme 404D resulted solely from an undesired conformational change, mutation of His 398 to glutamine would not be expected to restore activity and wild-type K_m values. That mutation of His 398 to glutamine complemented the mutation at position 404 thus is most simply interpreted in terms of a model that postulates an ionic interaction between the carboxylate anion of Asp 404 and the imidazolium cation of His 398 that renders it incapable of protonating the inhibitory CoAS⁻ thioanion (Fig. 3).

Materials and methods

Materials

Purchased materials included desthio-coenzyme A and mevaldic acid precursor (Sigma), Vent polymerase (New England Biolabs), (*R,S*)-[3-¹⁴C]-HMG-CoA (Amersham), [5-³H]-mevalonolactone (New England Nuclear), and silica gel thin-layer chromatography sheets (Kodak). Additional materials were purchased from previously listed sources (Bischoff & Rodwell, 1996). (*S*)-HMG-CoA (Bischoff & Rodwell, 1992) and the homogeneous catalytic domain of Syrian hamster HMG-CoA reductase (Frimpong et al., 1993) were prepared as previously described. For preparation of mevaldehyde, 60 mg of mevaldic acid precursor was dissolved in

<i>H. volcanii</i>	393-HLSSA H AELGR-403
Syrian hamster	860-HLDRS H <u>MVHNRSKINLQDLQGTCTKAA</u> -887
Chimera VH-1	393-HLSSA H AELGR <u>SKINLQDLQGTCTKAA</u> -420
Chimera VH-2	393-HLSSA H <u>MVHNRSKINLQDLQGTCTKAA</u> -420

Fig. 2. Amino-terminal sequences of wild-type *H. volcanii* and hamster wild-type enzymes, and of chimeric enzymes VH-1 and VH-2. Underlined residues are from the hamster enzyme. Shown in bold are the catalytic histidine and the target serine.

Fig. 3. Proposed mechanism by which insertion of negative charge at position 404 attenuates the activity of *H. volcanii* HMG-CoA reductase. The carboxylate anion of an aspartate at position 404 competes for the proton of the imidazolium ion on His 398, making the histidinium proton unavailable for protonation of the inhibitory CoAS⁻ thioanion.

1.5 mL of cold water and maintained on ice for 5 min. NH₄OH (1 N), 1.5 mL, was then added and the solution was extracted five times with equal volumes of toluene. The organic phase was discarded. Concentrated HCl, 0.15 mL, was added to the aqueous phase and dry N₂ was passed over the solution for 15 min to remove methanol and residual toluene. Following addition of K_xPO₄ to a concentration of 25 mM, the pH was adjusted to 6.6 with NaOH for use. Mevaldehyde concentration was determined by reaction with dinitrophenylhydrazine using mevaldehyde precursor as standard.

Assays of HMG-CoA reductase activities

Spectrophotometric assays for catalysis of Reactions [1], [2], and [3] were conducted as previously described (Bischoff & Rodwell, 1996), except that 1.0 mM desthio-coenzyme A replaced 1.0 mM coenzyme A for assay of Reaction [2]. Assays were initiated by the addition of (*S*)-HMG-CoA or (*R,S*)-mevaldehyde. For all three reactions, one enzyme unit (μ U) represents the turnover in one minute of 1 μ mol of NADPH. HMG-CoA reductase activity for Reaction [1] was also measured using a modification of the radioisotopic assay of Shapiro et al. (1974). Assays contained, in 30 μ L of 3.0 M KCl, 25 mM K_xPO₄, 5.0 mM dithiothreitol, pH 7.3, 0.5 mM (*R,S*)-[3-¹⁴C]-HMG-CoA (1.3 mCi/mmol), 1.0 mM NADPH, and 1.5 nCi of (*R,S*)-[5-³H]-mevalonate. Reactions were initiated by the addition of (*R,S*)-[3-¹⁴C]-HMG-CoA. After 30 min incubation at 37°C, reactions were terminated by adding 8 μ L of 6 N HCl. Mevalonate was then separated from HMG-CoA and HMG by silica gel thin-layer chromatography in toluene:acetone (1:1). Incorporation of ¹⁴C into mevalonate was measured using a Beckman model LS1801 scintillation counter. Product loss due to sample transfers was corrected by determining the recovery of the internal control, [5-³H]-mevalonate.

Construction and expression of mutant genes and purification of mutant enzymes

Oligonucleotides were synthesized at the Purdue University Laboratory for Macromolecular Structure. Construction of mutant genes employed the polymerase chain reaction (Griffin & Griffin, 1994) using wild-type *H. volcanii* HMG-CoA reductase DNA from plasmid pT7-Vred (Bischoff & Rodwell, 1996) as template. Used as primers were the N-terminal 29-mer 5'-GAAACGTCGGCATATGACAGACGCCGCGT-3' and C-terminal oligonucleotides that encode the indicated mutant enzymes: 5'-ATATGAATTCTTACC

GACCGAGTTCGGCCTGGGCGC-3' (enzyme H398Q), 5'-TGAAGAATTCTAGTCCCGACCGAGTTCGGC-3' (enzyme 404N), and 5'-TGAAGAATTCTAGTCCCGACCGAGTTCGGC-3' (enzyme 404D). Construction of the 404D/H398Q mutant gene employed the H398Q mutant gene as template and the 404D oligonucleotide as the C-terminal primer. Mutant genes were sequenced to confirm the presence of the desired mutation, then expressed in *E. coli* strain BL21 (DE3) cells. Wild-type and mutant enzymes were purified through the DEAE fraction as described previously (Bischoff & Rodwell, 1996).

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