Pseudomonas mevalonii 3-Hydroxy-3-methylglutaryl-CoA Lyase

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[15] *Pseudomonas mevalonii* 3-Hydroxy-3-methylglutaryl-CoA Lyase

By HENRY M. MIZIORKO and CHAKRAVARTHY NARASIMHAN

Introduction

$$\text{3-Hydroxy-3-methylglutaryl-CoA} \xrightarrow{M^{2+}} \text{acetoacetate} + \text{acetyl-CoA}$$

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) cleavage is a divalent cation-dependent irreversible reaction.[1] It has been well established to occur in the ketogenic tissues (e.g., liver, kidney) of animals, providing a freely soluble lipid-derived metabolic fuel.[2] Several bacterial DNA sequences encoding HMG-CoA lyase have been reported.[3,4] The requirement for this enzymatic reaction in prokaryotes has been most convincingly explained[5] in the case of the enzyme from *Pseudomonas mevalonii.* In this bacterium, which utilizes mevalonic acid as a carbon source, the production of acetyl-CoA by HMG-CoA lyase supports a variety of biosynthetic pathways. The isolated enzyme has demonstrated utility in preparation of (*R*)-HMG-CoA, a potent inhibitor of HMG-CoA reductase, because of its ability to quantitatively deplete the physiologically active metabolite, (*S*)-HMG-CoA, from the mixed isomers produced by chemical synthesis of HMG-CoA.[6] In addition, the recombinant form of *P. mevalonii* HMG-CoA lyase, as expressed and isolated from *Escherichia coli,* represents the protein first used in studies that identified components of the enzyme active site.[7] For mechanistic or protein chemistry work that must be performed in the absence of high levels of reducing compounds [e.g., dithiothreitol (DTT), mercaptoethanol], the preferred enzyme would be bacterial HMG-CoA lyase.

The procedure described here for preparation of recombinant *P. mevalonii* HMG-CoA lyase represents straightforward methodology that has been routinely employed for preparation of both wild-type[8] and mutant

[1] L. Stegink and M. J. Coon, *J. Biol. Chem.* **243,** 5272 (1968).
[2] A. M. Robinson and D. H. Williams, *Physiol. Rev.* **60,** 143 (1980).
[3] D. H. Anderson and V. W. Rodwell, *J. Bacteriol.* **171,** 6468 (1989).
[4] M. Baltscheffsky, M. Brosche, T. Hultman, L. Lundvik, P. Nyren, Y. Sakai-Nore, A. Severin, and A. Strid, *Biochim. Biophys. Acta* **1337,** 113 (1997).
[5] D. S. Scher and V. W. Rodwell, *Biochim. Biophys. Acta* **1003,** 321 (1989).
[6] K. M. Bischoff and V. W. Rodwell, *Biochem. Med. Metab. Biol.* **48,** 149 (1992).
[7] P. W. Hruz, C. Narasimhan, and H. M. Miziorko, *Biochemistry* **31,** 6842 (1992).
[8] C. Narasimhan and H. M. Miziorko, *Biochemistry* **31,** 11224 (1992).

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forms[9] of the enzyme. This strategy supports isolation of significant amounts of high specific activity enzyme with good homogeneity, assuming that robust expression of soluble, active enzyme has been achieved during bacterial propagation and that enzyme stability is maintained during protein isolation. Preparation of the recombinant protein from *E. coli* in which expression has been suboptimal produces enzyme of reasonably high purity and specific activity that is useful for many experimental applications; yield, however, will be substantially lower. Experimental details that are important to optimizing expression are included in the protocol described below.

Assay Methods

Principle

HMG-CoA cleavage may be followed spectrophotometrically by using coupled enzyme assays that measure production of either of the two reaction products, acetyl-CoA or acetoacetate. Alternatively, the reaction may be followed with [^{14}C]HMG-CoA, estimating substrate cleavage by measuring conversion of the original acid-stable radioactivity to a volatile radiolabeled product. Different experimental situations have demanded use of each of these assays.

Citrate Synthase-Coupled Assay of HMG-CoA Lyase Activity

Reagents

Tris-HCl (pH 8.2), 1.0 *M*
$MgCl_2$, 1.0 *M*
DTT, 100 m*M*
NADH, 5 m*M*
NAD, 30 m*M*
L-Malate, 50 m*M* (neutralized as potassium salt)
HMG-CoA (pH 4.5), 6 m*M* [synthetic HMG-CoA is a mixture of (*R,S*)-isomers]
Citrate synthase (pig heart), 215 U/mg
Malate dehydrogenase (pig heart), 900 U/mg

Procedure. This spectrophotometric assay represents a modification of the method of Stegink and Coon[1] in which acetyl-CoA, produced along with acetoacetate on HMG-CoA cleavage, is coupled to a citrate synthase assay. Reaction of acetyl-CoA to produce citrate requires oxaloacetate, generated in the cuvette by malate dehydrogenase-catalyzed oxidation of

[9] C. Narasimhan, J. R. Roberts, and H. M. Miziorko, *Biochemistry* **34**, 9930 (1995).

malate with the reduction of NAD^+ and the resulting increase in $A_{340\,nm}$. The rate of NAD^+ reduction is proportional to the amount of HMG-CoA lyase added to a limiting value of 1.5 ΔA/min and linear until a total absorbance increase of >0.4 has occurred. The complete reaction mixture (1.0 ml) contains 200 μmol of Tris-HCl (pH 8.2), 10 μmol of $MgCl_2$, 1.5 μmol of NAD^+, 0.05 μmol of NADH, 5.0 μmol of DTT, 2.5 μmol of L-malate, malate dehydrogenase (9 U), citrate synthase (4 U), and the sample containing HMG-CoA lyase. After the mixture has been incubated at 30° and a stable baseline recorded, HMG-CoA (0.12 μmol) is added and the rate of increase in $A_{340\,nm}$ is measured. An extinction coefficient of $6.2 \times 10^3\ M^{-1}$ is used to quantitate product acetyl-CoA formation. This assay is used routinely for measuring HMG-CoA lyase activity at stages of enzyme isolation where NADH oxidase contaminants do not require significant correction of the measured rate of absorbance increase. The small volumes of *E. coli* extracts needed to measure activity of highly overexpressed HMG-CoA lyase do not cause serious interference. This assay may be unsuitable for inhibition studies that involve acyl-CoA analogs that also significantly inhibit citrate synthase.

β-Hydroxybutyrate Dehydrogenase-Coupled Assay of HMG-CoA Lyase Activity

Reagents

Tris-HCl (pH 8.2), 1.0 *M*
$MgCl_2$, 1.0 *M*
DTT, 100 m*M*
NADH, 5 m*M*
HMG-CoA (pH 4.5), 6 m*M*
β-Hydroxybutyrate dehydrogenase (*Rhodopseudomonas sphaeroides*), 36 U/ml

Procedure. In this spectrophotometric assay,[10] the acetoacetate produced along with acetyl-CoA on HMG-CoA cleavage is coupled to the hydroxybutyrate dehydrogenase assay.[11] Tris-HCl (pH 8.2, 100 μmol), NADH (0.1 μmol), $MgCl_2$ (5.0 μmol),* DTT (2.0 μmol), β-hydroxybutyrate dehydrogenase (0.5 IU), and the sample containing HMG-CoA lyase are added to a 500-μl assay mixture and allowed to incubate at 30° for 10 min, during which time any baseline drift in $A_{340\,nm}$ may be observed. HMG-CoA (0.06 μmol) is added and the rate of decrease in $A_{340\,nm}$ is measured.

[10] P. W. Hruz, V. E. Anderson, and H. M. Miziorko, *Biochim. Biophys. Acta* **1162,** 149 (1993).
[11] M. N. Berry, *Biochim. Biophys. Acta* **92,** 156 (1964).
* $MnCl_2$ (0.4–0.6 μmol) can also be used as the activator divalent cation in this assay.[10]

An extinction coefficient of $6.2 \times 10^3\ M^{-1}$ is used to quantitate the production of acetoacetate. This assay is used to measure activity of alternative substrates that produce products that do not efficiently couple to citrate synthase or when measuring HMG-CoA lyase activity in the presence of acyl-CoA analogs that inhibit citrate synthase.

Radioactive Assay of HMG-CoA Lyase Activity

Reagents

Tris-HCl (pH 8.2), 1.0 *M*
$MgCl_2$, 1.0 *M*
DTT, 100 m*M*
[^{14}C]HMG-CoA (pH 4.5), 2.4 m*M* (~3 mCi/mmol)

Procedure. Improved sensitivity, required when sample activity is limited (e.g., in the assay of a catalytically impaired mutant HMG-CoA lyase), is afforded by measuring the cleavage of [^{14}C]HMG-CoA by the method of Clinkenbeard *et al.*[12] Although either commercially available, chemically synthesized [3-^{14}C]HMG-CoA or enzymatically synthesized [5-^{14}C]HMG-CoA is suitable for the assay, the chemically synthesized substrate is a mixture of (*R,S*)-isomers and only 50% of the total radioactivity will be volatilized on exhaustion of this substrate. The reaction mixture (0.2 ml) contains Tris-HCl (pH 8.2, 40 μmol), $MgCl_2$ (2 μmol), DTT (2 μmol), and the sample containing HMG-CoA lyase. The reaction, performed at 30°, is initiated by addition of [^{14}C]HMG-CoA (24 nmol; 6000 dpm/nmol). At various time points, aliquots (40 μl) are removed, pipetted into glass shell vials (15 × 45 mm; flat bottomed), and acidified with 6 *N* HCl (0.1 ml) prior to heating to dryness at 95°, using an aluminum heating block [LabLine (Melrose Park, IL) 2073 or equivalent] in a fume hood. The acid-stable radioactivity attributable to [^{14}C]HMG from unreacted substrate is determined by liquid scintillation counting. Depletion of acid-stable radioactivity is a measure of enzymatic cleavage of substrate to form a volatile radiolabeled product ([3-^{14}C]acetoacetate from [3-^{14}C]HMG-CoA; [1-^{14}C]acetoacetate from [5-^{14}C]HMC-CoA).

Units. A unit of enzymatic activity is defined as the amount of enzyme necessary to convert 1 μmol of HMG-CoA to products acetyl-CoA and acetoacetate in 1 min under the conditions described. Specific activity is expressed in units per milligram of protein. In the published reports from which these methods have been compiled, protein concentration has been

[12] K. D. Clinkenbeard, W. D. Reed, R. A. Mooney, and M. D. Lane, *J. Biol. Chem.* **250,** 3108 (1975).

determined by the Bradford method,[13] with bovine serum albumin used as a calibration standard.

Expression of Active HMG-CoA Lyase

Escherichia coli BL21 (DE3) transformed with the expression vector pT7-2600 containing the *P. mevalonii* HMG-CoA lyase gene (*mvaB*) is a generous gift of V. Rodwell (Purdue University, Lafayette, IN). The fraction of total *E. coli* BL21 (DE3) protein represented by the *mvaB* gene product is a function of bacterial growth conditions (namely, temperature).[8] For example, at 37° more than half of the total protein expressed is represented by HMG-CoA lyase. At 30 and 22°, 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 high at both 37 and 30°, the specific activities of enzyme in those fractions are comparatively low. On comparison of soluble extracts with the corresponding crude homogenates, it becomes apparent that lyase protein is largely insoluble when expressed under temperature conditions typical for bacterial propagation. Growth at 22°, however, markedly improves the expression of catalytically active enzyme. Most of the enzyme that has been expressed remains soluble after high-speed centrifugation of the crude extract. Therefore, the 22° growth condition is now routinely used to overexpress active *P. mevalonii* HMG-CoA lyase.

Purification of HMG-CoA Lyase

Bacterial Growth and Induction

Bacteria are grown in LB medium [10 g of Bacto tryptone (Difco, Detroit, MI), 5 g of Bacto yeast extract, and 5 g of NaCl per liter] at 22° in flasks shaken at 250 rpm. Growth is monitored by periodically measuring the optical density (OD) at 600 nm. Starter cultures (15 ml) are grown overnight in LB medium containing ampicillin (200 μg/ml). This overnight culture is diluted to 1.5 liters with LB medium containing ampicillin (200 μg/ml) and allowed to grow at 22° to an OD of 0.6. At this point, expression of HMG-CoA lyase is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* and growth continued until late-log phase (OD ~3.0). The cells are harvested by low-speed centrif-

[13] M. M. Bradford, *Anal. Biochem.* **72,** 248 (1976).

ugation (3000*g*, 10 min) at 4° and the pellets may be stored at −20° prior to cell lysis.

Bacteria are suspended in 50 ml of cold buffer that consists of 20 m*M* phosphate (pH 7.2), 1 m*M* EDTA, DNase I (10 μg/ml), RNase A (10 μg/ml), and 100 μ*M* phenylmethylsulfonyl fluoride (PMSF). The cells are lysed with a French pressure cell at 16,000 psi. This crude extract is centrifuged at 181,000*g* for 60 min and the resultant high-speed supernatant containing the active HMG-CoA lyase decanted and kept on ice. This high-speed supernatant is promptly brought to 40% $(NH_4)_2SO_4$ saturation by addition of the solid salt with slow stirring. The mixture is stirred slowly at 4° for an additional 4.5 hr. The precipitated protein is centrifuged at 12,100*g* for 20 min at 4°. After removing the supernatant from the pellet, the pellet is dissolved in a minimum volume of 10 m*M* phosphate buffer, pH 7.2, containing 20% (v/v) glycerol. At this point, the inclusion of glycerol in buffer is crucial to retention of enzyme activity. The dissolved ammonium sulfate fraction is desalted by rapidly passing the sample, divided into appropriately sized aliquots, over Sephadex G-50 centrifugal desalting columns[14] equilibrated with 10 m*M* phosphate buffer containing 20% (v/v) glycerol. The desalted sample is immediately applied to a Q-Sepharose Fast Flow anion-exchange column (1.5 × 45 cm) equilibrated with the desalting buffer. The bound material is eluted with a 10–100 m*M* phosphate gradient (1.0 liter), pH 7.2, containing 20% (v/v) glycerol. The protein peak containing the enzyme activity is pooled and concentrated with an Amicon (Danvers, MA) concentrator equipped with a PM30 membrane.

The pooled active fractions are essentially homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1). The purification steps are summarized in Table I. 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 high level of initial overexpression.

Characterization of *Pseudomonas Mevalonii* HMG-CoA Lyase

Enzyme Stability

The recombinant enzyme recovered from the Q-Sepharose anion-exchange column retains full activity for several months if stored at −80° in the phosphate buffer containing 20% (v/v) glycerol, pH 7.2. The recombinant enzyme exhibits, after early stages in the purification, a marked requirement for stabilizing agents such as glycerol or substrate. This situation

[14] H. S. Penefsky, *J. Biol. Chem.* **252,** 2891 (1977).

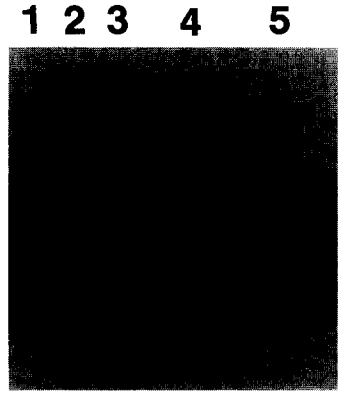

FIG. 1. SDS–PAGE of *P. mevalonii* HMG-CoA lyase at various stages of purification. Lane 1, molecular weight standards (14.4, 21.5, 31.0, 45.0, 66.2, and 97.4 kDa); lane 2, total bacterial cell extract; lane 3, high-speed supernatant obtained by centrifuging the extract at 181,000*g* for 60 min; lane 4, sample from ammonium sulfate precipitation (0–40% saturation); lane 5, HMG-CoA lyase after Q-Sepharose column chromatography. [Reprinted, with permission, from C. Narasimhan and H. M. Miziorko, *Biochemistry* **31,** 11224 (1992).]

contrasts sharply with the stability observed with the avian liver enzyme and can, in some cases, complicate experimental design. The bacterial enzyme is labile at ambient or higher temperatures. For example, even in the presence of 20% (v/v) glycerol, the enzyme loses 63% of its initial activity when incubated at ambient temperature over a period of 5 hr. Addition of 10 m*M* $MgCl_2$ to this sample does not improve the stability of

TABLE I
PURIFICATION OF RECOMBINANT *Pseudomonas mevalonii* HMG-CoA LYASE[a]

Purification step	Total units[b]	Total protein[c] (mg)	Specific activity (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

[a] Reprinted, with permission, from C. Narasimhan and H. M. Miziorko, *Biochemistry* **31,** 11224 (1992).
[b] Activity measured by the citrate synthase-coupled spectrophotometric assay.
[c] Protein measured by the Bradford dye-binding assay, employing bovine serum albumin as the calibration standard.

the enzyme at ambient temperature. The presence of 0.2 m*M* HMG-CoA helps, however, to maintain the activity (>90% after 8 hr) of the enzyme at 22°.

The recombinant *P. mevalonii* enzyme is attractive in that it does not exhibit the stringent requirement for exogenous thiol that characterizes the avian enzyme.[15,16] When isolated in buffers that contain no exogenous thiol, enzyme shows only about a twofold enhancement in activity when 5 m*M* DTT is added. Conversely, after separating DTT-preincubated enzyme from reducing agents by centrifugal gel filtration, about 50% of the optimal activity is retained. This property promises to facilitate physical and protein chemistry studies that would otherwise be hindered by the requirement to either maintain an elevated level of exogenous thiol or work under anaerobic conditions.

Molecular Properties

The native molecular weight and quaternary structure of recombinant *P. mevalonii* HMG-CoA lyase remain to be resolved. The enzyme has been subjected to gel filtration on a Superose 12 column (12 mm × 30 cm; Pharmacia, Uppsala, Sweden) equilibrated with 50 m*M* Tris, pH 8.00, containing 0.1 *M* NaCl, 10% (v/v) glycerol, and calibrated with appropriate molecular weight markers. Under these conditions, the native recombinant lyase, which elutes slightly before a carbonic anhydrase marker, exhibits a molecular weight approximating that reported for the HMG-CoA lyase protomer (Table II; 27 kDa,[15] 32 kDa[5]) and measured for the recombinant enzyme under denaturing SDS–PAGE conditions (Fig. 1). However, in subsequent spin-labeling studies,[9,17] a nitroxide-containing substrate analog has been demonstrated to bind the protein, exhibiting a rotational correlation time of 20 nsec. If enzyme is assumed to be spherical, a Stokes radius of 27 Å would be predicted from this rotational correlation time. This value agrees well with the estimate of 28.2 Å reported by Scher and Rodwell.[5] Using a partial specific volume of 0.75 ml/g, a protein with the predicted Strokes radius of 27 Å would have a molecular mass of 64 kDa, in good agreement with the value anticipated for a dimeric enzyme. On the basis of this estimate, the bacterial lyase would resemble the enzyme isolated from avian liver and the recombinant human enzyme, which have clearly been demonstrated by cross-linking studies to exist as dimers.

[15] P. R. Kramer and H. M. Miziorko, *J. Biol. Chem.* **255,** 11023 (1980).
[16] P. W. Hruz and H. M. Miziorko, *Protein Sci.* **1,** 1144 (1992).
[17] C. Narasimhan, W. E. Antholine, and H. M. Miziorko, *Arch. Biochem. Biophys.* **312,** 467 (1994).

TABLE II
PROPERTIES OF HMG-CoA LYASES[a]

Property	*P. mevalonii* lyase[b]		Avian lyase[c]
	Homologously expressed	Recombinant	
Reduced thiol requirement in assay	Marked dependency	Twofold 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	6[d]
K_a (Mg^{2+}, μM)	—	6,900	56[d]
Subunit	32,000[e]	32,000	27,000
Molecular mass (Da)	31,600[f]		

[a] Reprinted, with permission, from C. Narasimhan and H. M. Miziorko, *Biochemistry* **31,** 11224 (1992).
[b] For the homologously expressed *P. mevalonii* lyase, the data are from Scher and Rodwell[5]; data for the recombinant lyase are from Narasimhan and Miziorko.[8]
[c] Data for the avian lyase are from Kramer and Miziorko.[15]
[d] Apparent K_m values, estimated at saturating HMG-CoA concentration, are from Hruz *et al.*[10]
[e] Molecular weight determined by SDS–PAGE.
[f] From the cDNA sequence (Anderson and Rodwell[3]).

Catalytic Properties

The specific activity observed for the homogeneous recombinant protein is 70 U/mg, a value severalfold higher than reported for the homologously expressed *P. mevalonii* enzyme.[5] The presence of multiple protein components in that preparation may partially account for this discrepancy. In contrast, the avian[15] and human[18] enzymes exhibit specific activities approximately fivefold higher than measured with the prokaryotic enzyme. While such a difference may reflect actual higher catalytic efficiency in the eukaryotic enzymes, 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 II), 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

[18] J. R. Roberts, C. Narasimhan, P. W. Hruz, G. A. Mitchell, and H. M. Miziorko, *J. Biol. Chem.* **269,** 17841 (1994).

one isomer from an (*R,S*)-mixture of chemically prepared HMG-CoA.[1] As discussed by Higgins *et al.*,[19] lyase uses the same isomer that is metabolized by HMG-CoA reductase to form (*R*)-mevalonate. Because the reductase reaction does not affect the stereochemistry at C-3 and because the stereochemistry at C-3 of (*R*)-mevalonate is equivalent to that at C-3 of (*S*)-HMG-CoA, it follows that (*S*)-HMG-CoA is the substrate for reductase as well as lyase. This analysis has more recently been experimentally validated by Rodwell and colleagues.[5]

Isolated Enzyme Containing Tightly Bound Cations

Inhibition by Metal Chelators. Recombinant *P. mevalonii* HMG-CoA lyase is sensitive to metal chelators. On a 4-hr incubation with *o*-phenanthroline (4 m*M*), enzyme activity gradually decreases to 43% of its initial activity. That inhibition is attributable to chelation of a tightly bound cation is confirmed by control experiments that demonstrate the nonchelating isomer, *m*-phenanthroline, to be without effect. Attempts to restore enzyme activity by resupplementing the enzyme with exogenous cation after *o*-phenanthroline treatment have not been successful. Incubation of recombinant HMG-CoA lyase with 0.2 m*M* EDTA for up to 4 hr does not result in any significant inhibition of enzyme activity.

Metal Analyses. Enzyme purified after expression in *E. coli* grown in standard LB broth has been analyzed by atomic absorption and electron paramagnetic resonance (EPR) methodologies. Copper is the predominant tightly bound cation identified, although zinc and iron are also present at lower levels. The concentrations of cations associated with the isolated recombinant lyase do not approach the level expected for stoichiometric binding to enzyme protomer. A survey of 20 metals by inductively coupled plasma emission spectroscopy failed to detect significant levels of other cations. However, when growth medium is supplemented with 1 m*M* divalent copper ($CuSO_4$) prior to induction of HMG-CoA lyase synthesis, the enzyme subsequently isolated shows an order of magnitude increase in metal content, with bound cation approaching stoichiometric levels with respect to enzyme subunits. At this elevated level of tightly bound cation, there is good agreement between atomic absorption and EPR estimates, suggesting that all the bound copper is the divalent, paramagnetic species. Furthermore, the measured EPR parameters ($A_{\parallel} = 152$ G; $g_{\parallel} = 2.28$) suggest that enzyme liganding creates a type II copper center[20] that may involve protein nitrogen ligand(s). These observations make the possibility of adventitious cation binding appear unlikely.

[19] M. J. P. Higgins, J. A. Kornblatt, and H. Rudney, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd Ed., Vol. VII, pp. 432–434. Academic Press, New York, 1972.

[20] J. Peisach and W. Blumberg, *Arch. Biochem. Biophys.* **165,** 691 (1974).

On the basis of 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 observation that enzyme low in copper content is more unstable to thermal denaturation than enzyme that has been copper enriched. Clearly, 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 any precise role can be assigned.

Enzyme Activation by Dissociable Divalent Cations

While added Zn^{2+} or Cu^{2+} shows no stimulatory effects on activity of the isolated enzyme, micromolar concentrations of Mn^{2+} and millimolar concentrations of Mg^{2+} markedly stimulate enzyme activity. In the case of HMG-CoA lyase, Mn^{2+} supports enzyme activity at only about 40% of the optimal Mg^{2+}-supported level. Concentration dependence studies[21] for each of these cations have been performed[8] to determine activator constants (K_a values) of 2 μM and 6.8 mM, respectively. It has also been observed that Mn^{2+} at concentrations higher than 100 μM inhibits the enzyme activity while Mg^{2+} at concentrations lower than 300 μM has virtually 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 by induction in copper-supplemented LB broth.

The three orders of magnitude difference between the activator constants, which is much larger than the difference usually observed between 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.[22-24] Sussman and Weinstein[25] 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.

Acknowledgment

Pseudomonas mevalonii HMG-CoA lyase studies performed in the author's laboratory have been supported by NIH DK-21491.

[21] A. S. Mildvan and M. Cohn, *J. Biol. Chem.* **240,** 238 (1965).
[22] B. H. Lee and T. Nowak, *Biochemistry* **31,** 2165 (1992).
[23] M. E. Lee and T. Nowak, *Biochemistry* **31,** 2172 (1992).
[24] M. D. Denton and A. Ginsburg, *Biochemistry* **8,** 1714 (1969).
[25] F. Sussman and H. Weinstein, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7880 (1989).