4-Hydroxycinnamoyl-CoA: An Ionizable Probe of the Active Site of the Medium Chain Acyl-CoA Dehydrogenase†

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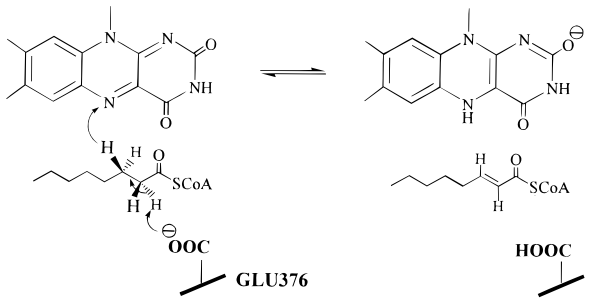
ABSTRACT: 4-OH-Cinnamoyl-CoA has been synthesized as a probe of the active site in the medium chain acyl-CoA dehydrogenase. The protonated form of the free ligand (λ_{max} = 336 nm) yields the corresponding phenolate (λ_{max} = 388 nm) with a p*K* of 8.9. 4-OH-Cinnamoyl-CoA binds tightly (K_d = 47 nM, pH 6) to the pig kidney dehydrogenase with a prominent new band at 388 nm, suggesting ionization of the bound ligand. However, this spectrum reflects polarization, not deprotonation, of the neutral form of the ligand. Thus, the 388 nm band is abolished as the pH is raised (not lowered), and analogous spectral and pH behavior is observed with the nonionizable analogue 4-methoxycinnamoyl-CoA. Studies with wild type, E99G, and E376Q mutants of the human medium chain acyl-CoA dehydrogenase showed that these two active site carboxylates strongly suppress ionization of the 4-OH ligand. Binding to the double mutant E99G/E376Q gives an intense new band as the pH is raised (p*K* = 7.8), with an absorbance maximum at 498 nm resembling the natural 4-OH-cinnamoyl-thioester chromophore of the photoactive yellow protein. Raman difference spectroscopy in water and D_2O, using the free ligand and wild-type and double-mutant enzyme·ligand complexes, confirms that the 4-OH group of the thioester is ionized only when bound to the double mutant. These data demonstrate the strong electrostatic coupling between ligand and enzyme, and the critical role Glu376 plays in modulating thioester polarization in the medium chain acyl-CoA dehydrogenase.

Activation of thioester substrates in the medium chain acyl-CoA dehydrogenase is initiated by removal of the *pro-R* α-proton with the concerted elimination of a hydride equivalent from the *β*-position to the N-5 position of the flavin ring (*1*−*6*; Scheme 1). The resulting complex, between reduced flavoprotein and tightly bound *trans*-2-enoyl-CoA product (*7*−*10*), is then reoxidized in two 1-electron steps by the electron-transferring flavoprotein (ETF)[1] (*7*, *11*−*13*). Chemical modification and crystallographic and mutagenesis studies show that the carboxylate of Glu376 is the catalytic base in the medium chain enzyme (*14*−*17*). Recent studies have shown that the p*K* of this residue is dramatically raised (to greater than 9) with the desolvation of the active site that accompanies binding a preferred substrate (*18*−*20*). While this effect goes some way to explaining rate enhancements in the acyl-CoA dehydrogenases, the stabilization of the developing negative charge on the substrate carbonyl oxygen in the transition state appears to be catalytically crucial (*19*, *20*−*22*). While there are no helix dipoles or metal ions directed toward this carbonyl group, two hydrogen bonds

Scheme 1



have been identified in the snugly fitting oxygen binding pocket (*16*, *20*, *22*, *23*). One H-bond is provided by the backbone N−H of Glu376 itself, the other from the 2′-OH− of the ribityl side chain of the bound flavin (*16*). Removing this latter interaction, by replacing the normal flavin with 2′-deoxy-FAD, raises the activation energy for substrate dehydrogenation by about 8−9 kcal (*20*, *23*). In addition, charge-transfer between a developing enolate donor stacked over the electron-deficient oxidized flavin acceptor might also contribute to lowering the activation energy barrier for deprotonation and flavin reduction.

The present study started with the aim of finding tight binding inhibitors of the acyl-CoA dehydrogenase based on the ability of the enzyme to stabilize anionic thioester analogues. Thus, for example, even the simple CoA persulfide, identified as the natural "greening ligand" of a number of acyl-CoA dehydrogenases (*24*), and CoA-sulfonate (*25*)

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[1] Abbreviations: ETF, electron-transferring flavoprotein; hMCAD, recombinant human medium chain acyl-CoA dehydrogenase; PYP, photoactive yellow protein.

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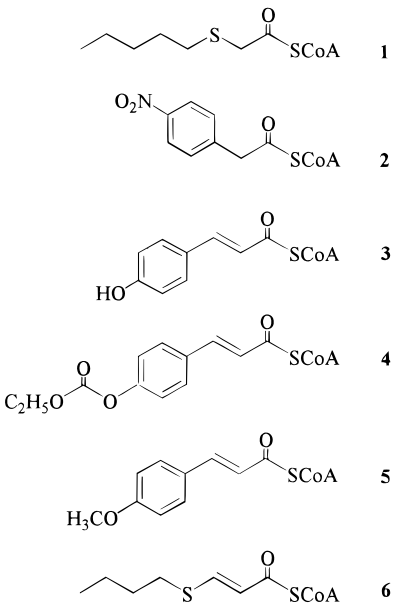
Chart 1: CoA Analogues Described in This Work[a]



[a] Compound **1**, 3-thiaoctanoyl-CoA; compound **2**, *p*-nitrophenylacetyl-CoA; compound **3**, 4-OH-cinnamoyl-CoA; compound **4**, 4-OH-cinnamoyl-CoA ethyl carbonate; compound **5**, 4-methoxycinnamoyl-CoA; compound **6**, 4-thia-*trans*-2-octenoyl-CoA.

bind much more tightly than CoASH itself. 3-Ketoacyl-CoA derivatives bind as their enolates (*25*−*31*), although the bulky and polar carbonyl oxygen at C-3 is likely to impair binding per se (*25*). 3-Thiaoctanoyl-CoA and related thioesters (compound **1**, Chart 1; *25*, *32*) are perhaps better examples, and provide a dramatic illustration of the enzyme's ability to lower the p*K* of the α-protons of these weakly acidic substrate analogues by up to 10 pH units (*20*−*22*, *32*, *33*). The large p*K* shifts correspond to a strong preferential binding of the enolate forms of these ligands (*22*, *25*, *32*, *33*). *p*-Nitrophenylacetyl-CoA (compound **2**, Chart 1) and related compounds synthesized by Ghisla and colleagues provide sensitive active site probes of the acyl-CoA dehydrogenases because their enolates are colored, and can therefore be visualized directly (*21*). *p*-Nitrophenylacetyl-CoA binds to the medium chain enzyme (K_d = 25 μM at pH 7.6; *21*) with an apparent p*K* of 4.9, some 8−9 pH units lower than that estimated for the free ligand (p*K* = 13.6; *21*). Despite their great utility, these colored analogues bind comparatively weakly to the dehydrogenase because the bulky substituent at C-2 impairs binding. Indeed, the parent analogue, phenylacetyl-CoA, binds to the medium chain dehydrogenase with a K_d of about 110 μM (Zhou and Thorpe, unpublished observations).

The early work of Murfin and Drysdale (*1*) identified a number of ring-substituted dihydrocinnamoyl-CoA analogues as substrates of the medium chain enzyme. These observations led to the idea that a corresponding enoyl-CoA product, 4-OH-cinnamoyl-CoA (Chart 1, compound **3**), might be an interesting ligand of the enzyme. First, the bulky benzene ring is now a substituent of the C-3 (not the C-2) position: this would be expected to be better tolerated by the enzyme. Second, other enoyl-CoA analogues have proved sensitive probes of the polarization[2] of the thioester carbonyl group (*18*, *19*, *34*, *35*). Third, the p*K* of the phenolic oxygen of this cinnamoyl derivative has been estimated at about 9.0 (*36*), and so we might have a relatively acidic ionizable probe of the enzyme. Fourth, 4-OH-cinnamoyl-CoA plays a central role in plant secondary metabolism, so a more detailed characterization of this thioester is of interest. For example, 4-OH-cinnamoyl-CoA was found to be an important metabolite in the biosynthesis of acylated flavanoids, alkaloids, lignins, and chlorogenic acid (*37*, *38*). Its role in plant-pathogen defense mechanisms includes the biosynthetic pathways for hydroxycinnamoyltyramines, cell wall reinforcement compounds (*39*−*42*), and the antifungal phytoalexins (*43*). Finally, a thioester between 4-OH-cinnamic acid and a cysteine residue in the photoactive yellow protein (PYP) is the chromophore responsible for blue-light photoavoidance in a number of halophilic purple phototrophic bacteria (*44*−*48*). The mode and consequences of cis/trans photoisomerization of this chromophore in PYP are under intensive study (*49*−*53*).

This work presents a convenient synthesis of 4-OH-cinnamoyl-CoA and a characterization of its properties both in free solution and bound to the medium chain acyl-CoA dehydrogenase. These studies reiterate the importance of electrostatic interactions in the active site of the acyl-CoA dehydrogenase and provide a dramatic example of the polarization of a thioester enone. Finally, 4-OH-cinnamoyl-CoA complexes of the acyl-CoA dehydrogenase are examined by Raman spectroscopy to allow the protonation state of the ligand to be determined and to provide a comparison to the corresponding thioester in PYP.

MATERIALS AND METHODS

Enzymes. Pig kidney medium chain acyl-CoA dehydrogenase (*54*) was purified following the modifications described in DuPlessis et al. (*55*). Recombinant human wild-type medium chain dehydrogenase, the E376Q and E99G single mutants (generous gifts from Dr. Sandro Ghisla), and the E99G/E376Q double mutant were purified by the methods described previously (*19*, *55*). Recombinant enzymes were de-greened to free them of CoA persulfide (*24*) as described earlier (*19*).

The double mutant was constructed as follows. pTrc/E376Q TG1 *Escherichia coli* cells were a generous gift of Dr. Sandro Ghisla. The additional E99G mutation was introduced using the oligonucleotide 5′-GGTTCAGACT-GCTATTGGTGGAAATTCTTTGGGG-3′ (sense) and the QuikChange (Statagene) site-directed mutagenesis kit. The mutation was verified by sequence analysis (Biology Core Facility, University of Delaware) using a 17-mer synthetic sequencing oligonucleotide. The expression plasmid pTRC carrying the double mutation was then transformed into *E. coli* BL21(DE3) cells.

Materials. 4-Methoxy- and 4-OH-cinnamic acids, octanoyl-CoA, coenzyme A, and porcine liver esterase were purchased from Sigma. 4-Methoxycinnamoyl-CoA and cinnamoyl-CoA were synthesized as described previously (*18*). BL21(DE3) competent cells were from Novagen.

General Methods. Unless otherwise stated, all UV−Vis titrations were done at 25 °C using an HP8452 diode array spectrophotometer in buffers containing 0.3 mM EDTA.

[2] Throughout this work, polarization refers to a red-shift in the enone absorbance of a bound ligand compared to the same protonation state in free solution.

Concentrations of wild-type and E376Q and E99G medium chain acyl-CoA dehydrogenases were determined using an extinction coefficient of 14.8 mM^{-1} cm^{-1} at 448 nm (*56*). The corresponding value for the double mutant was determined by the guanidine hydrochloride method (*54*) as 14.1 mM^{-1} cm^{-1}. 4-Methoxycinnamoyl-CoA was quantitated at 260 nm (20.1 mM^{-1} cm^{-1}; *57*). Ligand binding and pH titration experiments were performed as described previously (*19*).

Synthesis of 4-OH-Cinnamoyl-CoA. 4-OH-Cinnamoyl-CoA was synthesized using the mixed anhydride method (*58*). 4-OH-Cinnamic acid (0.11 mmol in 1.5 mL of dry tetrahydrofuran) was mixed at room temperature with 1 equiv of dry triethylamine followed by 1 equiv of ethyl chloroformate. The mixture was stirred under nitrogen for 5 min, and then 0.3 equiv of CoASH in 1.5 mL of 250 mM $NaHCO_3$ was added to the cloudy white suspension. The resulting clear, pale yellow, solution was stirred for 10 min and then adjusted to pH 5 with acetic acid. The crude thioester was purified on a semipreparative octadecylsilica HPLC column with an increasing gradient of methanol and 25 mM phosphate buffer, pH 5.3 (*59*). The major product was desalted by HPLC and concentrated by rotary evaporation. The material showed absorbance maxima at 262 and 308 nm, consistent with a CoA thioester, but showed no obvious pH dependence from 6 to 10 (data not shown). Proton NMR, recorded in D_2O using a Bruker 400 MHz spectrometer, showed, in addition to the expected CoA resonances, an additional triplet (three protons) at 1.2 ppm and a quartet (two protons) at 4.15 ppm. The resonances for trans 2-enoyl protons at 6.55 and 7.3 ppm and for aromatic protons at 7.05 and 7.4 ppm were observed. All these data are consistent with structure **4** (Chart 1). Compound **4** binds relatively weakly to the pig medium chain acyl-CoA dehydrogenase (K_d = 5.5 μM in 50 mM phosphate buffer, pH 6) with a small red-shift of the main absorbance peak (data not shown). The ethyl carbonate group was conveniently removed by diluting compound **4** to 30 μM with water and incubating 10 mL of the solution with 0.11 mg of porcine liver esterase for 10 min at 25 °C. The reaction was conveniently followed by the decrease at 308 nm and the increase in absorbance at 336 nm. The esterase was removed by ultrafiltration and the product concentrated by rotary evaporation. The resonances for the trans 2-enoyl protons at 6.26 and 7.45 ppm (scalar coupling of 16.2 Hz) and for the aromatic protons at 6.82 and 7.54 ppm were consistent with compound **4** (Chart 1) and the trans form of the PYP chromophore (*50, 60*).

The extinction coefficient for 4-OH-cinnamoyl-CoA was determined as described previously (*57*). The NMR spectrum of a solution of the thioester of known absorbance was integrated using potassium acetate as internal standard in D_2O. Spectra were recorded using a Bruker 250 MHz spectrometer with an Aspect 3000 computer. Extinction coefficients for 4-OH-cinnamoyl-CoA were $\epsilon_{262\ nm}$ = 20 mM^{-1} cm^{-1} and $\epsilon_{336\ nm}$ = 27.6 mM^{-1} cm^{-1}.

Raman Spectroscopy. Raman difference spectra were acquired using an instrument based, in part, on that described by Dong et al. (*61*). All the components have been optimized for operation in the near-IR to reduce problems associated with large fluorescence backgrounds. Near-IR excitation was provided by a model 890 Ti:sapphire laser (Coherent), pumped by an Innova 308C argon ion laser (Coherent; operated at about 800 mW). Excitation at 752 nm provided the best balance between Raman intensity, throughout the spectral region of interest, and obtainable laser power. A holographic band-pass filter (Kaiser Optical Systems, Inc.) was placed at the exit port of the Ti:sapphire laser to remove spurious lines that were found to interfere with the Raman difference spectra of strongly scattering samples. The laser beam was focused into the base of a rectangular quartz cell, and the Raman scattering was collected in a 90° geometry. Raman scattered light was collimated by an f/1 poly(methyl methacrylate) aspheric lens (US Precision Lens) before passing through a super notch plus holographic filter (Kaiser Optical Systems, Inc.) to suppress the Rayleigh scattered light. The collimated light was then focused onto the slit of the spectrograph by a near-IR-coated 85 mm f/1.4 camera lens (Kaiser Optical Systems, Inc.). To maximize throughput, a single grating f/1.4 Kaiser Holospec spectrograph was used to disperse the Raman scattered light. The advantages of this spectrograph, which employs an axially transmissive holographic grating, have been discussed elsewhere (*61*). Finally, a red-sensitive, back-thinned, back-illuminated CCD camera (Princeton Instruments, model no. EHRB1024) was used to detect the Raman scattered intensity. This detector had a peak quantum efficiency of about 80%, and allowed measurement of the spectral region between about 400 and 2500 cm^{-1} in a single run using 752 nm excitation. Under the conditions used here (see later), the resolution of our system was approximately 12 cm^{-1}. Data acquisition used WinSpec (Princeton Instruments) and spectral manipulation utilized Win-IR software.

Raman spectra were acquired for 4 min using 120 μL of enzyme (100−200 μM) in a 3 mm by 3 mm rectangular quartz cell. Ligand (typically 1 or 2 μL) was added to the solution and the spectrum re-recorded without making any changes to the optical alignment or the cell position. Difference spectra were then calculated by subtraction, choosing an appropriate scaling factor to remove any residual protein signals. Spectra were wavenumber-calibrated against cyclohexanone.

Modeling. Insight II (MSI 95.0) was run on a Silicon Graphics Indigo II workstation. Ligand Design and Discover programs were used to construct and model 4-OH-cinnamoyl-CoA into the active site of the B subunit of pig medium chain acyl-CoA dehydrogenase as described previously (*18, 62*).

RESULTS AND DISCUSSION

Synthesis and Characterization of 4-OH-Cinnamoyl-CoA. The synthesis of 4-OH-cinnamoyl-CoA presented under Materials and Methods provides a simple alternative procedure to several rather laborious earlier methods (*37, 63*−*67*). The new method directly affords the 4-OH-protected thioester shown in Chart 1 (compound **4**), and this derivative can be readily purified by HPLC and stored until needed. Although chemical methods for removing the protecting group proved problematic (not shown), the 4-OH-substituent is rapidly and quantitatively hydrolyzed by pig liver esterase (see Materials and Methods). Subsequently, the esterase can be removed by ultrafiltration and the ethanol, released from hydrolysis of the protecting group, by evaporation. The 4-OH material so obtained was pure by HPLC and gave the expected proton NMR spectrum (see Materials and Methods).

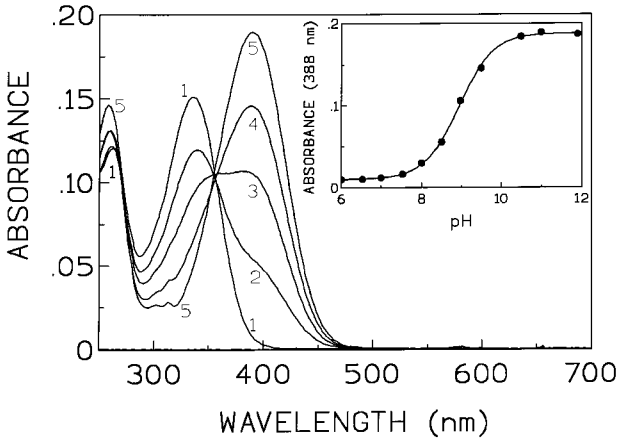


FIGURE 1: pH titration of free 4-OH-cinnamoyl-CoA. The thioester was diluted to a concentration of 6.03 μM in 0.7 mL aliquots of a mixture of 25 mM potassium phosphate and 25 mM sodium pyrophosphate (25 °C) adjusted to the pH values shown in the inset. Curves 1−5 correspond to pH 6, 8.5, 9, 9.5, and 11, respectively. The absorbance changes at 388 nm were fit a p*K* of 8.9 as seen in the inset.

Figure 1 shows a pH titration of 4-OH-cinnamoyl-CoA. At low pH, the thioester shows a prominent peak at 336 nm (curve 1; $\epsilon = 27.6$ mM^{-1} cm^{-1}) in addition to the expected adenine absorbance at 260 nm. The ratio of the absorbance at 336 and 260 nm (1.23, curve 1) is considerably higher than that observed with material synthesized by thioester exchange (*37*, *64*), by use of *N*-hydroxysuccinimide esters (*37*, *64*, *66*), via carbohydrate protection (*38*, *64*), or enzymatically by using either acyl-CoA synthetase from beef liver (*63*) or cinnamoyl-CoA synthetase from *Pseudomomas putida* (*65*). In Figure 1, deprotonation of 4-OH-cinnamoyl-CoA yields curve 5, with a red-shifted, more intense, band at 388 nm ($\epsilon = 34.2$ mM^{-1} cm^{-1}) and the appearance of additional low-intensity sharper peaks at 302 and 314 nm. The inset in Figure 1 shows the absorbance changes at 388 nm fit to the ionization of a single proton with a p*K* of 8.9. This agrees with the estimate of 9.0 ± 0.5 for the corresponding p*K* of the thioester chromophore in denatured PYP (*36*; see later). The deprotonated thioester is rather stable: less than a 3% decline in 388 nm absorbance occurs over 20 min in a mixture of 25 mM potassium phosphate and 25 mM pyrophosphate buffer adjusted to pH 11.9.

4-OH-Cinnamoyl-CoA Binding to Pig Kidney Medium Chain Acyl-CoA Dehydrogenase. Figure 2 shows the spectral changes produced on binding 4-OH-cinnamoyl-CoA to the pig kidney medium chain acyl-CoA dehydrogenase at pH 6.0. The changes are completed in less than 5 s and are stable apparently indefinitely. Upon thioester binding, the main flavin absorbance peak is red-shifted by 6 nm, and a shoulder appears at 492 nm as expected for ligand-induced desolvation of the active site (*18*, *25*). Since 4-OH-cinnamoyl-CoA binds very tightly to the dehydrogenase, an accurate estimate of the dissociation constant required the use of low enzyme concentrations in 5 cm path-length cells. The inset in Figure 2 represents such a titration with data fit to a K_d of 47 nM. The additional absorbance increase below 440 nm, upon the addition of 1 equiv of 4-OH-cinnamoyl-CoA (Figure 2, curve 3), is dominated by the contribution of the bound ligand.

Figure 3 compares the difference spectra (E·L − E) at two pH values. At pH 6, the bound 4-OH-cinnamoyl-CoA

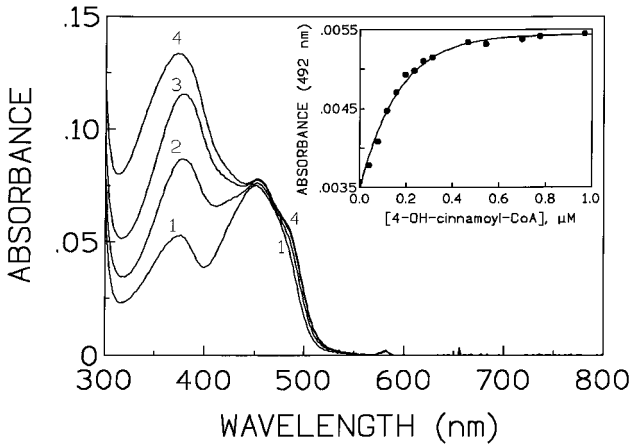


FIGURE 2: Spectral changes on the addition of 4-OH-cinnamoyl-CoA to pig kidney medium chain acyl-CoA dehydrogenase. The enzyme (4.84 μM in 0.7 mL of 50 mM phosphate buffer, pH 6, 25 °C; curve 1) was titrated with 0.52, 1.05, and 1.55 equiv of 4-OH-cinnamoyl-CoA (curves 2−4, respectively). Intermediate spectra are omitted for clarity. The inset is a titration repeated using 0.15 μM enzyme in a 5 cm path-length cell. The line corresponds to a K_d of 47 nM.

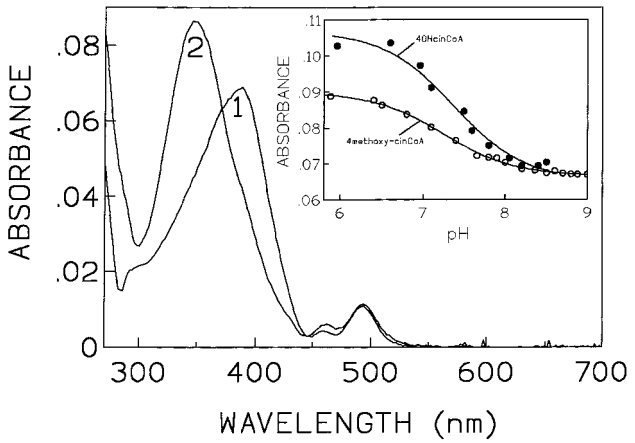


FIGURE 3: pH dependence of the difference spectrum of the pig kidney medium chain acyl-CoA dehydrogenase complexed with 4-OH-cinnamoyl-CoA and free enzyme. Main panel: the spectrum of the enzyme (4.4 μM) in 0.7 mL of 10 mM phosphate buffer, pH 6, 25 °C, was subtracted from that after the addition of 0.7 equiv (3.08 μM) of 4-OH ligand (curve 1). The pH of the solution was then increased by the careful addition of increments of 2 M Tris buffer (2 M, pH 9.5) up to a pH of 8.4 (curve 2). Intermediate spectra are omitted for clarity. The spectrum of the enzyme alone is essentially unchanged under these conditions. The closed circles in the inset show that the pH dependence at 388 nm fits a single proton slope with an apparent p*K* of 7.4. The experiment was repeated with 4-methoxycinnamoyl-CoA using 5.4 μM enzyme and 432 μM (92 equiv) ligand. This higher ligand concentration was used to ensure saturation as the pH is raised. To avoid excessive end-absorbance of the ligand, polarization was monitored, not at the maximum 364 nm (pH 6), but at 420 nm (open circles). The line is for a monoprotic ionization with an apparent p*K* of 7.2.

gives a maximum at 388 nm (curve 1) superimposed on the smaller perturbation of the flavin chromophore (maximum at 492 nm). The contribution of the flavin perturbation within these complexes can be estimated with some confidence. Thus, simple CoA thioester ligands, which do not themselves interact electronically with the oxidized enzyme by charge-transfer or net redox reactions (e.g., octyl-SCoA, 2-aza-octanoyl-CoA, and *trans*-2-octenoyl-CoA; *18*, *25*), all induce similar flavin difference spectra. Comparison of these spectra

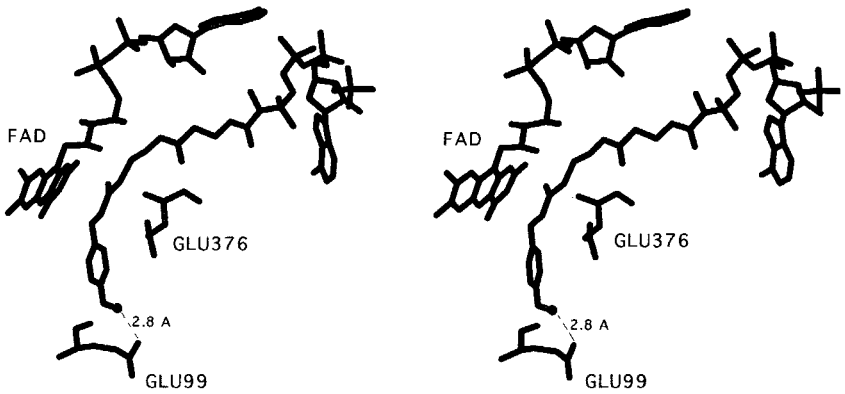


FIGURE 4: Model of 4-OH-cinnamoyl-CoA bound to the medium chain acyl-CoA dehydrogenase. The figure was prepared as described under Materials and Methods. For clarity, only FAD, Glu99, and Glu376 are shown from the enzyme.

with those in Figure 3 shows that curves 1 and 2 are dominated by the absorbance of the bound 4-OH-ligand.

Since the peak at 388 nm (curve 1, Figure 3) coincided with the absorbance of the deprotonated 4-OH-cinnamoyl-CoA (curve 5, Figure 1), we initially believed that the ligand was bound to the acyl-CoA dehydrogenase in its deprotonated form at pH 6. However, the work described below shows that this is not the case. The 388 nm peak reflects polarization of the bound neutral (protonated) form of the ligand, rather than ionization of 4-OH-cinnamoyl-CoA.

One observation not readily reconciled with ionization of the ligand at pH 6.0 is the pH dependence of the spectral changes. Solutions of the medium chain acyl-CoA dehydrogenase complexed with 0.7 equiv of 4-OH-cinnamoyl-CoA at pH 6.0 were titrated with base. The main panel in Figure 3 shows that increasing pH does not result in significant dissociation of the ligand from the enzyme, because the flavin difference spectrum at 492 nm remains substantially unchanged. The use of substoichiometric ligand concentrations ensures that the absorbance changes below 400 nm are not complicated by the contribution of free chromophore. In Figure 3, the peak at 388 nm (pH 6) shifts to 348 nm at pH 8.4 (curve 2, Figure 3). The inset fits these data to the ionization of a single group with an apparent p*K* of 7.4. Obviously, such changes are the exact opposite of those expected for a simple deprotonation, where the anionic form of the ligand should appear at high pH (see Figure 1).

Experiments with 4-methoxycinnamoyl-CoA (Chart 1, compound **5**; see Materials and Methods), a nonionizable analogue of the 4-OH derivative, confirm that the spectral changes in Figure 3 cannot be due to ionization of the ligand. The 4-methoxy analogue binds to the pig kidney medium chain dehydrogenase with spectral changes at pH 6.0 similar to those shown in Figure 2 ($K_d = 0.68\ \mu$M; data not shown). Thus, the free ligand has an absorbance maximum at 334 nm, and the bound ligand about 364 and 336 nm at pH 6 and 8.5, respectively (not shown). The apparent p*K* for this loss of red-shifted absorbance is 7.2 (inset, Figure 3, open circles), similar to the p*K* of 7.4 observed for the 4-OH analogue. Recent studies with 4-thia-*trans*-2-enoyl-CoA analogues (such as the octenoyl-CoA derivative, compound **6**, Chart 1; *18, 19*) help rationalize these data. These thioesters exhibit a pH-independent absorbance maximum for their free forms at 312 nm which is shifted to 342 nm when bound to the medium chain acyl-CoA dehydrogenase at low pH (*18*). With increasing pH, the ligand remains bound, but the red-shifted polarized spectrum of the chromophore disappears as the catalytic base Glu376 deprotonates (*19*). Furthermore, the modulation of the p*K* of Glu376 depends on the length of the 4-thiaenoyl-CoA analogue bound to the enzyme. Ligands with an optimal chain length exert the most effect, with p*K* values above 9 for C-8 chains (*18, 19*). The p*K* measured with the somewhat-oversized C-13 analogue is 7.5 (*19*), similar to that observed with 4-OH-cinnamoyl-CoA. Thus, the data presented in Figure 3 are consistent with a loss of polarization of bound 4-OH- and 4-methoxy-cinnamoyl-CoA derivatives with deprotonation of Glu376.

Figure 4 shows a structure of 4-OH-cinnamoyl-CoA complexed to the porcine medium chain acyl-CoA dehydrogenase (see Materials and Methods). During the dehydrogenation of a normal substrate, such as octanoyl-CoA, the side chain of the catalytic base, Glu376, is believed to rotate to allow abstraction of the *pro-R* α-proton. The data presented above suggest that the p*K* of Glu376 in the 4-OH-cinnamoyl-CoA complex (p$K = 7.4$; see earlier) is lower than that of the bound phenol, so that loss of ligand polarization occurs before deprotonation of the thioester. Once deprotonated, the negative charge on Glu376 would be expected to raise the p*K* of the ligand still further. Thus, we examined the behavior of the E376Q mutant of the medium chain enzyme because it might allow deprotonation of the 4-OH ligand at elevated pH values.

Ligand Binding to Recombinant Wild-Type, E99G, and E376Q Human Medium Chain Acyl-CoA Dehydrogenase. Since the human, but not the porcine, medium chain acyl-CoA dehydrogenase has been cloned and overexpressed (*15, 55, 68*), we repeated key experiments to ensure that both enzymes behaved comparably toward 4-OH-cinnamoyl-CoA. The enzymes show high sequence identity (*15, 17*), have essentially the same crystal structure (*16, 17, 56*), and show great mechanistic similarities (*23, 68, 69*). The recombinant human enzyme gave a somewhat smaller spectral shift on the addition of 4-OH-cinnamoyl-CoA at pH 6.0, with a difference maximum at 370 nm, not 388 nm (data not shown). However, it still binds the ligand tightly (K_d of 40 vs 47 nM for the pig kidney enzyme; Table 1). As expected, the human enzyme showed the same decrease in polarization as the pH is raised (with an apparent p*K* of 7.8 compared to 7.4; see earlier).

The inset to Figure 5 shows (E·L − E) difference spectra for the E376Q mutant of the human enzyme. In sharp contrast

Table 1: Binding Constants for 4-OH-Cinnamoyl-CoA in Complex with Medium Chain Acyl-CoA Dehydrogenase and Its Mutants[a]

enzyme	K_d (μM, pH 6)	K_d (μM, pH 8.3)
pig MCAD	0.047	0.295
human MCAD	0.040	1.45
E376Q hMCAD	0.560	1.15
E99G hMCAD	0.960	1.6
E99G/E376Q hMCAD	0.550	2.82 (pH 9)

[a] K_d values were determined as described previously (*19*).

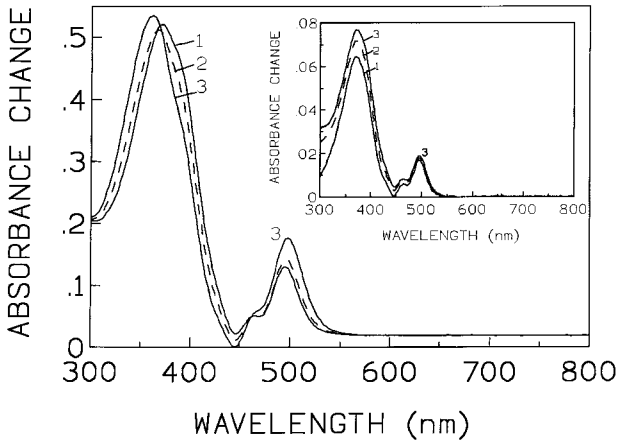


FIGURE 5: pH dependence of difference spectra for 4-OH-cinnamoyl-CoA binding to E99G and E376Q single mutants of the human medium chain acyl-CoA dehydrogenase. Curve 1 in the main panel is the difference spectrum for the E99G mutant (30.6 μM in 10 mM phosphate buffer, pH 5.8, 25 °C) after the addition of 0.86 equiv (26.2 μM) of 4-OH-cinnamoyl-CoA. Difference spectra at pH 6.6 (curve 2) and 9 (curve 3) were obtained upon the addition of Tris buffer (as in Figure 3). Curve 1 in the inset shows the difference spectra for the E376Q mutant (5.1 μM in 0.7 mL of 10 mM phosphate buffer, 25 °C, pH 6.6) on the addition of 3.6 μM (0.7 equiv) 4-OH ligand. Curves 2 and 3 correspond to pH values of 8 and 8.74. Intermediate spectra are omitted for clarity.

to the wild-type recombinant protein, or the pig enzyme shown in Figure 3, no loss of polarization is observed with increasing pH (curves 1−3). There is, in fact, a 23% increase in absorbance at 372 nm without significant shift in the wavelength maximum. These data suggest that the pH dependence observed in Figure 3 does indeed reflect the ionization of Glu376. However, although the 4-OH ligand remains polarized over the pH range shown in Figure 5, the lack of sizable additional spectral changes suggested that deprotonation of the 4-OH position was still not occurring.

The crystal structure of the medium chain dehydrogenase shows that, in both pig and human enzymes, an additional glutamate (Glu99) occupies a position at the bottom of the binding pocket (Figure 4; *16, 17*). The particular role of Glu99 in these enzymes is not clear: it is neither catalytically essential *(21, 23)* nor conserved in other acyl-CoA dehydrogenases. In the medium chain structure, Glu99 turns almost 90° to accommodate more bulky ligands (*16, 17*). The model in Figure 4 shows that Glu99 is comfortably within H-bonding distance of the 4-OH group of the bound ligand. Similar modeling experiments with 4-nitrophenylacetyl-CoA have reached the same conclusion (*21*). While the p*K* of Glu99 in the 4-OH ligand complexes is unknown, a carboxylate residue here might effectively suppress the ionization of the 4-OH group.

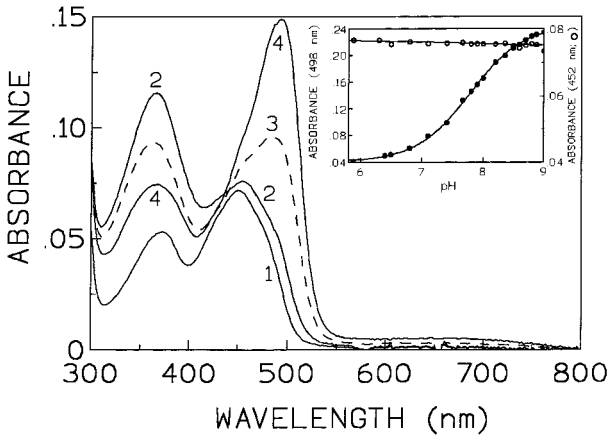


FIGURE 6: Spectral changes for the complex between 4-OH-cinnamoyl-CoA and the E99G/E376Q human medium chain acyl-CoA dehydrogenase double mutant. The double mutant (curve 1; 4.8 μM in 0.7 mL of 10 mM phosphate, pH 5.9, at 25 °C) was mixed with 3.74 μM (0.78 equiv) 4-OH-cinnamoyl-CoA (curve 2). The pH was raised with Tris buffer (see Figure 3), and spectra 3 and 4 were recorded at pH 7.4 and 8.7, respectively. Intermediate spectra are omitted for clarity. The inset plots the pH dependence of the spectral changes at 498 nm using excess ligand (4.95 μM enzyme with 45.2 μM 4-OH-cinnamoyl-CoA) to ensure saturation of the spectral changes over the entire pH range. Absorbance values at 498 nm were fit to a p*K* of 7.8 (inset, solid circles). The open circles in the inset correspond to an analogous pH titration for the double-mutant enzyme (4.89 μM) in the presence of 505 μM 4-methoxycinnamoyl-CoA in the same buffer system.

The main panel in Figure 5 shows that the E99G mutant does not suffer the dramatic loss of polarization observed with the 4-OH-cinnamoyl-CoA complex of the native enzyme (Figure 3). There is, however, an 8 nm blue-shift in the main peak of the difference spectrum as the pH is raised from 6.0 to 9. Over this pH range, there was also an unanticipated 34% increase in the difference spectrum at 492 nm. This hint of a new spectral feature suggested that it was worth examining the behavior of the double-mutant E99G/E376Q.

Polarization of the E99G/E376Q Double Mutant by 4-OH-Cinnamoyl-CoA. The double mutant was constructed, overexpressed, and purified as described under Materials and Methods. Figure 6 shows that E99G/E376Q behaves dramatically differently to single mutants or the wild-type enzyme upon the addition of 4-OH-cinnamoyl-CoA. The double mutant at pH 5.9 (curve 1) was complexed with 0.78 mol of 4-OH-cinnamoyl-CoA (curve 2), yielding a spectrum similar to that observed at low pH with the other enzymes (e.g., see curve 3, Figure 2). However, raising the pH effects a pronounced increase in absorbance at 498 nm (curve 4, Figure 6). These changes (solid circles in the inset) are fit to an apparent p*K* of 7.8. In comparison, the spectrum of the bound ligand in complexes of the wild type (data not shown) or single mutants is blue-shifted or almost unchanged with increasing pH (see Figure 5).

Separate titrations of the double mutant, following the absorbance changes upon the addition of excess 4-OH-cinnamoyl-CoA, gave an estimated extinction coefficient for the bound ligand of 37.4 mM^{-1} cm^{-1} at 498 nm at pH 9. This extinction coefficient increases to 39.2 mM^{-1} cm^{-1} on extrapolation to the high-pH limit shown in the inset to Figure 6. In addition to this striking new absorbance band, the main panel shows that a weak long-wavelength feature

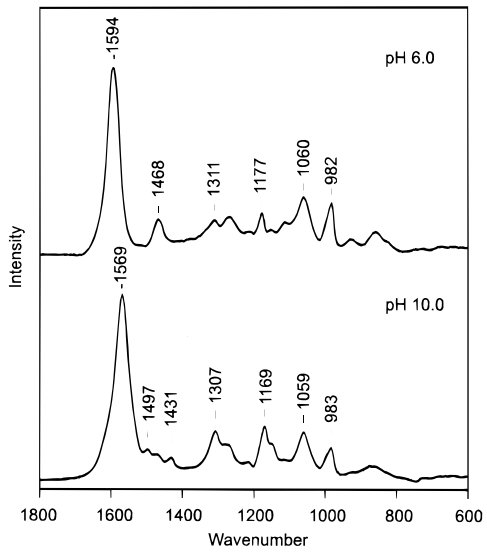


FIGURE 7: Raman spectra of free 4-OH-cinnamoyl-CoA. The spectrum of 4-OH-cinnamoyl-CoA (500 μM) was recorded in 140 μL of 20 mM phosphate buffer, pH 6, 25 °C, or in 20 mM bicarbonate buffer (pH 10). Spectra are presented after subtraction of the solvent contribution.

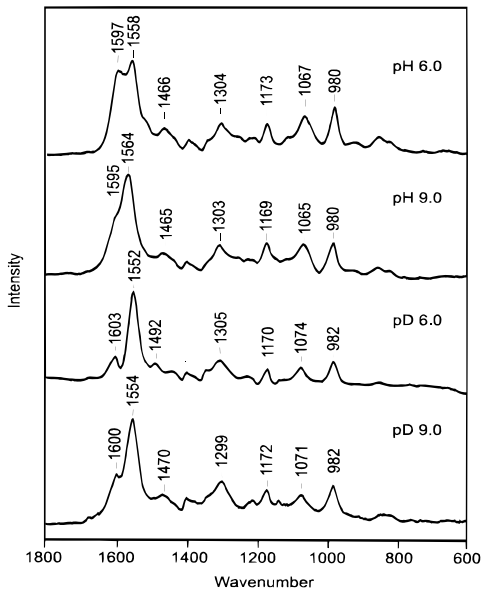


FIGURE 8: Raman difference spectra of 4-OH-cinnamoyl-CoA bound to the human medium chain acyl-CoA dehydrogenase. Spectra were acquired and manipulated as described under Materials and Methods using 134 μM human wild-type dehydrogenase with 0.9 equiv of 4-OH-cinnamoyl-CoA. The contribution of the free ligand, in the appropriate buffer, was subtracted to generate each difference spectrum.

centered at 660 nm appears as the pH is raised. This band is suggestive of a charge-transfer interaction (*26*, *27*, *70*) between 4-OH-cinnamoyl-CoA as a donor and the oxidized flavin as acceptor (see later).

The pH dependence in Figure 6 suggests that deprotonation of the phenol group of 4-OH-cinnamoyl-CoA occurs on binding to the double mutant. Such effects would not be expected with 4-methoxycinnamoyl-CoA. This is the case: no increase in 498 nm absorbance is observed over the pH range studied (open circles, inset, Figure 6). Throughout, 4-methoxycinnamoyl-CoA binds to the double mutant with the typically red-shifted flavin spectrum observed for the native enzyme and single mutants. No significant long-wavelength feature is observed.

Raman Difference Spectroscopy on 4-OH-Cinnamoyl-CoA Bound to Wild-Type and Double-Mutant MCAD. Raman difference spectroscopy can often provide valuable information regarding the structure of bound substrate analogues and the details of their interactions with enzyme active sites (*71*−*73*). The data on 4-OH-cinnamoyl-CoA bound to the wild-type and double-mutant human enzymes at pH 6 and 9 are fully consistent with the interpretations outlined in the preceding section.

The Raman difference spectra of phenol and phenolate forms of free 4-OH-cinnamoyl-CoA were recorded at pH 6 and 10 and provide a useful reference point for the interpretation of the vibrational data (Figure 7). The spectra are dominated by phenol ring modes, and some of these are sensitive to deprotonation, while others are not (*74*, *75*). In particular, the most intense band in the spectrum at pH 6 appears at 1594 cm^{-1} and shifts to 1569 cm^{-1} upon ionization. This band can be assigned to a normal mode similar to either the Y8a or the Y8b mode of tyrosine (1617 and 1601 cm^{-1}, respectively, shifting to 1603 and 1558 cm^{-1} upon deprotonation; *76*, *77*). In free 4-OH-cinnamoyl-CoA, these modes are not resolved. Here, the observed bands may correspond to a Y8b-like mode with the Y8a-like mode lying in the shoulder of this intense band to higher wavenumbers. Another possibility is that the Y8a-like mode is more intense than the Y8b-like mode for the un-ionized form, and that this situation is reversed for the ionized form. Whatever the assignments, our results are in qualitative agreement with preresonance Raman data on a free 4-OH-cinnamoyl thioester used to characterize the chromophore in PYP (*77*). Figure 7 shows that ionization of the CoA thioester results in a number of other minor changes in both position and intensity of the bands. For example, between 1400 and 1500 cm^{-1}, a single band appears at 1468 cm^{-1} for the un-ionized form to be replaced by a triplet of bands at 1431, 1474, and 1497 cm^{-1} at high pH. As expected (*77*), the phenol form of 4-OH-cinnamoyl-CoA is sensitive to deuteration, whereas the deprotonated phenolate species is not (data not shown). Finally, we note that there is no evidence of either the carbonyl or the ethylenic stretching modes that would be expected to contribute Raman intensity between 1600 and 1700 cm^{-1}. In the following section, we use the intense Y8a- and Y8b-like normal modes to assess protonation states of the bound ligands.

Raman difference spectra are presented for 4-OH-cinnamoyl-CoA bound to wild type and double mutant at pH 6 and 9 in Figures 8 and 9, respectively. Table 2 summarizes changes to the Y8a- and Y8b-like normal modes. The first point to note is that these bands become fully resolved on binding to the dehydrogenase. By comparison with the free ligand (Figure 7 and Table 2), one might expect Y8a and Y8b bands represent ionized and un-ionized forms, respectively. However, 4-methoxycinnamoyl-CoA, which cannot ionize, shows the same distinctive features. Thus, both ionized and un-ionized forms generate two Raman bands, corresponding to the Y8a- and Y8b-like modes, with the ionized form appearing at lower wavenumber (*73*, *76*, *77*).

Three main lines of evidence suggest that deprotonation of 4-OH-cinnamoyl-CoA requires both use of the double

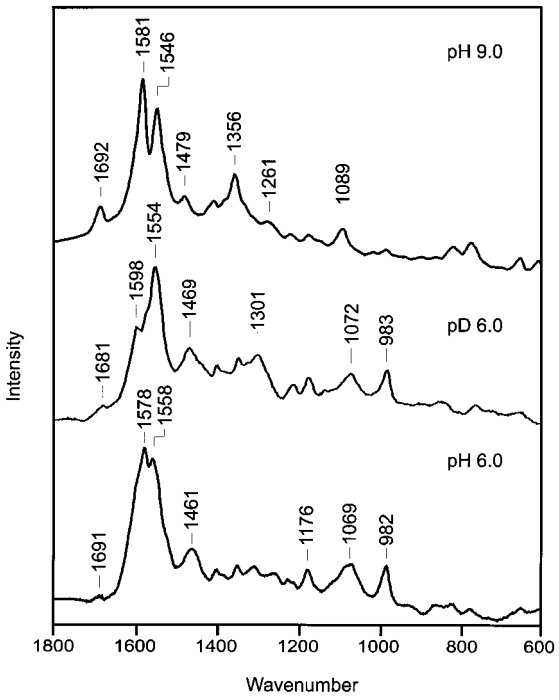


FIGURE 9: Raman difference spectra for the 4-OH-cinnamoyl-CoA complex of E99G/E376Q double mutant. Spectra were recorded as in Figure 8 using 100−140 μM enzyme.

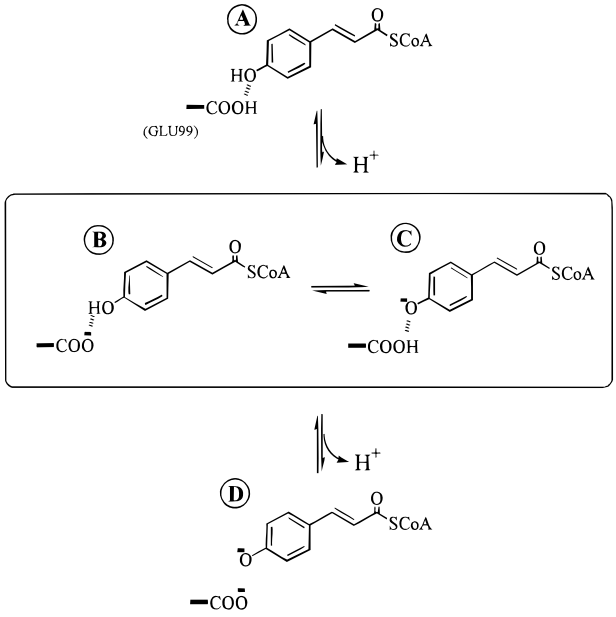
Table 2: Positions of Y8a- and Y8b-like Modes of 4-OH-Cinnamoyl-CoA and 4-Methoxycinnamoyl-CoA Ligands[a]

enzyme	pH	ligand	Y8a (cm^{-1})	Y8b (cm^{-1})
−	6	4-OH-cinnamoyl-CoA	1594	−
−	10	4-OH-cinnamoyl-CoA	−	1569
−	6	4-methoxycinnamoyl-CoA	1596	−
−	10	4-methoxycinnamoyl-CoA	1596	−
hMCAD	6	4-OH-cinnamoyl-CoA	1597	1558
hMCAD	9	4-OH-cinnamoyl-CoA	1595(sh)[b]	1564
hMCAD	6	4-methoxycinnamoyl-CoA	1601	1553
DM[c]	6	4-OH-cinnamoyl-CoA	1577	1558
DM	9	4-OH-cinnamoyl-CoA	1580	1545
DM	9	4-methoxycinnamoyl-CoA	1576	1559

[a] Raman data were obtained as described under Materials and Methods. [b] Shoulder. [c] E99G/E376Q double mutant of human medium chain acyl-CoA dehydrogenase.

mutant and an elevated pH. First, the Y8a and Y8b modes for 4-OH-cinnamoyl-CoA bound to the wild-type enzyme at pH 6 and pH 9 (Figure 8) are comparable to those for the bound 4-methoxycinnamoyl-CoA at pH 6 (data not shown). This suggests that 4-OH-cinnamoyl-CoA is not ionized in the wild-type enzyme over the pH range 6−9. With the double mutant, the spectrum of the 4-OH analogue at pH 6 is similar to that obtained with 4-methoxycinnamoyl-CoA at pH 9 (Table 2), again consistent with binding of the protonated 4-OH derivative at pH 6. However, with the double mutant at pH 9, a 14 cm^{-1} shift of the Y8b-like mode to lower wavenumber, relative to bound 4-methoxycinnamoyl-CoA at the same pH, is consistent with ligand deprotonation. A second line of evidence concerns Raman difference spectra acquired in D_2O (Figures 8 and 9). The spectrum of the 4-OH ligand is completely insensitive to deuteration when bound to the double mutant at pH 9, but becomes sensitive at pH 6 (as expected for protonation of a phenolate). With the wild-type enzyme, however, the corresponding bands are sensitive to deuteration at both pH 6 and pH 9 (Figure 8). Finally, the resonance Raman spectrum of the deprotonated 4-OH-cinnamoyl chromophore of PYP shows shifts, relative to an un-ionized model compound (*77*), which are qualitatively similar to our data with the double mutant at pH 9.

Scheme 2



Conclusions. This paper demonstrates that substantial deprotonation of 4-OH-cinnamoyl-CoA only occurs when this highly chromophoric ligand is bound to the double-mutant E99G/E376Q of the human medium chain acyl-CoA dehydrogenase. The resulting spectral changes are dramatic, and comparable to those encountered when the 4-OH-cinnamoyl moiety is covalently incorporated in PYP (*36*, *45*, *47*, *49*, *50*).

An important issue raised by this work is why the anion of 4-OH-cinnamoyl-CoA is only conspicuous in the double mutant, when much more weakly acidic ligands are fully deprotonated when bound to the wild-type protein. Thus, 3-thiaoctanoyl-CoA (p*K* ca. 15), *trans*-3-octenoyl-CoA (p*K* ca. 19), and *p*-nitrophenylacetyl-CoA (p*K* ca. 13.6) are deprotonated essentially completely in complex with the wild-type enzyme at neutral pH values (*21*, *23*, *25*, *32*, *33*). It is not simply that 4-OH-cinnamoyl-CoA binds weakly to the wild-type enzyme. In fact, the K_d of 40 nM at pH 6 (Table 1) is comparable to that of *trans*-3-octenoyl-CoA (*25*), and about 10- and >600-fold tighter than 3-thiaoctanoyl-CoA and *p*-nitrophenylacetyl-CoA, respectively (*21*, *32*). It might be argued that the 4-OH− moiety does not ionize because the catalytic base Glu376, whose normal function is in α-proton abstraction, is too far away from the phenolic hydroxyl of the cinnamoyl derivative. However, a model of the medium chain dehydrogenase complexed with 4-OH-cinnamoyl-CoA (see Materials and Methods) places Glu99 within H-bonding distance of the *p*-hydroxy group of the ligand. Glu99 is thus a potential recipient of the phenolic proton (Scheme 2).

The p*K* of Glu99 has been estimated as 7.3 in the free enzyme (*21*) and about 8.0 in complex with *p*-nitrophenylacetyl-CoA (*21*). While the microscopic p*K* of Glu99 in this complex is not known, it is difficult to argue, a priori, that

its p*K* would be higher than that of the phenol. Thus, the predominant state of the ligand would be expected to be the phenol (as in Scheme 2, form B), not the phenolate (form C). Thus, the spectrum of the bound ligand would resemble that of the polarized methoxy analogue, and not the highly polarized anionic chromophore in PYP. In principle, a second deprotonation would create the phenolate species (form D), but electrostatic repulsion between carboxylate and phenolate would be expected to be highly unfavorable.

Even if the p*K* values for Glu99 and the phenol are closer than envisaged, there is another reason the highly polarized anionic form of 4-OH-cinnamoyl-CoA is not clearly evident with the native enzyme. A body of evidence shows that the polarization of ligands such as 4-thia-*trans*-2-enoyl-CoA (*18*, *19*) and cinnamoyl-CoA (*19*, *57*) is lost when the catalytic base, Glu376, is deprotonated. This is also exactly what is seen in Figure 3 with the ionizable ligand, 4-OH-cinnamoyl-CoA (apparent p*K* of 7.4). Thus, as the pH of the enzyme•4-OH-cinnamoyl-CoA complex is raised, and before significant ionization of the ligand could be observed, Glu376 deprotonates and minimizes further chance for polarization. Thus, if the apparent p*K* of Glu376 is lower than that of the macroscopic p*K* for the first ionization (Glu99/4-OH-cinnamoyl-CoA), little spectral signature for the anionic 4-OH-cinnamoyl-CoA ligand would be evident. The larger issue of why the polarization of thioester ligands (with or without ionizable groups) is abolished on deprotonation of Glu376 is not understood at present. Perhaps the enhanced solvation of the carboxylate may disrupt polarization and orientation of the thioester carbonyl group (*19*).

In any event, experiments with the E376Q mutant (Figure 5, inset) suggest that the equilibrium between forms B and C in Scheme 2 strongly favors the neutral phenol (form B). Thus, little increase in 498 nm absorbance is observed as the pH is raised. There is, however, a hint of ionization of the ligand with the E99G mutant, and this prompted construction of the double mutant. When both potential negative charges are removed in the double mutant, the anionic form of the ligand can form unimpeded by these additional factors. The resulting pronounced polarization of the 4-OH-cinnamoyl-CoA phenolate anion presumably requires the H-bonded interactions to the thioester carbonyl group [i.e., from the N−H of Glu376 and from the 2′-OH− of the ribityl side chain (*16*)] that contribute to lowering the p*K* of the α-proton in normal substrates. In the case of PYP, the phenolate oxygen is also stabilized by an electrostatic interaction with Arg42 and H-bonds from protonated Tyr42 and Glu46 residues (*52*). Without a comparable compensating positive charge, the medium chain dehydrogenase requires removal of both Glu99 and Glu376 before significant ionization can be achieved.

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