Protein Engineering of the HMG-CoA Reductase of *Pseudomonas mevalonii*. Construction of Mutant Enzymes Whose Activity Is Regulated by Phosphorylation and Dephosphorylation†

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ABSTRACT: The activity of *Pseudomonas mevalonii* HMG-CoA reductase (EC 1.1.1.88) is not regulated by phosphorylation, presumably due to the absence of a suitable target serine and protein kinase recognition motif. We have engineered *P. mevalonii* HMG-CoA reductase to a form whose activity, like that of mammalian HMG-CoA reductases, is regulated by phosphorylation/dephosphorylation. We substituted serine for arginine 387, the residue that corresponds to the regulatory serine of the HMG-CoA reductases of higher eukaryotes. A recognition motif for cAMP-dependent protein kinase was added by replacing leucine 384 by histidine (enzyme L384H/R387S) and also valine 391 by leucine (enzyme L384H/R387S/V391L). The activity of *P. mevalonii* HMG-CoA reductase mutant enzymes L384H/R387S and L384H/R387S/V391L was attenuated by phosphorylation. Restoration of activity accompanied subsequent dephosphorylation catalyzed by lambda protein phosphatase. Incorporation and subsequent release of phosphate paralleled the attenuation and restoration of catalytic activity. Incorporation of 0.5 mol of phosphate per subunit was accompanied by an approximately 50% decrease in initial activity. As in the analogous Syrian hamster mutant enzyme S871D, *P. mevalonii* mutant enzyme R387D exhibited 10% wild-type activity, suggesting that the attenuation of activity that accompanies phosphorylation results at least in part from the introduction of negative charge. Engineering of *P. mevalonii* HMG-CoA reductase to forms whose activity is reversibly regulated by phosphorylation/dephosphorylation provides an attractive model for future structure-based mechanistic studies. Solution of the X-ray structure of phosphorylated and dephosphorylated forms of engineered *P. mevalonii* HMG-CoA reductase should then reveal interactions of the active site phosphoseryl residue that result in attenuation of catalytic activity.

Reversible phosphorylation plays critical roles in mediating cellular responses to extracellular or intracellular stimuli and initiates multiple levels of protein phosphorylation that activate or deactivate specific protein targets. Examples include trans-cytoplasmic signaling to the nucleus by the mitogen-activated protein kinase pathway (Marshall, 1995), cell cycle regulation mediated by the action of cyclin-dependent kinases (Pines, 1993), and the regulation of metabolic enzymes such as glycogen phosphorylase, glycogen synthase, and acetyl-CoA carboxylase through the second messenger cAMP,[1] produced in response to the binding of glucagon or epinephrine to β-adrenergic receptors. The importance of phosphorylation by protein kinases and dephosphorylation by protein phosphatases is underscored by a recent estimate of more than 2000 protein kinase genes and 1000 protein phosphatase genes in human subjects (Hunter, 1995).

In mammals, HMG-CoA reductase catalyzes the rate-limiting step in the biosynthesis of isoprenoids, isoprenoid derivatives, and cholesterol. As the control point in this critical metabolic pathway, HMG-CoA reductase is a target for cholesterol-lowering drugs used to treat hypercholesterolemia. On the cellular level, the activity of HMG-CoA reductases of higher eukaryotes is regulated post-translationally by reversible phosphorylation (Hunter & Rodwell, 1980; Kennelly & Rodwell, 1985). It was noted over two decades ago that incubation of washed rat liver microsomes with ATP, Mg^{2+}, and liver cytosol attenuated HMG-CoA reductase activity, and that subsequent treatment with a second cytosolic fraction restored activity (Beg *et al*., 1973; Nordstrom *et al*., 1977). This led to the identification and purification of HMG-CoA reductase kinase[2] (Harwood *et al*., 1984) and of HMG-CoA reductase phosphatase (Brown & Rodwell, 1983).

Rat (Clarke & Hardie, 1990) and hamster HMG-CoA reductase (Sato *et al*., 1993; Omkumar & Rodwell, 1994) are phosphorylated on Ser^{871}, located six residues from the catalytic histidine, His^{865} (Darnay & Rodwell, 1993; Frimpong & Rodwell, 1994), a spacing that is strictly conserved in the HMG-CoA reductases of all higher eukaryotes (Omkumar *et al*., 1994; Friesen & Rodwell, 1997). A novel

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[1] Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; CoASH, coenzyme A; PKA, cAMP-dependent protein kinase; hamster HMG-CoA reductase, the catalytic domain of Syrian hamster HMG-CoA reductase expressed in *Escherichia coli* and purified to homogeneity as previously described (Frimpong *et al*., 1993).

[2] HMG-CoA reductase kinase was later shown to phosphorylate acetyl-CoA carboxylase (Carling *et al*., 1989) and hormone-sensitive lipase (Garton *et al*., 1989) and to be activated by 5′-AMP. These observations prompted the renaming of HMG-CoA reductase kinase to AMP-activated protein kinase.

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Table 1: Experimental Design for Construction of Genes Encoding Enzymes Mutated at Positions 384, 385, 387, 390, and 391[a]

enzyme	381 His	382 Met	383 Ala	384 Leu	385 His	386 Ala	387 Arg	388 Asn	389 Ile	390 Ala	391 Val	392 Val	393 Ala	394 Gly
				*Sph*I				*Ssp*I						
wild-type	CAC	ATG	GCC	CTG	CAT	GCG	CGC	AAT	ATT	GCC	GTG	GTG	GCG	GGC
						*Nhe*I								
L384H/R387S	CAC	ATG	GCC	CAT	CAT	GCT	AGC	AAT	ATT	GCC				
						*Nhe*I								
H385N/R387S		ATG	GCC	CTG	AAT	GCT	AGC	AAT	ATT	GCC				
						*Nhe*I								
L384H/H385N/R387S	CAC	ATG	GCC	CAT	AAT	GCT	AGC	AAT	ATT	GCC				
						*Nhe*I				*Psp1406*I				
R387S/A390N			GCC	CTG	CAT	GCT	AGC	AAT	ATT	AAC	GTT	GTG	GCG	GGC
						*Nhe*I			*Eco47*III					
R387S/V391L			GCC	CTG	CAT	GCT	AGC	AAT	ATA	GCG	CTG	GTG	GCG	GGC
						*Nhe*I			*Ase*I					
R387S/A390N/V391L			GCC	CTG	CAT	GCT	AGC	AAT	ATT	AAT	CTG	GTG	GCG	GGC

[a] Lines over bases of the wild-type gene locate *Sph*I and *Ssp*I restriction sites. Underlined bases indicate where mutations eliminated these restriction sites or added new restriction sites.

mechanism has been proposed to explain why phosphorylation attenuates HMG-CoA reductase activity: an ionic interaction between the phosphoserine and the nearby catalytic histidine prevents the histidine from protonating the potentially inhibitory $CoAS^-$ thioanion formed during stage one of the overall reaction (Frimpong & Rodwell, 1994), hindering its release from the enzyme with resulting attenuation of activity (Omkumar & Rodwell, 1994).

To investigate the regulation of HMG-CoA reductase activity by reversible phosphorylation, we have engineered *P. mevalonii* HMG-CoA reductase, the only form of the enzyme whose structure has been solved (Lawrence *et al*., 1995), to forms whose activity is regulated by phosphorylation. In the *P. mevalonii* enzyme, the catalytic histidine His^{381} corresponds to His^{865} of the hamster enzyme (Darnay *et al*., 1992) and Arg^{387} to Ser^{871} of the hamster enzyme. By replacing Arg^{387} with a phosphoacceptor serine and construction of a cAMP-dependent protein kinase recognition motif, we have engineered forms of *P. mevalonii* HMG-CoA reductase whose catalytic activity is reversibly regulated by phosphorylation and dephosphorylation.

EXPERIMENTAL PROCEDURES

Materials. Purchased reagents included Blue Sepharose CL-6B (Pharmacia), (*R,S*)-HMG-CoA, DEAE Sepharose, and NADH (Sigma), T4 DNA ligase (Promega), an Isotherm DNA sequencing kit (Epicentre Technologies); restriction enzymes (New England Biolabs, Promega, or Gibco); Vent DNA polymerase (New England Biolabs); [α-^{35}S]dATP and [γ-^{32}P]ATP (Amersham); a Qiaex gel extraction kit (Qiagen); homogeneous cAMP-dependent protein kinase (Promega); and homogeneous lambda protein phosphatase (New England Biolabs).

Site-Directed Mutagenesis. Oligonucleotides were synthesized in the Purdue University Laboratory for Macromolecular Structure and were purified by thin-layer chromatography prior to use. The PCR-based overlap extension method of oligonucleotide-directed mutagenesis (Mikaelian & Sergeant, 1992) was employed to construct genes that encoded mutations at positions 384, 385, 387, 390, or 391. All mutations were verified by DNA sequencing (Sanger *et al*., 1977) using an Isotherm DNA sequencing kit and [α-^{35}S]-dATP. The oligonucleotides used to generate genes encoding mutant enzymes R387S and R387D changed the CGC codon to AGC or GAC and simultaneously eliminated a *BssH*II site. The genes for mutant enzymes L384H/R387S, H385N/R387S, L384H/H385N/R387S, R387S/A390N, R387S/V391L, and R387S/A390N/V391L contained a silent mutation encoding a unique *Nhe*I site at positions 386 and 387 (Table 1). The unique *Nhe*I sites were then used to create genes encoding enzymes L384H/R387S/A390N/V391L, H385N/R387S/A390N/V391L, L384H/H385N/R387S/A390N/V391L, L384H/R387S/A390N, L384H/R387S/V391L, H385N/R387S/A390N, L384H/H385N/R387S/V391L, H385N/R387S/V391L, and L384H/H385N/R387S/A390N by fusing DNA sequences encoding mutations at positions 384 and 385 to sequences encoding mutations at positions 390 and 391 at the unique *Nhe*I site.

Expression and Purification of Enzymes. Wild-type and mutant *P. mevalonii* HMG-CoA reductases were expressed behind the tac promoter of pHMGR (Beach & Rodwell, 1989). *Escherichia coli* BL21 cells harboring wild-type or mutant expression vectors were grown at 37 °C, with shaking at 300 rpm, in LB medium (Sambrook *et al*., 1989) that contained 50 μg/mL ampicillin. Purification of wild-type and mutant *P. mevalonii* enzymes to electrophoretic homogeneity was conducted as previously described for the wild-type enzyme (Wang *et al*., 1990).

Assay of HMG-CoA Reductase Activity. Spectrophotometric assay of the reductive deacylation of HMG-CoA employed a Hewlett-Packard model 8452A diode array spectrophotometer equipped with a cell holder maintained at 37 °C to monitor the HMG-CoA-dependent disappearance of NADH at 340 nm. Assays contained 0.3 mM (*R,S*)-HMG-CoA, 0.13 mM NADH, 0.1 M KCl, and 0.1 M K_xPO_4, pH 6.75, in a total volume of 150 μL. Reaction mixtures containing all components except HMG-CoA were first monitored to detect any HMG-CoA-independent oxidation

Table 2: Kinetic Parameters of Wild-type and Mutant *P. mevalonii* HMG-CoA Reductases

enzyme[a]	sequence from residues 381−391[b]	V_{max} (eu/mg)	K_m (μM) for HMG-CoA	K_m (μM) for NADH
wild-type *P. mevalonii* HMG-CoA reductase	**H**MALHARNIAV	32	45	33
L384H/R387S/V391L (HSL)	**H**MAHHA**S**NIAL	25	128	35
H385N/R387S/V391L	**H**MAL**N**A**S**NIAL	20	133	46
L384H/H385N/R387S/V391L	**H**MAH**N**A**S**NIAL	20	817	81
H385N/R387S	**H**MAL**N**A**S**NIAV	17	72	52
L384H/H385N/R387S	**H**MAH**N**A**S**NIAV	16	427	89
R387S/V391L	**H**MALHA**S**NIAL	14	99	41
L384H/R387S (HS)	**H**MAHHA**S**NIAV	6.6	74	30
R387S/A390N/V391L	**H**MALHA**S**NINL	1.3	65	50
R387S/A390N	**H**MALHA**S**NI**N**V	1.0	47	139

[a] No activity was detectable for enzymes L384H/H385N/R387S/A390N/V391L, H385N/R387S/A390N/V391L, L384H/H385N/R387S/A390N, H385N/R387S/A390N, L384H/R387S/A390N/V391L, or L384H/R387S/A390N/V391L. [b] Shown in bold face are the catalytic histidine, His^{381} (Darnay *et al.,* 1992), and the introduced target serine, Ser^{387}. Underlined residues differ from those of the wild-type enzyme.

of NADH. Reactions were then initiated by adding HMG-CoA. One unit of HMG-CoA reductase activity is defined as the amount of enzyme which oxidizes 1 μmol of NADH in 1 min.

RESULTS

Retention of Alanine at Position 390 is Accompanied by Retention of High Catalytic Activity. AMP-activated protein kinase proved unsuitable for investigating attenuation of the activity of *P. mevalonii* HMG-CoA reductase. Efficient phosphorylation by AMP-activated protein kinase of mutant forms of *P. mevalonii* HMG-CoA reductase with a target serine at position 387 requires an asparagine and leucine at positions 390 and 391, respectively (Friesen & Rodwell, 1997). However, of eight mutant enzymes with asparagine at position 390, only two were catalytically active. Even the activity of enzymes R387S/A390N and R387S/A390N/V391L did not exceed 4% wild-type activity (Table 2). By contrast, all seven mutant enzymes with alanine at position 390 had activities adequate for investigation of the attenuation of their activity that might accompany their phosphorylation (Table 2). We therefore asked whether a different protein kinase might be more suitable for investigating attenuation of the activity of *P. mevalonii* HMG-CoA reductase.

cAMP-Dependent Protein Kinase Readily Phosphorylates Mutant Enzymes L384H/R387S and L384H/R387S/V391L. Mutant enzymes L384H/R387S (HS)[3] and L384H/R387S/V391L (HSL) had high catalytic activity and essentially wild-type K_m values for HMG-CoA and NADH. These mutant enzymes possess a sequence around Ser^{387} that approximates a consensus kinase recognition sequence for cAMP-dependent protein kinase (PKA)[4] (Kennelly & Krebs, 1991). We therefore asked whether PKA could catalyze the incorporation of phosphate into mutant enzymes HS and HSL. Under conditions where no phosphate was incorporated into the wild-type enzyme, PKA indeed catalyzed the incorporation of ^{32}P from [γ-^{32}P]ATP into mutant enzymes HS and HSL. Phosphorylation of no other mutant enzyme exceeded 3.2%.[5] On the basis of phosphorylation of a single serine per subunit, 26% and 46% of the HS and HSL monomers were labeled (Figure 1).

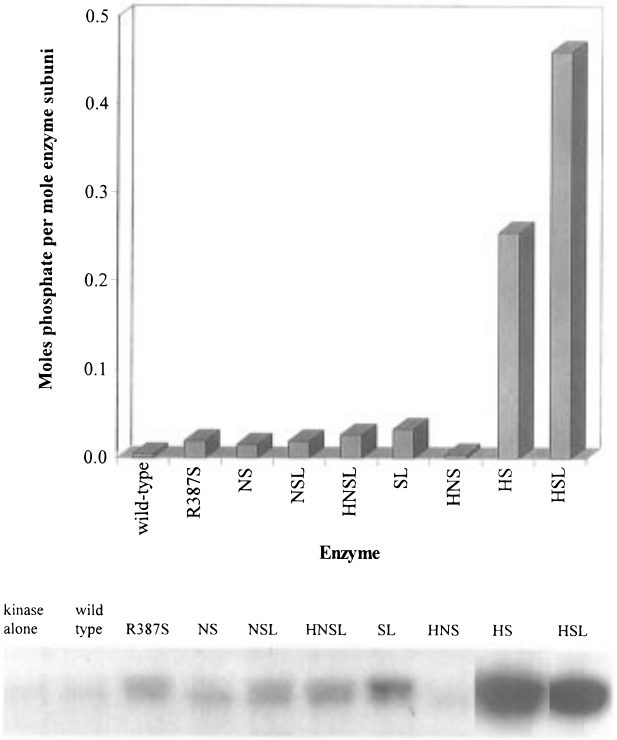

FIGURE 1: Susceptibility of wild-type and mutant enzymes to phosphorylation by cAMP-dependent protein kinase. Phosphorylation of 60 μg portions of purified HMG-CoA reductase mutant enzymes employed 13 μg (600 units) of PKA and 200 μM [γ-^{32}P]-ATP (specific activity 7−21 Ci/mmol) in 20 μL of 40 mM Tris-HCl and 20 mM magnesium acetate, pH 7.4. Portions were removed after 60 min and subjected to 12% SDS−PAGE. Following electrophoresis, gels were soaked in 10% methanol:10% acetic acid for 15 min, then dried under vacuum at 80 °C. Incorporation of ^{32}P into bands whose mobility corresponded to that of an HMG-CoA reductase subunit was determined in a Packard Instant Imager. For the abbreviations used to designate enzymes derived from mutant enzyme R387S, H refers to His^{384}, N to Asn^{385}, S to Ser^{387}, and L to Leu^{391}.

Phosphorylation Attenuates the Activity of Mutant Enzymes L384H/R387S and L384H/R387S/V391L. Attenuation of the catalytic activity of mutant enzymes HS and HSL accompanied their phosphorylation. In both instances, the decrease in activity paralleled the extent of phosphorylation

[3] For ease in discussion, we term the frequently cited mutant enzymes L384H/R387S and L384H/R387S/V391L mutant enzymes HS and HSL, respectively.

[4] His^{384} and His^{385} occupy positions in mutant enzymes HS and HSL analogous to that of the two arginine residues of many natural PKA substrates.

[5] These observations also confirm that the presence of two basic residues on the N-terminal side of the target serine promote phosphorylation by PKA (Kennelly & Krebs, 1991) and shows that histidines can substitute for the arginines of natural PKA substrates.

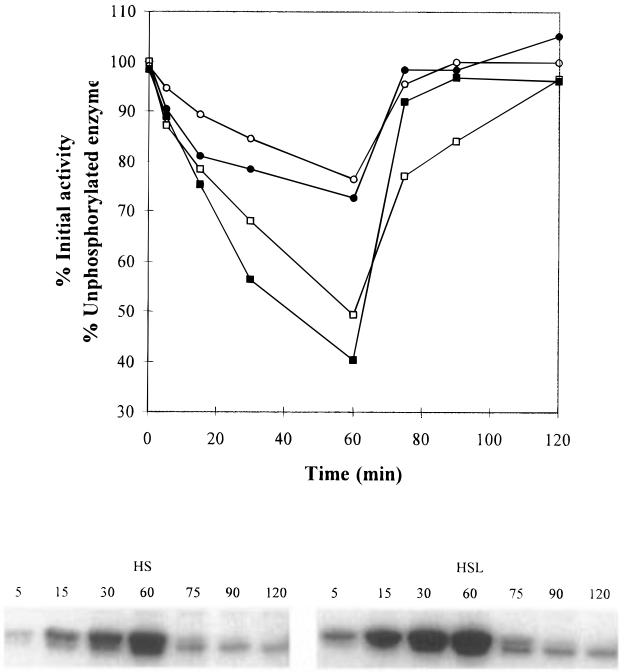

FIGURE 2: Phosphorylation attenuates and dephosphorylation restores the catalytic activity of mutant enzymes L384H/R387S (HS) and L384H/R387S/V391L (HSL). Parallel phosphorylations of 60 μg portions of purified HMG-CoA reductase mutant enzyme HS or HSL employed 13 μg (600 units) of PKA and 200 μM nonradioactive ATP or 200 μM [γ-^{32}P]ATP (specific activity 7−21 Ci/mmol) under conditions that otherwise were identical to those of Figure 1. Non-radioactive portions removed at the indicated times were assayed for catalytic activity. Radioactive portions were subjected to 12% SDS−PAGE. At the 60 min time point, 5 μL portions were mixed with 5 μL of a solution containing 1200 units of lambda protein phosphatase in 1 mM dithiothreitol, 4 mM $MgCl_2$, 10 mM Tris, pH 7.8. Incubation was continued at 30 °C. Portions removed at the indicated times were then assayed for catalytic activity and analyzed by SDS−PAGE. Top: Fraction of initial activity (open symbols) and fraction of enzyme remaining unphosphorylated (closed symbols) for enzymes HS (circles) and HSL (squares). Bottom: Autoradiogram of a time course for phosphorylation and subsequent dephosphorylation of enzymes HS and HSL. Numbers indicate time in minutes. The band of higher apparent mass represents the phosphorylated 45 kDa subunit of mutant enzymes HS and HSL, and the band of lower apparent mass represents the 42 kDa autophosphorylated catalytic subunit of cAMP-dependent protein kinase. As shown, lambda protein phosphatase efficiently dephosphorylated mutant enzymes HS and HSL, but not PKA. This confirms the known resistance of autophosphorylated Thr^{197} and Ser^{338} of PKA to dephosphorylation (Toner-Webb *et al*., 1992; Thomas *et al*., 1995).

with an approximate stoichiometry of 1 phosphate per subunit (Figure 2).

Dephosphorylation Restores Activity to Mutant Enzymes L384H/R387S and L384H/R387S/V391L. Lambda protein phosphatase removed the incorporated ^{32}P from previously phosphorylated mutant enzyme HS or HSL, and dephosphorylation was accompanied by complete restoration of initial activity (Figure 2).

Replacing Arginine 387 by Aspartate Mimics Attenuation of Activity by Phosphorylation. The attenuation of catalytic activity that accompanies phosphorylation of Ser^{871} of Syrian hamster HMG-CoA reductase was previously inferred to result at least in part from the introduction of negative charge. Syrian hamster mutant enzyme S871D bound substrates with wild-type affinities, but had only 10% wild-type activity (Omkumar *et al*., 1994). To inquire whether the introduction of negative charge at position 387 of *P. mevalonii* HMG-CoA reductase also contributed to the attenuation of activity that accompanies phosphorylation, we constructed *P. mevalonii* mutant enzyme R387D. Arg^{387} is not essential for catalysis since mutant enzyme R387S retained 84% wild-type activity and essentially wild-type K_m values for HMG-CoA and NADH (Table 3). Like its hamster counterpart, mutant enzyme R387D had essentially wild-type K_m values for HMG-CoA and NADH and exhibited 10% wild-type activity (Table 3). As for the hamster enzyme, the attenuation of activity that accompanies phosphorylation of the regulatory serine thus appears to result at least in part from the introduction of negative charge.

Table 3: Kinetic Parameters of Wild-type and Mutant *P. mevalonii* Enzymes R387S and R387D and of Wild-type and Mutant Hamster Enzyme S871D[a]

	V_{max} (eu/mg)	K_m (μM) HMG-CoA	NADH	NADPH
wild-type *P. mevalonii* HMG-CoA reductase	32	45	33	
R387S	27	47	32	
R387D	3.0	49	99	
wild-type hamster HMG-CoA reductase	16	22		42
S871D	1.6	14		57

[a] Data are for the reductive deacylation of HMG-CoA to mevalonate and for the Syrian hamster enzyme are from Omkumar *et al.* (1994).

DISCUSSION

The activity of HMG-CoA reductase, the catalyst of the rate-limiting step of cholesterol biosynthesis in mammals and the target of cholesterol-lowering drugs, is regulated *in vivo* by reversible phosphorylation. The structure of the wild-type *P. mevalonii* enzyme, a form whose activity is not naturally regulated by phosphorylation, has been solved at 3 Å resolution (Lawrence *et al*., 1995). The mechanism by which phosphorylation modulates activity has been postulated to involve an ionic interaction between the phosphoserine and the nearby catalytic histidine, blocking release from the enzyme of the inhibitory $CoAS^-$ thioanion. Our ultimate goal is to determine the structural basis for this regulation. Toward this goal, we have engineered wild-type *P. mevalonii* HMG-CoA reductase, a substrate for neither the AMP-activated nor the cAMP-dependent protein kinase, to forms whose activity is reversibly regulated by phosphorylation/dephosphorylation. Phosphorylation employed cAMP-dependent protein kinase. Dephosphorylation employed lambda phosphatase.

Site-directed mutagenesis was employed to insert a phospho-acceptor serine and a cAMP-dependent protein kinase recognition sequence into wild-type *P. mevalonii* HMG-CoA reductase. In all relevant properties, phosphorylation, subsequent dephosphorylation, and accompanying changes in catalytic activity, *P. mevalonii* HMG-CoA reductase mutant enzymes L384H/R387S (HS) and L384H/R387S/V391L (HSL) behaved in a manner analogous to that of mammalian HMG-CoA reductase. To our knowledge, this represents the first instance in which an enzyme has been engineered to a form that not only is phosphorylated at a specific residue, but whose activity is reversibly regulated by successive phosphorylation and dephosphorylation.

The same amino acids perform analogous functions in the bacterial and mammalian enzymes (Wang *et al*., 1990; Darnay *et al*., 1992; Darnay & Rodwell, 1993; Frimpong & Rodwell, 1994), implying a similar active site geometry for *P. mevalonii* and mammalian HMG-CoA reductases. The only three-dimensional HMG-CoA reductase structure is that from *P. mevalonii.* This enzyme, when phosphorylated at position 387, thus constitutes an attractive model for structural studies of the mechanism whereby phosphorylation attenuates activity. Ultimate solution of the structure of mutant *P. mevalonii* HMG-CoA reductases in their phospho- and dephospho-forms thus should reveal specific interactions of the phosphoserine residue in the active site that are critical for attenuation of HMG-CoA reductase activity.

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