Isothermal Titration Microcalorimetric Studies for the Binding of Octenoyl-CoA to Medium Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: We investigated the binding of octenoyl-CoA to pig kidney medium chain acyl-CoA dehydrogenase (MCAD) by isothermal titration microcalorimetry under a variety of experimental conditions. At 25 °C in 50 mM phosphate buffer at pH 7.6 (ionic strength of 175 mM), the binding is characterized by the stoichiometry (n) of 0.89 mole of octenoyl-CoA/(mole of MCAD subunit), $\Delta G° = -8.75$ kcal/mol, $\Delta H° = -10.3$ kcal/mol, and $\Delta S° = -5.3$ cal mol^{-1} K^{-1}, suggesting that formation of MCAD−octenoyl-CoA is enthalpically driven. By employing buffers with various ionization enthalpies, we discerned that formation of the MCAD−octenoyl-CoA complex, at pH 7.6, accompanies abstraction (consumption) of 0.52 ± 0.15 proton/(MCAD subunit) from the buffer media. We studied the effects of pH, ionic strength, and temperature on the thermodynamics of MCAD−octenoyl-CoA interaction. Whereas the ionic strength does not significantly influence the above interaction, the pH of the buffer media exhibits a pronounced effect. The pH dependence of the association constant of MCAD + octenoyl-CoA ⇄ MCAD−octenoyl-CoA yields a pK_a for the free enzyme of 6.2. Among thermodynamic parameters, whereas $\Delta G°$ remains invariant as a function of temperature, $\Delta H°$ and $\Delta S°$ both decrease with an increase in temperature. At temperatures of <25 °C, $\Delta G°$ is dominated by favorable entropic contributions. As the temperature increases, the entropic contributions progressively decrease, attain a value of zero at 23.8 °C, and then becomes unfavorable. During this transition, the enthalpic contributions become progressively favorable, resulting in an enthalpy−entropy compensation. The temperature dependence of $\Delta H°$ yields the heat capacity change (ΔC_p^0) of -0.37 ± 0.05 kcal mol^{-1} K^{-1}, attesting to the fact that the binding of octenoyl-CoA to MCAD is primarily dominated by the hydrophobic forces. The thermodynamic data presented herein are rationalized in light of structural−functional relationships in MCAD catalysis.

Medium chain acyl-CoA dehydrogenase (MCAD)[1] catalyzes the oxidation of a variety of acyl-CoA substrates into their enoyl-CoA products via two consecutive steps [for reviews, see Beinert (1963), Engel (1990), and Kim and Thorpe (1995); eq 1].

$$\text{E−FAD} + \text{acyl-CoA} \rightleftharpoons \text{E−FADH}_2\text{−enoyl-CoA} \quad (1A)$$

$$\text{E−FADH}_2\text{−enoyl-CoA} + \text{ETF−FAD} \rightleftharpoons \text{E−FAD−enoyl-CoA} + \text{ETF−FADH}_2 \quad (1B)$$

The first step (eq 1A), conventionally referred to as the "reductive half-reaction", involves the α−β dehydrogenation of acyl-CoA substrates, concomitant with reduction of the enzyme-bound FAD to $FADH_2$. This step produces a remarkably stable form of MCAD−$FADH_2$−enoyl-CoA as an intermediary complex, which is subsequently oxidized by "organic" electron acceptors, such as electron-transferring flavoprotein (ETF) or ferrocenium hexafluorophosphate ($FcPF_6$). The latter step (eq 1B) is referred to as the "oxidative half-reaction" (Beinert, 1963; Engel, 1990; Thorpe & Kim, 1995; Kumar & Srivastava, 1994, 1995).

By utilizing indolepropionyl-CoA (IPCoA) as a chromogenic substrate, as well as octanoyl-CoA as a physiological substrate, we provided evidence that the MCAD−$FADH_2$−enoyl-CoA complex predominates either as a Michaelis complex (in which the electronic structures of both flavin and enoyl-CoA species are the same as in aqueous solution) and a charge-transfer (CT) complex (in which the electronic structures of both flavin and enoyl-CoAs are perturbed; Johnson & Srivastava, 1993; Johnson et al., 1994; Kumar & Srivastava, 1994; Peterson et al., 1995). During the reductive half-reaction of the enzyme, the MCAD−$FADH_2$−enoyl-CoA CT complex is first formed, which is slowly converted to the MCAD−$FADH_2$−enoyl-CoA Michaelis complex (Johnson & Srivastava, 1993; Kumar & Srivastava, 1994, 1995). The latter complex readily dissociates into MCAD−$FADH_2$ and enoyl-CoA. Under physiological experimental conditions, the equilibrium distribution between these complexes lies predominantly in favor of the CT complex (Kumar & Srivastava, 1995). Since the organic electron acceptors preferentially accept electrons from the

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[1] Abbreviations: MCAD, medium chain acyl-CoA dehydrogenase; FAD, flavin adenine dinucleotide; OceCoA, 2-octenoyl coenzyme A; IPCoA, 3-indolepropionyl coenzyme A; IACoA, *trans*-3-indoleacryloyl coenzyme A; $FcPF_6$, ferrocenium hexafluorophosphate; ETF, electron-transferring flavoprotein; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine; $\Delta G°$, standard free energy change, $\Delta H°$, standard enthalpy change; $\Delta S°$, standard entropy change; $\Delta H°_{int}$, intrinsic enthalpy change, $\Delta H°_{obs}$, observed enthalpy change; $\Delta H°_{ion}$, ionization enthalpy change; p, number of protons consumed per mole of MCAD; ΔS_{rt}, rotational and translational entropy change; ΔS_v, vibrational entropy change; ΔS_{HE}, entropy change due to hydrophobic interaction; ΔS_{conf}, conformational entropy change; K_a, association constant; CT, charge-transfer; EDTA, ethylenediaminetetraacetic acid.

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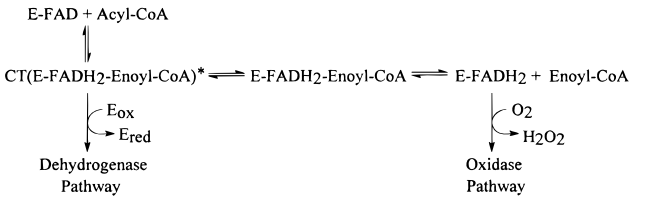
MCAD−$FADH_2$−enoyl-CoA CT complex (Johnson et al., 1993; McFarland et al., 1982; Gorelick et al., 1985), and O_2 accepts electrons only from MCAD−$FADH_2$, the dehydrogenase reaction of the enzyme remains as the preferred (physiologically desirable) pathway. The oxidase reaction of the enzyme originates due to a slow conversion of the CT complex to MCAD−$FADH_2$ (via the MCAD−$FADH_2$−enoyl-CoA Michaelis complex) followed by the transfer of electrons from MCAD−$FADH_2$ to O_2 (Johnson et al., 1994; Srivastava et al., 1995; Kumar & Srivastava, 1995; eq 2).



(2)

In eq 2, (E−$FADH_2$−enoyl-CoA)* and E−$FADH_2$−enoyl-CoA represent the two alternative conformational states of the enzyme, which differ with respect to the electronic structures of $FADH_2$ and enoyl-CoA species. These conformational states have been assigned as the CT and Michaelis complexes, respectively (Johnson & Srivastava, 1993; Johnson et al., 1994; Kumar & Srivastava, 1994, 1995).

The question as to how the CT complex is stabilized at the enzyme site arises. A cumulative account of all the previously published experimental data reveals that the single common factor which is responsible for the stabilization of the CT complex is the affinity of enoyl-CoA for the MCAD−$FADH_2$ site. Since the intrinsic stability of octenoyl-CoA within the CT complex was found to be remarkably similar to that found within the MCAD−FAD site (Kumar & Srivastava, 1994, 1995; Peterson et al., 1995), it occurred to us that the physical forces involved in stabilization of the above complexes must be the same. This was surprising since the formation of the CT complex involved "chemistry" (i.e., the reductive half-reaction), whereas the formation of the MCAD−FAD−octenoyl-CoA complex accompanied changes in the electronic structures of the individual components. Toward this end, we decided to compare the energetics of MCAD−FAD + octanoyl-CoA → MCAD−$FADH_2$−enoyl-CoA (CT complex) and MCAD−FAD + octenoyl-CoA → MCAD−FAD−octenoyl-CoA reactions via isothermal titration microcalorimetry. Such a comparison revealed that, although the overall free energy changes ($\Delta G°$) of these processes were more or less the same (Kumar & Srivastava, 1995), the enthalpy of the former process was about 10 kcal/mol more negative than that of the latter (K. L. Peterson and D. K. Srivastava, manuscript in preparation). Obviously, the similarity in the intrinsic binding affinity of octenoyl-CoA within the above complexes was due to a strong enthalpy−entropy compensation effect. While the above comparative studies are currently in progress in our laboratory, we investigated the thermodynamics of binding of octenoyl-CoA to MCAD−FAD, as elaborated below, under a variety of experimental conditions.

In recent years, due to the development of ultrasensitive microcalorimeters, the thermodynamics of binding of a variety of ligands with proteins have been investigated (Connelly & Thompson, 1992; Connelly et al., 1990; Chervenak & Toone, 1995; Lin et al., 1991; Jin et al., 1993; Bhatnagar et al., 1994). Such studies have enormously aided our understanding of the structural features of the protein−ligand complexes (Fisher & Singh, 1995; Bundle & Sigurskjold, 1994; Faergeman et al., 1996). However, the applications of titration microcalorimetry in investigating the binding of ligands to proteins have been sparse in the area of flavoproteins (Jelesarov & Bosshard, 1994; Palfey et al., 1997). To the best of our knowledge, the present investigation is the first detailed thermodynamic study of the binding of octenoyl-CoA to MCAD. As elaborated in the following sections, these studies have provided several new insights into the structural−functional and mechanistic aspects of MCAD catalysis.

MATERIALS AND METHODS

Materials

Octanoyl-CoA and EDTA were purchased from Sigma. All other reagents were of analytical reagent grade.

Methods

Unless stated otherwise, all experiments presented herein were conducted at 25 °C in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA (standard phosphate buffer). The ionic strength of the buffer was adjusted to 175 mM by addition of KCl. Medium chain acyl-CoA dehydrogenase (MCAD) was purified and assayed according to the procedure of Johnson et al. (1992). The site concentration of MCAD was determined by measuring the flavin content using an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979).

Octenoyl-CoA was prepared and assayed according to the procedure of Kumar and Srivastava, (1994). The extinction coefficient of octenoyl-CoA was taken to be 20.4 mM^{-1} cm^{-1} at 258 nm (Kumar & Srivastava, 1994).

Isothermal Titration Microcalorimetry. All calorimetric experiments were conducted on an MCS isothermal titration calorimeter (ITC) from Microcal, Inc. (Northampton, MA). A complete description of its predecessor instrument, OMEGA-ITC, experimental strategies, and data analyses are given by Wiseman et al. (1989). The calorimeter was calibrated by known heat pulses as described in the MCS-ITC manual. During titration, the reference cell was filled with a 0.03% azide solution in water. Prior to the titration experiment, both enzyme and octenoyl-CoA were thoroughly degassed under vacuum. The sample cell was filled either with 1.8 mL (effective volume = 1.36 mL) of buffer (for control) or with an appropriately diluted enzyme. The contents of the sample cell was titrated with 16−22 aliquots (4 μL each) of octenoyl-CoA. During the titration, the reaction mixture was continuously stirred at 400 rpm. The enzyme concentration was adjusted by 2% (as recommended by the manufacturer) to include a dilution effect of the enzyme solution, which occurs following a buffer rinse.

All calorimetric titration data were presented after subtracting the corresponding background. The background titration profiles, under identical experimental conditions, were obtained by injecting octenoyl-CoA into appropriate buffer solutions. The raw experimental data were presented as the amount of heat produced per second following each injection of ligand into the enzyme solution (minus the blank) as a function of time. The amount of heat produced per injection was calculated by integration of the area under

individual peaks by the Origin software, provided with the instrument. Final data are presented as the amount of heat produced per injection versus the molar ratio of octenoyl-CoA to MCAD. The data were analyzed by the Origin software as described by Wiseman et al. (1989). All parameters (viz. n, K_a, and $\Delta H°$) were allowed to vary during the curve fitting. The standard errors are derived from the best fit of the experimental data.

The data analysis produced three parameters, viz. stoichiometry (n), association constant (K_a), and the standard enthalpy changes ($\Delta H°$) for the binding of octenoyl-CoA to MCAD. The standard free energy change ($\Delta G°$) for the binding was calculated according to the relationship $\Delta G° = -RT \ln K_a$. Given the magnitudes of $\Delta G°$ and $\Delta H°$, the standard entropy changes ($\Delta S°$) for the binding process were calculated according to the standard thermodynamic equation, $\Delta G° = \Delta H° - T\Delta S°$.

The buffer components for different pH ranges were as follows: citrate phosphate (25 mM citric acid, 25 mM KH_2PO_4, pH adjusted with NaOH) for the pH range of 5.7−6.0, phosphate (50 mM KH_2PO_4, pH adjusted with NaOH) for the pH range of 6.5−8.0, and Tris phosphate (50 mM Tris, 50 mM KH_2PO_4, pH adjusted with NaOH) for the pH range of 8.3−8.7. The ionic strengths of the buffers were adjusted to 175 mM by addition of KCl. The following buffers (containing 0.3 mM EDTA at pH 7.6 and an ionic strength of 175 mM) were used for determining the stoichiometry of protons consumed upon binding of octenoyl-CoA to MCAD: 50 mM potassium phosphate ($\Delta H°_{ion} = 1.22$ kcal/mol), 100 mM MOPS ($\Delta H°_{ion} = 5.29$ kcal/mol), 100 mM Tricine ($\Delta H°_{ion} = 7.76$ kcal/mol), and 100 mM Tris ($\Delta H°_{ion} = 11.51$ kcal/mol). The ionization enthalpies ($\Delta H°_{ion}$) of the above buffer species were taken from Morin and Freire (1991).

The temperature-dependent experiments were conducted in the standard 50 mM phosphate buffer (pH 7.6) containing 0.3 mM EDTA (ionic strength = 175 mM). The calorimeter was equilibrated (for about 12 h) at temperatures 7 °C lower than the desired temperatures for the individual titration experiments.

RESULTS

In a preliminary experiment, we first noted that addition of a small aliquot of octenoyl-CoA to MCAD (oxidized form of the enzyme) results in an exothermic peak on our MCS-ITC system. This observation prompted us to undertake a complete titration of MCAD by increasing aliquots of octenoyl-CoA. Since the dissociation constant (K_d) of the pig kidney MCAD−octenoyl-CoA complex, determined by the spectrophotometric method, was 0.41 μM [Kumar & Srivastava, 1995; 90 nM according to Powell et al. (1987)], we decided to titrate 10 μM MCAD with increasing aliquots of octenoyl-CoA, to satisfy the c value in the range of 10−100 (Wiseman et al., 1989). The c value is defined as the product of the enzyme−ligand association constant (K_a) and the total concentration of the enzyme ($[E]_t$) (i.e., $c = K_a[E]_t$).

Figure 1 shows the titration of 10 μM MCAD with 22 aliquots (4 μL each) of octenoyl-CoA (stock concentration = 400 μM) in 50 mM phosphate buffer (ionic strength = 175 mM) at pH 7.6 at 25 °C. The top panel of Figure 1 shows the raw calorimetric data, denoting the amount of heat produced (negative exothermic peaks) following each injection of octenoyl-CoA. The area under each peak represents the amount of heat produced upon binding of octenoyl-CoA to MCAD. Note that, as the titration progresses, the area under the peaks progressively becomes smaller due to an increased occupancy of the enzyme by octenoyl-CoA. The bottom panel of Figure 1 shows the plot of the amount of heat generated per injection as a function of the molar ratio of octenoyl-CoA to enzyme. The solid, smooth line represents the best fit of the experimental according to Wiseman et al. (1989), yielding values for the stoichiometry (n) of the MCAD−octenoyl-CoA complex, the association constant (K_a), and the standard enthalpy change ($\Delta H°$) of 0.89 ± 0.007 mole of bound octenoyl-CoA/(mole of MCAD subunit), (2.6 ± 0.33) × 10^6 M^{-1}, and −10.3 ± 0.16 kcal/mol, respectively. Assuming that the standard state of octenoyl-CoA is 1 M, the free energy change ($\Delta G°$) for the binding of octenoyl-CoA to MCAD was calculated ($\Delta G° = -RT \ln K_a$) to be −8.75 ± 0.08 kcal/mol. Given the values of $\Delta G°$ and $\Delta H°$, $\Delta S°$ can be calculated to be 5.2 cal mol^{-1} K^{-1}.

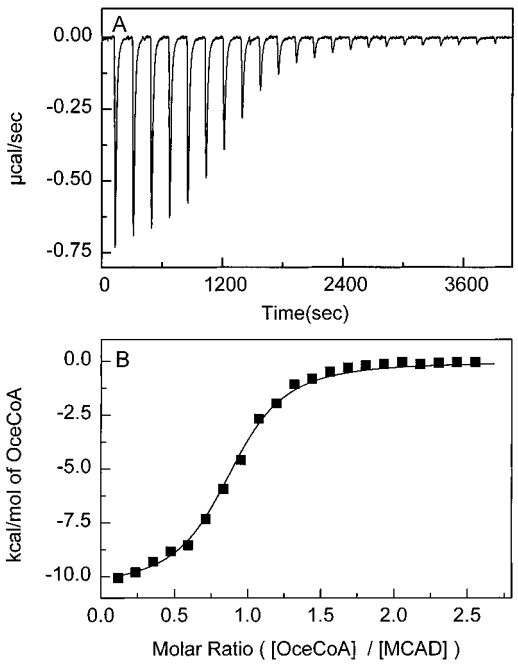


FIGURE 1: Titration of MCAD by octenoyl-CoA in 50 mM phosphate buffer (pH 7.6) containing 0.3 mM EDTA at 25 °C. Panel A shows the raw data, generated by titration of 1.8 mL of 10 μM MCAD by twenty-two 4 μL injection of 400 μM octenoyl-CoA. The area under each peak was integrated and plotted against the molar ratio of octenoyl-CoA to MCAD in panel B. The solid smooth line represents the best fit of the experimental data for the stoichiometry (n) of the MCAD−octenoyl-CoA complex (moles of bound octenoyl-CoA per mole of MCAD subunit) of 0.87, association constant (K_a) of 2.56 × 10^6 M^{-1}, and standard enthalpy changes ($\Delta H°$) of −10.3 kcal/mol.

To ascertain whether the stoichiometry of the MCAD−octenoyl-CoA complex and other thermodynamic parameters are affected by changes in the enzyme concentration, we performed the above experiment, utilizing 4.3 and 18 μM enzyme concentrations. The data analysis yielded values for n, K_a, and $\Delta H°$ of 0.73 ± 0.007, (1.53 ± 0.12) × 10^6 M^{-1}, and −11.8 ± 1.6 kcal/mol, respectively, at 4.3 μM enzyme and 0.85 ± 0.009, (3.1 ± 0.20) × 10^6 M^{-1}, and −10.7 ± 1.5 kcal/mol, respectively, at 18 μM enzyme. These data suggest that, within the limit of the experimental error, the above parameters remain unaffected upon changes in the enzyme concentration of 4.5-fold.

Table 1: Effect of Buffers on the Binding of Octenoyl-CoA to MCAD[a]

buffer	n	$K_a \times 10^6$ (M^{-1})	$\Delta H°_{obs}$ (kcal/mol)
phosphate	0.87 ± 0.007	2.6 ± 0.3	-10.3 ± 0.2
MOPS	0.81 ± 0.017	1.4 ± 0.2	-9.4 ± 0.3
Tricine	0.62 ± 0.008	3.3 ± 0.4	-6.2 ± 0.1
Tris	0.80 ± 0.045	1.1 ± 0.4	-5.5 ± 0.4

[a] At pH 7.6 and an ionic strength of 175 mM.

A casual perusal of the thermodynamic parameters (derived from the data of Figure 1) reveals that the magnitude of $\Delta G°$ (−8.75 kcal/mol) is dominated by the favorable contribution of $\Delta H°$ (−10.3 kcal/mol) and is offset by an unfavorable contribution of $T\Delta S°$ (−1.55 kcal/mol). Hence, under the experimental conditions of Figure 1, the overall binding of octenoyl-CoA to MCAD is considered to be enthalpically driven. As will be shown below, the above conclusion does not hold at temperatures lower than 25 °C.

Stoichiometry of Proton Consumption or Release upon Binding of Octenoyl-CoA to MCAD. It is known that the standard enthalpy changes ($\Delta H°$), derived from the binding isotherms, such as that of Figure 1, are not solely contributed by the physical forces governing the protein−ligand interactions. They often contain contributions from ionization enthalpy of the buffer species and/or changes in the protein conformations (Beschiaschvili & Seelig, 1992; Lin et al., 1991; Doyle et al., 1995). Although the enthalpic contributions of protein conformational changes can be taken as an integral component of the overall binding process, the enthalpic contributions due to protonation−deprotonation of the buffer species must be subtracted from $\Delta H°_{obs}$ to obtain the intrinsic enthalpy ($\Delta H°_{int}$) of the complex.

Previously, a number of investigators have determined the stoichiometry of protons consumed or released during the course of reaction or protein−ligand interaction by utilizing the buffers of known ionization enthalpies (Beschiaschvili & Seelig, 1992; Lin et al., 1991; Doyle et al., 1995; Morin & Freire, 1991; Jelesarov & Bosshard, 1994). The buffer species which have been customarily utilized for this goal are phosphate, MOPS, Tricine, and Tris. The corresponding ionization enthalpies of these buffers are 1.22, 5.29, 7.76, and 11.5 kcal/mol, respectively (Morin & Freire, 1991). Since the pK_a values of these buffers fall in the range of 7.2−8.3, they can be used as effective buffers at pH 7.6.

Table 1 summarizes the titration results of MCAD by octenoyl-CoA in different buffers (pH 7.6, ionic strength = 175 mM, and 25 °C). The data of Table 1 reveal that the association constant (K_a) of the MCAD−octenoyl-CoA complex varies between 1.14×10^6 M^{-1} (in the case of Tris buffer) and 2.56×10^6 M^{-1} (in the case of phosphate buffer), among different buffer systems. The above difference in K_a can be translated into a change in the $\Delta G°$ value of 0.48 kcal/mol. In contrast to this, there is a large buffer-dependent change in $\Delta H°$ for the binding of octenoyl-CoA to MCAD (see Table 1). For example, $\Delta H°_{obs}$ for the above interaction varies from −10.3 kcal/mol in the case of phosphate buffer to −5.5 kcal/mol in the case of Tris buffer. An examination of the data of Table 1 reveals that $\Delta H°_{obs}$ is directly related to the ionization enthalpy ($\Delta H°_{ion}$) of the buffer species. As the magnitude of $\Delta H°_{ion}$ increases, $\Delta H°_{obs}$ decreases, as demonstrated by the plot of Figure 2. These results suggest that the binding of octenoyl-CoA to MCAD involves

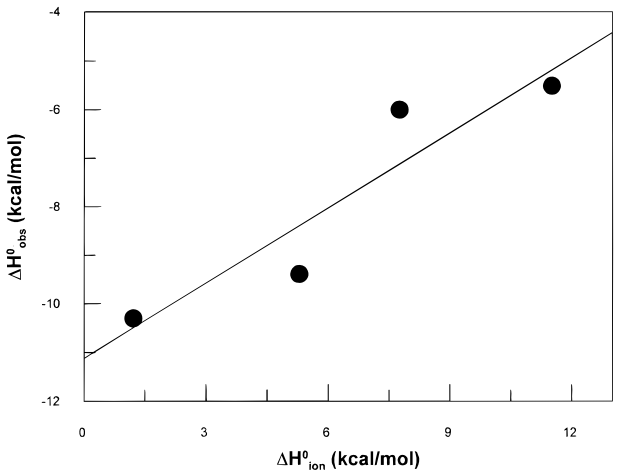


FIGURE 2: Dependence of $\Delta H°_{obs}$ (for the binding of octenoyl-CoA to MCAD) on $\Delta H°_{ion}$ of different buffers. The values of $\Delta H°_{obs}$ were taken from Table 1. The ionization enthalpy ($\Delta H°_{ion}$) of phosphate, MOPS, Tricine, and Tris buffers were taken to be 1.22, 5.29, 7.76, and 11.51 kcal/mol, respectively. Other conditions were the same as in Figure 1. The solid line is the linear regression analysis of the data for the slope (p, number of protons abstracted per mole of MCAD subunit) and intercept (intrinsic enthalpy for binding of octenoyl-CoA to MCAD, $\Delta H°_{int}$) of 0.52 and −11.1 kcal/mol, respectively.

abstraction of protons from the buffer media. In order to quantitate the amount of proton abstracted per mole of the MCAD−octenoyl-CoA complex, at pH 7.6, the data of Figure 2 were analyzed according to the following linear relationship (eq 3).

$$\Delta H°_{obs} = \Delta H°_{int} + p\Delta H°_{ion} \quad (3)$$

where p is the number of protons abstracted from the buffer media to stabilize the MCAD−octenoyl-CoA complex at pH 7.6. The best fit of the experimental data yields values for $\Delta H°_{int}$ and p of -11.0 ± 1.1 kcal/mol and 0.52 ± 0.15 proton consumed/(mole of the MCAD−octenoyl-CoA complex), respectively.

It should be pointed out that the stoichiometry of the MCAD−octenoyl-CoA complex measured in Tricine buffer ($n = 0.62$) is somewhat lower than that obtained in other buffers (see Table 1). In this regard, we must emphasize that among different parameters, derived from the best fit of the calorimetric data, stoichiometry (n) is most sensitive to variations in the enzyme concentrations. For example, if we analyze the above calorimetric data by inputting the total concentration of the enzyme as 8.5 μM (instead of 10 μM), the stoichiometry is increased to 0.703, without affecting the magnitudes of K_a and $\Delta H°_{obs}$. Hence, it appears that the lower stoichiometry of the MCAD−octenoyl-CoA complex in Tricine buffer is due to our inability to quantitate the effective enzyme concentration during the titration experiment. This may be due to precipitation or partial inactivation of the enzyme in Tricine buffer.

Effect of pH and Ionic Strength on MCAD−Octenoyl-CoA Interaction. We investigated the effect of pH on the binding isotherms for the titration of MCAD by octenoyl-CoA. To ensure a good buffering capacity, the following buffers were used during these experiments: citrate phosphate buffer for pH 5.7−6.0, phosphate buffer for pH 6.5−8.0, and Tris phosphate buffer for pH 8.3−8.7. The ionic strengths of

Table 2: Effect of pH on Microcalorimetric Titration Results for the Binding of Octenoyl-CoA to MCAD[a]

pH	*n*	$K_a \times 10^6$ (M^{-1})	$\Delta H°_{obs}$ (kcal/mol)
5.7	0.78 ±0.003	16.2 ± 1.4	−11.7 ± 0.1
6.0	0.86 ± 0.005	11.7 ± 1.3	−12.4 ± 0.1
6.5	0.96 ± 0.003	8.3 ± 0.4	−11.3 ± 0.1
7.0	0.97 ± 0.006	3.9 ± 0.3	−10.8 ± 0.1
7.6	0.89 ± 0.007	2.6 ± 0.3	−10.3 ± 0.1
8.0	0.92 ± 0.008	1.3 ± 0.1	−10.3 ± 0.1
8.3	0.91 ± 0.015	1.4 ± 0.2	−3.7 ± 0.1
8.7	0.87 ± 0.02	1.4 ± 0.2	−6.2 ± 0.2

[a] At 25 °C and with a 175 mM ionic strength. *n* represents the stoichiometry of bound octenoyl-CoA/MCAD subunit (mole/mole).

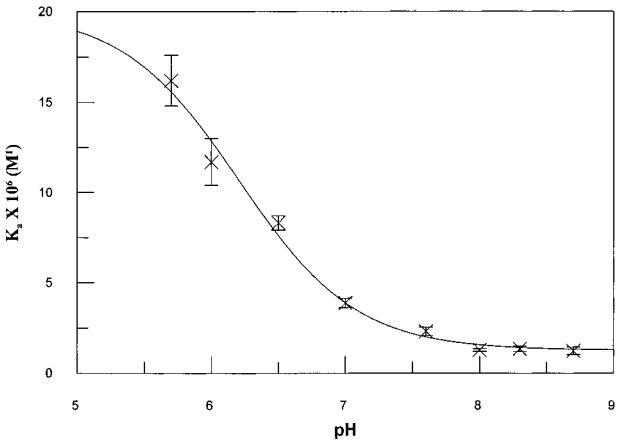


FIGURE 3: pH dependence of the association constant (K_a) of MCAD−octenoyl-CoA. The thermodynamic parameters for the titration of MCAD by octenoyl-CoA are contained in Table 2. The solid smooth line is the best fit of the experimental data according to the Henderson−Hasselbalch equation (for a single ionization site) with a pK_a of 6.2.

these buffers were maintained at 175 mM (see Materials and Methods). The experimental data are summarized in Table 2. These data confirm that the binding stoichiometry (moles of bound octenoyl-CoA per mole of the enzyme subunit) of the MCAD−octenoyl-CoA complex varies between 0.79 and 0.99 at different pH values. The above values can be taken to be representative of the 1/1 stoichiometry of the MCAD−octenoyl-CoA complex (i.e., 1 mole of octenoyl-CoA bound per mole of MCAD subunit). A similar stoichiometry was obtained for the above complex via the spectrophotometric methods (data not shown).

Table 2 shows that, as the pH increases, the affinity of octenoyl-CoA for MCAD (K_a value) decreases. This is consistent with our previous finding that the affinity of IACoA (a chromophoric enoyl-CoA) for MCAD decreases with an increase in pH of the buffer media (Johnson et al., 1992). Figure 3 shows the influence of pH on the binding affinity (K_a) of MCAD for octenoyl-CoA. The solid line is the best fit of the experimental data according to the Henderson−Hasselbalch equation, with a pK_a of 6.2.

Since the pH-dependent measurements of the association constant (K_a) at high (8.3 and 8.7) and low (5.7 and 6.0) pH values were performed in Tris phosphate and citrate phosphate buffers, respectively, we considered whether Tris and citrate had any influence on the above parameter. The fact that Tris has practically no influence on the binding of octenoyl-CoA to MCAD is clearly evident from the data of Table 1. Furthermore, the pK_a of 6.2 determined from the best fit of the experimental data of Figure 3 is unlikely to

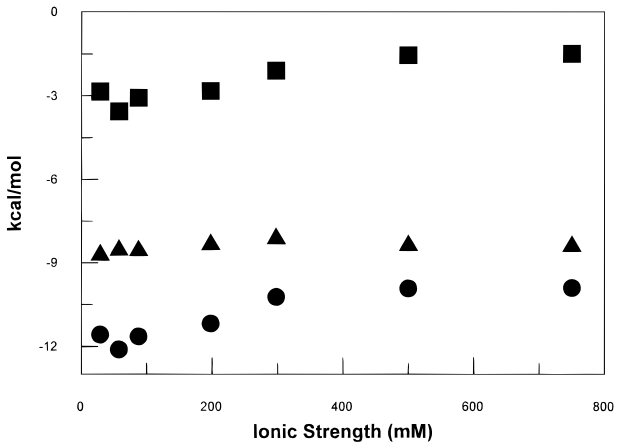


FIGURE 4: Effect of ionic strength on the thermodynamic parameters for the interaction of octenoyl-CoA with MCAD. $\Delta G°$, $\Delta H°$, and $T\Delta S°$ are represented by solid triangles, circles, and squares, respectively.

be affected by small changes in the K_a values at pH >8.0. To ascertain whether K_a was affected by citrate, we compared the titration results for the interaction of octenoyl-CoA with MCAD in the standard phosphate buffer at pH 6.3, in the absence and presence of 30 mM citrate. Values for *n*, K_a, and $\Delta H°$ in the absence of citrate were 0.85 ± 0.005, (9.1 ± 0.7) × 10^6 M^{-1}, and −13.3 ± 1.2 kcal/mol, respectively. The corresponding parameters in the presence of citrate were found to be 0.90 ± 0.004, (9.4 ± 0.6) × 10^6 M^{-1}, and −12.4 ± 0.7 kcal/mol, respectively. Clearly, like Tris, citrate exhibits no influence on the K_a value for the MCAD−octenoyl-CoA complex.

The pK_a derived from the data of Figure 3 can be taken as a measure of the pK_a of either octenoyl-CoA or the free enzyme (Tipton & Dixon, 1979). To resolve this, we titrated an aqueous solution of octanoyl-CoA (an analogue of octenoyl-CoA) with NaOH (data not shown). The titration results were consistent with at least three pK_as, of which the intermediary pK_a was found to be in the range of 6.8−7.2. The latter value is presumably due to ionization of the second proton from the phosphate groups of octenoyl-CoA. In light of these results, we are prompted to propose that the pK_a of 6.2 is representative of the pK_a of the free enzyme.

We further investigated the effects of ionic strength on binding of octenoyl-CoA to MCAD in the standard phosphate buffer at pH 7.6 and 25 °C. The derived thermodynamic parameters are plotted in Figure 4 as a function of ionic strength. These data suggest that there is no significant effect of ionic strength on $\Delta G°$, although $\Delta H°$ and $T\Delta S°$ show some variations as a function of ionic strength.

Effect of Temperature. We investigated the effect of temperature on binding of octenoyl-CoA to MCAD in the standard phosphate buffer (ionic strength = 175 mM) at pH 7.6. The thermodynamic parameters derived from the temperature-dependent titration results are presented in Table 3. A casual perusal of the data of Table 3 reveals that, whereas $\Delta G°$ remains practically invariant with changes in temperature, both $\Delta H°$ and $\Delta S°$ decrease with an increase in temperature. Figure 5 shows the plots of $\Delta G°$ and $\Delta H°$ as a function of $T\Delta S°$. Note that $\Delta H°$ increases linearly as a function of $T\Delta S°$, suggesting a strong enthalpy−entropy compensation for the binding of octenoyl-CoA to MCAD. From these data, it is apparent that the $\Delta H°$ versus $T\Delta S°$ plot intersects the $\Delta G°$ versus $T\Delta S°$ plot when the latter is

Table 3: Temperature Dependence of Thermodynamic Parameters for the Binding of Octenoyl-CoA to MCAD[a]

temperature (°C)	*n*	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
11.3	1.01 ± 0.004	−7.97 ± 0.17	−4.1 ± 0.2	3.81 ± 0.2
15.6	1.01 ± 0.01	−8.40 ± 0.05	−5.6 ± 0.1	2.77 ± 0.1
20.6	1.14 ± 0.01	−8.71 ± 0.06	−6.0 ± 0.1	2.67 ± 0.1
25.2	0.89 ± 0.007	−8.75 ± 0.08	−10.3 + 0.2	−1.58 ± 0.2
30.2	1.01 ± 0.013	−8.78 ± 0.07	−11.1 ± 0.2	−2.33 ± 0.2
35.3	0.99 ± 0.033	−8.67 ± 0.10	−12.5 ± 0.4	−3.88 ± 0.4

[a] In the standard phosphate buffer (ionic strength of 175 mM) at pH 7.6. *n* refers to the stoichiometry of octenoyl-CoA bound/MCAD subunit (mole/mole).

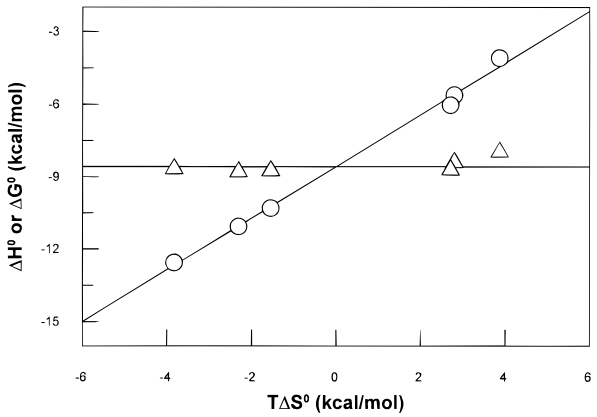


FIGURE 5: Enthalpy−entropy compensation plot for the binding of octenoyl-CoA to MCAD in 50 mM phosphate buffer (pH 7.6) containing 0.3 mM EDTA. The thermodynamic parameters for the binding of octenoyl-CoA to MCAD at different temperatures were calculated as described in Figure 1. The dependence of $\Delta G°$ and $\Delta H°$ on $T\Delta S°$ is shown by open triangles and open circles, respectively. The linear regression analysis for the data of $\Delta H°$ versus $T\Delta S°$ yields magnitudes of the slope and intercept of 1.07 ± 0.04 and −8.58 ± 0.11 kcal/mol, respectively.

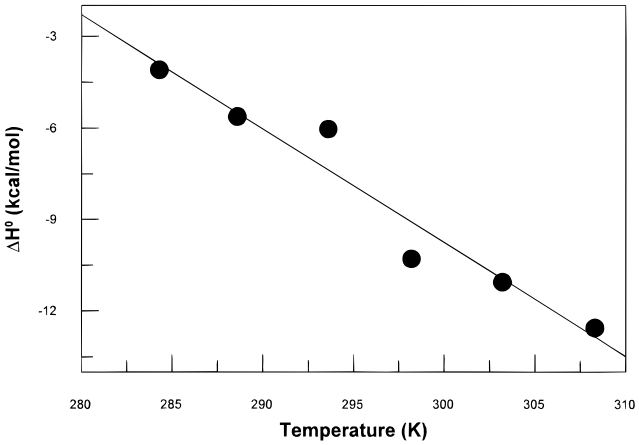


FIGURE 6: Temperature dependence of $\Delta H°$. Other conditions were the same as in Figure 5. The solid smooth line is the best fit of the experimental data for a $\Delta C_p°$ (slope) of −0.37 ± 0.05 kcal mol^{-1} K^{-1} and an intercept (at 0 K) of 102 ± 13.6 kcal/mol.

equal to zero. Clearly, the intersection point, $\Delta G° = \Delta H°$ (−8.58 kcal/mol), implies that the entropic contributions to MCAD−octenoyl-CoA binding are equal to zero.

We further analyzed the data of Table 3 by plotting $\Delta H°$ as a function of temperature (Figure 6). Note a linear dependence of $\Delta H°$ on temperature, with a slope of 0.37 ± 0.05 kcal mol^{-1} K^{-1}, and the *y*-axis intercept (at 0 K) of 102 kcal/mol. The slope of this plot serves as a measure of the heat capacity changes ($\Delta C_p°$) for MCAD−octenoyl-CoA binding. As discussed in the next section, a strong negative value for $\Delta C_p°$ suggests that the binding for octenoyl-CoA to MCAD is dominated by the hydrophobic forces. From this plot, we calculated the temperature at which $\Delta H° = -8.58$ kcal/mol (where the overall entropic contribution was zero; see Figure 5) to be 23.8 °C. Hence, at the latter temperature, $\Delta G°$ of MCAD + octenoyl−CoA binding is exclusively contributed by $\Delta H°$. At this temperature, the favorable entropic changes (i.e., hydrophobic and vibrational entropic effects) are negated by the unfavorable entropic changes (i.e., rotational, translational, and conformational entropic effects). The contributions of these entropic changes have been estimated in the next section.

DISCUSSION

We present, for the first time, a detailed thermodynamic analysis of the binding of octenoyl-CoA (the reaction product of the physiological substrate octanoyl-CoA) to pig kidney medium chain acyl-CoA dehydrogenase (MCAD), employing ultrasensitive isothermal titration microcalorimetry. To the best of our knowledge, this is the first microcalorimetric study involving MCAD.

The experimental data presented in the previous section provide the following new insights into the structural−functional and mechanistic aspects of the enzyme−octenoyl-CoA interaction. (1) The binding of octenoyl-CoA to MCAD accompanies abstraction of 0.52 proton/(mole of MCAD subunit) from the buffer media at pH 7.6. (2) The pH-dependent binding of octenoyl-CoA to MCAD conforms to a pK_a of 6.2, and this pK_a is representative of the free enzyme and not of octenoyl-CoA. (3) Whereas the binding of octenoyl-CoA to MCAD is entropically driven at lower temperatures (<25 °C), it is enthalpically driven at higher temperatures. There is a temperature-independent enthalpy−entropy compensation to the overall free energy of binding. (4) The binding of octenoyl-CoA to MCAD involves a strong negative change in heat capacity ($\Delta C_p° = -0.37$ kcal mol^{-1} K^{-1}), suggesting that the above process is primarily dominated by the hydrophobic forces, and is site-specific. (5) There is no significant effect of ionic strength on the thermodynamic parameters of the MCAD−octenoyl-CoA complex.

Modulation of pK_a of MCAD upon Binding of Octenoyl-CoA. The pK_a derived from the data of Figure 3 can be ascribed to either octenoyl-CoA or the free enzyme or both (Tipton & Dixon, 1979). Since our experimentally determined pK_a of octanoyl-CoA (an analogue of octenoyl-CoA) falls in the range of 6.8−7.2, about 0.6−1 pH unit higher than the pK_a of 6.2 obtained from the data of Figure 3, it appears to be evident that the observed pK_a is not conferred by octenoyl-CoA. Hence, we propose that the observed pK_a of 6.2 is due to some enzyme site group (see below).

One of the most interesting aspects of the present investigation is the demonstration that binding of octenoyl-CoA to MCAD accompanies abstraction of 0.52 proton from the buffer media at pH 7.6 upon formation of 1 mole of the MCAD−octenoyl-CoA complex. Since the stoichiometry of the MCAD−octenoyl-CoA complex (at saturating concentrations of octenoyl-CoA) remains more or less the same (e.g., 1/1 mole/mole) at different pH values (see Table 2), it is reasonable to assume that the total number of binding sites, responsible for the stabilization of the above complex, is

independent of pH. Assuming that only one enzyme site group needs to be protonated for the stabilization of the MCAD-octenoyl-CoA complex, the pK_a of such a group can be envisaged to fall in the vicinity of 7.6. Hence, the binding of octenoyl-CoA to MCAD results in an increase in the pK_a of free enzyme from 6.2 to around 7.6. A quantitative account of such changes and its implication in the enzyme catalysis will be presented in a separate communication.

Effect of Temperature on MCAD−Octenoyl-CoA Binding. It has been widely recognized that $\Delta C_p°$, derived from the temperature dependence of enthalpic changes for protein−ligand interactions, is one of the most valuable thermodynamic parameters for inferring the structural changes in proteins. Following the recognition by Edsall (1935) that transfer of nonpolar groups to aqueous solutions results in a large increase in $\Delta C_p°$, a number of workers subsequently recognized the relationships between $\Delta C_p°$ and the changes in the water accessible nonpolar surface areas of both model compounds and proteins (Privalov, 1979; Spolar et al., 1989, 1992; Livingstone et al., 1991). The exposure of nonpolar surface areas to water results in a positive increase in the $\Delta C_p°$ value, whereas the opposite (i.e., the burial of nonpolar surface areas from water) results in a negative decrease of the $\Delta C_p°$ value. A positive increase in $\Delta C_p°$ has also been considered to arise upon burial of polar surface areas, although the latter contribution is not as large as that contributed by the burial of water accessible nonpolar surface areas at least during protein folding (Makhatdze & Privalov, 1990, 1993; Murphy & Gill, 1991; Spolar et al., 1992). On the basis of the X-ray crystallographic data of several proteins, the changes in the water accessible surface areas of both nonpolar (ΔA_{np}) and polar (ΔA_p) residues, on protein folding, have been calculated. Such calculations reveal that the ratio of $\Delta A_{np}/\Delta A_p$ varies between 1.2 and 1.7 (Murphy & Freire, 1992). This range is comparable to a value for the ratio $\Delta A_{np}/\Delta A_p$ of 2.4, calculated for the binding of dodecanoyl-CoA to acyl-CoA binding protein (Faergeman et al., 1996). Assuming the above ratio holds for the interaction of octenoyl-CoA with MCAD, the magnitudes of ΔA_{np} and ΔA_p can be calculated according to the following empirical relationship (eq 4).

$$\Delta C_p° = C_{p,p}\Delta A_p + C_{p,np}\Delta A_{np} \quad (4)$$

The average contributions of $C_{p,np}$ and $C_{p,p}$ have been proposed to be 0.32 and −0.14 cal mol^{-1} K^{-1} Å^{-2}, respectively, by Spolar et al. (1992) and 0.45 and −0.26 cal mol^{-1} K^{-1} Å^{-2}, respectively, by Murphy et al. (1993). On the basis of these values, the magnitudes of ΔA_{np} and ΔA_p for the binding of octenoyl-CoA to MCAD can be calculated to be in the range of 1092−1425 and 455−594 Å^2, respectively. Further confirmations of these values must await calculations of ΔA_{np} and ΔA_p from the X-ray crystallographic structures of the MCAD−octenoyl-CoA complex.

The fact that $\Delta G°$ for the binding of octenoyl-CoA to MCAD remains unaffected by an increase in temperature, and there is a strong enthalpy−entropy compensation effect (Figure 5), is not novel to the present study. A number of previous investigators have found such properties with other protein−ligand complexes (Mukkur, 1978; Herron et al., 1986; Jin et al., 1993). The origin of the enthalpy−entropy compensatory effect presumably lies in the relative magnitudes of $\Delta S°$ and $\Delta C_p°$. Under the condition $\Delta C_p° > \Delta S°$ at all temperatures (as observed in the present case; see Table 3), the temperature-dependent changes in both $\Delta S°$ and $\Delta H°$ attain a constant value, which is equal to $\Delta C_p°$ (Ha et al., 1989).

From the enthalpy−entropy compensation plot, it is evident that, when $T\Delta S°$ is equal to zero, $\Delta H°$ and $\Delta G°$ attain a common value (−8.58 kcal/mol). Under the above situation, the overall entropic contributions to the overall free energy changes are zero. The temperature at which $\Delta H° = -8.58$ kcal/mol is found to be 296.8 K, from the data of Figure 6. Since the $\Delta S°$ is comprised of both favorable and unfavorable entropic contributions, it follows that at 296.8 K the former is balanced by the latter. The favorable entropic changes include solvent displacement (i.e., release of "frozen" water molecules in the bulk phase, often referred to as the hydrophobic effect; ΔS_{HE}) and an increase in vibrational modes (vibrational entropy; ΔS_v), whereas the unfavorable contributions include the loss in rotational and translational entropy (ΔS_{rt}) and conformational entropy (ΔS_{conf}) (eq 5).

$$\Delta S^0 = \underbrace{\Delta S^0_{HE} + \Delta S^0_V}_{\text{favorable}} + \overbrace{\Delta S^0 rt + \Delta S^0 conf}^{\text{unfavorable}} \quad (5)$$

Can the individual entropic contributions of eq 5 be ascertained for the binding of octenoyl-CoA to MCAD? In attempting to answering this question, we note that several investigators have predicted and/or calculated the individual entropic changes for interactions of a variety of ligands with their cognate proteins. However, such predictions have been based on several implicit assumptions. For example, Sturtevant (1977) argues that the overall entropic changes during protein−ligand interactions are dominated by hydrophobic ($\Delta S°_{HE}$) as well as vibrational ($\Delta S°_v$) entropic contributions. According to Sturtevant (1977), large temperature-dependent entropic changes during protein−ligand interactions are due to a delicate balance between $\Delta S°_{HE}$ and $\Delta S°_v$. In making quantitative predictions, this author assumes that the rotational-translational ($\Delta S°_{rt}$) and conformational ($\Delta S°_{conf}$) entropic changes are negligible. On the other hand, Spolar and Record (1994) argue that the contribution of the vibrational entropic changes ($\Delta S°_v$) is negligible during protein−ligand interactions. According to these authors, the favorable hydrophobic effect is balanced by the unfavorable, rotational and translational and conformational entropic changes, at temperatures where the overall entropic changes are zero. Such a temperature for the interaction of octenoyl-CoA to MCAD is 296.8 K (or 23.8 °C). The magnitude of ΔS_{HE} at the latter temperature can be calculated by the following relationship (eq 6; Spolar & Record, 1994).

$$\Delta S°_{HE}(T) = 1.35\Delta C_p° \ln(T/386) \quad (6)$$

The calculation yields a value for ΔS_{HE} for the binding of octenoyl-CoA to MCAD of 132 cal mol^{-1} K^{-1}. For a number of bimolecular association reactions, ΔS_{rt} has been thought to contribute −50 cal/mol of rotational and translation entropy (Spolar & Record, 1994). Hence, the remaining entropic loss of −82 cal mol^{-1} K^{-1} must be contributed by the loss in the conformational restrictions of MCAD and octenoyl-CoA molecules. The latter is a significant contribution of the total entropic changes, and its magnitude is only 38% lower than the favorable entropic contributions

due to the hydrophobic effect. Hence, unlike the other cases of protein−ligand interaction, the binding of octenoyl-CoA to MCAD cannot be taken as the "rigid body" interaction (Spolar & Record, 1994), i.e., the interaction involving no changes in the protein conformations (Kim et al., 1993). In other words, the interaction of octenoyl-CoA with MCAD must involve conformational changes in the protein and ligand structures, the fact consistent with our previously published kinetic and spectroscopic studies on the MCAD-catalyzed reaction (Johnson et al., 1992, 1993, 1994; Johnson & Srivastava, 1993; Kumar & Srivastava, 1994, 1995; Peterson et al., 1995; Srivastava et al., 1995).

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