Product Binding Modulates the Thermodynamic Properties of a *Megasphaera elsdenii* Short-Chain Acyl-CoA Dehydrogenase Active-Site Mutant†

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ABSTRACT: Previous work has shown that the redox properties of *Megasphaera elsdenii* short-chain acyl-CoA dehydrogenase (SCAD) are specifically modulated upon the binding of the substrate/product couple, allowing the reaction to proceed thermodynamically [Stankovich, M. T., & Soltysik, S. (1987) *Biochemistry 26,* 2627–2632]. The focus of this study on the Glu^{367}Gln SCAD mutant protein is to gain an understanding of this phenomenon. The active-site mutant Glu^{367}Gln SCAD inactivates the reductive and oxidative pathways and allows the effects of substrate (butyryl-CoA) and product (crotonyl-CoA) binding on the redox properties of the Glu^{367}Gln SCAD mutant protein to be determined separately. Red anionic semiquinone was found to be thermodynamically stabilized in coulometric/potentiometric reductions of both butyryl-CoA- and crotonyl-CoA-complexed Glu^{367}Gln SCAD. Reduction potential measurements showed that butyryl-CoA binding has little effect on the reduction potential of Glu^{367}Gln SCAD. Crotonyl-CoA complexation, however, shifted the reduction potential of the Glu^{367}Gln SCAD mutant protein by 30 mV in the positive direction. This modulation is similar to the 60-mV positive shift observed in native *M. elsdenii* SCAD upon complexation with the substrate/product couple [Stankovich, M. T., & Soltysik, S. (1987) *Biochemistry 26,* 2627–2632]. Thus, product binding and not substrate binding, thermodynamically regulates *M. elsdenii* SCAD. We propose that this observation is best explained by assuming that the product resembles an intermediate in the catalytic mechanism that is responsible for facilitating isopotential electron transfer from the substrate to the enzyme.

Short-chain acyl-CoA dehydrogenase (SCAD[1]) from the anaerobe *Megasphaera elsdenii* catalyzes the reversible 2,3-dehydrogenation of acyl-CoA thioesters to *trans*-2-enoyl-CoA thioester products. A similar two electron oxidation is also catalyzed by the mammalian acyl-CoA dehydrogenases as part of the mitochondrial fatty acid β-oxidation cycle. Because of its properties that are similar to those of the mammalian protein medium-chain acyl-CoA dehydrogenase (MCAD), *M. elsdenii* SCAD has been used as a model for the study of the mammalian acyl-CoA dehydrogenases.

Previously, it was shown that the redox properties of both *M. elsdenii* SCAD and mammalian MCAD are specifically modulated upon the binding of the substrate/product couple, allowing isopotential electron transfer from substrate to enzyme. The reduction potential values of the flavin cofactor in uncomplexed *M. elsdenii* SCAD (E_m = –0.079 V, pH 7.0) (Fink et al., 1986) and mammalian MCAD (E_m = –0.136 V, pH 7.6) (Lenn et al., 1990) become more positive upon complexation with their respective substrate/product couples. Complexed *M. elsdenii* SCAD shows a shift of 60 mV to a reduction potential value of –0.019 V (pH 7.0) (Stankovich & Soltysik, 1987), while complexed mammalian MCAD moves 110 mV to a reduction potential value of –0.026 V (pH 7.6) (Lenn et al., 1990). These electrochemical observations demonstrate that *M. elsdenii* SCAD and mammalian MCAD are regulated by substrate/product binding but the mechanism of this regulation has not been clarified.

The substrate turnover in SCAD and MCAD makes it difficult to directly distinguish between the effects of substrate and product binding on the observed thermodynamic regulation of SCAD and MCAD. Some structural portions of the ligand molecules, however, have been implicated in the thermodynamic regulation of SCAD and MCAD. This was accomplished by determining the effects of various substrate analogues on the redox properties of SCAD and MCAD. Since the analogues did not turn over with the enzyme, studies with only one species of ligand present could be conducted. Upon complexation of SCAD and MCAD with thioether analogues that lack the thioester carbonyl, 0–10- and 17–27-mV negative shifts in the redox potentials of SCAD and MCAD were observed, respectively (C. Pace and M. T. Stankovich, personal communication (1993); Johnson & Stankovich, 1993). These results implicated the thioester carbonyl as an important structural feature for the thermodynamic regulation of SCAD and MCAD. This was confirmed using analogues that retained the thioester carbonyl (e.g., 2-azaacyl-CoA ligands), through which large modulation of the redox potentials was observed in both SCAD and MCAD upon complexation (C. Pace and M. T. Stankovich, personal communication (1993); Johnson, 1993). In MCAD, the positive shift (70 mV) in the redox potential was greatest when it was complexed to 2-azaoctanoyl-CoA, which has the optimal chain length for MCAD (Johnson, 1993). As a result of these studies, it was concluded that the thioester carbonyl and the ligand chain length are essential structural features required for the thermodynamic regulation of SCAD and MCAD (C. Pace and M. T. Stankovich, personal communication (1993); Johnson & Stankovich, 1993). Both butyryl-CoA (BCoA) and crotonyl-CoA (CCoA) retain these important structural features.

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[1] Abbreviations: BCoA, butyryl-CoA; CCoA, crotonyl-CoA; CD, circular dichroism; $E_1°'$, formal potential of the first electron transfer; $E_2°'$, formal potential of the second electron transfer; E_m, midpoint potential; E-FAD, enzyme-bound FAD; FAD, flavin adenine dinucleotide; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase.

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To distinguish between the effects of substrate and product binding, we have characterized the thermodynamic properties of a site-directed $Glu^{367}Gln$ SCAD mutant protein complexed to substrate (BCoA) and product (CCoA). Previously, it was shown that the $Glu^{367}Gln$ SCAD mutant protein has minimal reductive and oxidative pathway activity with BCoA and CCoA, respectively (Becker et al., 1993). In this report, we demonstrate that product binding can regulate the thermodynamic properties of a mutant protein. Furthermore, we suggest that a resonance structure of product resembles an intermediate in the catalytic mechanism of native *M. elsdenii* SCAD which is responsible for the positive shift in the reduction potential of native *M. elsdenii* SCAD upon complexation with the substrate/product couple.

MATERIALS AND METHODS

Materials. The recombinant *M. elsdenii* wild-type and $Glu^{367}Gln$ mutant SCAD proteins were expressed from the *Escherichia coli* strain K19 and purified as previously described (Becker et al., 1993), except for the addition of the thiopropyl-Sepharose column step to remove the bound CoASH according to the method of Williamson and Engel (1982). CoASH-free SCAD protein was used in all of the spectroelectrochemical experiments. Both recombinant proteins were stored at −20 °C in 0.1 M potassium phosphate buffer at pH 7.0. The thiopropyl-Sepharose 6B and the butyryl-CoA and crotonyl-CoA ligands were purchased from Sigma Chemical Co. The following dyes were used: methyl viologen (Sigma), ferrocyanide (Fisher), pyocyanine made by photooxidation of phenazine methosulfate (Sigma), and indigo disulfonate (Aldrich). All experiments utilized glass-distilled water.

Methods. The concentrations of the oxidized and CoASH-free proteins were measured spectrophotometrically with the extinction coefficients determined by Engel's method (Williamson & Engel, 1984). The extinction coefficient measurements were performed at least four times to give an average value and a standard deviation. Extinction coefficients of 14.2 ± 0.4 cm^{-1} mM^{-1} at 451 nm and 13.9 ± 0.3 cm^{-1} mM^{-1} at 453 nm were determined for the CoASH-free recombinant *M. elsdenii* wild-type and $Glu^{367}Gln$ mutant SCAD proteins, respectively.

Potentiometric and coulometric measurements were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). The visible spectra in each experiment were obtained and stored on an Apple 2e-interfaced Cary 219 spectrophotometer Experimental conditions were 25 °C and pH 6.95–7.0 in 0.1 M potassium phosphate buffer. Methyl viologen (0.1 mM) was used as the mediator dye in both the potentiometric and coulometric titrations. The indicator dyes used in the potentiometric titrations were pyocyanine (E_m = −0.030 V, pH 7.0) (5–12 μM) and indigo disulfonate (E_m = −0.096 V, pH 7.0) (1–2 μM). E-FAD concentrations in the spectroelectrochemical experiments ranged from 10 to 28 μM. BCoA and CCoA concentrations were in 15–20 molar excess over that of E-FAD in the experiments with ligand-bound SCAD protein to ensure saturation. Using the dissociation constants determined in this work for BCoA (6.2 μM) and CCoA (2.2 μM) with the $Glu^{367}Gln$ SCAD protein, the amounts of enzyme bound to BCoA and CCoA during the experiments were calculated at lower limits of 96% and 98%, respectively.

The reduction potentials reported were determined by potentiometric measurements in the reductive direction. A test for the reversibility of the CCoA-complexed $Glu^{367}Gln$ SCAD protein system was performed by measuring potentials in the oxidative direction using ferrocyanide (0.1 mM) as the mediator dye. Equilibrium of the system in all potentiometric measurements was considered to be obtained when the ΔE was less than 1 mV/10 min; this was typically around 2 h. All potential values are reported versus the standard hydrogen electrode.

Table 1: Extinction Coefficients for Oxidized and Fully-Reduced $Glu^{367}Gln$ SCAD Complexed to Butyryl-CoA and Crotonyl-CoA

	wavelength and redox state					
	butyryl-CoA			crotonyl-CoA		
λ (nm)	457	387	570	459	387	570
oxidized (M^{-1} cm^{-1})	13400	9900	70	13500	9100	80
reduced (M^{-1} cm^{-1})	1200	3900	200	1600	3400	2900

Calculations. Quantitation of the four E-FAD redox species present during the course of the spectroelectrochemical titration was calculated by simultaneously solving four equations. The first equation was a mass balance equation relating the concentration of the four redox species to the total amount of SCAD in the experiment. The other three equations utilized Beer's law by relating the absorbance at three different wavelengths to the extinction coefficient of the four redox species and their corresponding concentrations in the experiment. It was assumed that the extinction coefficients for the red anionic semiquinone (19 000, 5710, and 300 cm^{-1} M^{-1} at 387, 450, and 570 nm, respectively) and the blue neutral semiquinone (4800, 5000, and 5600 cm^{-1} M^{-1} at 387, 450, and 570 nm, respectively) remained the same throughout all titrations. The extinction coefficient values for the red anionic semiquinone at 387 and 450 nm (Pace, personal communication (1993)) and the blue neutral semiquinone at 570 nm (Fink et al., 1986) have been determined for *M. elsdenii* SCAD. The extinction coefficient values at 570 nm for the red anionic semiquinone and at 387 and 450 nm for the blue neutral semiquinone were taken from MCAD (Gorelick et al., 1985; Lehman & Thorpe, 1990). The extinction coefficients for the oxidized and fully-reduced $Glu^{367}Gln$ SCAD mutant protein complexed to BCoA and CCoA were derived from spectral titrations in this work. The values used for data analysis are given in Table 1. Before quantitation of the 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:

$$E = E_m + (0.059/n) \log([ox]/[red]) \quad (1)$$

where E is the measured equilibrium potential at each point in the titration and n is the number of electrons. 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. The typical error in the reported midpoint potential values is ±1–3 mV. All midpoint potential value determinations exhibited Nernstian behavior as indicated by their n values.

The relationship between individual formal potential values ($E_1°'$ and $E_2°'$) and the midpoint potential value used to arrive at the maximal amount of semiquinone stabilized thermodynamically is the following:

$$E_1°' - E_2°' = 0.12 \log[2M/(1 - M)]$$

where M is the maximum fraction of semiquinone formed

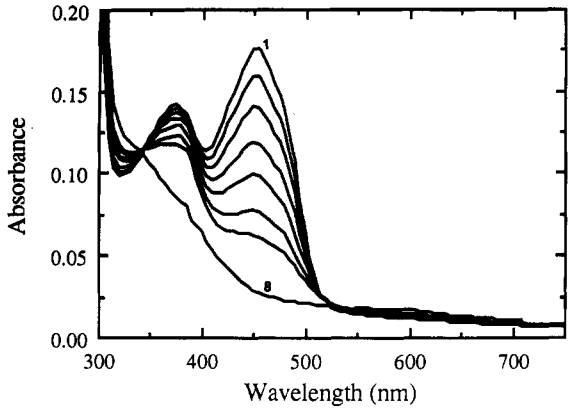

FIGURE 1: Coulometric reduction of uncomplexed Gln[367]Glu SCAD (12.6 μM) with 100 μM methyl viologen in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C (curves 1–8; n = 0.0, 0.27, 0.57, 0.91, 1.22, 1.57, 1.86, and fully reduced, respectively).

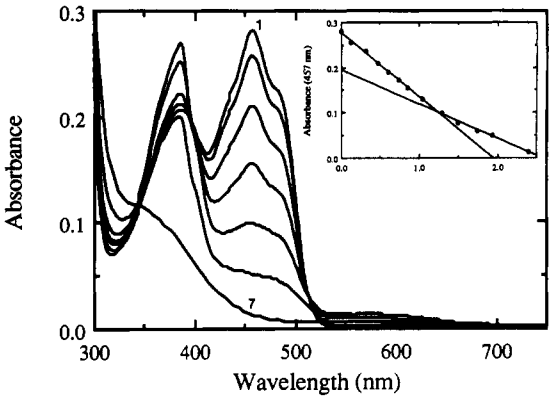

FIGURE 2: Coulometric reduction of butyryl-CoA-complexed Gln[367]-Glu SCAD (20.9 μM) with 100 μM methyl viologen in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C (curves 1–7; n = 0.0, 0.12, 0.32, 0.48, 0.89, 1.34, 2.0, and fully reduced, respectively). The inset is a plot of A_{457} versus the charge equivalents added.

(Clark, 1960; Einarsdottir et al., 1988). The relationship between binding constants and midpoint potentials under saturating conditions is the following equation:

$$E_{bound} = E_m - 0.056/n \log K_{d2}/K_{d1}$$

which has been reported previously (Clark, 1960; Einarsdottir et al., 1988).

The dissociation constants of butyryl-CoA and crotonyl-CoA to oxidized Glu[367]Gln SCAD at pH 7.0 were determined by a spectral titration, which measured the resulting perturbation of the enzyme absorbance spectrum. Data were analyzed by a computerized nonlinear regression method (Einarsdottir et al., 1989).

RESULTS

Uncomplexed Wild-Type and Glu[367]Gln Mutant SCAD. A coulometric titration of the Glu[367]Gln mutant showed that less than 10% was stabilized as the blue neutral semiquinone species (Figure 1). This is similar to the behavior of native *M. elsdenii* SCAD during a coulometric reduction, which also showed less than 10% blue neutral semiquinone and no red anionic semiquinone (Fink et al., 1986). The visible spectra of the wild-type and Glu[367]Gln mutant SCAD are similar during the coulometric titration, except for the red shift and the slightly higher resolution of the flavin maximum (453 nm) of the Glu[367]Gln SCAD mutant protein (Becker et al., 1993).

A reduction potential value of –0.075 V was determined for the recombinant wild-type *M. elsdenii* SCAD from potentiometric measurements. The difference in this value from that reported for native *M. elsdenii* SCAD (–0.079 V, pH 7.0) (Fink et al., 1986) is not significant and is probably due to the refinement in our experimental methods. The reduction potential value for the Glu[367]Gln mutant SCAD is –0.064 V (Table 2), which is 11 mV more positive than the reduction potential value for wild-type *M. elsdenii* SCAD. Less than 5% of the enzyme is in the blue neutral semiquinone form, and no red anionic semiquinone is evident during the potentiometric experiments for the wild-type and the Glu[367]Gln mutant *M. elsdenii* SCAD proteins. Thus, the individual formal potential values of $E_1°'$ and $E_2°'$ are separated by similar amounts in the two proteins.

BCoA-Complexed Glu[367]Gln SCAD. Complexation of BCoA to the Glu[367]Gln SCAD mutant protein leads to the appearance of shoulders and a red shift of the main flavin absorbance band from 453 to 457 nm. Coulometric reduction of the BCoA-bound Glu[367]Gln mutant demonstrated the maximum stabilization of close to 57% red anionic semiquinone, while only 7% blue neutral semiquinone was stabilized (Figure 2). The increase in absorbance at 370 nm indicates formation of the red anionic semiquinone, while the increased absorbance at 580 nm indicates the presence of blue neutral semiquinone. Thus, the main difference in the coulometric titration between uncomplexed and BCoA-complexed Glu[367]-Gln mutant SCAD proteins appears to be the stabilization of red anionic semiquinone, while the amount of blue neutral semiquinone is unchanged by complexation of the Glu[367]Gln mutant SCAD protein with BCoA. No charge-transfer species was detected during the experiment as the absorbance at 570 nm reached a maximum midway through the titration, indicative of blue neutral semiquinone.

Table 2: Comparison of Measured Thermodynamic Properties of Uncomplexed Glu[367]Gln SCAD to Those of Glu[367]Gln SCAD Bound to Butyryl-CoA and Crotonyl-CoA Ligands

ligand	% semi-quinone	E_m (V)	$E_1°'$ (V)[a]	$E_2°'$ (V)[a]	K_{dox} (μM)	K_{dsq} (μM)	K_{dred} (μM)
none	≤5	–0.064	≤–0.123	≥–0.005			
butyryl-CoA	34	–0.073	–0.072	–0.073	6.2	≤0.78	12.5
crotonyl-CoA	36	–0.034	–0.031	–0.037	2.2	≤0.05	0.19

[a] $E_1°'$ and $E_2°'$ were calculated from the measured reduction potentials and percent semiquinone species stabilized, as previously described (Clark, 1960; Einarsdottir et al., 1988).

The reduction potential value of the BCoA-complexed Glu[367]Gln SCAD mutant protein is –0.073 V (Table 2). This reduction potential is 11 mV more negative than the reduction potential for uncomplexed Glu[367]Gln SCAD. During the reduction potentiometric experiments, approximately 34% semiquinone was stabilized, indicating that the individual formal potentials were nearly isopotential (Table 2). A Nernst plot gave a 31-mV slope for the potentiometric titrations of BCoA-complexed Glu[367]Gln SCAD, which is close to the theoretical value of 30 mV for a two-electron transfer. The measured dissociation constant of BCoA to oxidized Glu[367]-Gln SCAD is 6.2 ± 0.67 μM. This measured value differs from the estimated dissociation constant of 1 μM for BCoA to oxidized native *M. elsdenii* SCAD (Fink, 1985). Possibly, the carboxylate group of the Glu[367] contributes to the strength of ligand binding, although it may be minimal. A study on the human Glu[367]Gln MCAD mutant protein with 4-aminobenzoyl-CoA showed that its binding strength was similar to that of the human wild-type MCAD protein (Ghisla et al.,

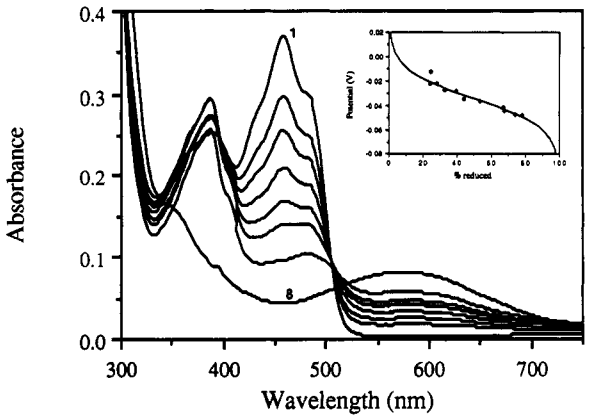

FIGURE 3: Coulometric reduction of crotonyl-CoA-complexed Gln^{367}-Glu SCAD (27.8 μM) with 100 μM methyl viologen in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C (curves 1–8; n = 0.0, 0.46, 0.70, 0.93, 1.17, 1.35, 1.71, and fully reduced, respectively). The inset is a best fit of the reduction potential data from potentiometric titrations of crotonyl-CoA-complexed Gln^{367}Glu SCAD.

1992). According to the measured reduction potential value, the strength of BCoA binding is 2-fold stronger to the oxidized form than to the reduced form of Glu^{367}Gln SCAD. From the calculated K_{dsq}, the greatest stabilization of BCoA binding is to the semiquinone form of Glu^{367}Gln SCAD.

CCoA-Complexed Glu^{367}Gln SCAD. The complexation of CCoA to Glu^{367}Gln SCAD is evident by the appearance of shoulders and a red shift of the flavin maximum to 459 nm. The spectral changes that occurred upon the coulometric reduction of CCoA-complexed Glu^{367}Gln SCAD are shown in Figure 3. During the titration, red anionic semiquinone (387 nm) and a protein to ligand charge-transfer band (570 nm) were formed. The amount of blue neutral semiquinone maximally stabilized is similar to that stabilized during the coulometric reduction of BCoA-complexed Glu^{367}Gln SCAD, while the maximum amount of red anionic semiquinone is less (≈42%).

A reduction potential value of –0.034 V (see Table 2 and the inset to Figure 3) was obtained for the CCoA-complexed Glu^{367}Gln SCAD mutant protein. A slope of 29 mV was obtained in the Nernst plot of E versus log([ox]/[red]), indicating that CCoA-complexed Glu^{367}Gln SCAD exhibited a two-electron transfer during the potentiometric titrations. The reduction potential value is 30 mV more positive than uncomplexed Glu^{367}Gln SCAD. About 36% semiquinone was formed during the potentiometric experiments of the CCoA-complexed Glu^{367}Gln SCAD. As a result, the individual formal potential values for the CCoA-complexed Glu^{367}Gln SCAD protein are almost isopotential, similar to BCoA-complexed Glu^{367}Gln SCAD. A potentiometric experiment of the CCoA-complexed Glu^{367}Gln SCAD in the oxidative direction showed that the midpoint potential value was –0.019 V and that a similar amount of semiquinone was stabilized. This demonstrates that the system is reasonably reversible, as the CCoA-complexed Glu^{367}Gln SCAD protein behaves similarly in both the reductive and oxidative directions.

The dissociation constant for CCoA with Glu^{367}Gln SCAD is 2.2 ± 0.85 μM. The calculated K_{dred} is 0.2 μM, indicating that the stabilization of the reduced form of Glu^{367}Gln SCAD upon product binding is almost 12-fold. Similar to BCoA-complexed Glu^{367}Gln SCAD, the semiquinone species of Glu^{367}Gln SCAD is stabilized the greatest (>40-fold) by CCoA binding.

DISCUSSION

The structural integrity of a mutant protein is normally in doubt until the X-ray crystallographic structure is determined. The X-ray crystallographic structure of the Glu^{367}Gln SCAD mutant protein has not been determined, but its completion is expected (J.-J. P. Kim, personal communication (1993)). In the meantime, alternative methods (e.g., UV–vis and UV–CD spectra, reduction potential, and binding constant measurements) have been used to test the structural integrity of the Glu^{367}Gln SCAD mutant protein (Becker et al., 1993). For example, reduction potential and dissociation constant measurements of the Glu^{367}Gln mutant and native *M. elsdenii* SCAD proteins complexed to 2-azabutyryl-CoA have shown that they behave similarly (C. Pace and M. T. Stankovich, personal communication (1993)). Upon complexation with 2-azabutyryl-CoA, the reduction potentials of both Glu^{367}-Gln and native *M. elsdenii* SCAD shift negatively. Since the behavior is similar between Glu^{367}Gln and native *M. elsdenii* SCAD with the 2-azabutyryl-CoA analogue, it is reasonable to expect that the thermodynamic modulation observed in Glu^{367}Gln SCAD by BCoA and CCoA binding would also occur in native *M. elsdenii* SCAD. Therefore, the thermodynamic characterization of the Glu^{367}Gln SCAD mutant protein has significant relevance to the molecular mechanism of regulation that occurs during catalysis in native *M. elsdenii* SCAD.

It is unclear why the uncomplexed Glu^{367}Gln SCAD mutant protein has a reduction potential value that is 11 mV more positive than that of wild-type *M. elsdenii* SCAD. The reduction potential value of the recombinant human Glu^{376}-Gln MCAD protein has been determined and it is also more positive than the respective recombinant human wild-type MCAD (G. Mancini-Samuelson, personal communication (1994)). The carboxylate group of Glu^{367} is over 7 Å away from the diazadiene portion of the flavin in SCAD (J.-J. P. Kim, personal communication (1993)). Despite this distance, an explanation for the more positive reduction potential of the Glu^{367}Gln SCAD mutant could be the substitution of a negative charge by a neutral residue at position 367 in the flavin environment.

Two similar effects of BCoA and CCoA binding on the redox properties of Glu^{367}Gln SCAD are evident from the spectroelectrochemical data. First, red anionic semiquinone is stabilized during the coulometric/potentiometric reductions of both BCoA- and CCoA-complexed Glu^{367}Gln SCAD, while the maximum amount of blue neutral semiquinone is virtually unchanged compared to uncomplexed Glu^{367}Gln SCAD. The stabilization of red anionic semiquinone by both BCoA and CCoA is not unexpected as both substrate and product binding have been reported to stabilize red anionic semiquinone in mammalian MCAD (Mizzer & Thorpe, 1981). Secondly, complexation of both BCoA and CCoA to Glu^{367}Gln SCAD moves the $E_1{}^{o\prime}$ formal potential positively (≈50 and 90 mV, respectively).

The common structural features between substrate and product are the thioester carbonyl and their chain lengths. As noted earlier, these structural features are needed for the interactions with the protein necessary to facilitate the thermodynamic regulation of SCAD and MCAD (C. Pace and M. T. Stankovich, personal communication (1993); Johnson & Stankovich, 1993). Interestingly, these features are also important for the kinetic modulation of electron transfer between reduced MCAD and oxygen (Wang & Thorpe, 1991). Another important function of the thioester carbonyl is to increase the acidity of the substrate α-proton

(Ghisla et al., 1992). Recent structural work with MCAD has identified some of the interactions between the thioester carbonyl and the protein that are responsible for these observed functions of the thioester carbonyl. Namely, the thioester carbonyl is hydrogen bonded to the 2-OH of the FAD ribityl chain and the main-chain amide nitrogen of Glu^{376} when substrate is complexed to MCAD (Kim et al., 1993; Ghisla et al., 1992). A similar interaction is expected in substrate-complexed native *M. elsdenii* SCAD (J.-J. P. Kim, 1993, personal communication). This is based on the 2.5-Å X-ray crystallographic structure of native *M. elsdenii* SCAD and the homology between SCAD and MCAD (36% identical residues), especially in the FAD binding domain (Becker et al., 1993). The homology in the structure and the amino acid sequence between SCAD and MCAD aided in the identification of the Glu^{367} catalytic base in SCAD, which is matched by the Glu^{376} catalytic base in MCAD. The hydrogen-bonding interaction of the protein with the thioester carbonyl is most likely responsible for the stabilization of red anionic semiquinone and the movement of the $E_1°'$ to a more positive value during the potentiometric measurements of BCoA- and CCoA-complexed Glu^{367}Gln SCAD.

The reduction potential (E_m) of Glu^{367}Gln SCAD is shifted 9 mV negative upon BCoA binding. This is in the opposite direction of the shift expected when the substrate/product couple is complexed to *M. elsdenii* SCAD. Small negative shifts in the reduction potential values for native *M. elsdenii SCAD* and mammalian MCAD were also observed upon complexation with thioether-CoA substrate analogues of varying lengths (C. Pace and M. T. Stankovich, personal communication (1993); Johnson & Stankovich, 1993). In MCAD, it was suggested that the thioether-CoA substrate analogues affected the reduction potential of MCAD in the same manner as substrate-complexed MCAD (Johnson & Stankovich, 1993). Consequently, the negative shift in the reduction potential of BCoA-complexed Glu^{367}Gln SCAD is not unexpected.

The 30-mV positive shift in the reduction potential (E_m) of the CCoA-complexed Glu^{367}Gln SCAD mutant protein is in agreement with previous studies on native *M. elsdenii* SCAD and mammalian MCAD. Stankovich and Soltysik (1987) found that decreased ratios of BCoA to CCoA complexed with native *M. elsdenii* SCAD increasingly shifted the measured reduction potential positively. In mammalian MCAD, binding studies indicated that product shifts the reduction potential in the positive direction: K_{dox} = 90 nM and K_{dred} = 0.013 nM (Cummings et al., 1992). Thus, the positive shift in the reduction potential of Glu^{367}Gln SCAD upon complexation with CCoA confirms that product binding modulates the reduction potential of native *M. elsdenii* SCAD. This regulation of the reduction potential by product complexation most likely occurs in MCAD as well.

Molecular insights into the different BCoA and CCoA complexation effects on the reduction potential of Glu^{367}Gln SCAD have been provided by a recent resonance Raman study on mammalian MCAD. An appreciable contribution from an ionic resonance structure for *trans*-2-octenoyl-CoA (product) exhibiting a partial negative charge on the thioester oxygen and a partial positive charge on carbon three was shown for reduced MCAD complexed to *trans*-2-octenoyl-CoA (Figure 4) (Nishina et al., 1992). This protein-stabilized ionic resonance structure is only possible with unsaturated product and not with substrate. Consequently, the same resonance structure formation may be occurring in CCoA-complexed Glu^{367}Gln SCAD. The partial positive charge developing

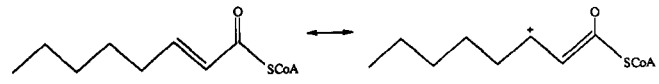

FIGURE 4: Protein-stabilized ionic resonance structure of *trans*-2-octenoyl-CoA complexed to MCAD, from Nishina et al. (1992).

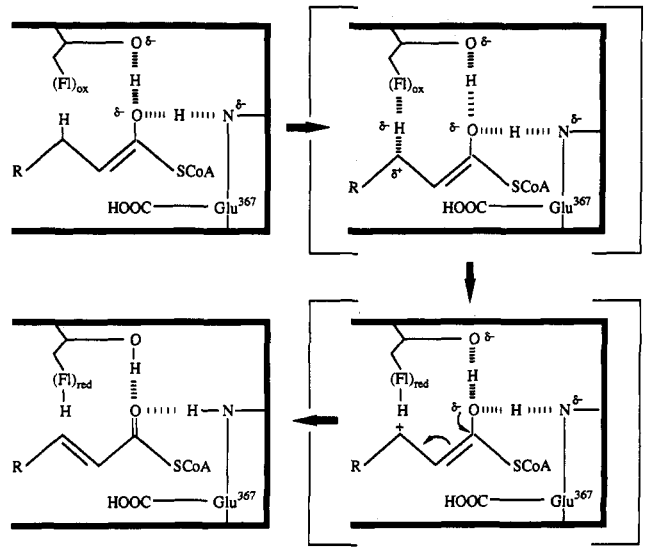

FIGURE 5: Proposed formation of the transition state in the *M. elsdenii* SCAD active site that modulates the redox potential of the flavin cofactor during the electron-transfer step.

around carbon three of the CCoA molecule could then influence the flavin electronic environment, causing its reduction potential to move in the positive direction as has been postulated for MCAD complexed to 2-azaacyl-CoA analogues (Johnson, 1993). X-ray crystallographic work with substrate-complexed MCAD has shown that the carbon three position of the *trans*-2-octenoyl-CoA is directly over the diazadiene portion of the flavin (Kim et al., 1993).

The hypothesis that product and not substrate binding modulates *M. elsdenii* SCAD seems contrary to its catalytic mechanism. A plausible explanation is that the product mimics some intermediate that occurs during catalysis. The formation of this intermediate would have to occur after or almost simultaneously with the initial α-proton abstraction from substrate, since substrate does not modulate the reduction potential of Glu^{367}Gln SCAD. This α-proton abstraction step is independent of the β-hydride transfer and would lead to the formation of an enolic intermediate, according to the proposed mechanism of Gerlt and Gassman (1993). The generation of an enolic intermediate with a partial negative charge on the thioester oxygen would require a strong hydrogen-bond interaction with the thioester carbonyl (e.g., hydrogen bonds with the 2-OH of the FAD ribityl chain and the main-chain amide nitrogen of the Glu^{367}), which is also needed to lower the p*K*a of the substrate α-proton (Gerlt & Gassman, 1993). From this enolic intermediate, a transition state could then form that would be characterized by a positive charge developing on carbon three (Figure 5). This charge could influence the redox potential of the flavin cofactor, eventually leading to the isopotential transfer of the substrate β-hydride to the flavin. Perhaps it is this intermediate which, like product, stabilizes a partial positive charge over the flavin ring and is the species responsible for the positive shift (60 mV) in the reduction potential of *M. elsdenii* SCAD when bound to the substrate/product couple. It is important to note that the thermodynamic modulation of the flavin is only required for the electron-transfer step but not the α-proton abstraction. Thus, the transition state that influences the redox potential

of the flavin does not facilitate its own formation, but rather is only a result of the preceding α-proton abstraction step.

When we consider the formation of an enolic intermediate according to the mechanism described by Gerlt and Gassman (1993), an explanation for the cause of red anionic semiquinone in both substrate- and product-complexed $Glu^{367}Gln$ SCAD becomes evident. It seems that in both substrate- and product-bound $Glu^{367}Gln$ SCAD a strong hydrogen bond would form between the protein and the thioester carbonyl, generating a partial positive charge on carbon one in substrate, and due to its conjugated system the partial positive charge in product would extend to carbon three. The generation of a partial positive charge in the active sites of both substrate- and product-complexed $Glu^{367}Gln$ SCAD would perhaps shift the $E_1°'$ formal potential positive and stabilize red anionic semiquinone. The extension of the partial positive charge onto carbon three in product, however, places the partial positive charge farther out over the flavin ring. Thus, product is able to move the reduction potential of $Glu^{367}Gln$ SCAD positively, while substrate is limited to only moving the $E_1°'$ formal potential positively. The X-ray crystallographic structure of $Glu^{367}Gln$ SCAD and further investigation into the molecular mechanism of the thermodynamic regulation in *M. elsdenii* SCAD by utilizing additional substrate/product analogues complexed to the $Glu^{367}Gln$ SCAD mutant protein are needed to test these postulates.

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