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Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase *in vitro* and in intact rat liver

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The intact, 100 kd microsomal enzyme and the 53 kd catalytic fragment of rat HMG-CoA reductase are both phosphorylated and inactivated by the AMP-activated protein kinase. Using the catalytic fragment, we have purified and sequenced peptides containing the single site of phosphorylation. Comparison with the amino acid sequence predicted from the cDNAs encoding other mammalian HMG-CoA reductases identifies this site as a serine residue close to the C-terminus (Ser872 in the human enzyme). Phosphopeptide mapping of native, 100 kd microsomal HMG-CoA reductase confirms that this C-terminal serine is the only major site phosphorylated in the intact enzyme by the AMP-activated protein kinase. The catalytic fragment of HMG-CoA reductase was also isolated from rat liver in the presence of protein phosphatase inhibitors under conditions where the enzyme is largely in the inactive form. HPLC, mass spectrometry and sequencing of the peptide containing Ser872 demonstrated that this site is highly phosphorylated in intact liver under these conditions. We have also identified by amino acid sequencing the N-terminus of the catalytic fragment, which corresponds to residue 423 of the human enzyme.

Key words: AMP-activated protein kinase/cholesterol synthesis/HMG-CoA reductase/phosphorylation sites/protein phosphorylation

Introduction

HMG-CoA reductase catalyses the principal regulatory step in the biosynthetic pathway of cholesterol and other isoprenoid compounds, i.e. the conversion of HMG-CoA to mevalonate (Gibson and Parker, 1987). Regulation of the enzyme has been the subject of great interest because of the involvement of cholesterol in the development of atherosclerosis and heart disease. Inhibitors of HMG-CoA reductase (Endo and Hasumi, 1989) dramatically lower plasma cholesterol levels in humans and are in clinical use as hypocholesterolaemic agents. Mevalonate derived products are also involved in the control of cell growth and proliferation. For example, the attachment of polyisoprene units derived from mevalonate is essential for the function of *ras* proteins (Hancock *et al.*, 1989), and it has been suggested that HMG-CoA reductase inhibitors may consequently allow the control of growth of certain tumours Lowy and Willumsen, 1989; Goldstein and Brown, 1990).

The mammalian enzyme is a glycoprotein of ~100 kd, with an amino-terminal domain containing seven hydrophobic regions which anchors the protein in the endoplasmic reticulum membrane, and a carboxy-terminal catalytic domain which projects into the cytosol (Liscum *et al.*, 1983). The membrane and catalytic domains are connected by a proteinase-sensitive linker region. Proteolysis of the native protein releases a soluble, catalytic fragment of ~53 kd from the membrane (Ness *et al.*, 1981).

HMG-CoA reductase is subject to complex multivalent control mechanisms (Goldstein and Brown, 1990). Negative feedback regulation of the amount of the enzyme protein occurs through repression of gene transcription (Edwards *et al.*, 1983), inhibition of mRNA translation (Nakanishi *et al.*, 1988) and stimulation of the rate of degradation (Gill *et al.*, 1985). The activity of the enzyme is regulated acutely by reversible phosphorylation (Gibson, 1985; Beg *et al.*, 1987a), with phosphorylation inactivating the enzyme. Many studies have addressed the *in vivo* phosphorylation state of the enzyme indirectly by measuring the activity before and after dephosphorylation by endogenous or exogenous protein phosphatases. Some investigators failed to find any changes in the proportion of the enzyme in the active state (e.g. Brown *et al.*, 1979), which called into question the physiological relevance of phosphorylation. However, it is now clear that these negative results can be explained by the failure to cool the tissue rapidly (e.g. by cold-clamping) during harvesting (Easom and Zammit, 1984a). Changes in the proportion of enzyme in the active state have been demonstrated in isolated hepatocytes (Ingebritsen *et al.*, 1979) and in rat liver *in vivo* (Easom and Zammit, 1984b). However, prior to the present study changes in phosphorylation state had not been measured directly, and the number and nature of phosphorylation sites had not been established.

HMG-CoA reductase is phosphorylated and inactivated *in vitro* by the AMP-activated protein kinase (formerly termed HMG-CoA reductase kinase), which is stimulated by 5′-AMP, and is itself regulated by reversible phosphorylation, being inactivated by dephosphorylation and reactivated by a kinase kinase (Hardie *et al.*, 1989). The AMP-activated protein kinase also phosphorylates and inactivates acetyl-CoA carboxylase (Carling *et al.*, 1987; Munday *et al.*, 1988; Davies *et al.*, 1989) and hormone-sensitive lipase/cholesterol esterase (Garton *et al.*, 1989), and may provide coordinate regulation of both fatty acid and cholesterol metabolism (Hardie *et al.*, 1989). The AMP-activated protein kinase is the major HMG-CoA reductase kinase in rat liver (when assayed in the absence of calcium and phospholipids) and copurifies with acetyl-CoA carboxylase kinase activity (Carling *et al.*, 1989). Purified HMG-CoA reductase can also be phosphorylated and inactivated *in vitro* by protein kinase C (Beg *et al.*, 1985) and a Ca^{2+}/calmodulin-dependent protein kinase (Beg *et al.*, 1987b).

In order to determine the precise role of phosphorylation of HMG-CoA reductase in the control of cholesterol biosynthesis it is necessary to identify which region of the protein is involved in the regulation of enzyme activity by

phosphorylation. In this paper, we report the identification of the regulatory site of phosphorylation in HMG-CoA reductase by the AMP-activated protein kinase. Phosphorylation at this site is also responsible for inactivation of the enzyme in intact liver. In addition, we have identified the site at which cleavage of the catalytic domain from the membrane domain occurs during isolation in the absence of proteinase inhibitors.

Results

Analysis of the site phosphorylated on the catalytic fragment of HMG-CoA reductase by the AMP-activated protein kinase

Purified catalytic fragment of HMG-CoA reductase was phosphorylated by incubation with [γ-^{32}P]ATP and the AMP-activated protein kinase to a stoichiometry of 0.8 mol phosphate/mol of 53 kd fragment, producing a 72% inactivation compared with controls lacking kinase. Digestion with cyanogen bromide plus Lys-C endoproteinase yielded a single radioactive peptide (Figure 1) that on reversed phase HPLC eluted at 22.5 min on the acetonitrile gradient used. Amino acid analysis of this peak indicated that the peptide had been purified in this single run, and had the composition Asx (1.2), Ser (0.9), His (0.8), Arg (1.1), Val (0.9) and Lys (1.0) (figures in parentheses show molar ratios with respect to [^{32}P]phosphate). This phosphopeptide had the amino acid sequence VHNRSK (Table I), which is identical to a region (residues 867–872) close to the C-terminus of Chinese hamster HMG-CoA reductase predicted from cDNA sequencing (Chin *et al.*, 1984), and apart from a conservative Val–Ile change, with residues 868–873 of the human sequence (Luskey and Stevens, 1985). Fast-atom bombardment mass spectrometry showed that the MH^+ ion had the expected mass of 820.

This peptide contains a single phosphorylatable residue, corresponding to Ser871 in the Chinese hamster enzyme and Ser872 in the human enzyme. In order to demonstrate conclusively that this residue was phosphorylated, the peptide was subject to β-elimination and derivatization with ethanethiol, which converts phosphoserine residues to the more stable, novel amino acid, S-ethylcysteine (Meyer *et al.*, 1986; Holmes, 1987). As expected, the phenylthiohydantoin derivative of S-ethylcysteine, but not that of serine or its dithiothreitol adduct, was detected at cycle 5 (Table I).

We also extended the sequence by isolating the peptide produced by digestion with cyanogen bromide alone (see Materials and methods). The sequence obtained was VHNRSKINLQDL (Table I), showing that the identity with the human and Chinese hamster sequences extends for a further six residues. This peptide required more purification steps than the cyanogen bromide/Lys-C endoproteinase peptide, and even on the first HPLC column was recovered in lower yield. The low yield may be explained by the fact that, based on the human and Chinese hamster sequences, this would represent the C-terminal cyanogen bromide peptide, and it is likely that a variable proteolytic cleavage of the carboxyl-terminus occurs during isolation.

Analysis of the site phosphorylated in native microsomal HMG-CoA reductase by the AMP-activated protein kinase

Microsomal HMG-CoA reductase was prepared using methods which prevent separation of the catalytic fragment from the membrane domain by proteolysis (Ness *et al.*, 1986). Incubation of the crude microsomes with unlabelled MgATP and the AMP-activated protein kinase produced an

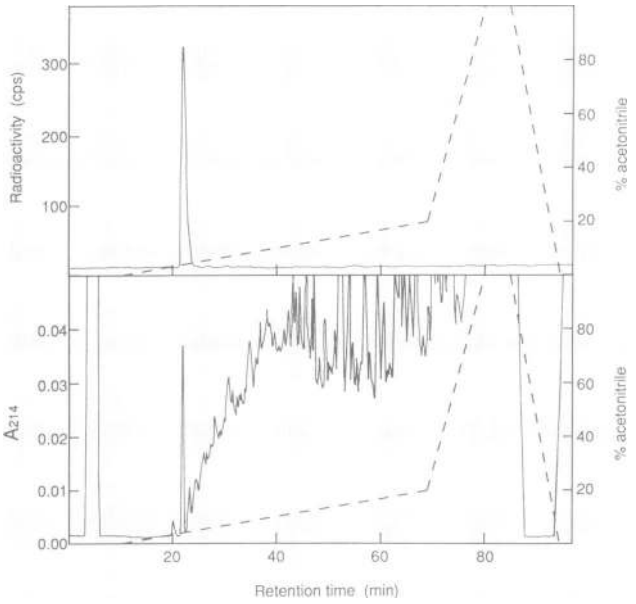

Fig. 1. Reversed-phase HPLC analysis of ^{32}P-labelled peptides derived from the catalytic fragment of HMG-CoA reductase after CNBr cleavage and Lys-C endoproteinase digestion. The top panel shows the radioactivity of the eluate and the bottom panel shows the absorbance at 214 nm. The fragment had been phosphorylated using [γ-^{32}P]ATP and the AMP-activated protein kinase.

Table I. Sequence analysis of phosphopeptides derived from the catalytic fragment of HMG-CoA reductase after phosphorylation by the AMP-activated protein kinase, or after isolation from intact liver in the presence of protein phosphatase inhibitors.

		AMP-activated protein kinase			Intact liver
Cycle	Xaa	CNBr	CNBr/Lys-C	CNBr/Lys-C (derivatized)	CNBr/Lys-C (derivatized)
1	Val	70.5	34.0	44.5	31.6
2	His	7.9	14.6	10.7	14.6
3	Asn	19.6	20.2	20.5	19.7
4	Arg	12.1	16.0	19.2	28.4
5	Ser	5.5	18.1	–	–
	EtCys	–	–	5.2	7.1
6	Lys	1.8	14.8	10.4	12.9
7	Ile	2.4			
8	Asn	9.9			
9	Leu	9.3			
10	Gln	5.5			
11	Asp	14.4			
12	Leu	3.5			

Peptides were isolated by digestion with cyanogen bromide (CNBr), with or without further digestion by Lys-C endoproteinase, with or without derivatization by ethanethiol. Columns show the amino acid identified (Xaa) and the amount of phenylthiohydantoin derivative recovered in pmol at each cycle. Serine was quantified as the serine derivative plus the dithiothreitol adduct. A dash indicates that no residue was detected above background.

85% inactivation of HMG-CoA reductase compared with controls incubated without kinase. The enzyme was phosphorylated under the same conditions using [γ-^{32}P]ATP, and then isolated by immunoprecipitation. Figure 2 shows that the 100 kd form of HMG-CoA reductase was the only major phosphorylated polypeptide present in the immunoprecipitate, while pre-immune serum did not precipitate any radioactive polypeptide. Digestion of the immunoprecipitate with cyanogen bromide and the Lys-C endoproteinase produced a single phosphopeptide, which comigrated on thin layer isoelectric focusing with the VHNRSK peptide produced following phosphorylation of the catalytic fragment (Figure 3). This confirms that the site corresponding to Ser872 is the only major site phosphorylated in intact, microsomal HMG-CoA reductase by the AMP-activated protein kinase.

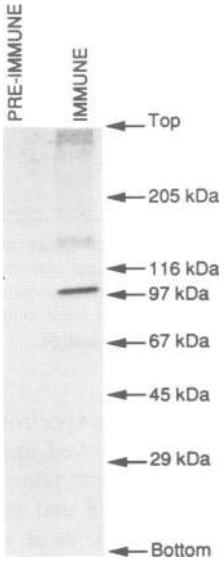

Fig. 2. Autoradiogram after SDS–PAGE of immunoprecipitates of rat liver microsomes made using pre-immune serum and anti-HMG-CoA reductase serum. The microsomes had been labelled using [γ-^{32}P]ATP and the AMP-activated protein kinase as described in the Materials and methods section. Arrows indicate the migration of marker proteins (Sigma high mol. wt markers, Sigma, Poole, Dorset, UK).

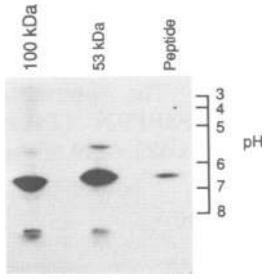

Fig. 3. Autoradiogram after thin layer isoelectric focusing of CNBr/Lys-C endoproteinase digests of intact microsomal HMG-CoA reductase (100 kd) or the 53 kd fragment (53 kd), both of which had been labelled using [γ-^{32}P]ATP and the AMP-activated protein kinase. The microsomal enzyme was isolated by immunoprecipitation as in Figure 2. We also analysed the labelled VHNRSK peptide isolated as in Figure 1 (peptide). The pH scale was drawn by reference to the migration of coloured isoelectric point markers (BDH, Poole, Dorset, UK) and is an approximate guide only.

The site phosphorylated by the AMP-activated protein kinase is phosphorylated in intact liver

The catalytic fragment of rat liver HMG-CoA reductase was prepared from livers which had been dissected without cold-clamping as before, except that protein phosphatase inhibitors (fluoride and pyrophosphate) were included in the homogenization buffer and throughout the preparation. Although the enzyme was homogeneous by SDS–gel electrophoresis, the specific activity was only ~10% of that of fully dephosphorylated enzyme, and the content of alkali-labile phosphate was 0.89 mol/mol. Figure 4 shows that this preparation of HMG-CoA reductase could be dramatically reactivated (after removal of fluoride and pyrophosphate) by incubation with the catalytic subunit of protein phosphatase-1, and could subsequently be inactivated by addition of the AMP-activated protein kinase in the presence of MgATP. For enzyme pretreated with protein phosphatase-1, the incorporation of phosphate by the AMP-activated protein kinase was 0.83 mol/mol 53 kd fragment, as against only 0.04 mol/mol for the enzyme not pretreated with phosphatase. These results indicated that the enzyme isolated from intact liver in the presence of protein phosphatase inhibitors was highly phosphorylated at a site labelled by the AMP-activated protein kinase.

10 nmol of the enzyme purified in the presence of protein phosphatase inhibitors, together with a trace amount (25 pmol) of the normal preparation which had been ^{32}P-labelled *in vitro* using the AMP-activated protein kinase, was digested with cyanogen bromide and Lys-C

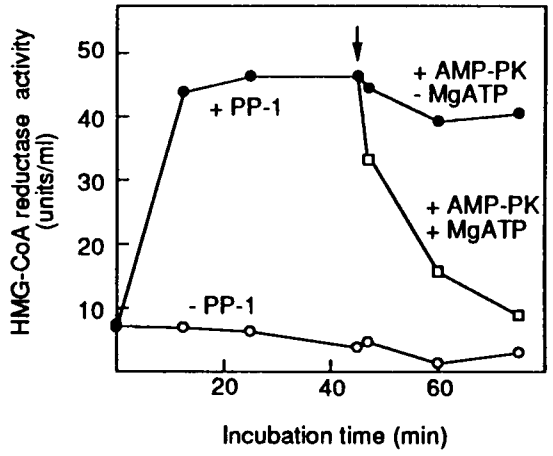

Fig. 4. Reactivation of HMG-CoA reductase phosphorylated in intact liver by protein phosphatase 1, and subsequent inactivation by the AMP-activated protein kinase. The catalytic fragment of HMG-CoA reductase was purified in the presence of 50 mM NaF and 5 mM Na pyrophosphate, concentrated and washed into 50 mM HEPES, pH 7.2, 5 mM dithiothreitol (without NaF and Na pyrophosphate). The specific activity was 720 units/mg. HMG-CoA reductase (120 units/ml) was incubated at 30°C with 0.1 vol of protein phosphatase 1 (50 units/ml in 50 mM Tris–HCl, 0.1 mM EGTA, 50% (by vol) glycerol) (+PP-1), or with phosphatase buffer alone (–PP-1). At the point indicated by the arrow, 0.1 vol of 1 M NaF (to inhibit the phosphatase), 0.1 vol of 1 mM ATP, 25 mM $MgCl_2$ 0.04 vol of 5 mM AMP and 0.025 vol of the AMP-activated protein kinase (60 units/ml) were added (+AMP-PK, +MgATP). One control incubation, treated with phosphatase, lacked ATP (+AMP-PK, –MgATP). Aliquots were removed at the times shown, diluted in 10 vol of 50 mM Na HEPES, pH 7.2, 50 mM NaF, 5 mM Na pyrophosphate, 5 mM dithiothreitol, and assayed for HMG-CoA reductase activity.

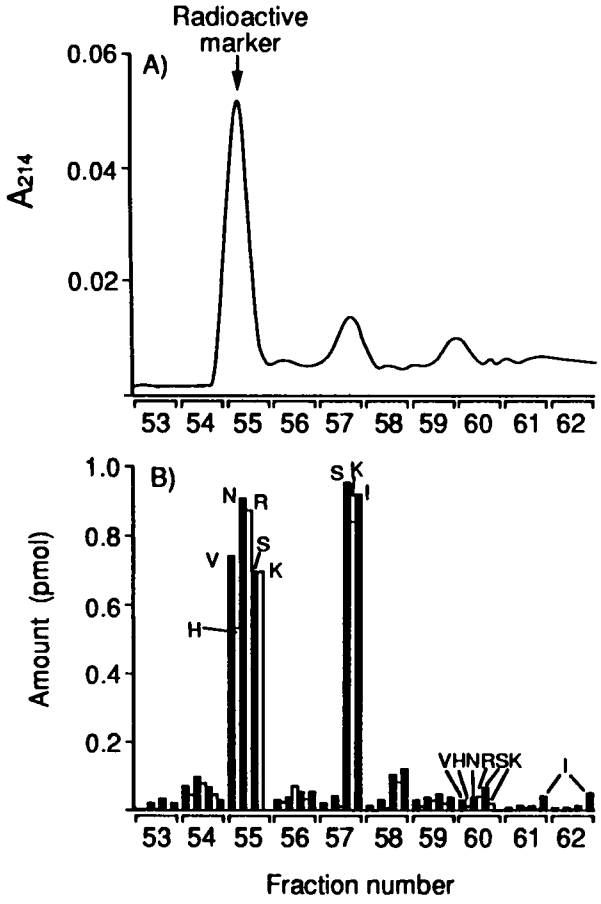

Fig. 5. (**A**) Absorbance at 214 nm and (**B**) amino acid analysis from an HPLC separation of cyanogen bromide/Lys-C endoproteinase peptides, derived from HMG-CoA reductase (53 kd fragment) purified in the presence of protein phosphatase inhibitors. The HPLC separation was as for Figure 1, but only the relevant portion of the eluate is shown. The elution position of a radioactive VHNRSK phosphopeptide marker is shown by an arrow. The amount of amino acid in each fraction is presented by vertical bars, with the order (left to right) being the same for each fraction: Val (V - filled bar); His (H - open bar); Asn (N - filled); Arg (R - open); Ser (S - filled); Lys (K - open); Ile (I - filled). Other amino acids were present at background levels (10–50 pmol) and did not significantly increase in any fraction.

endoproteinase, and the digest was fractionated by HPLC. Figure 5A shows that the radioactive marker co-eluted with an A_{214} peak at 22.5 min, and was followed by minor absorbance peaks at 23.5 and 25 min. Amino acid analysis of these fractions (Figure 5B) was consistent with the peak at 22.5 min (fraction 55) being the phosphorylated form of the VHNRSK peptide. The small peak at 23.5 min (fraction 57) had the composition Ile, Ser, Lys, and is probably derived from the sequence Met-Ile-Ser-Lys which is conserved in almost all HMG-CoA reductases (residues 658–661 in the Chinese hamster). Although present at low abundance, the small peak at 25 min (fractions 59/60) contained all the amino acids expected in the VHNRSK peptide (Figure 5B), and separate experiments (P.R.Clarke, unpublished) show that the dephospho- form of the peptide elutes in this position. Based on either the areas under the A_{214} peaks or the amino acid analysis, the proportion of the peptide in the phosphorylated form in Figure 5 was >90%. This is consistent with the enzyme activity results in Figure 4.

Table II. Sequence analysis of the N-terminus of the catalytic fragment of HMG-CoA reductase

Cycle	Xaa	Amount (pmol)
1	Thr	33.8
2	Pro	99.5
3	Pro	100.3
4	Leu	109.5
5	Ala	109.3
6	Val	107.4
7	Gly	91.6
8	Ala	93.6
9	Gln	61.2
10	Glu	72.6
11	Pro	61.9
12	Gly	76.0
13	Ile	51.4
14	Glu	50.3
15	Leu	57.8
16	Pro	47.2
17	Ser	45.4
18	Glu	41.8
19	Pro	34.3
20	Arg	19.5
21	Pro	33.4
22	Asn	28.4

Columns show the amino acid identified (Xaa), and the amount of phenylthiohydantoin derivative (PTH-Xaa) recovered at each cycle. Serine was quantified as the serine derivative plus the dithiothreitol adduct. A number of PTH-amino acids were evident in the first cycle and the identification of threonine is tentative.

Fast-atom bombardment mass spectrometry of the peak at 22.5 min from Figure 5 showed that it had the mass (MH^+) of 820, as expected for the phosphopeptide. It was also derivatized with ethanethiol and sequenced. Table I shows that its sequence was identical with that obtained previously for HMG-CoA reductase phosphorylated using purified AMP-activated protein kinase. The phenylthiohydantoin derivative of S-ethylcysteine, but not that of serine or its dithiothreitol adduct, was detected at cycle 5 as expected.

Identification of the amino-terminal sequence of the catalytic fragment

In order to ascertain where proteolytic cleavage of the catalytic domain from the membrane domain occurs during isolation, 1 nmol of the catalytic fragment was analysed by amino acid sequencing. The N-terminal sequence was TPPLAVGAQEPGIELPSEPRN (Table II) which is homologous with residues 423–449 of the Chinese hamster sequence.

Discussion

This study has definitively established the location of the single site phosphorylated by the AMP-activated protein kinase, and the N-terminus of the 53 kd catalytic fragment of rat HMG-CoA reductase. In Figure 6 these sites are superimposed on a model of the domain structure derived from analysis of cDNA sequences (Liscum *et al.*, 1985). The phosphorylation site is located right at the end of the conserved catalytic domain, and is followed by short C-

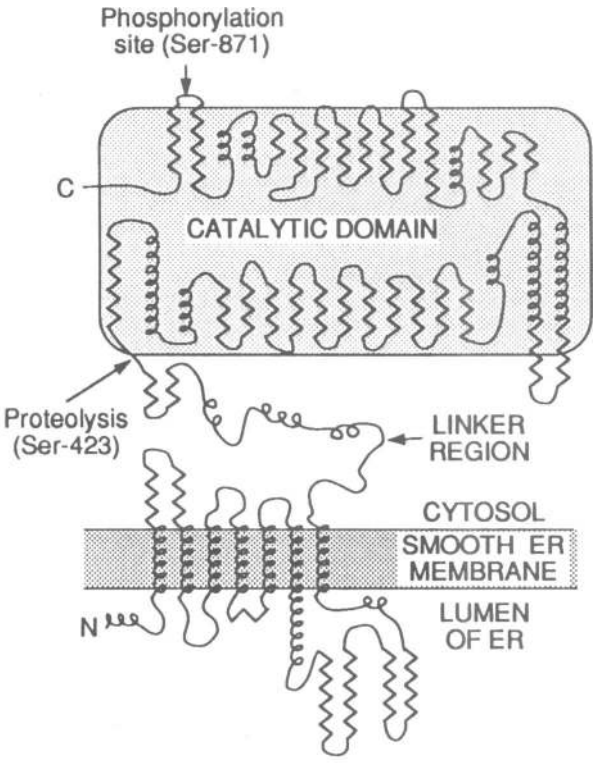

Fig. 6. Model for the domain structure and membrane topology of HMG-CoA reductase (based on Liscum *et al.*, 1985), showing the location of the phosphorylation site, and the N-terminus of the 53 kd fragment, defined in the present study.

A) C-terminal sequences:

	Phosphorylation ↓	
Rat	VH**M**R**S**KINLQDL	
Human	HLVK**S**H**M**IH**M**R**S**KINLQDLQGACTKKTA	
Chinese hamster	HLVR**S**H**M**VH**M**R**S**KINLQDLQGTCTKKSA	
Sea urchin	HLVK**S**H**M**KH**M**R**S**ALNIASPLPSIDEVAT..+28 aa	
Drosophila	DLVR**S**H**M**RH**M**R**S**SIAVNSANNPLNVTVSSCSTIS	
Arabidopsis	QLVR**S**H**M**KY**M**R**S**SRDISGATTTTTTTT	
Yeast 1	HLVQ**S**H**M**TH**M**R**K**PAEPTKPNNLDATDIN..+12 aa	
Yeast 2	HLVQ**S**H**M**TH**M**R**K**TNKANELPQPSNKGPPCKTSALL	
Schistosoma	DLVKA**H**M**H**F**M**R**A**KQSTNSHSCSHSTTTD..+57 aa	

B) N-terminus of catalytic domain:

	Proteolysis ↓	
Rat	TPPLAVGA**Q**E**P**GI**E**LP**S**E**P**RP**N**	
Chinese hamster	VLGA**S**-GTSPPVAART**Q**E**L**EI**E**LP**S**E**P**RP**N**	
Syrian hamster	VLGA**S**-GGCSPVAKGT**Q**E**P**EI**E**LP**S**E**P**RP**N**	
Human	VVGN**S**SLLDTSSVLVT**Q**E**P**EI**E**LP**R**E**P**RP**N**	

Fig. 7. Comparison of (**A**) the phosphorylation site sequence and (**B**) the N-terminal sequence, determined for the 53 kd fragment of rat liver HMG-CoA reductase in this study, and homologous sequences predicted from cDNA cloning of HMG-CoA reductases from other species. Residues conserved in all species are shown in bold. In (A), apart from the rat sequence, the complete predicted C-terminal sequences are shown unless stated otherwise. Sequence data are from: human (Luskey and Stevens, 1985); Chinese hamster (Chin *et al.*, 1984); Syrian hamster (Skalnik and Simoni, 1985); sea urchin (Woodward *et al.*, 1988); *Drosophila* (Gertler *et al.*, 1988); *Arabidopsis* (Learned and Fink, 1989); yeast (Basson *et al.*, 1988); *Schistosoma* (Rajkovic *et al.*, 1989).

		$10^4 \times Pt$
Rat HMG-CoA reductase	HMVH**N**R**S**K**I**NLQDL	2.027
Rat acetyl-CoA carboxylase (Ser-79)	HMRS**S**M**S**G**L**HLVKQ	1.257
Rat acetyl-CoA carboxylase (Ser-1200)	PTLNRM**S**F**A**S**N**LNH	0.934
Rat acetyl-CoA carboxylase (Ser-1215)	GMT**H**VA**S**VSDVLLD	1.275
Rat hormone-sensitive lipase (Ser565)	SMRRSV**S**EAALAQP	1.044
Rabbit muscle glycogen synthase (Ser7)	PLSRTL**S**VSSLPGL	0.763

Fig. 8. Amino acid sequences around six sites phosphorylated by the AMP-activated protein kinase on HMG-CoA reductase (this paper), acetyl-CoA carboxylase (Davies *et al.*, 1990), hormone-sensitive lipase (Garton *et al.*, 1989) and glycogen synthase (Carling and Hardie, 1989). The phosphorylated serine residue is indicated by bold type and the vertical arrow. Hydrophobic residues are underlined. Tetrapeptides having β-turn probabilities (Pt) $>0.75 \times 10^{-4}$ by the procedure of Chou and Fasman (1978) are indicated by boxes: the actual Pt values are shown on the right.

terminal sequences which are quite variable in HMG-CoA reductases from different species (Figure 7A). The phosphorylated serine residue itself (equivalent to Ser872 in the human sequence) is conserved in the sequence from rat, human, Chinese and Syrian hamster, *Drosophila melanogaster*, and the higher plant *Arabidopsis thaliana*, but not in the yeast *Saccharomyces cerevisiae*, the human parasite *Schistosoma mansoni* (Figure 7A) or the bacterium *Pseudomonas mevalonii* (Beach and Rodwell, 1989). Good evidence already exists for regulation of the enzyme by phosphorylation in all vertebrate species from fish to mammals (e.g. Hunter and Rodwell, 1980), but our data now suggest the possibility that it may also operate in invertebrates and higher plants. Interestingly, Hunter and Rodwell (1980) reported unpublished observations in which they could not obtain evidence for regulation of yeast HMG-CoA reductase by phosphorylation.

The N-terminus of the 53 kd catalytic fragment defined in the present study is equivalent to Ser423 in the human sequence. This lies right at the boundary between the highly conserved catalytic region and the central linker region, which is less well conserved even between the Syrian and Chinese hamsters (Figure 7B). The linker region as a whole is very hydrophilic, and Ser423 lies at a minimum point on the hydropathy plot of the Chinese hamster sequence (Liscum *et al.*, 1985). It is therefore probably exposed to the solvent, and the sequence around it is rich in proline, serine and threonine (Figure 7B). Such 'PEST' sequences have been reported to occur in other proteins which are degraded rapidly (Roger *et al.*, 1986). However, it must be stressed that this may not represent the site of initial cleavage, nor is it clear that the same cleavage site(s) is utilized during degradation *in vivo*.

Parker *et al.* (1984) reported that phosphorylation of the 100 kd microsomal form of rat liver HMG-CoA reductase increased its rate of conversion to the 53 kd fragment(s) by the intracellular Ca^{2+}-dependent proteinase, calpain II. This proteinase may be responsible for cleavage to the fragment during enzyme isolation, since cleavage can be prevented by EGTA and leupeptin (Ness *et al.*, 1986). In contrast to speculations made by Parker *et al.* (1989), our results show that the phosphorylation site is remote from the site of proteolysis in the linear sequence, and therefore could only contribute to the recognition site for the proteinase in an indirect manner via a conformational change. Once again it must be stressed that there is no evidence that calpain II

is the proteinase responsible for degradation of the enzyme *in vivo*.

The amino acid sequences around six sites phosphorylated by the AMP-activated protein kinase are shown in Figure 8. We previously suggested that a hydrophobic residue immediately N-terminal to the phosphorylated serine may be important for recognition (Carling and Hardie, 1989), but Figure 7 shows that the HMG-CoA reductase sequence does not agree with this hypothesis. However, close inspection reveals that all of the phosphorylated sequences have a number of hydrophobic side chains (M, V or L) particularly at $-1/-2$, $-4/-5$, $+1/+2$ and $+4/+5$ with respect to the phosphorylated serine. In addition, all six sequences contain at least one tetrapeptide, including the phosphorylated serine (Figure 6A), that is predicted to form a β-turn by the procedure of Chou and Fasman (1978). We have already shown that a peptide based on the sequence around Ser79 on acetyl-CoA carboxylase is a good substrate, and that replacement of the residue equivalent to Ser77 by alanine (which reduces the probability of a β-turn) also reduces the V_{max}/K_m by a factor of 5 (Davies *et al.*, 1989). One possibility is that the serine residue could lie exposed on a β-turn, while hydrophobic residues on the two β-strands connected by the turn would stabilize binding to the kinase. This hypothesis can now be addressed using variant synthetic peptides.

When rats are killed and the liver removed by dissection, HMG-CoA reductase becomes highly phosphorylated and inactivated (Easom and Zammit, 1984a). Under these conditions acetyl-CoA carboxylase is also highly phosphorylated at the sites phosphorylated *in vitro* by the AMP-activated protein kinase, and the kinase is persistently activated (S.P.Davies, unpublished). The inactivation of HMG-CoA reductase and acetyl-CoA carboxylase, and the activation of the AMP-activated protein kinase, can be prevented by freeze- or cold-clamping the liver prior to homogenization (Easom and Zammit, 1984a; S.P.Davies, unpublished). Although the highly inactive state of HMG-CoA reductase present in rat liver which has not been cold-clamped may therefore be somewhat non-physiological, we have investigated the phosphorylation state of this species by purifying the enzyme in the presence of protein phosphatase inhibitors. As expected, the site phosphorylated by the AMP-activated protein kinase, equivalent to Ser872 in the human enzyme, was found to be highly phosphorylated ($>90\%$) in the intact liver. This supports the idea that the inactivation of HMG-CoA reductase under these conditions is due to rises of AMP concentration, and consequent activation of the AMP-activated protein kinase, caused by interruption to the blood supply during dissection.

Identification of the regulatory site of phosphorylation on HMG-CoA reductase will allow the determination of changes in the phosphorylation state of the protein *in vivo* and in isolated cells by direct measurement. It also opens the way to site-directed mutagenesis experiments on the role of phosphorylation in intact cells, particularly its proposed role in regulating enzyme degradation.

Materials and methods

Materials

Cyanogen bromide and protein A–agarose were from Sigma, Poole, UK. Trifluoroacetic acid and acetonitrile were from Rathburn Chemicals, Winterburn, UK. Ethanethiol was from Pierce, Luton, UK. Lys-C endoproteinase (sequencing grade) was from Boehringer, Lewes, UK. All other chemicals were as in Carling *et al.* (1989).

For the purification of HMG-CoA reductase, rats were fed for 4 days on a diet containing 2% (by mass) cholestyramine and then 4 days on a diet containing 2% (by mass) cholestyramine and 0.04% (by mass) mevinolin (Lovastatin). Rats were maintained on a cycle of 12 h dark/12 h light, and were sacrificed 4 h into the dark period by stunning and cervical dislocation. The livers were dissected out as rapidly as possible without cold-clamping. The catalytic fragment was prepared by the method of Edwards *et al.* (1979), as described in Kleinsek *et al.* (1981). Where described in the text, 50 mM KCl in the buffer was replaced by 50 mM NaF and 5 mM Na pyrophosphate. Liver microsomes containing the intact form of HMG-CoA reductase were prepared by the method of Ness *et al.* (1986), as modified by Carling *et al.* (1989).

The AMP-activated protein kinase was purified from rat liver (Carling *et al.*, 1989). The catalytic subunit of protein phosphatase-1 was purified from rabbit skeletal muscle (Resink *et al.*, 1983).

Preparation of anti-HMG-CoA reductase sera

Adult male rabbits were inoculated by subcutaneous injection with 100 μg of homogeneous catalytic fragment of HMG-CoA reductase emulsified in Freund's complete adjuvant, followed by a further 100 μg in incomplete adjuvant after 4 weeks. Antisera were characterized by ability to cause inactivation and to detect HMG-CoA reductase on Western blots.

Enzyme assays

The catalytic fragment of HMG-CoA reductase was assayed by a spectrophotometric method (Carling *et al.*, 1989). Microsomal HMG-CoA reductase was assayed by a radiochemical method (Easom and Zammit, 1984b). The AMP-activated protein kinase was assayed as in Davies *et al.* (1989). Protein phosphatase-1 was assayed as in Resink *et al.* (1983).

Phosphorylation of the catalytic fragment of HMG-CoA reductase

The catalytic fragment of HMG-CoA reductase (0.1–0.2 mg/ml) was phosphorylated at 30°C in incubations which contained 50 mM Na HEPES, pH 7.2, 10% (by vol) glycerol, 100 mM KCl, 0.2 mM AMP, 4 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP ($2-5 \times 10^5$ c.p.m./nmol by Cerenkov counting) and the AMP-activated protein kinase. Total incorporation of ^{32}P was estimated by precipitation of aliquots with 25% (by mass) trichloroacetic acid (TCA) (Guy *et al.*, 1981). The inactivation of HMG-CoA reductase was assessed by removing aliquots of the phosphorylation mixture into 4 vols of 20 mM EDTA and assayed HMG-CoA reductase activity spectrophotometrically.

For analysis of the sites of phosphorylation, reactions were terminated by addition of 0.33 vol of 100% (by mass) TCA and allowed to stand on ice for 5 min. The precipitate was collected by centrifugation, washed three times with 25% (by mass) TCA to remove [γ-^{32}P]ATP, and subjected to cyanogen bromide cleavage and Lys-C endoproteinase digestion.

Cyanogen bromide cleavage and Lys-C endoproteinase digestion of HMG-CoA reductase

The TCA precipitated catalytic fragment (10 nmol) or the immunoprecipitated native form of HMG-CoA reductase was dissolved in 100 μl of 70% (by vol) formic acid and ~10 mg of cyanogen bromide added. After standing at 4°C for 16 h, the sample was dried in a vacuum concentrator, redissolved in 100 μl of H_2O, and dried again to remove cyanogen bromide. The resulting peptides were redissolved in 25% (by vol) acetonitrile, 0.2% (by vol) trifluoroacetic acid (TFA) by sonication for 10 min in a sonicating bath, diluted with 10 vols of 0.1% (by vol) TFA and applied to a SEP-PAK C18 (Waters/Millipore) cartridge. ^{32}P-labelled peptides were eluted with 50% (by vol) acetonitrile, 0.1% (by vol) TFA and concentrated to ~100 μl on a vacuum concentrator.

For proteolytic digestion, 100 μl of 25% (by vol) acetonitrile was added to an equal volume of the cyanogen bromide cleaved peptides in H_2O and the sample sonicated as before. 250 μl of 50 mM Tris–HCl, pH 8.5 at 37°C, containing Lys-C endoproteinase (at a mass ratio of 1:100 with the initial amount of HMG-CoA reductase used) was added. Digestion was carried out for 16 h at 37°C and the endoproteinase removed by using a SEP-PAK C18 cartridge as before. Recovery of [^{32}P]peptides was >85% at each stage.

Analysis and purification of phosphopeptides

Phosphopeptides were purified by reversed-phase HPLC using a Vydac C18 column (25 × 0.45 cm) equilibrated in 0.1% (by vol) TFA and developed

with a gradient of acetonitrile as shown in Figure 1. The eluates were monitored continuously by their absorbance at 214 nm and by their radioactivity detected by Cerenkov counting using a Reeve analytical monitor. In the case of cyanogen bromide cleaved peptides not digested with Lys-C endoproteinase, peptides were further purified on a C18 column equilibrated in 5 mM Pic A reagent (Waters/Millipore) developed with a gradient of 10–50% acetonitrile over 60 min. An additional HPLC run in the TFA system described above was subsequently used to remove Pic A before sequencing.

Amino acid composition and sequence analysis of peptides

Amino acid compositions were determined after hydrolysis for 24 h in 6 M HCl/2 mM phenol, using the Waters PICO-TAG system (Holmes *et al.*, 1986). Peptides were sequenced on an Applied Biosystems 470A gas-phase sequencer with automated phenylthiohydantoin amino acid analysis. In some cases described in the text, conversion of phosphoserine residues to S-ethylcysteine (Meyer *et al.*, 1986, as modified by Holmes, 1987) was carried out prior to sequencing.

Fast-atom bombardment mass spectrometry

Positive ion analysis of peptides was performed on a VG Analytical model 70-250SE mass spectrometer with a fast-atom bombardment Xenon source. Peptides were dissolved in 0.5 μl of 10% acetic acid and mixed with 0.5 μl of matrix (dithiothreitol/dithioerythritol, 3:1 by mass) on the probe tip before insertion into the machine.

Immunoprecipitation of the native (100 kd) form of HMG-CoA reductase

Microsomes prepared by the method of Ness *et al.* (1986) were incubated at 20 mg protein/ml in a reaction volume of 250 μl with the AMP-activated protein kinase, under the same conditions as used for the phosphorylation of the catalytic fragment, except that 1 mM phenylmethane sulphonyl fluoride (PMSF) and 0.1 mM leupeptin were included in the incubation buffers. The reaction was terminated by the addition of 3.5 ml of immunoprecipitation buffer (1% (by vol) Triton X-100, 0.5% (mass/vol) Na deoxycholate, 0.1% (mass/vol) sodium dodecyl sulphate, 20 mM Na HEPES, pH 7.2, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM leupeptin, 1 mM PMSF) and 500 μl of anti-HMG-CoA reductase serum was added. After standing at 4°C for 16 h, immunocomplexes were collected by the addition of 100 μl of Protein A–agarose, incubation at 20°C for 30 min, and centrifugation at 8000 *g* for 3 min. These precipitates were washed three times with 1 ml of immunoprecipitation buffer. For phosphopeptide analysis by isoelectric focusing, the precipitates were digested as described above. Alternatively, the immunocomplexes were eluted from the Protein A–agarose by heating at 50°C for 30 min in 62.5 mM Tris–HCl, pH 6.5, 5% (mass/vol) SDS, 10% (by vol) 2-mercaptoethanol. The Protein A–agarose was pelleted by centrifugation and the supernatant transferred to tubes containing glycerol, bromophenol blue and urea of final concentrations of 10% (by vol), 0.002% (mass/vol) and 8 M respectively. After heating for 5 min at 95°C, iodoacetamide was added to 100 mM and the samples applied to 5–15% gradient acrylamide gels. Electrophoresis in SDS was carried out using the buffer system of Laemmli (1970). The positions of radioactive polypeptides were detected by autoradiography with β-max film (Amersham) using intensifying screens.

Isoelectric focusing of phosphopeptides

Phosphopeptides produced by combined cyanogen bromide and Lys-C endoproteinase digestion were analysed by isoelectric focusing using a Biorad mini-IEF apparatus with ampholytes in the range pI 3.5–10 (Pharmacia). The gel was dried on its plastic backing, wrapped in clingfilm and autoradiographed as described above.

Other analytical procedures

Protein concentration was measured by the dye-binding method of Bradford (1976). Alkali-labile phosphate was measured as in Guy *et al.* (1981).

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