Investigation of Conserved Acidic Residues in 3-Hydroxy-3-methylglutaryl-CoA Lyase

IMPLICATIONS FOR HUMAN DISEASE AND FOR FUNCTIONAL ROLES IN A FAMILY OF RELATED PROTEINS*

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3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase catalyzes the divalent cation-dependent cleavage of HMG-CoA to form acetyl-CoA and acetoacetate. In metal-dependent aldol and Claisen reactions, acidic residues often function either as cation ligands or as participants in general acid/base catalysis. Site-directed mutagenesis was used to produce conservative substitutions for the conserved acidic residues Glu-37, Asp-42, Glu-72, Asp-204, Glu-279, and Asp-280. HMG-CoA lyase deficiency results from a human mutation that substitutes lysine for glutamate 279. The E279K mutation has also been engineered; expression in Escherichia coli produces an unstable protein. Substitution of alanine for glutamate 279 produces a protein that is sufficiently stable for isolation and retains substantial catalytic activity. However, thermal inactivation experiments demonstrate that E279A is much less stable than wild-type enzyme. HMG-CoA lyase deficiency also results from mutations at aspartate 42. Substitutions that eliminate a carboxyl group at residue 42 perturb cation binding and substantially lower catalytic efficiency (10^4-10^5-fold decreases in specific activity for D42A, D42G, or D42H versus wild-type). Substitutions of alanine for the other conserved acidic residues indicate the importance of glutamate 72. E72A exhibits a 200-fold decrease in k_{cat} and >10^6-fold decrease in k_{cat}/k_{m} for activator cation (26-fold for Mg^{2+}; >200-fold for Mn^{2+}). Similar, but less pronounced, effects are measured for the D204A mutant. E72A and D204A mutant proteins both bind stoichiometric amounts of Mn^{2+}, but D204A exhibits only a 2-fold inflation in K_D for Mn^{2+}, whereas E72A exhibits a 12-fold inflation in K_D (23 μM) in comparison with wild-type enzyme (K_D = 1.9 μM). Acidic residues corresponding to HMG-CoA lyase Asp-42 and Glu-72 are conserved in the HMG-CoA lyase protein family, which includes proteins that utilize acetyl-CoA in aldol condensations. These related reactions may require an activator cation that binds to the corresponding acidic residues in this protein family.

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HMG-CoA\(^{1}\) lyase catalyzes the cleavage of HMG-CoA into acetyl-CoA and acetoacetate (1) by a proposed general acid/base mechanism (Scheme 1). Enzyme activity is absolutely dependent on the presence of a divalent cation (e.g. Mg\(^{2+}\), Mn\(^{2+}\)) and is stimulated by reducing agents (e.g. DTT). Cleavage of HMG-CoA is involved in the generation of ketone bodies to maintain the energy requirements of non-hepatic tissues (2) as well as the terminal step of leucine catabolism (3).

HMG-CoA lyase is a member of the HMG-CoA lyase family of proteins that catalyze C-C cleavage/condensation reactions. Members of the HMG-CoA lyase family include homocitrinate synthase and isopropylmalate synthase; both catalyze the aldol condensation of acetyl-CoA with either α-ketoglutarate or α-ketoisovalerate, forming homocitrinate and α-isopropylmalate, respectively (4). A molecular structure has not yet been determined for any family member, and few details regarding the enzyme reaction chemistry have been reported for these family members. Therefore, studies of HMG-CoA lyase may improve our basic understanding of structure/function relationships among members of the HMG-CoA lyase family.

The importance of the ketogenic cycle is underscored in hereditary HMG-CoA lyase deficiency, which can result in hypoketotic hypoglycemia and a marked increase in serum levels of several organic acids. Uncontrolled, HMG-CoA lyase deficiency can result in mental retardation and episodes of seizures and coma (5). Several mutations in the HMG-CoA lyase gene correlating with deficiency have been identified (5), including the missense mutations: H233R (6), R41Q, D42E, D42H, D42G (7), and most recently, E279K (8). Modeling of human mutations using a recombinant expression system would allow for the more detailed characterization of the mutant enzyme, testing the correlation of the mutation with human disease and potentially identifying active site residues.

As noted above, HMG-CoA lyase activity requires the presence of a divalent cation, such as Mg\(^{2+}\) or Mn\(^{2+}\). Previous experiments have suggested that the divalent cation and substrate form a ternary complex with the enzyme. The metal has been proposed to ligate to oxygen atoms of the 1-carboxyl and 3-hydroxyl group of HMG-CoA, facilitating enolization and thereby, stabilization, of a carbamion intermediate (9). Two or more amino acid side chains are expected to function directly or indirectly in the octahedral coordination of the cation activator, but to date, only one amino acid, histidine 235, has been implicated as an enzyme ligand to the metal (10).

Cysteine 266 and histidine 233 have been identified as catalytic amino acids based on mutagenic and kinetic analyses (6).
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TABLE I

| Enzyme       | Specific activity | Butyryl-CoA modification stoichiometry |
|--------------|------------------|----------------------------------------|
| Wild-type    | 160              | 0.88 ± 0.01                            |
| D37D         | 140              |                                        |
| D42A         | 0.001            | 0.81 ± 0.05                            |
| D42E         | 18               | 0.90 ± 0.08                            |
| D42G         | 0.012            | 0.79 ± 0.02                            |
| D42H         | <0.01            | 1.1 ± 0.01                             |
| E27D         | 0.28             | 0.99 ± 0.07                            |
| E72A         | 0.14             | 1.0 ± 0.08                             |
| D204A        | 8                | 0.94 ± 0.03                            |
| E279A        | 160              |                                        |
| D280A        | 160              |                                        |

Fig. 1. Sequence alignment of the HMG-CoA lyase protein family. Acidic residues in HMG-CoA lyase were targeted for mutagenesis based on alignment of HMG-CoA lyase (HMGCoA lyase) amino acid sequences with homocitrate synthase (HCS), isopropylmalate synthase (IMS), 4-hydroxy-2-oxovalerate aldolase (HOA), and oxaloacetate decarboxylase α chain (OAA). Only a representative number of sequences have been shown for each enzyme. All sequences were obtained from public databases. Alignment was generated using the Pfam protein family database (26). Acidic residues that are invariant among HMG-CoA lyases and homologous in the HMG-CoA lyase protein family are in bold and underlined. Numbers appearing above the sequence alignment correspond to the amino acid numbering of human HMG-CoA lyase. Sequences from the following organisms and accession numbers are included: Homo sapiens, P35914; Pseudomonas mevalonii, P13703; Caenorhabditis elegans, AAJ73908; Streptomyces coelicolor, CAB57215; Arbidibacteriellia italiana, AAI5569; Bacillus subtilis, D69893; Pseudomonas putida, AAA5569; Homo sapiens, H11005; Lactobacillus casei, Q02151; E. coli, P09151; Buchnera aphidicola, P46571; Azobacter chroococcum, P23122; Rhodobacter sphaeroides, Q01181; Burkholderia cepacia, P51015; Pseudomonas putida, P51017; Klebsiella pneumoniae, P13187.

72, and aspartate 204 (Fig. 1). This alignment was generated using the Pfam data bank (26). Therefore, glutamate 37, glutamate 72, and aspartate 204 have also been investigated by mutagenesis approaches. The human mutations D42E, D42G, D42H, and E279K were modeled by producing recombinant forms of these proteins; other substitutions were produced as indicated below, a variety of substitutions (including the clinically detected mutations) can be engineered for this aspartate and the resulting mutant proteins remain sufficiently stable for characterization in some detail. As indicated in Table I, specific activity of any particular aspartate 42 mutant is strongly dependent on the nature of the substitution; any replacement of the acidic side chain results in a marked decrease in specific activity.

Expression and Isolation of Mutant HMG-CoA Lyase Proteins—With the exception of E279K and E37A, the mutant enzymes were expressed and purified using the protocol developed for wild-type mitochondrial HMG-CoA lyase (20). Specific activities of purified proteins are documented in Table I. As indicated in Fig. 2, the mutant enzymes that were subjected to detailed characterization were isolated in a highly purified form. In the case of either E279K or E37A, Western blot analysis detected protein in bacterial extracts but after subjecting purified E279A at 37 °C (Fig. 3) resulted in a significant decrease in measurable activity (t1/2 = 60 min), whereas comparable treatment of wild-type enzyme did not result in loss of activity. In fact, wild-type enzyme retained full activity after a 6-h incubation. Other HMG-CoA lyase mutants (e.g., E72A, D204A, and D280A) exhibit stability comparable with wild-type enzyme. On the basis of the results with E279K and E279A, the HMG-CoA lyase deficiency associated with mutation at this residue can be attributed to impaired stability of the enzyme.

Other reports of human HMG-CoA lyase deficiency (7) implicate highly conserved aspartate 42 as an important residue. In inherited human HMG-CoA lyase deficiency, the point mutations D42E, D42G, and D42H have been identified. As indicated below, a variety of substitutions (including the clinically detected mutations) can be engineered for this aspartate and the resulting mutant proteins remain sufficiently stable for characterization in some detail. As indicated in Table I, specific activity of any particular aspartate 42 mutant is strongly dependent on the nature of the substitution; any replacement of the acidic side chain results in a marked decrease in specific activity.

Substantial decreases in specific activity are also observed (Table I) upon substitutions that replace the acidic side chain of either glutamate 72 or aspartate 204, both of which are highly conserved in the HMG-CoA lyase family of enzymes. To test whether those mutants that exhibit large decreases in catalytic activity (e.g., D42A, D42G, D42H, D42N, E72A, D204A, D280A) retain significant structural integrity, modification of these proteins with the affinity label, 2-butyryl-CoA, was performed. The modification stoichiometry for these low activity mutants is not substantially different from the value measured for wild-type enzyme (Table I), suggesting that the active site of each of the mutants approximates the structure of wild-type enzyme and that these mutants are not significantly structurally perturbed. Thus, substantial differences in kinetic or equilibrium binding characteristics that these mutant enzymes exhibit can be interpreted without the concern that a major structural pertur-
Enzymological Characterization of Mutant HMG-CoA Lyase Proteins—More detailed analysis (Table II) of the kinetic properties of E37D, E279A, and D280A mutants suggested little difference in $V_{\text{max}}$ or $K_m$,HMG-CoA values in comparison with wild-type enzyme. For the clinically significant D42E mutant, there is little change in the apparent $K_m$,HMG-CoA but almost an order of magnitude decrease in $V_{\text{max}}$ when the amide side chain of asparagine 42, glutamate 72, and aspartate 204 mutants. Of those mutant HMG-CoA lyase enzymes that exhibit notable decreases in catalytic efficiency (and for which $K_m$ can be accurately estimated), only E72A is characterized by a substantial (1 order of magnitude) decrease in apparent $K_m$,HMG-CoA. To test whether the $K_m$ perturbation reflected a change in substrate affinity, the $K_s$ for the competitive inhibitor, 3-hydroxyglutaryl-CoA, was measured for this mutant (Fig. 4). This $K_s$ which should closely correlate with the intrinsic affinity of the inhibitor, is inflated for E72A (Table II) by approximately 1 order of magnitude (in good agreement with the magnitude of the $K_m$ effect) suggesting an active site function for the carboxyl side chain of glutamate 72.

Analysis of kinetic and equilibrium binding parameters has been performed to evaluate the interaction of divalent cation with the catalytically impaired mutants (Table III). D204A exhibits inflation of the apparent $K_m$ for Mg$^{2+}$ and Mn$^{2+}$ of ~20- and 21-fold, respectively. Inflations in apparent $K_m$ values are observed for D42E (25- and 17-fold) and D42N (80- and 19-fold) mutants. E72A also exhibits somewhat larger effects. For the E72A mutant, increases in the apparent $K_m$ values for Mg$^{2+}$ and Mn$^{2+}$ are ~26- and 237-fold, respectively.

The combination of catalytic and binding terms in $K_m$ estimates can complicate comparisons between wild-type and mutant enzymes, especially when mutants exhibit large changes in catalytic efficiency. A more direct evaluation of the ability of wild-type or mutant HMG-CoA lyases to specifically form a binary enzyme-metal complex is afforded using electron spin resonance to measure Mn$^{2+}$ binding to enzyme. Results of such experiments were subjected to Scatchard analyses (Fig. 5). D204A retains the ability to form stoichiometric levels of a binary enzyme-metal complex (Fig. 5A) with a $K_D$ (3.5 μM) that represents only a 2-fold increase over the value measured for wild-type enzyme (Table III) and is consistent with the apparent $K_m$ for Mn$^{2+}$. In contrast, E72A forms stoichiometric levels of a binary enzyme-metal complex (Fig. 5B) but exhibits >12-fold inflation in the measured $K_D$ (23 μM; Table III). Inclusion of the substrate analog hydroxyglutaryl-CoA does not substantially change the binding stoichiometry or affinity measured for E72A. In contrast, Mn$^{2+}$ binding studies on D42N fail to detect a binary enzyme-metal complex; only when hydroxyglutaryl-CoA (1 mM) is included does Mn$^{2+}$ bind at stoichiometric levels (Table III). The D42G mutation associated with human inherited disease also affects cation binding; no binary enzyme-metal complex is detectable. As in the case of D42N, binding studies performed in the presence of hydroxyglutaryl-CoA (1 mM) detected stoichiometric binding of cation to D42G but the measured $K_D$ (27 μM) is substantially larger than when such an experiment is performed for D42N ($K_D = 3.2$ μM). The more conservatively substituted D42E mutant forms a Mn$^{2+}$ enzyme-Mn$^{2+}$ complex with stoichiometry and affinity values that are comparable with wild-type enzyme (Table III).

**DISCUSSION**

The correlation of metabolic disease or dysfunction with mutations in HMG-CoA lyase that reduce enzyme activity by 10-fold or more has suggested that substantial activity is required for humans to function well under metabolic stress that leads to increased lipid catabolism. In the case of the E279K mutation, transient expression studies (8) suggested a residual level of activity of ~2%, but no data were reported on enzyme protein levels or stability. Our results on the bacterial expression of E279K and E279A mutants suggest that altered stability, rather than involvement of Glu-279 in reaction chemistry accounts for lack of activity in these mutants. Thus, this region of the protein may be structurally sensitive, but there is no indication that it contributes to catalytic or regulatory sites of the enzyme. In contrast, mutations at aspartate 42 do not seem to substantially destabilize protein but instead influence the efficiency of the reaction. The random human mutations that correspond to D42H and D42G proteins do not destabilize protein but do correlate with extremely low (<0.01%) activity. The human D42E mutation does not impede production of stable purified protein with substantial (10%) activity (Tables I and II) under optimized assay conditions. Although no large change in substrate $K_m$ is apparent, a significant increase in $K_m$ for the
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Table II

| Enzyme        | V_max (units/mg) | K_m, HMG-CoA (µM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ µM⁻¹) | K_m, HG-CoA (µM) |
|---------------|------------------|-------------------|-------------|-----------------------|------------------|
| Wild-type     | 191 ± 10         | 48 ± 6            | 108         | 2.27                  | 340 ± 48         |
| E37D          | 152 ± 6          | 36 ± 6            | 86.2        | 2.39                  |                  |
| D42E          | 20 ± 1           | 32 ± 6            | 11.3        | 0.35                  |                  |
| D42N          | 0.33 ± 0.17      | 43 ± 7            | 0.187       | 0.0044                |                  |
| E72A          | 1.0 ± 0.07       | 477 ± 80          | 0.570       | 0.0012                | 3300 ± 181       |
| D204A         | 9.6 ± 5.5        | 166 ± 31          | 5.5         | 0.033                 |                  |
| E279A         | 163 ± 9          | 27 ± 5            | 92.4        | 3.38                  |                  |
| D280A         | 195 ± 10         | 17 ± 3            | 110.5       | 6.39                  |                  |

* K_m, HMG-CoA is an apparent value, determined in the presence of 10 mM Mg²⁺.

³-Hydroxyglutaryl-CoA (HG-CoA) is a competitive inhibitor of HMG-CoA lyase.

activation cation (Table III) together with intrinsically lower catalytic efficiency might account for the decreased activity of D42E in a physiological context. This possibility is supported by the observed failure of D42N and D42G to bind activator cation in the absence of a substrate/substrate analog and may account for the observation of low activity in the other clinically detected Asp-42 mutants. Limited characterization of the D42G mutant has determined a K_equil, of 27 µM (14-fold inflation versus wild-type). However, as with D42N, this required the inclusion of hydroxyglutaryl-CoA (1 mM) to detect metal binding to enzyme.

In contrast to the results for glutamate 279 and aspartate 280, which suggest no substantial catalytic or regulatory function for these residues, the data for mutations that eliminate other highly conserved acidic side chains suggest that some of these residues (most notably aspartate 42 and glutamate 72) map within the catalytic site. The inflation in the K_equil, E72A for the binary complex with cation suggests that the carboxyl side chain is one ligand to the cation. The K_equil, for cation binding is not altered in the presence of a substrate analog. However, the affinity for substrate or substrate analog, hydroxyglutaryl-CoA, is weakened. Together, the data suggest that the cation activator bridges the carboxyl side chain and the substrate. Because this carboxyl side chain is conserved in the various enzymes included in the HMG-CoA lyase family, the corresponding acidic residues may support similar functions. Typically, divalent cations such as Mg²⁺ or Mn²⁺ exhibit octahedral coordination, with two or more amino acid side chains ligating metal directly or indirectly (e.g. with a water molecule interposed). Therefore, although glutamate 72 has been implicated in such a function, other candidates need to be identified. D204A exhibits inflated K_m values for cation (measured kinetically under turnover conditions) but this mutant exhibits equilibrium binding of Mn²⁺ that is not much different from observations made with wild-type enzyme. Thus, functional assignment of aspartate 204 remains somewhat ambiguous.

In contrast, an active site role for aspartate 42 seems more apparent. Whereas glutamate substitution for aspartate does not have a crucial effect on activity or formation of a binary enzyme-cation complex, substitutions that eliminate a charged carboxylate side chain dramatically lower catalytic efficiency. Precedent suggests that magnitude of the effect on catalysis attributable to elimination of a cation ligand can range from 1 to 4 orders of magnitude. In the case of phosphoenolpyruvate mutase (27), alanine substitution of any of three different acidic residues that function as cation ligands decreases activity by 200-, 500-, and 8900-fold, depending on which acidic function is eliminated. For acetohydroxyacid synthase (28), substitution of asparagine for an aspartate that ligands to the cation required for the reaction decreases activity by only 7-fold. A similar asparagine substitution for either of the two different asparagines that ligate to the metal bound in xylose isomerase (29) lowers activity by 20–50-fold. Because of such a range of effects on catalysis, the impact of mutagenesis on K_m values for cation can be variable and direct equilibrium binding measurements become useful. In the case of D42N and D42G, replacement of the acidic side chain abolishes the ability to detect a binary enzyme-Mn²⁺ complex. Only in the presence of substrate analog can Mn²⁺ binding be detected. This observation is analogous to results for histidine 235 (10). Elimination of this imidazole side chain disrupted binary enzyme-cation complex formation; a weak complex could be measured in the presence of substrate analog. Previous results (9) have suggested that both HMG-CoA lyase and its substrate may provide ligands to cation. It seems possible that both aspartate 42 and histidine 235 are involved directly or indirectly in cation ligation, which becomes impaired when either of these side chains is replaced. In the presence of the substrate/analog, additional cation ligands are present (e.g. the C3 hydroxyl or C1 thioester carbonyl groups) and the ability to bind cation improves.

Precedent for cation liganding to carboxyl side chains as well as to substrate in reactions that involve C-C bond formation or cleavage is available for both cation-dependent (class II) aldolases (15) and Claisen condensation reactions (13, 14). In particular, results for the analogous malate synthase reaction seem quite relevant. Whereas this reaction involves cation-dependent condensation of acetyl-CoA with glyoxylate to transiently form malyl-CoA that is hydrolyzed to the products malate and CoASH, the condensation is chemically similar to a
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TABLE III

| Parameter                     | Wild-type | D42E | D42N | E72A | D204A |
|------------------------------|-----------|------|------|------|-------|
| $K_M$ (mM)                   | 0.20 ± 0.02 | 5.1 ± 0.71 | 16 ± 1.8 | 5.2 ± 0.45 | 4.0 ± 0.30 |
| $K_M$ (µM)                  | 0.18 ± 0.04 | 3.2 ± 0.4  | 3.5 ± 0.5 | 42.7 ± 7.9  | 3.8 ± 0.5  |
| $K_M$ (µM)                   | 1.9 ± 0.3   | 25.2 ± 0.4 | ND       | 22.8 ± 5.8  | 3.5 ± 0.17 |
| $K_M$ (µM)                  | 1.3 ± 0.05  | 1.0 ± 0.03 | 1.1 ± 0.1 | 1.2 ± 0.01  | ND      |
| $K_M$ (µM)                  | 4.0 ± 0.8   | 3.2 ± 1.0  | 23.7 ± 2.0 | ND         | ND     |
| Stoichiometry                | 1.1 ± 0.05  | 1.1 ± 0.06 | ND       | 0.99 ± 0.04 | ND     |

* $K_M$ values were determined under standard assay conditions using 200 µM HMG-CoA. Therefore, these are apparent $K_M$ values.

Not detectable. D42N required the presence of HG-CoA to detect appreciable metal binding.

In additional experiments with the catalytically impaired D42G mutant, no binary enzyme-Mn$^{2+}$ could be detected, as is also the case for D42N. However, in the presence of 1 mM hydroxyglutaryl-CoA (HG-CoA), stoichiometric cation binding could be observed; the measured $K_M$ (27 µM) is inflated in comparison with the value measured for D42N under similar experimental conditions.

led to the suggestion that cation may also stabilize the enolate of acetyl-CoA, although this issue remains somewhat speculative. Although the malate synthase results argue that postulating a role for the activator cation of HMG-CoA lyase in ligating both carboxyl side chains and substrate oxygen-containing substituents is quite reasonable, the precedent also raises a question concerning general acid/base catalysts. Structural data for two different malate synthase proteins (13, 14) implicate an active site carboxyl side chain in proton abstraction from acetyl-CoA. A recent report of malate synthase mutagenesis experiments supports the importance of the acidic residue (30) but does not clearly establish the true magnitude of the contribution of this side chain. In the case of the HMG-CoA lyase reaction (Scheme 1), substrate cleavage is expected to generate a carbanionic (or enolate) form of acetyl-CoA that must be protonated at the C-2 position to complete the reaction. Thus, the side chain that functions as a surrogate to support (albeit poorly) the normal catalytic function of the carboxyl group of aspartate 42 (103-fold for H233A). Both of these residues are conserved in the entire HMG-CoA lyase family of enzymes, which catalyze various reactions that involve proton addition/removal at C-2 of acetyl-CoA. Whereas histidine 233 might function as a general acid at physiological pH, aspartate 42 would require an altered pK to fulfill such a function. Altered pK values for active site carboxyl groups are well preceded but such a possibility remains to be explored for HMG-CoA lyase. Because there is a significant (>100-fold) recovery of catalytic efficiency when a side chain with hydrogen bonding potential (D42N) is compared with the observations for a mutant (D42A,D42G) that lacks such potential, it remains possible that D42N can position the side chain of another residue that functions as a surrogate to support (albeit poorly) the normal catalytic function of the carboxyl group of aspartate 42. These various possibilities all argue for an active site location for aspartate 42, which is compatible with the strong correlation of human disease with mutations of this residue.

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Fig. 5. Scatchard plot of ESR data for manganese binding to D204A (panel A) and E72A (panel B) HMG-CoA lyase. Mn$^{2+}$ binding for E72A was determined in the absence (○) or presence (●) of the competitive inhibitor 3-hydroxyglutaryl-CoA. The binding of Mn$^{2+}$ to human HMG-CoA lyase was measured on a Varian Century Line 9 GHz spectrometer with a TEs20 cavity as described previously (10). Each spectrum was recorded at 22 °C with a modulation amplitude of 10 G, a modulation frequency of 100 kHz, a microwave power of 60 mW, a field sweep of 1000 G, and a time constant of 0.25 s. The ESR samples contained variable concentrations of Mn$^{2+}$ (10–150 µM) with constant HMG-CoA lyase sites (50 µM). The amount of bound Mn$^{2+}$ was determined by directly comparing the spectral amplitudes of samples containing HMG-CoA lyase to the corresponding amplitudes observed with a buffered solution containing an equal concentration of Mn$^{2+}$ in the absence of enzyme. The data were fit by linear regression analysis, and the binding constant ($K_M$) and stoichiometry (n) were determined from the slope and intercept, respectively.

reversal of the cation-dependent cleavage of HMG-CoA to form acetyl-CoA and acetooacetate. In recent work that demonstrates the importance of carboxyl groups as cation ligands (14), the importance of cation in polarizing the glyoxylate carbonyl and positioning substrates at the active site has also been indicated. Other work on another form of malate synthase (13) has

FIG. 5.
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