Pseudomonas mevalonii 3-Hydroxy-3-methylglutaryl-CoA Lyase: Characterization of the Isolated Recombinant Protein and Investigation of the Enzyme's Cation Requirements†

Chakravarthy Narasimhan and Henry M. Miziorko*

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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ABSTRACT: *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl-CoA lyase has been expressed in an active form in *Escherichia coli* and purified to homogeneity. Enzyme activity in crude extracts is 30-fold higher than reported for a homologous expression system. After Q-Sepharose fast-flow anion-exchange chromatography, the enzyme, which represents the first homogeneous preparation of a prokaryotic form of the protein, exhibits a specific activity of 70 units/mg. The purified enzyme is stable when stored in 20% glycerol at −80 °C. The recombinant bacterial enzyme cross reacts with antiserum produced against avian liver lyase, indicating some sequence homology between the two proteins. The enzyme exhibits a K_m = 20 μM for (*S*)-HMG-CoA. Divalent cations (Mg^{2+} and Mn^{2+}) markedly stimulate the enzyme activity under assay conditions; activity is only modestly increased by exogenous mercaptans. The activator constant, K_a, for Mg^{2+} (6.9 mM) is 3 orders of magnitude greater than that for Mn^{2+} (2.0 μM). While EDTA dos not affect activity, *o*-phenanthroline treatment markedly inhibits the enzyme. In contrast, *m*-phenanthroline is ineffective, suggesting that the ortho isomer's effect is attributable to chelation of a tightly bound metal ion. Atomic absorption and EPR analyses of isolated enzyme indicate the presence of tightly bound copper. In enzyme expressed using standard LB broth, copper is detected at stoichiometries of only 0.07–0.10. When the growth medium is supplemented with 1 mM $CuSO_4$, stoichiometry of copper binding increases to over 0.7 per enzyme subunit. Copper-enriched lyase displays enhanced thermal stability in comparison with enzyme that is low in metal content. Agreement between atomic absorption and EPR stoichiometry estimates suggests that all of the tighly bound cation is divalent copper. The EPR parameter $A_{\parallel}$ is estimated to be 152 G, compatible with assignment of type II copper. The measured $g_{\parallel}$ value of 2.28 suggests that cation binding involves nitrogen ligand(s).

3-Hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) catalyzes the terminal reaction in eukaryotic hepatic ketogenesis (Lynen et al., 1958), namely, the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into acetoacetate and acetyl-CoA. The enzyme thus plays a vital role in the generation of ketone bodies which are alternative sources of fuel during periods of starvation (Robinson and Williams, 1980). HMG-CoA[1] lyase also functions in the catabolic pathway of the amino acid leucine (Faull et al., 1976). Deficiency of human HMG-CoA lyase has been reported to cause severe metabolic acidosis following periods of fasting or infection (Gibson et al., 1988); the disease has proven fatal in a number of cases (Ozand et al., 1991). Although the importance of HMG-CoA lyase in metabolism and the consequences of enzyme deficiency are well understood (Robinson and Williams, 1980; Faull et al., 1976; Gibson et al., 1988; Ozand et al., 1991), characterization of the enzyme and details concerning the protein's structure are limited. The enzyme has been partially purified from many eukaryotic sources (Bachawat et al., 1955) and has been purified to homogeneity from avian liver (Kramer and Miziorko, 1980).

Recently, Scher and Rodwell (1989) partially purified the enzyme from a prokaryotic source, *Pseudomonas mevalonii*, where it functions in concert with HMG-CoA reductase to potentiate growth from the carbon source, mevalonic acid, by producing acetyl-CoA. Anderson and Rodwell have reported the nucleotide sequence of the second gene of the *mva* operon of *P. mevalonii*, *mvaB* (Anderson and Rodwell, 1989); this DNA was expressed in *E. coli* and was identified as the structural gene for HMG-CoA lyase. No heterologous expression of a eukaryotic lyase has been reported to date.

The prospect of overexpressing the *P. mevalonii* protein appears attractive in that it forms the basis for development of a model system that would facilitate detailed physical and structural investigation of the enzyme. We have investigated this recombinant system and now report the heterologous expression, purification to homogeneity, and partial characterization of a high specific activity *P. mevalonii* HMG-CoA lyase.

EXPERIMENTAL PROCEDURES

Materials. Sephadex G-50, NAD^+, NADH, and ampicillin were purchased from Sigma (St. Louis, MO). Isopropyl thiogalactoside (IPTG) was bought from US Biochemicals (Cleveland, OH). Bacto tryptone and yeast extract were purchased from Difco Laboratories (Detroit, MI). The Q-Sepharose fast-flow anion-exchange resin was supplied by Pharmacia (Uppsala, Sweden). All other chemicals were of high-quality reagent grade and were used without further purification.

Methods. Protein Assays. Determination of protein concentration was done following the method of Bradford (1976) using bovine serum albumin as the standard.

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* Address editorial correspondence to this author at Biochemistry Department, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Phone: 414-257-8437. FAX: 414-257-2008.

[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PMSF, phenylmethanesulfonyl fluoride; EPR, electron paramagnetic resonance; IPTG, isopropyl thiogalactoside.

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Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions with an 11% acrylamide running gel and a 4.5% acrylamide stacking gel (Laemmli, 1970). The molecular weight standards used were as follows: phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 000), and lactalbumin (14 400).

Preparation of HMG-CoA. Synthesis of HMG anhydride was done by the method of Goldfarb and Pitot (1971). The anhydride was reacted with the lithium salt of CoA at pH 8.0 under nitrogen at 4 °C for about 5 min with vigorous mixing. The completion of the reaction was verified qualitatively by the nitroprusside test (Simon and Shemin, 1953), and HMG-CoA was determined both spectrophotometrically as well as enzymatically (Knappe, 1963) at 30 °C using a millimolar extinction coefficient of 6.2 for NADH. The concentration of HMG-CoA determined by the enzymatic assay gave directly the concentration of the (*S*)-isomer, which is the substrate for HMG-CoA lyase (Higgins et al., 1972; Stegink and Coon, 1968; Scher and Rodwell, 1989). Unless stated otherwise, the concentrations of HMG-CoA reported here refer to the (*S*)-isomer.

Assay of HMG-CoA Lyase Activity. The activity of HMG-CoA lyase was measured by the citrate synthase coupled assay developed by Stegink and Coon (1968), as modified by Kramer and Miziorko (1980). The assays were done at 30 °C using a Cary 219 double-beam Spectrophotometer equipped with a thermostated sample compartment. The assay mixture (1 mL) contained 200 μmol of Tris/Cl, pH 8.2, 10 μmol of $MgCl_2$, 1.5 μmol of NAD^+, 0.05 μmol of NADH, 5.0 μmol of dithithreitol, 2.5 μmol of malate, 20 μg each of citrate synthase and malate dehydrogenase, the sample of HMG-CoA lyase to be assayed, and 0.11 μmol of HMG-CoA, which was added after all the components had been incubated at 30 °C for 10 min. Chelex 100-treated deionized water was used to prepare all assay reagents.

Bacterial Growth. A sample of *Escherichia coli* (*E. coli*) BL21(DE3) transformed with the expression vector pT7-2600 containing the *P. mevalonii* HMG-CoA lyase gene (Anderson and Rodwell, 1989) was a generous gift from Dr. Victor Rodwell (Purdue University). Bacteria were grown in LB media (10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 5 g of NaCl per liter) at 37 °C, 30 °C, or 22 °C in flasks shaken at 250 rpm. Growth was monitored by periodically measuring the optical density (OD) at 600 nm. Starter cultures (5 mL) were grown at 37 °C in LB containing 200 μg/mL ampicillin overnight. This overnight culture was diluted approximately 100-fold with LB containing 200 μg/mL ampicillin and allowed to grow at the appropriate temperature conditions to an OD of 0.6. At this point, expression of HMG-CoA lyase was induced by the addition of IPTG to a final concentration of 1 mM, and the growth was continued until late log phase. The cells were harvested by low-speed centrifugation (3000*g*, 10 min) at 4 °C and the pellets stored at −20 °C prior to cell lysis. For large-scale growth, the volumes of overnight and subsequent cultures were increased proportionately. Bacteria were lysed using a French pressure cell using a buffer that consisted of 20 mM phosphate, pH 7.2, 1 mM EDTA, 10 μg/mL DNAase I, 10 μg/mL RNAase A, and 100 μM PMSF. The buffer used for enzyme purification consisted of 10–100 mM phosphate, 20% glycerol, pH 7.2.

Atomic Absorption. Atomic absorption analysis was performed using an Instrumentation Laboratory Inc. (Allied Systems) Video 12 aa/ae spectrophotometer equipped with a hollow cathode lamp source. Air–acetylene flame was used for all analyses. Appropriate certified reference standard solutions were used for instrument calibration.

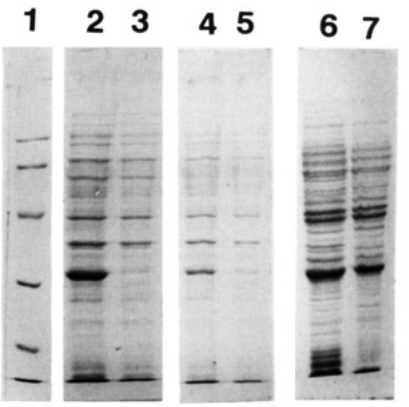

FIGURE 1: SDS-polyacrylamide gel of unfractionated extracts and high-speed supernatants of *E. coli* lysate containing HMG-CoA lyase. Lane 1: molecular weight standards. Lanes 2, 4, 6: crude extracts from 37, 30, and 22 °C. Lanes 3, 5, 7: soluble extracts from 37, 30, and 22 °C.

EPR Measurements. Conventional X-band EPR spectra were obtained with a Varian Century-Line 9-GHz spectrometer. The spectra were recorded at liquid nitrogen temperature with a frequency modulation of 100 KHz, microwave power of 5 mW, and modulation amplitude of 5 G. The field sweep was 1000 G and the time constant was 0.25 s. Spin quantitation was done using the SUMSPC90 program developed by Hyde et al. (1992). The *g* factors were calculated from the equation $h\nu = g\beta H$ where h is Planck's constant, ν is the spectral frequency, H is the static field, and β is the Bohr magneton.

RESULTS

Expression of Active HMG-CoA Lyase. The fraction of total *E. coli* BL21(DE3) protein represented by the *mvaB* gene product is a function of bacterial growth conditions (viz. temperature). For example, at 37 °C, over half of the total protein expressed is represented by HMG-CoA lyase. At 30 and 22 °C, the fraction of total expressed protein that is represented by lyase decreases somewhat although it remains the major component of the samples. Even more dependent on these expression conditions is the level of HMG-CoA lyase activity. Although the expression levels of lyase protein are very high at both 37 and 30 °C, as indicated by the gels of the crude extracts (Figure 1, lanes 2 and 4), the specific activities of enzyme in those fractions are rather low. Upon comparison of soluble extracts (high-speed supernatants, Figure 1, lanes 3 and 5) with the corresponding crude homogenates, it is apparent that lyase protein is largely insoluble when expressed under conditions typical for bacterial propagation. Although the specific activity of enzyme expressed at 30 °C is better by a factor of 5–6 over that at 37 °C (Table I), little of the total lyase protein represents active soluble enzyme (Figure 1, lanes 4, 5). Growth at 22 °C, however, markedly improves the expression of catalytically active enzyme (Table I). Most of the enzyme that has been expressed remains soluble after high-speed centrifugation of the crude extract (Figure 1, lanes 6 and 7). Therefore, the 22 °C temperature growth condition is now routinely used to overexpress and purify active *P. mevalonii* HMG-CoA lyase.

Purification of HMG CoA-Lyase. E. coli cells harvested in late log phase were pelleted from 1.5 L of culture by low-speed centrifugation (3000*g*, 10 min), and the cell pellet

Table I: Comparison of Expression of Recombinant *P. mevalonii* HMG-CoA Lyase under Various Growth Conditions[a]

temp, (°C)	activity (units/mg)	
	crude	supernatant
37	0.3	0.6
30	1.7	2.7
22	7.0	7.0

[a] For each of the growth conditions, 25 mL of culture was grown in LB medium, containing 200 μg/mL ampicillin, to an OD_{600} of 0.6 and then induced with 1 mM IPTG. The cells were grown to late log phase and then harvested. The cell pellet was suspended in 10 mL of lysis buffer and ruptured in a French pressure cell.

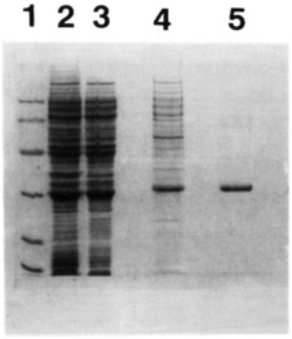

FIGURE 2: SDS-PAGE of HMG-CoA lyase at various stages of purification. The gel samples were prepared in a reducing buffer containing Tris, SDS, mercaptoethanol and bromophenol blue. Lane 1: molecular weight standards. Lane 2: total bacterial cell extract. Lane 3: high-speed supernatant obtained by centrifuging the extract at 181000*g* for 60 min. Lane 4: sample from ammonium sulfate precipitation (0–40% saturation). Lane 5: HMG-CoA lyase after Q-Sepharose column chromatography.

was homogenized in 50 mL of cold (0 °C) lysis buffer. The bacteria were ruptured in a French pressure cell at 16 000 psi. This crude extract was centrifuged at 181000*g* for 60 min, and the resultant high-speed supernatant containing the active HMG-CoA lyase was decanted and kept on ice. This high-speed supernatant was promptly brought to 40% $(NH_4)_2SO_4$ saturation by addition of the solid salt with slow stirring. The mixture was kept stirring slowly at 4 °C for 4.5 h. The precipitated protein was centrifuged at 12100*g* for 20 min at 4 °C. After removal of the supernatant from the pellet, the pellet was dissolved in a minimum volume of 10 mM phosphate buffer, pH 7.2, containing 20% glycerol. The dissolved ammonium sulfate fraction was desalted by rapidly passing the sample, divided into appropriately sized aliquots, over Sephadex G-50 centrifugal desalting columns (Penefsky, 1977) equilibrated with 10 mM phosphate buffer containing 20% glycerol. The desalted sample was immediately applied to a Q-Sepharose fast-flow anion-exchange column (1.5 cm × 45 cm) equilibrated with the desalting buffer. The bound material was eluted with a 10–100 mM phosphate gradient (1.0 L), pH 7.2, containing 20% glycerol. The protein peak containing the enzyme activity was pooled and concentrated using an Amicon concentrator equipped with a PM-30 membrane. The pooled active fractions were shown to be homogeneous by SDS-PAGE (Figure 2). The purification steps are summarized in Table II. The enzyme is recovered after the final step in about 41% yield. The preparation represents an approximate 10-fold purification from the crude extract to the homogeneous enzyme, reflecting the level of initial overexpression.

Enzyme Stability. The recombinant enzyme recovered from the Q-Sepharose anion-exchange column retains full activity for several months if stored at –80 °C in the phosphate buffer

Table II: Purification of Recombinant *P. mevalonii* HMG-CoA Lyase

purification step	total units	total protein (mg)	sp act. (units/mg)	% yield
crude extract	3416	490	7.0	100
soluble extract	3122	446	7.0	91
0–40% $(NH_4)_2SO_4$ fractionation	1990	95	21.0	58
Q-Sepharose chromatography	1384	19.8	70.0	41

containing 20% glycerol, pH 7.2. The enzyme was found to be extremely labile at ambient or higher temperatures in the absence of 20% glycerol and/or 0.2 mM substrate. For example, even in the presence of 20% glycerol, the enzyme lost 63% of its initial activity when incubated at ambient temperature over a period of 5 h. Addition of 10 mM $MgCl_2$ to this sample did not improve the stability of the enzyme at ambient temperature. The presence of 0.2 mM HMG-CoA helped, however, to maintain the activity (>90% after 8 h) of the enzyme at 22 °C.

The enzyme, isolated in buffers that contained no exogenous thiol, showed about a 2-fold enhancement in activity in the presence of 5 mM dithiothreitol (DTT). Conversely, after separation of enzyme that had been preincubated with excess reductant by centrifugal gel filtration, about 50% of the optimal activity was observed.

Characterization of P. mevalonii HMG-CoA Lyase. The specific activity of the homogeneous recombinant enzyme isolated as described above is 70 units/mg. Lineweaver–Burk analysis of data demonstrating the dependence of the recombinant enzyme's activity on the concentration of substrate, (*S*)-HMG-CoA, indicates a Michaelis constant, K_m = 20 μM (Table III).

Recombinant HMG-CoA lyase was subjected to gel filtration on a Superose 12 column (12 mm × 30 cm, Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris, pH 8.00, containing 0.1 M NaCl and 10% glycerol, and calibrated using appropriate molecular weight markers. Under these conditions, the native recombinant lyase, which elutes slightly before a carbonic anhydrase marker, exhibits the mobility expected of a protein with a native molecular weight very similar to that reported for the HMG-CoA lyase promoter (Table III: 27 kDa, Kramer and Miziorko, 1980; 32 kDa, Scher and Rodwell, 1989) and measured for the recombinant enzyme under denaturing SDS-PAGE conditions (Figure 2).

Antigenic Properties of Recombinant HMG-CoA Lyase. Figure 3 shows a western blot in which the recombinant bacterial lyase is detected using polyclonal antiserum prepared against avian liver lyase (Kramer and Miziorko, 1980). Although the sensitivity with which recombinant bacterial lyase is detected (Figure 3, lane 2) is reduced by at least 1 order of magnitude in comparison with the avian lyase (Figure 3, lane 1), the blot nevertheless indicates cross reaction between recombinant bacterial lyase and anti-avian lyase antibody. This suggests the presence of some sequence identity between the two lyases and represents the first report of cross reactivity of a bacterial lyase with antibody raised against the eukaryotic enzyme.

Isolated Enzyme Contains Tightly Bound Cations. Inhibition by Metal Chelators. Recombinant HMG-CoA lyase was preincubated with *o*-phenanthroline at ambient temperature; aliquots were withdrawn after specified time intervals and used to determine enzyme activity in the presence of 10 mM $MgCl_2$. As shown in Figure 4, at 4 mM *o*-phenanthroline concentration, enzyme activity gradually decreases and levels off at 43% of its initial activity after 4 h. That inhibition is

Table III: Properties of HMG-CoA Lyases

property	bacterial lyase[a] wild type	bacterial lyase[a] recombinant	avian lyase[b]
reduced thiol requirement in assay	marked dependency	2-fold increase	marked dependency
cation requirement	marked dependency	marked dependency	marked dependency
specific activity (units/mg)	22.1	70	351
K_m (HMG-CoA, μM)	100	20	8
K_a (Mn^{2+}, μM)		2	10[c]
K_a (Mg^{2+}, μM)		6900	50[c]
subunit molecular weight (dalton)	32000[d] 31600[e]	32000	27000

[a] For the wild type bacterial lyase, the data are from Scher and Rodwell (1989); data for the recombinant lyase are from the present study. [b] Data for the avian lyase are from Kramer and Miziorko (1980). [c] Apparent K_m, estimated at saturating HMG-CoA concentration (P. Hruz, unpublished observation). [d] Molecular weight determined from SDS-PAGE. [e] From cDNA sequence (Anderson and Rodwell, 1989).

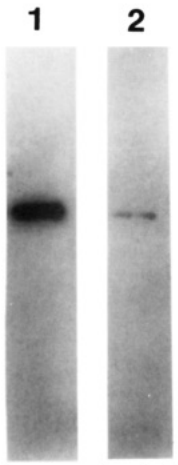

FIGURE 3: Western blot demonstrating the immunoreaction between recombinant *P. mevalonii* HMG-CoA lyase and antiserum (rabbit) prepared against avian lyase. One hundred nanograms of purified chicken liver lyase (lane 1) or 100 ng of recombinant *P. mevalonii* HMG-CoA lyase (lane 2) was used in the experiment. After transfer of protein to nitrocellulose and incubation with antiserum, the antigen–antibody complex was detected using ^{125}I-protein A.

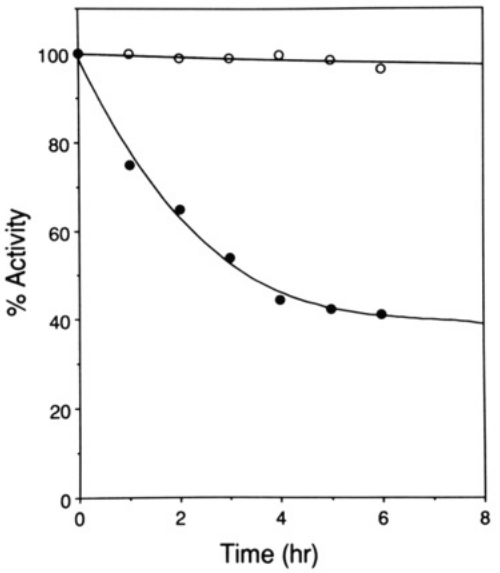

FIGURE 4: Effect of *o*-phenanthroline on the activity of recombinant *P. mevalonii* HMG-CoA lyase. Enzyme, in 10 mM potassium phosphate, pH 7.2, containing 20% glycerol and 0.2 mM HMG-CoA, was preincubated with 4 mM *o*-phenanthroline (●) at ambient temperature. Aliquots were drawn at indicated time intervals and assayed for lyase activity. As a control, *m*-phenanthroline (4 mM; ○) was used for incubation, which resulted in very little inhibition of enzyme activity over the time period of the experiment.

attributable to chelation of a tightly bound cation is confirmed by control experiments in which the nonchelating isomer, *m*-phenanthroline, is shown to be without effect (Figure 4). Incubation of recombinant HMG-CoA lyase with 0.2 mM EDTA for up to 4 h did not result in any significant inhibition of enzyme activity. Attempts to restore enzyme activity by resupplementing the enzyme with exogenous cation after *o*-phenanthroline treatment were not successful.

Metals Analyses. Enzyme purified after expression in *E. coli* grown in standard LB broth was analyzed by atomic absorption and EPR methodologies (Table IV, Figure 5). Copper was the predominant tightly bound cation identified, although zinc and iron were also present at lower levels. The concentrations of cations associated with the isolated recombinant lyase did not approach the level expected for stoichiometric binding to enzyme subunit. A survey of 20 metals by inductively coupled plasma emission spectroscopy failed to detect significant levels of other cations. When growth medium was supplemented (Omburo et al., 1992) with 1 mM divalent copper ($CuSO_4$) prior to induction of HMG-CoA lyase synthesis, expression levels of active, soluble lyase were not appreciably affected. However, the enzyme that was subsequently isolated showed 1 order of magnitude increase in metal content, with bound cation approaching stoichiometric levels with respect to enzyme subunits (Table IV). At this elevated level of tightly bound cation, there is good agreement between atomic absorption and EPR estimates, suggesting that all of the bound copper is the divalent, paramagnetic

Table IV: Copper Content of Recombinant *P. mevalonii* HMG-CoA Lyase

growth medium	Cu(II) (mol/mol) atomic absorption[a]	Cu(II) (mol/mol) ESR[b]
unsupplemented LB broth	0.07	0.17
1.0 mM $CuSO_4$ added to LB broth before induction	0.75	0.72
1.0 mM $CuSO_4$ added to LB broth at the time of induction	0.48	0.60

[a] Atomic absorption was performed on an Instrumentation Laboratory Inc. (Allied Analytical Systems) Video 12 aa/ae spectrophotometer equipped with hollow cathode lamp source. Other parameters were lamp current 5 mA, wavelength 324.7 nm, and air–acetylene flame. [b] Spin quantitation of each of these samples was done by double integration of the recorded EPR spectra against a 1 mM copper nitrate standard using the SUMSPC90 program developed by Hyde et al. (1992).

species. Furthermore, the measured EPR parameters ($A_\parallel$ = 152 G; $g_\parallel$ = 2.28) suggest that enzyme liganding creates a type II copper center that may involve protein nitrogen ligand(s) (Peisach and Blumberg, 1974). This observation prompts us to discount the possibility of adventitious cation binding.

If metal supplementation is delayed until the time at which enzyme induction is initiated, stoichiometry of bound cation

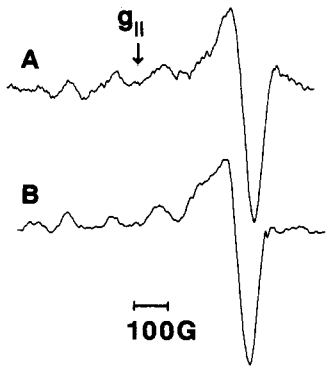

FIGURE 5: X-band EPR spectra of recombinant *P. mevalonii* HMG-CoA lyase, purified after expression in *E. coli*. (A) The enzyme was expressed in *E. coli* grown in unsupplemented LB broth. Enzyme concentration in the EPR sample was 206 μM. (B) Enzyme was expressed using LB broth supplemented with 1 mM $CuSO_4$; enzyme concentration in the EPR sample was 39 μM. The spectral parameters were scan range 1000 G, modulation amplitude 5 G, modulation frequency 100 KHz, temperature 77 K. The *g* factor and hyperfine constants, measured as described in Methods, are $g_{\|}$ = 2.28 and $A_{\|}$ = 152 G. Displayed spectra are averages of 25 scans.

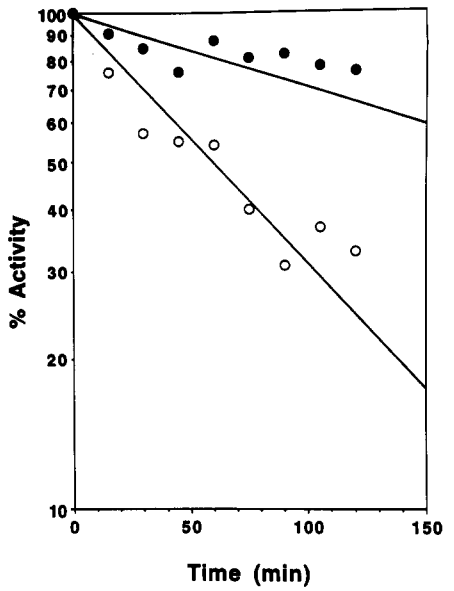

FIGURE 6: Thermal stability of recombinant *P. mevalonii* HMG-CoA lyase. Copper-enriched (●) or unsupplemented (○) enzyme in 10 mM potassium phosphate, pH 7.2, containing 20% glycerol and 0.2 mM HMG-CoA, was incubated at 30 °C. Aliquots were withdrawn at 15-min time intervals, over a period of 2 h, and assayed for lyase activity. The calculated $t_{1/2}$ values for copper-enriched and unsupplemented enzyme were 269 and 68 min, respectively.

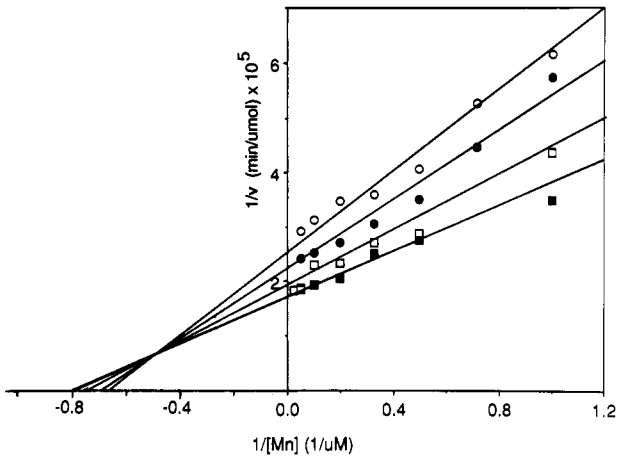

FIGURE 7: Double-reciprocal plot of the initial velocity of the HMG-CoA lyase reaction as a function of Mn^{2+} concentration. Concentrations of the substrate, HMG-CoA, were 26.6 μM (○), 39.9 μM (●), 53.2 μM (□), and 106.4 μM (■). The activator constant, K_a, was calculated from the intersection point, as described by Mildvan and Cohn (1965).

in isolated enzyme is increased over that measured when there is no supplementation. However, the observed levels are somewhat lower than detected when metal is added prior to induction. This finding is not unexpected, as production of the T7 RNA polymerase encoded by genomic DNA in the BL21(DE3) strain of *E. coli* is not stringently controlled by the *lac* promoter. Modest but easily detectable expression of the pT7-2600 plasmid-encoded lyase occurs even in the absence of IPTG. Thus, delay in copper supplementation probably results in a mixture of enzyme molecules, a fraction of which is low in metal content. Based on our observation that specific activity of freshly isolated HMG-CoA lyase does not strongly correlate with differences in copper content, assignment of a structural, rather than catalytic, role to tightly bound cation seems reasonable. This hypothesis is supported by the results of thermal denaturation studies (Figure 6), which indicate that enzyme low in metal content loses activity at a 4-fold faster rate than enzyme that is high in metal content as a result of copper supplementation. An expanded investigation of the function of bound cation in prokaryotic lyase, as well as a survey of the levels of bound cation in eukaryotic lyases, will be required before a more precise role for this metal can be assigned.

Enzyme Activation by Dissociable Divalent Cations. While added Zn^{2+} or Cu^{2+} showed no stimulatory effects on activity of the isolated enzyme, micromolar concentrations of Mn^{2+} and millimolar concentrations of Mg^{2+} markedly stimulated enzyme activity. Concentration-dependence studies (Mildvan and Cohn, 1965) for each of these cations were performed to determine activator constants (K_a) of 2 μM and 6.8 mM, respectively (Figure 7, Table III). It was also observed that Mn^{2+} at concentrations higher than 100 μM inhibited the enzyme activity while Mg^{2+} at concentrations lower than 300 μM virtually had no stimulatory effect on the enzyme activity. The 10^3-fold difference between affinity constants for Mg^{2+} and Mn^{2+} does not depend on content of tightly bound copper, as comparable differences have been measured with enzyme produced upon induction in copper-supplemented LB broth.

DISCUSSION

This report documents the overexpression in *E. coli* and isolation in homogeneous form of a bacterial HMG-CoA lyase in quantities significantly larger than those available from conventional sources. This accomplishment affords us a model system for exploring the structure/function relationships that account for HMG-CoA cleavage. In this context, it becomes important to compare the recombinant enzyme with the homologously expressed *P. mevalonii* lyase as well as with the avian liver enzyme.

While extensive immunochemical analyses of HMG-CoA lyases have not been reported, preliminary analyses of crude fibroblast extracts (Miziorko, 1985) suggest that antiserum prepared against the avian liver mitochondrial enzyme cross reacts with a human fibroblast protein of subunit molecular weight comparable to the avian enzyme. The observation that the recombinant enzyme also reacts with antiserum against avian enzyme indicates that the bacterial protein exhibits significant structural homology with the eukaryotic lyases.

The recombinant enzyme exhibits, after early stages in the purification, a marked requirement for stabilizing agents such as glycerol and substrate. This situation contrasts sharply with the stability observed with the avian liver enzyme and can, in some cases, complicate experimental design. However, the recombinant enzyme is attractive in that it does not exhibit the stringent requirement for exogenous thiol that characterizes the avian enzyme (Kramer & Miziorko, 1980). This property promises to facilitate certain physical and protein chemistry studies that would otherwise be hindered by the requirement to maintain an elevated level of exogenous thiol.

The specific activity observed for the homogeneous recombinant protein is 70 units/mg, a value severalfold higher than reported for the homologously expressed *P. mevalonii* enzyme (Scher and Rodwell, 1989). The presence of multiple protein components in that preparation would appear to account for this discrepancy. In contrast, the avian enzyme (Kramer and Miziorko, 1980) exhibits a specific activity approximately 5-fold higher than measured with the recombinant enzyme. While such a difference may reflect actual higher catalytic efficiency in the eukaryotic enzyme, the lability of the recombinant enzyme, despite precautions taken during isolation, may partially account for the observed differences.

In comparing the substrate binding properties of the various HMG-CoA lyases (Table III), variations comparable in magnitude to those observed for specific activities are apparent. Apparent K_m values for the (*S*)-isomer of HMG-CoA are in the 10^{-5}–10^{-4} M range. HMG-CoA lyase utilizes only one isomer from an (*R*,*S*)-mixture of chemically prepared HMG-CoA (Stegink & Coon, 1968). As discussed by Higgins et al. (1972), lyase uses the same isomer that is metabolized by HMG-CoA reductase to form (*R*)-mevalonate. Since the reductase reaction does not affect the stereochemistry at C3 and since the stereochemistry at C3 of (*R*)-mevalonate is equivalent to that at C3 of (*S*)-HMG-CoA, it follows that (*S*)-HMG-CoA is the substrate for reductase as well as lyase. This analysis has recently been experimentally validated by Rodwell and his colleagues (Scher and Rodwell, 1989).

In contrast to some variations that have been noted in specific activity and substrate affinity, the recombinant, avian, and *P. mevalonii* lyases all exhibit a marked activity stimulation by divalent cation. Although there have been no rigorous explanations for the requirement of these cations, Kramer and Miziorko advanced the hypothesis that HMG-CoA lyase is a metalloenzyme and that the bound metal is being slowly removed during purification procedure (Kramer and Miziorko, 1980). In the present study, *o*-phenanthroline, a metal chelator, did show an inhibitory effect on the enzyme activity (Figure 4). The anionic chelator, EDTA, showed minimal effect on activity of the prokaryotic enzyme. This would suggest that the loss of activity observed in the presence of *o*-phenanthroline may be due to efficient removal of bound metal by the less polar *o*-phenanthroline and that anionic chelators like EDTA have limited accessibility to the metal binding site (Dumas et al., 1989). The possibility that aqueous solvent has limited access to key structural or catalytic regions of the protein becomes even more attractive (vide infra) in view of the differential stimulation of enzyme activity by dissociable cations. While, as suggested by the thermal denaturation data (Figure 6), it is possible that tightly bound cation has only a structural role, it would be interesting to identify its ligands and to determine its proximity to the HMG-CoA binding site. The ability to populate the tight cation site with Cu^{2+} will facilitate a spectroscopic approach to these issues. The presence in HMG-CoA lyases of conserved –HXH– and –CXXXC– sequences is interesting and suggests potential cation ligands.

In investigating the requirement for a dissociable cation, it has been observed that the activator constant (K_a) for Mg^{2+} is 3 orders of magnitude greater than the K_a for Mn^{2+} (Table III). This discrepancy, which is considerably larger than the usual differences observed between the binding constants of Mg^{2+} and Mn^{2+} to small molecules, may have structural implications. Similar differences between Mn^{2+} and Mg^{2+} binding to other enzyme systems have been reported (Lee and Nowak, 1992a,b; Denton and Ginsburg, 1969). Sussman and Weinstein (1989) have investigated the marked discrimination that proteins may exhibit in binding cations of different ionic radii. Their study suggests that Mn^{2+} may preferentially be stabilized in a hydrophobic binding pocket within the HMG-CoA lyase active site. Mg^{2+}, which exhibits a strong aqueous solvation preference, would be expected to bind much less tightly in such a model. For enolase, depending on pH, K_m for Mg^{2+} binding was up to a factor of 100 greater than the K_m for Mn^{2+}. In this case, independent binding sites for these metals have been proposed, namely, catalytic sites, conformational sites, and inhibitor sites (Lee and Nowak, 1992a). Mn^{2+} has been shown to bind to the catalytic site and Mg^{2+} to occupy the conformational site (Lee and Nowak, 1992a). In the case of HMG-CoA lyase, Mn^{2+} supports enzyme activity at only about 40% of the optimal Mg^{2+}-supported level. The stimulatory effect of manganese is observed up to a concentration of about 100 μM; at higher concentrations, an inhibitory effect is observed. Magnesium shows a stimulatory effect only at millimolar concentrations (Table III). Based on these observations, it is not unreasonable to postulate the existence of multiple independent roles for cations in the HMG-CoA lyase reaction. Additional experiments are planned to evaluate whether this hypothesis, or the explanation offered above concerning preferences for hydrophobic/hydrophilic binding pockets, provides the most plausible explanation for the enzyme's discrimination between Mn^{2+} and Mg^{2+}.

In conclusion, the recombinant form of *P. mevalonii* HMG-CoA lyase represents a useful experimental model. Availability of homogeneous protein in substantial amounts has facilitated work on enzyme–cation interactions and will expedite more extensive structure/function investigation by spectroscopic, protein chemistry, and protein engineering approaches. The data that result from such experiments are likely to take on additional significance as studies on the structural basis for human HMG-CoA lyase deficiencies begin to explain the documented (Gibson et al., 1988) physiological consequences of the inability to metabolize HMG-CoA.

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REFERENCES

Anderson, D. H., & Rodwell, V. W. (1989) *J. Bacteriol. 171*, 6468–6472.

Bachawat, B. K., Robinson, W. G., & Coon, M. J. (1955) *J. Biol. Chem. 216*, 727–736.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Denton, M. D., & Ginsburg, A. (1969) *Biochemistry 8*, 1714–1725.
Dumas, D. P., Caldwell, S. R., Wild, J. R., & Raushel, F. M. (1989) *J. Biol. Chem. 264*, 19659–19665.
Faull, K. F., Bolton, P., Halpern, P., Hammond, J., Danks, D. M., Hahnel, R., Wilkinson, S. P., Wysocki, S. J., & Masters, P. L. (1976) *N. Engl. J. Med. 294*, 1013.
Gibson, K. M., Breuer, J., & Nyhan, W. L. (1988) *Eur. J. Pediatr. 148*, 180–186.
Goldfarb, S., & Pitot, H. C. (1971) *J. Lipid Res. 12*, 512–515.
Higgins, M. J. P., Kornblatt, J. A., & Rudney, H. (1972) In *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. VII, pp 432–434, Academic Press, New York.
Hyde, J. S., Jesmanowicz, A., Ratke, J., and Antholine, W. E. (1992) *J. Magn. Reson. 96*, 1–13.
Knappe, J. (1963) In Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.) p 445–448, Verlag Chemie, Academic Press, New York.
Kramer, P. R., & Miziorko, H. M. (1980) *J. Biol. Chem. 255*, 11023–11028.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lee, B. H., & Nowak, T. (1992a) *Biochemistry 31*, 2165–2171.
Lee, M. E., & Nowak, T. (1992b) *Biochemistry 31*, 2172–2180.
Lynen, F., Henning, U., Bublitz, C., Sorbo, B., & Kroplin-Rueff, L. (1958) Biochem. Z. *330*, 269–295.
Mildvan, A. S., & Cohn, M. (1965) *J. Biol. Chem. 240*, 238–246.
Miziorko, H. M. (1985) *Fed. Proc. 44*, 1413.
Omburo, G. A., Kuo, J. M., Mullins, L. S., & Raushel, F. M. (1992) *J. Biol. Chem. 267*, 13278–13283.
Ozand, P. T., al Ageel, A., Ascon, G., Brismar, J., Thomas, E., & Gleispach, H. (1991) *J. Inherited Metab. Dis. 14*, 174–188.
Peisach, J., & Blumberg, W. (1974) *Arch. Biochem. Biophys. 165*, 691–708.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Robinson, A. M., & Williams, D. H. (1980) *Physiol. Rev. 60*, 143–187.
Scher, D. S., & Rodwell, V. W. (1989) *Biochim. Biophys. Acta 1003*, 321–326.
Simon, E. J., & Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.
Stegink, L., & Coon, M. J. (1968) *J. Biol. Chem. 243*, 5272–5279.
Sussman, F., & Weinstein, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7880–7884.

Registry No. HMG-CoA lyase, 9030-83-5; Mg^{2+}, 7439-95-4; Mn^{2+}, 7439-96-5; glycerol, 56-81-5; *o*-phenanthroline, 66-71-7; copper, 7440-50-8.