Evidence for the Interaction of Avian 3-Hydroxy-3-methylglutaryl-CoA Synthase Histidine 264 with Acetoacetyl-CoA†

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ABSTRACT: Previous work on HMG-CoA synthase has implied the presence of a reactive active site histidine, prompting our examination of the possible function of invariant histidine residues by site-directed mutagenesis. Mutations encoding H197N, H264N/A, and H436N HMG-CoA synthases were constructed, and the mutant enzymes were overexpressed in *Escherichia coli* BL21(DE3). Kinetic characterization of the isolated synthase variants indicates that, while H197N and H436N enzymes behave similarly to wild-type synthase, H264N and H264A synthases exhibit significant differences. Although the K_m for acetyl-CoA is not substantially altered, H264N/A synthases catalyze production of HMG-CoA at a diminished (∼25-fold slower) rate. In contrast, H264N/A synthases can efficiently catalyze the acetyl-CoA hydrolysis partial reaction exhibiting a K_m for acetyl-CoA that, again, approximates the value obtained with the wild-type enzyme. These mutants also retain the ability to form significant levels of the acetyl-*S*-enzyme reaction intermediate. The functional catalysis of partial reactions argues that the H264 mutant proteins retain substantial structural integrity. In this context, it appears significant that the H264N/A synthases exhibit a ∼100-fold increase in the K_m for acetoacetyl-CoA. In order to test whether the two orders of magnitude effect may be largely attributed to a decreased affinity of acetoacetyl-CoA for these enzymes and, more specifically, whether H264 interacts with the carbonyl oxygen of acetoacetyl-CoA's thioester, turnover of *S*-(3-oxobutyl)-CoA, a thioether analog of acetoacetyl-CoA, was investigated. This alternative substrate, in which a methylene group replaces the thioester carbonyl, is utilized by wild-type synthase with an apparent V_{max} that is ∼100-fold lower and an apparent K_m that is 25-fold higher than the values obtained using the physiological substrate, acetoacetyl-CoA. H264A synthase also catalyzes the turnover of *S*-(3-oxobutyl)-CoA; the diminution in rate supported by the alternative substrate is comparable in magnitude to the effect observed for wild-type enzyme. In contrast, H264A exhibits *comparable* apparent K_m values for *S*-(3-oxobutyl)-CoA and acetoacetyl-CoA. Thus, unlike wild-type synthase, there is *no* penalty in terms of efficiency of H264A saturation when the alternative thioether substrate replaces the physiological substrate. These data suggest that the imidazole of H264 in avian enzyme may play a role in anchoring the second substrate, acetoacetyl-CoA, by interacting with the carbonyl oxygen of the thioester functionality.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)[1] synthase (EC 4.1.3.5) catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to produce HMG-CoA. Two distinct enzymes (Clinkenbeard et al., 1975; Reed et al., 1975) encoded by two separate genes (Kattar-Cooley et al., 1990; Ayte et al., 1990a,b) catalyze this reaction to generate HMG-CoA, a key intermediate in both cholesterogenic and ketogenic pathways. As anticipated for the enzymes that catalyze the first irreversible step in these metabolic pathways, HMG-CoA synthase has been implicated as a control point (Smith et al., 1988; Quant et al., 1989; Casals et al., 1992). Therefore, the cholesterogenic isozyme, located in the cytoplasm, has become a subject of increased interest as a target for antisteroidogenic agents (Omura et al., 1987; Greenspan et al., 1993; Hashizume et al., 1994).

Kinetic studies on the yeast (Middleton, 1972) and the ox liver (Lowe & Tubbs, 1985; Page & Tubbs, 1978) enzymes indicate that this enzyme follows a ping-pong bi-bi mechanism. Covalent acyl−enzyme reaction intermediates are expected for such a reaction mechanism. An acetylated enzyme species was implicated in studies on yeast synthase (Middleton & Tubbs, 1974). Work on avian synthase (Miziorko et al., 1975; Miziorko & Lane, 1977) established that an active site cysteine supports formation of both enzyme-*S*-acetyl and enzyme-*S*-HMG-CoA intermediates. These studies support the hypothesis that production of HMG-CoA follows a three-step process (eqs 1−3; Miziorko et al., 1975):

$$\text{EnzSH} + \text{acetyl-CoA} \rightleftharpoons \text{acetyl-SEnz} + \text{CoASH} \quad (1)$$

$$\text{acetoacetyl-CoA} + \text{acetyl-SEnz} \rightarrow \text{EnzS-HMG-CoA} \quad (2)$$

$$\text{EnzS-HMG-CoA} + H_2O \rightarrow \text{EnzSH} + \text{HMG-CoA} \quad (3)$$

According to this scheme, the first step involves transfer of acetyl group from acetyl-CoA to the cysteinyl sulfhydryl of the enzyme, to give rise to an acetyl-*S*-enzyme intermedi-

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; R·CoA, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA; oePCR, overlap extension PCR; DEPC, diethylpyrocarbonate.

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ate. Subsequent deprotonation of C2 of the acetyl moiety of the thioester intermediate gives rise to a carbanion, which attacks C3 of acetoacetyl-CoA to produce an enzyme-*S*-HMG-CoA intermediate. This is followed by a rapid hydrolysis of the second intermediate to release HMG-CoA. Affinity labeling (Miziorko & Behnke, 1985a) and peptide mapping work (Miziorko & Behnke, 1985b) identified C129 as a reactive active site cysteine. Protein chemistry (Vollmer et al., 1988) and mutagenesis studies (Misra et al., 1993) of this enzyme allowed the unambiguous functional assignment of C129 as the active site amino acid that forms the acetyl-*S*-enzyme intermediate (eq 1).

The identity of other amino acid residues involved in the reaction chemistry indicated by eqs 2 and 3 remains to be established. Recent work (Misra et al., 1995) ruled out participation of additional conserved cysteines in reaction chemistry but also indicated the presence of a reactive histidine in the active site. This observation, as well as other results that implicated the participation of a histidine in HMG-CoA synthesis, prompted our use of site-directed mutagenesis techniques to investigate possible roles for conserved histidine residues.

The results, as documented in this report, indicate a specific and significant role for an active site histidine.

EXPERIMENTAL PROCEDURES

Materials

Escherichia coli BL21(DE3) and the expression vector pET-3d were purchased from Novagen (Madison, WI). *E. coli* strain DH5α was obtained from Bethesda Research Laboratory (Gaithersburg, MD). Deoxyoligonucleotides were synthesized by the Protein/Nucleic Acid Facility at the Medical College of Wisconsin and were purified using C_{18} Sep-Pak cartridges from Millipore (Bedford, MA). Qiagen (Chatsworth, CA) plasmid mini kits were used to isolate plasmid DNA from bacterial cultures. Qiaex (Qiagen Inc.) reagents and protocols were used for extraction of nucleic acid fragments from agarose gels. The restriction enzymes were purchased from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA). DNA sequencing was performed using an ALF automated sequencer and the autoread kit and protocol provided by Pharmacia (Milwaukee, WI). Ampicillin and isopropyl β-D-thiogalactoside were provided by United States Biochemical (Cleveland, OH). All other reagents were purchased from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), Pharmacia (Milwaukee, WI), or Bio-Rad (Richmond, CA).

Methods

Strategy for Generating Mutant Alleles of HMG-CoA Synthase. Each of the histidines at positions 197, 264, and 436 (Figure 1) was individually replaced by an asparagine. At position 264, histidine was also replaced by an alanine. The sequences of the deoxyoligonucleotides used in this procedure are listed in Table 1. H197N and H264N/A mutations were generated by the overlap extension PCR technique (Ho et al., 1989). As two restriction sites closely flanked the H436 codon, cassette mutagenesis was employed for replacement of histidine at that position. For this technique (Lo et al., 1984), a cassette composed of synthetic double-stranded deoxyoligonucleotides encoding the desired mutation was utilized to replace the wild-type sequence. The synthetic duplex is designed with restriction overhangs at 5′ and 3′ ends that facilitate ligation of the cassette into appropriately digested wild-type plasmid to produce the mutagenic expression plasmid. As there are no unique restriction sites in pACS close to the mutations that were to be engineered, it became necessary to use restriction sites that were present more than once in the expression plasmid. In the case of H197N and H264N/A substitution, a strategy was designed that involved ligation of multiple DNA fragments. However, a subcloning strategy was adopted for H436N substitution.

H197N. A 650 bp DNA fragment (Figure 2, left) encoding the mutation was generated by oePCR using *Pfu* DNA polymerase according to manufacturer's protocol. The amplification procedure included 25 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 40 s) and extension (72 °C, 30 s) followed by a cycle of extension at 72 °C for 5 min. The fragment encoding the mutation was purified by a Sephacryl-400 (Pharmacia) centrifugal column. The product was digested with *Bbs*I and *Sph*I restriction endonucleases, and the resulting 282 bp fragment was purified by extracting the DNA from the 1.6% agarose gel used for electrophoretic separation of the fragments from the digest. The purified mutagenic fragment was mixed with 271 bp *Bst*XI−*Bbs*I, 654 bp *Sph*I−*Nsi*I, and 5.3 kb *Nsi*I−*Bst*XI fragments for a four-way ligation. The rest of the fragments used in the ligation mixture were obtained by appropriate restriction and gel purification of the wild-type sequences.

H264N/A. Histidine 264 is encoded in a region spanning 266 base pairs between *Sph*I and *Bsm*I restriction sequences (Figure 2, center). A 536 bp fragment encoding either an H264N or H264A substitution was produced by oePCR according to the same amplification protocol used for the H197N substitution. Restriction of the 536 bp mutagenic fragment with *Sph*I and *Bsm*I restriction enzymes resulted in a 266 bp DNA fragment. Gel-purified mutagenic 266 bp *Sph*I−*Bsm*I, 553 bp *Bst*XI−*Sph*I, 389 bp *Bsm*I−*Nsi*I, and 5.3 kb *Nsi*I−*Bst*XI fragments were ligated. The latter three deoxynucleic acid sequences were generated by restriction and gel purification of wild-type sequences from pACS.

H436N. This substitution was performed by replacing the wild-type sequences with a cassette encoding the mutation (Figure 2, right). As the size of the cassette to be used in these experiments exceeded the practical size limit for synthetic deoxyoligonucleotides, sets of overlapping duplexes were used to produce the desired cassette (Table 1). Although two sets of duplexes could have accommodated the 96 bases necessary for the complete cassette, three duplexes were used for the H436N substitution. This allows us to engineer other substitutions in the region by shuttling only the middle duplex (which encodes the substitution) to form the complete cassette. This cassette contains *Nsi*I and *Bgl*II restriction overhangs at 5′ and 3′ ends, respectively. The *Bgl*II recognition sequence occurs twice in the plasmid; therefore, either a multiple fragment ligation protocol or a subcloning procedure had to be developed. As a multiple ligation strategy would have involved a large (six) number of fragments, the subcloning approach was adopted for H436N substitution.

The mutagenic duplex was first cloned into pKK-syn, which contains unique *Nsi*I and *Bgl*II sites, and then a larger

fragment encoding the mutation was shuttled into pACS. In order to accomplish this, the 6.4 kb pKK-syn plasmid was digested with *Bgl*II and *Nsi*I restriction enzymes. The gel purified 6.3 kb *Bgl*II−*Nsi*I fragment was ligated to the cassette (formed by denaturation and subsequent annealing of six synthetic deoxyoligonucleotides). Plasmids were purified from the selected transformants and characterized by restriction analysis and DNA sequencing. The synthase-encoding 1.8 kb *Nco*I insert was excised from the confirmed pKK-syn-H436N plasmid, gel purified, and ligated into *Nco*I restricted pET-3d, and those plasmids containing the properly oriented coding insert were selected as follows. Competent DH5α cells were transformed with the ligation mixtures to allow propagation of mutagenic plasmids, which were purified from selected transformants and characterized by restriction analysis and DNA sequencing of both strands. The verified plasmids were transformed into competent BL21-(DE3) cells for subsequent expression of different synthase variants.

Isolation and Characterization of Mutant Synthases. The procedure (Misra et al., 1993) developed for purification of the wild-type enzyme was followed for isolation of the mutant enzymes from 800 mL bacterial cultures. Protein content of the purified enzyme was estimated by the Bradford assay (Bradford, 1976), using bovine serum albumin as the standard. The purity of the enzymes was assessed by SDS−polyacrylamide gel electrophoresis.

Kinetic Characterization of Mutant Synthases. The stoichiometry of acetyl-CoA binding and of covalent enzyme acylation was determined as reported previously (Miziorko et al., 1975; Misra et al., 1993); error in the stoichiometry estimates is <20%. A standard spectrophotometric assay (Clinkenbeard et al., 1975; Misra et al., 1993) was employed to generate the initial velocity data needed for estimation of K_m for acetyl-CoA. Reaction mixtures contained a fixed concentration (20 or 250 μM, as specified) of acetoacetyl-CoA and concentrations of acetyl-CoA ranging from 25 μM to 1 mM. For estimation of K_m for acetoacetyl-CoA, 40 mM magnesium chloride was added to the assay mixture (100 mM Tris-HCl/0.1 mM EDTA, pH 8.2) to afford a 5-fold improvement in sensitivity; the millimolar extinction coefficient (300 nm) increases from 3.6 to 18.0 under these conditions. K_m for acetoacetyl-CoA was measured in the presence of constant acetyl-CoA (200 μM) and varied concentrations of acetoacetyl-CoA ranging from 0.2 to 4 μM for the wild-type, H197N, and H436N synthases and 13−150 μM for H264N/A synthases.

Initial velocity data for determination of K_m for oxobutyl-CoA were generated using a standard radioactive assay (Clinkenbeard et al., 1975; Miziorko et al., 1982) that employed a fixed concentration of [^{14}C]acetyl-CoA (200 μM, 8000 dpm/nmol) and variable concentrations of oxobutyl-CoA (10 μM to 1 mM).

Acetyl-CoA hydrolase activity of different synthases was measured as reported previously (Miziorko et al., 1975) by monitoring depletion of [^{14}C]acetyl-CoA after conversion of residual substrate to acid stable [^{14}C]citrate, using excess citrate synthase and oxaloacetate. The concentrations of acetyl-CoA employed in the assay ranged from 6 to 200 μM.

Standard kinetic parameters were estimated using nonlinear regression analysis of rate data; error in these estimates is <15%.

CONSERVED HISTIDINES IN HMG-CoA SYNTHASES

	190	257	427
mmou[a]	EQGLRGTHME	DVQYMIFHTP	IMNQREQFYH
mrat[b]	EQGLRGTHME	DVQYMIFHTP	IMNQREQFYH
mhum[c]	ERGLRGTHME	DLQYMIFHTP	IMNQREQFYH
cham[d]	DRGLRGTHMQ	DFGFMISHSP	NMKLREDTHH
crat[e]	DRGLRGTHMQ	DFGFMIFHSP	NMKLREDTHH
chum[f]	ERGLRGTHMQ	DFGFMIFHSP	NMKLREDTHH
cavi[g]	ERGLRGTHMQ	DFGFMIFHSP	NMKIRQETHH
cbge[h]	DRGVRSSHMQ	RLDAVLFHAP	TMETREHNHH
xbge[i]	DRGVRASHMK	YFDAFVFHSP	IMEIREQNHH

FIGURE 1: Alignment of deduced amino acid sequences for HMG-CoA synthases to indicate the positions of conserved histidine residues. Shaded columns indicate histidine residues invariant among all sequences. The deduced amino acid sequences were reported as indicated: [a]mouse mitochondrial isozyme (Boukaftane et al., 1994); [b]rat mitochondrial isozyme, (Ayté et al., 1990b); [c]human mitochondrial isozyme (Boukaftane et al., 1994); [d]hamster cytosolic isozyme (Gil et al., 1986); [e]rat cytosolic isozyme (Ayté et al., 1990a); [f]human cytosolic isozyme (Russ et al., 1992); [g]avian cytosolic isozyme (Kattar-Cooley et al., 1990); [h]*Blattella germanica* cytosolic isozyme-1 (Martínez-Gonzalez et al., 1993); [i]*Blattella germanica* cytosolic isozyme-2 (Buesa et al., 1994). Numbers appearing above the sequence alignment correspond to sequence assignments in the full length cytosolic isoenzymes.

RESULTS

Rationale and Strategy for Mutagenesis of Histidine Residues. Two lines of protein chemistry evidence suggest the presence of a reactive histidine within the HMG-CoA synthase active site. The enzyme is rapidly inactivated by diethyl pyrocarbonate (DEPC) modification (at 2.4 mM reagent, $t_{1/2}$ = 4.5 min at pH 6.5 and 23 °C; C. E. Behnke, unpublished results). Inactivation is coincident with appearance of a 240 nm UV absorbance peak, indicating formation of carbethoxyhistidine. As enzyme activity is reduced to baseline levels, $A_{240\ nm}$ measurement (Miles, 1977) suggests that virtually complete modification of the enzyme's 13 histidines has occurred. While multiple histidine residues are accessible to DEPC, preincubation of enzyme with substrate acetoacetyl-CoA (100 μM) afffords substantial protection against inactivation ($t_{1/2}$ > 15 min), suggesting an active site location for at least one histidine residue. Moreover, our previous report (Misra et al., 1995) on modification of a synthase mutant (C129S) that lacked the preferred target of the mechanism-based inhibitor, chloropropionyl-CoA, indicated some modification of histidine by that active site-directed reagent. The above observations, together with the precedent establishing a role for histidine residues in the mechanistically analogous citrate synthase reaction (Alter et al., 1990; Pereira et al., 1994; Karpusas et al., 1990), led us to consider evaluating possible roles of conserved histidines in the HMG-CoA synthase reaction. Use of a standard protein chemistry approach to study histidine involvement in HMG-CoA formation seemed unattractive due to the multiplicity of reactive histidines. Mutagenesis appeared to represent a more attractive approach.

Alignment of deduced amino acid sequences (Figure 1) for nine eukaryotic synthases indicates that there are invariant histidine residues at positions 197, 264, and 436. Using the mutagenic oligonucleotides listed in Table 1, plasmids encoding H197N, H264N, and H264A were constructed using overlap extension PCR (Ho et al., 1989) while the plasmid encoding H436N was prepared with a synthetic cassette (Figure 2). H→N substitutions were designed since asparagine cannot participate in acid/base catalysis but

Table 1: Synthetic Deoxyoligonucleotides Used in Mutagenesis of the HMG-CoA Synthase Gene[a]

Enzyme	Sequences of primers used for mutagenesis	Type of primer
H197N	5′ G CGT GGA ACC AAC ATG CAG CAT GC 3′	mutagenic primer
	5′ GAT GGG CTT CGC CTC TGA CCG AGA GG 3′	upstream primer
	5′ TGT ACC AGT TTA GCG TAG GGA GAA TG 3′	downstream primer
H264N	5′ TC ATG ATC TTT AAC TCT CCC TAC TG 3′	mutagenic primer
	5′ GCT GTG TAT GCC ACT GGA 3′	upstream primer
	5′ GGA CAC AAG TAA GGA AGC 3	downstream primer
H264A	5′ TC ATG ATC TTT GCT TCT CCC TAC TG 3′	mutagenic primer
	upstream and downstream primers same as used for H264N	
H436N	5′ TTG CAC CTG ATG TCT TTG CTG AAA ACA TGA AGA TTA G 3′	the cassete is composed of
	3′ A CGT AAC GTG GAC TAC AGA AAC GAC TTT TG 5′	3 oligonucleotide duplexes
	5′ A CAG GAG ACA AAC CAC TTG GCC AAC TAT ATT CC 3′	
	3′ TAC TTC TAA TCT GTC CTC TGT TTG GTG AAC CGG 5′	
	5′ ACA GTG TTC AGT AGA A 3′	
	3′ TTG ATA TAA GGT TGT CAC AAG TCA TCT TCT AG 5′	

[a] The shaded boxes indicate the base changes that were made to substitute an asparagine for a histidine at positions 197, 264, and 436 as well as an alanine for histidine at position 264.

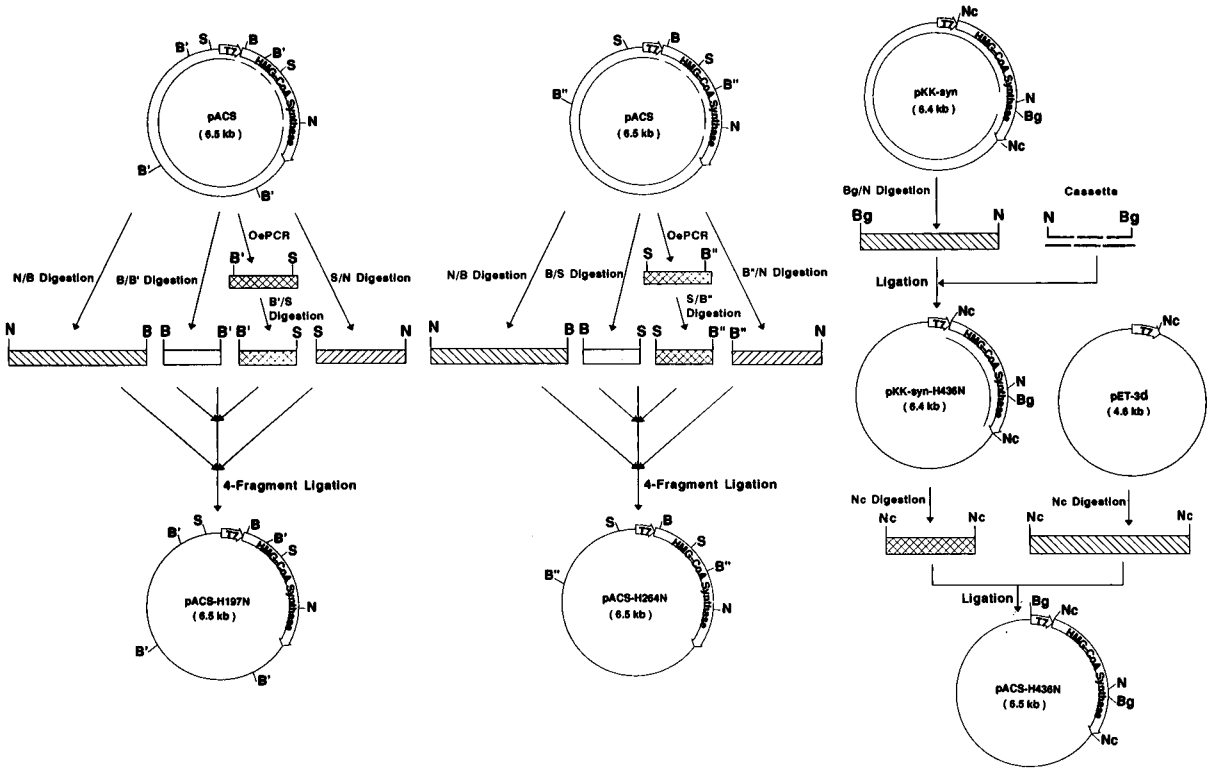

FIGURE 2: Construction of histidine mutants. For H197N (left panel), a four-fragment ligation strategy that includes 5.3 kb *Nsi*I−*Bst*XI, 272 bp *Bst*XI−*Bbs*I, 282 bp *Bbs*I−*Sph*I (encoding the H197N substitution), and 654 bp *Sph*I−*Nsi*I DNA fragments is illustrated. The restriction sites are represented as follows: B, *Bst*XI; B′, *Bbs*I; N, *Nsi*I; S, *Sph*I. For H264N/A (center panel), a four-fragment ligation that includes 5.3 kb *Nsi*I−*Bst*XI, 553 bp *Bst*XI−*Sph*I, 266 bp *Sph*I−*Bsm*I (encoding the H264N or H264A substitution), and 389 bp *Bsm*I−*Nsi*I DNA fragments is illustrated. The restriction sites are represented as follows: B, *Bst*XI; B″, *Bsm*I; N, *Nsi*I; S, *Sph*I. For H436N (right panel), the mutagenesis strategy involves replacement of wild type sequence with the mutagenic cassette (composed of three sets of overlapping duplexes) encoding the H436N substitution by initial subcloning of the cassette into pKK-syn and subsequent transfer of the 1.8 kb mutagenic *Nco*I insert into the expression plasmid (pACS). The restriction sites are represented as follows: Bg, *Bgl*II; N, *Nsi*I; Nc, *Nco*I.

maintains the potential for hydrogen bonding. Preliminary observations suggesting the value of more detailed investigation of H264 prompted engineering of an alanine substitution to eliminate any contribution to hydrogen bonding by the side chain of residue 264.

Expression and Isolation of Mutant HMG-CoA Synthases. Different HMG-CoA synthase mutant proteins encoded by various pET-3d derived expression plasmids were expressed in the bacterial host in a soluble form and at levels comparable to that observed for production of the wild-type

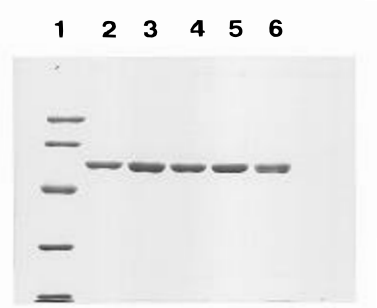

FIGURE 3: SDS−polyacrylamide gel electrophoresis of HMG-CoA synthase variants. Lane 1 contains the following molecular mass markers: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lanes 2−6 display 2 μg of the purified preparations of wild-type HMG-CoA synthase, H197N, H264N, H264A, and H436N, respectively.

Table 2: Kinetic Characterization of HMG-CoA Synthase Mutants

kinetic parameter	wild type	H197N	H264N	H264A	H436N
V_{max}[a], overall reaction (units/mg)	4.43	5.08	0.13	0.22	2.43
K_m[a], acetyl-CoA (μM)	294	440	172	122	221
K_m[b], acetoacetyl-CoA (μM)	0.85	0.55	58	115	0.62
V_{max}[c], overall reaction with oxobutyl-CoA (units/mg)	0.048	nd[d]	nd	0.002	nd
K_m[b], oxobutyl-CoA (μM)	21	nd	nd	116	nd

[a] Rates were measured in the presence of saturating levels of acetoacetyl-CoA and variable concentrations of acetyl-CoA employing a spectrophotometric assay. [b] K_m values were determined in the presence of 200 μM acetyl-CoA. [c] V_{max} was extrapolated from rates measured in the presence of 200 μM acetyl-CoA, using oxobutyl-CoA concentrations ranging from 10 uM to 1 mM. [d] nd indicates not determined.

enzyme. Mutant proteins, isolated by procedures developed for the wild-type enzyme, displayed similar chromatographic behavior and resulted in homogenous (Figure 3) and stable preparations.

Catalysis of HMG-CoA Formation by Wild-Type Enzyme and Histidine Mutants: Acetyl-CoA Dependence. In catalysis of the overall condensation reaction that produces HMG-CoA, H197N, and H436N synthases exhibit kinetic characteristics (V_{max}, K_m) very similar to those measured using wild-type enzyme (Table 2); H264N and H264A proteins, however, display some interesting differences. When measured under standard spectrophotometric assay conditions, in which [acetoacetyl-CoA] = 20 μM, the H264 mutants exhibit ~50-fold decreases in V_{max}; measurements at varying [acetyl-CoA] indicate an apparent K_m that is smaller by 10−15-fold than the value measured for wild-type enzyme. In the spectrophotometric assay (Clinkenbeard et al., 1974), progress of the condensation reaction to form HMG-CoA is monitored as disappearance of acetoacetyl-CoA ($\Delta A_{300\ nm}$). While the enzyme from a variety of sources is saturated at [acetoacetyl-CoA] $\leq 10^{-6}$ M, due to signal/noise considerations the assay is normally performed in the presence of $\geq$20 μM acetoacetyl-CoA. Such a concentration of acetoacetyl-CoA is somewhat inhibitory to the wild-type enzyme because this second substrate can compete with the first substrate, acetyl-CoA, for binding to the free enzyme; this inhibitory phenomenon is commonly observed in enzymes that follow classic ping-pong bi-bi mechanisms (Cleland, 1970). Therefore, $K_{m\ acetyl-CoA}$ determined for wild-type enzyme under these assay conditions is somewhat inflated over any intrinsic K_m value. One of the factors responsible for an *apparent* decrease in $K_{m\ acetyl-CoA}$ for the H264 mutants (as measured at an acetoacetyl-CoA concentration used in a standard assay) may be diminished binding affinity of the second substrate to the free enzyme, i.e., reduced substrate inhibition. When measured at [acetoacetyl-CoA] sufficiently elevated to saturate the H264 mutants, $K_{m\ acetyl-CoA}$ estimates rebound to values not more than 2.5-fold lower than observed with wild-type enzyme although V_{max} values remain depressed by ~25-fold (Table 2). These observations support the hypothesis that, in addition to accounting for modest effects on catalytic efficiency, H264 may selectively interact with one of the enzyme's two substrates. In order to better discriminate between H264's impact on either substrate, it appeared useful to measure and compare the K_m values for acetyl-CoA using an approach that would be uncomplicated by the presence of acetoacetyl-CoA.

Table 3: Partial Reactions Catalyzed by HMG-CoA Synthase Mutants

	wild type	H264N	H264A
V_{max}[a], acetyl-CoA hydrolysis (units/mg)	0.03	0.17	0.12
K_m[a], acetyl-CoA (μM, acetyl-CoA hydrolysis)	15	38	36
stoichiometry of acetyl-coa binding[b]	1.01	0.30	0.60
stoichiometry, covalent[c]	0.70	0.21	0.53

[a] Hydrolase activity was measured by converting the remaining [^{14}C]acetyl-CoA to acid stable citrate with excess citrate synthase and oxaloacetate. [b] Determined by rapid centrifugal gel filtration after incubation of enzyme with 1 mM acetyl-CoA. [c] Determined by TCA precipitation after incubation of enzyme with 1 mM acetyl-CoA.

Catalysis of Partial Reactions by Wild-Type and H264 Mutant HMG-CoA Synthases: Acetyl-CoA Dependence. In the absence of its second substrate, HMG-CoA synthase catalyzes hydrolysis of acetyl-CoA at a rate which is ~1% of the overall reaction rate (Miziorko et al., 1975). $K_{m\ acetyl-CoA}$, measured for this partial reaction, is 15 μM (Table 3). For acetyl-CoA hydrolysis, H264N/A synthases exhibit K_m values (36−38 μM; Table 3) approaching those measured for wild-type enzyme, suggesting the integrity of the acetyl-CoA binding site of these mutants.

An additional test of acetyl-CoA binding site integrity is afforded by binding experiments employing R·CoA, a spin labeled substrate analog (Weidman et al., 1973) that competes for the acetyl-CoA site (Miziorko et al., 1979). In binding experiments with H264N, Scatchard analysis indicates that the mutant contains a full complement (n = 1.07) of binding sites with affinity (K_d = 146 μM; C. Narasimhan, unpublished results) for R·CoA that approaches the value obtained with wild-type enzyme (K_d = 102 μM; Misra et al., 1993). EPR spectral parameters for the H264N-bound spin label suggest a rotational correlation time τ_c = 30 ns, which compares well with the estimate published for wild-type enzyme (τ_c = 35 ns; Misra et al., 1993). These observations suggests that replacement of the H264 imidazole perturbs neither the enzyme's overall tertiary structure nor the active site substrate binding pocket to any significant extent.

Another approach to evaluating interaction of acetyl-CoA with HMG-CoA synthase involves measurement of isolated

tight binary complexes or of covalently acetylated enzyme. Centrifugal gel filtration affords an estimate of the binding stoichiometry (Table 3) for radiolabeled acetyl-CoA which includes contributions both from the tight Michaelis complex as well as from covalently acetylated protein. The estimates for H264N and H264A (0.30 and 0.60 per subunit, respectively) are only modestly diminished from the stoichiometric labeling observed with wild-type enzyme. Likewise, the stoichiometry of covalent C129 acetylation of H264N (0.21) and H264A (0.53) enzymes differs by only a factor of 2−3 from the estimate for wild-type enzyme (0.70). Some diminution in binding stoichiometry in these experiments may be explained by the 4−6-fold enhancement of the competing abortive acetyl-CoA hydrolase activity in these mutants (H264N = 0.17 unit/mg; H264A = 0.12 unit/mg). Nonetheless, these collected observations reinforce the argument that replacement of H264 does not dramatically perturb the interaction between enzyme and acetyl-CoA.

Kinetic Characterization of Enzyme−Acetoacetyl-CoA Interactions. To test the hypothesis that the contrasts between H264N or H264A and the wild-type, H197N, and H436N enzymes are largely attributable to altered interaction with the *second* substrate, direct determination of the K_m for that substrate becomes necessary. In the case of acetoacetyl-CoA, the physiologically relevant second substrate for HMG-CoA synthase, experimental results support the hypothesis. There is a two orders of magnitude increase in the observed K_m values for H264N and H264A synthases (58 and 115 μM, respectively; Table 2) in comparison with the corresponding values for the wild-type enzyme (0.85 μM) or for the H197N and H436N mutants (0.55 and 0.62 μM, respectively).

The possibility that H264 interacts with the second substrate of HMG-CoA synthase can also be investigated using an alternative substrate. In previous studies with the liver enzyme (Miziorko et al., 1982), it has been documented that *S*-(3-oxobutyl)-CoA, the thio*ether* analog of acetoacetyl-CoA, functions as a second substrate for avian liver HMG-CoA synthase. The recombinant avian cytoplasmic enzyme employed in this study also has the ability to utilize *S*-(3-oxobutyl)-CoA to form a thioether analog of HMG-CoA, exhibiting apparent K_m (21 μM) and V_{max} (0.048 unit/mg) values in excellent agreement with those reported for the mitochondrial enzyme. The data (Table 2) indicate that there is almost a two orders of magnitude diminution in V_{max} and a 25-fold increase in K_m for oxobutyl-CoA in comparison with the values determined using acetoacetyl-CoA as a second substrate. These observations with the thioether analog suggest that, for wild-type HMG-CoA synthase, the presence of the thioester carbonyl may be important for anchoring of acetoacetyl-CoA. Turnover of *S*-(3-oxobutyl)-CoA with H264A synthase[2] allows us to directly test the more specific hypothesis that H264 ordinarily interacts with the thioester carbonyl of acetoacetyl-CoA. H264A utilizes *S*-(3-oxobutyl)-CoA at a rate that is also ∼100-fold lower than measured with acetoacetyl-CoA; this diminution is comparable in magnitude to that observed using wild-type enzyme. In contrast, with H264A there is no penalty in terms of the K_m parameter when the alternative thioether substrate is employed; $K_{m\ S-(3-oxobutyl)-CoA}$ (116 μM; Table 2) is indistinguishable from $K_{m\ acetoacetyl-CoA}$ (115 μM). If a hypothesis that binding energy derives from interaction of the second substrate's thioester carbonyl with H264's imidazole is indeed correct, it seems reasonable that H264A, which lacks the putative interacting imidazole, should not discriminate between the thioester-containing and the carbonyl-deficient thioether second substrates.

[2] H264A, rather than H264N, was selected for this experiment since it less actively catalyzes the hydrolysis of acetyl-CoA, thus minimizing the competition for that substrate when the slow condensation reaction with oxobutyl-CoA is measured.

DISCUSSION

The availability of a recombinant form of avian HMG-CoA synthase (Misra et al., 1993) has expedited our previous investigation of the initial steps in the enzyme-catalyzed reaction. For example, the function of cysteine-129, which had been proposed, on the basis of protein chemistry studies, to support formation of the acetyl-*S*-enzyme reaction intermediate (Vollmer et al., 1989), was confirmed by directed mutagenesis experiments (Misra et al., 1993). These experiments also demonstrated that a serine hydroxyl will not function as a replacement; the requirement for a cysteine thiol in supporting formation of this reaction intermediate is absolute. The identification of additional active site amino acids by traditional affinity labeling and protein chemistry approaches has been complicated by the presence of other reactive cysteines, which map in this region (Miziorko et al., 1990) but are not crucial to reaction chemistry (Misra et al., 1995). Our observations on the sensitivity of the enzyme to treatment with the histidine-directed group specific reagent, diethypyrocarbonate, and the fact that histidine is a secondary target of the mechanism-based inhibitor, 3-chloropropionyl-CoA, prompted us to now address the possible functions for imidazole groups in the HMG-CoA synthase reaction. The recent addition of sequences of invertebrate HMG-CoA synthases (Martinez-Gonzalez et al., 1993; Buesa et al., 1994) to the data base reduced the number of histidines that are invariant in homology alignments to three, making direct evaluation of histidine function by directed mutagenesis a manageable task.

In accounting for the results of H264 replacement, the partial reactions catalyzed by the enzyme represent useful tools that allow selective examination of several of the steps involved in HMG-CoA formation. The slow hydrolysis of acetyl-CoA in the absence of second substrate catalyzed by the purified liver enzyme (Miziorko et al., 1975) is a trait closely matched in terms of rate and K_m by the recombinant enzyme (Misra et al., 1993). This observation suggests that this partial reaction is indeed attributable to synthase and not due to a contaminant protein from liver or *E. coli*. In the context of the H264 mutants, lack of substantial perturbation of the acetyl-CoA hydrolysis partial reaction, as well as other evidence presented above, suggests that H264 is not critical to interaction with the first substrate. The slight hydrolysis rate increases that are supported by the mutants may suggest that, with these proteins, there may be enhanced access of solvent to the labile thioester linkage. The modest effects of H264 replacement on formation of the covalent acetyl-*S*-enzyme reaction intermediate also argue that this residue primarily influences a subsequent step in the reaction pathway. Any consideration of H264 as the catalytic base that deprotonates C2 of acetyl-*S*-enzyme would appear to be precluded by the expectation that elimination of such a residue should decrease k_{cat} by several orders of magnitude

(Alter et al., 1990), an effect much larger than observed upon H264 substitution.

These observations and the significant increases in the K_m for acetoacetyl-CoA argue that altered interaction with second substrate is the primary effect of H264 replacement. The other novel tool that proves quite valuable for testing this assertion is the alternative substrate, *S*-(3-oxobutyl)-CoA. The close agreement between H264A and wild-type synthases in magnitude of rate diminution when the thioether substrate replaces acetoacetyl-CoA contrasts sharply with the observation that H264A's K_m for oxobutyl-CoA is equivalent to that measured for the physiological second substrate. The empirically measured K_m for the second substrate in a ping-pong bi-bi reaction is, however, a complex parameter that includes both rate and binding constants. While this precludes straightforward extrapolation from observed K_m values to binding affinities, the collected data clearly support a postulate of interaction between H264's imidazole side chain and the thioester carbonyl of acetoacetyl-CoA. Future additional investigation of the proposed interaction would seem to be worthwhile.

While structural information for enzymes that catalyze related reactions is limited, results with citrate synthase offer some relevant precedent. In catalyzing the condensation of acetyl-CoA with oxaloacetate, citrate synthase's H274 interacts with the thioester carbonyl of acetyl-CoA in promoting enolization of this substrate (Karpusas et al., 1990). More recent data (Kurz et al., 1995) implicate citrate synthase's H320 in contributing not only to oxaloacetate carbonyl polarization but to acetyl-CoA enolization as well. No comparable chemical role is required for HMG-CoA synthase's H264, since the second substrate's thioester carbonyl is not directly involved in the chemistry of the condensation reaction. In fact, while H264 is conserved in the animal and invertebrate HMG-CoA synthases for which sequences have been deduced, its function could be served by other amino acids. Thus it remains possible that, as sequences are elucidated from lower organisms, residues other than histidine will be implicated in stabilization of acetoacetyl-CoA binding. Nonetheless, the citrate synthase example discussed above adds plausibility to the histidine−thioester carbonyl interaction currently proposed on the basis of the kinetic characterization of avian HMG-CoA synthase H264 mutants.

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