Effect of Transition-State Analogues on the Redox Properties of Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: The binding of substrate/product or transition-state intermediates modifies the properties of medium-chain fatty acyl-CoA dehydrogenase (MCAD) by causing the redox potential to shift positive and the oxygen reactivity to slow by 3000-fold. Two ligands, identified as being the most effective in slowing oxygen reactivity, were 2-azaoctanoyl-CoA and 3-thiaoctanoyl-CoA [Wang, R., & Thorpe, C. (1991) *Biochemistry 30,* 7895−7901]. We have measured the potential shifts caused by the binding of both ligands to determine which is most similar to the potential shift caused by substrate/product mixture, the assumption being that the best transition-state structural intermediate would give the potential shift most similar to that of substrate/product [Lenn, N. D., Stankovich, M. T., & Liu, H. (1990) *Biochemistry 29,* 10594−10602]. Both ligands shifted the potential positive, but the shift caused by 2-azaoctanoyl-CoA was 65% that of substrate/product, while 3-thiaoctanoyl-CoA was only 20% of that value. This positive shift is proposed to be caused by a resonance form stabilized by the interaction of the catalytically essential carbonyl of the acyl-CoA with two hydrogen bonds from the enzyme, which induces a partial negative charge on the carbonyl and a partial positive charge on carbon 2 of the ligand and carbon 3 of the substrate/product couple. The X-ray structure shows that carbons 2 and 3 of the substrate/product overlap the diazadiene portion of the flavin ring [Kim, J.-J. P., Wang, M., & Paschke, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 7523−7527]. The net positive charge near the flavin would make electron transfer easier. The importance of both hydrogen bonds to the potential shift was clearly demonstrated by using 2-deoxy-FAD MCAD which lacked the 2-hydroxyl group of the ribityl chain of the flavin, thus eliminating one hydrogen bond. In this case, when 2-azaoctanoyl-CoA was bound, the midpoint potential shift was only 30 mV positive as compared to a 70 mV positive shift observed with normal FAD MCAD. Thus both hydrogen bonds are required to generate the positive charge required for the full potential shift.

Medium-chain acyl-CoA dehydrogenase (MCAD)[1] catalyzes the two-electron oxidation of a broad range of fatty acids and is the initial enzyme in the fatty acid oxidation cycle, from which up to 40% of the total human energy requirement is derived (Sherratt, 1988; Bremer & Osmundson, 1984). Specifically, MCAD catalyzes the conversion of straight-chain fatty acyl-CoA thioesters to their corresponding α,β-enoyl-CoA products. MCAD consists of four identical monomers each containing one flavin adenine dinucleotide (FAD). The reaction catalyzed by the enzyme is believed to occur in two steps (Beinert, 1963; Thorpe et al., 1979):

$$\text{E-FAD}_{ox} + \text{SH}_2 \rightleftharpoons \text{E-FADH}_{2red}\text{-P} \quad (1)$$

$$\text{E-FADH}_{2red}\text{-P} + 2\text{ETF}_{ox} \rightleftharpoons \text{E-FAD}_{ox}\text{-P} + 2\text{ETF}_{1e^-} \quad (2)$$

The reductive half-reaction, the first step of dehydrogenation (1), is believed to involve the abstraction of both a proton and a hydride ion of the thioester-CoA by Glu^{376} and the flavin, respectively, to yield a fully reduced MCAD and product (Ghisla et al., 1984; Frerman et al., 1980; Powell & Thorpe, 1988). Earlier redox studies have shown that electron transfer from substrate ($E_m = -26$ mV) to uncomplexed MCAD ($E_m = -145$ mV) is thermodynamically unfavorable (Lenn et al., 1990; Johnson & Stankovich, 1993). However, upon substrate/product couple complexation the redox potential of MCAD is shifted positive by over 100 mV, providing the thermodynamic driving force for the reaction and thereby acting as an on/off switch for the entire electron-transfer process of fatty acid oxidation. Because of the experimental conditions, it could not be distinguished which specific ligand−protein interactions were critical in shifting the potential positive since many interactions were present.

The specific interactions at the molecular level responsible for the regulation of an enzyme's redox properties are usually unknown. However, from previous oxygen reactivity data and structure information it was concluded that the thioester carbonyl on the substrate is essential for catalysis (Wang & Thorpe, 1991a; Kim et al., 1993). Structural work has further shown that the thioester carbonyl is involved in two hydrogen

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[1] Abbreviations: ACD, acyl-CoA dehydrogenase; $E_1^{o\prime}$, formal potential of first electron transfer; $E_2^{o\prime}$, formal potential of second electron transfer; E_m, midpoint potential; E-FAD_{ox}, oxidized MCAD; E-$FADH_{1e^-}$, one-electron-reduced MCAD; E-$FADH_{2red}$, two-electron-reduced MCAD; ETF, electron-transferring flavoprotein; FAD, flavin adenine dinucleotide; K_d, dissociation constant for MCAD; MCAD, medium-chain acyl-CoA dehydrogenase; P, product (*trans*-2-enoyl-CoA); SH_2, substrate (thioester-CoA).

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bonds, the first to the 2-OH of the ribityl of the flavin and the second to the amide backbone of Glu^{376}. The inductive effect caused by the hydrogen bonds to the thioester carbonyl is the probable reason for the lowering of the pK_a of the α-proton of the substrate, making proton abstraction possible.

By using structurally well-defined intermediates, we can identify the important structural features which cause the potential to shift positive. We have already shown this to be true with the thioesters. These ligands which lack the crucial carbonyl caused the redox potential of MCAD to shift negative, a direction opposite to that of substrate/product (Johnson & Stankovich, 1993). For the work presented here the ligands 2-azaoctanoyl-CoA and 3-thiaoctanoyl-CoA[2] were chosen because they contain the thioester carbonyl which preserves the essential hydrogen bonds between the ligand and MCAD. In addition, the ligands lead to an approximately 3000-fold slowing of the rate of reoxidation of the uncomplexed two-proton fully reduced form of the enzyme with oxygen (Wang & Thorpe, 1991a). This is significant because MCAD bound to product also shows slower oxygen reactivity. In addition, the 3-thiaoctanoyl-CoA ligand was selected for study because it structurally resembles natural substrate except a thioether sulfur has replaced the methylene group at the C-3 position of the fatty acyl-CoA. This ligand binds and the α-proton deprotonates but does not transfer electrons to MCAD, making thermodynamic studies more defined, since there is only one form of the ligand present (Wang & Thorpe, 1991b). The second ligand, 2-azaoctanoyl-CoA, was chosen because it structurally resembles natural substrate except an amino group has replaced a methylene group at the C-2 position of the fatty acyl-CoA. It is thought that the α-hydrogen is not abstracted from this ligand because the ligand does not form a charge-transfer band with oxidized or reduced MCAD (Thorpe, personal communication).

Since MCAD in the presence of the substrate/product couple results in a large positive shift in redox potential, redox chemistry was utilized to monitor MCAD regulation. Spectroelectrochemical methods were used to measure the redox potentials of MCAD bound to two ligands. Ligands which cause the redox potentials to shift positive, mimicking substrate/product, will provide information about key structural features which result in regulation are possible transition-state analogues. In addition, the role of the 2-OH ribityl hydrogen bond to the thioester carbonyl of the substrate in the formation of a transition state was investigated using 2-deoxy-FAD MCAD.

MATERIALS AND METHODS

Materials. Medium-chain acyl-CoA dehydrogenase (MCAD) was purified from pig kidney according to the method of Thorpe et al. (1979), with the addition of an octyl-Sepharose column to aid in the removal of contaminating enoyl hydratase activity (Lau et al., 1986). Concentrations of oxidized uncomplexed MCAD were measured spectrophotometrically with an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm. MCAD was stored at −20 °C. The 2-deoxy-FAD MCAD was prepared according to published procedures (Engst, 1993). The 2-azaacyl-CoAs were synthesized according to the method of Thorpe (personal communication). 3-Thiaoctanoyl-CoA was synthesized according to the method of Lau et al. (1988). The CoA ligands were purified by HPLC (Corkey et al., 1982), desalted by a Bio-Gel P-2 (Bio-Rad) column, and characterized by NMR. All CoA ligands were lyophilized and stored at −20 °C.

All experiments were performed at 25 °C unless otherwise noted, and glass-distilled water was utilized. The following dyes were used: methyl viologen (Sigma); indigodisulfonate (Aldrich); 8-chlororiboflavin, the generous gift of Dr. J. P. Lambooy, University of Maryland; pyocyanine, photochemically synthesized by the method of McIlwain (1937) using phenazine methosulfate from Sigma as the starting material. Sodium pyrophosphate buffer was used for experiments done at pH 8.5, and potassium phosphate buffer was used for all other experiments.

Methods. Potentiometric and coulometric measurements were performed essentially as previously described (Stankovich, 1980; Stankovich & Fox, 1983). All MCAD potentiometric titrations were performed using dithionite as a reductant and methyl viologen to mediate electron transfer. Potentiometric titrations with 2-deoxy-FAD MCAD were performed electrochemically and also utilized methyl viologen to mediate electron transfer. The indicator dyes used in the potentiometric titrations and their redox potentials at pH 7.6 were 8-chlororiboflavin (−0.153 V), indigodisulfonate (−0.118 V), and pyocyanine (−0.019 V). Typical concentrations in the experimental solutions were 10 μM MCAD-FAD, 100 μM methyl viologen, and 2−4 μM indicator dyes (potentiometric titration). After each reductive experiment the experimental solution was reoxidized with 20 μL of 5 mM ferricyanide. This was done because of MCAD's extreme protection against reoxidation by molecular oxygen when complexed to the ligands used in this work (Wang & Thorpe, 1991a). This was not necessary for the 2-deoxy-FAD MCAD experiments. The reoxidation of the 2-azaoctanoyl-CoA complexed to 2-deoxy-FAD MCAD was significantly faster than MCAD complexed to 2-azaoctanoyl-CoA.

The potentiometric titrations were all performed in the reductive direction. However, for a few experiments with MCAD the solution was coulometrically reoxidized to a small extent (~5%) after a reductive potential was obtained. This was done for several points during the course of reduction to ensure that the potential measurements taken during the titration were at equilibrium and that the system was reversible. For all potentiometric experiments equilibrium of the system was considered to be obtained when the ΔE was less than 0.001 V/10 min; this was typically around 1−2 h. All potential values are reported versus the standard hydrogen electrode.

Dithionite titrations were performed as previously described (Einarsdottir et al., 1988). The dithionite solution was standardized with lumiflavin-3-acetate. Ligand incubation studies were performed by addition of a 30−40-fold excess of the ligand to MCAD or 2-deoxy-FAD MCAD at pH 7.6. The visible spectra of MCAD were monitored over a 24-h period at 25 °C. The visible spectra of 2-deoxy-FAD MCAD were monitored over a 19-h period at 25 °C.

Calculations. The concentrations of MCAD-FAD redox species present during the course of the spectroelectrochemical titration were calculated by simultaneously solving three or four equations depending upon the number of redox species present. The initial equation was a mass balance

[2] See Figure 4 for ligand structures.

Table 1: Extinction Coefficients for Oxidized and Fully Reduced MCAD Complexed to Three Different 2-Azaacyl-CoAs and 3-Thiaoctanoyl-CoA

wavelength and redox state	2-azabutyryl-CoA			2-azaoctanoyl-CoA			2-azatetradecanoyl-CoA			3-thiaoctanoyl-CoA		
λ (nm)	570	455	385	570	455	385	570	455	385	570	455	385
oxidized (M^{-1} cm^{-1})	100	15200	11400	100	14400	10600	100	15000	11000	500	9900	9700
reduced (M^{-1} cm^{-1})	200	1000	3900	200	1000	3900	200	500	3500	200	1200	3100

equation relating the concentration of the redox species to the total amount of MCAD in the experiment. The other equations utilized Beer's law by relating the absorbance at different wavelengths to the extinction coefficient of the redox species and to their corresponding concentrations in the experiment. It was assumed that the extinction coefficients for the blue neutral semiquinone (5000, 4800, and 5300 M^{-1} cm^{-1} at 385, 446, and 570 nm, respectively) (Gorelick et al., 1985; Lehman & Thorpe, 1990) and the red anionic semiquinone (19 000, 3000, and 300 M^{-1} cm^{-1} at 385, 446, and 570 nm, respectively) remained identical for all titrations. The extinction coefficients for the red anionic semiquinone were obtained from a dithionite titration of MCAD complexed with 2-azaoctanoyl-CoA at pH 8.5. The extinction coefficients for oxidized MCAD complexed to the 2-azaacyl-CoAs and to 3-thiaoctanoyl-CoA were determined from spectral titrations in the present work. The extinction coefficients for fully reduced MCAD complexed to the 2-azaacyl-CoAs and 3-thiaoctanoyl-CoA were determined at the end of the reductive experiments. The values used for data analysis are given in Table 1. The extinction coefficient used for oxidized 2-deoxy-FAD MCAD was estimated from the difference to the spectrum obtained upon denaturation with SDS (15400 M^{-1} cm^{-1}). The extinction coefficient used for oxidized 2-deoxy-FAD MCAD complexed to 2-azaoctanoyl-CoA was estimated to be 14400 M^{-1} cm^{-1}. Before quantitation of redox species, the spectra were corrected for turbidity which occurred during the titration.

The midpoint potentials (E_m) and n values were calculated by a computerized nonlinear regression fit (Duggleby, 1981) to a plot of E versus [ox]/[red] using the Nernst equation:

$$E = E_m + (0.059/n) \log([ox]/[red]) \quad (3)$$

where E is the measured equilibrium potential at each point in the titration and n is the number of electrons. The individual formal potential values for the first ($E_1°'$) and second ($E_2°'$) electron transfers were calculated in similar fashion. In arriving at a final reported midpoint potential value under specified conditions, data from several experiments were incorporated together and used in the nonlinear regression analysis. Typical error for a potentiometric analysis experiment was ±2−5 mV. All midpoint potential value determinations exhibited Nernstian behavior as indicated by their n values.

The relationship between individual formal potential values and the midpoint potential value used to arrive at the maximal amount of the semiquinone thermodynamically stabilized has been previously described (Clark, 1960; Einarsdottir et al., 1988). The relationship between binding constants and midpoint potentials under saturating conditions has also been previously described (Clark, 1960; Einarsdottir et al., 1988).

The dissociation constant of 2-azaoctanoyl-CoA to oxidized MCAD at pH 6.5 was determined by a spectral titration which measured the resulting perturbation of the enzyme

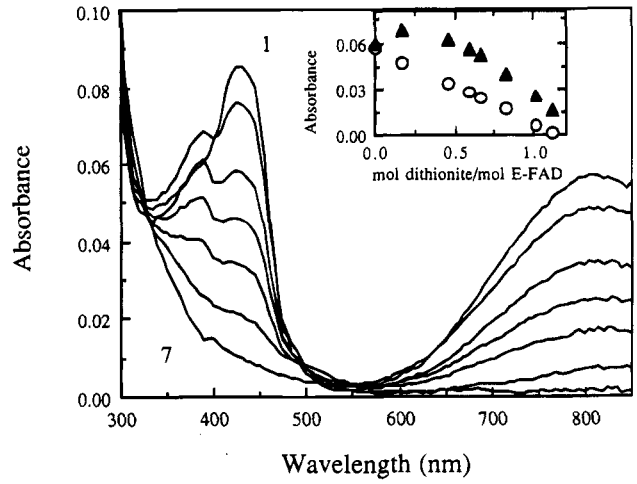

FIGURE 1: Dithionite titration of 6.48 μM MCAD complexed with 21 μM 3-thiaoctanoyl-CoA in 50 mM potassium phosphate buffer, pH 7.6 at 25 °C. Spectra 1−7 correspond to addition of respectively 0.0, 0.17, 0.46, 0.67, 0.81, 1.01, and 1.12 mol of dithionite/mol of E-FAD. The inset shows absorbance values from the two phases of the titration plotted at 804 (open circles) and 385 nm (solid triangles).

absorbance spectrum. Data were analyzed by a computerized nonlinear regression method (Einarsdottir et al., 1989).

RESULTS

3-Thiaoctanoyl-CoA-Complexed MCAD. No electron transfer between 3-thiaoctanoyl-CoA and MCAD occurred when the flavin chromophore was monitored over extended periods of incubation. Complexation of this ligand to oxidized MCAD leads to the appearance of an intense broad charge-transfer band (extinction coefficient of 8700 M^{-1} cm^{-1} at 804 nm) formed between the deprotonated ligand and flavin. In addition, there is a blue shift in the major flavin transition and a corresponding decrease in the intensity of the 370 nm peak (Lau et al., 1988).

Dithionite Titrations of 3-Thiaoctanoyl-CoA-Complexed MCAD. A dithionite titration was performed in order to initially characterize the spectra of the reduced species of MCAD complexed with 3-thiaoctanoyl-CoA. Figure 1 shows the dithionite titration while the inset is a plot of the absorbance at 804 and 385 nm versus moles of dithionite per mole of MCAD-FAD. From the reduction spectra at pH 7.6 it was evident that 16% red anionic semiquinone was formed. Due to the broad charge-transfer band it was difficult to determine whether small amounts of blue neutral semiquinone were also formed during the course of this titration. However, it appears that very little (<3%) blue neutral semiquinone was formed. Dithionite titrations were performed at pH 8.5 and 6.5, in order to examine the pH dependence of both the protonation state and the percentage of semiquinone formed. At pH 8.5, 40% red anionic semiquinone was formed with no visible formation of blue neutral semiquinone. At pH 6.5, 40% red anionic semi-

Table 2: Comparison of Measured Thermodynamic Properties of Uncomplexed MCAD and 2-Deoxy-FAD-MCAD, Natural Substrate/Product Couple-Complexed MCAD, MCAD Bound to Three Different 2-Azaacyl-CoA Ligands, and 3-Thiaoctanoyl-CoA and 2-Deoxy-FAD-MCAD Bound to 2-Azaoctanoyl-CoA All at pH = 7.6, 50 mM KP_i at 25 °C Unless Otherwise Noted

MCAD with ligand	% semiquinone/protonation state	E_m (V)	$E_1°'$ (V)	$E_2°'$ (V)	$K_{d_{ox}}$ (nM)	$K_{d_{sq}}$ (nM)	$K_{d_{red}}$ (nM)
none[a]	20/neutral	−0.145	−0.166	−0.129			
octanoyl-/*trans*-2-octenoyl-	−/anionic[b]	−0.026[c]					
2-azabutyryl-CoA	40/mixture	−0.112	−0.103	−0.119	4400[d]	450	350
2-azaoctanoyl-CoA	56/anionic	−0.071	−0.050	−0.094	50[e]	0.62	0.17
2-azatetradecanoyl-CoA	51/anionic	−0.114	−0.086	−0.125	420[d]	34	40
3-thiaoctanoyl-CoA	<5/anionic	−0.122[f]			470[g]		80
2-deoxy-FAD-MCAD with ligand	% semiquinone/protonation state	E_m (V)	$E_1°'$ (V)	$E_2°'$ (V)	$K_{d_{ox}}$ (nM)	$K_{d_{sq}}$ (nM)	$K_{d_{red}}$ (nM)
none	20/neutral	−0.173	−0.190	−0.156			
2-azaoctanoyl-CoA	30/mixture	−0.142	−0.111	−0.158			

[a] Johnson and Stankovich (1993). [b] Mizzer and Thorpe (1981). [c] Lenn et al. (1990). [d] Thorpe, personal communication. [e] Wang and Thorpe (1991). [f] Experimental conditions: 18 °C in 5% glycerol. [g] Lau et al. (1988).

quinone and 60% blue neutral semiquinone were formed. A pK_a value of 6.7 ± 0.1 was calculated for MCAD's flavosemiquinone complexed with 3-thiaoctanoyl-CoA from the amounts of semiquinone formed at pH 6.5.

Coulometric Titration of 3-Thiaoctanoyl-CoA-Complexed MCAD. A coulometric titration of 3-thiaoctanoyl-CoA-complexed MCAD was done at pH 7.6. No semiquinone of either protonation state was visible, and the spectral changes observed at all wavelengths were linear versus reducing equivalents added. The larger amounts of red anionic semiquinone stabilized in dithionite titrations are typical of flavoproteins. Both SCAD and flavoprotein oxidases stabilize larger amounts of red anionic semiquinone when reduced with dithionite versus electrochemical reduction mediated by methyl viologen (Fink et al., 1986; Stankovich et al., 1978). The reduction of red anionic semiquinones by negatively charged reducing agents such as dithionite tends to be kinetically slow even when thermodynamically feasible (Stankovich & Fox, 1983; Van den Berghe-Snorek & Stankovich, 1985). However, methyl viologen is a positively charged reductant, and electrochemical reduction in the presence of this mediator approaches thermodynamic reversibility.

Potentiometric Titration of 3-Thiaoctanoyl-CoA-Complexed MCAD. Potentiometric titrations were carried out in order to measure the midpoint potential value for MCAD complexed to 3-thiaoctanoyl-CoA. It was difficult to correct for turbidity because of the charge-transfer band which was present at longer wavelengths. Therefore, these measurements were done at 18 °C and in 5% glycerol in order to minimize turbidity. The following midpoint potential value for 3-thiaoctanoyl-CoA-complexed MCAD was measured at pH 7.6:

$$\text{E-FAD}_{ox} + 2e^- + H^+ \rightleftharpoons \text{E-FADH}_{red} \qquad E_m = -0.122 \text{ V} \tag{4}$$

The formal midpoint potential values for the individual electrons could not be calculated because no semiquinone is thermodynamically stabilized. This represents a positive potential shift of 23 mV with respect to free enzyme. However, using human MCAD, it has been observed that in the presence of glycerol and at lower temperatures the midpoint potential shifts 15 mV positive (G. Mancini-Samuelson, unpublished data). Therefore, a significant amount of the potential shift may be due to the conditions. If the positive shift is real and not an effect of the experimental conditions, the midpoint potential value indicates that 3-thiaoctanoyl-CoA binds tighter to reduced MCAD than to oxidized MCAD. The dissociation constant of 3-thiaoctanoyl-CoA to oxidized MCAD ($K_{d_{ox}}$) is 470 nM (Lau et al., 1988); thus, a dissociation constant of 80 nM was calculated for 3-thiaoctanoyl-CoA complexed to fully reduced MCAD ($K_{d_{red}}$). This corresponds to a 6-fold difference between $K_{d_{ox}}$ and $K_{d_{red}}$. The $K_{d_{red}}$ determined in this work is slightly smaller than the value of 120 nM determined by Wang and Thorpe (1991a). However, their value was obtained with 1,5-dihydrodeaza-FAD-substituted MCAD. The data are shown in Table 2.

2-Azaacyl-CoA-Complexed MCAD. Three different chain length 2-azaacyl-CoA compounds utilized in this study, butyryl-, octanoyl-, and tetradecanoyl-CoA, were complexed to oxidized MCAD, and the resulting spectra were examined using visible spectroscopy. Complexation of these ligands to oxidized MCAD caused spectral changes essentially the same as those which were caused upon complexation of the thioether-CoA class of ligands to MCAD (Powell et al., 1987). Specifically, complexation leads to the appearance of shoulders at 440 and 495 nm and a red shift of the major absorbance band. However, in contrast to the thioether-CoAs, the magnitude of spectral changes peaked for the eight carbon chain length ligand 2-azaoctanoyl-CoA. Both 2-azabutyryl- and 2-azatetradecaonyl-CoA induced less spectral perturbations than 2-azaoctanoyl-CoA.

As stated previously, it is important for this study that no electron transfer from the binding ligands to MCAD occurs. Thus, the 2-azaacyl-CoA ligands were complexed to oxidized MCAD, and the flavin chromophore was monitored over an extended period of time. Approximately 10% of the flavin chromophore was bleached in about 4 h, and about 66% was bleached after 24 h. 2-Azabutyryl-CoA and 2-azatetradecanoyl-CoA required 20 and 12 h, respectively, to bleach MCAD's flavin chromophore by only 10%. The resulting species could not be reoxidized either by exposure to air or by excess amounts of ferricyanide. Perhaps the flavin ring of MCAD is being covalently inactivated upon incubation with the 2-azaacyl-CoAs as similar spectral changes are observed upon inactivation with (methylenecyclopropyl)-acetyl-CoA (Ikeda & Tanaka, 1990; Lai et al., 1993). Therefore, all potentiometric experiments were accomplished during time periods in which less than 10% of the FAD had been bleached to minimize problems arising from the bleaching of E-FAD by the 2-azaacyl-CoA ligands.

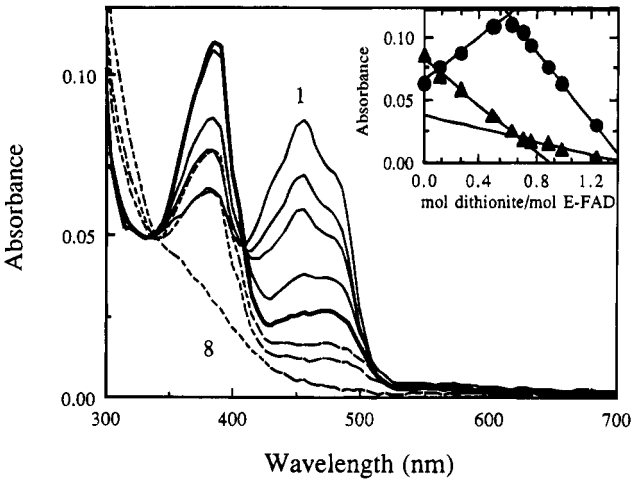

FIGURE 2: Dithionite titration of 5.96 μM MCAD complexed with 440 μM 2-azaoctanoyl-CoA in 20 mM potassium pyrophosphate buffer, pH 8.5 at 25 °C. Solid lines correspond to the first phase of reduction (curves 1−4; mol of dithionite/mol of E-FAD = 0.0, 0.12, 0.27, and 0.5, respectively). The bold solid line corresponds to the maximal amount of semiquinone generated (curve 5; mol of dithionite/mol of E-FAD = 0.62). Dashed lines correspond to the second phase of reduction (curves 6−8; mol of dithionite/mol of E-FAD = 0.90, 0.99, and 1.24, respectively). The inset shows absorbance values from the two phases of the titration plotted at 450 (solid triangles) and 385 nm (solid circles).

Dithionite Titrations of 2-Azaacyl-CoA-Complexed MCAD. Dithionite titrations were carried out in order to initially characterize the spectral course of reduction of MCAD complexed with the 2-azaacyl-CoA ligands. The reduction spectra at pH 7.6 revealed that a large amount of red anionic semiquinone was being formed. This is a distinct departure from the behavior observed with either uncomplexed MCAD or thioether-CoA-complexed MCAD where smaller amounts of blue neutral semiquinone are formed as the intermediate (Thorpe et al., 1979; Johnson & Stankovich, 1993). A dithionite titration was done at pH 8.5 in order to generate a maximal amount of red anionic semiquinone from which extinction coefficients could be calculated. The reduction spectra of 2-azaoctanoyl-CoA-complexed MCAD at pH 8.5 are shown in Figure 2. The inset to Figure 2 is a plot of the absorbance at both 455 and 385 nm versus moles of dithionite per mole of E-FAD. The intersection of the least-squares lines drawn through the data plotted at both wavelengths is assumed to be the theoretical absorbance of 100% red anionic semiquinone formation. The resulting extinction coefficients calculated for the red anionic semiquinone are 19 000, 3000, and 300 M^{-1} cm^{-1} at 385, 446, and 570 nm, respectively. These values are typical for the red anionic semiquinone (Massey & Palmer, 1966). From the calculated extinction coefficients it was determined that 95% red anionic semiquinone was formed at pH 8.5 and 83% red anionic semiquinone was formed at pH 7.6.

To determine a pK_a value for MCAD's flavosemiquinone complexed to 2-azaoctanoyl-CoA, a dithionite titration was performed at pH 6.5. In this titration 48% red anionic semiquinone and 31% blue neutral semiquinone were formed, resulting in a calculated pK_a value of 6.3. The 2-azaoctanoyl-CoA ligand may be binding weaker at pH 6.5 than at 7.6, resulting in the formation of less red anionic semiquinone. Therefore, the dissociation constant for this ligand complexed to oxidized MCAD was measured at pH 6.5. The resulting dissociation constant was 49 ± 2 nM and is consistent with the value of 50 nM previously determined at pH 7.6 (Wang & Thorpe, 1991a).

Table 3: Comparison of Amounts of Semiquinone Stabilized by Dithionite for Uncomplexed MCAD and MCAD Bound to Three Different 2-Azaacyl-CoA Ligands and 3-Thiaoctanoyl-CoA at pH 7.6

ligand	% anionic semi-quinone	% neutral semi-quinone	total % semi-quinone	pK_a (N-5)
none[a]		53	53	>10
2-azabutyryl-CoA	61	26	87	7.2
2-azaoctanoyl-CoA	83	<3	83	6.3
2-azatetradecanoyl-CoA	67	<3	67	<6.5
3-thiaoctanoyl-CoA	16	<3	16	6.7

[a] Thorpe et al. (1979).

Similar dithionite titrations of MCAD complexed to 2-azabutyryl- and 2-azatetradecanoyl-CoA were carried out at pH 7.6. A summary of the percentages of semiquinone formed and the calculated pK_a values as well as a comparison of these data with the dithionite titrations for MCAD complexed with the 3-thiaoctanoyl-CoA is given in Table 3. Note that the maximal amount of red anionic semiquinone is formed for 2-azaoctanoyl-CoA.

Previous studies have shown that the oxidation product from dithionite reduction, the sulfite ion, forms an adduct with the flavin prosthetic group in many flavoproteins, particularly those which form the red anionic semiquinone upon one-electron reduction (Massey et al., 1969). The formation of this adduct is characterized by the bleaching of the visible absorption spectrum of the oxidized flavoprotein during titration with sulfite ion. An incubation was done with sulfite to check for adduct formation because of the conversion of semiquinone from blue neutral to red anionic upon the complexation of the 2-azaacyl-CoA ligands. For this work, a 200-fold excess of sodium sulfite was added to 6 μM MCAD complexed with 100 μM 2-azabutyryl-CoA, and the mixture was incubated for 6 h at 25 °C. No change in the visible absorption spectrum was observed, which indicated that this adduct was not formed with the flavin in MCAD.

Potentiometric Titrations of 2-Azaacyl-CoA-Complexed MCAD. Potentiometric titrations were carried out in order to measure the formal potential values for the two redox couples of MCAD complexed to the 2-azaacyl-CoA ligands. The following formal potential values for MCAD complexed to 2-azaoctanoyl-CoA were measured at pH 7.6:

$$\text{E-FAD}_{ox} + e^- \rightleftharpoons \text{E-FAD}_{1e^-} \qquad E_1^{\circ\prime} = -0.050\ \text{V} \quad (5)$$

$$\text{E-FAD}_{1e^-} + e^- + H^+ \rightleftharpoons \text{E-FADH}_{red} \qquad E_2^{\circ\prime} = -0.094\ \text{V} \quad (6)$$

The midpoint potential was −0.071 V, and the maximal amount of red anionic semiquinone stabilized was 56%. The positive shift of the individual formal potential values when compared to uncomplexed MCAD indicates that 2-azaoctanoyl-CoA binds tighter to reduced MCAD than to either the oxidized or one-electron-reduced forms. The dissociation constant of 2-azaoctanoyl-CoA to oxidized MCAD ($K_{d_{ox}}$) is 50 nM (Wang & Thorpe, 1991a); thus dissociation constants of 0.62 and 0.17 nM were calculated for 2-azaoctanoyl-CoA complexed to the one-electron ($K_{d_{sq}}$) and two-electron-

reduced MCAD ($K_{d_{red}}$), respectively. This corresponds to a 300-fold difference between $K_{d_{ox}}$ and $K_{d_{red}}$. The $K_{d_{red}}$ calculated in this work does not agree with the value of 410 nM determined by Wang and Thorpe (1991a). However, their value was obtained with 1,5-dihydrodeaza-FAD-substituted MCAD.

Micelle formation was likely during the experiments using 2-azatetradecanoyl-CoA as the critical micelle concentration for palmitoyl-CoA is 40 μM (Smith & Powell, 1986). Thus, it might be postulated that this influenced the trend of redox potential versus chain length by not shifting MCAD's redox potential more positive when complexed to 2-azatetradecanoyl-CoA. However, all binding curves with this ligand to the oxidized form of MCAD (Lau et al., 1988) have shown a one to one stoichiometry, and no further spectral changes of the flavin chromophore were observed at higher concentrations (~200 μM) of ligand. This suggests that the micelle formation is not affecting the electrochemical results.

A summary of the thermodynamic properties of the 2-azaacyl-CoA ligands complexed to MCAD at pH 7.6 is given in Table 2. The trend in redox potentials determined for all of the 2-azaacyl-CoA-complexed MCAD systems was similar. The redox potentials are all shifted positive of the value for uncomplexed MCAD, and more semiquinone, primarily red anionic, is stabilized. The appearance of stoichiometric levels of anionic flavin semiquinone on binding the 2-azaacyl-CoA ligands may be due to the ligand's ability to stabilize a partial positive charge at nitrogen 2 of the ligand. This may also account for the more positive values for $E_1°'$ in Table 2 for the 2-azaacyl-CoA-complexed MCADs. It is interesting to note that the most positive shift in redox potential as well as the largest percentage of red anionic semiquinone formed is for the eight carbon chain length ligand 2-azaoctanoyl-CoA.

2-Deoxy-FAD MCAD Uncomplexed. The use of one ligand, 2-azaoctanoyl-CoA, and 2-deoxy-FAD MCAD allowed us to specifically probe the hydrogen bond that exists between the thioester carbonyl and the 2-OH ribityl of the flavin. For this study 2-deoxy-FAD was substituted for normal FAD in MCAD, thus removing one of the hydrogen bonds with the thioester carbonyl of the ligand. The 2-deoxy-FAD MCAD has been shown to have less than 5% of the activity of normal MCAD (Engst, 1993). The spectral and redox properties of the 2-deoxy-FAD MCAD were measured, in both the absence and presence of the ligand 2-azaoctanoyl-CoA, thus providing the unique ability to analyze the significance of a specific hydrogen bond. All experiments with 2-deoxy-FAD MCAD were performed at a pH of 7.6 in 50 mM KP_i buffer.

Potentiometric Titration of 2-Deoxy-FAD MCAD Uncomplexed. The midpoint potential value of 2-deoxy-FAD MCAD was determined to be −173 mV, and approximately 20% blue neutral semiquinone was formed.

$$\text{E-FAD}_{ox} + 2e^- + 2H^+ \rightleftharpoons \text{E-FADH}_{2red} \qquad E_m = -173 \text{ mV} \quad (7)$$

Previous work has shown the midpoint potential of MCAD to be −145 mV and to stabilize 20% blue neutral semiquinone (Johnson & Stankovich, 1993). The midpoint potential of 2-deoxy-FAD MCAD is shifted 28 mV negative compared to MCAD. Both MCAD and 2-deoxy-FAD MCAD thermodynamically stabilize the same amount of blue neutral semiquinone. The data are shown in Table 2.

2-Azaoctanoyl-CoA-Complexed 2-Deoxy-FAD MCAD. Complexation of 2-azaoctanoyl-CoA to oxidized 2-deoxy-FAD MCAD leads to absorbance changes similar to those of MCAD complexed with this ligand. Upon binding of 2-azaoctanoyl-CoA the major absorbance band is red shifted, and shoulders appear at 440 and 495 nm. The 2-azaoctanoyl-CoA ligand was complexed to 2-deoxy-FAD MCAD, and the flavin chromophore was monitored over an extended period of time. After 19 h only 8% of the flavin chromophore was bleached.

Dithionite Titration of 2-Deoxy-FAD-octanoyl-CoA-Complexed 2-Deoxy-FAD MCAD. A dithionite titration of the ligand-complexed 2-deoxy-FAD MCAD was necessary to initially characterize the spectral course of reduction. Approximately 10% blue neutral semiquinone and 20% red anionic semiquinone were formed during the reduction.

Potentiometric Titration of 2-Azaoctanoyl-CoA-Complexed 2-Deoxy-FAD MCAD. Potentiometric titrations were carried out in order to measure the midpoint potential value of 2-deoxy-FAD MCAD complexed to 2-azaoctanoyl-CoA. The midpoint potential determined was −142 mV.

$$\text{E-FAD}_{ox} + 2e^- + H^+ \rightleftharpoons \text{E-FADH}_{red} \qquad E_m = -142 \text{ mV} \quad (8)$$

The midpoint potential of the complexed 2-deoxy-FAD MCAD is shifted 31 mV positive compared to 2-deoxy-FAD MCAD uncomplexed. The comparison is shown in Table 2.

DISCUSSION

The main findings from our redox studies are as follows: First, the 3-thiaoctanoyl-CoA complexed with MCAD showed a positive shift in midpoint potential that was only 20% of that observed for natural substrate/product couple-complexed MCAD, but the shift may be due to the experimental conditions. Second, the 2-azaoctanoyl-CoA also showed a positive shift in midpoint potential when complexed to MCAD. However, the shift was much greater, representing 65% that of natural substrate/product couple-complexed MCAD. Finally, a less positive shift in midpoint potential was observed when the ligand 2-azaoctanoyl-CoA was complexed to 2-deoxy-FAD MCAD compared to the same ligand complexed to normal flavin. These data are consistent with the following postulate where a transition state causes MCAD regulation.

It has been shown in short-chain acyl-CoA dehydrogenase that product not substrate binding to the reduced form shifts the midpoint potential positive, enabling electron transfer to occur (Becker et al., 1994). This raises the question, how does product binding catalyze its own formation? A plausible explanation is that product mimics some transition state which occurs during catalysis. Further insight about the properties of the proposed transition state may be gained from resonance Raman data. A resonance Raman study on fully reduced MCAD complexed to the natural product *trans*-2-octenoyl-CoA, has shown that there is an appreciable contribution from a protein-stabilized ionic resonance structural conformation for *trans*-2-octenoyl-CoA exhibiting a partial negative charge on the thioester carbonyl and a partial

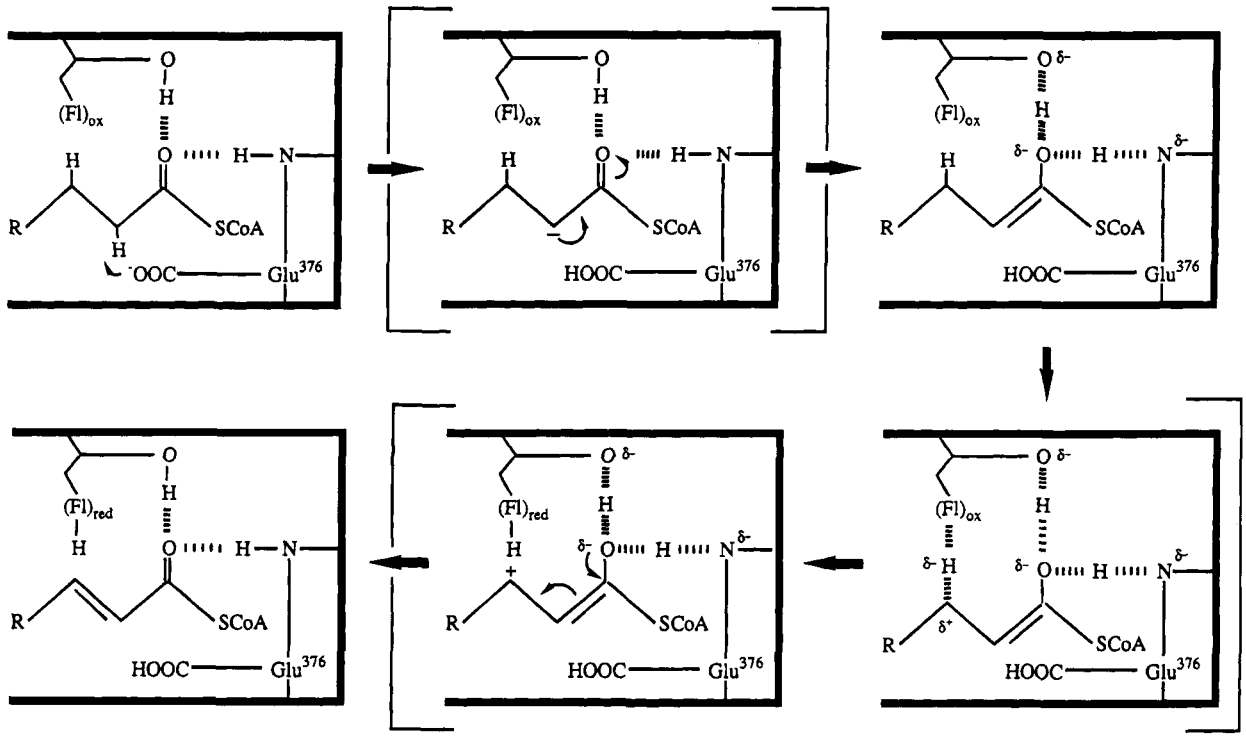

FIGURE 3: Postulated reaction mechanism for oxidation of substrate by MCAD showing the resonance structure of *trans*-2-octenoyl-CoA in the MCAD binding pocket.

positive charge on carbon 3 (Nishina et al., 1992). The structure of this resonance form of product is shown in the fifth frame of Figure 3. The electrophilic groups which are probably responsible for stabilizing the partial negative charge on the thioester carbonyl are the 2-OH ribityl of the flavin and the amide backbone of Glu^{376} since structural work has shown that a hydrogen bond exists between these groups and the thioester carbonyl (Ghisla et al., 1992; Kim et al., 1993). These hydrogen bonds may induce and stabilize a transition-state resonance structure similar to product.

Further, structural data have also shown that carbons 2 and 3 of *trans*-2-octenoyl-CoA lie directly over the diazadiene portion of the flavin ring, an area strongly associated with flavin reduction (Edmondson & Tollin, 1983; Nishimoto et al., 1985; Miura et al., 1993; Kim et al., 1993). Thus, a partial positive charge on carbon 3 of *trans*-2-octenoyl-CoA might contribute to the positive shift in redox potential and extreme tight binding of natural product to reduced MCAD.

A partial positive charge on the binding ligand stabilized by MCAD's active site centered over an electroactive area of the flavin ring is a reasonable explanation for the positive shift in redox potential (Ghisla & Massey, 1991; Distefano et al., 1989; Van den Berghe-Snorek & Stankovich, 1985). The induced partial positive charge affects the stability of reduced MCAD and shifts the redox potentials positive in a manner similar to that of the positive charges around N(1)–C(2)=O in lactate oxidase (Muh et al., 1994). It was found that when the positively charged Lys^{266} was mutated to a neutral residue in lactate oxidase, the midpoint potential of the flavin was shifted 30 mV negative. Current studies in our laboratory also support these findings. When the negatively charged active site base Glu^{376} was mutated to a neutral residue, glutamine, thus eliminating a negative charge approximately 7 Å away from the flavin, the potential shifted positive by 30 mV for human MCAD and shifted positive by 10 mV for SCAD (G. Mancini-Samuelson, unpublished data; Becker et al., 1994). Thus, it seems reasonable that a partial positive charge on the ligand which is only 3.3 Å away from the flavin would result in a large positive shift in the midpoint potential of the flavin (Kim et al., 1993). In addition, the data argue against "global effects" of substrate binding causing the midpoint potential to shift positive. X-ray crystal structure data of MCAD in both the absence and presence of substrate indicate that the overall structure changes very little when substrate binds (Kim et al., 1993). In fact, when the thioesters which lack the crucial carbonyl were bound to MCAD, the midpoint potential shifted negative (Johnson & Stankovich, 1993).

A model which may explain our observations is shown in Figure 3. The abstraction of the α-proton occurs via the Gerlt–Gassman mechanism (Gerlt et al., 1991, 1992; Gerlt & Gassman, 1993a,b). In this mechanism, a base and an acid (e.g., Glu^{376} and the 2-OH ribityl of the flavin for MCAD) bind to the carbonyl to generate and stabilize a transition state where the pK_a of the α-hydrogen of the fatty acyl-CoA is lowered so that it can be extracted by the base (note: the discussion is of transition states not intermediates). During β-bond cleavage, a partial positive charge may be generated at carbon 3 in the transition state, such that the partially positively charged species must be significant. This positively charged transition state in a hydrophobic environment may cause the enzyme potential to shift positive and thus may provide the driving force which allows the unfavorable electron transfer from the substrate to the flavin of MCAD occur. The structure of this possible transition state is shown in the fourth structure of Figure 3. It has many similarities to the resonance form of product shown in structure 5 of Figure 3.

Possibly, the 2-azaoctanoyl-CoA and 3-thiaoctanoyl-CoA ligands have structural similarities to that of the transition state. Both ligands contain the thioester carbonyl which preserves the hydrogen bonding. However, 2-azaoctanoyl-

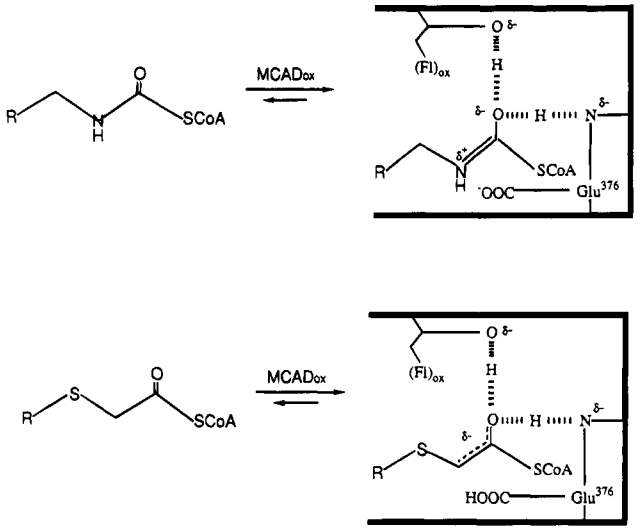

FIGURE 4: Proposed resonance structure of 2-azaoctanoyl-CoA in the MCAD binding pocket and the enolate structure of 3-thiaoctanoyl-CoA in the MCAD binding pocket.

CoA is likely not deprotonated, and thus when a negative charge is induced at the thioester carbonyl of the substrate, a positive charge develops at carbon 2 of the ligand. This leads to a separation of charge which is similar to that of the natural product (Figure 4). The partial positive charge near the diazadiene portion of the flavin ring results in the positive shift in redox potential and stabilization of the one-electron anionic reduced form. In contrast, the 3-thiaoctanoyl-CoA ligand can be deprotonated and forms a charge-transfer band between the negatively charged ligand and the FAD. The 3-thiaoctanoyl-CoA ligand preserves the thioester carbonyl functionality but does not stabilize a partial positive charge (Figure 4) and thus, according to thermodynamics, is not as good a model for the transition state as the 2-azaoctanoyl-CoA analogue.

A partial positive charge on the binding ligand induced by MCAD's active site centered over an electroactive area of the FAD ring appears to be a reasonable explanation for the positive shift in redox potential. However, it is of interest to consider why the 2-azaoctanoyl-CoA ligand does not shift the redox potential to the same extent as natural product. A likely difference in the two ligands is the extent to which the ionic resonance form contributes to their structures. The 2-azaoctanoyl-CoA ligand would appear to have a larger contribution to the ionic resonance structure because it contains a structure similar to a peptide bond. The carbon and nitrogen bonds of peptide linkages are known to have approximately 40% double bond character (Creighton, 1983). However, it is natural product and not 2-azaoctanoyl-CoA that causes the largest positive shift in the redox potential. For 2-azaoctanoyl-CoA the partial positive charge is located on carbon 2 instead of carbon 3; this may explain the smaller shift in the redox potentials. A possible explanation for the lack of deprotonation of the 2-azaoctanoyl-CoA ligand is, as discussed above, that 2-azaoctanoyl-CoA already possesses 40% double bond character; thus its geometry is probably planar. The α-proton on the ligand in the planar form may not be accessible to the Glu^{376}.

The 2-deoxy-FAD MCAD results provide further evidence that a partial negative charge develops on the thioester carbonyl of the ligand and is stabilized by two important hydrogen bonds; this development of a negative charge on the carbonyl leads to a partial positive charge on carbon 2 or 3 of the ligand which results in a positive shift in potential. When 2-deoxy-FAD MCAD was complexed with 2-azaoctanoyl-CoA, where only one hydrogen bond (the amide backbone of Glu^{376} to the thioester carbonyl) was present, the midpoint potential was shifted positive by 31 mV from uncomplexed 2-deoxy-FAD MCAD. This is a smaller shift than is observed when normal FAD MCAD is complexed with the same ligand (70 mV), where the two hydrogen bonds (the 2-OH ribityl and the amide backbone of Glu^{376} to the thioester carbonyl of the ligand) are intact. The smaller shift in potential is the result of only one hydrogen bond stabilizing the negative charge at the thioester carbonyl of 2-azaoctanoyl-CoA, thus resulting in less separation of charge and a smaller positive charge on carbon 2 of the ligand. The smaller positive charge on carbon 2 of the ligand, which lies directly over the diazadiene portion of the flavin ring, results in a smaller positive shift in potential compared to normal MCAD complexed with the ligand 2-azaoctanoyl-CoA. A 30 mV potential shift corresponds to a loss of 1 kcal, which is consistent with the loss of a hydrogen bond. Although we cannot rule out the possibility that water binds in the vicinity of the carbonyl in 2-deoxy-FAD MCAD, providing a second hydrogen bond to the carbonyl (the X-ray crystal structure is not known), the redox data are consistent with the loss of a hydrogen bond.

In summary, redox data from three kinds of experiments (previous work with short-chain acyl-CoA dehydrogenase mutants and the two types of MCAD studies presented here) support the idea that the formation of a product-like transition state causes the potential of MCAD to shift positive, providing the thermodynamic driving force for electron transfer. The thioester carbonyl plays an essential role because its hydrogen-bonding behavior causes the electron density to change by an inductive effect which causes the positive shift in redox potential.

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