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Human Cytoplasmic 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase: Expression, Purification, and Characterization of Recombinant Wild-Type and Cys[129] Mutant Enzymes[1]

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A cDNA for the human cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (EC 4.1.3.5) was subcloned and expressed from a T7-based vector in *Escherichia coli*. The over-produced enzyme was purified using a three-step protocol that generated 20 to 30 mg protein/liter cell culture. The physical and catalytic properties of the recombinant synthase are similar to those reported for the nonrecombinant enzymes from chicken liver [Clinkenbeard *et al.* (1975a) *J. Biol. Chem.* 250, 3124–3135] and rat liver [Mehrabian *et al.* (1986) *J. Biol. Chem.* 261, 16249–16255]. Mutation of Cys[129] to serine or alanine destroys HMG-CoA synthase activity by disrupting the first catalytic step in HMG-CoA synthesis, enzyme acetylation by acetyl coenzyme A. Furthermore, unlike the wild-type enzyme, neither mutant was capable of covalent modification by the β-lactone inhibitor, L-659,699 [Greenspan *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84, 7488–7492]. Kinetic analysis of the inhibition by L-659,699 revealed that this compound is a potent inhibitor of the recombinant human synthase, with an inhibition constant of 53.7 nM and an inactivation rate constant of 1.06 min^{-1}.

Animal cells obtain cholesterol either by receptor-mediated endocytosis of low density lipoproteins (LDL)[3] or by *de novo* synthesis (1). Cholesterol homeostasis is achieved through feedback regulation of both mechanisms; that is, when cellular cholesterol levels are high, the synthesis of LDL receptors and the rate of cholesterol biosynthesis are suppressed. Conversely, cells respond to an increased demand for cholesterol by increasing the production of LDL receptors and by increasing *de novo* cholesterol synthesis. An imbalance of these levels may lead to hypercholesterolemia and atherosclerosis (2).

The major rate-controlling step of cholesterol biosynthesis is the NADPH-dependent conversion of HMG-CoA to mevalonate by HMG-CoA reductase (EC 1.1.1.34). Although the activity of this enzyme exhibits the most profound changes in response to cellular cholesterol levels, the activity of several additional cholesterol biosynthetic enzymes are also affected by feedback regulation (3–7). Among them is HMG-CoA synthase, the enzyme that catalyzes the condensation of acetyl coenzyme A (Ac-CoA) with acetoacetyl coenzyme A (AcAc-CoA) to produce HMG-CoA, the substrate for the reductase-catalyzed reaction. Two distinct forms of this enzyme are capable of HMG-CoA synthesis, a cytosolic form and a mitochon-

[1] Sequence data from this article have been submitted to the National Center for Biotechnology Information (NCBI) and deposited with the GenBank/EMBL Data Libraries under Accession No. L25798.

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[3] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; Ac-CoA, acetyl coenzyme A; AcAc-CoA, acetoacetyl coenzyme A; kb, kilobase pairs; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; ORF, open reading frame; PCR, polymerase chain reaction; NBD-CoA, coenzyme A, (3-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)propionic acid ester; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; CD, circular dichroism; L-659,699, 3,5,7-trimethyl-12-hydroxy-13-hydroxymethyl-2,4-tetradecadienedioic acid 12,14-lactone; DTT, dithiothreitol; CoASH, coenzyme A.

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drial form (8–10). The cytosolic form, which can be found in most tissues, but is most abundant in the liver and adrenal cortex, participates in the synthesis of cholesterol and other polyisoprenoid products. The mitochondrial form, found solely in the liver and kidney, participates in the synthesis of ketone bodies (β-hydroxybutyrate and acetoacetate) in the ketogenic pathway.

The inhibition of HMG-CoA reductase activity has been a successful strategy to lower serum cholesterol (11, 12). Reductase inhibitors are therapeutically effective because reduced cellular cholesterol stimulates the production of LDL receptors which in turn enhances the rate of removal of LDL from the plasma (13). Since HMG-CoA synthase also catalyzes an important step in the cholesterol biosynthetic pathway, it is conceivable that a similar physiological effect may be achieved by inhibiting synthase activity. Thus, we wished to initiate detailed studies of the cytoplasmic human HMG-CoA synthase to evaluate several approaches toward drug development, including structure-based drug design. Such studies require large amounts (>50 mg) of pure protein. However, this enzyme is not readily available from human tissue. Isolation of large quantities from hamster, rat, or chicken liver would be difficult and impractical. Furthermore, the sequences of these enzymes are nonidentical to the human target. We have found, however, that large quantities of human HMG-CoA synthase can be obtained through heterologous expression in *Escherichia coli*. A cDNA clone for the human synthase, isolated from an adrenal cDNA library (14), was modified for subcloning into a T7-based expression plasmid and was used to transform *E. coli* strain BL21(DE3). Induction with isopropyl-β-D-thiogalactopyranoside (IPTG) resulted in high-level expression of a soluble protein that was subsequently purified and characterized. The physical and catalytic properties of the recombinant human enzyme are similar to those of the HMG-CoA synthases isolated from rat (6) and chicken liver (15).

The active-site nucleophile of the cytoplasmic synthase has previously been assigned to Cys^{129}, based on sequence comparisons and mechanistic studies with the mitochondrial enzyme from chicken liver (16–18). Through site-directed mutagenesis, we have confirmed the importance of this residue in catalysis. The mutants that were constructed, Cys^{129}→Ala and Cys^{129}→Ser, were also used to characterize the mechanism of inhibition by the fungal metabolite, L-659,699 (Fig. 1). Neither mutant was capable of HMG-CoA synthesis nor of covalent labeling by a fluorescent derivative of L-659,699.

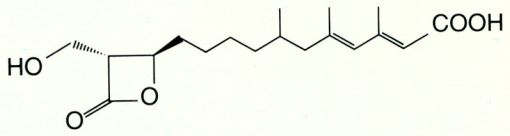

FIG. 1. L-659,699. 3,5,7-Trimethyl-12-hydroxy-13-hydroxymethyl-2,4-tetradecadienedioic acid 12,14-lactone.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized on a Milligen 7500 DNA synthesizer and purified using NENSORB minicolumns (NEN Research Products, Willmington, DE) as described by Johnson *et al.* (19). Polymerase chain reaction (PCR) reagents, including *Taq* polymerase, were obtained from Perkin–Elmer Cetus (Norwalk, CT). IPTG and MAX Efficiency DH5α competent cells were purchased from Gibco BRL (Gaithersburg, MD). *E. coli* strain BL21(DE3) was purchased from Novagen (Madison, WI) and strain MC1061 was purchased from Clontech (Palo Alto, CA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). T4 ligase and ATP were purchased from Stratagene (LaJolla, CA). DNase-free RNase A, aprotinin, pepstatin, and leupeptin were from Boehringer–Mannheim Biochemicals (Indianapolis, IN). Enzyme grade ammonium sulfate was obtained from Schwarz/Mann Biotech (ICN, Costa Mesa, CA). Hiload Superdex 200 PG 16/60, Mono-P HR 5/5, Q-Sepharose (Fast Flow), Superdex 6, and HR10/10 Fast Desalting FPLC columns, Sephadex G-25, and gel filtration molecular weight standard proteins were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). SDS–polyacrylamide gels, TBE running buffer, Tris–glycine–SDS running buffer, and Laemmli sample buffer were purchased from Novex (San Diego, CA). Protein molecular weight standards and Coomassie brilliant blue R-250 were from Bio-Rad (Richmond, CA). Purified bovine serum albumin was purchased from Pierce (Rockford, IL). [^{14}C]Acetyl-CoA was purchased from ICN (Irvine, CA). Coenyzme A, (3-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)propionic acid ester (NBD-CoA) was obtained from Molecular Probes, Inc. (Eugene, OR). All other reagents, including Ac-CoA and AcAc-CoA, were purchased from Sigma (St. Louis, MO). Immobilon P was purchased from Millipore (Milford, MA) and a Protoblot AP system for Western blotting was purchased from Promega (Madison, WI). Rabbit anti-rat HMG-CoA synthase antiserum for Western blotting was provided by M. Greenspan (Merck & Co., Inc.). L-659,699 was isolated from *Fusarium* sp. in the Merck Research Laboratories (Rahway, NJ).

General Methods

Mini plasmid preparations were prepared using a Qiagen (Chatsworth, CA) plasmid mini preparation kit according to the manufacturer's procedures. DNA sequencing was accomplished by the dideoxy chain termination method of Sanger *et al.* (20) using a Sequenase Version 2.0 reagent kit from United States Biochemical (Cleveland, OH).

Expression Plasmid Construction

The plasmid, pHSyn-22, contains a cDNA copy of the human HMG-CoA synthase mRNA which was isolated from a human fetal adrenal cDNA library (14). Both the coding and noncoding strands of this cDNA were sequenced and then modified for subcloning as follows: two synthetic oligonucleotide primers based on the HMG-CoA synthase sequence at the 5′ and 3′ ends were annealed to the pHSyn-22 template and filled in by PCR; thirty cycles (94°C, 30 s; 55°C, 1 min; 72°C, 2 min) of PCR were used to generate a 1648-bp strand encoding a 57-kDa polypeptide. Primer 1 (5′-TATACC**ATG**GCACCTGGATCACTTCCTTTGAATGC-3′) incorporates sequences for a unique *Nco*I site (underlined) and an initiating methionine (bold) at the 5′ end. Primer 2 (5′-TATAG-GATCCTCACAGAGTATCTTAATGTTCC-3′) introduces a unique *Bam*HI site (underlined) at the 3′ end and contains two stop codons. Primer 1 modifies the sequence so that the expressed protein begins with an alanine residue following cleavage at the N-terminal methionine by the bacterial methionyl aminopeptidase. The PCR fragment was di-

gested with *Nco*I and *Bam*HI and ligated into the *Nco*I and *Bam*HI sites of the T7 expression vector pET-8c (21). Initial clones were identified by transforming DH5α and using restriction digests to confirm the presence of a 6.2-kb plasmid, pET-HSyn. These clones were sequenced to confirm that the PCR reaction did not produce any errors. *E. coli* strain BL21(DE3) was then transformed with the modified vector to allow for T7 RNA polymerase-mediated transcription of the subcloned HMG-CoA synthase gene.

Site-Directed Mutagenesis

The coding sequence of the human HMG-CoA synthase open reading frame (ORF) was modified to convert Cys[129] to either a serine or an alanine by oligonucleotide-mediated mutagenesis using PCR. The mutagenesis primers used in the PCR reaction were

1. 5′-TATACC**ATGG**CACCTGGATCACTTCCTTTGAATGC-3′
2. 5′-CAATCCAGTTAACAGCATTGAAGACAGCAGCTGTGCC-TCCATA**AGA**TGCATTAGTTG-3′
3. 5′-CAATCCAGTTAACAGCATTGAAGACAGCAGCTGTGCC-TCCATA**AGC**TGCATTAGTTG-3′.

Primer 1 corresponds to the DNA sequence at the 5′ end of the synthase ORF which includes a unique *Nco*I site (underlined) and the initiating N-terminal Met codon (bold). Primers 2 and 3 are non-coding strand primers that convert the codon for Cys[129] to either serine or alanine (indicated in bold) and include a unique *Hpa*I site (underlined). Primers 1 and 2, or 1 and 3, were annealed to the pET expression vector containing the HMG-CoA synthase ORF, pET-HSyn, and filled in by PCR as described above to generate a 451-bp strand encoding the first 150 residues of the synthase ORF. The PCR fragments were digested with *Nco*I and *Hpa*I and ligated into the corresponding sites of pET-HSyn. Initial clones containing the modified HMG-CoA synthase sequence were obtained by transforming *E. coli* strain MC1061 with the ligation products, and the region amplified by PCR was sequenced to confirm that the PCR reaction did not introduce additional mutations.

Wild-Type and Mutant HMG-CoA Synthase Purification (Table I)

(i) Crude cell extract. A 1-liter culture of *E. coli* strain BL21(DE3) (pET-HSyn) was grown in M9 medium supplemented with 0.1% casamino acids and 200 μg/ml ampicillin at 37°C with shaking. When a cell density corresponding to an OD_{600} of 0.7 to 1.0 was reached, IPTG was added to a final concentration of 0.1 mM and the incubation was continued for another 4 h at 37°C. All further steps were performed at 4°C. Cells were pelleted at 4400*g* and resuspended in 20 ml of 20 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin (buffer A). The resuspension was stored frozen overnight, then thawed and lysed by two passes through a French press at 20,000 psi. Insoluble material was removed by centrifugation at 100,000*g* for 1 h and the resulting supernatant was used immediately for further purification or stored frozen for periods up to 1 month.

(ii) Ammonium sulfate precipitation. Ammonium sulfate (3.52 g) was slowly added to 20 ml of the clarified cell extract to achieve 30% saturation. This mixture was stirred for 10-min at 4°C and then centrifuged for 20 min at 28,000*g*. The supernatant from this step was adjusted to 45% saturation by adding 1.84 g ammonium sulfate. The pellet containing the synthase, obtained by centrifugation at 50,000*g* for 20 min, was resuspended to 3.0 ml in buffer A.

(iii) Gel filtration chromatography. A Pharmacia HiLoad Superdex 200 PG 16/60 column was equilibrated in 20 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol, and 0.1 mM EDTA (buffer B) at 1.0 ml/min. The resuspended ammonium sulfate pellet from (ii) was filtered through a Millipore Ultrafree-CL 0.45-μm filter and loaded on to the column at 0.25 ml/min using a 3.0-ml sample loop. The column was eluted in 150 ml equilibration buffer at 0.25 ml/min and 1.5-ml fractions were collected.

(iv) Q-Sepharose (Fast Flow) anion-exchange chromatography. A Pharmacia XK 16/40 column was packed with 20 ml Q-Sepharose (Fast Flow) and equilibrated in 20 mM Tris–HCl, pH 7.5, and 5.0 mM β-mercaptoethanol (buffer C). The column was loaded with 6 to 8 ml of the gel filtration pool from (iii) and then washed with 40 ml buffer C. The column was eluted with a 150-ml linear gradient of 0.0 to 0.3 M NaCl in buffer C and 2.0-ml fractions were collected. Gradient fractions were assayed for HMG-CoA synthase activity and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Circular Dichroism (CD)

Far uv CD spectra were measured with a Jasco J-720 spectropolarimeter. Samples at protein concentrations of 1.0 to 1.3 mg/ml were buffered with 20 mM Tris–HCl, pH 7.5, also containing 0.1 M NaCl and 1.0 mM dithiothreitol. A cylindrical quartz cuvette of 0.1 mm pathlength was used and the sample temperature was maintained at 10°C with a circulating water bath connected to the jacketed cuvette. For each sample an average of three spectra, each collected at 10 nm/min (response time = 4 s), was obtained. Corrections were made for background signal from buffer components. CD data were converted to mean residue ellipticities and analyzed for secondary structure contents by the variable selection method of Manvalan and Johnson (22).

TABLE I

Purification of Recombinant Human HMG-CoA Synthase from a 1-Liter Shake Flask Culture of *E. coli* BL21(DE3) (pET-HSyn)[a]

Purification step	Total activity (μmol/min)	Protein (mg)	Specific activity (μmol/min/mg)	Recovery (%)	Purification (-fold)
Cell extract	22.9	323	0.07	100	—
30–45% Ammonium sulfate pellet	25.1	111	0.23	100	3.2
Superdex-200 PG	16.4	31.8	0.52	72	7.3
Q-Sepharose FF	17.0	22.5	0.76	74	10.7

[a] Enzyme activity was quantitated by monitoring the decrease in absorbance at 303 nm of the magnesium enolate of AcAc-CoA upon condensation with Ac-CoA. Further details of this assay can be found under Materials and Methods.

Fluorescence Detection of Wild-Type and Mutant Synthase in the Presence of NBD-CoA

NBD-CoA (10 μM) was mixed with 20 μM wild-type or Cys^{129} mutant HMG-CoA synthase in 20 mM Tris–HCl, pH 8.0, containing 0.1 mM EDTA in a final volume of 400 μl. Fluorescence measurements were obtained using a Photon Technology International spectrofluorometer equilibrated to 25°C. Excitation was initiated at 468.5 nm and emission was measured from 490 to 650 nm.

Assay of HMG-CoA Synthase

HMG-CoA synthase was assayed using the spectrophotometric assay described by Lowe and Tubbs (23). In this assay the condensation of AcAc-CoA with Ac-CoA is followed by monitoring the decrease in absorbance at 303 nm of the magnesium enolate of AcAc-CoA at 25°C. The standard reaction mixture contains 0.1 M Tris–HCl, pH 8.0, 20 mM $MgCl_2$, 0.1 mM EDTA, 150 μM Ac-CoA, 7 μM AcAc-CoA, and 5 μg enzyme in a total volume of 1.0 ml. Deviations from these conditions are indicated in the figure legend of each experiment. Each assay is allowed to proceed for 2 min, which is within the linear time course of the assay. The apparent extinction coefficient for AcAc-CoA under these assay conditions is 12.2×10^3 M^{-1} cm^{-1}. The assays conducted in Fig. 8 also contained L-659,699 at the concentrations indicated in the figure legend. Inhibitor was added from a 1.0 mM stock solution made up in dimethyl sulfoxide and the reaction was initiated with the addition of enzyme. All other assays were initiated with the addition of Ac-CoA.

Isolation and Quantitation of [^{14}C]Acetyl-X-enzyme

The [^{14}C]acetyl-X-enzyme form of wild-type and Cys^{129} mutant HMG-CoA synthase was prepared using a modification of the procedure described by Vollmer *et al.* (18) as follows. Purified enzyme was adjusted to 100 mM potassium phosphate, pH 7.5 by running 1.5 mg through a Sephadex G-25 column (1.0 × 20 cm) equilibrated in this buffer. The pooled fractions were then concentrated to 1.5 mg/ml using a Centricon-10. One hundred twenty-five microliters of 500 μM [^{14}C]acetyl-CoA (52 μCi/μmol) was added to 300 μl (3.9 nmol) enzyme and incubated for 5 min at 30°C. This mixture was then quickly frozen on dry ice and then thawed for further workup. The thawed sample was maintained at 4°C during the separation of [^{14}C]acetyl-X-enzyme from CoASH and unreacted [^{14}C]acetyl-CoA by chromatography on a Sephadex G-25 column (1.0 × 20 cm) equilibrated in 10 mM potassium acetate, pH 5.0. Fractions (500 μl) were collected and 10 μl of each fraction was mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter. The stoichiometry of modification was determined by counting 10 μl of the pool of the fractions containing the peak protein fractions (based on A_{280}) and measuring the protein concentration of the pool by the Bradford method.

Fluorescence Labeling of L-659,699

The β-lactone acid L-659,699 (Fig. 1) was preactivated with one equivalent each of 1-hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in dimethylformamide. To the activated acid was added one equivalent of the amine, 5-dimethylaminonapthalene-1-(*N*-aminoethyl) sulfonamide, in one portion. The reaction conditions selectively produced the fluorescent amide at the carboxylic position of L-659,699, leaving the β-lactone ring intact. The reaction mixture was quenched with distilled, deionized water and extracted with ethyl acetate. The combined organic layers were dried (magnesium sulfate), suction filtered, and the filtrate evaporated *in vacuo* to yield the fluorescent product. The crude material was purified by C18 reverse-phase HPLC and characterized by fast atom bombardment mass spectrometry ($M + H = 599$).

Affinity Labeling of HMG-CoA Synthase

Wild-type HMG-CoA synthase (27 μM) and the Cys^{129} mutants (34 μM $Cys^{129} \rightarrow$Ser and 22 μM $Cys^{129} \rightarrow$Ala) were preincubated for 1 h at room temperature with a threefold molar excess of dansylated L-659,699. Approximately 1.0 mg of each protein was injected on to a HR 10/10 fast desalting column and eluted with 20 mM Tris–HCl, pH 8.0, at 0.5 ml/min. Fractions of 0.5 ml were collected. Fractions containing the protein peak were pooled and measured for protein content using the Bradford assay. The fluorescence of each pool was measured at 25°C using an excitation wavelength of 340 nm and an emission wavelength of 520 nm. Aliquots of each pool were also mixed with 2× Laemmli sample buffer and boiled. These samples were run out on a 14% SDS–PAGE gel. Fluorescent bands were detected using a long-range uv light source.

Calculation of Kinetic Constants

The kinetic parameters, K_m for Ac-CoA and K_i for AcAc-CoA, were obtained as described by Middleton (24). The initial-velocity equation compatible with substrate inhibition for a Bi Bi Ping Pong kinetic mechanism is

$$\nu/V_{max} = [A][B]/(K_{mA}[B](1 + [B]/K_i) + K_{mB}[A] + [A][B]), \quad [1]$$

where [A] represents the concentration of Ac-CoA, [B] represents the concentration of AcAc-CoA, K_{mA} and K_{mB} correspond to the Michaelis constants for Ac-CoA and AcAc-CoA, respectively, and K_i is the dissociation constant for an abortive complex between the free enzyme and AcAc-CoA. Since the value for K_{mB} is very small, Eq. [1] reduces to

$$\nu = V_{max}[A]/(K_{mA}(1 + [B]/K_i) + [A]), \quad [2]$$

which has the same form as the rate equation for competitive inhibition. The data were fit by nonlinear regression to Eq. [2] to generate values for K_{mA} and K_i.

The inactivation of HMG-CoA synthase by L-659,699 can not be studied by the measurement of initial inactivation rates under steady-state conditions because inactivation is too rapid and inhibitor concentrations change significantly during the assay period. Inhibition by L-659,699 was thus characterized by kinetic analysis of progress curves as described by Daniels *et al.* (25). The following derivation describes the time-dependent decrease in the rate of substrate consumption that occurs when an enzyme is exposed simultaneously to a substrate and an active-site directed inactivator:

$$E + I \overset{K_i}{\leftrightarrow} E \cdot I \overset{k_2}{\rightarrow} EI$$

$$E + S \overset{K_m}{\leftrightarrow} E \cdot S \overset{k_3}{\rightarrow} E + P$$

$$\ln(\nu/\nu_0) = [-k_2K_mI_0/(K_iK_m + S_0K_i + I_0K_m)]t = -\rho t, \quad [3]$$

where S_0 and I_0 represent the initial concentrations of substrate (AcAc-CoA) and inhibitor (L-659,699), ν_0 and ν are the velocity of the condensation reaction at initial time and time t, K_m is the Michaelis constant for the competing substrate (Ac-CoA), K_i is a binding constant which describes the affinity of the inhibitor for the enzyme, and k_2 represents the rate inactivation constant. Taking the derivative of Eq. [3] with respect to time, integrating to find product formation as a function of time and then rearranging to solve for substrate concentration at time t yields the equation

$$S_t = S_0 - \nu_0/\rho(1 - e^{-\rho t}), \quad [4]$$

where S_0 and S_t represent the concentration of AcAc-CoA at initial time and at time t, respectively, and ν_0 represents the initial velocity of the condensation reaction. Each progress curve can be fit by nonlinear regression analysis to Eq. [4] to obtain values for ρ. The inactivation constants, K_i and k_2, for L-659,699 were derived from a plot of the initial inhibitor concentration, I_0, versus I_0/ρ. This plot gives a straight line with a slope of $1/k_2$ and an X-intercept of $-K_i(1 + S_0/K_m)$, where S_0 and K_m are the initial concentration and Michaelis constant for Ac-CoA, respectively.

Protein Analysis

Protein concentrations were determined as described by Bradford (26). Protein samples were analyzed using Novex 14% SDS–polyacrylamide gels as described by Laemmli (27) and stained with Coomassie brilliant blue R-250. HMG-CoA synthase was detected by transferring the electrophoresed proteins to Immobilon P using a dry blotting apparatus from Integrated Separation Systems (Natick, MA) and immunoblotting with rabbit anti-rat HMG-CoA synthase antiserum. N-terminal amino acid sequencing was performed on proteins that were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore) using the procedure described by Towbin (28).

RESULTS

Sequencing of Human HMG-CoA Synthase cDNA

A cDNA clone (pHSyn-22) containing the human cytoplasmic HMG-CoA synthase coding region was subcloned as described under Materials and Methods. Sequencing of 1700 nucleotides downstream of the initiating ATG revealed an ORF of 1560 nucleotides (520 amino acids) (Fig. 2). Identification of an initiation and stop codon indicated that pHSyn-22 contains the entire coding sequence for the human enzyme. While this work was in progress the sequence of a human cytoplasmic synthase cDNA isolated from a fibroblast cDNA library was published (29). Comparison of the translated amino acid sequence of the two cytoplasmic human synthase cDNAs with those from Chinese hamster ovary (5), rat liver (9), and chicken liver (10) indicates that this enzyme is highly conserved between these species (Fig. 3). It is interesting to note that the two human synthases show a difference of seven amino acid residues (indicated by the closed circles in Fig. 3), four of which are completely conserved in human adrenal tissue, Chinese hamster ovary, rat liver, and chicken liver. We have also noted that there are eight silent substitutions in the nucleotide sequences of the two human cDNAs (not shown). This suggests that separate genes may exist for the human cytoplasmic synthase from different tissues, but there is no evidence to date, to confirm this hypothesis.

Miziorko and Behnke (16) and Vollmer *et al.* (18) sequenced the putative active site region of the mitochondrial synthase from chicken liver. Comparison of this sequence to the corresponding region of the human, hamster, chicken, and rat cytoplasmic synthase sequences indicates that this region is strictly conserved (region indicated by bold dashed line in Fig. 3). Additional sequence analyses using SWISSPROT, release 25 (30), PIR-Protein, release 36 (31), and GenPept (32) revealed that this region shows substantial similarity to the active sites of other condensing enzymes whose reactions proceed via acylation of cysteine thiols. For example, sequence comparisons of residues 107 to 162 of human adrenal HMG-CoA synthase show 57, 51, and 45% similarity to the active sites of the *laver* β-ketoacyl-ACP synthase III, stilbene synthase I from grape, and chalcone synthase F from petunia, respectively. No other regions of similarity, outside of the noted sequence that contains the active-site cysteine, were identified.

Subcloning the Human HMG-CoA Synthase

In order to express HMG-CoA synthase in *E. coli,* the 5′ and 3′ regions of the synthase cDNA in pHSyn-22 were modified for subcloning as described under Materials and Methods. The modified cDNA was subcloned into the expression vector pET-8c (21), and the resulting modified vector (pET-HSyn) was used to transform *E. coli,* strain BL21(DE3). Expression of HMG-CoA synthase was monitored by SDS–PAGE, and a time course of expression is shown in Fig. 4. A unique band at approximately 57 kDa is clearly visible after 30 min and the maximum level of expression of this polypeptide occurs within 3 to 4 h after addition of IPTG. This band is detected solely in the soluble fraction from the crude cell lysate by cross-reaction with rabbit anti-rat HMG-CoA synthase antiserum on Western blots and migrates identically to the rat enzyme (not shown). Furthermore, the size of this polypeptide is consistent with the theoretical molecular mass calculated from the amino acid sequence, 57,229 Da. N-terminal sequence analysis showed that the first nine amino acid residues of the unique 57 kDa band are as expected for the human HMG-CoA synthase. However, 75% of the expressed protein contains the N-terminal methionine, indicating incomplete removal by the *E. coli* methionyl aminopeptidase.

Purification and Characterization of HMG-CoA Synthase

HMG-CoA synthase was purified to homogeneity using a three-step protocol. The enzyme was first precipitated between 30 to 45% of saturated ammonium sulfate. A gel filtration column was used to remove residual ammonium sulfate from the solubilized pellet and lower molecular weight contaminants. The gel filtration pool was further purified by ion-exchange chromatography using Q-Sepharose. A SDS–polyacrylamide gel showing the protein composition of the pools from each step in the purification is shown in Fig. 5 and the corresponding purification table is shown in Table I. This procedure results in the recovery of 20 to 30 mg of purified synthase per liter of cell culture (Table I). The recovery of enzyme activity following purification is 74%. To confirm that the synthase exists as

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CCTTCACACAGCTCT TTCACCATGCCTGGA TCACTTCCTTTGAAT GCAGAAGCTTGCTGG CCAAAAGATGTTGGG      75
                   MetProGly SerLeuProLeuAsn AlaGluAlaCysTrp ProLysAspValGly

ATTGTTGCCCTTGAG ATCTATTTTCCTTCT CAATATGTTGATCAA GCAGAGTTGGAAAAA TATGATGGTGTAGAT    150
IleValAlaLeuGlu IleTyrPheProSer GlnTyrValAspGln AlaGluLeuGluLys TyrAspGlyValAsp

GCTGGGAAGTATACC ATTGGCTTGGGCCAG GCCAAGATGGGCTTC TGCACAGATAGAGAA GATATTAACTCTCTT    225
AlaGlyLysTyrThr IleGlyLeuGlyGln AlaLysMetGlyPhe CysThrAspArgGlu AspIleAsnSerLeu

TGCATGACTGTGGTT CAGAATCTTATGGAG AGAAATAACCTTTCC TATGATTGCATTGGG CGGCTGGAAGTTGGA    300
CysMetThrValVal GlnAsnLeuMetGlu ArgAsnAsnLeuSer TyrAspCysIleGly ArgLeuGluValGly

ACAGAGACAATCATC GACAAATCAAAGTCT GTGAAGACTAATTTG ATGCAGCTGTTTGAA GAGTCTGGGAATACA    375
ThrGluThrIleIle AspLysSerLysSer ValLysThrAsnLeu MetGlnLeuPheGlu GluSerGlyAsnThr

GATATAGAAGGAATC GACACAACTAATGCA TGCTATGGAGGCACA GCTGCTGTCTTCAAT GCTGTTAACTGGATT    450
AspIleGluGlyIle AspThrThrAsnAla CysTyrGlyGlyThr AlaAlaValPheAsn AlaValAsnTrpIle

GAGTCCAGCTCTTGG GATGGACGGTATGCC CTGGTAGTTGCAGGA GATATTGCTGTATAT GCCACAGGAAATGCT    525
GluSerSerSerTrp AspGlyArgTyrAla LeuValValAlaGly AspIleAlaValTyr AlaThrGlyAsnAla

AGACCTACAGGTGGA GTTGGAGCAGTAGCT CTGCTAATTGGGCCA AATGCTCCTTTAATT TTTGAACGAGGGCTT    600
ArgProThrGlyGly ValGlyAlaValAla LeuLeuIleGlyPro AsnAlaProLeuIle PheGluArgGlyLeu

CGTGGGACACATATG CAACATGCCTATGAT TTTTACAAGCCTGAT ATGCTATCTGAATAT CCTATAGTAGATGGG    675
ArgGlyThrHisMet GlnHisAlaTyrAsp PheTyrLysProAsp MetLeuSerGluTyr ProIleValAspGly

AAACTCTCCATACAG TGCTACCTCAGTGCA TTAGACCGCTGCTAT TCTGTCTACTGCAAA AAGATCCATGCCCAG    750
LysLeuSerIleGln CysTyrLeuSerAla LeuAspArgCysTyr SerValTyrCysLys LysIleHisAlaGln

TGGCAGAAAGAGGGA AATGATAAAGATTTT ACCTTGAATGATTTT GGCTTCATGATCTTT CACTCACCATATTGT    825
TrpGlnLysGluGly AsnAspLysAspPhe ThrLeuAsnAspPhe GlyPheMetIlePhe HisSerProTyrCys

AAACTGGTTCAGAAA TCTCTAGCTCGGATG TTGCTGAATGACTTC CTTAATGACCAGAAT AGAGATAAAAATAGT    900
LysLeuValGlnLys SerLeuAlaArgMet LeuLeuAsnAspPhe LeuAsnAspGlnAsn ArgAspLysAsnSer

ATCTATAGTGGCCTG GAAGCCTTTGGGGAT GTTAAATTAGAAGAC ACCTACTTTGATAGA GATGTGGAGAAGGCA    975
IleTyrSerGlyLeu GluAlaPheGlyAsp ValLysLeuGluAsp ThrTyrPheAspArg AspValGluLysAla

TTTATGAAGGCTAGC TCTGAACTCTTCAGT CAGAAAACAAAGGCA TCTTTACTTGTATCA AATCAAAATGGAAAT   1050
PheMetLysAlaSer SerGluLeuPheSer GlnLysThrLysAla SerLeuLeuValSer AsnGlnAsnGlyAsn

ATGTACACATCTTCA GTATATGGTTCCCTT GCATCTGTTCTAGCA CAGTACTCACCTCAG CAATTAGCAGGGAAG   1125
MetTyrThrSerSer ValTyrGlySerLeu AlaSerValLeuAla GlnTyrSerProGln GlnLeuAlaGlyLys

AGAATTGGAGTGTTT TCTTATGGTTCTGGT TTGGCTGCCACTCTG TACTCTCTTAAAGTC ACACAAGATGCTACA   1200
ArgIleGlyValPhe SerTyrGlySerGly LeuAlaAlaThrLeu TyrSerLeuLysVal ThrGlnAspAlaThr

CCGGGGTCTGCTCTT GATAAAATAACAGCA AGTTTATGTGATCTT AAATCAAGGCTTGAT TCAAGAACTGGTGTG   1275
ProGlySerAlaLeu AspLysIleThrAla SerLeuCysAspLeu LysSerArgLeuAsp SerArgThrGlyVal

GCACCAGATGTCTTC GCTGAAAACATGAAG CTCAGAGAGGACACC CATCATTTGGTCAAC TATATTCCCCAGGGT   1350
AlaProAspValPhe AlaGluAsnMetLys LeuArgGluAspThr HisHisLeuValAsn TyrIleProGlnGly

TCAATAGATTCACTC TTTGAAGGAACGTGG TACTTAGTTAGGGTG GATGAAAAGCACAGA AGAACTTACGCTCGG   1425
SerIleAspSerLeu PheGluGlyThrTrp TyrLeuValArgVal AspGluLysHisArg ArgThrTyrAlaArg

CGTCCCACTCCAAAT GATGACACTTTGGAT GAAGGAGTAGGACTT GTGCATTCAAACATA GCAACTGAGCATATT   1500
ArgProThrProAsn AspAspThrLeuAsp GluGlyValGlyLeu ValHisSerAsnIle AlaThrGluHisIle

CCAAGCCCTGCCAAG AAAGTACCAAGACTC CCTGCCACAGCAGCA GAACCTGAAGCAGCT GTCATTAGTAATGGG   1575
ProSerProAlaLys LysValProArgLeu ProAlaThrAlaAla GluProGluAlaAla ValIleSerAsnGly

GAACATTAAGATACT CTGTGAGGTGCAAGA CTTCAGGGTGGGGTG GGCATGGGGTGGGGG TATGGGAACAGTTGG   1650
GluHis
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FIG. 2. Complete nucleotide and deduced amino acid sequence of the human cytosolic HMG-CoA synthase. The cDNA clone, pHSyn-22, isolated by Gil *et al.* (14), was sequenced using the dideoxy chain termination method. The nucleotides are numbered on the right-hand side. The amino acid sequence homologous to the active site of the chicken mitochondrial HMG-CoA synthase (16, 18) is underlined.

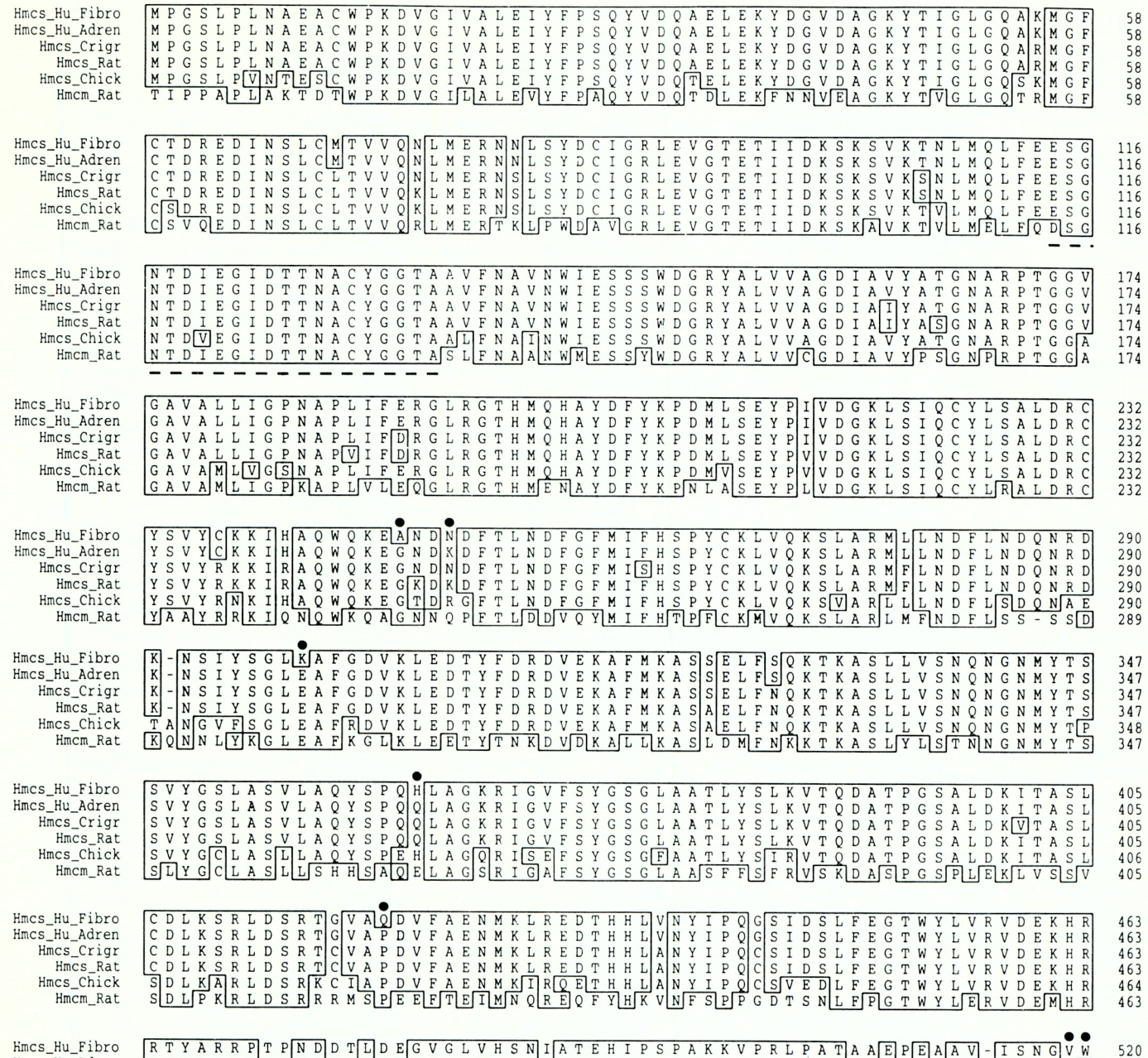

FIG. 3. Deduced amino acid sequence alignments. Deduced amino acid sequence alignment of the cytoplasmic HMG-CoA synthases from human fibroblast (Hmcs_Hu_Fibro), human adrenal (Hmcs_Hu_Adren), Chinese hamster ovary (Hmsc_Crigr), rat liver (Hmsc_Rat), and chicken liver (Hmsc_Chick), and the mitochondrial HMG-CoA synthase from rat liver (Hmcm_Rat). (●) Residues which differ between the human fibroblast and human adrenal enzyme. The boxed regions represent regions of identity between the species and the region underlined by the bold dashed line corresponds to the active site region identified for the mitochondrial HMG-CoA synthase from chicken liver as discussed in the text. The signal sequence of the mitochondrial enzyme is not included in the alignment.

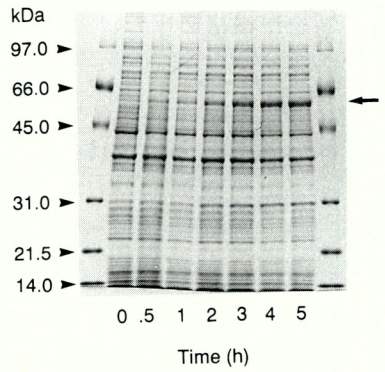

FIG. 4. Time course of IPTG induction of HMG-CoA synthase. Cells were grown in 150 ml M-9 media with ampicillin (200 μg/ml) for 3.5 h at 37°C. IPTG was added to a final concentration of 0.1 mM and 1.0-ml aliquots were removed from the shaker at the times indicated. These aliquots were centrifuged in a microfuge and the pellets were resuspended in Laemmli sample buffer to a final OD_{600} of 4.0. Ten microliters was loaded in each lane of a 14% SDS–polyacrylamide gel. The HMG-CoA synthase band at 57 kDa is indicated by the arrow.

a dimer, the purified protein was characterized by gel filtration chromatography using a Superose-6 column. The purified enzyme eluted from this column at a molecular weight of 120,000 (not shown), which is consistent with that found for the rat liver enzyme (6). Several studies have shown that the cytosolic synthases from chicken (15) and rat liver (6) have a p*I* of approximately 4.5. The same value was obtained for the recombinant human enzyme by chromatofocusing (not shown).

Activity of the Recombinant HMG-CoA Synthase

HMG-CoA synthase can be assayed using either a continuous spectrophotometric assay or a fixed-time radiochemical assay (15). The spectrophotometric assay is preferable since it is more convenient and provides a continuous assay of the condensation of the two substrates. Using the spectrophotometric assay, it was found that the recombinant synthase was purified over 10-fold from the cell extract and had a final specific activity of 0.76 μmol/min/mg (Table I). This value is consistent with that recently reported for a recombinant cytoplasmic HMG-CoA synthase from chicken liver (33).

Kinetic analyses of HMG-CoA synthase from chicken liver (15), ox liver (23), and yeast (24) are complicated by the substrate inhibition of AcAc-CoA. The same observation has now been made for the recombinant human enzyme. We have found, as has been shown for the mitochondrial synthase from ox liver (23) and the cholesterogenic enzyme from baker's yeast (24), that AcAc-CoA substrate inhibition is competitive with respect to Ac-CoA. Fitting the data by computer using nonlinear regression analysis to the rate equation for competitive inhibition (Eq. [2] under Materials and Methods), yielded a V_{max} of 0.7 ± 0.1 μmol/min/mg, a K_m for Ac-CoA of 29 ± 7 μM, and a K_i for AcAc-CoA of 12 ± 3 μM. The K_m for AcAc-CoA cannot be determined since the level of substrate below which inhibition does not occur can not be ascertained due to the limited sensitivity of the assay. Fitting the data to the rate equation for noncompetitive inhibition yielded ill-defined parameters with much higher standard error.

The fact that the kinetic data we have obtained fit the rate equation for competitive inhibition much better than that for noncompetitive inhibition is consistent with the hypothesis that two structurally related substrates compete for binding to the same site on the enzyme. If AcAc-CoA were a noncompetitive inhibitor it would imply that binding could occur somewhere other than at the active site of the enzyme. To date, there is no kinetic or biochemical evidence for any HMG-CoA synthase that supports this mechanism.

Characterization of Cys^{129} HMG-CoA Synthase Mutants

Vollmer *et al.* (18) have demonstrated that the catalytic nucleophile at the active site of the mitochondrial synthase from chicken liver is the cysteine thiol that corresponds to Cys^{129} in the sequence for the cytoplasmic enzyme from hamster. In order to demonstrate that this is truly the catalytic residue of the recombinant human enzyme, Cys^{129} was converted either to serine or to alanine using oligonucleotide-directed mutagenesis. These substitutions were chosen for mutagenesis because they alter the reactivity of this residue with minimal likelihood of gross conformational changes to the enzyme (34). Furthermore, a serine hydroxyl might be capable of acting as the catalytic nucleophile in the first step of the synthase reaction (i.e., enzyme acetylation by Ac-CoA). We found

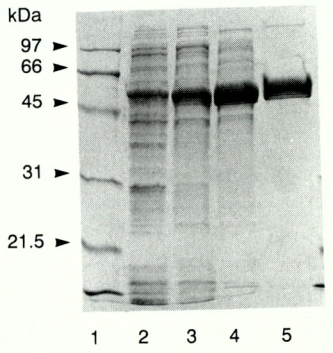

FIG. 5. Purification of HMG-CoA synthase as monitored by SDS–PAGE (14%). Lane 1, marker proteins; lanes 2–5, each contains approximately 10 μg of total protein; lane 2, whole-cell extract of BL21(DE3) (pET-HSyn) 4 h after induction by IPTG; lane 3, resuspended pellet of 30–45% ammonium sulfate precipitation; lane 4, pooled HMG-CoA synthase fractions from Superdex-200 PG column; lane 5, pooled HMG-CoA synthase fractions from Q-Sepharose (Fast Flow) column. Protein bands were detected by Coomassie staining.

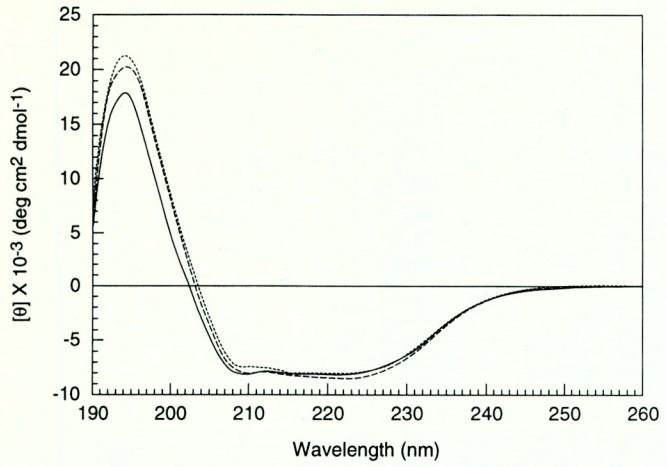

FIG. 6. Far uv CD spectra of wild-type (—), Cys[129]→Ser (---), and Cys[129]→Ala (· · ·) HMG-Co A synthase. Protein concentrations were in the range of 1.0–1.3 mg/ml and the samples were buffered at pH 7.5 with Tris–HCl containing 100 mM NaCl and 1.0 mM DTT. A cylindrical jacketed quartz cuvette of 0.1-mm pathlength was used and the temperature was maintained at 10°C. Data are expressed as mean residue ellipticities.

that these substitutions had no effect on either the level of expression or on the chromatographic properties of the synthase by gel filtration and ion-exchange chromatography (data not shown). The far uv CD spectra of the wild-type and mutant HMG-CoA synthases are shown in Fig. 6. The three samples exhibit very similar spectra, which contain minima near 208 and 222 nm, characteristic of an α-helical structure. Variable selection analysis (22) yielded nearly identical results for secondary structure contents for the wild-type and the two mutant proteins: approximately 29–32% α-helix, 10–16% β-sheet, 22–26% β-turn, and 26–32% unordered structure. CD spectra calculated from these estimates agreed well with experimentally determined spectra in the 190- to 260-nm range although deviations in the 184- to 190-nm range were observed due to relatively high noise level in experimental data below 190 nm (not shown).

Kinetic analyses, on the other hand, revealed that the activities of the two mutants were considerably reduced. Both mutants showed no detectable activity above background in the spectrophotometric assay, even when five times more mutant than wild-type enzyme was used.[4] This low level of activity suggested that it would be prudent to conduct a more stringent evaluation of the structural integrity of the mutants. This was accomplished by monitoring the fluorescence of the CoA analog, NBD-CoA, in the presence of the wild-type and mutant proteins. This compound was chosen for this experiment because CoA is a noncompetitive inhibitor of the enzyme (23, 24) and should therefore be capable of interacting with the wild-type as well as the mutant synthases if they are folded properly. We found that the fluorescence intensity of NBD-CoA at 544 nm increased 1.5-fold in the presence of either the wild-type or mutant synthases (Fig. 7). To confirm that this enhancement was not due to nonspecific hydrophobic interactions, the fluorescence of NBD-CoA was monitored in the presence of bovine serum albumin (BSA) instead of the recombinant HMG-CoA synthase and with synthase that was preincubated with 1.0 mM AcAc-CoA. In both cases no enhancement of fluorescence was observed (not shown).

Isolation of [^{14}C]Acetyl-S-enzyme

HMG-CoA synthesis occurs via a two-step reaction mechanism. In the first step, HMG-CoA synthase reacts with Ac-CoA forming a covalent acetyl-*S*-enzyme intermediate upon releasing CoA. This intermediate then condenses in a second step with AcAc-CoA, forming enzyme-bound product HMG-CoA, which is liberated by hydrolysis (17). We sought to determine whether a serine hydroxyl group could substitute for the sulfhydryl of Cys[129] as the nucleophile in the formation of the acetyl-enzyme. This was accomplished by determining whether [^{14}C]acetyl-labeled enzyme could be isolated from a gel filtration column following incubation with [^{14}C]Ac-CoA. The Cys[129]→Ala mutant was used as a control to correct for nonspecific binding of radiolabeled substrate. Isolation

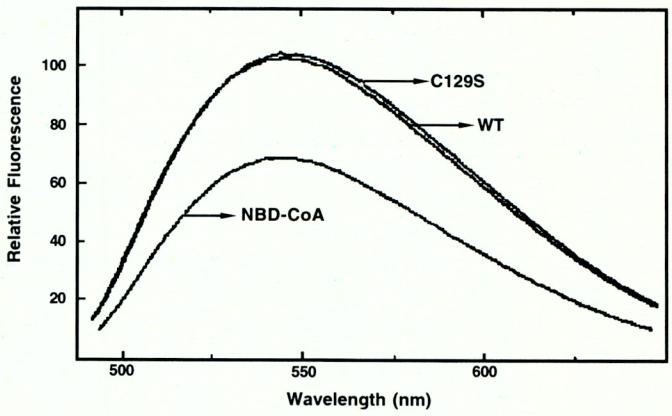

FIG. 7. Incubation of wild-type and Cys[129]→Ser HMG-CoA synthase with NBD-CoA. These scans represent the fluorescence emission of 10 μM NBD-CoA alone and in the presence of 20 μM wild-type (WT) and Cys[129]→Ser (C129S) HMG-CoA synthase using an excitation wavelength of 468.5 nm. The Cys[129]→Ala mutant induces the same fluorescence enhancement as the WT and C129S mutant (not shown). No fluorescence enhancement was observed when NBD-CoA was mixed with either 20 μM BSA or with wild-type or mutant synthase that was preincubated with 1.0 mM AcAc-CoA.

[4] The background rate of hydrolysis for this spectrophotometric assay corresponds to 2% of the activity observed for the wild-type HMG-CoA synthase. Since the observed rate with both Cys[129] mutants was less than 50% over background, we can conclude that the activity of these mutants is less than 1% of that of the wild-type synthase.

of the acetylated intermediate was accomplished using the procedure described by Vollmer *et al.* (18) which incorporates conditions for optimal acetylation and includes precautions to minimize the chemical and enzymatic hydrolysis of the ester linkages. The stoichiometry of modification for the wild-type enzyme was 0.75 mol of acetyl per mol of enzyme dimer, which is similar to that found for the mitochondrial enzymes from chicken (18) and ox liver (35). The stoichiometry of modification for the Cys^{129}→Ser mutant was 0.04 mol of acetyl per mol of enzyme dimer, which corresponds to 5% of the labeling observed for the wild-type synthase. This level of radioactivity even appears in fractions that are devoid of protein and therefore represents background. Extending the incubation period during the acetylation reaction from 5 to 30 min with the Cys^{129}→Ser mutant had no effect on the extent of labeling.

Characterization of HMG-CoA Synthase Inhibition by L-659,699

Mayer *et al.* (36) have shown that the cytosolic HMG-CoA synthase from rat liver is inhibited by L-659,699 (designated as 1233A in that paper) in a time-dependent, irreversible manner, indicative of active-site directed inhibition. They also report an IC_{50} for this compound of 10 nM. The efficiency of this compound hampers the analysis of inactivation kinetics because the inactivation is too rapid and inhibitor concentrations change significantly during the assay period (36). The rate of inactivation, however, can be slowed to a determinable rate by following progress curves of the inhibition in the presence of high concentrations of the competing substrate, Ac-CoA. This approach has successfully been used by Daniels *et al.* (25) to evaluate the inhibition of α-chymotrypsin by a series of haloenol lactones. Daniels *et al.* show how a series of equations, derived by Main (37), can be used to calculate an inhibition constant, K_i, and an inactivation rate constant, k_2 for extremely potent, active-site directed, irreversible inhibitors. These constants are obtained by analysis of the changing slopes of the progress curves with time as described under Materials and Methods. The progress curves of the inhibition of the human HMG-CoA synthase by L-659,699 are shown in Fig. 8 and a plot of I_0/ρ versus I_0 is shown in the inset. Through kinetic analysis of these curves we have found that the K_i for this inhibitor is 53.7 nM and the k_2 is 1.06 min^{-1}.

Labeling the Wild-Type and Cys^{129} Synthase Mutants with a Fluorescent Inhibitor

To determine if Cys^{129} is also the target for covalent modification by L-659,699, the carboxylate of this inhibitor was dansylated as described under Materials and Methods to make a fluorescent probe. This particular change was made because it has been shown by Mayer *et al.* (36) and by Greenspan *et al.* (38) that this portion of L-659,699 is not required for inhibition of the cytoplasmic synthase from rat liver. The wild-type synthase, as well as the synthase mutants, were incubated with the dansylated inhibitor and the labeled proteins were isolated on a desalting column. The protein peak from each labeling experiment was then analyzed by fluorescence spectroscopy and by SDS–PAGE as described under Materials and Methods. A fluorescence- and Coomassie-stained gel of these samples are shown in Fig. 9. The values given in the figure legend to Fig. 9 represent the relative fluorescence at 520 nm of the wild-type and mutant synthases. These measurements indicate that the relative fluorescence of the mutants is barely 1% that of the wild-type. The fluorescence gel shows that while fluorescence of the wild-type enzyme is readily detected, fluorescence of neither the Cys^{129}→Ala nor the Cys^{129}→Ser mutants could be detected. These results indicate that Cys^{129} is also essential for covalent modification by L-659,699.

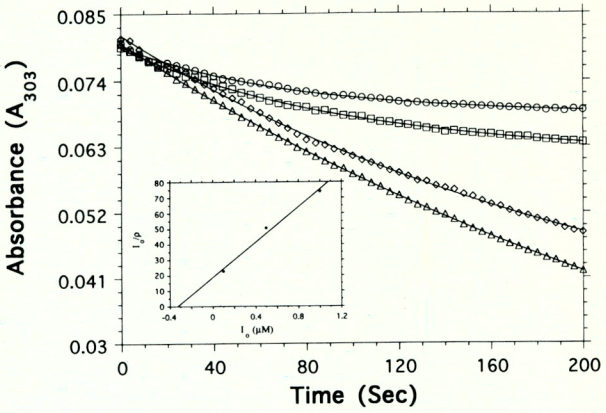

FIG. 8. Determination of K_i and k_2 for L-659,699. This figure depicts the inhibition progress curves for recombinant human HMG-CoA synthase in the presence of L-659,699. Each reaction contained 0.1 M Tris–HCl, pH 8.0, 0.2 mM EDTA, 20 mM $MgCl_2$, 150 μM Ac-CoA, 7.0 μM AcAc-CoA, and L-659,699 at the following concentrations: (○) 1.0 μM, (□) 0.5 μM, (◇) 0.1 μM, and (△) 0 μM. The progress curves were fit by nonlinear regression to Eq. [4] under Materials and Methods to obtain values for ρ. A plot of the initial inhibitor concentration (I_0) versus I_0/ρ is shown in the inset. This plot gives a straight line with a slope of $1/k_2$ and an X-intercept of $-K_i(1 + S_0/K_m)$ where S_0 and K_m are the initial concentration and Michaelis constant for Ac-CoA.

DISCUSSION

Cholesterogenic HMG-CoA synthases from animal tissues and cultured cells have been extensively characterized. The isolation and characterization of HMG-CoA synthase from human tissues has yet to be reported. Given the potential of this enzyme as a therapeutic target for lowering high serum cholesterol, we sought to overexpress and purify the human cytoplasmic HMG-CoA synthase

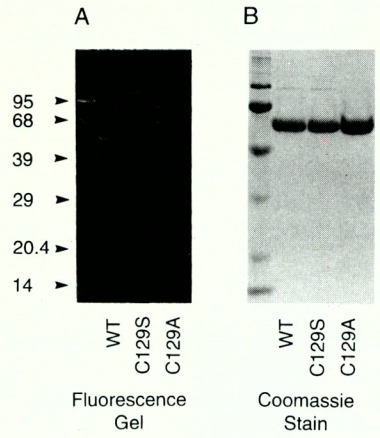

FIG. 9. Fluorescent labeling of wild-type and mutant HMG-CoA synthase. Wild-type HMG-CoA synthase and the Cys[129] mutants were preincubated with dansylated L-659,699 and then separated on a desalting column to remove the unbound drug. Approximately 4 μg of protein from the pool of each chromatographic run was loaded on a 14% SDS–polyacrylamide gel. The gel was first photographed using a long-range uv light source (A) and then immediately placed in Coomassie staining solution (B). The relative fluorescence at 520 nm of the pool for each protein sample is as follows: WT, wild-type, 9.2×10^6; C129S, Cys[129]→Ser, 1.0×10^5; C129A, Cys[129]→Ala, 6.6×10^4.

for biochemical and ultimately, structural studies. This task was made possible by the isolation of a cDNA for the enzyme from a human fetal adrenal cDNA library (14). This cDNA (pHSyn-22) was used by Gil *et al.* (14) to characterize the 5′ untranslated region of the human synthase mRNA. We have sequenced pHSyn-22 and have found that it contains the entire coding region of the synthase gene (Fig. 2). The recombinant HMG-CoA synthase generated from pHSyn-22 is biochemically indistinguishable from the nonrecombinant enzymes isolated from animal tissues and baker's yeast (6, 15, 24). This indicates that the recombinant enzyme provides a suitable alternative to the nonrecombinant enzyme for initiating drug discovery efforts. The procedure developed for the isolation of the recombinant enzyme is both efficient and economical, allowing for practical production of large quantities of protein needed for structural studies such as X-ray crystallography.

Kinetic and chemical experiments of HMG-CoA synthases isolated from chicken liver (17), ox liver (23, 35), and yeast (24, 39) have established that HMG-CoA synthesis proceeds via formation of a covalent acetyl-*S*-enzyme intermediate. The site of acetylation of the mitochondrial synthase from chicken liver has been identified by Vollmer *et al.* (18). This study reported that the cysteine residue corresponding to Cys[129] of the hamster liver cytoplasmic HMG-CoA synthase provides the nucleophile for attack at the thioester carbonyl carbon of Ac-CoA. Although the mitochondrial and cytoplasmic forms of HMG-CoA synthase are distinctly different (9, 10, 40), sequence comparisons with the human, hamster, chicken, and rat cytoplasmic enzymes reveal that this region of both forms of the enzyme is highly conserved (bold dotted line in Fig. 3).

One purpose in making the Cys[129] mutants was to confirm that this residue was the catalytic residue for the human cytoplasmic enzyme. Substitutions were based on residue exchange matrices constructed by Bordo and Argos (34) from homologous and known three-dimensional protein structures. These matrices suggest that the safest (i.e., least likely to affect folding or induce gross conformational changes) substitutions for exposed cysteine residues are replacements with either serine or alanine. Consistent with this hypothesis, we found that these mutants retain the ability to fold correctly and efficiently, as judged by their solubility, expression levels, CD spectra (Fig. 6) and similarity in purification (by both ion exchange and gel filtration chromatography) with the wild-type enzyme. They are also capable of binding a fluorescent CoA derivative (Fig. 7), but their ability to catalyze HMG-CoA synthesis is substantially reduced.

The question then arises; is the inability of these mutants to catalyze HMG-CoA synthesis due to their inability to form the acetylated intermediate or to their inability to participate in the aldol condensation leading to formation of enzyme-bound HMG-CoA? Interruption of the first step is conceivable since the hydroxyl group of serine is less nucleophilic than the sulfhydryl group of cysteine. On the other hand, acetyl oxoesters tend to be less acidic than acetyl thioesters (41, 42) which could prohibit the nucleophilic addition of the acetyl-enzyme α-carbanion to the C-3 carbonyl of AcAc-CoA. Toward answering this question, the ability to isolate the acetyl-X-enzyme for the wild-type and mutant synthases was tested. It was found that while the wild-type enzyme gave nearly stoichiometric labeling, no significant amount of acetylated enzyme could be detected for the serine or alanine mutant. Similar experiments were conducted with a recombinant chicken cytoplasmic HMG-CoA synthase (33), showing that mutation of Cys[129] to serine leads to expression of a catalytically inactive enzyme that is unable to form a covalent acetyl-*O*-enzyme species. This implies that either the rate of formation of the acetyl-*O*-synthase is insignificant over the 30 min incubation period, or hydrolysis of the acetyl-*O*-synthase intermediate is faster than its formation. This is in sharp contrast to the β-ketothiolase from *Zoogloea ramigera,* an enzyme which catalyzes a Claisen condensation via an acetyl-*S*-enzyme intermediate (43). Unlike the synthase Cys[129]→Ser mutant, stoichiometric levels of acetylated Cys[89]→Ser thiolase could be isolated following a 30-s incubation at 25°C with [^{14}C]Ac-CoA. From the experiments reported here, no conclusions regarding the ability of an acetyl-*O*-synthase intermediate, if formed, to participate in the condensation reaction with AcAc-CoA can be drawn. A more

detailed study of the partial reaction of the wild-type and mutant synthases is required to address the precise functional effects of the Cys to Ser mutation on the enzyme acetylation and adol condensation steps of the overall reaction.

To further probe the chemistry of the wild-type and mutant synthases we sought to determine if Cys^{129} could be covalently modified by a fluorescent derivative of the β-lactone inhibitor, L-659,699 (Fig. 1). L-659,699 was first isolated as an antibiotic from *Cephalosporin* sp. (44) and was later isolated independently from *Scopulariopsis* sp. and *Fusarium* sp. (45). This fungal metabolite affects cholesterol biosynthesis by specifically inhibiting HMG-CoA synthase (38, 45, 46). The structural features of L-659,699, important for inhibition of the rat liver HMG-CoA synthase, have been studied in detail (36, 38). These studies showed that the integrity of the lactone ring was essential for inhibition, whereas changes in the carboxyl end could be tolerated. By analogy with the initial step of HMG-CoA synthesis, it is likely that inactivation by L-659,699 occurs through nucleophilic attack by the active-site cysteine thiol at the carbonyl carbon of the β-lactone (36). This theory has been supported by Greenspan *et al.* (47), who showed that a radiolabeled analog of L-659,699 could be released from the rat liver synthase by treatment with hydroxylamine. Our experiments with the dansylated inhibitor (Fig. 9) are the first studies to show that Cys^{129} is essential for covalent labeling by this inhibitor.

The potency of L-659,699 was assessed using kinetic analyses for enzyme-activated irreversible inhibitors. Our data shows that it is a very potent inhibitor possessing an inhibition constant, K_i, of 53.7 nM and an inactivation rate constant, k_2, of 1.06 min^{-1}. Also, L-659,699 has sufficient cell permeability to inhibit [^{13}C]acetate incorporation into sterols in cultured Hep G2 cells (47). Unfortunately, the β-lactone moiety of L-659,699 introduces two complications that limit its use as a therapeutic; the covalent interaction of the inhibitor with enzyme may make L-659,699 immunogenic, and the intrinsic reactivity of the β-lactone may lead to nonspecific modification of other essential proteins. Determination of the structure of the active site of an inhibited HMG-CoA synthase complex could provide insight into the design of a reversible, noncovalent inhibitor, eliminating these concerns. This task can now be made possible through the use of the described bacterial expression system, which results in the production of quantities of pure human synthase sufficient to support a crystallographic approach to structure determination.

ACKNOWLEDGMENTS

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