In order to evaluate the potential contribution of conserved aromatic residues to the hydrophobic active site of 3-hydroxy-3-methylglutaryl-CoA synthase, site-directed mutagenesis was employed to produce Y130L, Y163L, F204L, Y225L, Y346L, and Y376L proteins. Each mutant protein was expressed at levels comparable with wild-type enzyme and was isolated in highly purified form. Initial kinetic characterization indicated that F204L exhibits a substantial (>300-fold) decrease in catalytic rate ($k_{cat}$). Upon modification with the mechanism-based inhibitor, 3-chloropropionyl-CoA, or in formation of a stable binary complex with acetoacetyl-CoA, F204L exhibits binding stoichiometries comparable with wild-type enzyme, suggesting substantial retention of active site integrity. Y130L and Y376L exhibit inflated values (80- and 40-fold, respectively) for the $K_m$ for acetyl-CoA in the acetyl-CoA hydrosylation partial reaction; these mutants also exhibit inverse magnitude decrease in $k_{cat}$. Formation of the acetyl-S-enzyme reaction intermediate by Y130L, F204L, and Y376L proceeds slowly in comparison with wild-type enzyme. However, solvent exchange into the thioester carbonyl oxygen of these acetyl-S-enzyme intermediates is not slow in comparison with previous observations for D159A and D203A mutants, which also exhibit slow acetyl-S-enzyme formation. The magnitude of the differential isotope shift upon exchange of $H_2$-18O into [13C]acetyl-S-enzyme suggests a polarization of the thioester carbonyl and a reduction in bond order. Such an effect may substantially contribute to the upfield [13C]NMR shift observed for [13C]acetate-S-enzyme. The influence on acetyl-S-enzyme formation, as well as observed $K_m$ (F204L) and $K_{cat}$ (Y130L; Y376L) effects, implicate these invariant residues as part of the catalytic site. Substitution of phenylalanine (Y130F, Y376F) instead of leucine at residues 130 and 376 diminishes the effects on catalytic rate and substrate affinity observed for Y130L and Y376L, underscoring the influence of aromatic side chains near the active site.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)\textsuperscript{1} synthase catalyzes a committed step in the pathways for isoprenoid, cholesterol, and ketone body production. The cytosolic isomerase, involved in isoprenoid/cholesterol biosynthesis, is transcribedentially regulated (1) by mechanisms distinct from those that mediate transcriptional control of the mitochondrial isoform (2) that supports ketogenesis.

The condensation of substrates acetyl-CoA and acetoacetyl-CoA (AcAc-CoA) to form HMG-CoA may be viewed as a three-step process (3) involving formation of an acetyl-S-enzyme intermediate, condensation to form a transient enzyme-S-HMG-CoA intermediate, and hydrolysis to release product HMG-CoA (Scheme 1).

Early mechanistic and protein chemistry studies led to selective modification (4) and mapping (5) of the cytochrome that forms the acetyl-S-enzyme intermediate. Development of a recombinant form of the avian cytosolic enzyme (6) allowed the demonstration of the strict requirement for cysteine in formation of this intermediate. Evaluation of invariant residues implicated as active site residues not only a histidine (7) but also several acidic residues that influence either formation of the acetyl-S-enzyme intermediate (8) or condensation of this intermediate with the second substrate (9).

Recent work on the acetyl-S-enzyme intermediate (9, 10) has suggested that the active site has substantial hydrophobic character. A report on acetoacetyl-CoA binding to acyl-CoA dehydrogenase (11) documented large NMR shifts for the bound metabolite that are comparable in magnitude with those observed for HMG-CoA synthase’s acetyl-S-enzyme intermediate (10, 12). Model studies and molecular orbital calculations (11) suggested that the effect was potentially attributable to stacking of metabolite with the heterocyclic flavin cofactor of acyl-CoA dehydrogenase. These observations suggested the potential influence of hydrophobic, aromatic residues at the active site of HMG-CoA synthase and prompted evaluation of the importance of aromatic residues that are conserved in this protein. This report documents the characterization of mutant enzymes in which aromatic side chains have been replaced and indicates that several conserved aromatic residues influence either the catalytic rate or enzyme-substrate interactions.

**EXPERIMENTAL PROCEDURES**

Materials

*Escherichia coli* BL21 (DE3) and the expression vector pET-3d were purchased from Novagen (Madison, WI). Deoxyoligonucleotides were purchased from Operon Technologies (Alameda, CA). QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Qiagen (Chatsworth, CA) plasmid kits were used to isolate plasmid DNA. DNA ligase were purchased from New England Biolabs (Beverly, MA) and Amersham Biosciences. DNA sequencing was performed on an ABI 3100 Genetic Analyzer at the Protein/Nucleic Acid Facility of the Medical College of Wisconsin. Ampicillin and isopropylthiogalactoside were purchased from U.S. Biochemical Corp. [1-14C]Acetyl-CoA and ethyl[3-14C]Acetate were products of Moravek Biochemicals (Brea, CA). 3-Chloro-[1-14C]propionic acid was purchased from American Radiolabeled Chemicals (St. Louis, MO). [1,2-13C]Acetic anhydride, used for the production of [1,2-13C]Acetyl-CoA (13), was a product of IsoTech.

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**The Influence of Conserved Aromatic Residues in 3-Hydroxy-3-methylglutaryl-CoA Synthase**

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1 The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; AcAc, acetoacetyl; ACP, acyl carrier peptide.
(Miamisburg, OH). All other reagents were purchased from Sigma, Aldrich, or Amersham Biosciences.

**Methods**

**Sequence Homology Analysis**—All sequences used in the alignment analysis are defined in the published data bases as HMG-CoA synthases. Only sequences encoding full-length proteins were included in the analysis. Amino acid sequences were aligned using the Pileup program in the Genetics Computer Group-Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI).

**Synthesis of Radiolabeled 3-Chloropropionyl-CoA—**Synthesis was performed according to the procedures of Mizioriko and Behnke (4) except that ethylchloroformate was used to activate the 3-chloro-[1-14C]propionic acid to the mixed anhydride, and this intermediate was used to thioesterify CoASH. The product was precipitated from cold methanol/acetone (1:4). The purified product was assessed for concentration and purity by UV spectroscopy and reverse-phase high pressure liquid chromatography.

**DNA was isolated from selected transfectants and analyzed by restriction mapping and DNA sequencing. The verified mutant clones were transformed into competent BL21(DE3) cells for expression and isolation as previously described for wild type HMG-CoA synthase (6).**

**Isolation of HMG-CoA Synthase—**The procedure developed for purification of the wild-type enzyme (6) was followed for isolation of the aromatic substituted enzymes from isopropyl thiogalactoside-induced bacterial cultures. Protein content of the purified enzymes was estimated by the Bradford assay (15), using bovine serum albumin as the standard. The purity of the enzymes was assessed by SDS-polyacrylamide gel electrophoresis.

**Activity Assay and Enzymological Characterization of HMG-CoA Synthase Mutants—**Either the standard spectrophotometric assay (6, 16) or the more sensitive radioisotopic assay (16) was used to measure activity. In the spectrophotometric assay, the reaction mix included 100 mM Tris-Cl, pH 8.2, 100 μM EDTA, appropriate amounts of HMG-CoA synthase (~6 μg for wild-type enzyme), 20 μM acetoacetyl-CoA, and 200 μM acetyl-CoA (unless varied concentrations were used for Kₗ determinations). The reaction was performed at 30 °C, and acetyl-CoA-dependent loss of acetoacetyl-CoA was measured as a decrease in 300-nm absorbance, using a millimolar extinction coefficient of 3.6. For improved sensitivity, the spectrophotometric assay can be performed in the presence of 40 mM MgCl₂ in such cases, the millimolar extinction coefficient of 20.0 is used for acetoacetyl-CoA.

For the radioisotopic assay, the reaction mixture included 100 mM Tris-HCl, pH 8.2, 100 μM EDTA, 20 μM acetoacetyl-CoA, 200 μM [1-14C]acetyl-CoA (8600–10,000 dpm/nmol) (unless reagent concentration was varied for Kₗ determinations), and appropriate amounts of wild-type or mutant HMG-CoA synthase. The reaction was initiated by the addition of radiolabeled acetyl-CoA to the assay mixture containing the rest of the components at 30 °C. At specified time intervals, 40-μl aliquots were removed from the incubation mixture and acidified with 6 N HCl. The mixture was heated to dryness, and acid-stable radioactivity due to [1-14C]-HMG-CoA was measured by liquid scintillation counting.

Acetyl-CoA hydrolyase activity of wild type and mutant synthases was measured as reported previously (17) by monitoring enzyme-dependent depletion of [1-14C]acetate by conversion of residual substrate to acid-stable [1-14C]citrate, using excess citrate synthase and oxaloacetate.

**Stoichiometry of covalent acetylation (i.e. acetyl-S-enzyme formation) was determined according to the procedure described by Mizioriko et al. (17) with minor modifications.** 45-μl (90 μg of enzyme) aliquots of incubation mixture containing 1 mM [1-14C]acetyl-CoA (8600–10,000 dpm/nmol) and enzyme (2 mg/ml) in 100 mM sodium phosphate buffer, pH 7.0, were treated with 1 ml of ice-cold 10% trichloroacetic acid. The denatured protein was transferred to a glass fiber filter. The filters were washed extensively with ice-cold 10% trichloroacetic acid, followed...
Aromatic Residues in HMG-CoA Synthase

by 50 mm sodium pyrophosphate in 500 mm HCl and once with cold absolute ethanol. Filters were dried, and radioactivity was determined by liquid scintillation counting. Rates of covalent acetylation were determined by taking aliquots at specified times after initiation of the reaction by [1-14C]acetyl-CoA addition and isolating covalent acetyl-S-enzyme as described above. Rates were determined by linear regression analysis of the initial linear region of the time course.

Modification stoichiometry upon inactivation of HMG-CoA synthase by the mechanism-based inhibitor, 3-chloro-[1-14C]propionyl-CoA, was measured according the following protocol. 25 μl aliquots of an incubation mixture containing 3-chloro-[1-14C]propionyl-CoA (126 nmol, 10,000 dpm/nmol) and enzyme (6 nmol) in 100 mM sodium phosphate, pH 7.5, were removed and treated with 1 ml of ice-cold 10% trichloroacetic acid, followed by 50 mM sodium pyrophosphate in H2O, 10% D2O and 0.5 mM EDTA. A minimum of two spectra were consecutively run for each spectrum, except for samples containing F204L, where 2500 transients were recorded. Spectra were recorded at 23°C. Chemical shifts are not corrected for dilution of 18O-enriched solvent by inclusion of 10% D2O in the sample.

The stoichiometry of noncovalent acetoacetyl-CoA binding was determined using a modification of the procedure of Vollmer et al. (18). The enzyme (150 μg) in 100 mM sodium phosphate, pH 7.0, was incubated for 5 min at 30°C. [1-14C]acetoacetyl-CoA (2500 dpm/nmol) was added to bring the final concentration to 1000 μM. Unbound acetoacetyl-CoA was removed using a Sephadex G-50 centrifugal column equilibrated with 100 mM sodium phosphate buffer, pH 7.0. Protein in the recovered samples was estimated by the Bradford assay, and radioactivity bound to enzyme was determined by liquid scintillation counting.

13C NMR Measurements—For measurement of the chemical shifts of [1-12C]acetyl-S-enzyme or [1-13C]acetyl-S-CoA, proton-decoupled experiments were performed using a Bruker AC-300 instrument operating at 75.5 MHz for 13C. Spectra were recorded at 21°C. Chemical shifts are referenced to TMS. 16,000 data point spectra were recorded using a 35° pulse angle and a 2-s pulse delay. 6000 transients were acquired for each spectrum. Samples contained 0.5 mM enzyme sites and 1.5 mM [1-13C]acetyl-CoA in 20 mM potassium phosphate, pH 7.0, containing 50% D2O for a lock signal.

For measurement of 15O differential isotope shifts of the mutant or wild-type [1-12C]acetyl-S-enzymes, a Bruker DRX 600 spectrometer, equipped with a QNP probe operating at 151 MHz for 13C was used. Proton-decoupled spectra (32,000 data points) were acquired using a

Table 1

Comparison between catalytic properties of wild-type and aromatic mutant HMG-CoA synthases

| Enzyme | Vmax (overall reaction) | Km of acetyl-CoA (overall reaction) | Km of acetyl-CoA (hydrolysis partial reaction) |
|--------|-------------------------|------------------------------------|-----------------------------------------------|
| Wild type* | 4.40 ± 0.39 mmol/mg | 290 ± 22 μM | 14 ± 2 μM |
| Y130L*   | 0.30 ± 0.03 mmol/mg | 992 ± 233 μM | 1268 ± 484 μM |
| Y163L    | 0.78 ± 0.06 mmol/mg | 200 ± 40 μM | 12 ± 2 μM |
| F204L    | 0.012 ± 0.001 mmol/mg | 260 ± 59 μM | 22 ± 3 μM |
| Y225L    | 1.66 ± 0.06 mmol/mg | 125 ± 12 μM | 2 ± 0.3 μM |
| Y346L    | 0.38 ± 0.01 mmol/mg | 604 ± 40 μM | 20 ± 4 μM |
| Y376L*   | 0.44 ± 0.01 mmol/mg | 604 ± 36 μM | 608 ± 110 μM |

* Data for wild-type enzyme are taken from Ref. 8.

For Y130F, Vmax = 4.93 ± 0.22 units/mg; Km of acetyl-CoA = 565 ± 65 μM (overall reaction); Km of acetyl-CoA = 28 ± 8 μM (hydrolysis partial reaction).

For Y376L, Vmax = 4.95 ± 0.04 units/mg; Km of acetyl-CoA = 307 ± 8 μM (overall reaction).

45° pulse angle and a 2-s pulse delay. 1500 transients were recorded for each spectrum, except for samples containing F204L, where 2500 transients were recorded. Spectra were recorded at 23°C. Samples were prepared by dissolving matched, buffered, freeze-dried proteins in either reagent grade 18O water, supplemented with 10% D2O, or 1H-18O-enriched (95%) water supplemented with 10% D2O. Final samples (0.5 ml) contained 0.6 mM enzyme sites and 1.3 mM [1-12C]acetyl-CoA in 20 mM potassium phosphate, pH 7.0, supplemented with 0.5 mM dithiothreitol and 0.5 mM EDTA. A minimum of two spectra were consecutively run for each 15O- or 1H-18O-containing sample. Additional spectra for 18O samples were run after overnight incubation (minimum 12 h) with no further changes in isotope shifts being detected. Reported isotope shifts are not corrected for dilution of 1H-18O-enriched solvent by inclusion of 10% D2O in the sample.

RESULTS

Strategy for Identification of Mutagenesis Targets—Alignments of 28 deduced sequences for HMG-CoA synthases indicated that six aromatic residues are invariant or highly conserved. Fig. 1 depicts regions from an alignment of 10 representative sequences, selected from fungal, plant, animal, and bacterial proteins. These regions include Tyr130, Tyr163, Phe204, Tyr225, Tyr346, and Tyr376 (residue numbering corresponds to animal cytosolic proteins). The lineups also contain residues Cys129, Asp159, and Asp252, which have been demonstrated (6, 8) to have a large impact on catalysis. The sequences flanking Tyr246 and Tyr276 are among the most conserved regions observed upon alignment of HMG-CoA synthases. In order to eliminate side chain aromaticity without changing the hydrophobicity, plasmids encoding leucine substitutions for each of these aromatic residues were constructed by mutagenesis of the pET-3d-derived plasmid that encodes wild-type avian HMG-CoA synthase. These plasmids were validated by
DNA sequencing to ensure the presence of the mutated codon and the absence of PCR artifacts.

Expression, Isolation, and Preliminary Characterization of Mutant HMG-CoA Synthases—Plasmids encoding leucine substitutions for the aromatic residues were transformed into E. coli BL21 (DE3) and used for protein expression. Soluble mutant proteins were produced at levels comparable with those achieved when expressing wild-type avian cytosolic enzyme. Purification of the mutants employed the procedure described for wild-type enzyme (6). Each of the six mutant enzymes was recovered from anion exchange chromatography (Amersham Biosciences fast-Q resin) in substantially homogeneous form (Fig. 2), eluting at an ionic strength comparable with that used for isolation of wild-type enzyme.

Each of the six mutants was characterized by a variety of kinetic and binding stoichiometry measurements. Table I summarizes the measured values for \( V_{\text{max}} \) in the overall condensation reaction, \( K_m \) for acetyl-CoA in the overall condensation reaction, and \( K_m \) for acetyl-CoA in the partial reaction in which acetyl-CoA hydrolysis occurs in the absence of the second substrate, acetoacetate-CoA. Due to substrate inhibition by acetoacetate-CoA, the \( K_m \) value for acetyl-CoA in the overall reaction does not correlate as well with intrinsic binding affinity as does the corresponding \( K_m \) value measured in the hydrolysis partial reaction (6, 17).

Upon comparison of the \( V_{\text{max}} \) values in Table I, it is apparent that, with the exception of F204L, the substitution of aromatic side chains has only modest effects on catalysis, with changes of approximately 1 order of magnitude or less being observed. In the case of F204L, \( V_{\text{max}} \) is decreased by over 300-fold; this effect seems substantial for substitution of a side chain that should not be involved in reaction chemistry or traditional hydrogen bonding. Little effect of this substitution is observed for \( K_m \) acetyl-CoA values in the overall reaction or the hydrolysis partial reaction. The other contrasts that appear in Table I include a 2–3-fold inflation in the \( K_m \) acetyl-CoA values for Y130L, Y346L, and Y376L, as measured in the overall condensation reaction. \( K_m \) of acetyl-CoA in the hydrolysis partial reaction correlates more closely with the saturation of enzyme by acetyl-CoA to form the acetyl-S-enzyme (Fig. 3), providing a better estimate of intrinsic affinity of enzyme for this substrate. Upon inspection of the values measured for \( K_m \) of acetyl-CoA in the hydrolysis partial reaction, substantial inflation of \( K_m \) is observed for Y130L and Y376L, respectively, in comparison with the value for wild-type enzyme. Thus, these substitutions most notably influence acetyl-CoA saturation and have a smaller, albeit significant, effect on catalytic rate. In order to evaluate whether the loss of tyrosine’s alcohol substituent or the aromatic ring accounted for the effects observed with the leucine-substituted proteins, Y130F and Y376F mutant enzymes were also constructed, expressed, and isolated. Characterization of these phenylalanine substituted enzymes indicated catalytic rates that are comparable with wild-type enzyme (Y130F: \( V_{\text{max}} = 4.93 \) units/mg; Y376F: \( V_{\text{max}} = 4.95 \) units/mg). Changes in \( K_m \) acetyl-CoA in the overall reaction are also less pronounced (Y130F: \( K_m \) of acetyl-CoA = 565 ± 65 \( \mu \)M (overall reaction; this value diminishes to 28 ± 8 \( \mu \)M for the hydrolysis partial reaction); Y376F: \( K_m \) of acetyl-CoA = 307 ± 8 \( \mu \)M (overall reaction)). On the basis of the contrasts measured for F204L, Y130L, and Y376L in comparison with wild-type enzyme, these three mutants were subjected to more detailed enzymological and biophysical characterization.

![Fig. 3. Dependence of acetyl-S-enzyme formation on the concentration of acetyl-CoA. Recombinant avian cytosolic HMG-CoA synthase (1.4 \( \mu \)M) is incubated in 100 mM sodium phosphate buffer, pH 7.0, for 5 min at 23 °C prior to the addition of \( ^{14} \text{C} \) acetyl-CoA (46,000 dpm/nmol) at the concentrations indicated. After 20 s, the reaction was quenched by the addition of cold 10% trichloroacetic acid. Denatured \( ^{14} \text{C} \) acetyl-S-enzyme intermediate is trapped on glass fiber filters and measured by liquid scintillation counting after the removal of noncovalently bound radioactivity by the filter washing procedure described under “Methods.”](image-url)

**Table II**

Kinetic parameters and binding properties of wild-type and aromatic mutant HMG-CoA synthases

Procedures for assay of overall and partial reactions, as well as binding/modification stoichiometry experiments are described under “Methods.” \( V_{\text{max}} \) and \( K_m \) estimates were determined by nonlinear regression analysis of data fit to a hyperbolic saturation curve.

| Parameter | Wild type | Y130L | F204L | Y376L |
|-----------|-----------|-------|-------|-------|
| \( V_{\text{max}} \) (Overall reaction) (units/mg) | 4.40 ± 0.39 | 0.30 ± 0.03 | 0.012 ± 0.001 | 0.44 ± 0.01 |
| \( K_m \) of AcAc-CoA (\( \mu \)M) | 0.9 ± 0.1 | 3.3 ± 0.4 | <5 | 3.1 ± 0.2 |
| \( V_{\text{max}} \) (hydrolysis) (units/mg) | 0.018 ± 0.002 | 0.012 ± 0.003 | 0.0020 ± 0.0001 | 0.018 ± 0.001 |
| Acetylation rate (units/mg) | 1.59 ± 0.16 | 0.0031 ± 0.0003 | 0.0016 ± 0.0001 | 0.0050 ± 0.0001 |
| Acetylation stoichiometry | 0.62 ± 0.03 | 0.54 ± 0.02 | 0.35 ± 0.05 | 0.54 ± 0.01 |
| Cl-prop-CoA modification stoichiometry | 0.7 ± 0.07 | — | 0.8 ± 0.05 | — |
| AcAc-CoA binding stoichiometry | 0.8 ± 0.05 | — | 1.0 ± 0.08 | — |

* Data taken from Ref. 8.

* Data taken from Ref. 7.

* Data taken from Ref. 6.

* Value not determined for this mutant.
Aromatic Residues in HMG-CoA Synthase

have a large influence on interaction with this second substrate. Upon comparison of the mutants’ ability to catalyze the acetyl-CoA hydrolysis partial reaction, the most notable contrast with the value measured for wild-type enzyme occurs for F204L (Table II), once again reflecting a difference in catalytic efficiency as a dominant characteristic of this mutant.

Initial measurements of the formation of the covalent acetyl-S-enzyme reaction intermediate upon short (5-s) incubations with acetyl-CoA indicated that each of these mutants exhibited lower levels of trichloroacetic acid-precipitable adduct than measured for wild-type enzyme. This prompted experiments (Fig. 4) that measured the time course of covalent acetyl-S-enzyme formation. Initial rates for formation of this reaction intermediate (Table II) could be measured due to the slow kinetics of this process. Comparison of these rates with steady state rates of condensation or hydrolysis reactions is not straightforward due to differences in pH and other reaction conditions used in the various assays (a correction factor of >9-fold is required (8)). Nonetheless, the acetylation rates of Y130L, F204L, and Y376L mutants are significantly lower than the value measured for wild-type enzyme. A similar effect has been observed (8) upon substitution to eliminate the carboxyl side chain of Asp159 also slows covalent acetylation (8). In contrast, Y163L (which is found in the same conserved sequence as Asp159, Fig. 1) exhibits a rapid formation of covalent intermediate (data not shown). Rapid acetylation also occurs for Y130F and Y376F mutants, indicating the importance of an aromatic side chain at residues 130 and 376.

Upon incubation with acetyl-CoA until limiting covalent acetylation occurs, the stoichiometry of covalent intermediate formation by the aromatic mutants Y130L (0.5 per site) and Y376L (0.6 per site) approximates the value measured for wild-type avian cytosolic enzyme (Table II). The value measured for F204L reflects a 40% decrease. Such a value may reflect an altered equilibrium between the noncovalent Michaelis acetyl-CoA complex and the covalent reaction intermediate. Nonetheless, in view of the reduced acetylation stoichiometry, it seemed important to determine whether the F204L mutant retains a full complement of functional binding sites, so additional studies were performed. Incubation of enzyme with the mechanism-based inhibitor, 3-chloropropionyl-CoA (4), results in comparable modification (alkylation) stoichiometries for wild-type (0.7 per site) and F204L (0.8 per site) proteins (Table II). Additionally, formation of a binary noncovalent complex between avian HMG-CoA synthase and [14C]AcAc-CoA can be detected by centrifugal gel filtration. The binding stoichiometries measured for wild-type (0.8 per site) and F204L (1.0 per site) enzymes are comparable (Table II). Thus, on the basis of these comparable modification and binary complex stoichiometry results, the active site of F204L seems to be largely intact. Consequently, the decrease in catalytic activity exhibited by this mutant appears to be significant.

Biophysical Characterization of HMG-CoA Synthase Mutants—To determine whether aromatic side chains in HMG-CoA synthase might influence the large upfield chemical shift observed for C-1 of acetyl-S-enzyme (10, 12), NMR measurements of wild-type and aromatic mutants were performed. As documented in Table III, a single replacement of the aromatic side chain of Tyr130, Phe204, or Tyr376 does not influence the magnitude of the large (20 ppm) upfield shift of the C-1 acetyl thioester carbonyl resonance. The smaller (7 ppm) upfield shift of the C-2 methyl signal is similarly unaffected. Measurements performed on Y163L, F225L, and Y346L mutants also reflect no impact of single aromatic substitutions on the peak positions of the acetyl group in the covalent intermediate. Previous work (12) suggested that the active site environment in the acetyl-S-enzyme intermediate might be sheltered from solvent since, upon introduction of paramagnetic Mn2+, no broadening of the acetyl C-1 peak was observed. Using the aromatic mutants and wild-type protein, each exhaustively diazylized prior to formation of acetyl-S-enzyme, we observed substantial peak broadening upon Mn2+ addition. The negative observation previously reported may have resulted from carry over of residual EDTA from enzyme storage buffer, since that protein sample was buffer exchanged by repeated concentration/dilution rather than by exhaustive dialysis.

The aromatic mutants Y130L, F204L, and Y376L only slowly form the covalent acetyl-S-enzyme reaction intermediate. Comparable behavior has been reported for D159A and D203A catalytic mutants (8). Moreover, these carboxyl-substituted mutants only slowly exchange solvent into the thioester carbonyl of the acetyl-S-enzyme adduct (8). On the basis of these observations, the ability of Y130L, F204L, and Y376L enzymes to exchange H218O solvent into the [1,2-13C]acetyl-S-enzyme intermediate (Scheme 2) was investigated. Measurements were performed at a higher field (151 vs 75.5 MHz) and instrument sensitivity than available for previous studies, facilitating isotope shift measurements over shorter time intervals. The results of this study (Table IV) confirm the earlier report of a differential 18O isotope shift on the C-1 resonance. Comparable upfield shifts (4.0–5.0 Hz) of the C-1 signal are

| Peak assignment | Wild type | Y130L | F204L | Y376L |
|----------------|-----------|-------|-------|-------|
| C-1 thioester carbonyl (ppm) | 183.9 | 183.9 | 183.9 | 183.9 |
| C-2 methyl (ppm) | 25.7 | 25.7 | 25.7 | 25.7 |

Table III
13C NMR shifts of [1,2-13C]acetyl-S-enzyme intermediates
Measurements were performed as previously described (10) using a Bruker AC-300 NMR spectrometer, equipped with a QNP probe operating for 13C at 75.5 MHz.
observed for wild-type, Y130L, F204L, and Y376L proteins. Since the C-2 methyl group has no oxygen substituents, little effect is expected for the C-2 methyl signal, and, as previously reported, only minimal changes in methyl peak position are observed (Table IV). Due to improved instrumental sensitivity, the C-1 upfield shift for the aromatic mutants is detectable within 60–100 min of spectrum acquisition, which is initiated after appropriate preincubation (30 min minimum) of enzyme with acetyl-CoA to maximize acetyl-S-enzyme formation. These observations contrast with the delayed development of the 18O shift observed for D159A and D203A, which required between 5 and 10 h to reach maximum exchange/upfield shift. Such a difference may reflect a more direct influence of Asp159 and Asp203 carboxyls on solvent water polarization and hydroxyl attack to form tetrahedral adduct or on the subsequent collapse of the tetrahedral adduct that accounts for solvent exchange and reformation of acetyl-S-enzyme (Scheme 2). The aromatic residues Tyr130, Phe204, or Tyr376 do not seem to greatly influence the efficiency of such solvent addition/exchange.

**DISCUSSION**

The possibility that aromatic side chains within the HMG-CoA synthase active site significantly influence substrate binding or catalysis derives from several observations or precedents. Previous work (9, 10) has demonstrated that, for the acetyl-S-enzyme reaction intermediate, both the acetyl carbon atoms and the C-2 methyl protons exhibit upfield shifts in their NMR signals in comparison with signals measured for acetyl-CoA in buffer. Such upfield shifts may reflect a hydrophobic, low dielectric local environment for this intermediate. For example, ∼40% of the upfield shift effect measured for C-1 of acetyl-S-enzyme is observed for C-1 of the acetyl groups of N,S-diacectyleysteamine when this acetyl-CoA analog is measured in benzene rather than aqueous solvent (10). Additionally, ring current effects from π electrons of aromatic rings may influence observed NMR shifts. In fact, the large shift observed for the thioester carbon of AcAc-CoA upon binding to acetyl-CoA dehydrogenase has prompted molecular orbital calculations on a lumilavin/5-thiahexanedione model (11) that suggest the importance of orbital overlap of the stacked molecules. That hypothesis has never been tested by a control with flavin-depleted protein. However, our results indicate that no single aromatic side chain among those targeted for mutagenesis makes a dominant contribution to the upfield shift. It remains possible that, in aggregate, these residues contribute to a low dielectric local environment that accounts for part of the upfield shifts (10) observed for the acetyl-S-enzyme intermediate of HMG-CoA synthase.

A more likely explanation for a larger component of the upfield shift for the thioester carbonyl is suggested by the magnitude of the observed 18O-induced isotope shifts for acetyl-S-enzyme reaction intermediates prepared with wild-type and mutant proteins. The data (Table IV) qualitatively confirm earlier measurements performed at a lower magnetic field (12). However, the magnitude of measured upfield shifts (∼4.5 Hz; Table IV) corresponds to an effect (equal to 0.03 ppm (uncorrected for dilution by 10% D2O)) that is somewhat smaller than the value (0.05 ppm) previously measured under the more difficult conditions that exist at lower field and sensitivity. Although the magnitude (0.03 ppm) of this 18O-induced shift for the thioester carbonyl is intermediate between the results reported (19) for bond orders of 1.5 (carboxyl oxygen; 0.025 ppm) and 2 (carboxyl oxygen; 0.05 ppm), the current results are closer to the value for a carboxyl oxygen. Due to the sulfur atom in the thioester linkage, the thioesterified carbonyl carbon has more ketone character than expected for the comparable carbon in oxoesters or carboxylic acids; this accounts for the 204-ppm signal for [1,13C]acetyl-CoA in solution. Such effects on bond order also account for a 25-ppm difference between chemical shifts for the oxoesterified C-1 carbonyl of phenyl acetate (169 ppm) in comparison with that of the homologous thioester, S-phenyl thioacetate (194 ppm). Similarly, the C-5 carbonyl peak (183 ppm) of HMG-CoA is 20 ppm upfield of the C-1 thioester carbonyl (203 ppm) (12). Effects of such a magnitude (>16 ppm) have also been observed in nonenzymatic model experiments (11) when bond order in ketone carbons (e.g. ethyl acetooacetate) changes upon enolization. The current results raise the possibility that, upon formation of the acetyl-S-enzyme intermediate, an active site residue interacts to polarize the C-1 carbonyl, withdrawing electron density and decreasing bond order to that more typical of an oxoester or carboxylic acid. Polarization of the thioester carbonyl upon binding of acyl-CoA molecules to enzymes that catalyze their metabolism is well preceded (20, 21). Our previous observation (12) of a deuteration-induced shift in the 13C NMR signal of the thioester carbonyl when HMG-CoA synthase’s acetyl-S-enzyme intermediate is prepared in deuterated solvent is in accord with polarization that involves hydrogen bond formation.

The potential importance of aromatic residues in HMG-CoA synthase is also suggested by the observation of domain homology between microbial HMG-CoA synthase and ketoacyl-ACP synthase III, which catalyzes a similar condensation reaction. Inactivation of HMG-CoA synthase by cerulenin, an antibiotic well established as a ketoacyl-ACP synthase inhibitor, reinforces the prediction of domain homology between these proteins. The active site of ketoacyl-ACP synthase is situated at the end of a tunnel lined with conserved aromatic and other
hydrophilic residues (22). The C-terminal regions of both proteins contain homologous sequences which include an aromatic residue (corresponding to Tyr\textsuperscript{376} in avian HMG-CoA synthase) followed by a glycine-serine-rich sequence. In ketoacyl-ACP synthase, this aromatic residue (Phe\textsuperscript{303}) is situated close to the cysteine involved in acyl-enzyme intermediate formation (22). Observed homologies suggest that a comparable juxtaposition of HMG-CoA synthase Tyr\textsuperscript{376} and Cys\textsuperscript{129} may occur.

Previous work on HMG-CoA synthase has implicated active site residues (e.g., Cys\textsuperscript{129}, Asp\textsuperscript{159} and Asp\textsuperscript{203}) located in regions that also include highly conserved aromatic residues (e.g., Tyr\textsuperscript{130}, Tyr\textsuperscript{163}, Phe\textsuperscript{204}). Of the three remaining aromatic residues targeted for mutagenesis, Tyr\textsuperscript{346} and Tyr\textsuperscript{376} map in regions that are among the most highly conserved in HMG-CoA synthase proteins. On this basis, both the large \( k_{cat} \) effect observed for F204L as well as the \( k_{cat} \) acetyl-CoA and smaller \( k_{cat} \) effects observed for Y130L and Y376L seem reasonable and support the hypothesis that these aromatic residues are part of the active site cavity.

Speculation concerning potential functional assignments may be influenced by the hydrogen bonding potential of Tyr\textsuperscript{130} and Tyr\textsuperscript{376}, which contrasts with the situation for Phe\textsuperscript{204}. The active site of ketoacyl-ACP synthase contains an oxyanion cavity (22, 23) predicted to be important for deacylation to free the active site cysteine for the next round of catalysis. In the case of HMG-CoA synthase, a similar hydrolytic deacylation of the transient CoA-S-HMG-CoA-enzyme intermediate must also occur (Scheme 1, part 3). An oxyanion cavity similar in character to that observed for ketoacyl-ACP synthase and other acyl-CoA metabolizing enzymes (21) also exists in acetylcholinesterase (24). Demonstration of the importance of aromatic residues in acetycholinesterase (25) required simultaneous substitution of two residues, which have been assigned the function of restricting nonproductive mobility of a histidine component of a catalytic triad. In the case of HMG-CoA synthase, a substantial effect (300-fold diminution in \( k_{cat} \) of the overall reaction) results from the single F204L substitution. On this basis, a comparable function for Phe\textsuperscript{204} in orienting or restricting nonproductive motion of one of the polar residues implicated in catalysis seems plausible. The results of emerging structural work on a prokaryotic HMG-CoA synthase (26, 27) confirm that the phenyl ring of the homolog of avian synthase Phe\textsuperscript{204} (Fig. 1) closely interacts with an acidic side chain implicated as a general acid/base catalyst (9).

In the case of HMG-CoA synthase Y130L and Y376L, participation in a hydrogen bonding network would be lost upon leucine substitution. This could allow side chain or backbone movement or alter cysteine shape and/or negatively impact substrate affinity due to steric hindrance. In the case of ketoacyl-ACP synthase (23), several aromatic residues contribute to the substrate pocket, supporting such a hypothesis. An alternative explanation derives from the observation that the oxyanion pockets of both ketoacyl-ACP synthase and acetylcholinesterase are characterized by an extensive hydrogen bonding network. It remains possible that elimination of any hydrogen bonds that might be contributed by HMG-CoA synthase Tyr\textsuperscript{130} and/or Tyr\textsuperscript{376} disrupts the proper orientation of polar side chains that are more directly involved in interactions with acetyl-CoA. Emerging structural data for prokaryotic HMG-CoA synthase (26, 27) confirm a hydrogen bonding interaction between the homolog of avian synthase Tyr\textsuperscript{376} (Fig. 1) and an invariant acidic residue that profoundly effects catalysis and acetyl-S-enzyme intermediate formation (8). Additionally, disruption of a hydrogen bond in which the homolog of avian synthase Tyr\textsuperscript{310} (Fig. 1) participates (26, 27) could remove a constraint on peptide backbone motion that influences orientation of the adjacent crucial Cys\textsuperscript{129} sulfhydryl. Although these hydrogen bonds characterize prokaryotic HMG-CoA synthase, their status remains uncertain in the animal enzyme. Our results for phenylalanine substitutions for Tyr\textsuperscript{310} and Tyr\textsuperscript{376} of the avian enzyme suggest that the presence of an aromatic ring is quite important. The effect of minimizing any change in accessible surface area at these residues may outweigh any hydrogen bonding contributions in the animal enzyme. Alternatively, the phenylalanine ring might support the type of CH-O interaction which is increasingly recognized as significant in proteins (27).

The influence of aromatic residues on the function of other condensing enzymes (e.g., ketoacyl-ACP synthase, chalcone synthase) that exhibit domain homology with HMG-CoA synthase has not been extensively studied. As additional investigation confirms the prediction of domain homology, it will be interesting to determine whether effects of the type observed for aromatic substituted mutants of HMG-CoA synthase are also observed upon comparable substitutions to eliminate aromatic side chains in other condensing enzymes.

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