Energetic Consequences of Accommodating a Bulkier Ligand at the Active Site of Medium Chain Acyl-CoA Dehydrogenase by Creating a Complementary Enzyme Site Cavity†

Karen M. Peterson and D. K. Srivastava*

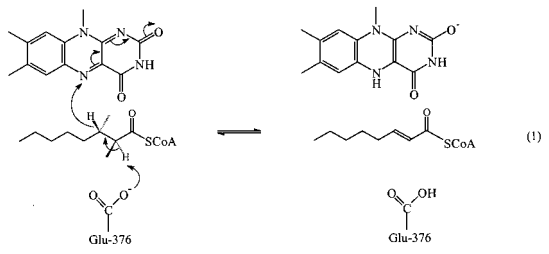
Department of Biochemistry and Molecular Biology, North Dakota State University, Fargo, North Dakota 58105

Received June 8, 2000; Revised Manuscript Received August 9, 2000

ABSTRACT: The substitution of the C═O by the C═S group in 2-azaoctanoyl-CoA increases the volume of the ligand by 11 Å^3, and the excision of a methylene group from Glu-376, via Glu-376 → Asp (E376D) mutation in medium chain acyl-CoA dehydrogenase (MCAD), creates a complementary cavity of 18 Å^3 dimension, just opposite to the ligand's carbonyl group. We investigated whether the newly created cavity would facilitate accommodation of the bulkier (C═O → C═S substituted) ligand within the active site of the enzyme. To ascertain this, we determined the binding affinity and kinetics of association and dissociation of 2-azaoctanoyl-CoA and the C═O → C═S substituted ligand, 2-azadithiooctanoyl-CoA, involving the wild-type and Glu-376 → Asp mutant enzymes. The experimental data revealed that the binding of 2-azadithiooctanoyl-CoA to the wild-type enzyme was energetically unfavorable as compared to 2-azaoctanoyl-CoA. However, such an energetic constraint was alleviated for the binding of the former ligand to the E376D mutant enzyme site. A detailed account of the free energy and enthalpic profiles for the binding of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and Glu-376 → Asp mutant enzymes throws light on the flexibility of the enzyme site cavity in stabilizing the ground and transition states of the enzyme−ligand complexes.

In pursuit of delineating the structural−functional relationships during the course of the medium chain acyl-CoA dehydrogenase (MCAD)[1] catalyzed reactions (*1*−*9*), we became interested in investigating the molecular basis of the enzyme−ligand complementarity. Due to the lack of strict substrate specificity of the enzyme for different chain lengths of acyl-CoAs (*10*−*12*), as well as its ability to bind differently substituted ligands (*1*, *13*−*17*), the variation in the ligand structure on the kinetic and thermodynamic properties of the enzyme could be easily investigated. In certain cases, such studies have been complemented by creating site-specific mutations in the vicinity of the ligand binding site of the enzyme (*9*, *17*−*19*).

The catalytic cycle of MCAD is initiated by the abstraction of the α-proton from the acyl-CoA substrate by the carboxyl group of Glu-376, followed by the transfer of the β-hydrogen to the isoalloxazine ring of FAD (*20*, *21*; eq 1). The reduced flavin ($FADH_2$) thus generated at the active site of the enzyme is reoxidized via the transfer of electrons to external electron acceptors, such as electron-transferring flavoproteins (ETF) in the physiological system (*20*, *21*).



(1)

On consideration that Glu-376 serves as a crucial residue during the enzyme catalysis, we and others created selected site-specific mutations of the above residue and compared the kinetic properties of the wild-type and mutant enzymes (*8*, *22*, *23*). Utilizing one such mutation, namely, Glu-376 → Asp (E376D), we demonstrated that the transfer of the α-proton of octanoyl-CoA to the carboxyl group of Glu/Asp-376 proceeded directly (i.e., specific acid−base catalysis) without involvement of an intervening solvent molecule (i.e., general acid−base catalysis; *8*). The above mutation was found to impair the octanoyl-CoA-dependent rate of the reductive half-reaction of the enzyme (which is initiated via the abstraction of the α-proton) by about 800-fold and that for the octynoyl-CoA-dependent inactivation of the enzyme (initiated via the abstraction of the γ-proton) by 16-fold (*8*). Both of these effects were ascribed to be due to the increase in distance between the corresponding proton donor (α- and γ-carbon atoms of C_8-CoA's) and proton acceptor (the

† This work was supported by National Science Foundation Grant MCB-9904416.

* To whom correspondence should be addressed. Tel: (701) 231-7831. Fax: (701) 231-9657. E-mail: DK_Srivastava@ndsu.nodak.edu.

[1] Abbreviations: MCAD, human liver medium chain acyl-CoA dehydrogenase; FAD, flavin adenine dinucleotide; $FcPF_6$, ferricenium hexafluorophosphate; $\Delta G°$, standard free energy change; $\Delta H°$, standard enthalpy change; $\Delta S°$, standard entropy change; $\Delta G^{\ddagger}$, transition state free energy change; $\Delta H^{\ddagger}$, transition state enthalpy change; $\Delta S^{\ddagger}$, transition state entropy change; E_a^f, energy of activation for the forward reaction; E_a^r, energy of activation for the reverse reaction; k_f, forward rate constant; k_r, reverse rate constant; K_c, dissociation constant of the enzyme−ligand collision complex; K_d, overall dissociation constant; EDTA, ethylenediaminetetraacetic acid.

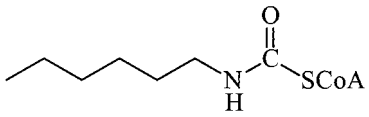
10.1021/bi001317e CCC: $19.00
Published on Web 09/21/2000

carboxyl group of Asp-376) residues within the active site of the enzyme (*8*, *18*). A further interesting feature of the E376D mutation was the lack of coupling between the protein conformational changes and catalysis, the phenomenon observed for the binding/reactivity of a selected substrate/product pair with the wild-type enzyme (*3*−*6*). Whereas the transient kinetic profiles for the association of octenoyl-CoA to both wild-type and E376D mutant enzymes conformed to the biphasic kinetics, the octanoyl-CoA-dependent reductive half-reaction of the wild-type and mutant enzymes emerged out to be biphasic and tetraphasic in nature, respectively (*8*). In the case of E376D, the first two phases of the octanoyl-CoA-dependent reaction were found to be essentially identical to those observed for the binding of octenoyl-CoA (*8*), but the subsequent two phases were considerably slower, and their origins were found to be due to the impairment of the chemical transformation step of the mutant enzyme. The overall comparative analysis of the experimental data led to the conclusion that the E376D mutation abolished the intrinsic coupling between the chemical transformation and protein conformational change steps (*8*).

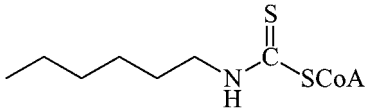
As considered by others, Glu-376 to Asp mutation has the potential to create a "void"/"cavity" of dimension equal to that of a methylene group (*24*−*26*). To ascertain how such a void would adjust within the enzyme site upon binding of ligands, we undertook comparative studies for the binding of structurally diverse ligands, namely, octenoyl-CoA, indoleacryloyl-CoA, and acetoacetyl-CoA, to the wild-type and E376D mutant enzymes (*18*). Such studies involved UV/vis spectroscopy and stopped-flow-based transient kinetic, microcalorimetry-based thermodynamic, and computer graphic model-building approaches. The experimental data revealed that the E376D mutation did not affect any of the above properties for the binding of octenoyl-CoA, but it produced opposite effects for the binding of the other two ligands, viz., indoleacryloyl-CoA and acetoacetyl-CoA (*18*). Whereas the E376D mutation enhanced the magnitude of spectral changes for the binding of indoleacryloyl-CoA to the enzyme, it diminished the magnitude of the above changes for the binding of acetoacetyl-CoA (*18*). The above effects were found to be due to changes in the isomerization equilibrium of the enzyme−ligand collision complexes, and the latter influenced the overall enthalpic changes ($\Delta H°$) of the enzyme−ligand complexes (*18*). For example, whereas the E376D mutation did not have any influence on $\Delta H°$ for the binding of octenoyl-CoA, it decreased (i.e., became more favorable) and increased (i.e., became less favorable) the $\Delta H°$ value for the binding of indoleacryloyl-CoA and acetoacetyl-CoA, respectively (*18*). These experimental data were explained in the light of the proximity between the isoalloxazine ring of FAD and the ligand molecule via the model-building studies of the individual enzyme−ligand complexes (*18*).

On the basis of these results, we became interested in determining the energetic consequences for the binding of minimally altered ligands to the wild-type and E376D mutant enzymes. In this endeavor, we deliberated to undertake a comparative kinetic investigation for the binding of 2-azaoctanoyl-CoA and its C═O → C═S substituted derivative, namely, 2-azadithiooctanoyl-CoA (27; Chart 1). Both of these ligands have been judged to be redox inactive, and thus they do not undergo the enzyme-catalyzed oxido-reductive reaction (*27*, *28*).

Chart 1



2-Azaoctanoyl-CoA



2-Azadithiooctanoyl-CoA

As will be detailed in the Results section, the C═O → C═S substitution increases the bulkiness of the CoA−ligand by 11 Å^3, and the replacement of Glu-376 by the Asp residue (via E376D mutation) creates a 14 Å^3 cavity on the opposite side of the carbonyl group. The purpose of this study has been to ascertain the thermodynamic consequences of accommodating the bulkier ligand within the enzyme site that harbored a cavity of complementary dimension. The experimental results and the derived conclusion are presented in the subsequent sections.

MATERIALS AND METHODS

Materials. 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, and hexyl isothiocyanate was purchased from Lancaster Synthesis. All other reagents were of analytical grade.

General. Unless otherwise stated, all experiments were performed in 50 mM potassium phosphate, pH 7.6, containing 10% glycerol and 0.3 mM EDTA, referred to as the standard phosphate buffer. The site-specific mutation of the enzyme's active site residue Glu-376 to Asp was performed as described previously (*8*). Both the wild-type and mutant forms of human MCAD were expressed and purified according to the method of Peterson et al. (*6*) and assayed by monitoring the reduction of ferricenium hexafluorophosphate ($FcPF_6$) at 300 nm ($\epsilon = 4.3$ mM^{-1} cm^{-1}) in a reaction mixture containing 100 μM octanoyl-CoA and 350 μM $FcPF_6$ (*29*). The wild-type and E376D mutant enzymes were purified to an A_{280}/A_{450} ratio of about 5. The extinction coefficient of the wild-type and mutant enzymes was taken to be 15.4 mM^{-1} cm^{-1} at 446 nm (*30*).

The CoA derivatives, 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA, were prepared essentially as described by Trievel et al. (*27*). These ligands were prepared by the reactions of coenzyme A with *n*-hexyl isocyanate and *n*-hexyl isothiocyanate, respectively. In a typical preparation, a solution of isocyanate or isothiocyanate (in THF) was allowed to react with coenzyme A in 0.25 M $NaHCO_3$ under the stream of nitrogen. The reaction times for preparations of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA were selected to be 30 min and 2.5 h, respectively. After that, the pH of the mixture was adjusted to 5.5 with dilute acetic acid. The products were purified on a C_{18} reverse-phase HPLC

column, equilibrated with 20 mM ammonium acetate, pH 5.5, and eluted with an increasing gradient of methanol. These CoA derivatives were stored as lyophilized powders at −70 °C. The extinction coefficients of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA were taken to be 16.0 and 22 mM^{-1} cm^{-1} at 260 nm, respectively (*27*).

Transient Kinetic Experiments. Single-wavelength transient kinetic experiments were performed on an Applied Photophysics SX-18 MV stopped-flow system (optical path length 10 mm, dead time 1.3−1.5 ms). The stopped-flow system was configured in a single mixing mode so that the contents of both syringes, A and B, were diluted by 50%. The stopped-flow kinetic traces were analyzed by the data analysis package provided by Applied Photophysics.

The rate constants for the association of the respective ligands to the wild-type and mutant enzymes were determined by mixing the above species via the stopped-flow syringes (under the pseudo-first-order conditions; [E−FAD] ≪ [ligand]) and monitoring the time-dependent absorbance changes at 438 nm. The rate constants for the dissociation of the ligands from their respective enzyme sites were determined by mixing 20 μM enzyme + 20 μM 2-azaoctanoyl-CoA or 50 μM 2-azadithiooctanoyl-CoA (syringe A) with 2 mM acetoacetyl-CoA (syringe B) via the stopped-flow syringes and monitoring the increase in absorbance at 545 nm as described by Kumar and Srivastava (*5*).

Spectrophotometric Titrations for the Binding of CoA−Ligands to the Wild-Type and Mutant Enzymes. The spectrophotometric titrations were performed either on a Perkin-Elmer Lambda-3B or on a Beckman 7400 diode array spectrophotometer. The difference spectra of the enzyme−ligand complexes were generated by subtracting the spectra of the individual components from their mixture (after dilution corrections). The binding isotherms of the enzyme−ligand complexes were constructed by titration of the wild-type and E376D mutant enzymes with 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA at 438 nm. The dissociation constants and the overall amplitudes of the absorbance changes during the titrations were discerned by analyzing the experimental data by a complete solution of the quadratic equation describing the enzyme−ligand interactions, as detailed by Qin and Srivastava (*31*).

Molecular Modeling Studies. The molecular modeling studies were performed using the X-ray crystallographic structural coordinates of pig liver MCAD in the presence of bound C_8-CoA (3MDE), obtained from the Brookhaven Protein Data Bank. The enzyme-bound C_8-CoA structure was modified to adopt the structures of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA by the help of the Biopolymer module of Insight-II [98]. The Glu-376 → Asp mutation was constructed by replacing the side chain of Glu-376 with Asp by the aid of Homology-98 software. Prior to the calculation of molecular volumes of the CoA−ligands and Glu/Asp-376 residues, these species were unmerged from the protein structure and saved as individual files in the pdb format. The molecular volumes of 2-azaoctanoyl-CoA, 2-azadithiooctanoyl-CoA, Glu-376, and Asp-376 were calculated by the aid of the Connolly's MSROLL software, which runs on the SGI-Unix workstation (*32*). The above calculation was performed by computationally rolling a spherical probe of either 1.2 or 1.4 Å radius on the surface of the individual species. The water-accessible surface areas (Connolly surface) of the above species were calculated by the aid of Insight-II [98] software, using a probe radius of 1.4 Å (*33*).

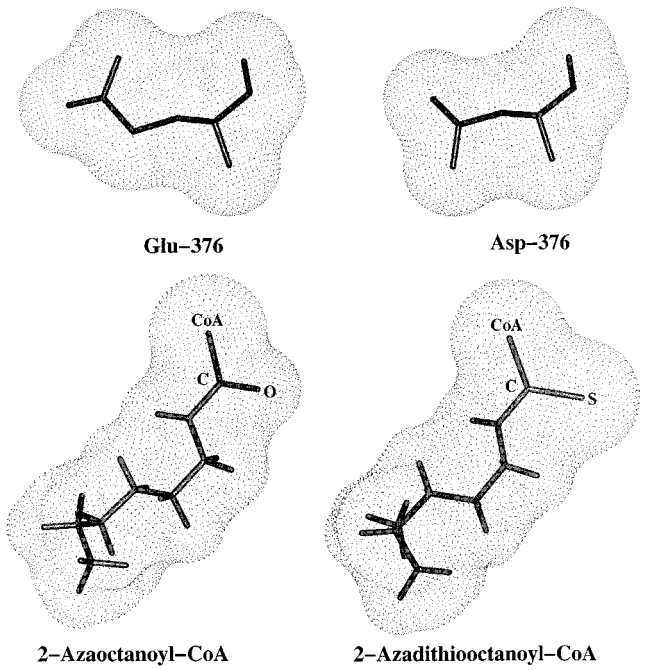


FIGURE 1: Connolly surfaces around the selected residues of medium chain acyl-CoA dehydrogenase. These surfaces denote the qualitative difference in volumes upon altering Glu-376 (top, left) by Asp (top, right) and upon substitution of the C═O group (in 2-azaoctanoyl-CoA) by the C═S group (yielding 2-azadithiooctanoyl-CoA). For clarity, the extraneous regions of the coenzyme A structure have been deleted.

RESULTS

The volumes of 2-azaoctanoyl-CoA, 2-azadithiooctanoyl-CoA, Glu-376, and Asp-376, calculated by the aid of MSROLL software (see Materials and Methods), using the probe radius of 1.2 Å, were found to be 870, 859, 125, and 107 $Å^3$, respectively. The above values calculated using the probe radius of 1.4 Å were found to be 868, 879, 126, and 108 $Å^3$, respectively. From these data, the change in volume for the C═O → C═S substitution in the ligand structure and that for the replacement of Glu-376 by Asp-376 were determined to be about +11 and −18 $Å^3$, respectively. (The + and − signs reflect the increase and decrease in volumes, respectively). To visualize the extent of the above changes, we present the Connolly surfaces around the isolated Glu-376, Asp-376, and the truncated regions of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA in Figure 1. Note the difference in the surface density contours in the regions around carboxyl groups of the amino acid residues and C═O/C═S groups of the CoA−ligands.

To further probe whether the replacement of Glu-376 by Asp creates a cavity within the enzyme site, we utilized the MSROLL software for calculating the volumes of internal cavities within the single subunit of pig liver MCAD, with and without replacement of Glu-376 by Asp. The theoretical data revealed that, by utilizing a 1.4 Å probe, 19 internal cavities within the individual subunits of the wild-type and E376D mutant enzymes were detected. Of these, one cavity, in the vicinity of Glu/Asp-376, showed a volume of 72 $Å^3$ in the case of the wild-type (i.e., Glu-376 containing) enzyme and 86 $Å^3$ in the case of the E376D mutant enzyme. From

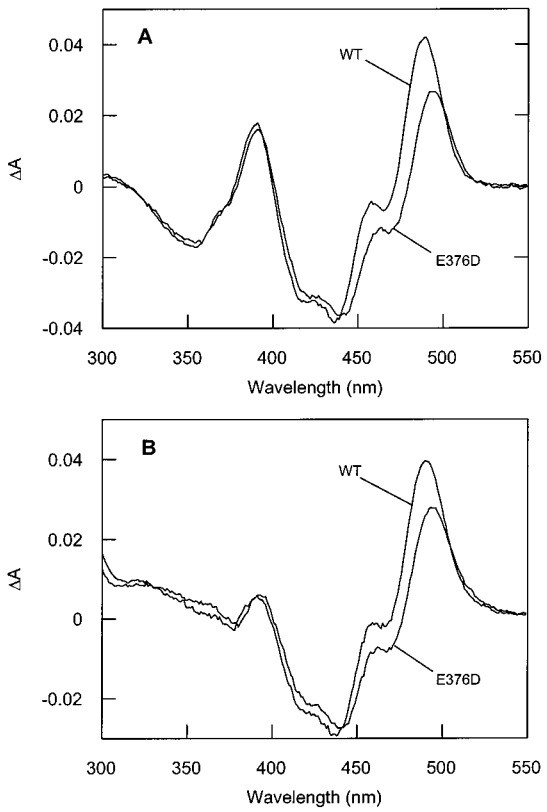


FIGURE 2: Spectral changes upon binding of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes. The difference spectra (i.e., the spectra of the enzyme−ligand complexes minus the individual species) for the binding of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes are shown in panels A and B, respectively. The spectral acquisitions have been performed in 50 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA and 10% glycerol at 25 °C. [Wild type] or [E376D] = 14.9 μM, [2-azaoctanoyl-CoA] = 70.6 μM, and [2-azadithiooctanoyl-CoA] = 123.3 μM.

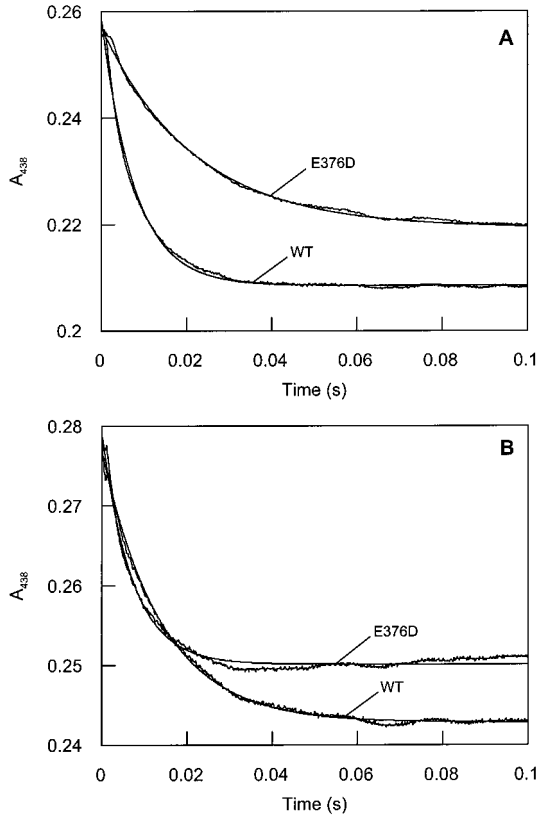


FIGURE 3: Representative stopped-flow kinetic traces for the association of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA with wild-type and E376D mutant enzymes at 5 °C. Other experimental conditions were the same as in Figure 2. Panel A shows the kinetic traces for the association of 400 μM 2-azaoctanoyl-CoA with 40 μM (premixing concentrations) wild-type or E376D mutant enzyme at 438 nm. The solid, smooth lines are the best fits of the experimental data according to the single exponential rate equation with relaxation constants ($1/\tau_{association}$) of 129 s^{-1} and 46.3 s^{-1} and amplitudes of 0.050 and 0.038 absorbance unit for the wild-type and E376D mutant enzymes, respectively. Panel B shows the stopped-flow traces for the association of 400 μM 2-azadithiooctanoyl-CoA with 40 μM (premixing concentrations) wild-type or E376D mutant enzymes at 438 nm. The solid, smooth lines are the best fits of the experimental data according to the single exponential rate equation with relaxation constants ($1/\tau_{association}$) of 71.6 s^{-1} and 140 s^{-1} and amplitudes of 0.029 and 0.034 absorbance unit for wild-type and E376D mutant enzymes, respectively.

these data, the cavity created via the Glu-376 → Asp mutation was discerned to be 14 Å^3 dimension. Note that the cavity volume thus calculated is comparable to the difference in volume (18 Å^3) between Glu-376 and Asp residues (Figure 1). It should be pointed out that Matthews and his collaborators argue that the cavity calculations are more reliable by using a probe radius of 1.2 Å instead of 1.4 Å (*34*). When we used the probe of 1.2 Å radius, the cavity volume was found to be 13 Å^3, more or less the same as discerned by using the probe radius of 1.4 Å.

Prior to performing the transient kinetic experiments for the association and dissociation of ligands, we investigated the spectral features of the enzyme−ligand complexes. Although the electronic spectra of the enzyme-bound 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA have been reported for pig kidney MCAD (*27*), no spectral features of the above ligands at the resident sites of the recombinant human liver MCAD and/or its Glu376 → Asp (E376D) mutations have been known. Figure 2 shows the difference spectra for the binding of saturating concentrations of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes. Although the spectral data for the binding of the above ligands to the wild-type human liver MCAD were found to be similar to those obtained with the pig kidney enzyme, they were slightly blue shifted in the case the E376D mutant enzyme. The mutant enzyme also showed a bathochromic effect (vis à vis the wild-type enzyme) at wavelengths greater than 400 nm. The molecular basis of the (above-noted) mutation and ligand-induced spectral changes of the enzyme−ligand complexes will be published elsewhere.

By utilizing the changes in the spectral signals of Figure 2, we could determine the kinetics of association of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes. Figure 3 shows the comparative transient kinetic traces (recorded at 438 nm) for the association of saturating concentrations of the above ligands with wild-type and E376D mutant enzymes, at 5 °C, under pseudo-first-order conditions ([ligand] ≫ [enzyme]). To facilitate comparison, the reaction traces of Figure 3 are normalized with respect to the concentrations of the absorbing species. The solid, smooth lines are the best fit of the experimental data for the association of 2-azaoctanoyl-CoA to the wild-type and E376D mutant enzymes, according to

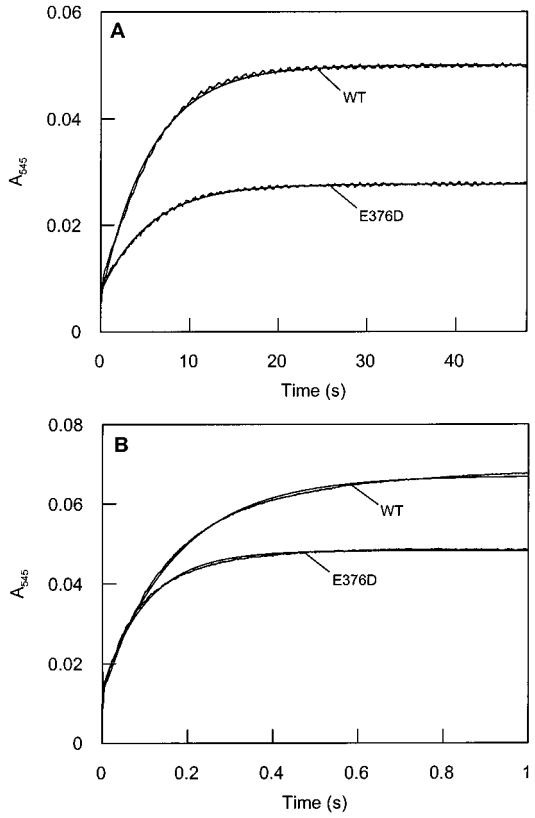


FIGURE 4: Representative stopped-flow kinetic traces for the dissociation of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA from the wild-type and E376D mutant enzymes at 5 °C. Other conditions were the same as in Figure 2. Panel A shows the kinetic traces at 545 nm upon mixing (premixing concentrations) of 40 μM wild-type or E376D mutant enzyme + 40 μM 2-azaoctanoyl-CoA (syringe 1) with 2 mM acetoacetyl-CoA (syringe 2) via the stopped-flow syringes. The solid, smooth lines are the best fits of the experimental data according to the single exponential rate equation with relaxation constants ($1/\tau_{dissociation}$) of 0.18 s^{-1} each and amplitudes of 0.044 and 0.020 absorbance unit for the wild-type and E376D mutant enzymes, respectively. Panel B shows the stopped-flow kinetic traces at 545 nm upon mixing of 40 μM wild-type or E376D mutant enzyme + 100 μM 2-azadithiooctanoyl-CoA (syringe 1) with 2 mM acetoacetyl-CoA (syringe 2) via the stopped-flow syringes. The solid, smooth lines are the best fits of the experimental data according to the single exponential rate equation with relaxation constants ($1/\tau_{dissociation}$) of 5.7 s^{-1} and 9.7 s^{-1} and amplitudes of 0.057 and 0.035 absorbance unit for the wild-type and E376D mutant enzymes, respectively.

the single exponential rate equation, with relaxation rate constants ($1/\tau_{association}$) of 129 s^{-1} and 46.3 s^{-1}, respectively. The corresponding parameters for the binding of 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes were found to be 71.6 s^{-1} and 128 s^{-1}, respectively. In each case, the association rate constant ($1/\tau_{association}$) exhibits saturation kinetics (data not shown), consistent with a two-step enzyme−ligand interaction as observed previously (*1*). From the data of Figure 3, it is evident that the magnitude of $1/\tau_{association}$ for the binding of 2-azadithiooctanoyl-CoA to the wild-type enzyme is about 45% lower than that obtained for the binding of 2-azaoctanoyl-CoA. On the other hand, $1/\tau_{association}$ for the binding of 2-azadithiooctanoyl-CoA to the E376D mutant enzyme is 2.8-fold higher than that obtained for the binding of 2-azaoctanoyl-CoA to the mutant enzyme. On assessing the effect of the E376D mutation, it is evident that whereas the mutation increases the magnitude of $1/\tau_{association}$ for the binding of 2-azadithiooctanoyl-CoA by 1.8-fold, it impairs the above parameter for the binding of 2-azaoctanoyl-CoA by 2.8-fold. As will be elaborated below, the above difference becomes more pronounced at higher temperature due to a marked difference in the energies of activation of the corresponding isomerization steps of the individual enzyme−ligand complexes. However, on qualitative grounds, it appears, although the cavity formed via the E376D mutation is not favorable for the association of 2-azaoctanoyl-CoA, that it is favorable for the association of the bulkier ligand, 2-azadithiooctanoyl-CoA.

To ascertain the reversibility of the above-noted enzyme−ligand complexes, as well as their facile dissociability from the corresponding enzyme sites, we determined the dissociation "off-rates" ($1/\tau_{dissociation}$) of the enzyme−ligand complexes via the acetoacetyl-CoA displacement method, as elaborated by Johnson et al. (*1*). Figure 4 shows the representative kinetic traces upon mixing a particular enzyme−ligand complex ([enzyme] ≈ [ligand]) with a high concentration of acetoacetyl-CoA at 5 °C. Like the data of Figure 3, these traces have also been normalized to facilitate the comparison. The solid, smooth lines are the best fit of the experimental data for the dissociation of the individual enzyme−ligand complexes. Such analyses yielded the dissociation off-rates of 2-azaoctanoyl-CoA from the wild-type and E376D mutant enzymes as each being equal to 0.18 s^{-1}, and the dissociation off-rates of 2-azadithiooctanoyl-CoA from the wild-type and E376D mutant enzymes as being equal to 5.7 s^{-1} and 9.7 s^{-1}, respectively. From these data, it is evident that the dissociation off-rates of 2-azadithiooctanoyl-CoA from the wild-type and E376D mutant enzymes are about 2-orders of magnitude faster than those obtained with 2-azaoctanoyl-CoA. Clearly, the C═O → C═S substitution in the ligand structure promotes its facile dissociation from the enzyme sites.

All of our previous transient kinetic studies for the binding of CoA−ligands to pig kidney and recombinant human liver enzymes have been consistent with at least a two-step binding process (*1*, *4*, *31*). Of these, the first (fast) step involves the formation of the enzyme−ligand collision complex with a dissociation constant of K_c, followed by the slow isomerization (via the protein conformational changes) of the collision complex with forward and reverse rate constants of k_f and k_r, respectively (eq 2). On the basis of the rapid

$$\underset{}{\text{E−FAD + ligand}} \underset{K_c}{\rightleftharpoons} \underset{\text{collision complex}}{\text{E−FAD−ligand}} \overset{k_f}{\underset{k_r}{\rightleftharpoons}} \underset{\text{isomerized complex}}{\text{(E−FAD−ligand)*}} \quad (2)$$

$$1/\tau_{association} = k_f + k_r$$

$$1/\tau_{dissociation} = k_r$$

equilibrium assumption, the observed relaxation rate constant for the enzyme−ligand association ($1/\tau_{association}$) is given by the sum of the forward (k_f) and reverse (k_r) rate constants of the enzyme−ligand isomerization step, and the dissociation off-rate of the enzyme−ligand complex ($1/\tau_{dissociation}$) serves as a measure of k_r (eq 2). From the above relationships, the magnitude of k_f is ascertained by subtracting $1/\tau_{dissociation}$ from $1/\tau_{association}$.

To discern the energetic contributions during the course of the binding of 2-azaoctanoyl-CoA and 2-azadithiooc-

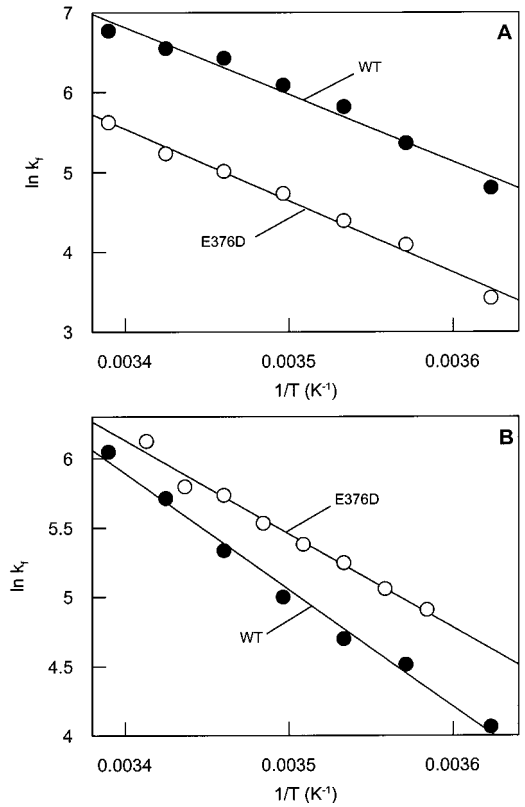


FIGURE 5: Arrhenius plots for the forward (k_f) rate constants of the isomerization of the enzyme−ligand complexes. Panel A shows the temperature dependence of k_f for the interaction of 2-azaoctanoyl-CoA with the wild-type (closed circles) and E376D (open circles) mutant enzymes, respectively. The solid lines are linear regression fit of the experimental data for the energy of activation of 16.6 and 17.8 kcal/mol for the wild-type and E376D mutant enzymes, respectively. Panel B shows the temperature dependence of k_f for the interaction of 2-azadithiooctanoyl-CoA with wild-type (closed circles) and E376D mutant MCAD (open circles). The solid lines are the linear regression analysis of the experimental data for the energy of activation of 16.7 and 12.7 kcal/mol for the wild-type and E376D mutant enzymes, respectively.

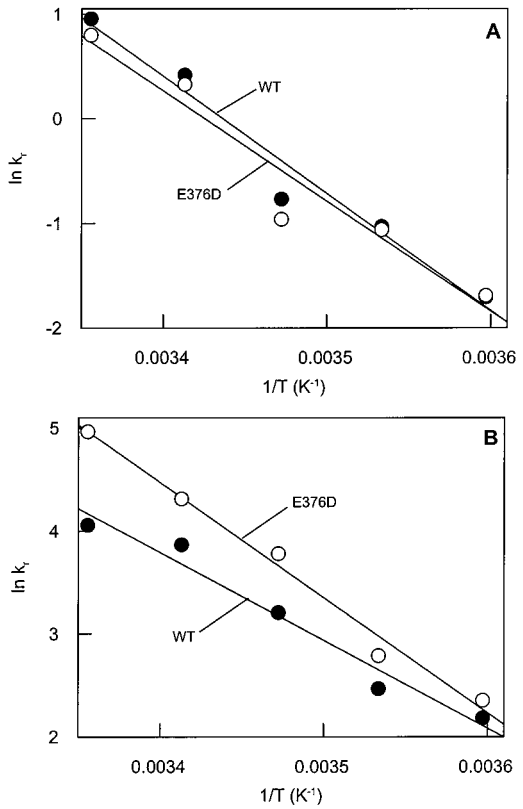


FIGURE 6: Arrhenius plots for the dissociation off-rates (k_r) of the enzyme−ligand complexes. Panel A shows the temperature dependence of the dissociation off-rates of 2-azaoctanoyl-CoA from the wild-type (closed circles) and E376D (open circles) mutant enzymes, respectively. The solid lines represent the linear regression analysis of the experimental data for the energy of activation of 22.2 and 20.9 kcal/mol for the wild-type and E376D mutant enzymes, respectively. Panel B shows the temperature dependence of the dissociation off-rates of 2-azadithiooctanoyl-CoA from the wild-type (closed circles) and E376D (open circles) mutant enzymes, respectively. The solid lines are the linear regression analysis of the experimental data for the energy of activation of 17.0 and 22.2 kcal/mol for the wild-type and E376D mutant enzymes, respectively.

tanoyl-CoA to the wild-type and E376D mutant enzymes, we investigated the temperature dependence of the forward (k_f) and reverse (k_r) rate constants, calculated from the data of Figures 3 and 4. Figure 5 shows the Arrhenius plots for the forward rate constants (k_f) of association of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes. The solid, straight lines are the best fit of the experimental data according to the Arrhenius equation in the following format:

$$\ln k = \ln A - \frac{E_a}{RT} \tag{3}$$

where A, E_a, R, and T represent the preexponential factor, the energy of activation, the universal gas constant, and the absolute temperature, respectively.

According to eq 3, the Y-axis intercepts and slope values of Figure 5 serve as the measures of ln A and E_a/R, respectively. From these data, the energies of activation of the forward isomerization steps (E_a^f) of the wild-type−2-azaoctanoyl-CoA and E376D−2-azaoctanoyl-CoA complexes were discerned to be 16.6 and 17.8 kcal/mol, respectively. The corresponding parameters for the wild-type and E376D mutant enzyme−ligand complexes involving 2-azadithiooctanoyl-CoA were discerned to be 16.7 and 12.7 kcal/mol, respectively. Given these, it is noteworthy that whereas the magnitude of E_a^f for the 2-azaoctanoyl-CoA-dependent forward isomerization step of the enzyme is increased by 1.2 kcal/mol upon E376D mutation, it is decreased by 4 kcal/mol in the case of 2-azadithiooctanoyl-CoA under an identical experimental condition. Clearly, the increase in bulkiness of the ligand is enthalpically better tolerated in the transition state (of the isomerization step) in the case of the E376D mutant vis à vis the wild-type enzyme (see below).

We further analyzed the temperature dependence of the reverse rate constants (k_r) for the dissociation of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA from the wild-type and E376D mutant enzymes. Figure 6 shows the Arrhenius plots for the dissociation off-rates (k_r) of the enzyme−ligand complexes. The experimental data were analyzed according to eq 3. The energies of activation for the reversal of the isomerization step (E_a^r) of the wild-type−2-azaoctanoyl-CoA and E376D−2-azaoctanoyl-CoA complexes were determined to be 22.2 and 20.9 kcal/mol, respectively. The corresponding parameters involving the 2-azadithiooctanoyl-CoA−ligand were found to be 17.0 and 22.2 kcal/mol, respectively. From

these data, it is apparent that the E_a^r values for the dissociation of 2-azaoctanoyl-CoA from the wild-type and E376D mutant enzymes are 5.2 kcal/mol higher and 1.3 kcal/mol lower, respectively, than those obtained for the dissociation of 2-azadithiooctanoyl-CoA from the above enzyme sites. On the other hand, whereas the E376D mutation decreases the magnitude of the E_a^r value for the dissociation of 2-azaoctanoyl-CoA by 1.3 kcal/mol, it increases the E_a^r value for the dissociation of 2-azadithiooctanoyl-CoA by 5.2 kcal/mol. Clearly, the energy of activation for the dissociation of the C═O → C═S substituted ligand from the cavity-harboring E376D mutant enzyme is considerably higher than that from the wild-type enzyme.

By utilizing the changes in the spectral signals of Figure 2, we performed the spectrophotometric titrations for the binding of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes. As noted by us as well as by others, following the initial (rapid) spectral changes upon binding of the above ligands to their enzyme sites, the enzyme−ligand complexes exhibit slow electronic structural changes in the flavin region (*27*, *28*, *35*). Although the latter changes do not influence the $\Delta G°$ values of the enzyme−ligand complexes ($\Delta G°_{WT-2-azaoctanoyl-CoA} = -8.7$ kcal/mol, $\Delta G°_{E376D-2-azaoctanoyl-CoA} = -8.8$ kcal/mol; $\Delta G°_{WT-2-azadithiooctanoyl-CoA} = -6.4$ kcal/mol, $\Delta G°_{E376D-2-azadithiooctanoyl-CoA} = -6.8$ kcal/mol), as corroborated by the independent microcalorimetric data (to be published subsequently), the overall spectrophotometric titrations were performed within 10 min to avoid the absorption artifacts due to the slow (secondary) spectral changes. Figure 7 shows the binding isotherms for the interactions of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA with the wild-type and E376D mutant enzymes. As expected from the spectral data of Figure 2, the overall amplitude of the absorption changes (at 438 nm) is higher with the wild-type enzyme as compared to the E376D mutant enzyme, involving both 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA ligands. The solid, smooth lines are the best fit of the experimental data for the dissociation constants of 0.44 μM and 0.50 μM for the binding of 2-azaoctanoyl-CoA to the wild-type and E376D mutant enzymes (Figure 7A) and for the dissociation constants of 18.3 μM and 6.1 μM for the binding of 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes, respectively. From these data, it is further evident that although the binding of 2-azadithiooctanoyl-CoA to either the wild-type or E376D mutant enzyme is not as favorable as that of 2-azaoctanoyl-CoA, the cavity created via the E376D mutation favorably accommodates the former (the C═O → C═S substituted bulkier) ligand as compared to the unsubstituted (i.e., 2-azaoctanoyl-CoA) ligand.

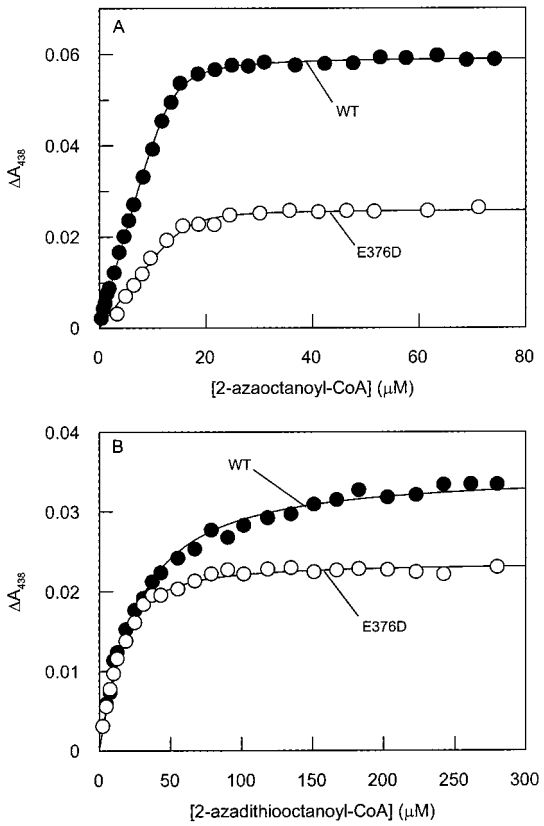


FIGURE 7: Binding isotherms for the interactions of 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA with the wild-type and E376D mutant enzymes. Other experimental conditions are the same as in Figure 2. Panel A shows the titration of 19.6 μM wild type (closed circles) and 20.7 μM E376D (open circles) with 2-azaoctanoyl-CoA at 438 nm. The solid, smooth lines are the best fit of the experimental data for the dissociation constants of 0.44 ± 0.06 μM and 0.50 ± 0.22 μM and stoichiometries of 0.68 ± 0.01 and 0.77 ± 0.04 mol of 2-azaoctanoyl-CoA/mol of MCAD subunit for the wild type and E376D mutant, respectively. Panel B shows the titration of 13.4 μM wild type and 14.7 μM E376D with 2-azadithiooctanoyl-CoA at 438 nm. The solid, smooth lines are the best fit of the experimental data with dissociation constants of 0.18.26 ± 1.18 μM and 6.1 ± 0.80 μM and stoichiometries of 1.01 ± 0.16 and 0.98 ± 0.11 mol of 2-azadithiooctanoyl-CoA/mol of MCAD subunit for the wild-type and E376D mutant enzymes, respectively.

DISCUSSION

The present work describes the energetic consequences of accommodating a slightly bulkier ligand at the medium chain acyl-CoA dehydrogenase (MCAD) site by creating a complementary cavity via the site-specific mutagenesis. Due to ease in synthesis, discernible UV/vis spectral features, and reasonably tight binding affinities to the enzyme sites (*27*), two ligands, namely, 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA, were selected toward these studies. These ligands differ with respect to C═O versus C═S substitution at the CoA−thioester linkage, and the latter is 11 Å^3 larger (bulkier) than 2-azaoctanoyl-CoA. On the other hand, the Glu-376 → Asp mutation at the enzyme site creates a cavity of about 14 Å^3 dimension. The experimental data presented in the previous section support the view that the bulkier ligand is easily accommodated at the cavity-harboring E376D mutant enzyme site as compared to the wild-type enzyme site.

As elaborated in the Results section, the binding of both 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA to the wild-type and E376D mutant enzymes conforms to the two-step mechanism (see eq 2). Since the equilibrium constant for the dissociation of the first step (K_c) could not be accurately determined by investigating the ligand concentration dependencies of the relaxation rate constants for the association ($1/\tau_{association}$) of the ligands to their cognate enzyme sites, recourse was made to subtract the contributions of the second step from the overall dissociation constants (K_d) of the enzyme−ligand complexes. Given the magnitudes of the forward (k_f) and reverse (k_r) rate constants for the isomerization step and the overall dissociation constants of the

Table 1: Thermodynamic Parameters for the Binding of 2-Azaoctanoyl-CoA and 2-Azadithiooctanoyl-CoA to the Wild-Type and E376D Mutant Enzymes[a]

parameters	WT−aza[b] (kcal/mol	E376D−aza (kcal/mol)	WT−azadi (kcal/mol)	E376D−azadi (kcal/mol)
$\Delta G°$ (overall)	−8.67	−8.59	−6.46	−7.12
$\Delta G°$ (collision complex)	−4.95	−5.56	−5.12	−6.39
$\Delta G^{‡}$ (forward)	13.1	13.9	13.7	13.8
$\Delta H^{‡}$ (forward)	16.0	17.2	16.1	12.1
$\Delta G^{‡}$ (reverse)	16.9	17.0	15.0	14.5
$\Delta H^{‡}$ (reverse)	21.6	20.3	16.3	21.6
$\Delta G°$ (k_f/k_r)	−3.71	−3.03	−1.34	−0.73
$\Delta H°$ (k_f/k_r)	−5.6	−3.1	−0.2	−9.5

[a] Calculated at 25 °C. [b] aza and azadi refer to 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA, respectively.

enzyme−ligand complexes (K_d), the magnitudes of K_c were determined from the relationship:

$$K_c = K_d \frac{k_f}{k_r} \tag{4}$$

Given the above parameters, the ground ($\Delta G°$) and transition state ($\Delta G^{‡}$) free energy changes for the binding and isomerization of the individual enzyme−ligand complexes were determined from the relationships:

$$\Delta G°_c = -RT \ln(1/K_c)$$
$$\Delta G^{‡}_f = -RT \ln\left(\frac{k_f h}{k_B T}\right) \tag{5}$$
$$\Delta G^{‡}_r = -RT \ln\left(\frac{k_r h}{k_B T}\right)$$

In eq 5, R is the gas constant (1.986 cal K^{-1} mol^{-1}), T is the absolute temperature, h is Plank's constant (1.58×10^{-34} cal s), and k_B is Boltzmann's constant (3.3×10^{-24} cal K^{-1}). The calculated parameters of eq 5 are summarized in Table 1.

Given the energy of activation parameters for the forward (E_a^f) and reverse (E_a^r) isomerization steps of the individual enzyme−ligand complexes, the enthalpic ($\Delta H^{‡}$) contributions for the above steps were derived from the relationship:

$$\Delta H^{‡} = E_a - RT \tag{6}$$

The magnitudes of $\Delta H^{‡}$ for the forward and reverse isomerization steps of the individual enzyme−ligand complexes are also summarized in Table 1.

Figure 8 shows the influence of the Glu-376 → Asp mutation on the free energy profiles for the binding and isomerization of the enzyme−ligand complexes, involving 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA as ligands. The data of Figure 8 reveal that the E376D mutation stabilizes the ground states of both enzyme−ligand complexes. However, the magnitudes of $\Delta\Delta G°_c$ (i.e., difference in the free energy changes between the wild-type and mutant enzyme−ligand complexes) involving 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA are 0.61 and 1.27 kcal/mol, respectively. Hence, the E376D mutation favorably stabilizes the ground state of the enzyme−ligand collision complex involving the bulkier ligand, 2-azadithiooctanoyl-CoA, as

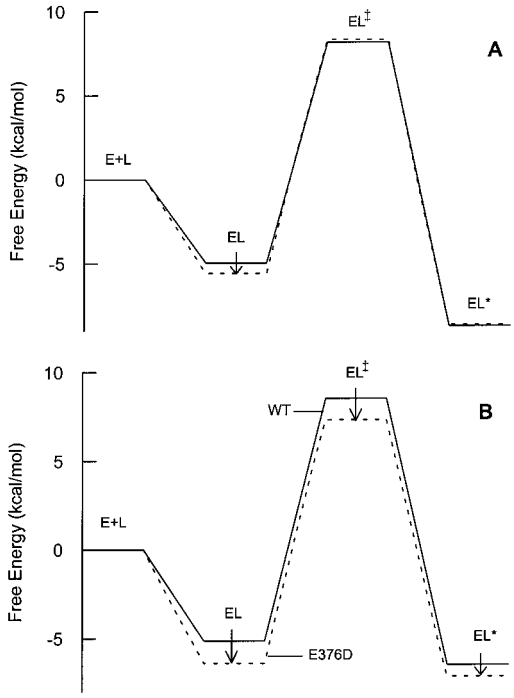


FIGURE 8: Effect of the Glu-376 → Asp mutation on the free energy profiles for the binding of 2-azaoctanoyl-CoA (top panel) and 2-azadithiooctanoyl-CoA (bottom panel) to the enzyme. E, L, EL, and EL* represent the free enzyme, CoA−ligand, enzyme−ligand collision complex, and enzyme−ligand isomerized complex, respectively. The free energies of the enzyme and ligands are taken as being equal to zero.

compared to that of 2-azaoctanoyl-CoA. On the other hand, whereas the E376D mutation exhibits a miniscule effect on stabilizing the transition state formed during the 2-azaoctanoyl-CoA-dependent isomerization step, the above mutation stabilizes the transition state formed during the 2-azadithiooctanoyl-CoA-dependent process by 1.2 kcal/mol. Likewise, whereas the E376D mutation exhibits practically no influence in stabilizing the ground state of the enzyme−ligand isomerized complex, formed with 2-azaoctanoyl-CoA, it stabilizes the latter complex formed with 2-azadithiooctanoyl-CoA by 0.7 kcal/mol. Clearly, the cavity created via the E376D mutation stabilizes both the ground and transition state energies of the bulkier ligand by more or less an equal magnitude. This is presumably since the cavity formed upon E376D mutation widens the enzyme's active site such that the thio-substituted (bulkier) ligand is easily accommodated within the collision complex. Such a cavity also facilitates the isomerization of the enzyme−ligand collision complex (involving the thio-substituted ligand), since the isomerization step requires adjustment of the ligand structure with respect to the isoalloxazine ring of the enzyme-bound FAD (*18*).

From the energy of activation parameters of Figures 5 and 6, we discern the magnitudes of enthalpic changes ($\Delta H^{‡}$) during the course of isomerization of the individual enzyme−ligand complexes. Figure 9 shows the enthalpic profiles for the influence of the E376D mutation on the 2-azaoctanoyl-CoA- and 2-azadithiooctanoyl-CoA-dependent isomerization of the enzyme−ligand complexes. Since we could not determine the enthalpic changes for the formation of the enzyme−ligand collision complexes, the enthalpic profiles are represented with respect to the enthalpies of the enzyme−ligand collision complexes being equal to zero. As evident

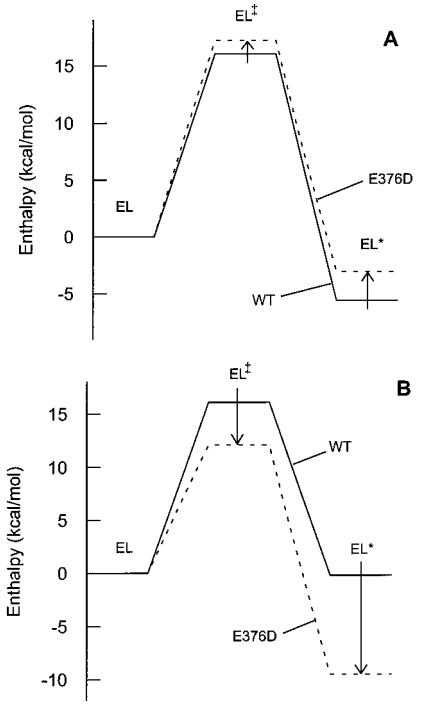


FIGURE 9: Effect of the Glu-376 → Asp mutation on the enthalpic profiles for the isomerization of the enzyme−ligand complexes. The top and bottom panels show the 2-azaoctanoyl-CoA- and 2-azadithiooctanoyl-CoA-dependent processes. The enthalpies of the enzyme−ligand collision complexes are taken as being equal to zero.

from the data of Figure 9 and Table 1, the E376D mutation exhibits a more pronounced effect on the enthalpic changes (vis à vis the free energy changes) during the course of the isomerization of the enzyme−ligand complexes. Interestingly, unlike the free energy profiles, the enthalpic profiles exhibit opposite (mutation-dependent) effects for the isomerization of the enzyme−ligand complexes. For example, whereas the E376D mutation increases the $\Delta H^{\ddagger}$ value for the 2-azaoctanoyl-CoA-dependent isomerization step by 1.2 kcal/mol, it decreases the $\Delta H^{\ddagger}$ value for the 2-azadithiooctanoyl-CoA-dependent isomerization step by about 4 kcal/mol. The enthalpic profile of Figure 9 further suggests that whereas the enthalpic difference between the ground states of the enzyme−2-azaoctanoyl-CoA collision and isomerized complexes is unfavorable by 2.5 kcal/mol upon E376D mutation, the above parameter involving 2-azadithiooctanoyl-CoA is favorable by 9.3 kcal/mol upon E376D mutation. On the basis of these data, it is apparent that the cavity formed upon E376D mutation is enthalpically more advantageous for the isomerization of the thio-substituted enzyme−ligand complex, as well as for the stabilization of the final enzyme−ligand isomerized complex, as compared to those obtained with the normal ligand, 2-azaoctanoyl-CoA. In fact, with the latter ligand, the E376D mutation enthalpically destabilizes both the transition state and the ground state of the enzyme−ligand isomerized complex by 1.2 and 2.5 kcal/mol, respectively. Clearly, the entropic contribution plays a major role in stabilizing/destabilizing the ground and transition states of the individual enzyme−ligand complexes.

On the basis of the X-ray crystallographic data, it is evident that the carbonyl oxygen of octenoyl-CoA is within 2.9 Å bonding distance from the 2′-ribityl hydroxyl oxygen of FAD (*36*). Since 2-azaoctanoyl-CoA exhibits a marked structural similarity with octenoyl-CoA, the former ligand is expected to bind at the enzyme site with more or less the same conformation and without exerting any steric constraint. Given that the van der Waals contact radii of oxygen and sulfur atoms are 1.45 and 1.9 Å, respectively, the C═O → C═S substitution in 2-azaoctanoyl-CoA would pose a strong steric repulsion between the thio group of the ligand and the 2′-ribityl hydroxyl group of FAD. This would necessitate readjustment of the protein and/or ligand conformation to accommodate the thio-substituted ligand within the wild-type enzyme site. At this point, it should be mentioned that since the electronegativity of sulfur is considerably lower than that of oxygen (2.58 versus 3.44 on the Pauling scale; *37*), the possibility of forming a hydrogen bond between the thio group of the ligand and 2′-ribityl hydroxyl oxygen of FAD is miniscule. Hence, the readjustment in the protein and/or the ligand structure to accommodate 2-azadithiooctanoyl-CoA within the wild-type enzyme site is unlikely to be dominated by the energetics of the potential hydrogen bonding between the thio group of the ligand and 2′-ribityl hydroxyl group of FAD (*18*). It should be further pointed out that the E376D mutation would be unlikely to cause a major change in the protein structure. This is particularly true since the global structure of the wild-type human liver MCAD is not significantly altered upon E376G/T255E double mutation (*44*). Hence, the observed kinetic and thermodynamic properties of the enzyme−ligand complexes are unlikely to be due to the major changes in the protein conformation upon E376D mutation.

However, irrespective of the nature of the protein structural changes, the experimental data of Table 1 reveal that the overall ΔG° (i.e., including the isomerization equilibrium) for the binding of the thio-substituted ligand (2-azadithiooctanoyl-CoA) is 2.2 kcal/mol less favorable than that of the normal (2-azaoctanoyl-CoA) ligand. On the other hand, there is no energetic penalty for accommodation of the thio-substituted (bulkier) ligand within the wild-type enzyme−ligand collision complex. Hence, the main energetic constraint for the binding of the bulkier (thio-substituted) ligand to the wild-type enzyme site lies in the isomerization step. Since the latter involves the changes in the protein conformation (*35*), it seems likely that the steric constraint in accommodating the bulkier ligand at the enzyme site is maximally realized during the course of the protein structural changes. We surmise that, prior to the ligand binding, the active site pocket of the wild-type enzyme is wide enough to accommodate either 2-azaoctanoyl-CoA or 2-azadithiooctanoyl-CoA (as the enzyme−ligand collision complex) with equal facility. However, as the enzyme−ligand collision complex undergoes the isomerization (via the protein conformational changes) process, the active site cavity of the (wild-type) enzyme is narrowed down, and thus, the bulkier ligand experiences more steric constraint than the normal ligand, both at the transition state and at the subsequent ground state levels. Since the creation of the enzyme site cavity, via the E376D mutation, alleviates such steric constraint, the energetics of the 2-azadithiooctanoyl-CoA-dependent isomerization process becomes favorable.

The ability of an enzyme protein to accommodate the structurally altered substrate/ligand species at its active site is dependent upon the flexibility of the amino acid residues that create the substrate/ligand binding pocket. There has

been growing evidence that the cavity-creating mutations (or the presence of naturally occurring cavities) in the vicinity of the ligand binding pockets facilitate accommodations of structurally different types of ligand species at the active sites of enzymes (*38*−*42*). On the basis of their extensive studies on the cavity-creating mutations in T-4 lysozyme, Matthews and his collaborators argue that the internal cavities are comprised of both flexible and rigid parts (*43*). Whereas the flexible region moves to various extents in response to the binding of structurally altered ligands, the rigid region remains more or less unperturbed. The relative proportion of the rigid and flexible regions in cavities is presumably dependent upon the spatial locations of the cavities within the protein structures, and they may be responsible for accommodating differently substituted ligands at their cognate enzyme sites.

REFERENCES

1. Johnson, J. K., Wang, Z.-X., and Srivastava, D. K. (1992) *Biochemistry 31*, 10564−10575.
2. Johnson, J. K., and Srivastava, D. K. (1993) *Biochemistry 32*, 8004−8013.
3. Kumar, N. R., and Srivastava, D. K. (1994) *Biochemistry 33*, 8833−8841.
4. Srivastava, D. K., Kumar, N. R., and Peterson, K. L. (1995) *Biochemistry 34*, 4625−4632.
5. Kumar, N. R., and Srivastava, D. K. (1995) *Biochemistry 34*, 9434−9443.
6. Peterson, K. L., Sergienko, E. E., Wu, Y., Kumar, N. R., Strauss, A. W., Oleson, A. E., Muhonen, W. W., Shabb, J. B., and Srivastava, D. K. (1995) *Biochemistry 34*, 14942−14953.
7. Peterson, K. L., and Srivastava, D. K. (1997) *Biochem. J. 325*, 751−760.
8. Peterson, K. L., Galitz, D. S., and Srivastava, D. K. (1998) *Biochemistry 37*, 8446−8456.
9. Peterson, K. L., Peterson, K. M., and Srivastava, D. K. (1998) *Biochemistry 37*, 12659−12671.
10. Beinert, H., and Page, E. (1957) *J. Biol. Chem. 225*, 479−497.
11. Hall, C. L., Lambeth, J. D., and Kamin, H. (1979) *J. Biol. Chem. 254*, 2023−2031.
12. Nandy, A., Kieweg, V., Krautle, F. G., Vock, P., Kuchler, B., Bross, P., Kimm, J. J., Rashed, I., and Ghisla, S. (1996) *Biochemistry 35*, 12402−12411.
13. Murfin, W. W. (1974) Ph.D. Dissertation, Washington University, St. Louis, MO.
14. McFarland, J. T., Lee, M., Reinsch, J., and Raven, W. (1982) *Biochemistry 21*, 1224−1229.
15. Powell, P. J., Lau, S. M., Kilian, D., and Thorpe, C. (1987) *Biochemistry 26*, 3704−3710.
16. Lau, S.-M., Brantley, R. K., and Thorpe, C. (1988) *Biochemistry 27*, 5089−5095.
17. Vock, P., Engst, S., Eder, M., and Ghisla, S. (1998) *Biochemistry 37*, 1848−1860.
18. Srivastava, D. K., and Peterson, K. L. (1998) *Biochemistry 37*, 8446−8456.
19. Rudik, I., Ghisla, S., and Thorpe, C. (1998) *Biochemistry 37*, 8437−8445.
20. Engel, P. C. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. III, pp 597−655, CRC Press, Inc., London.
21. Thorpe, C., and Kim, J.-J. P. (1995) *FASEB J. 9*, 718−725.
22. Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I., and Ghisla, S. (1990) *J. Biol. Chem. 265*, 7116−7119.
23. Mancini-Samuelson, G. J., Kieweg, V., Sabaj, K. M., Ghisla, S., and Stankovich, M. T. (1998) *Biochemistry 37*, 14605−14612.
24. Nishikawa, S., Moroika, H., Fuchimura, K., Tanaka, T., Uesugi, S., Ohtuska, E., and Ikehara, M. (1986) *Biochem. Biophys. Res. Commun. 138*, 789−794.
25. Loll, P. L., and Lattman, E. E. (1990) *Biochemistry 29*, 6866−6873.
26. Raines, R. T., Sutton, E. L., Strauss, D. R., Gilbert, W., and Knowles, J. R. (1986) *Biochemistry 25*, 7142−7154.
27. Trievel, R. C., Wang, R., Anderson, V. E., and Thorpe, C. (1995) *Biochemistry 34*, 8597−8605.
28. Johnson, B. D., Mancini-Samuelson, G. J., and Stankovich, M. T. (1995) *Biochemistry 34*, 7047−7055.
29. Lehman, T. C., Hale, D. E., Bhala, A., and Thorpe, C. (1990) *Anal. Biochem. 186*, 280−284.
30. Thorpe, C., Matthews, R. G., and Williams, C. W., Jr. (1979) *Biochemistry 18*, 331−337.
31. Qin, L., and Srivastava, D. K. (1998) *Biochemistry 37*, 3499−3508.
32. Connolly, M. L. (1985) *J. Am. Chem. Soc. 107*, 1118−1124.
33. Connolly, M. L. (1983) *Science 221*, 709−713.
34. Eriksson, A. E., Baase, W. A., Zhang, X.-J., Heinz, D. W., Blaber, M., Baldwin, E. P., and Matthews, B. W. (1992) *Science 255*, 178−183.
35. Peterson, K. M., Gopalan, K. V., and Srivastava, D. K. (2000) *Biochemistry* (in press).
36. Kim, J.-J. P., Wang, M., and Paschke, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7523−7527.
37. Oxtoby, D. W., and Nachtrieb, N. H. (1985) in *Principles of Modern Chemistry*, p 696, Saunders College Publishing, Philadelphia, PA.
38. Wick, M. J., Mihic, S. J., Ueno, S., Mascia, M. P., Trudell, J. R., Brozowski, S. J., Ye, Q., Harrison, N. L., and Harris, R. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6504−6509.
39. Stoll, V. S., Simpson, S. J., Krauth-Siegel, R. L., Walsh, C. T., and Pai, E. F. (1997) *Biochemistry 36*, 6437−6447.
40. Sha, R. S., Kane, C. D., Xu, Z., Banaszak, L. J., and Bernlohr, D. A. (1993) *J. Biol. Chem. 268*, 7885−7892.
41. Baldwin, E., Xu, J., Hajiseyedjavadi, O., Baase, W. A., and Matthews, B. W. (1996) *J. Mol. Biol. 259*, 542−559.
42. Morton, A., Baase, W. A., and Matthews, B. W. (1995) *Biochemistry 34*, 8564−8575.
43. Morton, A., and Matthews, B. W. (1995) *Biochemistry 34*, 8576−8588.
44. Lee, H. J., Wang, M., Paschke, R., Nandy, A., Ghisla, S., and Kim, J.-J. P. (1996) *Biochemistry 35*, 12412−12420.

BI001317E