Influence of Glu-376 → Gln mutation on enthalpy and heat capacity changes for the binding of slightly altered ligands to medium chain acyl-CoA dehydrogenase

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Abstract

We showed that the $\alpha\text{-CH}_2 \rightarrow \text{NH}$ substitution in octanoyl-CoA alters the ground and transition state energies for the binding of the CoA ligands to medium-chain acyl-CoA dehydrogenase (MCAD), and such an effect is caused by a small electrostatic difference between the ligands. To ascertain the extent that the electrostatic contribution of the ligand structure and/or the enzyme site environment modulates the thermodynamics of the enzyme–ligand interaction, we undertook comparative microcalorimetric studies for the binding of 2-azaoctanoyl-CoA ($\alpha\text{-CH}_2 \rightarrow \text{NH}$ substituted octanoyl-CoA) and octenoyl-CoA to the wild-type and Glu-376 → Gln mutant enzymes. The experimental data revealed that both enthalpy (ΔH°) and heat capacity changes (ΔC_p°) for the binding of 2-azaoctanoyl-CoA ($\Delta H_{298}^\circ = -21.7 \pm 0.8$ kcal/mole, $\Delta C_p^\circ = -0.627 \pm 0.04$ kcal/mole/K) to the wild-type MCAD were more negative than those obtained for the binding of octenoyl-CoA ($\Delta H_{298}^\circ = -17.2 \pm 1.6$ kcal/mole, $\Delta C_p^\circ = -0.526 \pm 0.03$ kcal/mole/K). Of these, the decrease in the magnitude of ΔC_p° for the binding of 2-azaoctanoyl-CoA (vis-à-vis octenoyl-CoA) to the enzyme was unexpected, because the former ligand could be envisaged to be more polar than the latter. To our further surprise, the ligand-dependent discrimination in the above parameters was completely abolished on Glu-376 → Gln mutation of the enzyme. Both ΔH° and ΔC_p° values for the binding of 2-azaoctanoyl-CoA ($\Delta H_{298}^\circ = -13.3 \pm 0.6$ kcal/mole, $\Delta C_p^\circ = -0.511 \pm 0.03$ kcal/mole/K) to the E376Q mutant enzyme were found to be correspondingly identical to those obtained for the binding of octenoyl-CoA ($\Delta H_{298}^\circ = -13.2 \pm 0.6$ kcal/mole, $\Delta C_p^\circ = -0.520 \pm 0.02$ kcal/mole/K). However, in neither case could the experimentally determined ΔC_p° values be predicted on the basis of the changes in the water accessible surface areas of the enzyme and ligand species. Arguments are presented that the origin of the above thermodynamic differences lies in solvent reorganization and water-mediated electrostatic interaction between ligands and enzyme site groups, and such interactions are intrinsic to the molecular basis of the enzyme–ligand complementarity.

Keywords: Medium chain acyl-CoA dehydrogenase; isothermal microcalorimetry; thermodynamics of ligand binding; Glu-376 → Gln mutation; 2-azaoctanoyl-CoA; octenoyl-CoA

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Abbreviations: MCAD, human liver medium-chain acyl-CoA dehydrogenase; FAD, flavin adenine dinucleotide; FcPF₆, ferricinium hexafluoro phosphate; ΔG° , standard free energy change; ΔH° , standard enthalpy change; ΔS° , standard entropy change; K_a , association constant; ΔC_p° ,

standard heat capacity change; OcaCoA, octanoyl-CoA; OceCoA, 2-octenoyl-CoA; aza-CoA, 2-azaoctanoyl-CoA; THF, tetrahydrofuran; EDTA, ethylenediamine tetraacetic acid; 3',5'-ADP, 3',5'-adenosine diphosphate; $C_{p,p}$, heat capacity for changes in the polar surface areas; $C_{p,np}$, heat capacity for changes in the nonpolar surface areas; ΔA_p , changes in the water accessible polar surface areas; ΔA_{np} , changes in the water accessible nonpolar surface areas.

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Medium-chain acyl-CoA dehydrogenase (MCAD) shows a wide range of substrate specificity, and it interacts with a variety of differently substituted CoA-derivatives (for reviews, see Beinert 1963; Engel 1990; Thorpe and Kim 1995). This feature of the enzyme provided an opportunity to investigate the influence of a small variation in the ligand structure on the kinetic and thermodynamic properties of the enzyme–ligand complexes, as well as during the enzyme catalysis (Engel 1990; Srivastava et al. 1997; Peterson and Srivastava 1997; Peterson et al. 1998b; Vock et al. 1998; Cummings and Thorpe 1994; Powell et al. 1987; McFarland et al. 1982). In the past, a few such studies involved the influence of deletion of distal fragments (e.g., 3'-phosphate and 3',5'-ADP) of the coenzyme-A structure (Srivastava et al. 1995; Peterson and Srivastava 1997; Peterson et al. 1998b), utilization of increasing chain lengths of acyl/enoyl-CoAs (Beinert 1963; Engel 1990; Peterson et al. 1995; Kumar 1997; Nandy et al. 1996), and the effect of hetero-atom substitution in the fatty acid regions of CoA-ligands on the spectroscopic, kinetic, and thermodynamic properties of the enzyme (Engel 1990; Thorpe and Kim 1995; Cummings and Thorpe 1994; Powell et al. 1987; Trievel et al. 1995). These studies have sometimes been corroborated, but at other times opposed by the known structural features of the enzyme–ligand complexes (Peterson and Srivastava 1997; Peterson et al. 1998; Kim et al. 1993; Lee et al. 1996).

The X-ray crystallographic structures of both pig liver and recombinant human liver enzymes in the absence and presence of bound product (octenoyl-CoA) have been solved to atomic resolutions (Kim et al. 1993; Lee et al. 1996; Kim and Wu 1988). The structural data reveal that the ligand-binding pocket of the enzyme traverses almost to the center of the protein structure, and is predominantly comprised of hydrophobic amino acid residues (Kim et al. 1993). The binding of octenoyl-CoA results in exclusion of at least four structured water molecules from the enzyme site (Kim et al. 1993). These features are corroborated by the detailed microcalorimetric studies for the binding of octenoyl-CoA to both pig kidney and recombinant human liver MCADs (Srivastava et al. 1997; Peterson et al. 1998b). Consistent with the principle of the classic hydrophobic effect (Tanford 1980), the binding of octenoyl-CoA (a predominantly hydrophobic ligand) to the hydrophobic enzyme site pockets of pig kidney and human liver enzymes yields large negative ΔC_p° values, albeit of substantially different magnitudes (Srivastava et al. 1997; Peterson et al. 1998b).

We recently undertook a comparative transient kinetic investigation for the binding of octenoyl-CoA and 2-aza-octenoyl-CoA (a redox inactive ligand in which the α -CH group is replaced by NH) via the stopped-flow method (Peterson et al. 2000). Such studies revealed that although the above substitution did not influence the overall binding affinity of the ligands to the enzyme, it influenced the micro-

scopic steps during the overall binding process (Peterson et al. 2000). The binding of these as well as other ligands to the enzyme was found to proceed via two steps (Johnson et al. 1992; Kumar and Srivastava 1994, 1995; Srivastava et al. 1995): The first (fast) step involved the formation of the enzyme–ligand collision/Michaelis complex, followed by the isomerization of the latter complex during the second (slow) step. The latter step was found to be coupled to the protein conformational change:

A comparative account of the binding studies for the above ligands led to the proposition that the electrostatic field involving the carboxyl group of Glu-376 plays an important role in stabilizing the ground and transition state structures during the isomerization steps of the cognate enzyme–ligand complexes (Peterson et al. 2000). With these features in mind, we proceeded to determine the thermodynamic consequences of α -CH₂ $\rightarrow$ NH substitution in octenoyl-CoA on binding of ligands to the wild-type and E376Q mutant enzymes. The results and conclusion derived from such studies are contained in the following sections.

Results

Figure 1 shows the isothermal microcalorimetric data for the titration of wild-type MCAD with increasing concentrations of 2-aza-octenoyl-CoA in 50 mM phosphate buffer at pH 7.6, containing 0.3 mM EDTA and 10% glycerol. The top panel shows the effect of injecting 4 μ L aliquots (the first aliquot being 1 μ L) of 205 μ M stock solution of 2-aza-octenoyl-CoA into 1.8 mL of 10.1 μ M solution of wild-type MCAD. The addition of each aliquot follows the emergence of a negative (exothermic) peak because of the enzyme–ligand interaction. The area under each peak serves as a measure of the amount of heat produced on ligand binding. Note that as the titration progresses, the area under the peak becomes smaller because of increased occupancy of the enzyme site by 2-aza-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 2-aza-octenoyl-CoA to the wild-type MCAD. The solid smooth line is the best fit of the experimental data according to Wiseman et al. (1989) for the values of stoichiometry (n), the association constant (K_a), and the standard enthalpic changes (ΔH°) of the enzyme-2-aza-octenoyl-CoA complex of 0.88 ± 0.01

Fig. 1. Isothermal microcalorimetric titration data for the binding of 2-aza-octanoyl-CoA with wild-type MCAD at 25°C. The *top* panel shows the raw calorimetric data generated by titration of 1.8 mL of 10.13 μ M wild-type enzyme with 60 injections (first injection: 1 μ L, subsequent injections: 4 μ L each) of 205.1 μ M 2-aza-octanoyl-CoA. The area under each peak was integrated and plotted against the molar ratio of 2-aza-octanoyl-CoA to the enzyme in the *bottom* panel. The solid smooth line is the best fit of the data for the stoichiometry (n) of the enzyme-2-aza-octanoyl-CoA complex (moles of bound 2-aza-octanoyl-CoA per mole of the enzyme subunit) of 0.88 ± 0.01 , association constant (K_a) of $(3.1 \pm 0.21) \times 10^6 \text{ M}^{-1}$, and the standard enthalpic changes (ΔH°) of $-21.1 \pm 0.2 \text{ kcal/mole}$.

(moles of bound ligand per mole of MCAD subunit), $(3.1 \pm 0.2) \times 10^6 \text{ M}^{-1}$, and $-21.1 \pm 0.2 \text{ kcal/mole}$, respectively. These values are similar to the preliminary microcalorimetric data of Peterson et al. (2000). The magnitude of K_a , discerned from the data of Figure 1, is translated to the standard free energy changes ($\Delta G^\circ = -RT \ln K_a$) of -8.9 kcal/mole , on consideration of the standard state being equal to 1 M. Given the enthalpy (ΔH°) and free energy (ΔG°) changes for the binding of 2-aza-octanoyl-CoA to the enzyme, the entropic contribution ($T\Delta S^\circ$) for the above process can be calculated ($T\Delta S^\circ = -\Delta G^\circ + \Delta H^\circ$) to be -12.2 kcal/mole . It should be emphasized that the titration experiment of Figure 1 was performed in triplicate, and the average values of n , ΔG° , ΔH° , and $T\Delta S^\circ$ were discerned to be 0.85 ± 0.04 , $-8.7 \pm 0.2 \text{ kcal/mole}$, $-21.7 \pm 0.2 \text{ kcal/mole}$, and $-12.9 \pm 0.9 \text{ kcal/mole}$, respectively. The microcalorimetric titration data for the binding of octenoyl-CoA to MCAD yielded the average values of n , ΔG° , ΔH° , and

$T\Delta S^\circ$ to be 0.98 ± 0.06 , $-8.3 \pm 0.2 \text{ kcal/mole}$, $-17.2 \pm 1.6 \text{ kcal/mole}$, and $-8.9 \pm 1.8 \text{ kcal/mole}$, respectively (Peterson et al. 1998). A comparative account of these data reveals that whereas the magnitudes of n and ΔG° for the binding of 2-aza-octanoyl-CoA are similar to those obtained for the binding of octenoyl-CoA, the ΔH° and $T\Delta S^\circ$ values are considerably different (Peterson et al. 2000). Of the latter parameters, the ΔH° and $T\Delta S^\circ$ values for the binding of 2-aza-octanoyl-CoA to MCAD are 4.5 and 4.1 kcal/mole more negative, respectively, (i.e., enthalpically more favorable, but entropically less favorable) than those obtained for the binding of octenoyl-CoA to the enzyme.

Effect of Glu-376 $\rightarrow$ Gln (E376Q) mutation

It has been shown previously that the carbonyl groups of the CoA-thioesters are polarized on binding to the enzyme site (Murfin 1974; Johnson et al. 1992; Rudik et al. 1998; Engst et al. 1999). Such a polarization is expected to generate partial positive charges at the α -NH group of 2-aza-octanoyl-CoA and at the γ -CH₂ group of octenoyl-CoA, resulting in coulombic interactions with the neighboring carboxyl group ($\sim 4.6 \text{ \AA}$ away) of Glu-376 (Gilson and Honig 1988; Xiao and Honig 1999). To probe the energetic contribution of the above interaction, we performed microcalorimetric studies for the binding of the above ligands to the Glu-376 $\rightarrow$ Gln mutant enzyme (data not shown). The analysis of the experimental data (performed in duplicate) yielded magnitudes of n , K_a , and ΔH° for the binding of 2-aza-octanoyl-CoA to the E376Q mutant enzyme as being equal to 0.96 ± 0.05 , $(3.4 \pm 0.3) \times 10^6 \text{ M}^{-1}$ and $-13.3 \pm 0.7 \text{ kcal/mole}$, respectively. The corresponding values for the binding of octenoyl-CoA to the E376Q mutant enzyme were found to be 1.1 ± 0.06 , $(2.2 \pm 1.4) \times 10^6 \text{ M}^{-1}$ and $-13.2 \pm 0.6 \text{ kcal/mole}$, respectively. Note that unlike the wild-type enzyme, the ΔH° values for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the E376Q mutant enzyme are remarkably the same. Assuming that the E376Q mutation did not significantly alter the protein conformation (as suggested by the model building studies), it appears evident that the origin of the enthalpic and entropic differences for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the wild-type enzyme in part lies in the electrostatic interaction between the carboxyl group of Glu-376 and the partial positive charges generated at the α -NH and γ -CH₂ groups of 2-aza-octanoyl-CoA and octenoyl-CoA, respectively.

Temperature dependence of the thermodynamic parameters

We investigated the temperature dependence of the thermodynamic parameters for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the wild-type and E376Q mutant enzymes. All these experiments were repeated two to five

times, often using different batches of enzyme and ligand preparations, and the standard deviations have been calculated from the average of replicate experiments (Table 1). A casual look at the experimental data of Table 1 reveals that, except for a few instances, the stoichiometry of the enzyme–ligand complexes is close to unity. We believe the deviation in stoichiometry from unity is attributable to our inability to determine precisely the “intact” active site concentrations of the enzymes from different batches. We noted that if the experimental data were reanalyzed after adjusting the concentrations of the enzyme (based on the observed stoichiometries of the enzyme–ligand complexes from initial analyses), the derived thermodynamic parameters were not altered significantly. Such parameters were, however, altered when we deliberately fixed the stoichiometry of the enzyme–ligand complexes (without adjusting the enzyme concentrations) as being equal to 1. Hence, we opted to analyze all the microcalorimetric titration data presented herein as such without any preconceived bias.

Figure 2 shows the temperature dependence of ΔH° for the binding of 2-azaocetanoyl-CoA and octenoyl-CoA to the wild-type enzyme. Note that as the temperature increases, the magnitude of ΔH° decreases linearly, that is, it becomes more favorable. Such a pattern is suggestive of the classic

hydrophobic effect (Tanford 1980; Dill 1990; Baldwin 1986; Livingstone et al. 1991; Spolar et al. 1989; Privalov 1979). The solid lines are the linear regression analysis of the experimental data for the slope and intercept values of -0.627 ± 0.050 kcal/mole/K and 167 ± 14 kcal/mole, respectively. The corresponding parameters for the binding of octenoyl-CoA were found to be -0.526 ± 0.03 kcal/mole/K and 140 ± 9 kcal/mole, respectively. Of these, whereas the slopes serve as the measure of the heat capacity changes (ΔC_p°), the intercept values are the measures of the enthalpic changes at 0 K. From the data of Figure 2, it is apparent that the ΔC_p° value for the binding of 2-azaocetanoyl-CoA is 101 cal/mole/K more negative than that for the binding of octenoyl-CoA. According to the classic paradigm of the hydrophobic effect, the above difference would imply that the binding of 2-azaocetanoyl-CoA to the enzyme had a more pronounced hydrophobic effect than that of octenoyl-CoA (Edsall 1935; Tanford 1980). Such a deduction appeared unlikely because 2-azaocetanoyl-CoA is a more polar ligand than octenoyl-CoA.

It should be pointed out that the temperature dependence of ΔG° allowed us to determine the van't Hoff enthalpy ($\Delta H^\circ_{\text{vH}}$) and heat capacity (ΔC_p°) changes for the binding of 2-azaocetanoyl-CoA and octenoyl-CoA to the wild-type

Table 1. Temperature-dependence of thermodynamic parameters for the binding of 2-azaocetanoyl-CoA and octenoyl-CoA to the wild-type and E376Q mutant enzymes[#]

Enzyme + ligand	Temp. (K)	# Expt.	<i>n</i>	K_a ($\times 10^6 \text{ M}^{-1}$)	ΔH° (kcal/mole)	ΔG° (kcal/mole)	$T\Delta S^\circ$ (kcal/mole)
WT + Oce-CoA*	284.3	3	0.89 ± 0.03	2.6 ± 0.9	-9.5 ± 0.8	-8.3 ± 0.2	-1.2 ± 0.9
	287.5	3	0.93 ± 0.06	2.6 ± 1.0	-10.9 ± 2.1	-8.4 ± 0.3	-2.6 ± 2.4
	293.5	3	1.0 ± 0.02	1.3 ± 0.7	-13.3 ± 1.2	-8.2 ± 0.3	-5.1 ± 1.3
	298.2	3	0.98 ± 0.06	1.3 ± 0.5	-17.2 ± 1.6	-8.3 ± 0.2	-8.9 ± 1.8
	303.1	5	1.0 ± 0.1	1.6 ± 0.9	-19.6 ± 1.9	-8.5 ± 0.4	-11.1 ± 2.2
	308.2	4	1.2 ± 0.06	0.8 ± 0.4	-21.5 ± 1.3	-8.2 ± 0.3	-13.3 ± 1.6
WT + Aza-CoA	283.4	2	0.85 ± 0.004	6.9 ± 0.9	-10.7 ± 0.1	-8.9 ± 0.1	-1.8 ± 0.1
	288.3	2	0.83 ± 0.002	7.3 ± 1.5	-13.4 ± 0.2	-9.0 ± 0.1	-4.4 ± 0.3
	293.5	2	0.79 ± 0.03	4.2 ± 0.3	-17.4 ± 1.2	-8.9 ± 0.03	-8.5 ± 1.2
	298.1	2	0.85 ± 0.04	2.6 ± 0.7	-21.7 ± 0.8	-8.7 ± 0.2	-12.9 ± 0.9
	303.1	3	0.83 ± 0.04	1.9 ± 0.4	-23.1 ± 0.8	-8.7 ± 0.1	-14.4 ± 0.9
	308.1	3	0.79 ± 0.03	1.3 ± 0.2	-25.6 ± 0.9	-8.6 ± 0.1	-17.0 ± 0.9
E376Q + Oce-CoA	288.0	2	1.1 ± 0.01	7.0 ± 1.2	-8.0 ± 0.04	-9.0 ± 0.1	1.1 ± 0.1
	293.2	2	0.95 ± 0.05	9.2 ± 1.4	-11.3 ± 0.6	-9.3 ± 0.1	-1.9 ± 0.6
	298.0	2	1.1 ± 0.06	2.2 ± 1.4	-13.2 ± 0.6	-8.6 ± 0.4	-4.6 ± 1.1
	303.1	3	1.1 ± 0.07	2.9 ± 1.5	-15.9 ± 0.5	-8.9 ± 0.4	-6.9 ± 0.8
	308.1	3	1.3 ± 0.04	0.87 ± 0.6	-18.7 ± 0.1	-8.3 ± 0.5	-10.4 ± 0.4
E376Q + Aza-CoA	283.8	3	1.1 ± 0.03	3.7 ± 1.02	-5.7 ± 0.2	-8.5 ± 0.2	2.8 ± 0.4
	288.3	3	0.85 ± 0.02	7.5 ± 3.7	-8.7 ± 0.8	-9.0 ± 0.3	3.1 ± 0.9
	293.2	4	0.86 ± 0.04	4.8 ± 1.8	-12.2 ± 1.0	-8.9 ± 0.2	-3.3 ± 1.1
	298.1	2	0.96 ± 0.05	3.4 ± 0.3	-13.3 ± 0.7	-8.9 ± 0.1	-4.4 ± 0.7
	303.1	3	0.89 ± 0.02	3.9 ± 0.2	-16.3 ± 0.2	-9.1 ± 0.03	-7.2 ± 0.2
	308.2	3	0.91 ± 0.04	2.4 ± 0.20	-18.5 ± 0.5	-9.0 ± 0.1	-9.5 ± 0.5

[#]The stock concentrations of enzymes (wild-type and E376Q mutant) and ligands (octenoyl-CoA and 2-azaocetanoyl-CoA) in these experiments varied between 8 and 12 μM and 230 and 350 μM , respectively.

*From Peterson et al. (1998).

Fig. 2. Temperature dependence of ΔH° for the interactions of 2-aza-octanoyl-CoA and octenoyl-CoA with wild-type MCAD. The experimental data for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA are represented by the closed and open circles, respectively. The solid smooth lines are the linear regression analysis of the experimental data for slope (ΔC_p°) of -0.627 ± 0.050 kcal mole $^{-1}$ K $^{-1}$ and -0.526 ± 0.03 kcal mole $^{-1}$ K $^{-1}$, and intercept (at 0.0 K) of 167 ± 14 kcal/mole and 140 ± 9 kcal/mole for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA, respectively.

enzyme by analyzing the experimental data according to the integrated van't Hoff equation (Naghibi et al. 1995). Such analyses yielded the magnitudes of $\Delta H^\circ_{\text{vH}}$ and ΔC_p° to be -13.9 ± 1.6 kcal/mole and -0.415 ± 0.366 kcal/mole/K for the binding of 2-aza-octanoyl-CoA, and -9.0 ± 1.8 kcal/mole and 0.235 ± 0.432 kcal/mole/K for the binding of octenoyl-CoA to the enzyme site, respectively (figures not shown). Note that these values are substantially different from those obtained from the data of Figures 2 and 3 (see below). It is noteworthy that the ΔC_p° values derived from the integrated van't Hoff equation have unusually high standard errors, and in the case of enzyme-octenoyl-CoA interaction, its magnitude is positive. According to Chaires (1997), the origin of such discrepancies lies in the small changes in the heat capacity on binding of ligands to their cognate protein sites, and such changes cannot be reliably determined by van't Hoff analyses.

We considered whether or not the observed difference in the ΔC_p° values for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the wild-type enzyme was attributable to the carboxyl group of Glu-376 at the wild-type enzyme site. To probe this, we performed temperature-dependent thermodynamic studies for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the E376Q mutant enzyme (Fig. 3). The data in Figure 3 yielded the magnitudes of the ΔC_p° values for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the enzyme to be -0.511 ± 0.03 kcal/mole/K, and -0.520 ± 0.02 kcal/mole/K, respectively. Hence, unlike the marked difference in the ΔC_p° values for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the wild-type en-

Fig. 3. Temperature dependence of ΔH° for the interaction of 2-aza-octanoyl-CoA and octenoyl-CoA with E376Q mutant MCAD. The experimental data for 2-aza-octanoyl-CoA and octenoyl-CoA are represented by the closed and open circles, respectively. The solid smooth lines are the linear regression analysis of the experimental data for slope (ΔC_p°) of -0.511 ± 0.03 kcal mole $^{-1}$ K $^{-1}$ and -0.520 ± 0.02 kcal mole $^{-1}$ K $^{-1}$, and intercept (at 0.0 K) of 139 ± 10 kcal/mole and 142 ± 5.6 kcal/mole for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA, respectively.

zyme (see Fig. 2), the above parameter for the binding of 2-aza-octanoyl-CoA and octenoyl-CoA to the E376Q mutant enzyme is remarkably the same. Therefore, the ligand-dependent discriminations in the ΔH° and ΔC_p° values, as discerned with the wild-type enzyme, are abolished on Glu-376 $\rightarrow$ Gln mutation.

From the thermodynamic data in Table 1, it should be noted further that irrespective of the type of ligand or enzyme, the temperature-dependent decrease in ΔH° is compensated by the corresponding increase in the $T\Delta S^\circ$ value, such that the ΔG° practically remains constant over the entire temperature range. Clearly, like other enzyme–ligand interacting systems (Herron et al. 1986; Jin et al. 1993; Mukkur 1978; Sigurskjold et al. 1994), the enthalpy-entropy compensation effect is prevalent for the binding of both 2-aza-octanoyl-CoA and octenoyl-CoA to the wild-type and E376Q mutant enzymes, and its origin may lie in the weak interaction (presumably mediated via hydrogen bonding) between the enzyme–ligand species (Dunitz 1995).

Model building studies and the changes in the water accessible surface areas on enzyme–ligand interactions

The molecular model building studies were performed by using the coordinates of the dimeric form of pig liver MCAD (obtained from the Brookhaven Protein Data Bank) in the absence (file name: pdb3mdd.pdb, to be referred herein as the apo enzyme) and the presence of C₈CoA (file

name: 3pdbmde.pdb the holo enzyme). A casual look at these coordinate files revealed that the number of crystallographically identifiable water molecules in the dimeric forms of the apo and holo structures were 166 and 182, respectively. Evidently, the binding of C_8CoA to the apo enzyme site accompanies incorporation of 16 additional water molecules, and most of these water molecules were confined either to the subunit contact region or to the coenzyme-A binding region. If the above information is realistic (particularly because many "mobile" water molecules remain undetected by the X-ray crystallographic technique), it would strengthen our overall thermodynamic conclusion (see Discussion).

Figure 4 shows the location of water molecules within a 10 Å perimeter of the C_8CoA binding site in both apo and holo structures of the enzyme. This was accomplished by superimposition of the backbones of the above protein structures followed by interfacing the water molecules around the holo-enzyme bound C_8CoA via the aid of the Insight-II (98) software. Selected water molecules from the apo (light circle) and holo (dark circle) enzyme structures are labeled as A and H, respectively (Fig. 4), following their residue numbers (found in the respective coordinate files). Note that whereas most of the water molecules occupy the same or similar positions in both apo and holo structures, some water molecules are displaced from their original po-

sition in the apo structure to a new position in the holo structure. In Figure 4, the water molecule 803H appears to be a new water molecule in the holo structure of the enzyme. It is noteworthy that four water molecules, 802A to 805A, present in the apo structure (in place of the tail end region of C_8CoA) is removed on binding of the ligand (Kim et al. 1993). Because no high resolution X-ray crystallographic data are available for either wild-type enzyme-2-aza-octanoyl-CoA complex or of E376Q mutant enzyme in the absence or presence of any of the ligands used herein, it would be difficult to predict, a priori, the distribution of water molecules in these unknown enzyme-ligand structures. However, on energy minimization of different enzyme-ligand complexes (see below), it has been noticed that the water molecule, 803H, appear to move (~ 1 Å) closer to the amide nitrogen of 2-aza-octanoyl-CoA vis-à-vis its position in the octenoyl-CoA bound structure of the wild-type enzyme (data not shown). Based on these cursory observations, it is tempting to speculate that in the case of the wild-type enzyme-2-aza-octanoyl-CoA complex, a water molecule (possibly 803H) is involved in the hydrogen bonding between the carboxyl group of Glu-376 and the amide nitrogen of the ligand (see Discussion).

To ascertain the influence of $\alpha\text{-CH} \rightarrow \text{NH}$ substitution on the protein and/or ligand conformations, we performed the model building studies of the enzyme-2-aza-octanoyl-CoA

Fig. 4. Location of water molecules within 10 Å perimeter of the enzyme-bound C_8CoA . The water molecules present in the pdb3mde.pdb (holo structure) and pdb3mdd.pdb (apo structure) data files are shown by the light and dark circles, respectively. Some of the water molecules have been denoted by their residue numbers. The suffixes -A and -H represent the water molecules of the apo and holo structures, respectively.

and enzyme-octenoyl-CoA complexes, using the structural coordinates of pig liver MCAD- C_8 CoA complex (Kim et al. 1993). Because the electron density maps of both octanoyl-CoA and octenoyl-CoA could be fitted at the ligand-binding site, the latter CoA-derivatives are cumulatively referred to as C_8 -CoA (Kim et al. 1993). However, before undertaking the energy minimization approach (as described in the Materials and Methods section), we introduced an α - β double bond in C_8 -CoA to generate octenoyl-CoA and retained all the crystallographically identified water molecules in the protein structure. This was followed by the minimization of the enzyme-octenoyl-CoA complex (along with the water molecules) using the consistent valence force field (CVFF) by the aid of Discover-98 software (Dauber-Osguthorpe et al. 1988). The overall process was accomplished via a combination of steepest descent and conjugate gradient approaches, until the maximum derivative on the order of 10^{-4} to 10^{-5} kcal/mole/Å was achieved. To obtain the model of the enzyme-2-azaoctanoyl-CoA complex, the structure of octenoyl-CoA (from the energy minimized coordinates of the enzyme-octenoyl-CoA complex) was modified to generate 2-azaoctanoyl-CoA, and the resultant enzyme-ligand structure (along with the water molecules) was subjected to further energy minimization by adopting the above protocol. The Glu-376 $\rightarrow$ Gln mutation was created in both enzyme-octenoyl-CoA and enzyme-2-azaoctanoyl-CoA energy-minimized complexes before subjecting the resultant complexes to final energy minimizations. In the case of

wild-type enzyme, the rms deviation (for the protein backbone) between the minimized enzyme-octenoyl-CoA and the enzyme-2-azaoctanoyl-CoA structures was 0.0017 Å, and the root mean square deviation (RMSD) between the enzyme-bound octenoyl-CoA and 2-azaoctanoyl-CoA moieties was 0.25 Å. Figure 5 shows the spatial relationship among the enzyme-bound FAD, CoA-ligands, and Glu-376 residue within the energy-minimized structures of wild-type enzyme-octenoyl-CoA and enzyme-2-azaoctanoyl-CoA complexes. A casual look at the structural data in Figure 5 reveals the following features: (1) Whereas the fatty acid regions of octenoyl-CoA and 2-azaoctanoyl-CoA show some configurational differences, the coenzyme-A regions of both these ligands are essentially identical. (2) The 2'-ribityl hydroxyl and the isoalloxazine ring of FAD move toward the CoA ligand in the case of the enzyme-2-azaoctanoyl-CoA (vis-à-vis the enzyme-octenoyl-CoA) complex. (3) The distances between the carbonyl oxygen of octenoyl-CoA and 2'-ribityl hydroxyl group of FAD and the amide nitrogen of Glu-376 (the interactions responsible for the polarization of the carbonyl group) are lower as compared to those obtained with 2-azaoctanoyl-CoA. These features lead to the suggestion that the carbonyl group of octenoyl-CoA is more polarized than that of 2-azaoctanoyl-CoA. (4) The α -NH group of 2-azaoctanoyl-CoA (vis-à-vis the α -CH of octenoyl-CoA) moves toward the carboxyl oxygens (OE1 and OE2) of Glu-376. This is presumably an indication that the negatively charged carboxyl group of

Fig. 5. Spatial relationships among selected residues in the energy-minimized structures of the wild-type MCAD-ligand complexes. The relative positions of Glu-376 and the enzyme-bound FAD and CoA-ligands (2-azaoctanoyl-CoA and octenoyl-CoA) are shown. Note that the 2'-ribityl (hydroxyl) oxygen and the isoalloxazine ring of FAD move toward the CoA-ligand in the case of the enzyme-2-azaoctanoyl-CoA vis-à-vis the enzyme-octenoyl-CoA complex. Besides, the α -NH group of 2-azaoctanoyl-CoA moves toward the carboxyl oxygens (OE1 and OE2) of Glu-376 as compared to the α -CH group of octenoyl-CoA.

Glu-376 electrostatically attracts the partially positively charged α -NH group of 2-azaocetanoyl-CoA.

By using the energy minimized coordinates of the wild-type and E376Q mutant enzyme-octenoyl-CoA and enzyme-2-azaocetanoyl-CoA complexes (along with the associated water molecules), we could calculate the changes in the water-accessible polar and nonpolar surface areas on binding the corresponding ligands to their enzyme sites (Lee and Richards 1971) and predict the heat capacity changes for the enzyme–ligand complexes according to empirical relationships of Spolar et al (1992) and Murphy et al. (1993). The results are summarized in Table 2. It should be noted that because of the presence of the α -NH group, the water-accessible polar and nonpolar surface areas of 2-azaocetanoyl-CoA are slightly higher and lower, respectively, than those obtained with octenoyl-CoA. A small variation in the water-accessible surface areas of the apo (in the absence of any bound CoA-ligand) and holo (in the presence of bound CoA-ligand) enzymes are presumably because of small changes in enzyme conformation during the course of the energy minimizations of the corresponding enzyme–

ligand complexes. The magnitudes of ΔA_p (changes in the water-accessible polar surface areas) and ΔA_{np} (changes in the water-accessible nonpolar surface areas) were used to predict the heat capacity changes for the binding of octenoyl-CoA and 2-azaocetanoyl-CoA to the enzyme according to the following relationship:

$$\Delta C_p^\circ = C_{p,p} \Delta A_p + C_{p,np} \Delta A_{np}$$

in which $C_{p,p}$ and $C_{p,np}$ are the heat capacity functions for the changes in the polar and nonpolar surface areas, respectively. The magnitudes of $C_{p,p}$ and $C_{p,np}$ have been proposed to be -0.14 and 0.32 cal/mole/K/Å², respectively, by Spolar et al (1992), and -0.26 and 0.45 cal/mole/K/Å², respectively, by Murphy et al (1993).

Taking into account the magnitudes of ΔA_p and ΔA_{np} for the binding of 2-azaocetanoyl-CoA and octenoyl-CoA to the wild-type enzyme (Table 2), the corresponding ΔC_p° values for the binding of the above ligands can be predicted to be -0.172 and -0.178 kcal/mole/K, respectively, according to Spolar et al. (1992), and -0.202 and -0.212 kcal/mole/K,

Table 2. Changes in the solvent-accessible surface areas upon binding of 2-azaocetanoyl-CoA and octenoyl-CoA to pig liver wild-type and E376Q mutant MCADs, and the predicted ΔC_p° values

Enzyme/Ligand	A_p (Å ²)	A_{np} (Å ²)	ΔC_p° (kcal/mole/K) (Spolar et al 1992)	ΔC_p° (kcal/mole/K) (Murphy et al. 1993)
Wild-type MCAD-2-Azaocetanoyl-CoA				
Wild-type MCAD (without ligand)	7605	10168		
2-Azaocetanoyl-CoA	594	527		
Wild-type MCAD-2-azaocetanoyl-CoA	7567	9881		
$\Delta A_p = -632$ Å ² , $\Delta A_{np} = -814$ Å ²				
Predicted ΔC_p° for wild-type MCAD-2-azaocetanoyl-CoA			-0.172	-0.202
Wild-type MCAD-Octenoyl-CoA				
Wild-type MCAD (without ligand)	7593	10174		
Octenoyl-CoA	582	545		
Wild-type MCAD-Octenoyl-CoA	7555	9890		
$\Delta A_p = -620$ Å ² , $\Delta A_{np} = -829$ Å ²				
Predicted ΔC_p° for wild-type MCAD-octenoyl-CoA			-0.178	-0.212
E376Q MCAD-2-Azaocetanoyl-CoA				
E376Q MCAD (without ligand)	7600	10170		
2-Azaocetanoyl-CoA	593	526		
E376Q MCAD-2-Azaocetanoyl-CoA	7561	9879		
$\Delta A_p = -632$ Å ² , $\Delta A_{np} = -817$ Å ²				
Predicted ΔC_p° for E376Q MCAD-2-Azaocetanoyl-CoA			-0.173	-0.203
E376Q MCAD-Octenoyl-CoA				
E376Q MCAD (without ligand)	7598	10163		
Octenoyl-CoA	581	545		
E376Q MCAD-Octenoyl-CoA	7552	9873		
$\Delta A_p = -627$, $\Delta A_{np} = -835$				
Predicted ΔC_p° for E376Q MCAD-Octenoyl-CoA			-0.179	-0.213

The ΔC_p° values for the binding of 2-azaocetanoyl-CoA and octenoyl-CoA to the wild-type enzyme were determined to be -0.627 ± 0.05 kcal mole⁻¹ K⁻¹ and -0.526 ± 0.03 kcal mole⁻¹ K⁻¹, respectively. The ΔC_p° values for the binding of above ligands to the E376Q mutant enzymes were determined to be -0.511 ± 0.03 kcal mole⁻¹ K⁻¹ and -0.520 ± 0.02 kcal mole⁻¹ K⁻¹, respectively.

respectively, according to Murphy et al. (1993). Likewise, the ΔC_p° values for the binding of 2-azaoctanoyl-CoA and octenoyl-CoA to the E376Q mutant enzymes could be predicted to be -0.173 and -0.179 kcal/mole/K, respectively, according to Spolar et al. (1992), -0.203 and -0.213 kcal/mole/K, respectively, according to Murphy et al. (1993). It should be noted that these predicted values are considerably smaller than those obtained experimentally (see footnote in Table 2). Besides, the predicted values for the individual enzyme–ligand complexes are not as diverse as those determined experimentally. Clearly, the experimentally determined ΔC_p° values for the binding of the above ligands to both wild-type and E376Q mutant enzymes are not accountable on the basis of the changes in the water-accessible surface areas of interacting species. A qualitatively similar conclusion has been obtained for the binding of different types of ligands to their cognate enzyme sites (Holdgate et al. 1997; Rowe et al. 1998; Ladbury et al. 1994; Raman et al. 1995; Jin et al. 1993; Guinto and Di Cera 1996; Lundbak et al. 2000). The plausible reasons for such discrepancies are elaborated in the following section.

Discussion

The experimental data presented in the previous section lead to the following conclusions: (1) The binding affinities of both wild-type and Glu-376 $\rightarrow$ Gln (E376Q) mutant medium-chain acyl-CoA dehydrogenases (MCAD) for 2-azaoctanoyl-CoA and octenoyl-CoA are essentially identical. Hence, neither the α -CH $\rightarrow$ NH substitution in C₈-CoA nor the removal of a net negative charge from the enzyme site residue Glu-376 (i.e., via E376Q mutation) influences the ΔG° value of the enzyme–ligand complexes. (2) The ΔH° value for the binding of 2-azaoctanoyl-CoA to the wild-type enzyme is 4.5 kcal/mole more negative (favorable) than that for the binding of octenoyl-CoA, suggesting that the favorable enthalpic contribution for the binding of 2-azaoctanoyl-CoA is offset by the unfavorable entropic contribution by an equal magnitude. (3) The discriminatory feature in ΔH° for the binding of 2-azaoctanoyl-CoA and octenoyl-CoA to the enzyme is abolished on E376Q mutation; both these ligands yield ΔH° of ~ -13 kcal/mole on binding to the E376Q mutant enzyme site. (4) Whereas the heat capacity change (ΔC_p°) for the binding of 2-azaoctanoyl-CoA to the wild-type enzyme is 101 cal/mole/K more negative than that of octenoyl-CoA, the ΔC_p° values for the binding of the above ligands to the E376Q mutant enzyme are essentially identical (i.e., ~ -0.52 kcal/mole/K). However, in neither case could the experimentally determined ΔC_p° value be predicted on the basis of the changes in the water-accessible polar and nonpolar surface areas.

The comparative microcalorimetry-based ligand binding studies presented herein emphasize the importance of detailed thermodynamic evaluations to discern the molecular

basis of enzyme–ligand interactions. The physical forces that dictate enzyme–ligand complementarity remains elusive if only binding constants of the enzyme–ligand complexes are determined. The fact that the ΔG° values for the binding of 2-azaoctanoyl-CoA and octenoyl-CoA to the wild-type and E376Q mutant enzymes are equal provides no information about the influence of either α -CH $\rightarrow$ NH substitution or the elimination of the negative charge of Glu-376 in stabilizing the corresponding enzyme–ligand complexes. The role of the above variants starts emerging on comparison of the ΔH° and ΔC_p° values of the enzyme–ligand complexes.

Because the ΔH° value for the binding of 2-azaoctanoyl-CoA to the wild-type enzyme is 4.5 kcal/mole more negative (favorable) than that of octenoyl-CoA, and the above parameter remains invariant for the binding of both these ligands to the E376Q mutant enzyme (~ -13 kcal/mole), it implies that the coulombic interaction in part plays an important role in stabilizing the enzyme–ligand complexes (Gilson and Honig 1988; Xiao and Honig 1999). The structural, spectroscopic, and model building data all suggest that the carbonyl groups of octenoyl-CoA and 2-azaoctanoyl-CoA are polarized on binding to the enzyme sites, resulting in generation of an initial partial positive charge at the carbonyl carbon, which can be delocalized to the neighboring atoms (Engel 1990; Thorpe and Kim 1995; Kim et al. 1993; Srivastava and Peterson 1998; Johnson et al. 1995; Nishina et al. 1992). In the case of 2-azaoctanoyl-CoA, the partial positive charge can be stabilized at the α -NH center, but in the case of octenoyl-CoA, it can at best be stabilized at the γ carbon center. Given that both carboxyl oxygens (i.e., OE1 and OE2) of Glu-376 are closer to the α than the γ centers (Fig. 5), the columbic interaction between the negatively charged carboxyl group of Glu-376 and the partial positive charges of the above ligands is likely to be more pronounced in the case of 2-azaoctanoyl-CoA as compared to octenoyl-CoA. This may be responsible for the 4.5 kcal/mole enthalpic advantage for the binding of 2-azaoctanoyl-CoA (vis-à-vis octenoyl-CoA) to the wild-type enzyme. Because Glu-376 $\rightarrow$ Gln mutation eliminates the net negative charge of the carboxyl group of Glu-376, the discriminatory contribution of ΔH° for the binding of the above ligands to the enzyme is lost, yielding a nearly identical ΔH° (~ -13 kcal/mole).

To substantiate or refute the above simplistic explanation, we considered the possibility of whether or not the higher enthalpic changes during the course of titration of the wild-type enzyme with 2-azaoctanoyl-CoA was because of slow oxidase activity (Johnson et al. 1992; Johnson et al. 1994; McFarland et al. 1982) of the enzyme. Such a possibility seemed likely because we observed previously that the titration of human liver enzyme with octanoyl-CoA yielded about 10 kcal/mole more negative ΔH° value than that obtained with octenoyl-CoA (Srivastava et al. 1997). Further-

more, the experimental factor (e.g., the substrate type), which enhanced the rate of the oxidase reaction of MCAD, was also found to yield a more negative ΔH° value. This was further supported by an independent demonstration that the yeast acyl-CoA oxidase catalyzed conversion of hexanoyl-CoA to hexenoyl-CoA, coupled with the reduction of O_2 to H_2O_2 , was an exothermic process (unpubl. results). If 2-azaocanoyl-CoA underwent a slow oxidase reaction during the course of the enzyme–ligand titration, it would yield a more negative ΔH° value, and the magnitude of the latter would increase with an increase in temperature, yielding a higher ΔC_p° value (as observed experimentally). To probe this possibility, we measured the amount of H_2O_2 (Gopalan and Srivastava 1997) formed during the time regime of the microcalorimetric titration of MCAD by 2-azaocanoyl-CoA. The experimental data provided no evidence of formation of H_2O_2 on incubation of 2-azaocanoyl-CoA with either wild-type or E376Q mutant enzyme for up to 3 to 4 hr. Hence, the enthalpic contribution for the binding of 2-azaocanoyl-CoA is not because of some unforeseen artifact of the enzyme-catalyzed oxidase reaction.

A quantitative evaluation of the ΔG° and ΔH° values for the binding of 2-azaocanoyl-CoA and octenoyl-CoA to the wild-type and E376Q mutant enzymes reveals that the binding of these ligands is primarily enthalpically driven. Depending on the type of ligand, the favorable enthalpic contribution is offset by unfavorable entropic contributions of different magnitudes, yielding nearly identical ΔG° values for all the enzyme–ligand complexes. As discussed by Dunitz (1995), such an enthalpy-entropy compensation is a common property for the binding of ligands to proteins (Herron et al. 1986; Jin et al. 1993; Mukkur 1978; Sigurskjold et al. 1994; Srivastava et al. 1997; Peterson et al. 1998b; Qin and Srivastava 1998), and its prevalence is believed to be dictated by weak interactions (often mediated via the hydrogen bonding) between the cognate species (Dunitz 1995).

It is evident that the entropic loss for the binding of 2-azaocanoyl-CoA to the wild-type enzyme is maximum (i.e., -12.9 kcal/mole), followed by that for the binding of octenoyl-CoA to the wild-type enzyme (-8.9 kcal/mole), and it is minimum for the binding of these ligands (with equal magnitudes) to the E376Q mutant enzyme. At this point, without taking into account of the ΔC_p° values, the compensatory entropic contributions (for the individual enzyme–ligand complexes) can be attributed to the loss in rotational and translational freedoms of the interacting species. However, such a simplistic explanation fails to account for the temperature dependence of enthalpic changes and the relative magnitudes of the ΔC_p° values for the individual enzyme–ligand complexes (see below).

Following the proposal of Edsall (1935) that the solvation/desolvation of nonpolar molecules in aqueous phase is the major contributor of the heat capacity changes (ΔC_p°), sev-

eral investigators provided molecular interpretation of the hydrophobic effect for several processes such as partitioning of nonpolar molecules between organic and aqueous phases, protein unfolding, binding of ligands to proteins, and others (Tanford 1980; Privalov 1979; Spolar et al. 1992; Murphy et al. 1993; Makhatadze and Privalov 1990, 1993). A large negative ΔC_p° value for the protein–ligand interaction has been attributed to the predominance of the hydrophobic (yielding negative ΔC_p°) over the polar (yielding positive ΔC_p°) interactions (Livingstone et al. 1991; Spolar et al. 1989; Privalov 1979; Makhatadze and Privalov 1990, 1993). According to the classic view of the hydrophobic effect involving the enzyme–ligand interaction, the water molecules surrounding the hydrophobic region of the ligand, as well as those resident within the enzyme site pocket, are stripped out/excluded to the exterior bulk phase. These water molecules have low entropy and high heat capacity, and thus the release of such water molecules on enzyme–ligand interaction results in an increase in entropy and a decrease in heat capacity (Tanford 1980; Dill 1990; Rowe et al. 1998).

The magnitude of ΔC_p° for protein unfolding, as well as in selected cases of enzyme–ligand interactions, has been predicted successfully on the basis of the changes in the water-accessible polar and nonpolar surface areas of both enzyme and ligand species (Spolar et al. 1992; Murphy et al. 1993). If the structural data of the enzyme–ligand complexes were known to atomic resolutions, then their ΔC_p° values could be predicted easily by determining the solvent-accessible surface areas of the individual enzymes, ligands, and the enzyme–ligand complexes (Peterson et al. 1998b). When such an approach was undertaken for predicting the ΔC_p° values for the binding of 2-azaocanoyl-CoA and octenoyl-CoA to wild-type and E376Q mutant enzymes (see Table 2), the calculated ΔC_p° values were found to be considerably different from those obtained experimentally. In fact, the predicted ΔC_p° values were more or less the same for binding of the above ligands to the wild-type and mutant enzymes (Table 2). This is in marked contrast to the observation that the binding of a more polar ligand, namely, 2-azaocanoyl-CoA, to the wild-type enzyme yields 101 cal/mole/K more negative ΔC_p° value than that obtained for the binding of a less polar ligand, octenoyl-CoA. However, the ΔC_p° values for the binding of both these ligands to the E376Q mutant enzyme were essentially the same. Clearly, these results are not explainable in light of the classic paradigm of the hydrophobic effect, according to which the magnitude of ΔC_p° (for the binding of ligands to their cognate enzyme sites) should be related directly to the extent of hydrophobic interactions (Tanford 1980; Dill 1990). Several investigators have arrived at a similar conclusion in explaining protein–ligand, membrane–ligand, and protein–protein interaction data (Holdgate et al. 1997; Rowe et al. 1998; Ladbury et al. 1994; Raman et al. 1995;

Jin et al. 1993; Guinto and Di Cera 1996; Lundbak et al. 2000).

Freire and his collaborators (Gomez et al. 1995) have pointed out that the absolute heat capacity of a protein is comprised of the contributions from (1) the covalent primary structure (containing the contributions from vibrational frequencies, arising from the stretching and bending modes of each valence bond and internal rotations), (2) the noncovalent secondary and tertiary structures, and (3) the hydration/dehydration of individual atoms. Of these, although the primary (covalent) structural terms contribute maximally to the absolute heat capacity (C_p) of proteins, the changes in heat capacity (ΔC_p) on protein-folding and/or the protein–ligand interactions are dictated primarily by the hydration/dehydration terms. However, in the case of protein–ligand interactions, sometimes it may be difficult to sort the contributions of one heat capacity term from the other. For example, if the ligand binding alters the protein conformation, and the latter proceeds in concomitance with the changes in the hydration/dehydration properties of certain residues, it would be difficult to assign unambiguously the heat capacity contributions from the noncovalent versus hydration terms (Ayala et al. 1995; Ladbury et al. 1995; Lundback et al. 2000). Likewise, if the ligand binding accompanies sequestration of water molecules (which are highly ordered and possess reduced mobility) in the vicinity of the ligand binding site, the observed ΔC_p value would be different from that predicted on the basis of hydration/dehydration features of the ligand and enzyme sites (Guinto and Di Cera 1996; O'Brien et al. 1998)). A qualitatively similar conclusion can be drawn if the protein ligand interaction is mediated via a water molecule (see below). Evidently, the generalization derived from solvation/desolvation of small molecular weight compounds, as well as from the protein folding/unfolding data, may not be universally applicable for predicting the ΔC_p values of the protein–ligand interacting systems.

The question arises as to what other structural feature or features contribute to the negative magnitudes of ΔH° and ΔC_p on protein–ligand interactions, such as described herein. Although there is no definitive answer to this question, circumstantial evidence corroborates the notion that the origin of large negative ΔH° and ΔC_p values lies partly in the solvent-mediated interaction between the charged species (Ladbury 1996; Morton and Ladbury 1996; Tame et al. 1996; Sleight et al. 1999). Ladbury (1996) argues that the favorable enthalpic gain attributable to the water mediated (protein–ligand) interaction often overweighs the entropic penalty because of restrictions on the degree of freedom of the water molecule, thus resulting in a favorable free energy of interaction. In their detailed studies, Holdgate et al. (1997) described that the Arg136 $\rightarrow$ His mutation in DNA gyrase creates a cavity that sequesters a water molecule, which mediates the interaction between novobiocin and the

enzyme via hydrogen bonding. Such an arrangement yields large negative ΔH° and ΔC_p values. Likewise, Bhat et al. (1994) reported that the water molecules entrapped between the hen egg white lysozyme antigen–antibody interfaces promote their interactions and yield large negative ΔC_p values. In light of these observations, it is tempting to speculate that the discriminatory features in thermodynamic parameters for the binding of 2-azaoctanoyl-CoA and octenoyl-CoA to the wild-type enzyme are because of solvent-mediated interaction (albeit of different magnitudes) between the negatively charged carboxyl group of Glu-376 and the partially positively charged ligand species. We propose that the water molecules, which remain bound to the enzyme site phase, are reorganized on binding of ligands, and depending on their orientation, they bridge between the charged ligand and enzyme site residues (see Results). This may lead to a decrease in the heat capacity of water molecules on transition from their (initially resident) hydrophobic phase (possessing higher heat capacity) to the hydrogen-bonded phase involving the charged residues (possessing lower heat capacity). Because the E376Q mutation abolishes the net negative charge from the enzyme site, the solvent-mediated charge–charge interaction is not as pronounced as observed with the wild-type enzyme, and thus the ligand-dependent discrimination in ΔH° and ΔC_p values for the binding of 2-azaoctanoyl-CoA and octenoyl-CoA is abolished. We are currently testing this hypothesis by using Glu-376 $\rightarrow$ Asp mutation of MCAD (Peterson et al. 1998a), which creates an 18 Å³ cavity (Peterson and Srivastava 2000) and has the potential to entrap a water molecule, on the magnitudes of ΔH° and ΔC_p for the binding of above ligands, and we will report on these findings subsequently.

Materials and methods

Coenzyme A was purchased from Life Science Resources. Trans-2-octenoic acid was purchased from Pfaltz and Bauer; n-hexyl isocyanate was purchased from Acros. All other reagents were of analytical grade. Unless otherwise stated, all experiments were performed in 50 mM potassium phosphate at pH 7.6, containing 10% glycerol and 0.3 mM EDTA.

The expression system for the Glu-376 $\rightarrow$ Gln mutation of MCAD was obtained initially as a gift from Dr. Sandro Ghisla's laboratory. Subsequently, this and a variety of other site-specific mutations of MCAD were created in our laboratory using the Quick Change site-directed mutagenesis kit from Stratagene. As will be elaborated elsewhere, the overall procedure involved the annealing of two (5' and 3') synthetic primers containing the mutation site on to the double-stranded expression vector of wild-type MCAD, followed by extension of the primers by thermal cycling using PFU DNA polymerase. The reaction products were treated with Dpn-I (specific for methylated DNA) to cleave the parental (nonmutated) DNA template. The nicked vector DNA, incorporating the desired mutation, was transformed into *Epicurian Coli* XL1-Blue super-competent cells, and the selection was performed via antibiotic (ampicillin) resistance. Finally, the plasmid contain-

ing the desired mutations was transformed into *E. coli* TG1 cells for expression.

The expression and purification of wild-type and Glu-376 → Gln mutant human liver MCADs were performed as described by Peterson et al. (1995). The wild-type enzyme was assayed by monitoring the reduction of ferricinium hexafluorophosphate (FcPF₆) at 300 nm ($\epsilon = 4.3 \text{ mM}^{-1}\text{cm}^{-1}$; Powell and Thorpe 1988) in a reaction mixture containing 100 μM octanoyl-CoA and 350 μM FcPF₆. The A_{280}/A_{450} ratio of the purified enzyme was ~5. Glu-376 → Gln mutant enzyme was essentially inactive. The extinction coefficients of both wild-type and mutant enzymes were taken to be $15.4 \text{ mM}^{-1}\text{cm}^{-1}$ at 446 nm (Thorpe et al. 1979).

Octenoyl-CoA was prepared by the mixed anhydride method of Bernert and Sprecher (1977) and purified on a C₁₈ reverse-phase, high pressure liquid chromatography (HPLC) column, equilibrated with 20 mM potassium phosphate at pH 7.0, and eluted with an increasing gradient of methanol as described by Kumar and Srivastava (1994). The ligand, 2-azaoctanoyl-CoA, was prepared essentially as described by Trievel et al. (1995). Briefly, a solution of n-hexyl isocyanate dissolved in THF was added, under a nitrogen stream, to a solution of coenzyme A in 0.25 M NaHCO₃. The mixture was stirred under nitrogen for 30 min, after which the pH was adjusted to 5.5 with acetic acid. The products were purified on a C₁₈ reverse-phase HPLC column equilibrated with 20 mM ammonium acetate at pH 5.5, developed with an increasing gradient of methanol. All ligands were stored as lyophilized powders and eluted as a single peak at 260 nm. The extinction coefficients of octenoyl-CoA and 2-azaoctanoyl-CoA were taken to be $20.4 \text{ mM}^{-1}\text{cm}^{-1}$ and $16.0 \text{ mM}^{-1}\text{cm}^{-1}$ at 258 nm and 260 nm, respectively (Trievel et al. 1995; Kumar and Srivastava 1994).

Isothermal titration microcalorimetry

The isothermal titration microcalorimetry experiments were performed on an MCS isothermal titration microcalorimeter from Microcal as described by Srivastava et al (1997). The sample cell was filled with 1.8 mL (1.36 mL effective volume) of the buffer (control) or enzyme solution (experiment). The injector was filled with 250 μL of the appropriate ligand. The titration was initiated by a preliminary first injection of 1 μL followed by 59 injections of 4 μL each. During the experiment, the enzyme solution was stirred at a constant rate of 400 rpm.

All calorimetric data were presented after correction for the background as described previously (Srivastava et al. 1997). The experimental data were analyzed according to Wiseman et al. (1989).

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

Molecular model-building studies

All the molecular model building studies were performed on a Silicon Graphics (SGI)-O2 molecular modeling workstation with the aid of Insight-II (98), Biopolymers-98, Homology-98, and Discover-98 software developed by Molecular Simulations. The coordinates for the X-ray crystallographic structure of pig liver medium chain acyl-CoA dehydrogenase in the absence

(pdb3mdd.pdb) and presence of C₈-CoA (pdb3mde.pdb) were downloaded from the Brookhaven Protein Data Bank. The structural data of these proteins were manipulated and displayed with the aid of Insight-II. All modeling studies were performed in the presence of the crystallographically identified water molecules in the protein structures. The enzyme-bound C₈-CoA structure was modified to adopt the structures of octenoyl-CoA and 2-azaoctanoyl-CoA with the help of the Biopolymer module of Insight-II. The Glu-376 → Gln mutation was constructed by replacing the side chain of Glu-376 with Gln with the aid of Homology-98 software.

The energy minimizations of the enzyme-ligand complexes were performed using the consistent valence force field (CVFF) with the aid of Discover-98 software (Dauber-Osguthorpe et al. 1988). The overall protocol involved a combination of the steepest descent and conjugate gradient approach via the following sequence of steps: (1) By fixing all atoms, except for hydrogen, the appropriate structure was subjected to 200 iterations of steepest descent followed by 100 iterations of conjugate gradient until a derivative of 1 kcal/mole/Å was achieved. (2) By fixing the protein backbone, FAD, and CoA-ligands, 2000 iterations were performed via the above minimization steps until a derivative of 0.01 kcal/mole/Å was achieved. (3) Finally, by restraining the backbone structure, the energy minimization (15,000 iterations) was performed via the conjugate gradient approach until an absolute derivative in the range of 10^{-4} to 10^{-5} kcal/mole/Å was achieved.

Strategically, the wild-type-octenoyl-CoA complex was first minimized followed by changing the octenoyl-CoA structure to 2-azaoctanoyl-CoA and reminimization of the resultant complex. The Glu-376 → Gln mutation was created in the minimized wild-type enzyme-ligand complexes before subjecting the corresponding structures to further energy minimization via the above sequence of steps. The maximum derivative of all the minimized structures decreased to a level of 10^{-4} to 10^{-5} kcal/mole/Å, which is considered to be ideal for the globally minimized structure of the protein-ligand complexes of comparable dimensions (Roberts et al. 1986).

Using the energy-minimized structures of the individual enzyme-ligand complexes, we determined the solvent-accessible nonpolar and polar surface areas of the enzymes in the absence and presence of bound ligands, and those of the isolated ligands were determined according to Lee and Richards algorithm (Lee and Richards 1971), using a probe with a radius 1.4 Å via the ProState module of Homology-98 (Molecular Simulations).

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