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Purification, Characterization, and Cloning of a Eubacterial 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase, a Key Enzyme Involved in Biosynthesis of Terpenoids

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The eubacterial 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) was purified 3,000-fold from *Streptomyces* sp. strain CL190 to apparent homogeneity with an overall yield of 2.1%. The purification procedure consisted of $(NH_4)_2SO_4$ precipitation, heat treatment and anion exchange, hydrophobic interaction, and affinity chromatographies. The molecular mass of the enzyme was estimated to be 41 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 100 to 105 kDa by gel filtration chromatography, suggesting that the enzyme is most likely to be a dimer. The enzyme showed a pH optimum of around 7.2, with apparent K_m values of 62 μM for NADPH and 7.7 μM for HMG-CoA. A gene from CL190 responsible for HMG-CoA reductase was cloned by the colony hybridization method with an oligonucleotide probe synthesized on the basis of the N-terminal sequence of the purified enzyme. The amino acid sequence of the CL190 HMG-CoA reductase revealed several limited motifs which were highly conserved and common to the eucaryotic and archaebacterial enzymes. These sequence conservations suggest a strong evolutionary pressure to maintain amino acid residues at specific positions, indicating that the conserved motifs might play important roles in the structural conformation and/or catalytic properties of the enzyme.

A biosynthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) utilizes two NADPH equivalents to reductively deacylate the thioester group of HMG-CoA to the primary alcohol of mevalonate as follows: HMG-CoA + 2NADPH + $2H^+$ → mevalonate + CoASH + $2NADP^+$.

An extensive body of information concerning HMG-CoA reductase exists in eucaryotes. This enzyme performs the rate-limiting step in the biosynthesis of terpenoids and is regulated by a variety of steps, including transcription, translation, and enzyme degradation and phosphorylation (9, 21). The eucaryotic enzyme is a transmembrane glycoprotein located in the endoplasmic reticulum (32). Modeling studies of the deduced primary sequence of HMG-CoA reductase from a variety of organisms suggest that it consists of eight membrane-spanning regions. The N-terminal region has been shown to be involved in the regulated degradation of HMG-CoA reductase. The C-terminal domain projects into the cytosol and contains the catalytic site (31).

In archaebacteria, HMG-CoA reductases from *Haloferax volcanii* (5, 28) and *Sulfolobus solfataricus* (7), which belong to divergent kingdoms of the *Euryarchaeota* and the *Crenarchaeota* (49), respectively, have been cloned and sequenced. Each gene has been expressed in *Escherichia coli*, and these recombinant enzymes have been purified and characterized.

In eubacteria, however, no biosynthetic HMG-CoA reductase gene has been cloned. Only a biodegradative HMG-CoA reductase (EC 1.1.1.88), mevalonate + CoASH + $2NAD^+$ ⇄ HMG-CoA + 2NADH + $2H^+$, from *Pseudomonas mevalonii*, which can grow on mevalonate as the sole carbon source, has been extensively characterized (4, 20, 48). In contrast to the biosynthetic HMG-CoA reductase, this enzyme utilizes NAD(H) rather than NADP(H) as an oxidoreductant. The reaction is reversible and favors mevalonate production (39). Thus, the enzyme has served as a model for elucidating the active site structure and the catalytic mechanism of biosynthetic HMG-CoA reductases, and some functional motifs have been recently proposed from its three-dimensional structure analysis (29). However, the *P. mevalonii* HMG-CoA reductase had less than 25% sequence identity with eucaryotic HMG-CoA reductase and lacked several regions conserved in both eucaryotic and archaebacterial HMG-CoA reductases. Therefore, a comprehensive understanding of functional motifs of the biosynthetic HMG-CoA reductase is still lacking.

In order to elucidate the catalytic domains of the biosynthetic HMG-CoA reductase, it is important to obtain the enzyme from an additional kingdom, *Eubacteria*. In some eubacteria, such as *Myxococcus fulvus*, *Lactobacillus plantarum*, and *Staphylococcus carnosus*, involvement of the enzymes concerning the mevalonate pathway has been demonstrated by an incorporation experiment with [^{14}C]acetyl-CoA, [^{14}C]HMG-CoA, or [^{14}C]mevalonate (22). Furthermore, several terpenoid and hemiterpenoid metabolites, such as naphterpin (43, 44), furaquinocin (19), napyradiomycin (45), and terpentecin (23), produced by the genus *Streptomyces*, have been proved to be synthesized through the ubiquitous mevalonate pathway by incorporation experiments with ^{13}C-labeled acetate. However, no biosynthetic HMG-CoA reductase has been purified from these eubacteria. Thus, we decided to clone the HMG-CoA reductase gene from *Streptomyces* sp. strain CL190, which produces naphterpin (44), because we were able to detect the enzyme activity with this organism.

Considering codon usage specific to *Streptomyces* sp. strain

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CL190, we first designed several sets of PCR primers based on the highly conserved amino acid sequences between the eucaryotic and archaebacterial HMG-CoA reductases to isolate the HMG-CoA reductase gene from CL190. Contrary to our expectation, however, we were unable to obtain a specific DNA fragment by PCR with these primers. These findings indicated that the amino acid sequence of *Streptomyces* HMG-CoA reductase was somewhat different from eucaryotic and archaebacterial amino acid sequences. Thus, purification of the CL190 HMG-CoA reductase followed by the determination of its N-terminal amino acid sequence was essential for cloning the corresponding gene.

In this study, we report the purification, characterization, molecular cloning, and sequence of HMG-CoA reductase from *Streptomyces* sp. strain CL190. Moreover, the corresponding gene, named *hmgr*, was overexpressed in *E. coli*, and its recombinant HMG-CoA reductase was purified and characterized.

MATERIALS AND METHODS

Materials. Materials from commercial sources included *R*,*S*-[3-^{14}C]HMG-CoA and [γ-^{32}P]ATP (American Radiolabeled Chemicals, St. Louis, Mo.); *R*,*S*-[2-^{14}C]mevalonolactone (Amersham Corp.); phenylmethylsulfonyl fluoride (PMSF) (Sigma); Butyl Toyopearl 650M and Toyopearl HW65 (TOSOH, Tokyo, Japan); DEAE Sephacel, Blue Sepharose CL-6B, Mono Q HR5/5, Superdex 200, epoxy-activated Sepharose 6B, and a high- and low-molecular-weight electrophoresis calibration kit (Pharmacia Biotech, Uppsala, Sweden); Coomassie brilliant blue R-250 (Fluka, Buchs, Switzerland); and polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.). Pravastatin was a gift from Sankyo Co. Ltd. A CoA affinity column was prepared by using epoxy-activated Sepharose 6B as described by the manufacturer. All other reagents were of the highest analytical grade available.

Conditions for growth. *Streptomyces* sp. strain CL190 was cultured as previously described (43).

Buffered solutions. Buffer A, used throughout purification, contained 25 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol.

Buffer B contained buffer A plus 400 mM KCl.

Buffer C, used for heat treatment, contained 25 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 1 mM dithiothreitol, 1 M KCl, and 33% glycerol.

Buffer D contained buffer B plus 30% saturation $(NH_4)_2SO_4$.

Buffer E, used for Blue Sepharose CL-6B column chromatography, contained 50 mM potassium phosphate buffer (pH 7.2), 30 mM EDTA, 1 mM dithiothreitol, 200 mM KCl, and 100 mM sucrose.

Buffer F, used for Ni-nitrilotriacetic acid agarose column chromatography, contained 50 mM potassium phosphate buffer (pH 7.4), 200 mM KCl, and 10% glycerol.

Sodium dodecyl sulfate (SDS) sample buffer contained 1% SDS, 1% β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% bromphenol blue in 60 mM Tris-HCl (pH 6.8).

Radiometric assay of HMG-CoA reductase activity. The radiometric assay was conducted by the method described by Bach et al. (3) with some modifications. The assay system consisted of 25 mM potassium phosphate (pH 7.2), 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM glucose 6-phosphate, 14 mU of yeast glucose 6-phosphate dehydrogenase, 0.6 mM NADPH, 14 μM *R*,*S*-[3-^{14}C]HMG-CoA, and 10% (vol/vol) glycerol in a final volume of 50 μl. The reaction was initiated by adding HMG-CoA to the complete assay mixture. The reaction was terminated by adding 5 μl of HCl, and the mixture was allowed to lactonize for an additional 30 min. Mevalonolactone was extracted with 100 μl of ethyl acetate, and an aliquot of the supernatant was subjected to thin-layer chromatography on a silica gel. The plate, developed with a mixture of toluene, acetone, and triethylamine (80:40:1), was exposed to an imaging plate (Fujifilm), in which the intensity of photostimulated luminescence was proportional to the adsorbed radiation energy on the imaging plate (2). The amount of mevalonolactone was determined by the estimation of photostimulated luminescence of the corresponding spot with a BAS-1500 (Fujifilm). One pU of HMG-CoA reductase activity was defined as the amount of enzyme that formed 1 pmol of mevalonolactone per min at 30°C.

Spectrophotometric assay of HMG-CoA reductase activity. The spectrophotometric assay was conducted by the method described by Kleinsek et al. (26), with a slight modification. The assay system consisted of 25 mM potassium phosphate (pH 7.2), 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 0.3 mM NADPH, and 0.3 mM *R*,*S*-HMG-CoA in a final volume of 1 ml. The reaction was initiated by adding HMG-CoA to the complete assay mixture. The HMG-CoA-dependent oxidation of NADPH was monitored in a Shimadzu UV-160 spectrophotometer equipped with a cell holder adjusted at 30°C. One microunit of HMG-CoA reductase activity is defined as the amount of the enzyme that caused oxidation of 1 μmol of NADPH per min.

Purification of HMG-CoA reductase from *Streptomyces* sp. strain CL190. Unless otherwise stated, all the purification procedures were done in a cold room maintained at 4°C, and centrifugation was done at 10,000 $\times$ *g* for 30 min.

The mycelial cake (500 g) collected by centrifugation from the whole fermentation broth (10 liters) was washed by resuspending it in 20 mM potassium phosphate buffer (pH 7.2) and frozen at −30°C. Freezing the cells had no adverse effect on subsequent recovery of the enzyme. Frozen mycelia were thawed overnight, suspended in buffer A (3 ml per mycelial weight) containing 1 mM PMSF, and ruptured by ultrasonication. The cell lysate was centrifuged, and the supernatant was retained as a crude extract.

The crude extract (1.8 liters) was applied to a DEAE cellulose column (5 by 20 cm) previously equilibrated with buffer A containing 1 mM PMSF. After the column extract was washed with 8 column volumes of the same buffer described above, elution was carried out with buffer B at a flow rate of 1 ml/min. Active fractions were combined to give a DEAE fraction (103 ml).

This sample was adjusted to 45% saturation in $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$. The mixture was cooled on ice for an hour and centrifuged. The precipitate was dissolved and dialyzed against buffer C and centrifuged to give an ammonium sulfate fraction (13 ml).

To stabilize HMG-CoA reductase for heat treatment, NADPH was added to the ammonium sulfate fraction to give a final concentration of 2 mM (46). Then the enzyme solution was maintained at 60°C for 10 min, placed on ice, and centrifuged to remove denatured protein. The precipitate was washed with 2 volumes of buffer B. Following centrifugation, the initial supernatant liquid and wash were combined to give a heat fraction (29 ml).

This sample was dialyzed against buffer D and then applied to a Butyl-Toyopearl 650M column (2 by 30 cm) previously equilibrated with the same buffer. After the column was washed with 10 column volumes of buffer D, elution was carried out at 1 ml/min by mixing buffer D with a 300-min linear gradient of buffer B. The active fractions were combined to give a Butyl Toyopearl fraction (51 ml).

This sample was dialyzed against buffer E and applied to a Blue Sepharose CL-6B column (2 by 11 cm) previously equilibrated with buffer E. After washing with 5 column volumes of buffer E, elution was carried out at 1 ml/min by mixing buffer E with a 100-min linear gradient of 2.5 M KCl in buffer E. The active fractions were combined to give a Blue Sepharose CL-6B fraction (51 ml).

This sample was concentrated in a dialysis sac against polyethylene glycol 20,000, dialyzed against buffer A, and then applied to a Mono Q column (0.5 by 10 cm) (fast protein liquid chromatography system) previously equilibrated with buffer A. The column was washed with buffer A for 5 min, and then a linear KCl concentration gradient from 0 to 1 M was established over the next 60 min at a flow rate of 1 ml per min. The active fractions were combined to give a Mono Q fraction (2 ml).

This sample was dialyzed against buffer A and then applied to a CoA affinity column (0.6 by 2 cm) previously equilibrated with buffer A. Undesired proteins were removed from the column by washing with buffer A. The enzyme was then eluted at a flow rate of 0.1 ml/min with buffer A containing 250 mM KCl. The most active fraction (1 ml) was pooled and stored at 4°C.

Protein determination. The protein concentration was measured by the method of Bradford (8), with bovine serum albumin as the standard.

Determination of molecular mass. The molecular mass of the native HMG-CoA reductase was estimated by gel filtration on a Toyopearl HW65 (2 by 90 cm) and a Superdex 200 (1.6 by 60 cm) column which were equilibrated in buffer B. The column was eluted at a flow rate of 0.3 ml/min, and fractions of 2 ml were collected. Molecular mass was estimated by comparing the elution of HMG-CoA reductase with those of the standard proteins: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (66 kDa).

pH-dependent activity of HMG-CoA reductase. The radiometric assay was conducted as described above. Assay solutions consisted of 25 mM potassium phosphate at pH 6.0 to 7.6, 75 mM Tris-HCl at pH 7.6 to 9.0, 100 mM HEPES-KOH at pH 6.8 to 8.2, and 50 mM Gly-KOH at pH 8.5 to 9.5. Contained in the buffers used were 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, and 10% glycerol.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) and native PAGE were performed in slab gels of 8 to 25% polyacrylamide gradient gel with PhastSystem (Pharmacia). Protein was visualized by Coomassie brilliant blue R-250 staining.

Amino-terminal sequence determination. Purified HMG-CoA reductase was applied to SDS-PAGE. Electrophoretic transfer to polyvinylidene difluoride membrane after SDS-PAGE was performed by the method of Towbin et al. (47). Amino acid sequencing was performed on a protein sequencer 492A (Applied Biosystems). The first 30 residues of the amino-terminal sequence were determined to be TETHAIAGVPMRWVGPLRISGNVAETETQV.

Cloning and DNA sequencing of the *hmgr* gene from *Streptomyces* sp. strain CL190 genome. On the basis of the partial N-terminal sequence HAIAGVP MRW of HMG-CoA reductase from CL190, an oligonucleotide probe, 5′-CAC GCSATCGCSGGGGTSCCSATGCGSTGG-3′, was synthesized (Amersham and Pharmacia) and used for Southern hybridization blotting with total DNA of CL190 and for colony hybridization with a genomic library of CL190. DNA manipulations and transformation in *E. coli* were done as described by Sambrook

TABLE 1. Purification of HMG-CoA reductase

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude extract	5,500	2,900	0.53	100	1
DEAE Sephacel	2,200	2,900	1.3	100	2.5
Ammonium sulfate (0–45%)	900	2,700	3.0	93	5.7
Heat treatment (60°C, 10 min)	570	2,900	5.1	100	9.6
Butyl Toyopearl 650M	44	1,400	32	48	60
Blue Sepharose CL-6B	2.3	620	270	21	510
Mono Q	0.50	250	500	8.6	940
CoA affinity column	0.040	62	1,600	2.1	3,000

et al. (40). Chromosomal DNA extracted from *Streptomyces* sp. strain CL190 was partially digested with *Sau*3AI and size fractionated (2 to 4 kbp) by agarose gel electrophoresis. The 2- to 4-kbp DNA fragments were ligated to *Bam*HI- and phosphatase-treated pUC118 (Takara). The resulting plasmids were used as a genomic library of *Streptomyces* sp. strain CL190.

DNA sequence analysis. DNA sequence was determined by the dideoxy chain termination method (41) with an automated sequencer (model 4000L; Li-Cor) and the protocol of the supplier. A homology search with protein databases was performed by the FASTA program (30, 38). Amino acid sequences aligned by the GENETYX program (Software Development) were then edited visually to align consensus motifs.

Construction of the plasmid for overexpression in *E. coli* of the *hmgr* gene. On the basis of the entire nucleotide sequence of HMG-CoA reductase from *Streptomyces* sp. strain CL190, two oligonucleotide primers, 5′-GGGGGATCCAGC CGCTCGGTTCTCGTCACC-3′ (5′ of the *hmgr* gene) and 5′-CCCAAGCTTG TGACCCATTCCCAGTCCGCC-3′ (3′ of the *hmgr* gene), including *Bam*HI and *Hin*dIII restriction sites (underlined), were synthesized (Pharmacia) and used together with total DNA from *Streptomyces* sp. strain CL190 to amplify the *hmgr* gene. With *Taq* DNA polymerase (Boehringer) and the protocol of the supplier, a 1,074-bp fragment was amplified. The PCR fragment was cleaved with *Bam*HI and *Hin*dIII and cloned into pUC118 (Takara). Strain JM109 (Takara) was used as a recipient in the transformation. Clones were analyzed for the correct insert by DNA sequencing as described above. A correct fragment was cloned into the multicloning site of the expression vector pQE30 (Qiagen) to give pQHR30. It was designed to have an affinity tag consisting of just six consecutive histidine residues in the N-terminal region of the recombinant enzyme. Ni-nitrilotriacetic acid resin has a strong affinity for protein which has such histidine residues.

Expression and purification of the recombinant HMG-CoA reductase. *E. coli* M15 containing pREP4 [*neo lacI*] (Qiagen) was used as a host for expression of the *hmgr* gene. M15 (pREP4, pQHR30) was cultured at 37°C in 100 ml of Luria-Bertani medium containing 25 μg of kanamycin (Nacalai) per ml and 200 μg of ampicillin (Sigma) per ml for 5 h with 2 mM isopropyl-β-D-thiogalactoside (IPTG) added when an optical density at 660 nm of 0.8 was attained. Cells were harvested by centrifugation and resuspended in buffer F containing 1 mM PMSF. After brief sonication, the lysate was centrifuged at 10,000 × *g* for 20 min, and the supernatant was retained. The crude extract was applied to a Ni-nitrilotriacetic acid agarose column (1.3 by 2 cm) (Qiagen) previously equilibrated with buffer F containing 1 mM PMSF at a flow rate of 0.5 ml/min. Passing fractions (5 ml) were concentrated in a dialysis sac against polyethylene glycol 20,000, dialyzed against buffer A, and then applied to a Mono Q column (0.5 by 10 cm) previously equilibrated with buffer A. Elution was performed as described for the purification of the *Streptomyces* sp. strain CL190 enzyme.

Nucleotide sequence accession number. The nucleotide sequence of the *hmgr* gene has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB013457.

RESULTS

Purification of HMG-CoA reductase. A summary of the procedure used to purify HMG-CoA reductase from *Streptomyces* sp. strain CL190 is presented in Table 1. Interestingly, after the first three steps, consisting of DEAE Sephacel column chromatography, ammonium sulfate precipitation, and heat treatment, no decrease of the total HMG-CoA reductase activity was observed, probably due to the removal of inhibitory compounds present in enzyme solution. These steps had relatively little influence on the purity of HMG-CoA reductase. On the other hand, Butyl Toyopearl and Blue Sepharose chromatographies resulted in an increase in the purification fold from 9.6 to 510, although recoveries decreased to only 21% compared with the activity present in the Blue Sepharose pool. After Mono Q and CoA affinity chromatographies, the enzyme was purified 3,000-fold from the initial step, with a yield of 2.1% (Table 1). The purified HMG-CoA reductase showed a single band by SDS-PAGE and native PAGE (Fig. 1).

Determination of molecular mass of the HMG-CoA reductase from CL190. The apparent molecular mass of the HMG-CoA reductase was estimated to be 105 kDa by Toyopearl HW 65 and 100 kDa by Superdex 200 gel filtration. SDS-PAGE showed a subunit molecular mass of 41 kDa. Native PAGE gave a single protein band with a mobility corresponding to 120 kDa (Fig. 1). Native PAGE depends on both charge and size. Thus, based on the gel filtration results, CL190 HMG-CoA reductase is most likely to form a dimer.

Optimum pH. HMG-CoA reductase was most active in phosphate buffer. The optimum activity of the enzyme occurred at around pH 7.2.

Reaction temperature and heat stability. The effect of temperature on the activity of the enzyme was investigated over the range of 15 to 60°C. Maximum activity was observed at around 35 to 60°C. The activation energy was estimated to be 200 kJ per mol by an Arrhenius plot whose curve was straight over the range of 20 to 30°C (data not shown). This value was higher than those reported for *Sulfolobus solfataricus* (7) and *Raphanus sativus* (3) (Table 2). The purified enzyme was heat

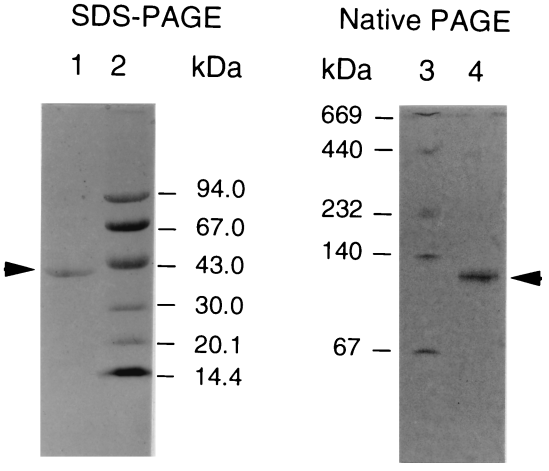


FIG. 1. Electrophoresis of the purified HMG-CoA reductase. Purified HMG-CoA reductase was analyzed by SDS–8 to 25% PAGE (left) and native 8 to 25% PAGE (right). Lanes: 1, SDS-treated enzyme (0.8 μg); 2 and 3, molecular mass standard; 4, native enzyme (1.0 μg). Proteins were stained with Coomassie brilliant blue R-250.

TABLE 2. Comparison of the enzymatic properties of *Streptomyces* sp. strain CL190 HMG-CoA reductase with those of other biosynthetic HMG-CoA reductases

Sample source	V_{max} (μU/mg of protein)	K_m (μM)		K_i (nM)	K_m/K_i	pH	Activation energy (kJ/mol)	Reference or source
		NADPH	HMG-CoA					
Eucaryotes								
Yeast		89	2.4			6.5		24[d]
Rat			4.0	0.64,[a] 1.4[b]	6,300,[a] 2,900[b]			1[g]
		30	17		6.3–7.3			25[h]
		30	0.5					34[d]
Syrian hamster	37	35	4.3	5.5[a]	780	6.2–6.8		18[f]
		62	8.2			6.8		17[f]
Human								
58 kDa			2.0	0.2[a]	10,000	7.2		33[f]
52 kDa			2.5	0.3[a]	8,300			
Raphanus sativus		27	1.5			7.5	92	3[d]
Archaebacteria								
Haloferax volcanii	34	66	60	15[a]	4,000	7.3		5[f]
Halobacterium halobium			20	20[a]	1,000			10[g]
Sulfolobus solfataricus	17	23	17			5.5	47	7[f]
Eubacterium								
Streptomyces sp. strain CL190								
		62	7.7	5.5[c]	1,400	7.2	200	This study[d]
		52	7.3					This study[e]
	3.5	20	2.7					This study[f]

[a] Lovastatin was used as the inhibitor.
[b] ML-236B was used as the inhibitor.
[c] Pravastatin was used as the inhibitor.
[d] Radiometric assay (purified enzyme).
[e] Radiometric assay (recombinant enzyme).
[f] Spectrophotometric assay (recombinant enzyme).
[g] Radiometric assay (partially purified enzyme).
[h] Spectrophotometric assay (purified enzyme).

stable at 55°C, but heating at 70°C led to 55% loss of the activity.

Kinetic parameters of the HMG-CoA reductase from CL190. K_m values calculated from double-reciprocal plots were 62 μM for NADPH and 7.7 μM for HMG-CoA, and V_{max} was 3.3 nU per mg of protein at 30°C. No activity was detectable when NADH was substituted for NADPH. Pravastatin (1, 10), which competitively inhibits HMG-CoA reductase activity, inhibited the HMG-CoA reductase from CL190. From Fig. 2, summarizing pravastatin's mode of inhibition, a K_i value of 5.5 nM was calculated for pravastatin. Thus, CL190's K_m (HMG-CoA)/K_i (pravastatin) ratio of 1,400 was similar to the K_m (HMG-CoA)/K_i (mevinolin) ratio for *Halobacterium halobium* (10) and rat liver HMG-CoA reductase (1) (Table 2).

Comparison of amino acid sequences of the HMG-CoA reductases from various origins. The *hmgr* gene of CL190 encodes 353 amino acid residues with a predicted molecular mass of 37,723 Da, which is the smallest value that has been reported so far. The deduced amino acid sequence of the *hmgr* gene showed significant sequence similarity to the catalytic domains of HMG-CoA reductase from eucaryote and to archaebacterial HMG-CoA reductases in the databases of the DDBJ (Fig. 3).

Purification and enzymatic properties of the recombinant enzyme. We assumed that the recombinant HMG-CoA reductase from CL190 had a strong affinity for Ni-nitrilotriacetic acid resin. However, the enzyme activity was found only in the passing fraction. The proteins eluted from the column with buffer F containing 200 mM imidazole showed no HMG-CoA reductase activity. Steric hindrance around the N-terminal region may weaken the binding between a 6-His tag and a Ni-nitrilotriacetic acid resin. Thus, the passing fraction was further purified by Mono Q column chromatography. The purified enzyme afforded a homogeneous protein band by SDS-PAGE with a molecular mass of 41 kDa. To compare the properties of the recombinant enzyme with those of the CL190 enzyme, we

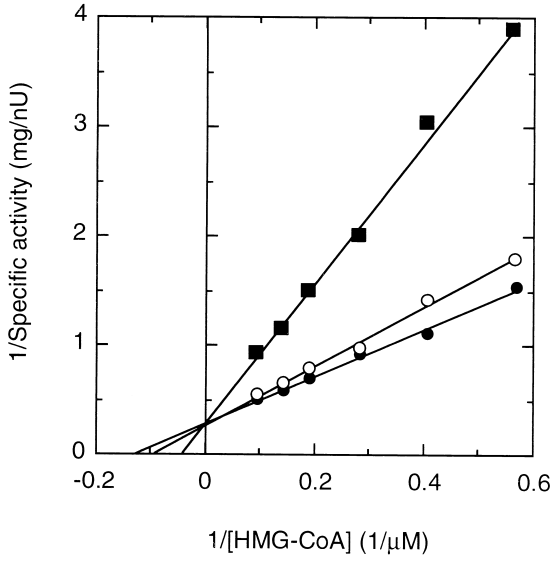
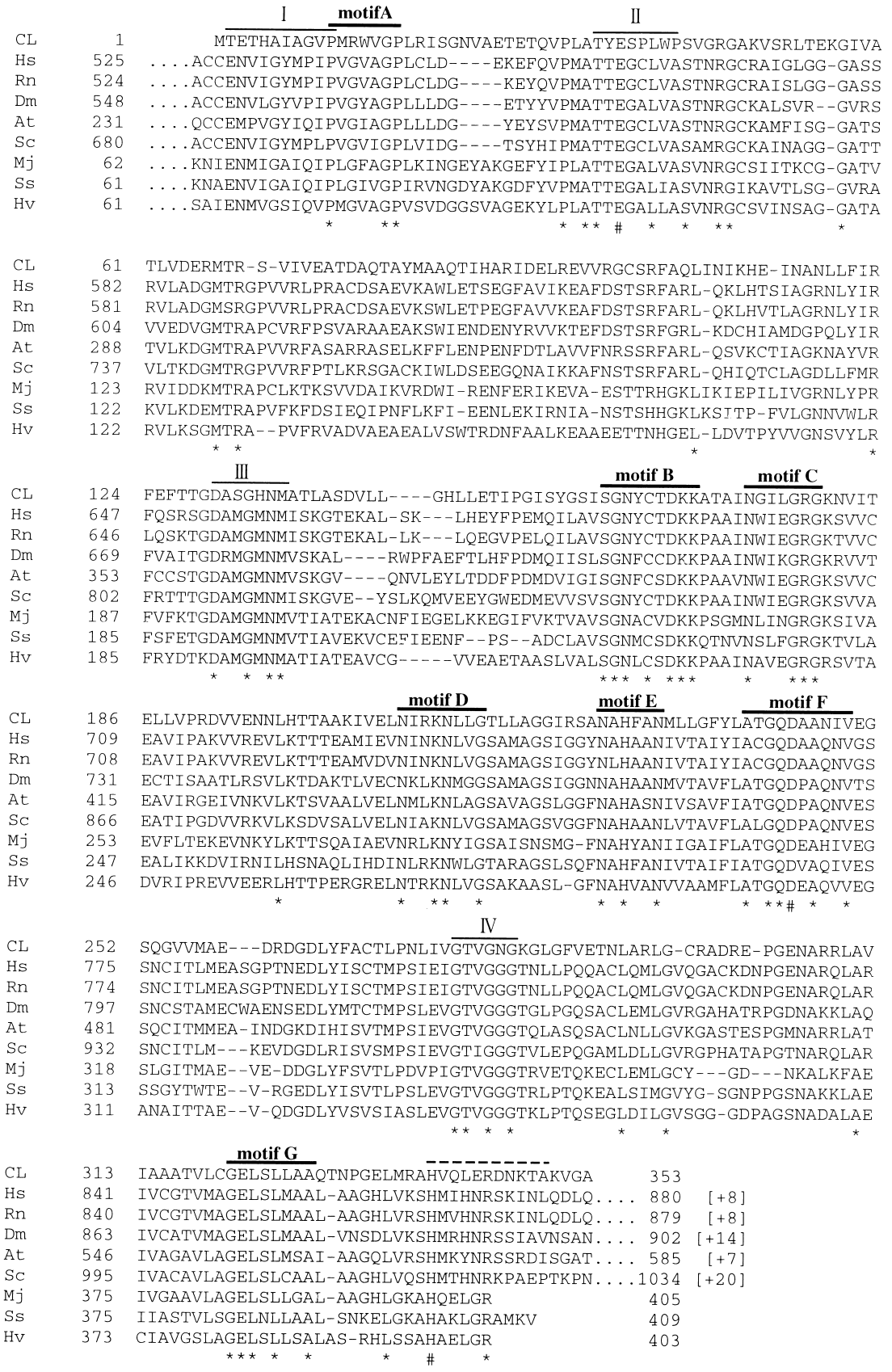


FIG. 2. Double-reciprocal plots for inhibition by pravastatin. HMG-CoA reductase activity of the purified CL190 enzyme was assayed in the presence of 0 μM (●), 1 μM (○), or 10 μM (■) pravastatin as described in Materials and Methods, except that the assay was initiated by adding NADPH. Each point represents the average of two determinations.


I
motifA
II
CL 1 MTETHAIAGVPMRWVGPLRISGNVAETETQVPLATYESPLWPSVGRGAKVSRLTEKGIVA
Hs 525 ....ACCENVIGYMPIPVGVAGPLCLD----EKEFQVPMATTEGCLVASTNRGCRAIGLGG-GASS
Rn 524 ....ACCENVIGYMPIPVGVAGPLCLDG----KEYQVPMATTEGCLVASTNRGCRAISLGG-GASS
Dm 548 ....ACCENVLGYVPIPVGYAGPLLLDG----ETYYVPMATTEGALVASTNRGCKALSVR--GVRS
At 231 ....QCCEMPVGYIQIPVGIAGPLLLDG----YEYSVPMATTEGCLVASTNRGCKAMFISG-GATS
Sc 680 ....ACCENVIGYMPLPVGVIGPLVIDG----TSYHIPMATTEGCLVASAMRGCKAINAGG-GATT
Mj 62 ....KNIENMIGAIQIPLGFAGPLKINGEYAKGEFYIPLATTEGALVASVNRGCSIITKCG-GATV
Ss 61 ....KNAENVIGAIQIPLGIVGPIRVNGDYAKGDFYVPMATTEGALIASVNRGIKAVTLSG-GVRA
Hv 61 ....SAIENMVGSIQVPMGVAGPVSVDGGSVAGEKYLPLATTEGALLASVNRGCSVINSAG-GATA
III
motif B
motif C
CL 124 FEFTTGDASGHNMATLASDVLL----GHLLETIPGISYGSISGNYCTDKKATAINGILGRGKNVIT
Hs 647 FQSRSGDAMGMNMISKGTEKAL-SK---LHEYFPEMQILAVSGNYCTDKKPAAINWIEGRGKSVVC
Rn 646 LQSKTGDAMGMNMISKGTEKAL-LK---LQEGVPELQILAVSGNYCTDKKPAAINWIEGRGKTVVC
Dm 669 FVAITGDRMGMNMVSKAL----RWPFAEFTLHFPDMQIISLSGNFCCDKKPAAINWIKGRGKRVVT
At 353 FCCSTGDAMGMNMVSKGV----QNVLEYLTDDFPDMDVIGISGNFCSDKKPAAVNWIEGRGKSVVC
Sc 802 FRTTTGDAMGMNMISKGVE--YSLKQMVEEYGWEDMEVVSVSGNYCTDKKPAAINWIEGRGKSVVA
Mj 187 FVFKTGDAMGMNMVTIATEKACNFIEGELKKEGIFVKTVAVSGNACVDKKPSGMNLINGRGKSIVA
Ss 185 FSFETGDAMGMNMVTIAVEKVCEFIEENF--PS--ADCLAVSGNMCSDKKQTNVNSLFGRGKTVLA
Hv 185 FRYDTKDAMGMNMATIATEAVCG-----VVEAETAASLVALSGNLCSDKKPAAINAVEGRGRSVTA
motif D
motif E
motif F
CL 186 ELLVPRDVVENNLHTTAAKIVELNIRKNLLGTLLAGGIRSANAHFANMLLGFYLATGQDAANIVEG
Hs 709 EAVIPAKVVREVLKTTTEAMIEVNINKNLVGSAMAGSIGGYNAHAANIVTAIYIACGQDAAQNVGS
Rn 708 EAVIPAKVVREVLKTTTEAMVDVNINKNLVGSAMAGSIGGYNLHAANIVTAIYIACGQDAAQNVGS
Dm 731 ECTISAATLRSVLKTDAKTLVECNKLKNMGGSAMAGSIGGNNAHAANMVTAVFLATGQDPAQNVTS
At 415 EAVIRGEIVNKVLKTSVAALVELNMLKNLAGSAVAGSLGGFNAHASNIVSAVFIATGQDPAQNVES
Sc 866 EATIPGDVVRKVLKSDVSALVELNIAKNLVGSAMAGSVGGFNAHAANLVTAVFLALGQDPAQNVES
Mj 253 EVFLTEKEVNKYLKTTSQAIAEVNRLKNYIGSAISNSMG-FNAHYANIIGAIFLATGQDEAHIVEG
Ss 247 EALIKKDVIRNILHSNAQLIHDINLRKNWLGTARAGSLSQFNAHFANIVTAIFIATGQDVAQIVES
Hv 246 DVRIPREVVEERLHTTPERGRELNTRKNLVGSAKAASL-GFNAHVANVVAAMFLATGQDEAQVVEG
IV
CL 252 SQGVVMAE---DRDGDLYFACTLPNLIVGTVGNGKGLGFVETNLARLG-CRADRE-PGENARRLAV
Hs 775 SNCITLMEASGPTNEDLYISCTMPSIEIGTVGGGTNLLPQQACLQMLGVQGACKDNPGENARQLAR
Rn 774 SNCITLMEASGPTNEDLYISCTMPSIEIGTVGGGTNLLPQQACLQMLGVQGACKDNPGENARQLAR
Dm 797 SNCSTAMECWAENSEDLYMTCTMPSLEVGTVGGGTGLPGQSACLEMLGVRGAHATRPGDNAKKLAQ
At 481 SQCITMMEA-INDGKDIHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGASTESPGMNARRLAT
Sc 932 SNCITLM---KEVDGDLRISVSMPSIEVGTIGGGTVLEPQGAMLDLLGVRGPHATAPGTNARQLAR
Mj 318 SLGITMAE--VE-DDGLYFSVTLPDVPIGTVGGGTRVETQKECLEMLGCY---GD---NKALKFAE
Ss 313 SSGYTWTE--V-RGEDLYISVTLPSLEVGTVGGGTRLPTQKEALSIMGVYG-SGNPPGSNAKKLAE
Hv 311 ANAITTAE--V-QDGDLYVSVSIASLEVGTVGGGTKLPTQSEGLDILGVSGG-GDPAGSNADALAE
motif G
CL 313 IAAATVLCGELSLLAAQTNPGELMRAHVQLERDNKTAKVGA 353
Hs 841 IVCGTVMAGELSLMAAL-AAGHLVKSHMIHNRSKINLQDLQ.... 880 [+8]
Rn 840 IVCGTVMAGELSLMAAL-AAGHLVRSHMVHNRSKINLQDLQ.... 879 [+8]
Dm 863 IVCATVMAGELSLMAAL-VNSDLVKSHMRHNRSSIAVNSAN.... 902 [+14]
At 546 IVAGAVLAGELSLMSAI-AAGQLVRSHMKYNRSSRDISGAT.... 585 [+7]
Sc 995 IVACAVLAGELSLCAAL-AAGHLVQSHMTHNRKPAEPTKPN....1034 [+20]
Mj 375 IVGAAVLAGELSLLGAL-AAGHLGKAHQELGR 405
Ss 375 IIASTVLSGELNLLAAL-SNKELGKAHAKLGRAMKV 409
Hv 373 CIAVGSLAGELSLLSALAS-RHLSSAHAELGR 403

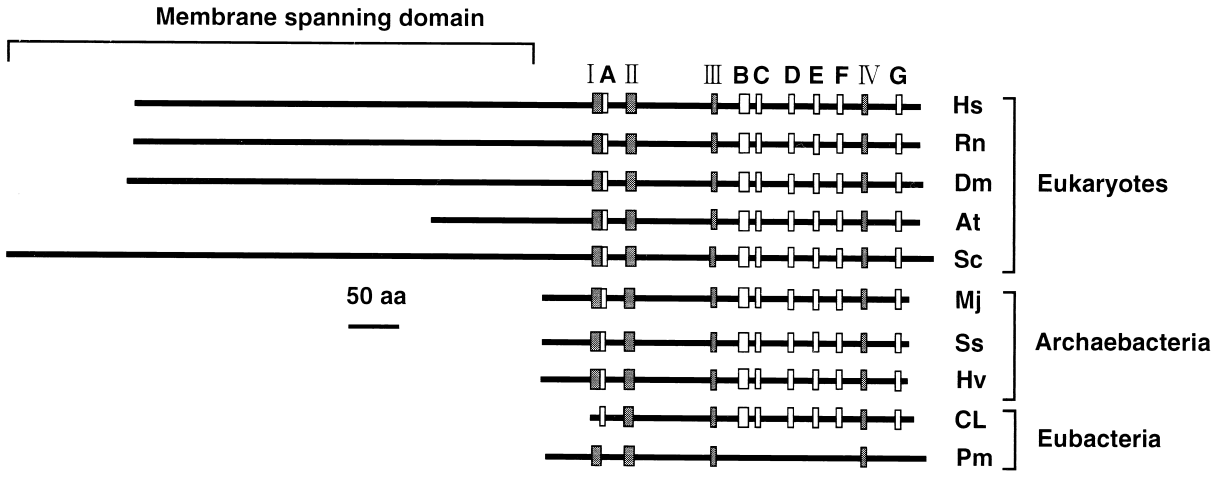


FIG. 4. Structures and conserved motifs of the HMG-CoA reductase proteins. Location of conserved motifs I to IV and A to G are indicated by shaded and open boxes, respectively. Pm indicates the HMG-CoA reductase from *P. mevalonii*. Other abbreviations used are as in Fig. 3.

carried out the HMG-CoA reductase assay by a radiometric method. The optimal activity of the HMG-CoA reductase expressed in *E. coli* occurred between pH 7.0 and 7.4 in potassium phosphate buffer, with a V_{max} of 3.1 nU per mg of protein. The K_m values calculated from double-reciprocal plots were 52 μM for NADPH and 7.3 μM for HMG-CoA. The enzyme showed no activity when NADH was substituted for NADPH. Thus, it was proven that the recombinant enzyme had the same nature as the CL190 enzyme.

Comparison of kinetic parameters. To compare the kinetic parameters from the recombinant enzyme with those from well-characterized eucaryotic or archaebacterial HMG-CoA reductases, we conducted the HMG-CoA reductase assay by the spectrophotometric method. The K_m (20 μM) of the recombinant enzyme for NADPH parallels the K_m values for archaebacterial HMG-CoA reductases. The K_m (2.7 μM) of the recombinant enzyme for HMG-CoA was close to those for eucaryotic HMG-CoA reductases. However, the V_{max} (3.5 μU/mg of protein) of the recombinant enzyme was lower than those of the enzymes from eucaryotes and archaebacteria by a factor of 5 to 10 (Table 2).

DISCUSSION

We successfully purified the biosynthetic HMG-CoA reductase from *Streptomyces* sp. strain CL190 by a procedure consisting of $(NH_4)_2SO_4$ precipitation, heat treatment and anion exchange, hydrophobic interaction, and affinity chromatographies. The final products appeared to be homogeneous as judged by SDS-PAGE.

The purified enzyme showed enzymatic properties similar to those of other biosynthetic HMG-CoA reductases as summarized in Table 2. A significant difference from other HMG-CoA reductases was observed in the low V_{max} value of the CL190 enzyme. We speculate that this may result, at least in part, from the presence of a negatively charged amino acid (Asp) at position 345, located only six residues after the predicted catalytic His^{339} in the amino acid sequence of CL190, because in both Syrian hamster and *P. mevalonii* the mutant enzymes introduced with Asp at the corresponding position exhibited only 10% of the parent enzymatic activity (15, 36).

The *hmgr* gene encodes a 353-residue HMG-CoA reductase with a predicted molecular mass of 37,723 Da. It should be noted that the subunit molecular mass of the enzyme was smaller than any other eucaryotic and archaebacterial enzymes reported so far. The membrane anchor domain, which is not essential for catalytic activity, consists of as many as eight transmembrane helices in mammals (27, 34), whereas it is truncated in plant HMG-CoA reductases and absent from the known noneucaryote HMG-CoA reductases (Fig. 4). The N-terminal sequence of the CL190 enzyme also lacks the multiple hydrophobic segments. Furthermore, it has been reported that phosphorylation-mediated regulation of HMG-CoA reductase activity by AMP-activated protein kinase involves a single serine, Ser^{871}, of rat (11) and hamster enzymes (37, 42) or Ser^{577} of isoform 1 of *Arabidopsis thaliana* HMG-CoA reductase (12) (Fig. 3). However, amino acid residues 339 to 349 of CL190 HMG-CoA reductase indicate the absence of a suitable target Ser for the kinase and putative kinase recognition sequence (15, 16). On the other hand, the entire amino acid sequence of the CL190 enzyme showed significant sequence similarity (37 to 40%) to the catalytic domains of the HMG-CoA reductases from the eucaryotes and archaebacteria (Fig. 3). Furthermore, three amino acid residues (E, D, and H), which have been implicated by mutagenesis and kinetic analysis as functioning in catalysis by *H. volcanii* (6), Syrian hamster (13, 18), and *P. mevalonii* (14, 48) HMG-CoA reductases, were also conserved in the CL190 enzyme (Fig. 3).

FIG. 3. Comparison of amino acid sequences of HMG-CoA reductase. A multiple alignment of the amino acid sequences was determined by using the GENETYX program. Identical amino acids among nine proteins are marked by asterisks and sharps. Dashes indicate gaps introduced for optimization of the alignment. Indicated numbers refer to amino acid positions. CL, *Streptomyces* sp. strain CL190; Hs, *Homo sapiens* (SWISS-PROT, P04035); Rn, *Rattus norvegicus* (SWISS-PROT, P51639); Dm, *Drosophila melanogaster* (SWISS-PROT, P14773); At, *A. thaliana* (SWISS-PROT, P14891); Sc, *Saccharomyces cerevisiae* (SWISS-PROT, P12683); Mj, *Methanococcus jannaschii* (SWISS-PROT, Q58116); Ss, *Sulfolobus solfataricus* (DAD, U95360); Hv, *Haloferax volcanii* (SWISS-PROT, Q59468). I, II, III, and IV indicate proposed binding sites for HMG-CoA (I and II) and NAD(P) (III and IV) (29). Sharps indicate amino acids that have been proposed to function in catalysis (6, 13, 14, 18, 48). Motifs A to G indicate regions in which the sequences were highly conserved. The dotted line over the sequences after motif G indicates putative kinase recognition sequences that are conserved in higher eucaryotes.

As shown in Fig. 3, the HMG-CoA binding motif of an E83 loop (motif II) and NAD(P) binding motifs of DAMGXN (motif III) and GX_2G_2XT (motif IV), which had been proposed on the basis of the crystal structure analysis of the HMG-CoA reductase from *P. mevalonii* (29), were found in the amino acid sequence deduced from the CL190 *hmgr* gene. However, an EX_3GX_4P motif (motif I), which had been reported for the HMG-CoA binding site (29), was not conserved in the amino acid sequence of the CL190 enzyme. In addition, the amino acid sequence of the CL190 HMG-CoA reductase revealed several additional limited motifs (A to G) which were highly conserved and common to other biosynthetic HMG-CoA reductases (Fig. 3). However, these motifs are not found in the amino acid sequence of the *P. mevalonii* HMG-CoA reductase (Fig. 4). These sequence conservations in the biosynthetic HMG-CoA reductases suggest strong evolutionary pressure to maintain these amino acid residues at specific positions, thus indicating that seven motifs (A to G) might play important roles in the structural conformation and/or catalytic properties of the enzyme. Crystal structure analysis of the HMG-CoA reductase from CL190 is indispensable for a thorough understanding of the functional motifs. Since the CL190 enzyme showed high heat stability, its three-dimensional structure is suitable for analysis by X-ray crystallography.

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