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Ring Current Effects in the Active Site of Medium-Chain Acyl-CoA Dehydrogenase Revealed by NMR Spectroscopy

Jiaquan Wu,† Alasdair F. Bell,‡ Andrew A. Jaye, and Peter J. Tonge*

Contribution from the Department of Chemistry, Stony Brook University, Stony Brook, New York 11794-3400

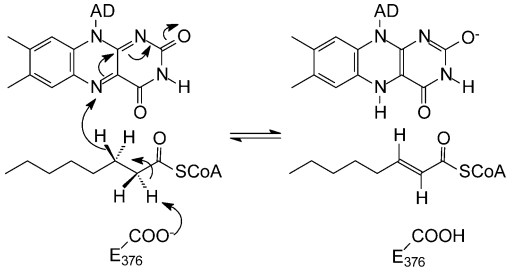
Received January 6, 2005; E-mail: peter.tonge@sunysb.edu

Abstract: Medium-chain acyl-CoA dehydrogenase (MCAD) catalyzes the flavin-dependent oxidation of fatty acyl-CoAs to the corresponding *trans*-2-enoyl-CoAs. The interaction of hexadienoyl-CoA (HD-CoA), a product analogue, with recombinant pig MCAD (pMCAD) has been studied using ^{13}C NMR and $^{1}H-^{13}C$ HSQC spectroscopy. Upon binding to oxidized pMCAD, the chemical shifts of the C1, C2, and C3 HD carbons are shifted upfield by 12.8, 2.1, and 13.8 ppm, respectively. In addition, the ^{1}H chemical shift of the C3-H is also shifted upfield by 1.31 ppm while the chemical shift of the C4 HD-CoA carbon is unchanged upon binding. These changes in chemical shift are unexpected given the results of previous Raman studies which revealed that the C3═C2−C1═O HD enone fragment is polarized upon binding to MCAD such that the electron density at the C3 and C1 carbons is reduced, not increased (Pellet et al. *Biochemistry* **2000**, *39*, 13982−13992). To investigate the apparent discrepancy between the NMR and Raman data for HD-CoA bound to MCAD, ^{13}C NMR spectra have been obtained for HD-CoA bound to enoyl-CoA hydratase, an enzyme system that has also previously been studied using Raman spectroscopy. Significantly, binding to enoyl-CoA hydratase causes the chemical shifts of the C1 and C3 HD carbons to move downfield by 4.8 and 5.6 ppm, respectively, while the C2 resonance moves upfield by 2.2 ppm, in close agreement with the alterations in electron density at these carbons predicted from Raman spectroscopy (Bell, A. F.; Wu, J.; Feng, Y.; Tonge, P. J. *Biochemistry* **2001**, *40*, 1725−33). The large increase in shielding experienced by the C1 and C3 HD carbons in the HD-CoA/MCAD complex is proposed to arise from the ring current field from the isoalloxazine portion of the flavin cofactor. The flavin ring current, which is only present when the enzyme is placed in an external magnetic field, also explains the differences in ^{13}C NMR chemical shifts for acetoacetyl-CoA when bound as an enolate to MCAD and enoyl-CoA hydratase and is used to rationalize the observation that the line widths of the C1 and C3 resonances are narrower when the ligands are bound to MCAD than when they are free in the protein solution.

Introduction

Fatty acid β-oxidation is initiated by the conversion of fatty acyl-CoAs to the corresponding 2-enoyl-CoAs by the flavin-dependent acyl-CoA dehydrogenases (ACDs).[1,2] Several ACDs with varying substrate chain length specificity exist, and medium chain acyl-CoA dehydrogenase (MCAD) is the most heavily studied isozyme.[3] The ACD reaction proceeds via the stereospecific abstraction of the substrate's C2 proton by the active site base (E376 in MCAD) with concomitant transfer of a hydride from C3 to the flavin N5 (Scheme 1). Experimental and theoretical studies support either a concerted[4] or stepwise reaction,[5] respectively, and in either case stabilization of negative charge accumulation on the thioester carbonyl is critical for catalysis.[6,7] A large number of studies have been performed in order to determine how the enzyme overcomes the two thermodynamic challenges to substrate oxidation, namely the mismatch in pK_a's between substrate (21) and general base (5) as well as the difference in redox potential between free enzyme (−145 mV) and substrate (−38 mV).[8,9] These barriers to the

Scheme 1. Oxidation of Octanoyl-CoA by MCAD



† Present address: Chemistry Department, Brown University, Providence, RI 02912.

‡ Present address: Medarex inc., 1324 Chesapeake Terrace, Sunnyvale, CA 94089.

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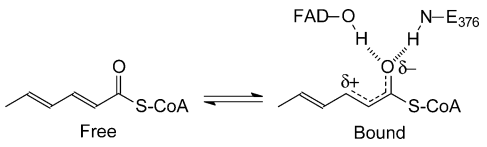
Scheme 2. Polarization of HD-CoA by MCAD



reaction are dealt with by substantial changes in both the α-proton acidity and redox potential of the substrate upon binding to the enzyme. The nature of these binding-induced changes in substrate reactivity is of central importance in MCAD enzymology.

Elegant studies utilizing ionizable substrate analogues indicate that enzyme−substrate interactions stabilize negative charge accumulation on the thioester carbonyl thereby reducing the pK_a of the acyl-CoA α-protons.[10,11] This stabilization is accomplished, in part, via hydrogen bonds from the enzyme and cofactor to the thioester carbonyl oxygen.[6,9,12] These interactions also serve to shift the redox potential of the flavin positive by over 100 mV, such that hydride transfer from substrate to flavin is now thermodynamically favored.[8,13] In addition to these interactions, desolvation of the active site upon substrate binding is also thought to be important for raising the pK_a of the catalytic base,[2,10,14,15] while Thorpe and colleagues have speculated that charge-transfer interactions in the enzyme−substrate complex may also play a role in transition state stabilization.[16,17]

Further insights into the enzyme substrate interactions in the MCAD active site have been gained from Raman and NMR studies. Nishina et al.[18] reported resonance Raman (RR) data for the crotonyl-CoA product bound to MCAD and concluded that the enzyme polarized the ground state of the ligand. We have since extended these studies to include the product analogue hexadienoyl-CoA[19] (HD-CoA) as well as the ionizable ligand 4-hydroxycinnamoyl-CoA.[20] Using 752 nm excitation we obtained Raman spectra of unlabeled and isotopically labeled HD-CoA bound to oxidized and reduced pig MCAD (pMCAD) and concluded that the enzyme polarized the C3═C2−C1═O enone portion of the HD moiety by stabilizing a withdrawal of negative charge from the β-carbon onto the carbonyl oxygen (Scheme 2).

The importance of hydrogen bonding interactions with the substrate carbonyl in ligand polarization was subsequently demonstrated using 2′-deoxyFAD-substituted enzyme.[6,7] The changes in electronic structure in these analogues report on the forces in place to stabilize the transition state(s) for substrate oxidation.[21,22] These interactions also potentially activate the ground state of the substrate, since accumulation of partial negative charge at the α-carbon will facilitate α-proton abstraction, while the partial positive charge at the β-carbon will favor elimination of the hydride.

The structures of two ionizable substrate analogues that form enolates upon binding to MCAD has also been examined by both RR and ^{13}C NMR spectroscopies. Nishina et al. reported RR data for labeled and unlabeled acetoacetyl-CoA (AcAc-CoA) bound to MCAD and concluded that the acetoacetyl enolate was localized on the C1 carbon while the C3 carbon remained a ketone (C═O).[23] These studies were subsequently extended using ^{13}C NMR spectroscopy in which it was determined that both the C1 and C3 carbons experienced significantly upfield chemical shifts while C2 shifted downfield.[24] Similar changes in C1 and C2 chemical shifts were observed for the substrate analogue 3-thiaoctanoyl-CoA.[25]

While the above NMR data are consistent with the expectation that the bound AcAc-CoA enolate is localized primarily on the C1═O, it is curious why C3 also experiences a large upfield chemical shift. The previous Raman studies on this ligand bound to MCAD support a decrease in electron density at C3,[23] which would cause a deshielding of the carbon and hence a downfield change in the ^{13}C chemical shift. Since AcAc-CoA is an ionizable probe of the active site, we have now extended our own studies on the nonionizable product analogue HD-CoA to include ^{13}C NMR and ^{13}C−^{1}H HSQC (heteronuclear single quantum correlation) spectroscopy. While our Raman studies on HD-CoA bound to MCAD indicate that the bound ligand is polarized as shown in Scheme 2, such that there is a decrease in electron density at C1 and C3, the NMR studies reveal substantial upfield chemical shifts for both the C1 and C3 HD carbons (−13 to −14 ppm) as well as the C3-H proton (−1.3 ppm), rather than the anticipated downfield change in chemical shift. To investigate the origin of these effects, we have revisited the ^{13}C NMR studies reported by Anderson and colleagues for HD-CoA bound to enoyl-CoA hydratase[26] and have also obtained ^{13}C NMR spectra of AcAc-CoA bound to enoyl-CoA hydratase. Significantly, the ^{13}C NMR data on HD-CoA bound to enoyl-CoA hydratase are consistent with our previous Raman studies which show that enoyl-CoA hydratase causes similar polarization of the hexadienoyl group as described above for MCAD in which there is a decrease in electron density at C1 and C3 (Scheme 2). We conclude that the substantial differences in the ^{13}C chemical shifts for the C3 and C1 carbons of the ligands bound to the two enzymes arise as a result of the ring current from the flavin cofactor in the MCAD active site. In support of this conclusion, preliminary Density Functional Theory (DFT) calculations on an isolated HD group in proximity to an isoalloxazine ring analogue reproduce the direction although not the full magnitude of the experimentally observed changes in NMR chemical shift.

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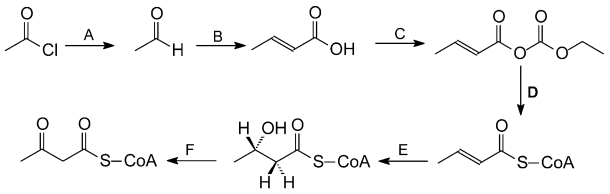
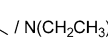
Scheme 3. Synthesis of Isotopically Labeled AcAc−CoA



(A) $(Bu)_3SnH$ / $Pd(0)(PPh_3)_4$; (B) $CH_2(COOH)_2$ / Py; (C)  / $N(CH_2CH_3)_3$ / THF;
(D) CoASH / pH 8.3; (E) Enoyl-CoA hydratase; (F) HAD / NAD^+/ LDH / Pyruvate

Experimental Procedures

Chemicals. Hexadienoic acid, 1,1′-carbonyldiimidazole, acetaldehyde, 1-^{13}C-acetyl chloride, coenzyme A (lithium salt; CoA[1]), L-3-hydroxyacyl-CoA dehydrogenase, lactate dehydrogenase and pyruvate were purchased from Sigma Chemical Co. [1,3-$^{13}C_2$] Malonic acid (99% ^{13}C), and [2-^{13}C] malonic acid (99% ^{13}C) were purchased from Cambridge Isotope Labs. A plasmid carrying the cDNA for bovine liver enoyl-CoA hydratase was a generous gift from Professor Hung-wen Liu.

Isotopically Labeled Hexadienoyl-CoA and Acetoacetyl-CoA. The synthesis and purification of C1, C2, C3, and C4 ^{13}C-labeled hexadienoyl-CoA (HD-CoA) has been described elsewhere.[27] C1, C2, and C3 ^{13}C-labeled acetoacetyl-CoA (AcAc-CoA) were synthesized as shown in Scheme 3. Crotonic acid labeled with ^{13}C at the C1, C2, or C3 position was synthesized as described in Bell et al.[27] (Scheme 3A and B) and then converted to the corresponding ^{13}C-labeled crotonyl-CoA as described (Scheme 3C and D).[28] The labeled crotonyl-CoAs were then converted enzymatically to the respective AcAc-CoAs using enoyl-CoA hydratase and L-3-hydroxylacyl-CoA dehydrogenase (HAD).[29,30] Crotonyl-CoA was first converted to 3-hydroxybutyl-CoA (HB-CoA) by enoyl-CoA hydratase (Scheme 3E). In a typical reaction, 10 μL of 0.1 mM enoyl-CoA hydratase was added to a 3 mM crotonyl-CoA solution in 5 mL of 50 mM potassium phosphate buffer, pH 7.6. The conversion was followed by monitoring the decrease in absorbance at 260 nm. No discernible absorbance change was observed after ca. 30 min, indicating that the majority of the crotonyl-CoA had been hydrated to HB-CoA. The HB-CoA was then oxidized to AcAc-CoA in situ by adding HAD and NAD^+ to final concentrations of 0.1 μM and 60 μM, respectively (Scheme 3F). The reaction was driven to completion by the addition of lactate dehydrogenase (LDH) (70 unit) and pyruvate (60 mM). After overnight incubation at RT, the resulting AcAc-CoA was purified by reverse phase HPLC. Conditions were the same as that used in the purification of crotonyl-CoA[28] except that the gradient of buffer B was from 0 to 20% in 60 min. AcAc-CoA eluted at 45 min. ^{1}H NMR (500 MHz, D2O): δ 0.78 (s, 3H, 11′ CH3), 0.91 (s, 3H, 10′ CH3), 2.32 (s, 3H, CH3Ac), 2.44 (t, 2H, H6″), 2.65 (s, 2H, CH2Ac, split into 2 singlets at 2.50 and 2.80 ppm upon ^{13}C labeling at the C2 position), 3.05 (t, 2H, H9″), 3.37 (t, 2H, H8″), 3.46 (t, 2H, H5″), 3.58 (q, 1H, H1″B), 3.86 (q, 1H, H1″A), 4.04 (s, 1H, H3″), 4.25 (s, 2H, H5′), 4.60 (s, 1H, H4′), 6.15 (d, 1H, H1′), 8.24 (s, 1H, H2), 8.54 (s, 1H, H8). The CoA H2′ and H3′ resonances are obscured by the solvent resonance. ^{13}C NMR (125 MHz, D2O): δ 21.13 (C11″), 23.74 (C10″), 30.76 (C9″), 38.30 (C6″), 41.15 (C2″), 41.26 (C5″), 41.60 (C8″), 57.70 (C2Ac), 68.44 (C5′), 74.71 (C1″), 76.79 (C2′), 77.03 (C3′ + C3″), 86.64 (C4′), 89.34 (C1′), 121.51 (C5), 142.68 (C8), 152.18 (C4), 155.72 (C2), 158.46 (C6), 176.87 (C7″), 177.61 (C4″), 196.00 (C1Ac), 201.40 (C3Ac).

Enzymes. Recombinant pig kidney MCAD (pMCAD) was expressed and purified as described previously.[7] The concentration of pMCAD was determined spectrophotometrically using an extinction coefficient of 15 400 M^{-1} cm^{-1} at 446 nm.[31]

Bovine liver enoyl-CoA hydratase was expressed in BL21(DE3)-pLysS cells following induction with 1 mM IPTG. The protein was purified using ethanol precipitation as described previously.[32] In most cases an additional precipitation procedure was performed in order to remove trace amounts of contaminating proteins. The concentration of enoyl-CoA hydratase was determined by active site titration with 4-dimethylaminocinnamoyl-CoA using an extinction coefficient of 48 48 000 $M^{-1}cm^{-1}$ at 496 nm.[33]

Preparation of NMR Samples. For the ^{13}C NMR experiments, enzymes were prepared in 50 mM potassium phosphate, 0.3 mM EDTA, pH 7.6 buffer containing 50% D_2O. Enzyme samples containing labeled HD-CoA were prepared by dissolving lyophilized HD-CoA directly in the enzyme solution. Initially, one equiv of ligand was added followed by subsequent additions of up to 6 equiv of ligand. For the ^{13}C−^{1}H HSQC NMR experiments, the pMCAD enzyme was exchanged into 50 mM potassium phosphate buffer prepared from P_2O_5 dissolved in 99.7% D_2O and adjusted to pD 7.6 with anhydrous K_2CO_3.[34] The concentration of pMCAD in all NMR experiments was in the range of 500 to 600 μM and that of enoyl-CoA hydratase was 1 to 2 mM. A total volume of 0.5 mL of enzyme was used in each sample.

^{13}C NMR Spectroscopy. The ^{13}C NMR spectra were obtained in 5 mm NMR tubes at the SUNY at Stony Brook NMR center on a Varian Inova 500 MHz instrument operating at 125.711 MHz using a Nalorac gradient dual band probe. The T_1 (spin−lattice relaxation time) of the carbonyl carbon of HD-CoA was determined to be 3.1 s using a standard two-pulse sequence in an inversion−recovery T_1 experiment. All spectra of either pure enzymes or enzyme−ligand complexes, consisting of 25 000−30 000 scans for pMCAD or 15 000−20 000 scans for enoyl-CoA hydratase, were acquired with broad band ^{1}H decoupling (decoupler power 45 W), a relaxation delay of 1.0 s, and an acquisition time of 1.3 s. These spectra were processed with 25 Hz of line broadening. A flip angle of 20° was used in each case, except for the complex of enzyme with ^{13}C1-labeled ligand where a flip angle of 10° was used to compensate for the relatively short relaxation delay of the carbonyl carbon. Spectra of pMCAD or enoyl-CoA hydratase and their complexes were obtained at 25 °C. The spectrum of pure enzyme was obtained prior to the addition of ligand. Spectra of ^{13}C-labeled ligands in the same buffer as that used for the enzyme samples were recorded separately using 500−600 transients, processed with 5 Hz of line broadening and calibrated using TSP. Spectra of the enzyme-ligand complexes were calibrated using chemical shift values of the unbound ligand.

^{1}H−^{13}C HSQC Spectroscopy. In addition to the direct detection of carbon chemical shift changes upon binding to pMCAD, indirect detection was also used to measure the chemical shift of both the carbon and the attached proton using ^{1}H−^{13}C HSQC spectroscopy. Sensitivity-enhanced gradient two-dimensional ^{1}H−^{13}C HSQC spectra[35] were recorded at 25 °C. Spectral widths for the proton and carbon dimensions were 8000 and 25 000 Hz, respectively. A total of 256 scans were collected for each t_1 increment and a total of 128 t_1 increments were acquired, each with 2048 points. Spectra were referenced to the chemical shifts of free ligand in both carbon and proton dimensions. The spectrum of pure enzyme was obtained prior to the addition of a ligand. Subsequently, spectra were obtained following each addition of one equiv of the lyophilized ligand. Spectra of ^{13}C-labeled HD-

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CoAs were recorded in the same buffer as that used for the enzyme samples using 32 scans for each t_1 increment and a total of 32 t_1 increments, each with 2048 points. The free ligand spectra were calibrated with TSP and spectra of the enzyme−HD-CoA complexes were calibrated using the chemical shift values observed for the free ligand. Data were processed with Felix 98 (Insight II, Accelrys).

Density Functional Theory Calculations. ^{13}C and ^{1}H NMR chemical shifts were calculated on a model system using Density Functional Theory (DFT) and Gaussian 03.[36] This method of gauging the effect of ring currents on chemical shifts directly from DFT calculations has been applied previously.[37] We used the B3LYP functional with a 6-31G(d,p) basis set for optimization and chemical shift calculation and found along with others[38] that the 'Continuous Set of Gauge Transformations' (CSGT) produced ^{13}C chemical shifts more in agreement with experiment than 'Gauge-Including Atomic orbitals' (GIAO). Initial calculations involved the HD-CoA model compound *trans*,*trans*-2,4-hexadienoyl ethyl thioester (HET) and utilized a benzene ring as a flavin analogue. The benzene was constrained to be parallel to the HET molecule at a distance of 3 Å from the C3 HD carbon. As is usual in active site calculations,[26] the geometry was optimized before calculating the ^{13}C and ^{1}H NMR chemical shifts.

Results

^{13}C NMR Titration of pMCAD with ^{13}C-Labeled HD-CoAs. Figure 1 contains the ^{13}C NMR spectra of pMCAD titrated with C1, C2, C3, and C4 ^{13}C-labeled HD-CoAs. The ^{13}C NMR spectrum of pMCAD prior to the addition of ligand is shown for each spectral region (i) and reveals signals that arise from the protein. For example, in the region from 160 to 200 ppm (Ai), the predominant signals arise from the carboxylate and carbonyl resonances of the amino acids,[39−41] while the signal at 160.4 ppm can be assigned to the resonance of either the arginine guanidino carbons or of the tyrosine C_ξ.[42] In addition, the strong signal around 130 ppm (B−D, i) arises from the aromatic amino acids in the protein.[42] As labeled HD-CoA is titrated into the protein (spectra ii−iv), new signals can be seen to grow in that can be assigned to the enzyme bound population of HD-CoA (Table 1). These signals are at 181.4, 124.5, and 129.5 ppm for the $^{13}C1$-, $^{13}C2$-, and $^{13}C3$-labeled HD-CoAs, respectively. Furthermore, as the titration continues, a second resonance can be observed in each case that has the same chemical shift as the unbound ligand (spectrum v). The signals for unbound HD-CoA appear at 193.9 (A: C1), 126.8 (B: C2) and 143.3 (C: C3) ppm, and thus binding to the enzyme has caused a large upfield shift in the resonances arising from the HD-CoA C1 (12.5 ppm) and C3 (13.8 ppm) carbons, and a smaller upfield shift (2.3 ppm) in the C2 HD-CoA resonance (Table 1). In contrast to the C1−C3 carbons, the chemical shift of the C4 HD-CoA carbon (133.4 ppm) remains unperturbed upon binding of the ligand to pMCAD (Figure 1D).

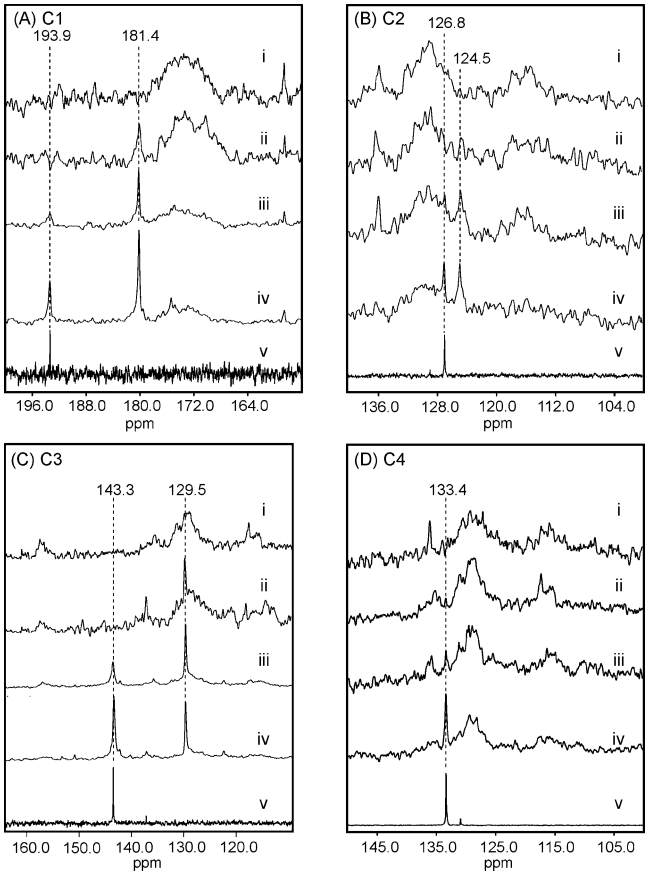

Figure 1. ^{13}C NMR titration of pMCAD with ^{13}C-labeled HD-CoAs. (A) Titration of pMCAD (540 μM) with (i−iv) 0, 2, 4 and 6 equiv of $^{13}C1$-HD-CoA, (v) 0.2 mM $^{13}C1$-HD-CoA. (B) Titration of pMCAD (560 μM) with (i−iv) 0, 1, 2, and 4 equiv of $^{13}C2$-HD-CoA, (v) 1 mM $^{13}C2$-HD-CoA. (C) Titration of pMCAD (540 μM) with (i−iv) 0, 1, 2, and 4 equiv of $^{13}C3$-HD-CoA, (v) 0.2 mM $^{13}C3$-HD-CoA. (D) Titration of pMCAD (540 μM) with (i−iv) 0, 1, 2, and 4 equiv of $^{13}C4$-HD-CoA, (v) 2 mM $^{13}C4$-HD-CoA in buffer.

Table 1. ^{13}C and ^{1}H Chemical Shift Values for HD-CoA Free and Bound to pMCAD

	free[a]		bound		
HD-CoA	δ (ppm)[b]	$\Delta\nu_{1/2}$[c] (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	$\Delta\delta$, bound − free (ppm)
$^{13}C1$[d]	193.9 (186.3)	29 (2)	181.4	16	−12.5
$^{13}C2$[d]	126.8 (127.3)	30 (5)	124.5	38	−2.3
$^{13}C3$[d]	143.3 (139.5)	40 (5)	129.5	21	−13.8
$^{13}C4$[d]	133.4 (131.0)	15 (5)	133.4	15	0.0
$^{13}C2$-H[e]	6.08 (5.84)	ND[f]	6.18	ND[f]	0.1
$^{13}C3$-H[e]	7.14 (7.35)	ND[f]	5.83	ND[f]	−1.3
$^{13}C4$-H[e]	6.18 (6.55)	ND[f]	6.12	ND[f]	−0.06

[a] Values are for unbound ligand in the presence of pMCAD. [b] Values in parentheses are calculated chemical shifts based on substrate analogue; *trans*,*trans*-2,4-hexadienoyl ethyl thioester. ^{13}C calculated using CSGT and ^{1}H using GIAO (see Experimental Procedures). [c] Values in parentheses are for the free ligand in the absence of protein. [d] ^{13}C NMR chemical shifts from the direct detection experiments. [e] ^{1}H NMR chemical shifts from the HSQC experiments. [f] ND, not determined.

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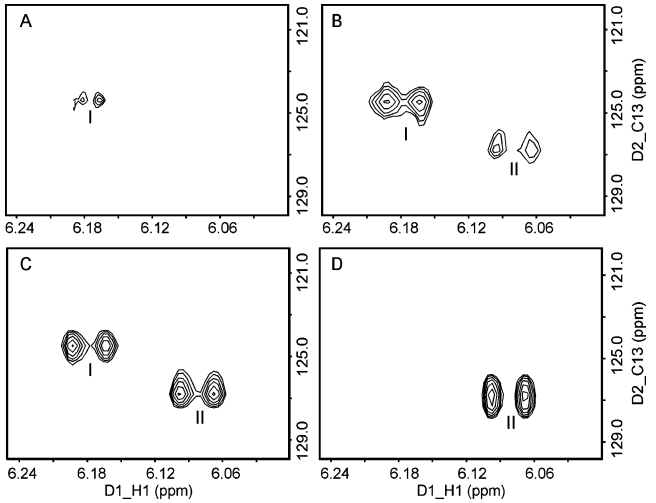

Figure 2. $^{13}C-^{1}H$ HSQC spectra of $^{13}C2$-HD-CoA free and bound to pMCAD. pMCAD (410 μM) with (A−C) 0.8, 1.6, and 3 equiv of $^{13}C2$-HD-CoA. (D) 2 mM $^{13}C2$-HD-CoA in buffer. Cross-peak I (^{1}H: 6.18 ppm, ^{13}C: 124.3 ppm) is from bound ligand while cross-peak II (^{1}H: 6.08 ppm, ^{13}C: 126.4 ppm) arises from free ligand.

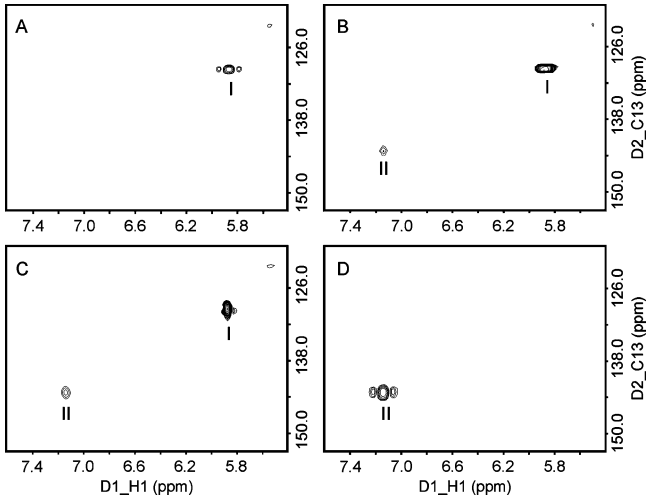

Figure 3. $^{13}C-^{1}H$ HSQC spectra of $^{13}C3$-HD-CoA free and bound to pMCAD. pMCAD (410 μM) with (A−C) 1, 2, and 4 equiv of $^{13}C3$-HD-CoA. (D) 2 mM $^{13}C2$-HD-CoA in buffer. Cross-peak I (^{1}H: 5.83 ppm, ^{13}C: 129.5 ppm) is from bound ligand while cross-peak II (^{1}H: 7.14 ppm, ^{13}C: 143.3 ppm) arises from free ligand.

The observation of ^{13}C resonances arising from both the free and bound ligand (Figure 1A−C) indicates that the two forms of HD-CoA are in slow exchange on the NMR time scale.[43] In addition, as expected the resonances of both free and bound HD-CoA are broader in the presence of protein compared to the signals arising from ligand in buffer alone (Table 1). Interestingly, the line width ($\Delta\nu_{1/2}$, defined as the width of the peak at half-height in Hz) for the C1 and C3 resonances are actually narrower (13 and 19 Hz, respectively) when HD-CoA is bound to pMCAD than when the ligand is free in the protein solution (Table 1). Finally, for $^{13}C4$-labeled HD-CoA (Figure 1D), where no change in chemical shift is observed upon binding, the resonance arising from the bound ligand population is 10 Hz broader than that for the ligand in the absence of protein (Table 1). The line broadening for unbound HD-CoA in the presence of pMCAD results from chemical exchange between the free and bound HD-CoA populations. As noted by Anderson and co-workers,[26] this difference in line width can be used to calculate a dissociation rate constant (k_{-1}) using eq 1:

$$\pi\Delta\nu_{1/2,\mathrm{obsd}} = \pi\Delta\nu_{1/2,\mathrm{free}} + k_{-1} \qquad (1)$$

where $\Delta\nu_{1/2,\mathrm{obsd}}$ and $\Delta\nu_{1/2,\mathrm{free}}$ are the line widths of the free ligand in the presence and absence of protein, respectively. For $^{13}C1$-, $^{13}C2$-, and $^{13}C3$-labeled HD-CoAs, line broadening of 27, 25, and 35 Hz provides k_{-1} estimates of 80−110 s^{-1}. Using a K_d value of 3.5 μM,[19] this gives an association rate constant for HD-CoA of 2.3−3.1 × 10^7 M^{-1} s^{-1}.

$^{1}H-^{13}C$ HSQC Spectroscopy. In addition to the direct detection ^{13}C NMR experiments, the HD-CoA/pMCAD complex has also been characterized using $^{1}H-^{13}C$ HSQC spectroscopy in order to extend our analysis to the protons attached to the ^{13}C-labeled carbons. Figures 2 and 3 contain the NMR data for the titration of pMCAD with $^{13}C2$- and $^{13}C3$-HD-CoA, respectively. For $^{13}C2$-HD-CoA cross-peak I arising at low ligand concentration (Figure 2A) is a doublet with a coupling constant (J) of 14 Hz. The intensity of this cross-peak increases as further ligand is added (Figure 2B and C), indicating that it arises from C2 of the bound ligand. Observation of cross-peak II after the addition of 1.6 equiv of ligand (Figure 2B) indicates that peak I is from the bound ligand while peak II arises from the free ligand. The 2.3 ppm difference between these two cross-peaks in the carbon dimension is identical to that observed for bound and free $^{13}C2$-HD-CoA in the direct detection experiment (see above, Table 1). In addition, the HD-CoA C2-H proton is deshielded 0.1 ppm on binding to the enzyme, as revealed by the change in this resonance from 6.08 ppm in the free ligand to 6.18 ppm in the bound ligand (Table 1).

Similar to the HSQC experiment with $^{13}C2$-HD-CoA, titration of pMCAD with 1 equiv of $^{13}C3$-HD-CoA gave one cross-peak at 129.5 ppm in the carbon dimension and 5.83 ppm in the proton dimension arising from the bound ligand (Figure 3A). Addition of further ligand resulted in the bound ligand peak intensifying and a second cross-peak appearing at 143.3 ppm in the carbon dimension and 7.14 ppm in the proton dimension (Figure 3B−D). The 13.8 ppm upfield shift in the $^{13}C3$ ligand resonance on binding is in agreement with the results of the direct detection experiment (see above). In addition, the HSQC spectrum of the $^{13}C3$-HD-CoA also reveals a 1.31 ppm upfield shift in the ^{1}H resonance of the C3-H proton upon binding to pMCAD (Table 1).

Titration of pMCAD with $^{13}C4$-HD-CoA was also monitored by HSQC spectroscopy (data not shown). Consistent with the ^{13}C NMR direct detection experiment, the $^{13}C4$ chemical shift of HD-CoA did not change upon binding to the enzyme (Table 1). However, careful scrutiny of spectra obtained from pMCAD samples containing excess ligand revealed two sets of cross-peaks that differed by about 0.06 ppm in the ^{1}H dimension (6.18 ppm free; 6.12 ppm bound). Thus, while binding does not perturb the $^{13}C4$ chemical shift, the attached proton experiences a slight upfield change in chemical shift.

^{13}C NMR Titration of Enoyl-CoA Hydratase with ^{13}C-Labeled HD-CoAs. Recombinant bovine liver enoyl-CoA hydratase was titrated with $^{13}C1$-, $^{13}C2$-, and $^{13}C3$-labeled HD-CoA. The results obtained are in good agreement with the data reported previously,[26] and consequently the NMR spectra are

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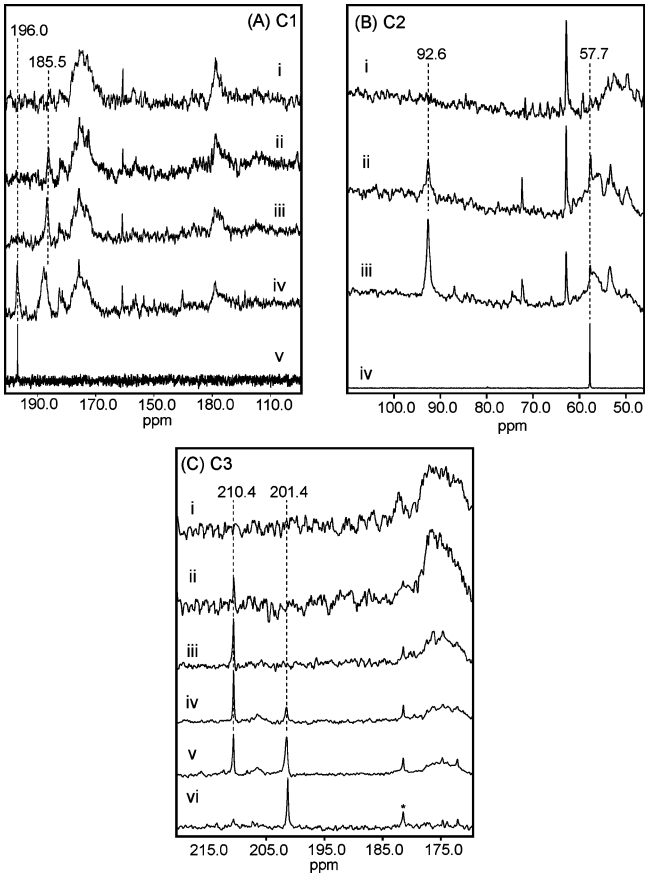

Figure 4. ^{13}C NMR titration of enoyl-CoA hydratase with ^{13}C-labeled AcAc-CoAs. (A) Titration of enoyl-CoA hydratase (1.3 mM) with (i−iv) 0, 1, 2, and 4 equiv of ^{13}C1−AcAc-CoA, (v) 2 mM ^{13}C1−AcAc-CoA in D_2O buffer. (B) Titration of enoyl-CoA hydratase (1.2 mM) with (i−iii) 0, 1.2, and 3.6 equiv of ^{13}C2−AcAc-CoA, (iv) 3 mM ^{13}C1−AcAc-CoA in buffer. (C) Titration of enoyl-CoA hydratase (1.2 mM) with (i−v) 0, 1, 2, 4, and 6 equiv of ^{13}C3−AcAc-CoA, (vi) Sample from (v) after removing enzyme. ∗, acetate contamination in the AcAc-CoA sample.

Table 2. ^{13}C Chemical Shift Values for HD-CoA Free and Bound to Enoyl-CoA Hydratase

	free		bound		
HD-CoA	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	$\Delta\delta$ (ppm)
^{13}C1	193.9	20	198.7	40	+4.8
^{13}C2	126.8	40	124.6	40	−2.2
^{13}C3	142.8	20	148.4	40	+5.6

not presented here. In each case, titration of the enzyme with ligand results in the initial appearance of a broad resonance that gradually shifts toward the position observed for the unbound ligand as more HD-CoA is added. For ^{13}C1-, ^{13}C2-, and ^{13}C3-labeled HD-CoAs the chemical shift of the bound ligand population is observed at 198.7, 124.6, and 148.4 ppm, respectively (Table 2). Thus, binding to enoyl-CoA hydratase causes the C1 and C3 HD-CoA resonances to move downfield by 4.8 and 5.6 ppm, respectively, while the C2 resonance moves upfield by 2.2 ppm. The chemical shift changes for the C1 and C2-labeled HD-CoAs are close to the values of 4.3 and −2.2 ppm reported by D'Ordine et al.[26]

^{13}C NMR Titration of Enoyl-CoA Hydratase with ^{13}C-Labeled Acetoacetyl-CoAs. Figure 4 contains the ^{13}C NMR spectra of enoyl-CoA hydratase titrated with ^{13}C1-, ^{13}C2-, and ^{13}C3-labeled acetoacetyl-CoAs (AcAc-CoAs). The observation of separate resonances for free and bound ligand in each case

Table 3. ^{13}C Chemical Shift Values for AcAc-CoA Free and Bound to Enoyl-CoA Hydratase

	free		bound		
AcAc-CoA	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	$\Delta\delta$ (ppm)
^{13}C1	196.0	20	185.5	40	−10.5
^{13}C2	57.7	ND[a]	92.6	40	+35.1
^{13}C3	201.4	20	210.4	15	+9.0

[a] ND, not determined. The ^{13}C2 resonance of the unbound ligand overlaps with the protein aliphatic resonances.

indicates that the two forms of AcAc-CoA are in slow exchange on the NMR time scale.[43] For ^{13}C1, ^{13}C2-, and ^{13}C3-labeled AcAc-CoAs the chemical shift of the bound ligand population is observed at 185.5, 92.6, and 210.4 ppm, respectively, while the corresponding resonances for free ligand appear at 196.0, 57.7, and 201.4 ppm, respectively (Figure 4 and Table 3). Thus, binding to enoyl-CoA hydratase causes the C1 AcAc-CoA resonance to move upfield by 10.5 ppm, while the C2 and C3 resonances move downfield by 35.1 and 9.0 ppm, respectively.

Density Functional Calculations. In an effort to try and predict how the ring current of FAD may affect the chemical shift of the substrate, we calculated ^{13}C and ^{1}H NMR chemical shifts on a model system using Density Functional Theory (DFT). The calculated chemical shifts of the isolated model compound *trans,trans*-2,4-hexadienoyl ethyl thioester (HET) are given in Table 1. Small differences between the values calculated for HET and the experimentally observed chemical shifts for the free HD-CoA ligand likely arise from interactions of the HD group with solvent water. As a first approximation of a 'bound' model, we used a benzene ring as a flavin analogue and constrained it to be parallel with HET at a distance of 3 Å from the HD C3 carbon. The differences in the chemical shifts (bound − free) were −0.4, +1.3, −3.8, −1.2, and +3.6 for C1, C2, C3, H3, and C4, respectively. The direction of these chemical shift changes are in agreement with experiment (except for C4, the chemical shift of which is not observed to change when bound to MCAD). We subsequently found that the degree and direction of shift was highly dependent on the position of the substrate relative to the aromatic ring. For example, by moving the benzene ring 0.5 Å closer to C3, the chemical shift differences became −2.6, +5.0, and −12.2 for C1, C2, and C3, respectively. These results suggest that the predicted chemical shifts are very sensitive to protein environment and that a geometrically accurate model of the experimental system is required. In an attempt to improve our model, we replaced the benzene ring with the more structurally relevant flavin analogue, methylisoalloxazine, and used the geometry in Figure 5 as initial coordinates for optimization. However, we were still unable to predict successfully both the direction and magnitude of all the observed chemical shifts.

Discussion

Spectroscopic Probes of Enzyme Mechanisms. The chemical transformation of a substrate into a product involves electronic rearrangement in the substrate during the reaction, which necessarily involves alterations in charge density on the reactant's functional groups. A critical aspect of an enzyme's catalytic power stems from the ability of the active site to cause and stabilize this charge rearrangement. Since the seminal studies of Knowles and colleagues,[44] vibrational and NMR spectroscopies have been used extensively to probe the structure

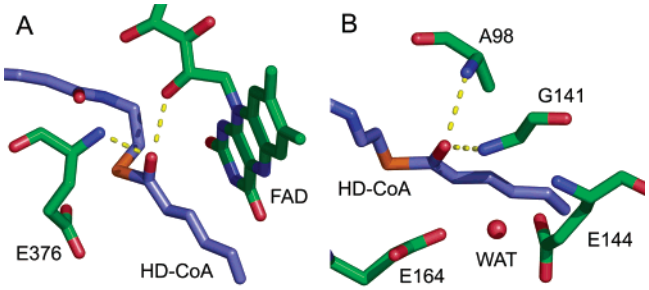

Figure 5. Active sites of MCAD and enoyl-CoA hydratase. (A) Structure of MCAD complexed with hexadienoyl-CoA (HD-CoA). The HD-CoA was modeled into the active site using the structure of 3-thiaoctanoyl-CoA bound to MCAD (1UDY.pdb).[51] The ligand is in close proximity to the isoalloxazine ring of the flavin (FAD) and is hydrogen bonded to the flavin's 2′-ribityl hydroxyl group and the backbone NH of the active site base E376. (B) Structure of HD-CoA bound to enoyl-CoA hydratase from 1MJ3.pdb.[21] The HD-CoA carbonyl group is shown hydrogen bonded to the backbone NH groups of A98 and G141. Also shown are the two catalytic glutamates (E144 and E164) as well as the water molecule that hydrates the substrate (WAT).

of ligands bound to enzymes.[45,46] In many cases these methods reveal subtle changes in the electronic structure of the ligand upon binding.[44,47,48] These perturbations in ground-state structure are a consequence of placing the ligand in an environment that has evolved to stabilize the transition state for the reaction.[21,22] Consequently, the distortions in structure reveal interactions in place to stabilize the transition state for the reaction. In addition, the ground state structural changes may also directly contribute to the catalytic process by destabilizing the reactant ground state.[21,49] In this paper, we extend our spectroscopic studies into the mechanism of the reaction catalyzed by medium chain acyl-CoA dehydrogenase (MCAD) by reporting ^{13}C NMR and ^{1}H−^{13}C HSQC spectroscopy for the product analogue HD-CoA bound to MCAD.

Previous Studies on the Interaction of HD-CoA with MCAD and Enoyl-CoA Hydratase. MCAD and enoyl-CoA hydratase, the first and second enzymes in the fatty acid β-oxidation pathway, share a number of similarities in their active site architecture (Figure 5). Both enzymes provide two hydrogen bonds to the acyl-CoA thioester C═O group in order to stabilize the carbanionic (like) transition states in each reaction. These hydrogen bonds are provided by the backbone amide of E376 and the 2′ ribityl hydroxyl of the flavin in MCAD,[12] and the backbone amides of A98 and G141 in enoyl-CoA hydratase.[50] In addition, both enzymes have an active site acid/base to catalyze protonation/deprotonation of the substrate's α-carbon. In MCAD this residue is E376, in enoyl-CoA hydratase it is E164. From this point the active sites diverge. In enoyl-CoA hydratase there is a second Glu (E144) that is involved in addition of an OH group to the substrate's β-carbon, while MCAD possesses a flavin to accept a hydride from the β-carbon. We have used hexadienoyl-CoA (HD-CoA), a product analogue for the reaction catalyzed by MCAD and a substrate analogue for the reaction catalyzed by enoyl-CoA hydratase, to probe the active sites of both enzyme. Using Raman spectroscopy coupled with isotope labeling, normal mode calculations, model studies, and site-directed mutagenesis, we have developed a detailed picture of the alterations in structure of the hexadienoyl group on binding to both enzymes.[7,19,21,27] Raman spectra of HD-CoA are dominated by bands associated with the −C═C−C═C− double bond stretching vibrations of the hexadienoyl moiety. These modes undergo shifts to lower wavenumber when HD-CoA binds to MCAD or enoyl-CoA hydratase due to altered vibrational coupling between the C═C stretching coordinates and the thioester C═O stretching mode. The alteration in coupling occurs primarily due to a decrease in wavenumber of the C═O mode caused by hydrogen bonding interactions between the enzyme and the HD carbonyl group. Both MCAD and enoyl-CoA hydratase provide two hydrogen bond donors to the substrate/HD C═O that play critical roles in catalysis by stabilizing the accumulation of negative charge on the substrate carbonyl in the carbanionic (like) transition state for the reactions catalyzed by both enzymes.[12,21] In the ground state, these interactions polarize the ligand such that there is a small increase in electron density on the C═O oxygen and C2 carbon and a decrease in electron density at C1 and C3 (Scheme 2). The changes in electron density at C2 and C3 will promote the reaction catalyzed by MCAD by facilitating hydride transfer from C3 and proton abstraction from C2.

The structure of HD-CoA bound to enoyl-CoA hydratase has also previously been studied using ^{13}C NMR spectroscopy by Anderson and co-workers.[26] These studies reveal a downfield shift of the C1 resonance on binding to enoyl-CoA hydratase and an upfield shift at C2, consistent with the Raman studies in which electron density is reduced at C1 and C3, and increased at C2 (Table 2). We have now supplemented these data by observing that the C3 HD-CoA resonance is also shifted downfield on binding, as expected from previous NMR data on other α,β-unsaturated acyl-CoAs[26] and from the Raman studies. On the basis of computational studies, Anderson and co-workers estimated that the ground-state strain revealed by the ligand polarization contributed 3.2 kcal/mol to catalysis[26] while our Raman studies suggest that up to 7 kcal/mol may be available for the enoyl-CoA hydratase reaction.[27] Analogous studies on MCAD suggest similar energetic consequences for the hydrogen bonding interactions in this enzyme. Specifically, the use of 2-deoxy FAD-reconstituted enzyme, where one of the oxyanion hydrogen bond donors has been removed, indicates that this hydrogen bond may be worth up to 4 kcal/mol in ground-state destabilization or a rate increase of 10^3 out of a total rate increase $>10^{11}$ for MCAD.[7]

^{13}C NMR Spectroscopy of HD-CoA Bound to MCAD. On the basis of the above similarities in the structure of HD-CoA bound to both MCAD and enoyl-CoA hydratase revealed by Raman spectroscopy, we anticipated similar correspondence between the ^{13}C NMR data for HD-CoA bound to both enzymes. While the change in chemical shift of the C2 HD carbon (−2.3 ppm) on binding to MCAD is similar to that observed for enoyl-CoA hydratase (−2.2 ppm), interaction with MCAD causes the C1 and C3 HD carbon chemical shifts to move upfield by 12−14 ppm (Table 1) in contrast to the ∼5 ppm downfield shift observed for the binding of HD-CoA to enoyl-CoA hydratase (Table 2). In addition, the ^{1}H chemical shift of the C3-H is also

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moved upfield by 1.3 ppm on binding to MCAD. If we assume that HD-CoA binds to MCAD in an analogous fashion to that observed for octanoyl-CoA[12] or 3-thiaoctanoyl-CoA[51] (Figure 5), then the C1 and C3 HD carbons will be in close proximity to the isoalloxazine ring. Consequently, we believe that the changes in ^{13}C and ^{1}H NMR chemical shifts observed for HD-CoA in complex with MCAD are a result of the isoalloxazine ring current. Importantly, these ring current effects only occur in the presence of an external magnetic field (i.e. during the NMR experiment but not during Raman data collection).

Ring current effects on ^{1}H and ^{13}C NMR chemical shifts in proteins arising from aromatic amino acids have been extensively studied both experimentally and computationally.[52−59] Application of an external magnetic field causes the delocalized electrons in the aromatic ring to circulate which in turn results in a local magnetic field from the ring. The local field is antiparallel to the applied field at the center of the ring and parallel to the applied field outside the ring. Consequently, nuclei occupying positions above or below the plane of the aromatic ring will experience a local field opposing the applied field and hence undergo an upfield change in chemical shift. In contrast, nuclei in the plane of the aromatic ring where the local field reinforces the external field will experience a downfield change in chemical shift.[58] In lysozyme, the ring current effect induced a 3 ppm downfield change in the chemical shift of the M105 β-H due to deshielding of this nucleus by the aromatic amino acids Y23, W108, and W111.[56] Ring current effects due to enzyme-bound aromatic cofactors such as porphyrins also cause large effects on the chemical shifts of nearby residues. In the light harvesting complex 2 from *Rhodopseudomonas acidophila*, H_δ and H_γ in the His residues that coordinates the porphyrin Mg (β-H30 and α-H31) are shifted upfield 3.50 and 3.70 ppm, respectively.[60] Additionally, in cytochrome c_2 from *Rhodospirillum rubrum*, M91 is one of the heme axial ligands and is therefore situated directly under the heme ring. As a result, resonances of the C_β, C_γ, and C_ϵ of Met-91 are shifted upfield by 5.7, 3.3, and 1.6 ppm, respectively, from their random coil values.[61] Of particular relevance to this work, Giessner-Prettre and Pullman[59] calculated the magnitude of the chemical shift brought about by a flavin ring at a distance of 3.4 Å from an intermolecular proton. Their work showed that at this distance, the chemical shift of the proton could be changed by as much as 1.2 ppm.

Returning to our studies of HD-CoA bound to MCAD, the structural data (Figure 5) indicate that the HD moiety is below the flavin ring and thus occupies a position where a ring current would cause an upfield change in chemical shift, consistent with the NMR data for the bound ligand. However, the ring current shifts we observe due to the flavin ring are significantly larger than any that have so far been reported. Indeed, if we assume that the C1 and C3 HD carbons would have chemical shifts similar to those observed for HD-CoA bound to enoyl-CoA hydratase in the absence of the applied magnetic field, then the ring current from the isoalloxazine ring causes an upfield shift of around 18 ppm for the C1 and C3 carbons. These large ring current effects are undoubtedly a consequence of the close proximity of the ligand and flavin. Attempts to reproduce the experimental changes in NMR chemical shift using DFT calculations on a simple model system established that (1) large chemical shifts, of the order and direction observed experimentally, can be predicted and that (2), the NMR spectrum depends highly on the model geometry. However, closer agreement between theory and experiment will undoubtedly require methods that allow inclusion of a greater portion of the protein, such as would be possible using a hybrid QM/MM approach.

Table 4. ^{13}C Chemical Shift Values for AcAc-CoA and 3-Thiaoctanoyl-CoA Free and Bound to MCAD

		free		bound		
ligand		δ (ppm)	$\Delta\nu_{1/2}$[c]	δ (ppm)	$\Delta\nu_{1/2}$[c]	$\Delta\delta$ (ppm)
AcAc−CoA[a]	$^{13}C1$	198.5	b	181.3	n	−17.2
	$^{13}C2$	59.9	n	103.4	b	+43.5
	$^{13}C3$	208.8	b	192.3	n	−16.5
3-thiaoctanoyl-CoA[b]	$^{13}C1$	203.3	b	163.7	n	−39.6
	$^{13}C2$	44.3	n	101.2	b	+56.9

[a] Data from Miura et al.[24] [b] Data from Tamaoki et al.[25] [c] The line width was not reported. However, the line shapes of the free and bound species were compared and denoted as "b" if the band was relatively broad and "n" if the band were narrower.

Charge transfer bands have been observed in many MCAD−ligand complexes and it has been argued that these bands are evidence not only for electronic interaction between flavin and ligand but also have catalytic consequence by stabilizing the transition state(s) for the reaction.[17,51] This interaction between ligand and flavin is exquisitely sensitive to subtle perturbations. For example, Thorpe and co-workers have noted that 5-deaza flavin-reconstituted MCAD is incapable of catalyzing α-proton exchange in a bound substrate.[16] On the basis of this information, we can now reexamine previous ^{13}C NMR studies on ionizable ligands bound to MCAD.

AcAc-CoA and 3-Thiaoctanoyl-CoA Bound to MCAD. Binding to MCAD causes the ^{13}C NMR chemical shifts of the C1 and C3 AcAc-CoA carbons to move upfield by around 17 ppm, while the C2 carbon resonance moves downfield by over 40 ppm.[24] Similar changes are observed for the binding of 3-thiaoctanoyl-CoA where binding causes changes of −40 and 57 ppm for the C1 and C2 carbons, respectively (Table 4).[25] These alterations in chemical shift have been ascribed to proton abstraction from C2 with the formation of an enzyme-stabilized enolate. However, while enolate formation is undoubtedly responsible for some of the chemical shift changes observed, we believe that the flavin ring current is also playing a key role. Specifically, for AcAc-CoA two enolate species can be formed (Scheme 4). In both cases the C2 carbon undergoes a change in hybridization from sp^3 to sp^2, in line with the large downfield change in ^{13}C chemical shift for the C2 carbon.[24,62]

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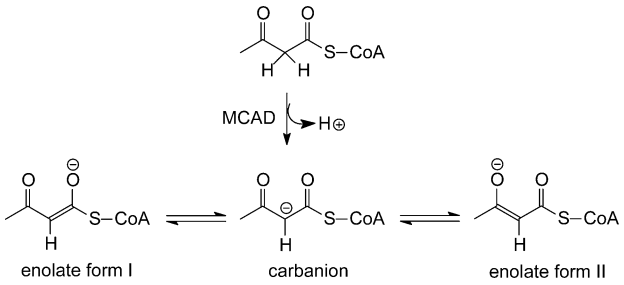
Scheme 4. AcAc−CoA Enolate Tautomers



However, on the basis of the fact that the enzyme active site is designed to stabilize charge accumulation on the carbonyl oxygen, we believe that enol form I will be preferentially present in the AcAc-CoA/MCAD complex.

To provide a reference point for this discussion, we thus examined the ^{13}C NMR spectrum of AcAc-CoA bound to enoyl-CoA hydratase. AcAc-CoA is a μM inhibitor and binds to enoyl-CoA hydratase as the enolate.[63,64] Our NMR data show chemical shift changes of −10.5, 35.1, and 9 ppm for the AcAc-CoA C1, C2, and C3 resonances, respectively, upon binding to enoyl-CoA hydratase (Table 3). Thus, the C1 and C2 AcAc carbons experience changes in chemical shift that are in the same direction, albeit smaller, than those observed in MCAD. However, the C3 AcAc carbon moves downfield 9 ppm upon binding to enoyl-CoA hydratase in contrast to the −16.5 ppm change upon binding to MCAD. On the basis of these data, we believe that both MCAD and enoyl-CoA hydratase bind AcAc-CoA as enol form I (Scheme 4) and that the upfield change in the C3 resonance in the MCAD complex is a result of the flavin ring current. This conclusion is supported by data on ethyl acetoacetate where keto to enol(ate) tautomerism causes an upfield change in chemical shift of the carbonyl carbon involved in the tautomerism, but a downfield change in chemical shift of the second carbonyl carbon.[24,62] Finally, the fact that the change in ^{13}C1 chemical shift is larger in MCAD than in enoyl-CoA hydratase (−17.2 vs −10.5 ppm) suggests that the flavin ring current is also partly responsible for the chemical shift of this carbon in the MCAD complex.

Ring Current Effects on Line Width. From Table 2 we can see that C1 and C3 HD-CoA resonances are broader when HD-CoA is bound to enoyl-CoA hydratase than the signals arising from the unbound ligand, reflecting the decreased transverse relaxation (*T2*) delays as a result of the decreased tumbling rate of the bound ligand. In contrast, when HD-CoA binds to MCAD, the bands arising from the C1 and C3 HD carbons are narrower than the corresponding bands from the unbound ligand (Table 1). This binding-induced decrease in line width is also observed for the ^{1}H signal arising from the proton attached to C3. Transformation of the first FID block from the HSQC experiment of ^{13}C3-HD-CoA bound to MCAD gives the C3-H proton spectrum of HD-CoA bound to MCAD (data not shown) where it can be seen that the peak arising from the bound ligand at 5.83 ppm is narrower (12 Hz) than that for the unbound ligand at 7.14 ppm (24 Hz). This decrease in line width is not observed for the C2 HD-CoA carbon, which experiences a similar change in chemical shift in complexes with both MCAD and enoyl-CoA hydratase (−2 ppm). Similar trends in line widths are also observed when AcAc-CoA and 3-thiaoctanoyl-CoA are bound to MCAD (Table 3) and thus it appears that only those nuclei in the ligand that experience the flavin ring current in MCAD show a decrease in line width upon binding. This suggests that an increase in *T2* accompanies the upfield change in chemical shift caused by the flavin's isoalloxazine ring.

Conclusion

^{13}C and ^{1}H NMR data have been obtained for HD-CoA bound to MCAD and enoyl-CoA hydratase. Binding causes a large upfield change of −13 to −14 ppm in the chemical shift for the C1 and C3 HD carbons upon binding to MCAD whereas smaller downfield chemical shift changes of 4 to 5 ppm are observed when HD-CoA is bound to enoyl-CoA hydratase. These data are in contrast to the picture provided by Raman spectroscopy which indicates that the electronic structure of the hexadienoyl group is similar when HD-CoA is bound to both enzymes. The discrepancy between the NMR and Raman data for ligands bound to MCAD is rationalized by proposing that the isoalloxazine ring of the MCAD flavin exerts a strong ring current effect on the C1 and C3 HD carbons. The local field produced by the isoalloxazine ring occurs only in the presence of an external magnetic field and causes the C1 and C3 HD resonances to move upfield by as much as 18 ppm. DFT calculations on a simple model system reproduce the direction though not the full magnitude of the experimentally observed changes in chemical shift and pave the way for a more detailed theoretical analysis using hybrid QM/MM methodology. Our conclusions are substantiated by ^{13}C NMR data for complexes of AcAc-CoA with MCAD and enoyl-CoA hydratase, which show that this ligand is bound as an enolate to both enzymes with the enol tautomer localized on C1 in each case.

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