Influence of Two Substrate Analogues on Thermodynamic Properties of Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: The objective of this work was to identify the key structural functionalities of substrate or product that modulate the thermodynamic properties of medium-chain acyl-CoA dehydrogenase (MCAD). In order to achieve this, two classes of substrate analogues, acetyl-CoA and thioether-CoAs, were complexed with MCAD and their effects on the redox properties of MCAD were measured. A pH dependence study of the redox potential of uncomplexed MCAD allowed us to compare redox properties between complexed and uncomplexed MCAD and to calculate the dissociation constants of the analogues to the three redox states of MCAD. The results from this work indicate that these analogues are not influencing the thermodynamic behavior of MCAD in the same way as natural substrate. Thus, we propose that the following two key structural features of the binding ligand are necessary for mimicking the thermodynamic effects natural substrate has on MCAD: a thioester carbonyl on carbon 1 and a fatty acyl-CoA chain length around 8 carbon units. Furthermore, with the advent of structural knowledge, insights into the interactions of these structural features with MCAD and their influence on MCAD's highly regulated dehydrogenation mechanism are discussed.

Mitochondrial fatty acid oxidation (β-oxidation) provides up to 40% of the total human energy requirement (Sherratt, 1988). The first step of this enzymatic oxidation cycle is catalyzed by a group of enzymes exhibiting varying chain-length specificities, the fatty acyl-CoA dehydrogenases. Medium-chain acyl-CoA dehydrogenase (MCAD)[1] is the most prominent enzyme of this group found in the mammalian kidney cortex (Thorpe et al., 1979). MCAD catalyzes the two-electron oxidation of a broad range of fatty acids with chain lengths from 4 to 16 carbon atoms, with optimal activity for fatty acyl-CoAs of 8-carbon chain length (Hall et al., 1979; Thorpe et al., 1979). MCAD consists of four identical monomers, each containing one flavin adenine dinucleotide (FAD). The reaction catalyzed by this enzyme is believed to occur in two steps (Beinert, 1963):

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

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

Studies on each of these steps have revealed several aspects of acyl-CoA dehydrogenase (ACD) regulation upon substrate or product binding.

The reductive half-reaction, the first step of dehydrogenation (eq 1), involves the abstraction of both a proton and a hydride ion from the α- and β-carbons of the thioester-CoA substrate, respectively, to yield the fully-reduced ACD and enoyl-CoA (Ghisla et al., 1984; Frerman et al., 1980). Redox studies have shown that substrate/product couple binding modulates the redox properties of MCAD and short-chain acyl-CoA dehydrogenase (SCAD) from *Megasphaera elsdenii*, causing their midpoint potentials to become significantly more positive (Lenn et al., 1990; Stankovich & Soltysik, 1987). This modulation in redox potential facilitates the isopotential delivery of reducing equivalents from the thioester-CoA to ACD. Furthermore, the magnitude of this shift depends, to some extent, on the chain length of the substrate/product couple (Lenn et al., 1990).

The oxidative half-reaction, the second step of the ACD's catalyzed reaction (eq 2), involves the oxidation of product-complexed reduced MCAD by 2 equiv of electron-transferring flavoprotein (ETF) and the subsequent release of the enoyl-CoA product (Crane & Beinert, 1956). Kinetic and binding studies have shown that product complexation affects the protonation state, efficiency, and sequential delivery of electron transfer between MCAD and ETF (Mizzer & Thorpe, 1981; Thorpe & Massey, 1983; Gorelick et al., 1985). MCAD appears to simultaneously accept two electrons from the substrate, but product-complexed MCAD transfers the reducing equivalents in two separate steps to ETF.

The focus of this research is to identify the key structural functionalities of the substrate, product, or possible intermediate(s) that play a crucial role in modulating the electron transfer properties of MCAD. Through the use of substrate and product analogues, we can determine the effects specific structural features have on modulating the thermodynamic properties of MCAD. Analogues are used since they bind but do not turn over with MCAD, making thermodynamic studies more defined, since there is only one species of ligand present. In this work, we have probed the initial binding interaction between substrate and MCAD using two classes of substrate analogues, acetyl-CoA and thioether-CoAs. These

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[1] Abbreviations: ACD, acyl-CoA dehydrogenase; AMPD, 2-amino-2-methyl-1,3-propanediol; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; $E_1^{o\prime}$, 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$_{ox}$, oxidized electron-transferring flavoprotein; ETF$_{1e^-}$, one-electron-reduced electron-transferring flavoprotein; FAD, flavin adenine dinucleotide; GCD, glutaryl-CoA dehydrogenase; K_d, dissociation constant for MCAD; MCAD, medium-chain acyl-CoA dehydrogenase, formerly named general acyl-CoA dehydrogenase (GAD); P, product (*trans*-2-enoyl-CoA); $K_{a,ox}$, ionization constant for amino acid when E-FAD$_{ox}$; $K_{a,red}$, ionization constant for amino acid when E-FAD$_{red}$; SCAD, short-chain acyl-CoA dehydrogenase, formerly named butyryl-CoA dehydrogenase (BCD); SH$_2$, substrate (thioester-CoA).

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analogues allowed us to study the impact which both the thioester carbonyl and different carbon chain lengths have on modulating the thermodynamic properties of MCAD. Furthermore, with the advent of structural knowledge (Kim & Wu, 1988; Kim et al., 1992), insights into the interactions of these structural features with MCAD and their influence on MCAD's highly regulated dehydrogenation mechanism are discussed.

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 enoylhydratase activity (Lau et al., 1986). Concentrations of oxidized uncomplexed MCAD were measured spectrophotometrically with an extinction coefficient of 15.4 M^{-1} cm^{-1} at 446 nm. MCAD was stored at −20 °C. Butyl- and octyl-CoA were synthesized according to the method of Blaschkowski et al. (1979). The thioether-CoAs were purified by HPLC or by anion-exchange chromatography (Corkey et al., 1982; Lau et al., 1977), desalted by a Bio-Gel P-2 (Bio-Rad) column, and characterized by NMR. Hexadecyl-CoA was the generous gift of Dr. Colin Thorpe. All thioether-CoAs were lyophilized and stored at −20 °C. Acetyl-CoA was purchased from Sigma.

All experiments were performed at 25 °C and utilized glass-distilled water. The following dyes were used: methyl viologen (Sigma), indigo disulfonate (Aldrich), 2-hydroxy-1,4-naphthoquinone (Eastman Kodak), 9-azariboflavin, the generous gift of Dr. D. Graham at Merck, Sharp and Dohme, and 8-chlororiboflavin, the generous gift of Dr. J. P. Lambooy, University of Maryland. The following buffer systems were used: potassium phosphate, sodium bicarbonate, sodium pyrophosphate, CHES (Sigma), and AMPD (Sigma).

Methods. Potentiometric and coulometric measurements were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). Experiments performed at pH values 6.5–8.5 were in 50 mM potassium phosphate buffer. Experiments performed at pH 9.0 and 10.0 were in four different buffering systems: 50 mM potassium phosphate in 20 mM CHES, 20 mM sodium pyrophosphate, 50 mM carbonate buffer, and 50 mM AMPD. To minimize the effects of ions when redox values were compared, the ionic strength (μ) was kept at 100 mM for the majority of the buffering systems used. The indicator dyes used in the potentiometric titrations and their potentials at pH 7.6 were 8-chlororiboflavin (−0.153 V, 35 mV/pH unit), 9-azariboflavin (−0.134 V, 26 mV/pH unit), indigo disulfonate [−0.118 V, pH dependence as reported by Clark (1960)], and 2-hydroxy-1,4-naphthoquinone [−0.189 V, pH dependence as reported by Clark (1960)]. Typical concentrations in the experimental solutions were 10 μM E-FAD, 100 μM methyl viologen, and 2–4 μM indicator dyes (potentiometric titration).

The potentiometric titrations were all performed in the reductive direction. However, for several experiments, 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. 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. The HPLC gradient used to check for turnover products was the same as that used by Lenn et al. (1990). The activity of the uncomplexed MCAD was monitored before and after spectroelectrochemical titration using the assay of Thorpe et al. (1979).

Table I: Extinction Coefficients for Oxidized and Fully-Reduced MCAD Complexed to Three Different Thioether-CoAs

wavelength and redox state	butyl-CoA		octyl-CoA		hexadecyl-CoA	
λ (nm)	449	570	457	570	457	570
oxidized (M^{-1} cm^{-1})	15100	100	14900	100	15300	100
reduced (M^{-1} cm^{-1})	900	200	1000	200	1500	200

Calculations. Quantitation of the three E-FAD redox species present during the course of the spectroelectrochemical titration was calculated by simultaneously solving three equations. The first equation was a mass balance equation relating the concentration of the three redox species to the total amount of MCAD in the experiment. The other two equations utilized Beer's law by relating the absorbance at two different wavelengths to the extinction coefficient of the three redox species and their corresponding concentrations in the experiment. It was assumed that the extinction coefficient for the blue neutral semiquinone (4800 and 5300 M^{-1} cm^{-1} as 446 and 570 nm, respectively) remained the same throughout all titrations and at all pH values. The extinction coefficient utilized for the three redox species of the uncomplexed and acetyl-CoA-complexed MCAD were the same as reported by Gorelick et al. (1985) and Lehman and Thorpe (1990). The extinction coefficients for oxidized MCAD complexed to various thioether-CoAs were derived from spectral titrations done in the present work. The extinction coefficients for fully-reduced MCAD complexed to various thioether-CoAs were determined at the end of the reductive experiments. The values used for data analysis are given in Table I. Before quantitation of redox species, the spectra were corrected for turbidity that 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 a specified condition, 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 semiquinone thermodynamically stabilized has been previously described (Clark, 1960; Einarsdottir et al., 1988). The relationship between binding constants and midpoint potentials under both saturating and nonsaturating conditions has also been previously described (Clark, 1960; Einarsdottir et al., 1988).

The determination of the pK_a value for an amino acid in close proximity to the FAD in MCAD was also calculated by a computerized nonlinear regression fit. The equation used in the regression analysis to simulate the overall redox system of MCAD at a range of pH values was taken from Williamson and Edmondson (1985).

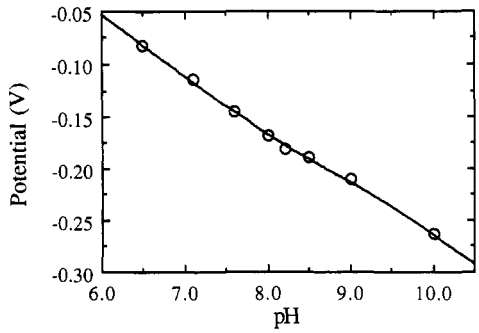

FIGURE 1: pH dependence of midpoint potential values (O) for uncomplexed MCAD. The line through data points has been obtained by a nonlinear regression analysis. The ionization constants found in the regression analysis were $pK_{a,ox}$ = 9.2 ± 0.1 and $pK_{a,red}$ = 8.3 ± 0.1 for the amino acid. The ionization constants assumed in the regression analysis were $pK_{a,ox}$ = 10.0 and $pK_{a,red}$ = 10.0 for E-FAD.

RESULTS

pH Dependence of Uncomplexed MCAD. We studied the pH dependence of the redox potential for uncomplexed MCAD in order to better compare the redox potentials and protonation states of uncomplexed MCAD with analogue-complexed MCAD. Spectroelectrochemical titrations were done at pH 7.6 to refine the previously determined values of both the redox potential and the amount of semiquinone stabilized. The following formal potential values were determined at pH 7.6:

$$\text{E-FAD}_{ox} + e^- + H^+ \rightleftharpoons \text{E-FADH}_{1e^-} \quad E_1^{\circ\prime} = -0.166\text{V} \quad (4)$$

$$\text{E-FADH}_{1e^-} + e^- + H^+ \rightleftharpoons \text{E-FADH}_{2red} \quad E_2^{\circ\prime} = -0.129\ \text{V} \quad (5)$$

The midpoint potential was –0.145 V and the maximal amount of blue neutral semiquinone stabilized was 20%.

The values correlate with the previously determined midpoint potential of –0.136 V at pH 7.6 by Lenn et al. (1990). Possible reasons for the refinement in the redox values are the use of an improved extinction coefficient for the blue neutral semiquinone (Gorelick et al., 1985; Lehman & Thorpe, 1990) and the use of an alternate indicator dye, 9-azariboflavin, that does not absorb in the same wavelength regions as the semiquinone. To draw further comparisons, Gustafson et al. (1986) determined a midpoint potential of –0.138 V for uncomplexed MCAD at pH 7.1 as compared to a value of –0.114 V determined in this work. The refinement in redox potential is most likely due to improved quantitation for previously described reasons.

The pH range of the uncomplexed enzyme characterized potentiometrically was from 6.5 to 10.0. The range of pH values studied was dictated by the stability of MCAD, since below pH 6.0 the enzyme precipitates and above pH 10.0 it degrades (Niu, 1990). Using a linear regression analysis, the pH dependence of the midpoint potential of uncomplexed MCAD, as shown in Figure 1, reflects a potential/pH slope of 51 mV/pH unit. This correlates to a pH dependence of two protons transferred per two electrons transferred. At all of the pH values only blue neutral semiquinone was observed. Table II shows that the amount of blue neutral semiquinone stabilized was dependent on pH, with values varying from 17% at pH 6.5 to 33% at pH 8.2. Mizzer and Thorpe (1981) also saw only blue neutral semiquinone up to a pH of 9.6. They also observed an increase in the amount of blue neutral semiquinone with increasing pH during the course of a series of dithionite titrations.

Table II: Redox Properties of Uncomplexed MCAD at a Range of pH Values

pH	% semiquinone	E_m (V)	$E_1^{\circ\prime}$ (V)	$E_2^{\circ\prime}$ (V)
6.5	17	–0.083	–0.112	–0.065
7.1	19	–0.114	–0.134	–0.094
7.6	20	–0.145	–0.166	–0.129
8.0	25	–0.168	–0.177	–0.155
8.2	33	–0.180	–0.180	–0.179
8.5	32	–0.189	–0.191	–0.189
9.0	32	–0.210	–0.211	–0.208
10.0	18	–0.263	–0.287	–0.245

Both the nonlinearity of Figure 1 and the varying amounts of blue neutral semiquinone with pH indicate a redox-linked pK_a around a pH value of 8. The likely identity of the species exhibiting this pK_a is an amino acid in close proximity to E-FAD, because E-FAD's spectral properties do not change. A nonlinear regression analysis making this assumption was applied to fit the redox data. The results of this analysis gave pK_a values of 9.2 ± 0.1 for the amino acid in the oxidized active site ($pK_{a,ox}$) and 8.3 ± 0.1 for the same amino acid in the reduced active site ($pK_{a,red}$).

For all of the buffering systems used in the pH study, the ionic strength (μ) was kept at 100 mM to minimize the effects of ions when comparing redox values. However, in a potentiometric titration at pH 9.0 where AMPD (μ = 30 mM) was utilized, the overall formal midpoint potential was found to be –0.169 V. This is a positive shift of 40 mV in comparison to the redox value, –0.210 V, obtained where μ = 100 mM at pH 9.0. The general trend of a negative shift in protein redox values with increasing ionic strength is consistent with the observations of others (Pace & Stankovich, 1986; Einarsdottir et al., 1988; Wang & Stankovich, 1991). The increased ionic strength tends to shield charged residues which are exposed to solvent, and the extent to which these residues affect the redox potential is dependent on the proximity of the redox group to solvent and to the solvent-exposed residues.

Thioether-CoA-Complexed MCAD. Thioether-CoA analogues structurally mimic natural substrate except they lack the thioester carbonyl. Because of this, the α-proton is not acidic enough to become abstracted in MCAD's active site; thus no redox reaction between ligand and MCAD can take place (Frerman et al., 1980; Thorpe et al., 1981). Note the designation of these ligands as substrate analogues and not product analogues because of their fully saturated hydrocarbon chains. The nonreactivity of the thioether-CoAs with MCAD was verified by observing that no E-FAD reduction occurred as monitored by visible absorbance measurements over extended periods of incubation. In addition, no turnover products were observed as monitored by HPLC.

Three different chain length thioether-CoAs were utilized in this study: butyl-, octyl-, and hexadecyl-CoA. Complexation of these ligands to oxidized MCAD leads to the appearance of shoulders at 435 and 480 nm and a red shift of the major absorbance band (Powell et al., 1987). As the carbon chain length of the thioether-CoA is extended, the magnitude of these spectral changes maximizes at the 8-carbon chain length thioether-CoA (Wang & Thorpe, 1991a). The spectral changes that occur upon coulometric reduction of octyl-CoA-complexed MCAD are shown in Figure 2. It is evident that the reduction proceeds in a fashion similar to that of uncomplexed MCAD, i.e., with the generation of a small amount of blue neutral semiquinone. The following formal potential values were measured at pH 7.6:

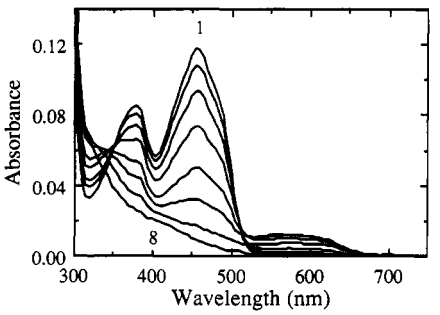

FIGURE 2: Coulometric titration of 7.75 μM MCAD with 124 μM octyl-CoA and 100 μM methyl viologen in 50 mM potassium phosphate buffer, pH 7.6, and at 25 °C (curves 1–8; n = 0.0, 0.11, 0.51, 0.90, 1.30, 1.70, 2.08, and fully reduced, respectively).

$$\text{E-FAD}_{ox} + e^- + H^+ \rightleftharpoons \text{E-FADH}_{1e^-} \quad E_1^{\circ\prime} = -0.167 \text{ V} \tag{6}$$

$$\text{E-FADH}_{1e^-} + e^- + H^+ \rightleftharpoons \text{E-FADH}_{2red} \quad E_2^{\circ\prime} = -0.156 \text{ V} \tag{7}$$

The midpoint potential was –0.162 V and the maximal amount of blue neutral semiquinone stabilized was 29%. The negative shift of the individual formal potential values indicates that octyl-CoA binds more tightly to oxidized MCAD than to either the one-electron or fully-reduced forms. The dissociation constant of octyl-CoA to oxidized MCAD ($K_{d_{ox}}$) is 4 μM (Powell et al., 1987); thus dissociation constants of 4.2 μM and 15 μM were calculated for octyl-CoA complexed to the one-electron ($K_{d_{sq}}$) and fully reduced MCAD ($K_{d_{red}}$), respectively. The $K_{d_{red}}$ calculated in this work does not agree with the value of 1.45 μM determined by Wang and Thorpe (1991b). However, their value was obtained with 1,5-dihydrodeaza-FAD-substituted MCAD.

A summary of the thermodynamic characterization of the thioether-CoA analogues complexed to MCAD at pH 7.6 is given in Table III. The spectral changes that occur upon coulometric/potentiometric reduction for all of the thioether-CoA-complexed MCAD systems were similar. Further, the redox potentials determined for all of the thioether-CoA-complexed MCAD systems were similar, although the amount of semiquinone stabilized increased with increasing carbon chain length of the thioether-CoAs. Note that the experiments with butyl-CoA complexed to MCAD were not done under saturating conditions. Therefore, the reported redox value has been corrected to take this into account.

The midpoint potentials of MCAD complexed to octyl-CoA were measured at three different pH values. The trend observed in the pH dependence of the midpoint potential was essentially identical to that of uncomplexed MCAD. Thus the two-electron reduction of E-FAD is accompanied by two protons. Also, varying amounts of blue neutral semiquinone with pH were observed.

Acetyl-CoA-Complexed MCAD. Acetyl-CoA does contain the thioester carbonyl; however, it is unable to undergo oxidation by MCAD because it lacks a β-carbon. Complexation of these ligands to oxidized MCAD leads to spectral changes similar to those induced by the thioether-CoAs. However, the magnitude of these spectral changes is very small, as also reported by Powell et al. (1987). Further, the spectral changes upon coulometric/potentiometric reduction are essentially the same as those of the uncomplexed MCAD. The measured midpoint potential was –0.144 V and the maximal amount of blue neutral semiquinone stabilized was approximately 20% at pH 7.6. These potentiometric measurements were not done under saturating conditions and the reported redox potential has been corrected to take this into account. The redox measurement for acetyl-CoA-complexed MCAD is isopotential to that of uncomplexed MCAD. Therefore, acetyl-CoA exhibits binding with equal strength to the two redox states of MCAD (see Table III).

DISCUSSION

pH Dependence of Uncomplexed MCAD. The first goal of this research was to fully characterize the pH dependence of the redox potential values of MCAD. Not only do the results from this type of study provide redox measurements, but they also provide information as to the protonation states of the one- and two-electron-reduced species. Information of this type is important, as other workers have shown that this is one of the primary properties of MCAD that is modulated upon natural substrate or product complexation (Mizzer & Thorpe, 1981; Thorpe & Massey, 1983; Gorelick et al., 1985). These results serve as a foundation with which to compare the thermodynamic properties, i.e., binding constants and redox potentials, of natural substrate-, product-, or analogue-complexed MCAD, both in this paper and in work that will follow.

The pH dependence of the midpoint potential of uncomplexed MCAD from pH 6.5 to 10.0 revealed the two-electron transfer is accompanied by two protons. Therefore, both the one- and two-electron-reduced E-FAD species exist in their neutral protonation states. This is quite different from natural substrate- or product-complexed MCAD, where the reduction of the FAD in MCAD is likely accompanied by only one proton. It is known that the one-electron-reduced MCAD complexed to natural substrate or product is in its anionic protonation state (Mizzer & Thorpe, 1981). Thus, it is likely that the two-electron-reduced MCAD is also in its anionic protonation state.

It is of interest is to compare the pH dependence of the midpoint potentials of MCAD with those of two similar dehydrogenase proteins, short-chain acyl-CoA dehydrogenase (SCAD) and glutaryl-CoA dehydrogenase (GCD). SCAD and GCD are both isolated from bacterial sources, *Megasphaera elsdenii* and *Paracoccus denitrificans*, respectively, and they are also tetramers containing one FAD group per monomer (Engel & Massey, 1971; Engel, 1981; Husain & Steenkamp, 1985). However, SCAD and GCD differ from

Table III: Comparison of Measured Thermodynamic Properties of Uncomplexed MCAD to Those of MCAD Bound to Three Different Thioether-CoA Ligands and Acetyl-CoA at pH 7.6

CoA ligand	% semiquinone	E_m (V)	$E_1^{\circ\prime}$ (V)	$E_2^{\circ\prime}$ (V)	$K_{d_{ox}}$[a] (μM)	$K_{d_{sq}}$ (μM)	$K_{d_{red}}$ (μM)
none	20	–0.145	–0.166	–0.129			
octanoyl/*trans*-2-octenoyl		–0.026[b]					
butyl	~20	–0.172			100		790
octyl	29	–0.162	–0.167	–0.156	4	4.2	15
hexadecyl	31	–0.164	–0.167	–0.159	0.04	0.042	0.17
acetyl	~20	–0.144			450		450

[a] Powell et al. (1987). [b] Lenn et al. (1990).

MCAD in the number of protons that are transferred upon the reduction of their FAD groups. Uncomplexed SCAD and GCD have only one proton accompanying their two-electron reduction (Fink et al., 1986; Byron et al., 1990). In addition, no semiquinone is thermodynamically stabilized, unlike MCAD, where up to 33% blue neutral semiquinone is observed. Similar to MCAD, dithionite titrations of SCAD have shown the formation of small amounts of blue neutral semiquinone (Fink et al., 1986). Therefore, the fully-reduced FAD species in uncomplexed SCAD must be of the anionic type. This is a distinct departure from MCAD, where the anionic fully-reduced FAD species appears to exist only upon natural substrate or product complexation.

Similar to MCAD, though, when SCAD is complexed with natural substrate or product the protonation state of the semiquinone is altered from blue neutral to red anionic (Stankovich & Soltysik, 1987; Becker, personal communication). The fully-reduced FAD species for both of these dehydrogenases complexed to natural substrate or product then likely exists in the anionic protonation state. The link for these dehydrogenases as to how structurally this change in protonation occurs and why it is important is not currently understood. Studies have illustrated anionic flavins' higher reactivity and lower redox potentials in comparison to their protonated counterparts (Edmondson & Tollin, 1983; Przysiecki et al., 1985). From this information it has been postulated that the anionic protonation states of MCAD's flavin group explain its increased electron-donating efficiency to ETF (Lehman & Thorpe, 1990).

Camilo et al. (1985) found a pK_a of 8.0 associated with MCAD's activity. This was determined by monitoring the steady-state parameters for the reaction of a substrate analogue, β-(2-furyl)propionyl-CoA, with ETF catalyzed by MCAD over a series of pH values. It was concluded that the identity of the residue displaying this pK_a was the abstractor of the C-2 proton from substrate in MCAD's active site. The identity of this amino acid is now known to be Glu^{376} through the use of mechanistic inhibitors, mutants, and X-ray structure data (Powell & Thorpe, 1988; Bross et al., 1990; Kim et al., 1992). We believe Glu^{376} is the same residue responsible for the redox-linked pK_a found in this work because it exhibits a similar pK_a value and it is near the N-5 position of the flavin ring, an area of high electron density in both the one- and two-electron-reduced species (Kim et al., 1992; Nishimoto, 1985; Edmondson & Tollin, 1983). An amino acid residue affecting the redox potential of a flavoprotein is not a unique discovery. There are other examples of redox-state-dependent changes of protonation of amino acids near a flavin that do not necessarily cause changes in spectral properties (Pace & Stankovich, 1986; Williamson & Edmondson, 1985).

Thioether-CoA-Complexed MCAD. To mimic the thermodynamic effects substrate has upon complexation to MCAD, the thioether-CoA class of analogues was used. Structurally, the thioether-CoAs appeared to be ideal candidates to test the impact of how both varying chain length and the lack of a thioester carbonyl modulate the thermodynamic properties of MCAD. The overall trend for all chain lengths of these analogues was to shift the redox potential in the negative direction, the opposite direction of natural substrate/product couple-complexed MCAD. Currently, it is not known whether the product, substrate, or both act to shift the redox potential in the positive direction. However, electrochemical (Stankovich & Soltysik, 1987; Becker, personal communication) and binding studies (Cummings et al., 1992; Wang & Thorpe, 1991b) suggest product shifts the redox potential in the positive direction.

Also from the measured redox values we calculated that the thioether-CoAs act to stabilize the oxidized form of MCAD versus the one- and two-electron-reduced MCAD. This stabilization is weak, as displayed by the nearly isopotential values of $E_1°'$ and $E_2°'$. Estimation of the binding constants between octanoyl-CoA and both oxidized and reduced forms of MCAD (Cummings et al., 1992; Wang & Thorpe, 1991b) suggests the same type of behavior, i.e., substrate binds equally well to both redox states of MCAD. Therefore, solely from the redox potentials and binding constants it appears that the thioether-CoA class of analogues is affecting MCAD in the same manner as natural substrate-complexed MCAD.

All thioether-CoAs stabilized more blue neutral semiquinone than the uncomplexed enzyme. We emphasize this is the blue neutral semiquinone, not the red anionic semiquinone which natural product and substrate binding appear to stabilize (Mizzer & Thorpe, 1981). Further, the fully-reduced FAD in the thioether-CoA-complexed MCAD is in the neutral protonation state. This is also in contrast to both natural product- and substrate-complexed MCAD that likely stabilize the anionic protonation state. Presently, the link between the protonation of the various redox states of MCAD and the overall shift in redox potential is not understood.

Finally, there appears to be no obvious thermodynamic preference for the 8-carbon chain thioether-CoA by any redox state of MCAD; i.e., the redox potentials are independent of thioether-CoA carbon chain length. Powell et al. (1987) previously arrived at the same conclusion in a binding study between thioether-CoA ligands and oxidized MCAD. No discontinuities or preference was observed for oxidized MCAD to bind an 8-carbon chain length thioether-CoA over the longer chain length thioether-CoAs. This is in direct contrast with electrochemical, activity, and structural studies on MCAD complexed with ligands that have the thioester carbonyl. Electrochemical work by Lenn et al. (1990) using saturating levels of various chain length natural substrate/product couples complexed to MCAD showed that the most positive shift in the redox potential was for the octanoyl/*trans*-2-octenoyl-CoA couple. Activity studies with natural substrates have shown an optimal specificity for the 8-carbon chain length (Hall et al., 1979; Thorpe et al., 1979). Finally, the structural work of Kim et al. (1992) has shown destructive interactions of the binding pocket with natural substrate of carbon chain lengths longer than 12 methylene units to exist, also suggesting an optimal chain length specificity.

Micelle formation was likely during the experiments using hexadecyl-CoA, as the critical micelle concentration is 20 μM (Wang & Thorpe, 1991a). Thus, it might be postulated that this may have influenced the electrochemical measurements and, therefore, disguised a trend for the longer chain length thioether-CoAs. However, all binding curves with this ligand to the oxidized form of MCAD in this work and others (Powell et al., 1987; Wang & Thorpe, 1991a) have shown a one-to-one relationship, and no further spectral changes of the flavin chromophore were observed at higher concentrations ($\sim$200 μM) of ligand. This suggests that the micelle formation is not affecting the electrochemical results.

It is now apparent that the thioester carbonyl is a key structural functionality in modulating the thermodynamic properties of MCAD. The same conclusion is supported kinetically where it was found that the thioether-CoA analogues do not kinetically modulate electron transfer between reduced MCAD and various oxidants (i.e., oxygen

and ferricenium ion) in the same manner as analogues with the thioester carbonyl (i.e., 3-thiaoctanoyl-CoA and 3-oxaoctanoyl-CoA) or natural product (Lehman & Thorpe, 1990; Wang & Thorpe, 1991b). The important function of the thioester carbonyl is to increase the acidity of the α-proton, facilitating its abstraction in the active site of MCAD. Researchers have proposed that the α-proton is further "activated" by a stabilizing interaction between the thioester carbonyl and the protein (Camilo et al., 1985; Engst & Ghisla, 1990; Johnson et al., 1992; Nishina et al., 1992). Recently, structural work has shown that a hydrogen bond exists between the 2-OH of the FAD ribityl chain and the thioester carbonyl, confirming what had been postulated (Kim et al., 1992; Ghisla et al., 1992). With this important interaction missing between the thioether-CoA and the FAD in the binding pocket, it can be speculated that the binding interactions of thioether-CoAs to MCAD are of a different type than those of ligands possessing a thioester carbonyl. The thioether-CoAs bind in a less specific manner, perhaps actually slipping in and out of the binding pocket (Kim, personal communication). However, the overall binding becomes stronger with an increasing number of methylene groups, because the binding interaction between MCAD and the thioether-CoAs is known to be of moderate hydrophobic strength (Powell et al., 1987). Thus, the interaction between the thioester carbonyl and the 2-OH from the FAD ribityl chain must be important for positioning the substrate near the FAD to thermodynamically modulate MCAD.

Acetyl-CoA-Complexed MCAD. Acetyl-CoA structurally appeared to be an ideal candidate to test the impact of chain length on modulating the thermodynamic properties of MCAD. It was found that acetyl-CoA did not affect the midpoint potential of MCAD, and thus, acetyl-CoA binds equally well to the two redox states of MCAD. Like the thioether-CoAs complexed to MCAD, solely from the redox potentials and binding constants it appears that acetyl-CoA is affecting MCAD in the same manner as natural substrate complexed to MCAD.

Also similar to the thioether-CoAs, acetyl-CoA-complexed MCAD stabilizes blue neutral semiquinone. This protonation state of the semiquinone is opposite to what natural substrate or product stabilizes. Therefore, acetyl-CoA must not be exerting the same type of influence upon MCAD's active site as natural substrate or product. The same conclusion is again supported by the finding that acetyl-CoA does not kinetically modulate electron transfer between reduced MCAD and oxygen in the same manner as analogues with the thioester carbonyl (i.e., 3-thiaoctanoyl-CoA and 3-oxaoctanoyl-CoA) or natural product (Wang & Thorpe, 1991b). Therefore, it is now apparent that fatty acyl-CoA chain length is also a key structural functionality in modulating the thermodynamic properties of MCAD.

Results from electrochemical, kinetic, and binding studies confirm the structural importance of fatty acyl-CoA chain length. The electrochemical work of Lenn et al. (1990) illustrated MCAD's preferential positive redox shift was for the 8-carbon chain over the 4-carbon chain substrate/product couple. Also, the kinetic reoxidation studies of Wang and Thorpe (1991b) found that preferential protection from oxygen was provided by MCAD complexed with longer chain length substrate analogues. Finally, the dissociation constants of acetyl-CoA to the two redox states of MCAD (see Table III) are nearly 100 times weaker than that of either natural substrate or product complexed to MCAD (Cummings et al., 1992; Wang & Thorpe, 1991b). This illustrates that the important interaction between the thioester carbonyl and the 2-OH of the FAD ribityl chain is not the sole interaction contributing to the ligand modulation of the thermodynamic properties of MCAD. Thus, the hydrophobic interactions provided by the increased chain length must provide improved binding stability of the ligand to the active site that serves to modulate the thermodynamic properties of MCAD.

The results from our redox studies with MCAD complexed to thioether-CoAs and acetyl-CoA, i.e., the stabilization of the blue neutral semiquinone and the nonthermodynamic specificity for the 8-carbon chain length thioether-CoA, suggest that these analogues are not influencing MCAD's thermodynamic behavior in the same way as natural substrate. Therefore, the carbonyl functionality missing in the thioether-CoAs and the fatty acyl-CoA chain length around 8 carbon units missing from acetyl-CoA are crucial not only for kinetic reasons in the turnover of MCAD with its natural substrate but also for thermodynamic reasons. With the knowledge gained from this study and through the use of other analogues in future work, we hope to fully understand the structural functionalities on the substrate and product ligands and their corresponding interactions with the active site which are responsible for the modulation of MCAD's thermodynamic behavior in this highly regulated reaction.

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