

# Protonic Equilibria in the Reductive Half-Reaction of the Medium-Chain Acyl-CoA Dehydrogenase<sup>†</sup>

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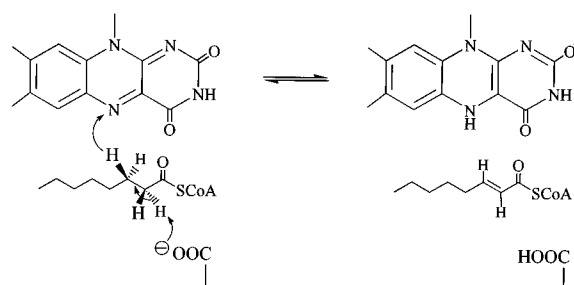
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Received February 18, 1998; Revised Manuscript Received April 9, 1998

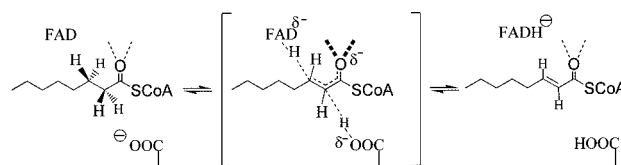
**ABSTRACT:** Oxidation of thioester substrates in the medium-chain acyl-CoA dehydrogenase involves  $\alpha$ -proton abstraction by the catalytic base, Glu376, with transfer of a  $\beta$ -hydride equivalent to the flavin prosthetic group. Polarization of bound acyl-CoA derivatives by the recombinant human liver enzyme has been studied with 4-thia-*trans*-2-enoyl-CoA analogues. Polarization is maximal at low pH, with an apparent  $pK$  of 9.2 for complexes with the C8 analogue, and progressively lower  $pK$  values as the length of the chain increases. This pH effect reflects ionization of the catalytic base, since polarization of a variety of enoyl-CoA analogues by the Glu376Gln mutant is pH independent. Binding of these ligands is accompanied by uptake of about 1 proton with the wild-type enzyme, but only about 0.1 proton with the Glu376Gln mutant. Rapid reaction studies show that proton uptake with the wild-type enzyme occurs at the same rate as polarization of the enoyl-CoA thioester, but is much slower than the initial ligand binding step. Studies with 6-OH-FAD-substituted enzyme show that this isomerization reaction also influences the flavin prosthetic group inducing deprotonation to the green anionic form. The failure of the bound thioether analogue, octyl-SCoA, to elicit  $pK$  shifts to flavin and Glu376 shows the importance of the thioester carbonyl oxygen in modulating key properties of the medium-chain enzyme. The role of thioester-mediated desolvation within the active site of the acyl-CoA dehydrogenases is discussed.

The reductive half-reaction in the acyl-CoA dehydrogenases involves abstraction of the *pro-R*- $\alpha$ -proton of a bound acyl-CoA thioester with elimination of the *pro-R*- $\beta$ -hydrogen as a hydride equivalent to the N-5 position of the flavin. The reaction appears concerted with normal (Scheme 1) substrates (1–5). The transition state for the dehydrogenation reaction is likely to have appreciable enolate character as negative charge migrates from the carboxylate base through the thioester to the flavin prosthetic group (6–10) (Scheme 2). Substitution of the C-3 methylene group in substrate analogues such as compounds **1** and **2** in Chart 1 precludes hydride transfer, but proton abstraction leads to the generation of strongly absorbing enolate to flavin charge-transfer complexes (7, 11, 12). These studies illustrate the profound stabilization of the enolates of these weakly acidic substrate analogues (with  $pK$  values lowered by 8–12 units; 7, 11, 12) on binding to the enzyme. The acidification of normal thioester substrates has been suggested to be even greater (12). The crystal structure of the medium-chain acyl-CoA dehydrogenase complexed with acyl-CoA substrates (13) suggests that the developing enolate might be stabilized in part by two hydrogen bonds: one from the 2'-OH of the ribityl side chain of FAD and one from a peptide N–H group to the substrate carbonyl oxygen (7, 10, 13; heavy dashed

Scheme 1



Scheme 2



lines in Scheme 2). Thus, removal of one of these interactions by reconstitution of the enzyme with 2'-deoxy-FAD leads to a profound ( $\geq 10^6$ -fold) slowing of the rate of reduction of the enzyme by octanoyl-CoA (7). Thus, this H-bond appears very important in lowering the activation energy of the transition state for normal catalysis (14–19).

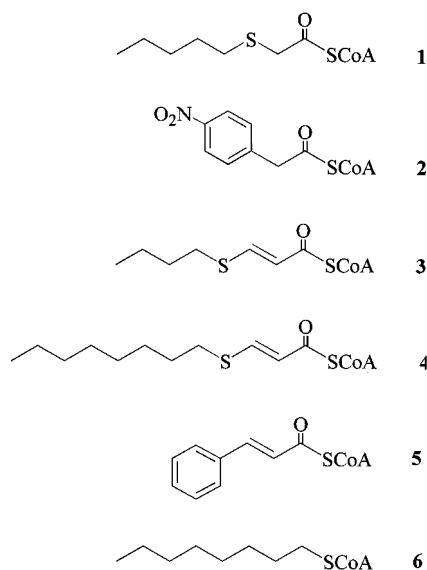
In addition to the enzyme-mediated acidification of the bound thioester mentioned above, raising the  $pK$  of the catalytic base (Glu376; 13, 20–22) would be a second strategy for rate enhancement of the reductive half-reaction. The higher the  $pK$ , the better able Glu376 would be expected to abstract an  $\alpha$ -proton from substrate (23–26). Desolvation

<sup>†</sup> This work was supported in part by NIH Grant GM26643 (C.T.) and Deutsche Forschungsgemeinschaft Grants Gh 2/4-7 and Gh 2/6-1 (S.G.) and by Training Grant USPHS 1-T32-GM-08550 to I.R.

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Chart 1: CoA Derivatives Described in This Work<sup>a</sup>

<sup>a</sup> Compound 1: 3-thia-octanoyl-CoA; compound 2: *p*-nitro-phenylacetyl-CoA; compound 3: 4-thia-*trans*-2-octenoyl-CoA; compound 4: 4-thia-*trans*-2-dodecenoyl-CoA; compound 5: cinnamoyl-CoA; compound 6: octyl-SCoA.

provides a simple and well-precedented means to elevate the *pK* of carboxylate residues. Such desolvation in the medium-chain acyl-CoA dehydrogenase occurs on ligand binding as progressively longer acyl chains displace more and more ordered water molecules from the active site (10, 13).

The present paper provides experimental support for the expectation that the *pK* of the catalytically essential base, Glu376, would be elevated on ligand binding. Further, the apparent *pK* of Glu376 is dependent on the structure and chain length of the acyl-CoA ligand used. These *pK* shifts are largely accomplished during some form of structural readjustment or isomerization after formation of the encounter complex. This work compares the effect of ligation on the *pK* of Glu376 and on the ionization state of the flavin prosthetic group. Finally, these studies reiterate the critical role of the thioester carbonyl group in facilitating these interrelated phenomena in the acyl-CoA dehydrogenases.

## MATERIALS AND METHODS

**Materials.** Recombinant human medium-chain acyl-CoA dehydrogenase (hwt-MCAD)<sup>1</sup> was isolated and purified as described previously (27, 28) with some modifications (Duplessis, unpublished observations). In brief, bacteria were grown overnight at 25 °C in the absence of isopropylthiogalactoside. Cells were disrupted in 20 mM phosphate buffer, pH 7.6, containing 50 μM FAD and 0.3 mM EDTA using a French press. The crude extract was sonicated for five 1 min intervals with cooling on ice to reduce viscosity. The extract was centrifuged and the 40–80% ammonium sulfate fraction dialyzed versus 20 mM phosphate buffer, pH 7.2, containing 5 μM FAD and 0.3 mM EDTA. Subsequently, Q-Sepharose (fast-flow), followed by Source 15Q and Source 15PHE FPLC columns, gave wild-type enzyme with a 280/446 nm ratio of 5.0. The Glu376Gln mutant was isolated

in a green CoA-persulfide form (29) as observed earlier (21). Where appropriate, enzyme was degreened before each experiment by the addition of a few grains of solid sodium dithionite (21, 29). Excess reagents and bound ligand were removed by washing with three 1 mL volumes of 50 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA in a Centricon-30 ultrafiltration unit (Amicon Co.).

Phenol red (sodium salt) indicator dye was purchased from Sigma. 4-Thia-*trans*-2-pentenoyl-, -octenoyl-, -dodecenoyl-, and -tridecenoyl-CoA were made by acyl-CoA-mediated oxidation of the corresponding CoA substrates (30). Cinnamoyl-CoA (9) and octyl-SCoA (31) were prepared as described previously. 6-OH-FAD was obtained as described previously (32).

**General Methods.** Concentrations of wild-type and Glu376Gln recombinant human medium-chain acyl-CoA dehydrogenase were determined using an extinction coefficient of 14.8 mM<sup>-1</sup> cm<sup>-1</sup> at 448 nm (33). CoA thioesters were quantified using the following extinction coefficients: 4-thiaenoyl-CoA analogues (22 mM<sup>-1</sup> cm<sup>-1</sup> at 312 nm; 30); cinnamoyl-CoA (17.6 mM<sup>-1</sup> cm<sup>-1</sup> at 260 nm; 34); octyl-SCoA (15.4 mM<sup>-1</sup> cm<sup>-1</sup> at 260 nm; 31).

All UV–Vis spectrophotometric titrations were performed at 25 °C using an HP8452 diode array spectrophotometer. General binding experiments utilized 50 mM phosphate buffer containing 0.3 mM EDTA and 1 or 5 cm path length cuvettes. The longer pathlength cuvettes were utilized for tightly binding ligands using enzyme concentrations of about 0.2 μM. For these experiments, the 5 cm cell was secured into a custom holder, and each increment of ligand was gently mixed with a small magnetic stirrer without moving the cuvette. Unless otherwise stated, pH titrations were performed in 10 mM phosphate buffer with 0.3 mM EDTA, pH 6, in a 1 cm pathlength cuvette. Enzymes were equilibrated with the same buffer system before each experiment. Changes in pH were effected by the addition of small increments of freshly prepared 2 M Tris-HCl buffer, pH 9.5, to the cuvette and compared to an identical titration using a pH electrode. Spectrophotometric data were analyzed using Enzfitter or InPlot.

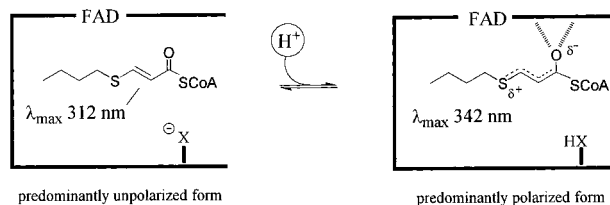
Rapid reaction experiments were performed using a Kinetic Instruments stopped-flow spectrophotometer with peripherals and software for data acquisition and fitting from Online Instruments Systems. All experiments were performed at 4 °C.

**6-Hydroxy-FAD-Reconstituted Enzyme.** Apoenzyme was prepared from wild-type and Glu376Gln human medium-chain acyl-CoA dehydrogenase using the acid ammonium sulfate/charcoal procedure (32, 35). Apoprotein was quantitated using an extinction coefficient of 63 mM<sup>-1</sup> cm<sup>-1</sup> at 280 nm (35). The apoprotein was incubated for 30 min with 2 equiv of oxidized 6-hydroxy-FAD first at 4 °C and then at 25 °C for 1 h. Unbound flavin was removed by washing the enzyme with 50 mM phosphate buffer, pH 7.6, 0.3 mM EDTA, in a Centricon-30 microconcentrator. Recovery of holoenzyme was between 40 and 65%. Concentrations of 6-hydroxy-FAD-dehydrogenase were estimated using an extinction coefficient of 18.5 mM<sup>-1</sup> cm<sup>-1</sup> at 422 nm for the yellow form of the enzyme (32).

**Proton Uptake.** Preliminary experiments established a *pK* of 7.8 for phenol red in 1 mM phosphate buffer at 25 °C, in good agreement with that determined earlier (36). The ratio

<sup>1</sup> Abbreviation: hwt-MCAD, recombinant human medium-chain acyl-CoA dehydrogenase.

Scheme 3



of 558/438 nm observed during these titrations was then used to determine the pH of solutions of enzyme containing the indicator. Control pH titrations showed that the spectrum and the  $pK$  of 4.0  $\mu\text{M}$  phenol red were unaffected by the presence of 5  $\mu\text{M}$  medium-chain dehydrogenase in 1 mM phosphate at 25 °C.

For the static proton uptake experiments, wild type or the Glu376Gln mutant was concentrated in 1 mM phosphate buffer, pH 7.0, and diluted with degassed water to give final concentrations of 10  $\mu\text{M}$  enzyme and 100  $\mu\text{M}$  phosphate buffer. Spectra were recorded in a cuvette flushed with nitrogen before the addition of 10  $\mu\text{M}$  phenol red through a small hole in the stopper. After the addition of a total of 10.5  $\mu\text{M}$  CoA ligands, the absorbance changes at 558 nm were returned to their previous values by the addition of standardized dilute HCl. Control experiments were performed in the absence of enzyme to correct for proton uptake due to the ligand alone (ranging from 0.007 to 0.5 proton).

Rapid mixing experiments to measure proton uptake were performed using the stopped-flow instrument by following absorbance changes at 558 nm. The dehydrogenase (10  $\mu\text{M}$  in 100  $\mu\text{M}$  degassed phosphate buffer, pH 7.0) was mixed with a solution of 10  $\mu\text{M}$  phenol red in 100  $\mu\text{M}$  degassed phosphate buffer, pH 7.0, containing either 15  $\mu\text{M}$  4-thia-dodecenoyl-CoA or 20  $\mu\text{M}$  cinnamoyl-CoA. Control experiments were performed by diluting phenol red with 100  $\mu\text{M}$  buffer in the presence or absence of enzyme. These traces showed a small, reproducible, absorbance increase at 558 nm (0.003) which was complete in 80 ms. These changes were subtracted from the mixing experiments performed in the presence of enoyl-CoA ligands.

## RESULTS

**4-Thia-*trans*-2-enoyl-CoA Analogues: Active Site Probes for the Medium Chain Acyl-CoA Dehydrogenase.** In free solution, 4-thia-*trans*-2-enoyl-CoA product analogues (e.g., compounds **3** and **4**, Chart 1) show a strong absorbance maximum at 312 nm (11, 30). When bound to the dehydrogenase (Scheme 3), they can exist either in a largely unpolarized form, absorbing at the same wavelength, or in a largely polarized form peaking at 342 nm (9). This interconversion is controlled by an unidentified titrating group, X, whose apparent  $pK$  was sensitive to the chain length of the ligand used. The present work, using the human liver medium-chain enzyme, shows that this group is the catalytic base itself (9).

Since all prior work with the 4-thia-enoyl-CoA ligands was done with enzyme isolated from pig kidney, and because of the minor differences in chain length specificity between the porcine and human enzymes (7, 9, 22, 33, 37), it was first necessary to repeat key control experiments with the

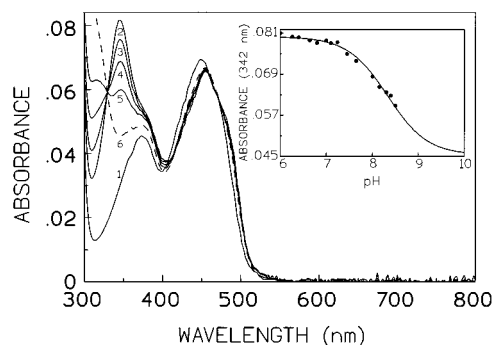


FIGURE 1: pH dependence of the polarization of 4-thia-*trans*-2-dodecenoyl-CoA bound to human wild-type medium-chain acyl-CoA dehydrogenase. The dehydrogenase (curve 1; 4.6  $\mu\text{M}$  in 0.7 mL of 10 mM phosphate buffer, pH 6 at 25 °C) was mixed with 0.87 equiv of 4-thia-*trans*-2-dodecenoyl-CoA (curve 2) and titrated with increasing amounts of Tris-HCl buffer (2 M, pH 9.5 at 25 °C). Curves 3–5 are selected for illustration and correspond to pH values of 7.45, 8.0, and 8.5, respectively. The limiting high pH value in the inset is taken from a comparable titration using the C-13 analogue and is shown in curve 6 (see the text). The solid line in the inset is fit to a monoprotic equilibrium with a  $pK$  of 8.3 and low and high pH limits of 0.08 and 0.047, respectively.

recombinant human protein. Figure 1 shows a pH titration of the human wild-type medium-chain acyl-CoA dehydrogenase complexed with 0.87 equiv of 4-thia-*trans*-2-dodecenoyl-CoA. Substoichiometric levels of this tightly binding thioester ( $K_D = 19$  nM at pH 6; see Materials and Methods) ensure that the observed changes are dominated by the spectrum of the complexed species. As seen with the pig enzyme, the polarized form of the ligand absorbing at 342 nm predominates at low pH (curve 2) and is replaced by a largely unpolarized form at 312 nm at high pH (Figure 1 and Scheme 3). Note that while pH effects a marked change in the spectrum of the complex below 360 nm, it does not make major changes to the flavin envelope where this can be seen unobstructed by the absorbance contribution of the ligand (e.g., at wavelengths  $>400$  nm; Figure 1). Thus, the ligand remains tightly bound over the pH range of these experiments. Because of the elevated  $pK_{\text{app}}$  observed with the C-12 complex, the high pH limit was estimated from analogous pH titrations using the C-13 ligand (where the unpolarized form can be obtained essentially quantitatively at high pH; curve 6, Figure 1). The data in the inset are fit to a monoprotic ionization with a  $pK_{\text{app}}$  of 8.3.

The pH titration in Figure 1 results in an increase in ionic strength of approximately 3-fold as the pH is raised from 6 to 8.5 (11 to 37 mM, respectively). These changes in ionic strength do not strongly affect the apparent  $pK$ . Thus, repeating the titration starting with 30 mM phosphate buffer, pH 6.0, gave an apparent  $pK$  of 8.1, similar to that observed in Figure 1. Similarly, the  $K_D$  for binding *trans*-2-octenoyl-CoA to the pig kidney medium-chain acyl-CoA dehydrogenase showed no significant ionic strength dependence (38).

The titration in Figure 1 was repeated with 4-thia-*trans*-2-enoyl-CoA ligands of various chain lengths, and the resulting  $pK$ s are compared with the pig medium-chain enzyme in Table 1. Both enzymes show a similar chain length dependent decrease in the  $pK_{\text{app}}$  going from 4-thia-C8 to 4-thia-C13 (Table 1; 9). Thus, these data are consistent with the close enzymological and structural similarities between these medium-chain enzymes (7, 22, 33, 37; this work).

Table 1:  $pK_{app}$  Values for Loss of Polarization for 4-Thia-*trans*-2-enoyl-CoA Complexes of Pig Kidney and Human Liver Medium-Chain Acyl-CoA Dehydrogenases<sup>a</sup>

ligand	$pK$ (pig)	$pK$ (human)
4-thiapentenoyl-CoA	nd <sup>b</sup>	8.9
4-thiaoctenoyl-CoA	9.3	9.2
4-thiadodecenoyl-CoA	8.4	8.3
4-thiatriecenoyl-CoA	7.5	7.1
4-thiatetradecenoyl-CoA	6.6	nd <sup>b</sup>

<sup>a</sup> Apparent  $pK$  values were determined as described under Materials and Methods. Values for the pig kidney medium-chain acyl-CoA dehydrogenase are taken from Trievel et al. (9). <sup>b</sup> Not determined.

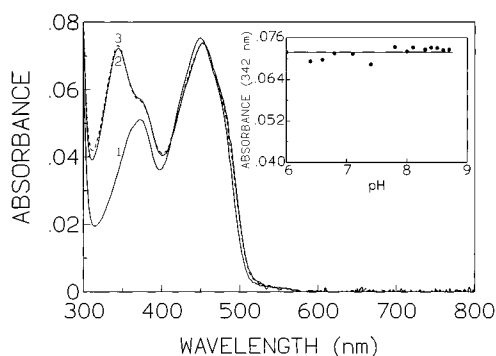


FIGURE 2: pH dependence of the polarization of 4-thia-*trans*-2-dodecenoyl-CoA bound to Glu376Gln mutant of the human medium-chain acyl-CoA dehydrogenase. The mutant enzyme (curve 1; 5  $\mu$ M in 0.7 mL of 10 mM phosphate at 25 °C, pH 6) was mixed with 3.2  $\mu$ M (0.65 equiv) of 4-thia-*trans*-2-dodecenoyl-CoA (curve 2). The pH was raised with Tris to pH 8.7 (curve 3) as in Figure 1. The absorbance changes at 342 nm are plotted in the inset.

The catalytic base, Glu376, was shown to be responsible for the pH dependence observed in Figure 1 using the human Glu376Gln mutant prepared earlier (21). This mutant is essentially catalytically inactive toward the normal substrate, octanoyl-CoA, with a rate for the reductive half-reaction less than 0.02% that of the wild type (21). However, the mutant does retain the ability to bind thioester ligands such as octanoyl-CoA, *p*-nitrophenylacetyl-SCoA, and *p*-aminobenzoyl-SCoA with comparable or higher affinities to those of the wild-type enzyme (7). Binding of the 4-thia-C12 analogue to the mutant enzyme is tight with a  $K_D$  of 185 nM at pH 6 (data not shown) and a red shift of the main absorbance envelope (Figure 2; see Materials and Methods). These spectral changes are similar to, but slightly less pronounced than, those observed for the wild-type enzyme (Figure 1). The prominent peak at 342 nm on the addition of 0.7 equiv of ligand at pH 6 shows that polarization of the enone chromophore does not require the presence of a catalytic base. However, in marked contrast to the wild-type enzyme, there is no significant loss of this polarization up to pH 8.7 (curve 3 and inset of Figure 2). These data strongly suggest that the  $pK$  observed in Figure 1 is coupled to the protonation state of Glu376-COOH.

**Ionization State of Glu376 Modulates the Polarization of Other Enone Ligands.** Although the heteroatom substitution in 4-thia-enoyl-CoA analogues appears to be a conservative change which is readily tolerated by the medium-chain acyl-CoA dehydrogenase (9, 30), it might be argued that the effects seen in Figures 1 and 2 are peculiar to these ligands. However, data with the pig kidney medium-chain enzyme and the rather bulky indoleacryloyl-CoA (39) can be

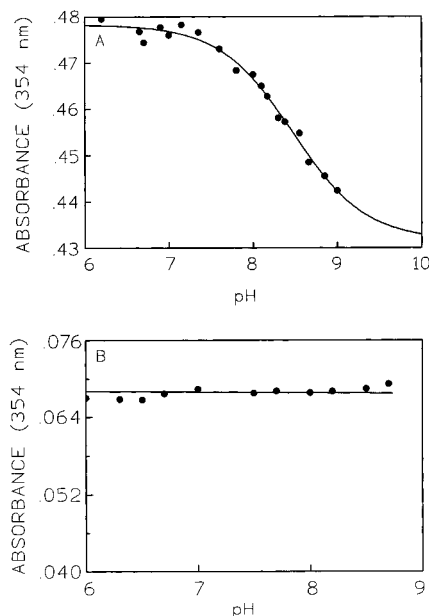


FIGURE 3: pH dependence of the polarization of cinnamoyl-CoA bound to wild-type and Glu376Gln mutant human liver medium-chain acyl-CoA dehydrogenase. Panel A represents absorbance changes at 354 nm taken from spectral titrations performed using 38  $\mu$ M wild-type enzyme in 0.7 mL of 10  $\mu$ M phosphate buffer, pH 6.2 at 25 °C. The solid line is fit to a  $pK$  of 8.5 with low and high pH limits of 0.478 and 0.432. Panel B is the corresponding spectral changes for the Glu376Gln mutant (3.6  $\mu$ M in 0.7 mL of 10 mM phosphate buffer, pH 6 at 25 °C).

interpreted in terms of just such a pH-dependent change in polarization (9). Figure 3A summarizes a pH titration with a smaller and more tightly binding chromophoric analogue, cinnamoyl-CoA (compound 5, Chart 1), to the wild-type enzyme ( $pK_{app} = 8.5$ ). In solution, free cinnamoyl-CoA shows an enone absorption maximum at 308 nm in good agreement with the spectrum reported by Murfin (1). The analogue binds tightly to the human medium-chain enzyme ( $K_D = 22$  nM at pH 6; data not shown). Again, the Glu376Gln mutant dehydrogenase shows practically no pH-dependent change in the spectrum of the bound ligand (Figure 3B). Thus, the apparent  $pK$  observed in Figure 3A with cinnamoyl-CoA is also associated with the ionization of Glu376. Comparable experiments with 4-methoxy- and 4-hydroxy-cinnamoyl-CoA support these conclusions (data not shown).

**Proton Uptake Experiments.** The experiments described above show that the polarization of a variety of enoyl-CoA product analogues depends on the protonation state of the catalytic base Glu376. Further, they allow a  $pK$  of greater than 9 to be assigned to Glu376 in complexes of the enzyme with enoyl-CoA products of optimal chain length. Since the  $pK_{app}$  of Glu376 in the free enzyme has been estimated as about 6 (12), thioester binding at pH 7 would be expected to lead to proton uptake. The extent and rate of this process are described below.

Proton uptake experiments utilized phenol red as a pH indicator in lightly buffered phosphate buffer. Control experiments showed that no significant binding of phenol red was detected spectrophotometrically (see Materials and Methods). Figure 4 (curve 1) shows the wild-type human medium-chain acyl-CoA dehydrogenase in 100  $\mu$ M phosphate buffer, pH 7.0. After the addition of phenol red (curve

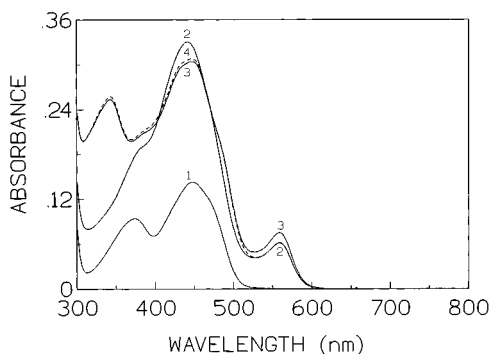


FIGURE 4: Proton uptake accompanies binding of 4-thia-*trans*-2-dodecenoyl-CoA to wild-type human medium-chain acyl-CoA dehydrogenase. The dehydrogenase (curve 1, 9.7  $\mu$ M in 0.7 mL of degassed 100  $\mu$ M phosphate buffer, pH 7 at 25  $^{\circ}$ C) was mixed with 9.6  $\mu$ M phenol red (curve 2; see Materials and Methods) followed by 1.02 equiv of 4-thia-*trans*-2-dodecenoyl-CoA (curve 3). Curve 4 is after the addition of 12  $\mu$ L of 0.92 mM HCl to return the 558 nm absorbance to that of curve 2 (see Materials and Methods).

2), 4-thia-*trans*-2-octenoyl-CoA was added, yielding curve 3. While the combined spectrum is dominated by phenol red absorbance, ligand binding to the enzyme is shown by the shoulder that appears between curves 2 and 3 at 490 nm. The increase in absorbance of phenol red at 558 nm (curves 2 to 3) lies outside the flavin envelope of either the free or the ligated enzyme and reflects an increase in pH due to proton uptake from the solution. The original absorbance of the dye was recovered on the addition of HCl, amounting to 1.05 protons per flavin (curve 4, after correction for a small proton uptake by the ligand alone; see Materials and Methods). Recently, Srivastava and colleagues (38) have reported proton uptake of  $0.52 \pm 0.15$  on binding *trans*-2-octenoyl-CoA to pig kidney medium-chain acyl-CoA dehydrogenase, based on analysis of microcalorimetric titrations.

When the experiment shown in Figure 4 was repeated with the Glu376Gln mutant, only 0.1 proton was taken up at pH 7 on binding the 4-thiaenoyl-CoA ligand. These experiments were extended with octenoyl-CoA and cinnamoyl-CoA and gave proton uptake values of 0.79 and 0.86 for the wild type, and 0.0 and 0.14 for the mutant, respectively (not shown; see Materials and Methods). These data confirm that the  $pK$  of the carboxylate base in the human medium-chain acyl-CoA dehydrogenase is raised markedly on binding a variety of thioester ligands. However, studies with octyl-SCoA, a thioether analogue lacking the thioester carbonyl oxygen (Chart 1, compound 6), show that proton uptake is not a general consequence of ligand binding.

Earlier studies with these alkyl-SCoA thioethers showed that while they can bind tightly to the pig kidney dehydrogenase, they fail to appropriately modulate key properties of the enzyme. These effects include an inability to raise the redox potential of the dehydrogenase upon binding (40), a failure to stabilize the anionic semiquinone (41) and to suppress oxygen reactivity of the reduced enzyme (42), and an anomalous response to hydrocarbon chain length (31). Spectrophotometric titrations of the human wild-type enzyme with octyl-SCoA at pH 7 in the absence of phenol red showed a  $K_D$  of 400 nM with spectral perturbations analogous to those seen with the pig kidney dehydrogenase (31). However, proton uptake experiments performed with the human enzyme gave a stoichiometry of only 0.11 proton per

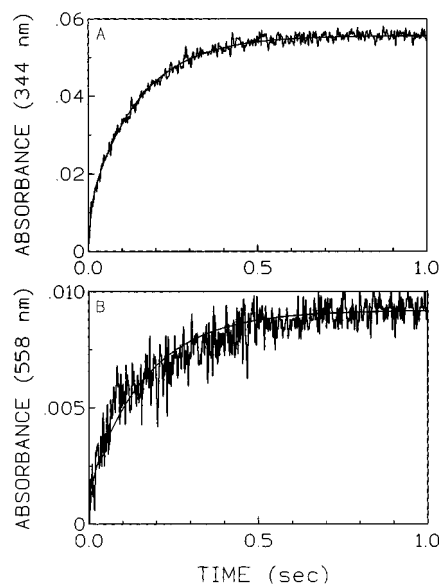
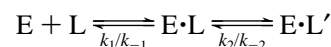


FIGURE 5: Comparison of the rates of polarization and proton uptake upon binding of 4-thia-*trans*-2-dodecenoyl-CoA to human wild-type medium-chain enzyme. Panel A shows the increase in polarization at 344 nm upon mixing ligand and enzyme to final concentrations of 15 and 10  $\mu$ M, respectively, in 100  $\mu$ M phosphate buffer, pH 7 at 4  $^{\circ}$ C (see Materials and Methods). Proton uptake was followed at 558 nm in panel B with the inclusion of 10  $\mu$ M phenol red with the enzyme (see Materials and Methods). Both curves were fit to two exponentials with major phases of 7.7/s (90% of total change; panel A) and 5.8/s (72%; panel B).

thioether bound compared to the approximately 1 proton taken up with thioester ligands. Thus, octyl-SCoA, while binding quite tightly to the medium-chain enzyme, again fails to elicit the response expected for thioester binding to the dehydrogenase.

**Kinetics of Proton Release.** Binding of thioester ligands to the medium-chain dehydrogenase first involves a diffusional encounter complex ( $E \cdot L$ ) followed by an isomerization step leading to the appearance of a polarized spectrum with chromophoric ligands such as indoleacryloyl-CoA ( $E \cdot L'$ ; 39; see below).



It was therefore of interest to see at what stage proton uptake occurs. Accordingly, the wild-type dehydrogenase in lightly buffered solution at pH 7.0, 4  $^{\circ}$ C, was mixed in the stopped-flow spectrophotometer with 1.5 equiv of 4-thia-*trans*-2-dodecenoyl-CoA in the same buffer containing phenol red. Control experiments were run in the absence of the pH indicator and showed no significant effect of phenol red on the rate of ligand binding (not shown). The formation of the encounter complex ( $E \cdot L$ ; judged by the absorbance increase at 492 nm, Figure 1) was essentially complete in the dead-time of the stopped-flow under these conditions. In contrast, the polarization of the ligand is much slower than binding (Figure 5A; with 90% of the absorbance changes at 344 nm occurring at 7.7/s). Figure 5B shows that the proton uptake using phenol red occurs with a similar rate constant (5.8/s; see legend) and corresponds to a stoichiometry of 1.06 protons/active site. In comparable experiments, polarization of the C-8 analogue proved too fast to measure accurately, and so kinetic proton uptake measurements were not pursued.

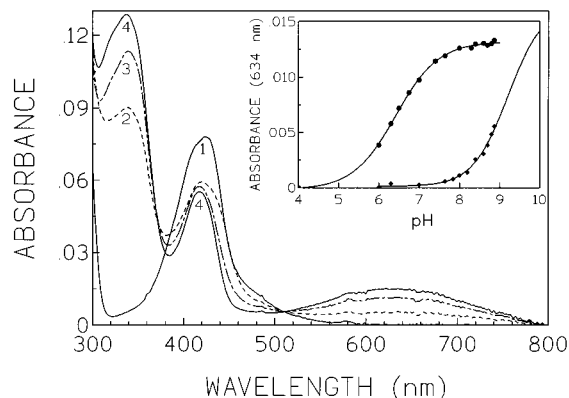
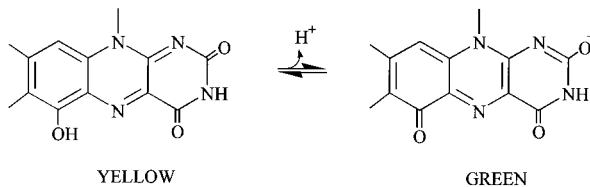


FIGURE 6: Effect of 4-thia-*trans*-2-enoyl-CoA binding on the ionization of 6-OH-FAD-substituted wild-type medium-chain acyl-CoA dehydrogenase. The 6-OH-FAD-substituted enzyme (curve 1; 4.2  $\mu\text{M}$  in 0.7 mL of 10 mM phosphate buffer, pH 6 at 25  $^{\circ}\text{C}$ ) was mixed with 6.8  $\mu\text{M}$  4-thia-*trans*-2-dodecenoyl-CoA (curve 2) and titrated with Tris as in Figure 1. Intermediate spectra in the titration are shown: curves 3, pH 7; curve 4, pH 8.9. The  $pK$  of 6.3 for the complex (solid circles) was obtained assuming a lower limit of zero for the 634 nm band (main figure). The diamonds in the inset represent a corresponding titration of the free enzyme with a  $pK$  of 9.1 using an upper pH limit set by the maximal absorbance seen for the corresponding pig kidney enzyme (32;  $pK_{\text{free}} = 8.7$ ).

#### Scheme 4



**6-Hydroxy-FAD-enzyme.** The experiments described above show that an isomerization of the enzyme·ligand complex affects not only the  $pK$  of the catalytic base but also the polarization of the substrate carbonyl group. Here we show that a third participant in catalysis, the flavin itself, undergoes modulation during this isomerization of the enzyme. Early work demonstrated that binding of crotonyl-CoA or octanoyl-CoA to the 6-OH-FAD-substituted pig kidney medium chain induces deprotonation of the neutral form of this oxidized flavin species (32). This effect is comparable to that observed with the blue neutral semiquinone of the unsubstituted enzyme (32, 41). Figure 6 shows a pH titration of the 6-OH-FAD-human enzyme complexed with 4-thia-*trans*-2-dodecenoyl-CoA. Preliminary experiments established that at pH 7.3 binding of this ligand is too tight to measure in a 1 cm pathlength cell, and so the concentration of 1.5 equiv used for the titration should be comfortably saturating. The yellow neutral form in Figure 6 is converted to the green anionic species (Scheme 4; 32, 43, 44) with a  $pK_{\text{app}}$  of 6.3 for the ligated form compared to a value estimated as 9.1 for the free enzyme. The corresponding free  $pK$  for the pig kidney enzyme is 8.7 (32).

The sizable spectral changes observed in Figure 6 allow the kinetics of this deprotonation to be followed when 4-thia-*trans*-2-dodecenoyl-CoA is added to the 6-OH-FAD-substituted medium-chain enzyme. The longer analogue is again used, rather than the C-8 derivative, to permit the reaction to be followed conveniently with the stopped-flow instrument. Figure 7A follows the absorbance at 634 nm upon mixing with 50  $\mu\text{M}$  4-thia-*trans*-2-dodecenoyl-CoA.

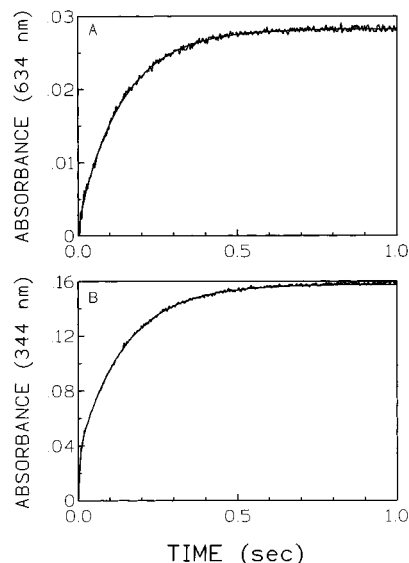


FIGURE 7: Kinetics of flavin deprotonation and enone polarization on binding 4-thia-*trans*-2-dodecenoyl-CoA to the 6-OH-FAD-enzyme. The wild-type enzyme (10  $\mu\text{M}$ ) was mixed with 4-thia-*trans*-2-dodecenoyl-CoA (50  $\mu\text{M}$ ) in 50 mM phosphate buffer, pH 7.3 at 4  $^{\circ}\text{C}$ , and the absorbance changes at 634 nm (A) and 344 nm (B) were measured. Data in panel A was fit to a single exponential of 7/s and to two exponentials with a major phase of 5.4/s in panel B (70% of total change).

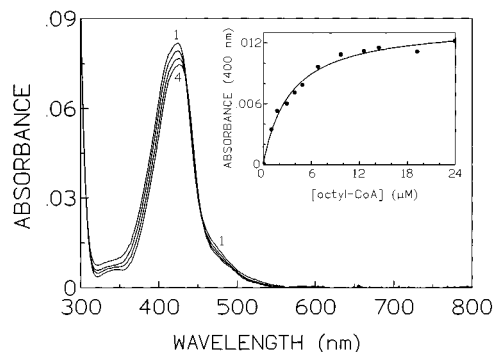


FIGURE 8: Octyl-SCoA binding to the 6-OH-FAD-substituted wild-type human medium-chain acyl-CoA dehydrogenase. The enzyme (4.7  $\mu\text{M}$  in 50 mM phosphate buffer, pH 7.3 at 25  $^{\circ}\text{C}$ ; curve 1) was mixed with 0.2, 1.0, and 3 equiv of 4-thia-*trans*-2-dodecenoyl-CoA (curves 2–4, respectively). Intermediate spectra are omitted for clarity. Absorbance changes at 400 nm (see inset) were fit to a dissociation constant of 1.1  $\mu\text{M}$ .

The observed rate constant of 7/s is comparable to polarization of the enone chromophore measured in the same experiment (5.4/s at 344 nm; Figure 7B). The spectral changes at 344 nm are dominated by the polarized enone absorbance. These rate constants are again comparable to those obtained for polarization of the C-12 ligand with the unsubstituted wild-type enzyme (7.7/s) and for proton uptake (5.8/s; see earlier). Thus, 6-OH-FAD substitution does not appear to adversely affect the isomerization reaction.

The marked effect of enoyl-CoA analogues on the  $pK$  of 6-OH-flavin might again be attributed to a simple binding of a CoA derivative. This is not the case. Octyl-SCoA binds relatively tightly to the 6-OH-FAD enzyme (Figure 8;  $K_D = 1.1 \mu\text{M}$ ) but without any significant shift in the  $pK$  of the flavin ( $pK$  of 9.2, data not shown, compared to 9.1 for the free enzyme; Figure 6). Note that the only difference between octyl-SCoA and octanoyl-CoA is the replacement of the thioester carbonyl with a methylene group. Clearly,

the thioester is a crucial determinant for the attainment of the catalytically competent state in the medium-chain enzyme.

## DISCUSSION

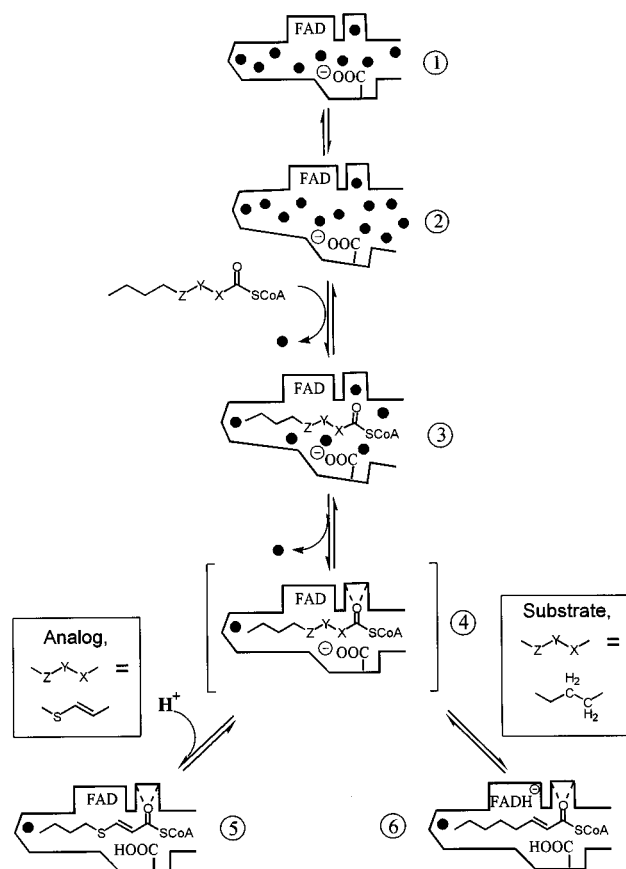
This paper shows that the apparent  $pK$  of the catalytic base, Glu376, is elevated from about 6.3 in the free enzyme (12) to greater than 9 in complexes with medium chain length enoyl-CoA analogues (Table 1). The progressive ligand-induced desolvation of the active site observed crystallographically (10, 13, 22) provides a ready explanation for this marked increase in  $pK$ . Thus, model studies show that a  $pK$  of  $>9$  is easily attained by a carboxyl group in a medium of low dielectric (23–26): the  $pK$  of acetic acid increases from 4.7 in water to 12 in dimethyl sulfoxide (45). Further, elevated  $pK$  values for carboxyl residues have been reported for a number of proteins including reduced thioredoxin,  $pK >9$  (46), 3-oxo- $\Delta$ -steroid-isomerase,  $pK$  of 9.5 (47), and 4-oxalocrotonate tautomerase,  $pK$  of 9 (48). Raising the  $pK$  of the catalytic base by ligand-induced desolvation thus appears to be an important catalytic strategy utilizing some of the intrinsic binding energy of thioester substrates (19, 23, 49).

While the medium-chain dehydrogenase has a broad chain length specificity (C-4 to C-16; 50), it shows optimal activity around C-8 (9, 27, 33, 51).  $V_{max}$  for octanoyl-CoA in the ferricenium assay increases with pH with an apparent  $pK$  of 8.2–8.3 (7, 33, 52). As might be expected from the present work, this  $pK_{app}$  is chain length dependent (7); however, the complexity of overall turnover in the acyl-CoA dehydrogenases complicates detailed analysis of these pH effects. In the static experiments described here, medium chain length ligands elevate the apparent  $pK$  of Glu376 most markedly, suggesting that they are the most effective at desolvating key regions of the active center. Table 1 shows that the  $pK$  observed with complexes of the somewhat oversized C-12 enoyl-CoA analogue is 0.9 unit lower than for the C-8 derivative. With further lengthening of the chain to C-14, the apparent  $pK$  for Glu376 in the C-14 complex drops a total of 2.7 pH units (approximating that of the free enzyme; Table 1). Since bulky substrates can only be accommodated by buckling the chain and/or enlargement of the binding cavity (10, 13), a poorer match between ligand and enzyme would be expected to lead to a less effective net desolvation of the active site and hence a more normal  $pK$  for Glu376.

Although C-12 chains can be accommodated in the active site of the medium-chain dehydrogenase without cracking the crystal (13, 22), they are slightly oversized from an enzymological perspective. Thus, the C-12 analogue is a poorer substrate than octanoyl-CoA in overall turnover with both pig and human enzymes (33, 51). Similarly, the longer analogue is a significantly slower reductant of both enzymes in the reductive half-reaction (by about 5-fold; 51; this work, data not shown). Further, the rate of polarization of enoyl-CoA product analogues is even more strongly chain length dependent, being some 30-fold slower with the C-12 chain compared to the C-8 chain (see earlier).

Scheme 5 is a schematic representation of the steps leading to the polarization of thioester ligands incorporating the earlier studies using 4-thia-enoyl-CoA analogues (9). Forms 1–4 (Scheme 5) are common to substrate and enoyl-CoA

Scheme 5



product analogues. Form 2 depicts an open conformation of the free enzyme (closed circles represent water molecules), because it is difficult to see from the crystal structure how such a large segmented CoA ligand could bind hydrocarbon-end first into a twisted narrow well without some breathing motion (13, 22). Form 3 is the encounter complex, but the critical desolvation step occurs subsequently (forms 4–6) with the alignment of the carbonyl oxygen to the FAD-ribityl-2'-OH and Glu376-N-H groups (13). Form 5 shows the polarized 4-thiaenoyl-CoA analogue in the closed conformation. Form 6 is the corresponding reduced enzyme–enoyl-CoA product complex. The desolvation step (forms 3 to 4) would both raise the  $pK$  of Glu376 and strengthen those H-bonds involved in polarization of the thioester carbonyl group (form 4; 19). In the case of enoyl-CoA analogues, the proton required for the neutralization of the Glu376 carboxylate (forms 4 and 5) comes from solvent. With good substrates, efficient sequestration of the active site from bulk solvent ensures that the polarized and activated thioester serves as a facile proton donor for the adjacent catalytic base (forms 4 and 6). The closed conformation minimizes a competing reprotonation of form 4 from solvent that would otherwise short-circuit catalysis.

While the crystal structure does not reveal major changes in tertiary or quaternary structure of free and bound enzyme (e.g., forms 1 and 5 or 6 in Scheme 5; 13, 22), there is strong evidence for a catalytically essential isomerization or conformational change following the initial encounter complex (forms 3 to 4/5 in Scheme 5). The kinetics of this isomerization have been extensively examined by Srivastava and colleagues, following the polarization of the enone

chromophore, indoleacryloyl-CoA (39, 53). The evidence presented above shows the sensitivity of this isomerization reaction to the size of the acyl chain. Thus, the rate at 25 °C for indoleacryloyl-CoA is about 6.7/s (39) compared to 7.7/s at 4 °C for 4-thia-*trans*-2-dodecenoyl-CoA and >300/s for the less bulky 4-thia-C8 analogue. Whatever the precise nature of this structural adjustment in enzyme–ligand complexes (schematically depicted as a closure of the active site and docking of the carbonyl group), it appears to influence multiple properties of the enzyme. For example, the rate of polarization of 4-thia-C12-enoyl-CoA (7.7/s) appears to be essentially the same as that of proton uptake by Glu376 (5.8/s) and of proton release from the 6-OH-FAD substituted enzyme (7/s).

The issue of how an enoyl-CoA ligand can induce proton uptake by Glu376 but proton release by both 6-OH-FAD (this work) and normal reduced flavin species (41) deserves comment. Scheme 2 shows that negative charge flows from carboxylate through an enolate-like transition state to the flavin. The accommodation of two negative charges distributed between Glu376, ligand, and flavin would be disfavored in a medium of low dielectric because of strong electrostatic coupling between participants (8, 10). Thus, protonation of Glu376 should lower the p*K* of 6-OH-FAD. Ligand-induced desolvation would also be expected to strengthen critical H-bonds to the pyrimidine ring of the bound isoalloxazine (13, 54, 55) especially when they involve anionic loci such as the C-2 oxygen (Scheme 4; 19). Thus, desolvation per se could favor anionic flavin species if H-bonds are important determinants in their stabilization.

The thioether analogue octyl-SCoA offers a striking contrast to the thioester derivatives used in this study. While thioether analogues bind quite tightly to the medium-chain dehydrogenase, they behave anomalously in several important respects (9, 31, 40–42). This work provides key additional information: octyl-SCoA binding does not induce a significant change in the p*K* of Glu376, and it cannot effect the deprotonation of 6-OH-FAD seen with the thioesters described above. Further, octyl-SCoA does not induce ionization of the blue neutral semiquinone of the unsubstituted enzyme (41). These data are consistent with the key role that the thioester carbonyl interactions play in the stabilization of the closed, catalytically competent, state for both reductive and oxidative halves of the reaction (9, 12, 56).

Polarization of the thioester carbonyl group has been observed by vibrational and NMR spectroscopies for a number of CoA-dependent enzymes (34, 57–61) including the acyl-CoA dehydrogenases (54, 55). In particular, the polarization seen in this work shows striking parallels with that observed for the binding of cinnamoyl-CoA analogues to enoyl-CoA hydratase (34, 59) and benzoyl-CoA derivatives to 4-chlorobenzoyl-CoA dehalogenase (60, 61). Strong, ground-state polarization of the carbonyl was observed via Raman and <sup>13</sup>C NMR with accompanying sizable red shifts in the UV/VIS spectrum of the bound ligands. Further, the pH dependence of the polarization observed with enoyl-CoA hydratase disappears upon mutation of the active site base, Glu164, to a Gln residue (59). Loss of polarization on deprotonation of Glu164 in the hydratase might involve unfavorable electrostatic repulsion between the polarized thioester carbonyl group and the carboxylate of Glu164 (34,

59). In addition to these local effects, the equilibrium between species 3 and 5 in the acyl-CoA dehydrogenases (Scheme 5) would also result in modulation of polarization. If the polarization seen in the present work with the enoyl-CoA analogues is of relevance to catalysis in the acyl-CoA dehydrogenase (6, 8, 9, 12, 56, 62), it would be expected to be present to some degree with true substrates and to be maximized in the transition state as Glu376 is neutralized by the *pro-R*- $\alpha$ -proton (Scheme 2). A future challenge will be to assess the polarization of the carbonyl group of actual substrates while bound to the oxidized dehydrogenase. In principle, 5-deaza-FAD-substituted enzyme (3, 32) should allow this complex to be examined in the absence of significant reduction of the flavin prosthetic group.

## REFERENCES

- Murfin, W. W. (1974) Ph.D. Thesis, Washington University, St. Louis, MO.
- Reinsch, J., Katz, A., Wean, J., Aprahamian, G., and McFarland, J. T. (1980) *J. Biol. Chem.* 255, 9093–9097.
- Ghisla, S., Thorpe, C., and Massey, V. (1984) *Biochemistry* 23, 3154–3160.
- Pohl, B., Raichle, T., and Ghisla, S. (1986) *Eur. J. Biochem.* 160, 109–115.
- Schopfer, L. M., Massey, V., Ghisla, S., and Thorpe, C. (1988) *Biochemistry* 27, 6599–6611.
- Engel, P. C. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. III, pp 597–655, CRC Press, Boca Raton, FL.
- Ghisla, S., Engst, S., Vock, P., Kieweg, V., Bross, P., Nandy, A., Rasched, I., and Strauss, A. W. (1993) in *Flavins and Flavoproteins* (Yagi, K., Ed.) pp 283–292, Walter de Gruyter, New York.
- Johnson, B. D., Mancini-Samuels, G. J., and Stankovich, M. T. (1995) *Biochemistry* 34, 7047–7055.
- Triebel, R. C., Wang, R., Anderson, V. E., and Thorpe, C. (1995) *Biochemistry* 34, 8597–8605.
- Thorpe, C., and Kim, J.-J. P. (1995) *FASEB J.* 9, 718–725.
- Lau, S.-M., Brantley, R. K., and Thorpe, C. (1988) *Biochemistry* 27, 5089–5095.
- Vock, P., Engst, S., Eder, M., and Ghisla, S. (1998) *Biochemistry* 37, 1848–1860.
- Kim, J.-J. P., Wang, M., and Paschke, R. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 7523–7527.
- Gerlt, J. A., and Gassman, P. G. (1993) *Biochemistry* 32, 11943–11952.
- Gerlt, J. A., and Gassman, P. G. (1993) *J. Am. Chem. Soc.* 115, 11552–11568.
- Cleland, W. W., and Kreevoy, M. M. (1994) *Science* 264, 1887–1890.
- Frey, P. A., Whitt, S. A., and Tobin, J. B. (1994) *Science* 264, 1927–1930.
- Tobin, J. B., Whitt, S. A., Cassidy, C. S., and Frey, P. A. (1995) *Biochemistry* 34, 6919–6924.
- Shan, S.-O., and Herschlag, D. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 14474–14479.
- Powell, P. J., and Thorpe, C. (1988) *Biochemistry* 27, 8022–8028.
- Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I., and Ghisla, S. (1990) *J. Biol. Chem.* 265, 7116–7119.
- Lee, H.-J. K., Wang, M., Paschke, R., Nandy, A., Ghisla, S., and Kim, J.-J. P. (1996) *Biochemistry* 35, 12412–12420.
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* 43, 219–410.
- Rose, I. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* 43, 491–517.
- Thibblin, A., and Jencks, W. P. (1979) *J. Am. Chem. Soc.* 101, 4963–4973.



26. Gilbert, H. F. (1981) *Biochemistry* 20, 5643–5649.
27. Thorpe, C., Matthews, R. G., and Williams, C. H. (1979) *Biochemistry* 18, 331–337.
28. Kieweg, V., Krautle, F.-G., Engst, S., Nandy, A., Vock, P., Abdel Ghani, A.-G., Bross, P., Rasched, I., Strauss, A. W., and Ghisla, S. (1997) *Eur. J. Biochem.* 246, 548–556.
29. Williamson, G., Engel, P. C., Mizzer, J. P., Thorpe, C., and Massey, V. (1982) *J. Biol. Chem.* 257, 4314–4320.
30. Lau, S.-M., Brantley, R. K., and Thorpe, C. (1989) *Biochemistry* 28, 8255–8261.
31. Powell, P. J., Lau, S.-M., Killian, D., and Thorpe, C. (1987) *Biochemistry* 26, 3704–3710.
32. Thorpe, C., and Massey, V. (1983) *Biochemistry* 22, 2972–2978.
33. Nandy, A., Kieweg, V., Krautle, F. G., Vock, P., Kuchler, B., Bross, P., Kim, J.-J. P., Rasched, I., and Ghisla, S. (1996) *Biochemistry* 35, 12402–12411.
34. D'Ordine, R. L., Tonge, P. J., Carey, P. R., and Anderson, V. E. (1994) *Biochemistry* 33, 12635–12643.
35. Mayer, E. J., and Thorpe, C. (1981) *Anal. Biochem.* 116, 227–229.
36. Fitzpatrick, P. F., and Massey, V. (1982) *J. Biol. Chem.* 257, 9958–9962.
37. Peterson, K. L., Sergienko, E. E., Wu, Y., Kumar, N. R., Strauss, A. W., Oleson, A. E., Muhonen, W. W., Snabb, J. B., and Srivastava, D. K. (1995) *Biochemistry* 34, 14942–14953.
38. Srivastava, D. K., Wang, S., and Peterson, K. L. (1997) *Biochemistry* 36, 6359–6366.
39. Johnson, J. K., Wang, Z.-X., and Srivastava, D. K. (1992) *Biochemistry* 31, 10564–10575.
40. Johnson, B. D., and Stankovich, M. T. (1993) *Biochemistry* 32, 10779–10785.
41. Mizzer, J. P., and Thorpe, C. (1981) *Biochemistry* 20, 4965–4970.
42. Wang, R., and Thorpe, C. (1991) *Biochemistry* 30, 7895–7901.
43. Mayhew, S. G., Whitfield, C. D., Ghisla, S., and Schuman-Jorns, M. (1974) *Eur. J. Biochem.* 44, 570–591.
44. Massey, V., and Hemmerich, P. (1980) *Biochem. Soc. Trans.* 8, 246–257.
45. Clare, B. W., Cook, D., Ko, E. C. F., Mac, Y. C., and Parker, A. J. (1966) *J. Am. Chem. Soc.* 88, 1911–1922.
46. Wilson, N. A., Barbar, E., Fuchs, J. A., and Woodward, C. (1995) *Biochemistry* 34, 8931–8939.
47. Wu, Z. R., Ebrahimian, S., Zawrotny, M. E., Thornburg, L. D., Perez-Alvarado, G. C., Brothers, P., Pollack, R. M., and Summers, M. F. (1997) *Science* 276, 415–418.
48. Stivers, J. T., Abeygunawardana, C., Mildvan, A. S., Hajjipour, G., and Whitman, C. P. (1996) *Biochemistry* 35, 814–823.
49. Page, M. I., and Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 1678–1683.
50. Crane, F. L., Mii, S., Hauge, J. G., Green, D. E., and Beinert, H. (1956) *J. Biol. Chem.* 218, 701–716.
51. Hall, C. L., Lambeth, J. D., and Kamin, H. (1979) *J. Biol. Chem.* 254, 2023–2031.
52. Kumar, N. R., Peterson, K. L., and Srivastava, D. K. (1996) in *Flavins and Flavoproteins* (Stevenson, K. J., Massey, V., and Williams, C. H., Eds.) pp 641–644, University of Calgary Press, Calgary, Alberta, Canada.
53. Johnson, J. K., and Srivastava, D. K. (1993) *Biochemistry* 32, 8004–8013.
54. Nishina, Y., Sato, K., Shiga, K., Fujii, S., Kuroda, K., and Miura, R. (1992) *J. Biochem. (Tokyo)* 111, 699–706.
55. Nishina, Y., Sato, K., Hazekawa, I., Fujii, S., Kuroda, K., Miura, R., and Shiga, K. (1993) in *Flavins and Flavoproteins* (Yagi, K., Ed.) pp 303–311, Walter de Gruyter, New York.
56. Sabaj, K. M., Stankovich, M. T., and Anderson, V. (1996) in *Flavins and Flavoproteins* (Stevenson, K. J., Massey, V., and Williams, C. H., Eds.) pp 645–648, University of Calgary Press, Calgary, Alberta, Canada.
57. Kurz, L. C., Ackerman, J. J., and Drysdale, G. R. (1985) *Biochemistry* 24, 452–457.
58. Kurz, L. C., and Drysdale, G. R. (1987) *Biochemistry* 26, 2623–2627.
59. D'Ordine, R. L., Bahnson, B. J., Tonge, P. J., and Anderson, V. E. (1994) *Biochemistry* 33, 14733–14742.
60. Taylor, K. L., Liu, R.-Q., Liang, P.-H., Price, J., Dunaway-Mariano, D., Tonge, P. J., Clarkson, J., and Carey, P. R. (1995) *Biochemistry* 34, 13881–13888.
61. Clarkson, J., Tonge, P. J., Taylor, K. L., Dunaway-Mariano, D., and Carey, P. R. (1997) *Biochemistry* 36, 10192–10199.
62. Johnson, B. D., and Stankovich, M. T. (1993) in *Flavins and Flavoproteins* (Yagi, K., Ed.) pp 323–326, Walter de Gruyter, New York.