Identification of Elements Critical for Phosphorylation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase by Adenosine Monophosphate-Activated Protein Kinase: Protein Engineering of the Naturally Nonphosphorylatable 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase from *Pseudomonas mevalonii*†

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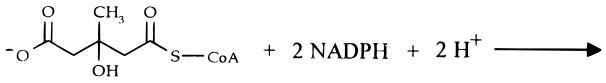
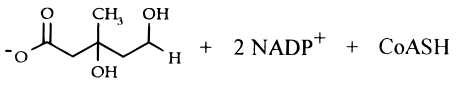
ABSTRACT: The initially nonphosphorylatable 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of *Pseudomonas mevalonii* (E.C. 1.1.1.88) was engineered to phosphorylatable forms in order to identify elements critical for phosphorylation of HMG-CoA reductase by AMP-activated protein kinase. *P. mevalonii* mutant enzymes phosphorylatable by AMP-activated protein kinase were engineered by substituting cognate residues from the kinase recognition sequence of Syrian hamster HMG-CoA reductase (E.C. 1.1.1.34). Various combinations of residues 381−391, which correspond to the kinase recognition sequence of the hamster enzyme, were mutated. *P. mevalonii* mutant enzyme R387S, in which a serine had been inserted at position P, which corresponds to that of the regulatory serine of the hamster enzyme, was only weakly phosphorylated. Genes that encoded thirty-six additional mutant enzymes containing various portions of the hamster kinase recognition sequence were constructed. Following expression, purified mutant enzymes were assayed as substrates for AMP-activated protein kinase. Identified as critical for phosphorylation was the simultaneous presence of aspartate or asparagine at position P+3 and of leucine at position P+4, three and four residues on the C-terminal side of the phosphorylatable serine, respectively. Two basic residues at positions P−1, P−2, or P−3 also appeared to be critical for phosphorylation when present in combination with aspartate or asparagine at P+3 and leucine at P+4.

In yeast and all higher eukarya, the enzyme HMG-CoA[1] reductase (E.C. 1.1.1.34) catalyzes the reductive deacylation of HMG-CoA to mevalonate, an early and committed step in the biosynthesis of isoprenoids:



(*S*)-HMG-CoA



(*R*)-Mevalonate

As for several key regulated enzymes of lipid and carbohydrate metabolism, the activity of the HMG-CoA reductases of higher eukarya is regulated by reversible phosphorylation (Hunter & Rodwell, 1980; Kennelly & Rodwell, 1985). It was noted over 2 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 (Harwood *et al*., 1984) and HMG-CoA reductase phosphatase (Brown & Rodwell, 1983). The first protein kinase shown to require a nucleotide as an allosteric effector (Harwood *et al*., 1984), HMG-CoA reductase kinase also phosphorylates acetyl-CoA carboxylase (Carling *et al*., 1989) and hormone-sensitive lipase (Garton *et al*., 1989) and is optimally activated by 5′-AMP. These observations prompted the renaming of HMG-CoA reductase kinase to AMP-activated protein kinase (AMPK). A participant in a protein kinase cascade that responds to the intracellular AMP/ATP ratio rather than to extracellular signals, AMPK appears to regulate fatty acid and cholesterol biosynthesis by inactivating HMG-CoA reductase and acetyl-CoA carboxylase when intracellular ATP levels are depleted (Carling *et al*., 1989).

Phosphorylation-mediated regulation of HMG-CoA reductase activity by AMPK involves a single serine, Ser^{871} of the rat (Clarke & Hardie, 1990) and hamster enzymes (Sato *et al*., 1993; Omkumar & Rodwell, 1994) and Ser^{577} of isoform 1 of *Arabidopsis thaliana* HMG-CoA reductase (Dale *et al*., 1995). While Ser^{871}, the regulatory serine of the Syrian hamster enzyme, plays no role in catalysis or

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[1] Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; CoASH, coenzyme A; hamster HMG-CoA reductase, the recombinant catalytic domain of Syrian hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase; AMPK, adenosine monophosphate-activated protein kinase.

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Mesocricetus auratus (Syrian hamster)	H	M	V	H	N	R	S	K	I	N	L
Mus musculus (Mouse)	H	M	V	H	N	R	S	K	I	N	L
Ricetulus grisieus (Chinese hamster)	H	M	V	H	N	R	S	K	I	N	L
Homo sapiens (Human)	H	M	I	H	N	R	S	K	I	N	L
Rattus norvegicus (Rat)	H	M	V	H	N	R	S	K	I	N	L
Xenopus laevis (Frog)	H	M	V	H	N	R	S	K	I	N	L
Strongylocentrus purpuratus (Sea urchin)	H	M	K	H	N	R	S	A	L	N	I
Blatella germanica (Cockroach)	H	M	R	H	N	R	S	S	V	S	T
Drosophila melanogaster (Fruit fly)	H	M	R	H	N	R	S	S	I	A	V
Arabidopsis thaliana 1	H	M	K	Y	N	R	S	S	R	D	I
Arabidopsis thaliana 2	H	M	K	Y	N	R	S	S	R	D	I
Raphanus sativus 1 (Radish)	H	M	K	Y	N	R	S	S	R	D	I
Raphanus sativus 2	H	M	K	Y	N	R	S	S	R	D	I
Solanum tuberosum 1	H	M	K	Y	N	R	S	I	K	D	I
Solanum tuberosum 2 (Potato)	H	M	K	Y	N	R	S	T	K	A	S
Solanum tuberosum 3	H	M	K	Y	N	R	S	C	K	D	V
Lycopersicon esculentum 1 (Tomato)	H	M	K	Y	N	R	S	I	K	D	I
Lycopersicon esculentum 2	H	M	K	Y	N	R	S	T	K	D	V
Lycopersicon esculentum 3	H	M	K	Y	N	R	S	S	K	D	V
Nicotiana sylvestris (Tobacco)	H	M	K	Y	N	R	S	T	K	D	V
Hevea brasiliensis 1 (Rubber tree)	H	M	K	Y	N	R	S	S	K	D	M
Hevea brasiliensis 2	H	M	K	Y	N	R	S	S	K	D	V
Hevea brasiliensis 3	H	M	K	Y	N	R	S	A	K	D	V
Camptotheca acuminata (Magnolia)	H	M	K	Y	N	R	S	N	K	D	V
Catharanthus roseus (Periwinkle)	H	M	K	Y	N	R	S	S	K	D	I
Dictyostelium discoideum (Slime mold)	H	L	Q	Y	N	R	A	K	T	N	
Saccharomyces cerevisiae 1 (Yeast)	H	M	T	H	N	R	K	P	A	E	P
Saccharomyces cerevisiae 2	H	M	T	H	N	R	K	T	N	K	A
Haloferax volcanii (Archaebacterium)	H	A	E	L	G	R					
Sulfolobus solfataricus (Archaebacterium)	H	A	K	L	G	R	A	M	K	V	
Schistosoma mansoni (Schistosome)	H	M	H	F	N	R	A	K	Q	S	T
Pseudomonas mevalonii (Bacterium)	H	M	A	L	H	A	R	N	I	A	V

FIGURE 1: Alignment of HMG-CoA reductase sequences. Shown is the region that extends for up to 10 residues beyond the catalytic histidine (black), positions P−6 to P+4. Where present, the putative phosphorylated serine, position P, is also boxed in black. The *S. solfataricus* sequence was kindly provided by James R. Brown and W. Ford Doolittle of Dalhousie University, Halifax, Nova Scotia. All other gene sequences were obtained from GenBank. Alignments were produced by the Pileup program of the Wisconsin package (Devereaux *et al.*, 1984).

substrate recognition (Omkumar & Rodwell, 1994), it is located only six residues from the catalytic histidine, His[865] (Darnay *et al.*, 1992), a His to Ser spacing that is strictly conserved in the HMG-CoA reductases of all higher eukarya (Figure 1).

Analysis of the sequences of substrates (Kennelly & Krebs, 1991) and studies using synthetic peptide substrates (Weekes *et al.*, 1993; Dale *et al.*, 1995) have implicated a consensus recognition motif for AMPK that includes hydrophobic residues at positions P−5 and P+4 and a basic residue at position P−2 or P−3.[2] While peptides provide useful information, their small size and unknown conformation limit their utility as models for proteins. We therefore engineered *P. mevalonii* HMG-CoA reductase which lacks an appropriately located serine and hence is not phosphorylated by AMPK, to produce phosphorylatable substrates and to identify elements critical for phosphorylation of HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Materials. Purchased reagents include DEAE-Sepharose (Sigma), Blue Sepharose CL-6B (Pharmacia), 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), and a Qiaex gel-extraction kit (Qiagen). AMP-activated protein kinase from rat liver was purified through the Blue Sepharose

[2] We refer to residues 381−391 of *P. mevalonii* HMG-CoA reductase, which correspond to residues 865−875 (the putative kinase recognition sequence) of hamster HMG-CoA reductase, as positions "P−6 through P+4", relative to the phosphorylatable serine as position "P".

	P-6	P-5	P-4	P-3	P-2	P-1	P	P+1	P+2	P+3	P+4
P. mevalonii	H	M	A	L	H	A	R	N	I	A	V
	CAC	ATG	GCC	CTG	CAT	GCG	CGC	AAT	ATT	GCC	GTG
Hamster	H	M	V	H	N	R	S	K	I	N	L
	CAC	ATG	GTT	CAC	AAC	AGA	TCG	AAG	ATA	AAT	TTA
Mutant	H	M	A/V	L/H	H/N	A/R/G/P	S	N/K	I	A/N/D/T	V/L
enzymes	CAC	ATG	G(C/T)C	C(T/A)T	(C/A)AT	(G/C)(G/C)T	TCG	AA(T/G)	ATT	(G/A)(C/A)C	(G/C)TG

FIGURE 2: Mutagenesis of residues at positions P−6 to P+4 of *P. mevalonii* HMG-CoA reductase. Positions P−6 to P+4 correspond to residues 381−391 of *P. mevalonii* HMG-CoA reductase and to residues 865−875 of the hamster enzyme. The DNA sequence is that of bases 5−37 of the degenerate 50-mer oligonucleotide employed for mutagenesis.

fraction (Carling *et al*., 1989). The method of Bradford (1976) and a Bio-Rad protein assay kit were used to determine protein concentration. Assays were performed in triplicate.

Expression and Purification of Enzymes. The catalytic domain of Syrian hamster HMG-CoA reductase (E.C. 1.1.1.34) was purified as previously described (Frimpong *et al*., 1993). 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 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 was conducted as previously described for the wild-type enzyme (Wang *et al*., 1990). The purity of the mutant enzymes was judged to be no less than 99% as judged by SDS−PAGE, a purity equal to that of the purified wild-type enzyme.

Site-Directed Mutagenesis. All mutant genes were constructed using the PCR-based overlap extension method of oligonucleotide-directed mutagenesis (Mikaelin & Sergeant, 1992). Oligonucleotides synthesized in the Purdue University Laboratory for Macromolecular Structure were purified by thin-layer chromatography or polyacrylamide gel electrophoresis prior to use.

The oligonucleotide used to generate the gene encoding mutant enzyme R387S changed the CGC codon to AGC and simultaneously removed a *Bss*HII site. The oligonucleotide used to generate the gene for mutant enzyme 1 encoded hamster residues at positions P−4 through P+4 and simultaneously inserted an *Nde*I site. "Random directed" PCR mutagenesis of residues 382−391 of *P. mevalonii* HMG-CoA reductase, which correspond to residues 866−875 of the Syrian hamster enzyme, employed a degenerate 50-mer oligonucleotide (Figure 2) that encoded a serine in place of arginine 387, and any combination of the *P. mevalonii* or the hamster residues at positions 382−386 and 388−391. Since positions 382 and 389 are methionine and isoleucine in both forms of the enzyme, these residues were not changed. Due to the degeneracy of the oligonucleotide, glycine and proline were also inserted at position 386 and aspartate and threonine at position 390.

Mutant genes encoding enzymes 24 and 31−35 introduced silent mutations encoding an *Nhe*I site at the codons for positions P and P−1. These *Nhe*I sites were employed to create genes encoding enzymes 21−23 and 25−30 by fusing DNA sequences encoding mutations at positions P−2 and P−3 (enzymes 31−33) to sequences encoding mutations at positions P+3 and P+4 (enzymes 24, 34, and 35). All mutations were verified by the dideoxy chain-termination method of DNA sequencing (Sanger *et al*., 1977) using an Isotherm DNA sequencing kit and [α-^{35}S]dATP.

Screening of Mutant Enzymes for Phosphorylation by AMP-Activated Protein Kinase. Ten-milliliter cultures of log-phase *E. coli* BL21 cells that harbored plasmids encoding mutant *P. mevalonii* HMG-CoA reductases were lysed by passage through a French pressure cell. The enzymes were then purified through the ammonium sulfate fraction (Wang *et al*., 1990) prior to analysis for their ability to be phosphorylated by AMPK. Promising candidate enzymes revealed by initial screenings were then purified to homogeneity and further characterized.

Phosphorylation by AMP-Activated Protein Kinase and Calculation of Phosphorylation Index. Assay mixtures contained, in 10 μL of 50 mM NaCl, 100 mM sucrose, 5 mM dithiothreitol, 10% glycerol, 50 mM Tris-HCl, pH 7.3, 200 μM [γ-^{32}P]ATP (specific activity 1000−3000 Ci/mmol), 200 μM AMP, 0.2 μg of AMPK, and 1.0 μg of HMG-CoA reductase. Experimental assays contained the mutant protein of interest. Control assays contained wild-type hamster or *P. mevalonii* HMG-CoA reductase. Incubations were for 30 min at 37 °C. Following addition of 10 μL of 2× SDS loading buffer (63 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.001% bromophenol blue), 10-μL portions were taken for SDS−12% PAGE. Following electrophoresis, gels were soaked in 10% methanol/10% acetic acid for 15 min and then dried under vacuum at 80 °C for 30 min. Incorporation of ^{32}P into bands whose mobility corresponded to that of an HMG-CoA reductase subunit was determined in a Packard instant imager. Relative levels of phosphorylation are expressed as the phosphorylation index, the incorporation of ^{32}P into a given form of the enzyme multiplied by 100 and then divided by the incorporation into the hamster enzyme control.

RESULTS

Phosphorylation of Wild-Type P. mevalonii HMG-CoA Reductase. No incorporation of ^{32}P from [γ-^{32}P]ATP was

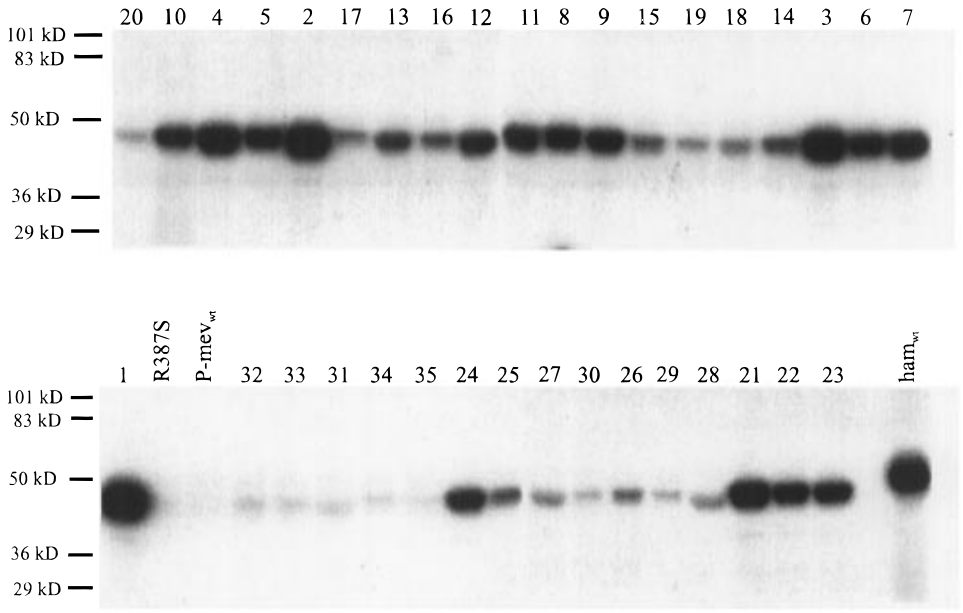

FIGURE 3: Autoradiogram showing the phosphorylation of wild-type and mutant enzymes by AMP-activated protein kinase. Incubation of each wild-type or mutant protein with AMPK was for 30 min at 37 °C. Portions, 0.5 μg, were then subjected to SDS−12% PAGE. Incorporation of ^{32}P into bands whose mobility corresponded to that of an HMG-CoA reductase subunit was determined in a Packard instant imager.

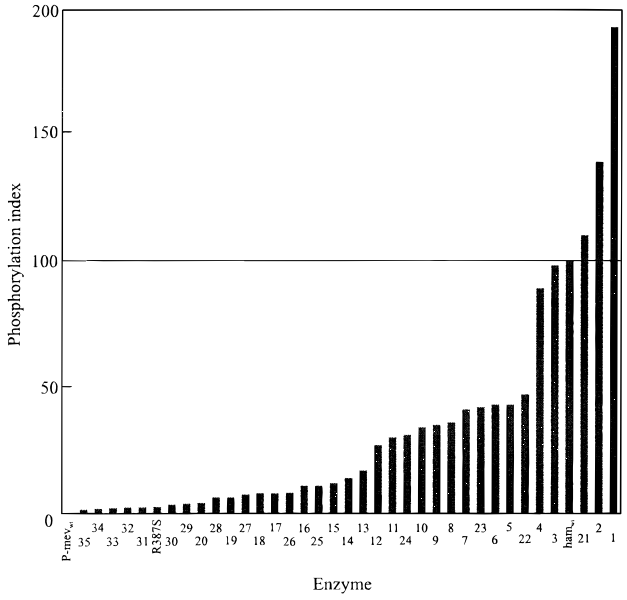

FIGURE 4: Graphical representation of the phosphorylation indices of wild-type and mutant enzymes. Phosphorylation index, the phosphorylation of a mutant enzyme relative to the hamster enzyme, was determined as described in Experimental Procedures. The line at a phosphorylation index of 100 represents the phosphorylation index of the hamster enzyme.

detected for wild-type *P. mevalonii* HMG-CoA reductase (P-mev$_{wt}$) (Figures 3 and 4), which has the sequence HMALHARNIAV at positions P−6 through P+4 (Figure 5, left).

Phosphorylation of Mutant Enzyme R387S. Replacement of Arg^{387} (position P) by serine formed enzyme R387S, which, while phosphorylatable, had a phosphorylation index of only 3 [Figures 4 and 5 (left)].

Phosphorylatable Mutants of P. mevalonii HMG-CoA Reductase. Replacement of all residues from positions P−6 to P+4 of *P. mevalonii* HMG-CoA reductase (HMALHARNIAV) by the corresponding residues of Syrian hamster HMG-CoA reductase (HMVHNR**S**KINL)[3] yielded a readily phosphorylatable substrate, enzyme 1 (Figures 3 and 4), whose phosphorylation index was 185 (Figure 5).

We next employed a degenerate oligonucleotide (Figure 2) to engineer genes encoding mutant *P. mevalonii* enzymes that contained either the *P. mevalonii* or the corresponding hamster residue at positions P−6 to P+4. Following expression and purification through the ammonium sulfate fraction, 118 mutant enzymes were screened as substrates for AMPK. Of the 30 enzymes that were phosphorylated, 11 appeared to be degraded or did not purify to homogeneity and were not characterized further. The other 19 enzymes, numbers 2−20 (Figure 5, left), were purified to homogeneity and their phosphorylation indices were determined. All nineteen had phosphorylation indices less than mutant enzyme 1 but greater than enzyme R387S (Figure 5, left). Of these 19, the most readily phosphorylated were enzymes 2 (HMAHHG**S**KIDL), 3 (HMVHHG**S**KIDL), and 4 (HMAHNR**S**NIDL), whose phosphorylation indices were 140, 95, and 85, respectively.

Identification of Residues Critical for Phosphorylation. The residues present at positions P−1 through P−4, P+1, P+3, and P+4 of enzymes 2−20 (Figure 5, left) were examined to identify positions critical for phosphorylation. Position P−4: In 13 of 19 mutant enzymes, but not in enzyme 2, whose phosphorylation index was 140, Val replaced the Ala of P-mev$_{wt}$. A Val at P−4 thus would not appear to be critical for phosphorylation. Position P−3: His at P−3 may be important for phosphorylation since in 12 of 19 mutant enzymes, including the readily phosphorylated enzymes 2, 3, and 4, His replaced the Leu of P-mev$_{wt}$. Position P−2: Asn replaced the His of P-mev$_{wt}$ in 14 of 19 mutant enzymes. While these 14 did not include enzyme 2 or 3, all mutants that lacked His at P−3 had Asn at P−2,

[3] For the sequence of wild-type and mutant enzymes at positions P−6 through P+4, the putative phosphorylatable serine is listed in boldface type and additional residues that differ from those of wild-type *P. mevalonii* HMG-CoA reductase are underlined.

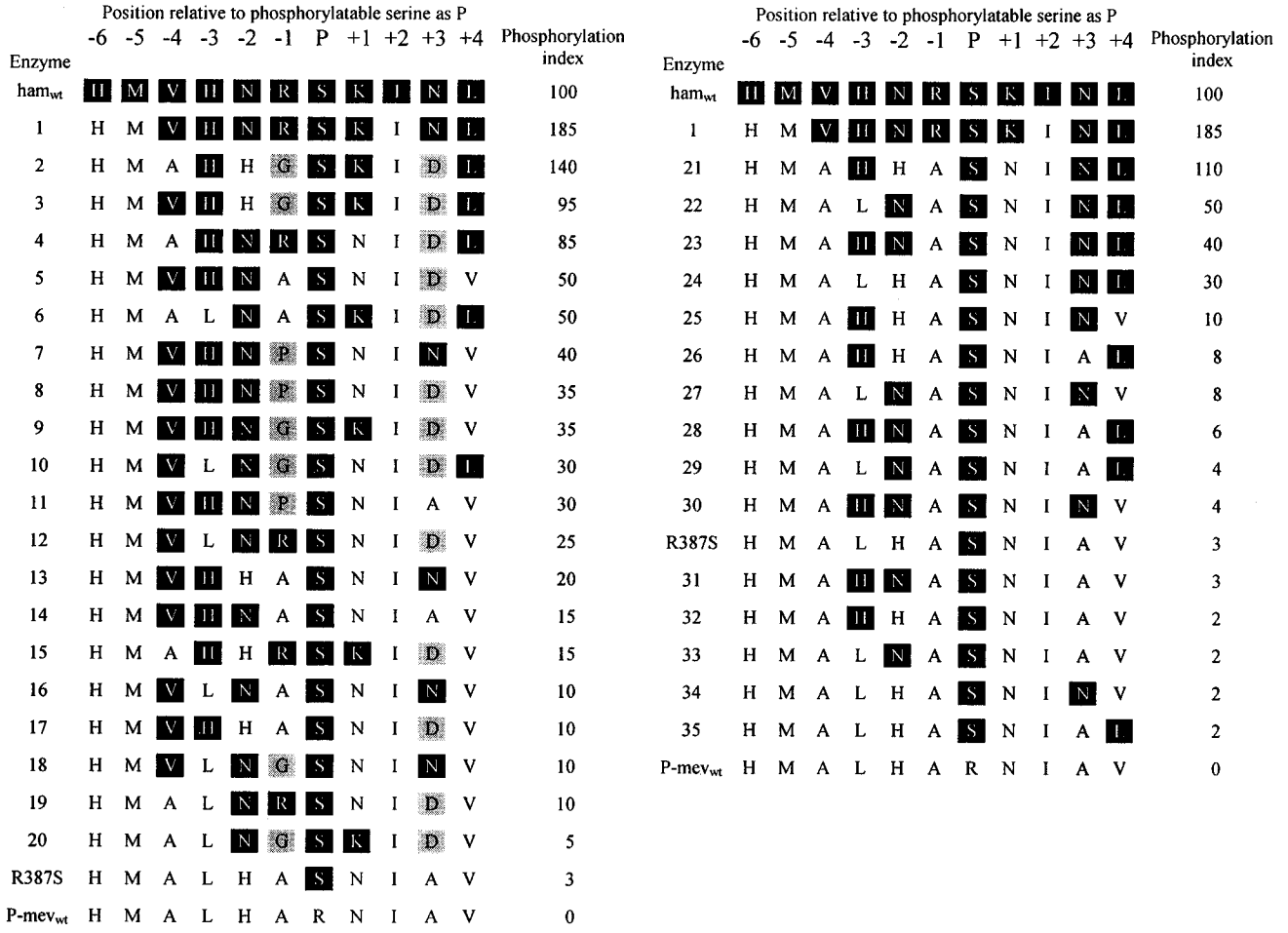

FIGURE 5: Sequences and phosphorylation indices of wild-type and mutant HMG-CoA reductases. Residues are numbered from the position of the putative phosphorylatable serine as P and are identified as being present in the hamster enzyme (ham$_{wt}$) (black), the wild-type *P. mevalonii* enzyme (P-mev$_{wt}$) (white), or neither ham$_{wt}$ nor P-mev$_{wt}$ (gray). Sequences shown are for ham$_{wt}$, P-mev$_{wt}$, and 21 mutant *P. mevalonii* HMG-CoA reductases (left) and for derivatives of enzyme R387S mutated at positions P−3, P−2, P+3, and P+4 (right).

suggesting that either His at P−3 or Asn at P−2 is important for phosphorylation. Position P−1: The presence of 6 Gly, 6 Ala, 4 Arg, and 3 Pro in the 19 mutant enzymes suggested that the residue present at P−1 is not a strong determinant of phosphorylation. Position P+1: In 6 of 19 mutant enzymes Lys replaced the Asn of P-mev$_{wt}$. Thus, while Lys was present in both enzymes 2 and 3, either Lys or Asn at P+1 appears to be suitable for phosphorylation. Position P+3: Asp replaced the Ala of P-mev$_{wt}$ in 13 of 19 mutant enzymes, including enzymes 2, 3, and 4. In addition, 4 of 19 mutants had Asn at this position. Replacing Ala at P+3 by either Asp or Asn thus appeared to be important for phosphorylation by AMPK. Position P+4: The 5 of 19 mutant enzymes in which Leu replaced Val of P-mev$_{wt}$ included enzymes 2, 3, and 4, suggesting that Leu at P+4 is advantageous for phosphorylation.

Phosphorylation of Enzyme R387S Mutated at Positions P−3, P−2, P+3, and P+4. Inspection of the sequences of enzymes 2−20 implicated positions P−3, P−2, P+3, and P+4 as possible determinants for phosphorylation of *P. mevalonii* HMG-CoA reductase by AMPK. We therefore next engineered derivatives of enzyme R387S in which positions P−3, P−2, P+3, and P+4 contained, individually and in combination, the residue present at the analogous position in the hamster enzyme. These 15 mutant enzymes were then purified to homogeneity and analyzed as substrates for AMPK (Figures 3 and 4).

A single amino acid substitution at position P−3, P−2, P+3, or P+4 did not improve phosphorylation of enzyme R387S. None of the phosphorylation indices of mutant enzymes 32−35 exceeded that of enzyme R387S. Several dual substitutions also had little or no effect. The phosphorylation index of enzyme 31 (HMAHNA**S**NIAV) was 3, and those of enzymes 25, 26, 27, and 29 (HMAHHA**S**NINV, HMALNA**S**NINV, HMAHHA**S**NIAL, and HMALNA**S**NIAL,) ranged from 10 to 4. The triple substitutions in enzymes 28 (HMAHNA**S**NIAL) and 30 (HMAHNA**S**NINV) also did not significantly increase the phosphorylation index relative to that of enzyme R387S.

The most striking improvement in phosphorylation by AMPK was for mutant enzymes that contained the combination of Asn at P+3 and Leu at P+4. Enzymes 22 (HMALNA**S**NINL), 23 (HMAHNA**S**NINL), and 24 (HMALHA**S**NINL) had phosphorylation indices of 50, 40, and 30, respectively. A combination of His, Ala, and Leu at positions P−3, P+3, and P+4 yielded enzyme 21 (HMAHHA**S**NINL), whose phosphorylation index of 110 was 37-fold greater than that of enzyme R387S. Enzyme 21 thus was phosphorylated by AMPK as readily as the hamster enzyme.

DISCUSSION

The high substrate specificity of AMP-activated protein kinase is shown by its ability to phosphorylate only Ser[871]

of the 27 Ser and 26 Thr residues of Syrian hamster HMG-CoA reductase (Clarke & Hardie, 1990; Sato *et al*. 1993; Omkumar & Rodwell, 1994) and by its inability to phosphorylate any of the 18 Ser or 15 Thr solvent-accessible residues (Lawrence *et al*., 1995) of the *P. mevalonii* enzyme.

To investigate elements critical for phosphorylation of a physiologic substrate by AMPK, we employed as a scaffold the HMG-CoA reductase of *P. mevalonii* upon which to engineer phosphorylatable forms of this naturally nonphosphorylatable enzyme. To establish the feasibility of our proposed approach, we first replaced residues 381−391 of the *P. mevalonii* enzyme by analogous residues 865−875 of the hamster enzyme. That this converted the enzyme to a readily phosphorylatable form suggested both that the elements necessary for kinase recognition are present in this region and that its insertion into the *P. mevalonii* enzyme was not accompanied by conformational changes unfavorable to recognition by AMPK.

Enhanced phosphorylation accompanied the presence of Asp or Asn at position P+3 in combination with Leu at position P+4. This combination thus appears to constitute one critical component of the AMPK recognition sequence. The importance of Asp or Asn at position P+3 is supported by the presence of Asn at P+3 in all five mammalian HMG-CoA reductases and of Asp at P+3 in 15 of 16 plant enzymes (Figure 1). The importance of Leu is suggested by its presence at P+4 in both mammalian and *Xenopus* HMG-CoA reductases and by the observation that substitution of Val at position P+4 of the Chinese hamster enzyme (Ching *et al*., 1996) significantly decreased phosphorylation by AMPK. By contrast, the HMG-CoA reductases of sea urchin, cockroach, and higher plants lack Leu at position P+4. Just as the plant AMPK homologue, unlike rat liver AMPK, does not require AMP (Ball *et al*., 1994), the kinase recognition motifs of the putative AMPK homologues of these organisms thus may differ slightly from that of rat liver AMPK.

Protein kinase recognition motifs frequently contain basic residues on the N-terminal side of the target serine (Kennelly & Krebs, 1991), and investigations using synthetic peptides (Weekes *et al*., 1993; Dale *et al*., 1995) have implicated basic residues in substrate recognition by AMPK. In addition, all HMG-CoA reductases with a putative phosphorylatable Ser have at least two basic amino acids at positions P−1 through P−4 (Figure 1). The mammalian enzymes all have His at P−3 and Arg at P−1, the plant enzymes have Lys at P−4 and Arg at P−1, and the HMG-CoA reductases from sea urchin, cockroach, and fruit fly have His, Lys, or Arg at P−1, P−3, and P−4.

The data presented here further document the importance for an AMPK recognition motif of basic residues on the N-terminal side of the phosphorylatable serine. While enhanced phosphorylation did not accompany the presence of His at P−2 or P−3 in *P. mevalonii* mutant enzymes with Asp or Asn at P+3 and Leu at P+4, the presence of His at P−2 and P−3, or of His at P−3 and Arg at P−1, yielded enzymes phosphorylated at least as readily as the hamster enzyme. In addition, we have shown that it is possible to employ protein engineering to convert a naturally nonphosphorylatable form of HMG-CoA reductase to forms that are excellent substrates for AMPK.

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