Structure of Hexadienoyl-CoA Bound to Enoyl-CoA Hydratase Determined by Transferred Nuclear Overhauser Effect Measurements: Mechanistic Predictions Based on the X-ray Structure of 4-(Chlorobenzoyl)-CoA Dehalogenase†

Wen-Jin Wu,‡ Vernon E. Anderson,§ Daniel P. Raleigh,*,‡,||,⊥ and Peter J. Tonge*,‡,||

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400, Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106-4935, Graduate Program in Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794-3400, and Graduate Program in Molecular and Cellular Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

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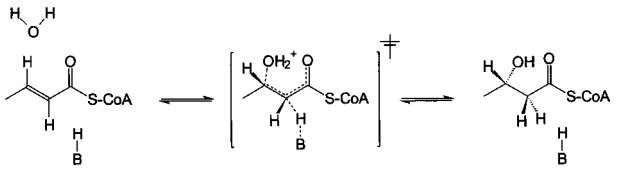
ABSTRACT: The structure of the substrate analog 2,4-hexadienoyl-coenzyme A (HD-CoA) bound to the enzyme enoyl-CoA hydratase has been determined using transferred nuclear Overhauser enhancement (TRNOE) spectroscopy. NOEs between the adenine H8 proton and several pantetheine protons in the bound form of HD-CoA indicate that the overall structure of the CoA molecule is bent, while NOEs between adenine and ribose protons indicate that the conformation about the glycosidic bond is *anti*. The absence of long range NOEs along the pantetheine moiety is consistent with this region of the molecule being bound in an extended conformation. In addition, NOEs between the vinylic protons indicate that the HD moiety is *s-trans* about C3−C4. The conformation of the CoA portion of bound HD-CoA is strikingly similar to that of the CoA portion of 4-(hydroxybenzoyl)-CoA bound to the active site of 4-(chlorobenzoyl)-CoA dehalogenase [Benning, M. M., et al. (1996) *Biochemistry 35*, 8103−8109]. The structural similarity of the ligands along with the primary sequence homology validates the modeling of the enoyl-CoA hydratase structure with the 4-(chlorobenzoyl)-CoA dehalogenase backbone. The homology modeling allows the prediction that the enoyl-CoA substrates are bound in an *s-cis* conformation about C1−C2 and that Glu 144 is present at the active site and can function as a general acid/base.

Enoyl-CoA hydratase (EC 4.2.1.17) catalyzes the *syn* addition of water across the C=C bond of α,*β*-unsaturated fatty acid CoA thioesters (Willadsen & Eggerer, 1975). On the basis of kinetic isotope effect studies, it has been proposed that the mechanism is concerted (Bahnson & Anderson, 1989, 1991), with both C2−H and C3−O bonds being formed in the same transition state. In Scheme 1, an enzyme group (B-H) supplies the required proton at C2 concomitant with the unassisted attack of water at C3.

Combined NMR, Raman, and UV−visible absorption studies have demonstrated that the enzyme active site causes ground state electron reorganization in conjugated substrate analogs bound to the enzyme (D'Ordine et al., 1994a). Specifically, using ^{13}C NMR, we have observed decreased shielding at C1 and C3 and an increased shielding at C2 for cinnamoyl-CoA (D'Ordine et al., 1994b; D'Ordine, 1993) and a similar effect at C1 and C2 of 2,4-hexadienoyl-CoA (HD-CoA)[1] (D'Ordine, 1993) when these substrates are bound to the enzyme active site. These observations

Scheme 1: Concerted Reaction



demonstrate that the *π*-electrons are polarized and suggest that the enzyme activates the ground state of the substrate to the chemical steps of catalysis. In addition, vibrational spectroscopic data indicate that the enzyme polarizes the ground state of the conjugated substrate analogs and that this polarization is limited to the C3=C2−C1=O portion of the acyl group (Tonge et al., 1995; D'Ordine et al., 1994a). Thus, for HD-CoA, C5=C4 appears unaffected upon binding to the enzyme, while canonical structures such as the one shown in Scheme 2 must make important contributions to the ground state structure of the molecule to account for the observed polarization of the C2=C3 double bond.

This selective polarization could result as a consequence of the alignment of the hexadienoyl (HD) molecule with the enzyme's electric field or via geometric distortion of the HD molecule so as to isolate C5=C4. In order to extract further

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* Authors to whom correspondence should be addressed. E-mail: (D.P.R.) Daniel.Raleigh@sunysb.edu and (P.J.T.) Peter.Tonge@sunysb.edu.

‡ Department of Chemistry, SUNY.

§ Case Western Reserve University.

|| Graduate Program in Biophysics, SUNY.

⊥ Graduate Program in Molecular and Cellular Biochemistry, SUNY.

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[1] Abbreviations: CPMG, Carr−Purcell−Meiboom−Gill; DQF-COSY, double-quantum-filtered correlation spectroscopy; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HD-CoA, 2,4-hexadienoyl-coenzyme A; NOESY, nuclear Overhauser effect spectroscopy; PCR, polymerase chain reaction; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TRNOE, transferred nuclear Overhauser effect; TRROE, transferred rotating frame nuclear Overhauser effect; TSP, 3-(trimethylsilyl) propionate; 1D, one-dimensional, 2D, two-dimensional.

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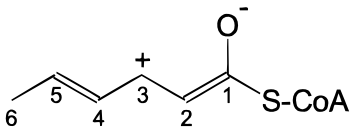
Scheme 2: Canonical Resonance Structure for the HD Molecule



information on the structure of bound HD-CoA, we undertook a transferred NOE (Balaram et al., 1972a,b; Clore & Groneborn, 1982, 1983) study of HD-CoA bound to enoyl-CoA hydratase. Many NOEs are observed for the bound ligand, allowing structural information to be extracted on both the CoA and HD portions of the ligand. In addition, the observed NOEs for the CoA portion of HD-CoA are consistent with the structure of CoA in the complex of 4-(hydroxybenzoyl)-CoA with 4-(chlorobenzoyl)-CoA dehalogenase. As the hydratase and dehalogenase share 28% sequence identity (50% similarity), the similarity in structure of the bound CoAs provides confidence that modeling the enoyl-CoA hydratase active site onto the framework provided by the X-ray structure of 4-(chlorobenzoyl)-CoA dehalogenase is justified. On the basis of these modeling studies, we propose both the identity and function of three catalytic moieties in the active site of enoyl-CoA hydratase.

MATERIALS AND METHODS

Chemicals. Coenzyme A (CoA) lithium salt was purchased from Sigma Chemical Co. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Labs.

Preparation of the CoA-Sepharose Affinity Column. A CoA-Sepharose affinity column was constructed by incubating 500 mg of CoA dissolved in 50 mM sodium borate at pH 10.0 (incubation buffer) with 5 g of epoxy-activated Sepharose 6B (Pharmacia) for 48 h at room temperature. Subsequently, the Sepharose was washed successively with incubation buffer, 0.1 M bicarbonate (pH 8.0), 0.1 M acetate (pH 4.0), and H_2O and incubated overnight with 0.1 M ethanolamine. Finally, the Sepharose was washed with 20 mM sodium phosphate (NaH_2PO_4), and 3 mM EDTA, at pH 7.4 (buffer A) and packed into a 1 × 10 cm column.

Preparation of Hexadienoyl-Coenzyme A. trans,trans-2,4-Hexadienoyl-CoA (HD-CoA) was synthesized and purified as described (Tonge et al., 1995). The concentration of the substrate stock solution was determined using an ϵ_{260} of 19 500 M^{-1} cm^{-1}.

Preparation and Purification of Enoyl-CoA Hydratase. A pUC18 plasmid containing the cDNA for rat mitochondrial enoyl-CoA hydratase was a kind gift from Professor Osumi at the Himeji Institute of Technology (Minami-Ishii et al., 1989). The cDNA encoding the mature hydratase protein was subcloned out of the pUC18 plasmid using PCR. In this amplification reaction, the forward primer used was 5′-CCTGGCATC**CATATG**GGTGCTAACTTTCAG-TACATC-3′ and the reverse primer was 5′-CGCTGGATC-CTGCAG**AAGCTT**TCAGTGGTCTTTGAAGTTGGC-3′. The regions of overlap with the DNA encoding the N and C termini of the enzyme are underlined. The forward primer was designed to introduce an *Nde*I restriction site and codon for methionine (bold) immediately preceding the GGT codon for the N-terminal glycine. The reverse primer was designed to introduce a *Hin*dIII site (bold) immediately following the TGA stop codon. Twenty cycles of PCR were performed using 0.6 μg of the pUC18 plasmid, 70 pmol of each primer, and an annealing temperature of 65 °C. The PCR product was isolated by ethanol precipitation, digested with *Nde*I and *Hin*dIII, gel purified, and ligated into the *Nde*I and *Hin*dIII sites of pET20b(+) (Novagen). The resulting construct (designated pET20ech1) was sequenced and subsequently used for protein expression in BL21(DE3)pLysS cells (Novagen).

Cultures of BL21(DE3)pLysS cells carrying the wild type plasmid were grown in 1 L of LB-ampicillin at 37 °C to an OD_{600} of 0.8. The cells were harvested by centrifugation and resuspended in an equal volume of fresh LB-ampicillin containing 1 mM isopropyl thiogalactoside. After a further 3 h of growth at 37 °C, the cells were harvested by centrifugation and the cell pellets frozen at −20 °C overnight. After thawing, the cells were resuspended in 30 mL of buffer A containing 1 mM DTT, briefly sonicated to shear the DNA and ensure complete cell lysis, and centrifuged at 17 000 rpm for 20 min. The enzyme was crystallized from the resulting supernatant by addition of ethanol while cooling in a dry ice/ethanol bath (Steinman & Hill, 1975). After standing for 48 h at 4 °C, the resulting white precipitate was collected by centrifugation, dissolved in a minimal volume of buffer A (20−25 mL), and dialyzed against 2 × 2 L of buffer A. The dialyzed solution was clarified by centrifugation (20 min at 17 000 rpm), filtered through a 0.2 μm filter (Millex), and applied to the CoA-sepharose affinity column equilibrated with buffer A. After the column was washed with buffer A, enzyme was eluted from the column using buffer A containing 0.3 M KCl. Fractions containing enzyme were pooled and concentrated using centricon-10 (Amicon) concentrators for the NMR experiments. For experiments requiring the enzyme in D_2O, enoyl-CoA hydratase was equilibrated with 20 mM NaH_2PO_4, and 3 mM EDTA in D_2O at pD 7.4 (pD = meter reading + 0.4) prior to elution from the CoA-sepharose affinity column. Subsequently, the enzyme was eluted from the column using the D_2O buffer containing 0.3 M KCl. The enzyme concentration was determined using the extinction coefficient of 16 000 M^{-1} cm^{-1} at 280 nm for the crystalline protein (Hass & Hill, 1969).

Samples for NMR Experiments. The NMR samples all contained 0.3 M KCl, 20 mM NaH_2PO_4, and 3 mM EDTA in either 100% D_2O at pD 7.4 or 92% H_2O/8% D_2O at pH 7.0. For the TRNOE experiments, 0.63 mM enoyl-CoA hydratase was titrated with a 205 mM HD-CoA stock solution. The final concentrations of HD-CoA and enzyme were 17 and 0.58 mM, respectively.

NMR Spectroscopy. The ^1H NMR spectra were recorded at the SUNY at Stony Brook NMR Center on a Bruker AMX-600 spectrometer and a Varian Inova 500 MHz spectrometer. All experiments in this study were performed at 298 K, unless otherwise specified. The two-dimensional experiments were acquired in the phase-sensitive mode with time proportional phase increment (TPPI) (Marion & Wüthrich, 1983).

Assignment of the Proton Spectra of the Free Substrate. The ^1H spectrum of free HD-CoA was assigned using DQF-COSY (Piantini et al., 1982; Rance et al., 1983) and ROESY (Bothner-By et al., 1984) spectra and by comparison with published assignments of CoA derivatives (Lee & Sarma, 1975; D'Ordine et al., 1995). A DQF-COSY spectrum was collected using a 512 (real) × 4096 (complex) data matrix.

The acquisition parameters for the DQF-COSY experiment were 32 scans per t_1, a 1.5 s relaxation delay, and a spectral width of 6944.4 Hz in both dimensions. No significant cross-peaks were observed in a NOESY (Kumar et al., 1980) spectrum collected with a 300 ms mixing time. A ROESY spectrum with a 300 ms mixing time was recorded to obtain structural information for the free ligand, and a ROESY experiment with a 75 ms mixing time was also performed to serve as a control for the transferred ROE (TRROE) experiments of the enzyme−ligand complex. *J* coupling constants for the hexadienoyl portion were obtained either directly from a 1D spectrum recorded with 64K data points or from the DQF-COSY spectrum mentioned above (for J_{ab} only). The final digital resolution of the DQF-COSY spectrum was 0.85 Hz.

Transferred NOE Experiments. The conformation of bound HD-CoA was studied by two-dimensional transferred NOE (TRNOE) experiments. Immediately prior to the start of the 2D experiments, the titration of the enzyme by HD-CoA was followed by 1D NMR spectroscopy. Spectra were recorded using a conventional 1D pulse sequence with water presaturation and by applying a CPMG sequence prior to acquisition in order to attenuate broad lines due to the enzyme.

The two-dimensional TRNOE experiments consisted of a series of NOESY spectra with mixing times of 25, 50, and 75 ms. In order to achieve flat baselines, several precautions were taken. A spin echo sequence was inserted after the first 90° excitation pulse to reduce the resonances due to the enzyme, and the pulse sequence used was presat-90°$_{ø1}$-Δ-180°$_{ø2}$-Δ-t_1-90°$_x$-τ-90°$_{ø3}$-acq. (Glaudemans et al., 1990). In addition, first-order phase corrections were avoided by carefully adjusting the delay time before starting acquisition and a large spectral width and a large filter band width were used in the F_2 dimension. Short mixing times were used to reduce spin diffusion and to achieve better linearity for the NOE volume buildup curves. A TRNOE experiment with a 75 ms mixing time was also performed for the samples in a 92% H_2O/8% D_2O solvent mixture in order to observe NOEs from the exchangeable protons. Transferred ROE (TRROE) experiments with a mixing time of 75 ms were performed with the D_2O and H_2O samples to distinguish between direct cross relaxation and two step spin−spin diffusion. The peaks due to the H2′ and H3′ protons of the ribose overlapped with the HOD peak at 298 K but are resolved at 277 K. The resonances of the hexadienoyl portion are also better resolved at 277 K; consequently, a set of TRNOE experiments with mixing times of 30, 50, 75, and 175 ms were performed at 277 K to study the conformation about the glycosidic bond and the conformation of the hexadienoyl region.

The parameters for the TRNOE study at 298 K were as follows: 80 scans per t_1 increment for mixing times of 50 and 75 ms, 96 scans per t_1 increment for the 25 ms mixing time, a 12 200 Hz sweep width in F_2, a 8400 Hz sweep width in F_1, 25 000 Hz filter width, 512 real points in F_1, 4096 complex points in F_2, and a 2 ms spin echo delay time in total. Low-power presaturation during the 2.4 s relaxation delay was used to suppress the residual HOD signal. Eighty scans per t_1 increment were collected for the TRNOE spectra recorded at 277 K. The other acquisition parameters were identical to those used for the 298 K experiments.

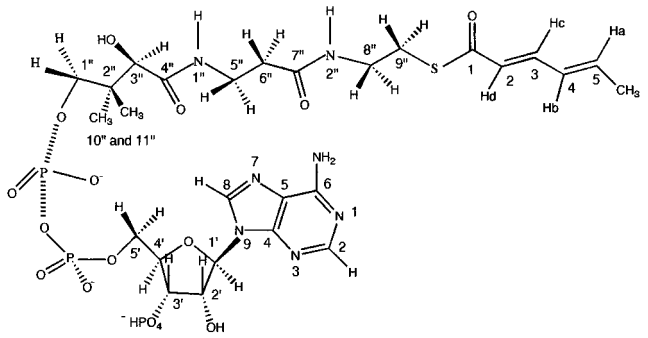


FIGURE 1: Covalent structure of hexadienoyl-coenzyme A showing the numbering system used.

Data were processed on a Silicon Graphics workstation with the program FELIX (Hare, Inc.). Zero filling was performed twice in F_1 and once in F_2 to give the final data matrices of 2048 (F_1) by 4096 (F_2) real data points. A cosine bell window function was applied in both dimensions prior to Fourier transformation, and the first t_1 point was multiplied by 0.5 to reduce t_1 noise for all data sets. Baseline correction was performed by using a fifth-order polynomial. All chemical shifts were referenced to the internal standard, TSP at 0.0 ppm.

NOE cross-peak volumes were measured using the standard FELIX integration routine. The spectra were scaled by the intensity of the TSP peak volume. The TRNOE cross-peak volumes were then normalized by the product of the number of protons ($n_a n_b$, where *n* is the number of protons of spin a or spin b) contributing to the cross-peaks (Yip, 1990). Cross-peaks which were too close to the diagonal peaks or with poor signal to noise were not used for distance calculations.

Determination of Interproton Distances. Interproton distances were determined by the method of initial slopes using the two-spin approximation (Hyberts & Wagner, 1989). The initial slopes were obtained by fitting the plots of NOE cross-peak volumes versus mixing time to a second-order polynomial. The initial slope for a given proton pair (σ_{a-b}) was then compared to the reference initial slope and converted into relative distances using an r^{-6} dependence, where *r* is the interproton distance. The diastereotopic 1″ methylene protons of pantoic acid were used as the distance reference with the distance between these two geminal protons assumed to be 1.8 Å. None of the conclusions discussed below depends on exact distance measurements. The same conclusions are reached if the observed NOEs are classified as strong, medium, and weak.

RESULTS AND DISCUSSION

Proton Assignment. A schematic diagram of HD-CoA is shown in Figure 1 and indicates the numbering system used. The chemical shifts of HD-CoA in 92% H_2O/8% D_2O at pH 5.2 , and 298 K are given below: H1′ 6.15, H2′ and H3′ (obscured by the solvent resonance), H4′ 4.60, H5′ 4.25, H1″$_A$ 3.86, H1″$_B$ 3.58 (1″$_A$, the upfield 1″ proton; 1″$_B$, the downfield 1″ proton), H3″ 4.04, H5″ 3.46, H6″ 2.44, H8″ 3.37, H9″ 3.05, C2″ (methyl groups) (10″ CH3 and 11″ CH3) 0.91, 0.78, H8 8.54, H2 8.24, H_a 6.32, H_b 6.18, H_c 7.14, H_d 6.08, CH3 1.84, N1″H 8.03, N2″H 8.20.

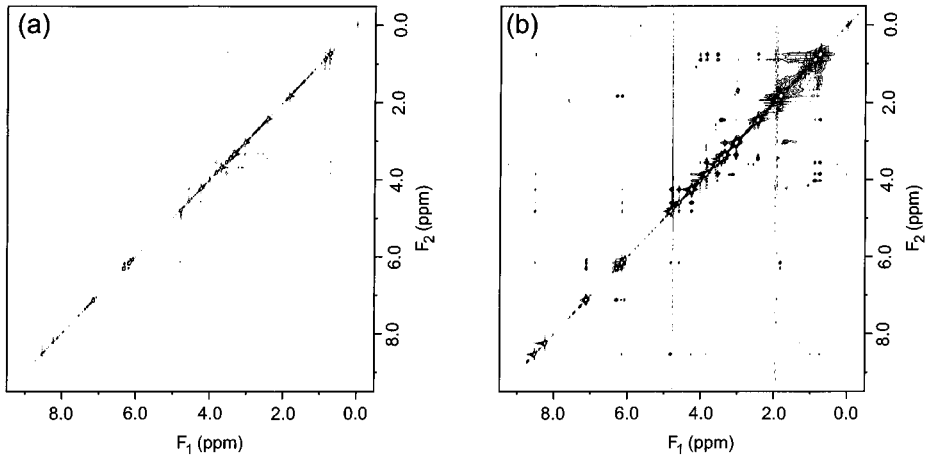


FIGURE 2: (a) 2D NOESY spectrum of HD-CoA recorded with a 300 ms mixing time in the absence of enzyme. (b) 2D NOESY spectra of HD-CoA (17 mM) recorded in the presence of 0.58 mM enoyl-CoA hydratase using a 75 ms mixing time. Both spectra were acquired in D_2O at 298 K and pD 7.4. Only positive contour levels are shown.

Conformation of the Free Ligand. The conformation of the hexadienoyl portion of the free ligand was analyzed by measuring $^1H-^1H$ scalar coupling constants. Coupling constants of 15.0 Hz (H_c and H_d) and 14.7 Hz (H_a and H_b) indicate that the hexadienoyl C═C double bonds are both *trans*. The 11.0 Hz coupling constant for proton c and proton b indicates that the C═C−C═C portion is in the planar *s-trans* configuration[2] (Patel, 1969).

A NOESY spectrum of the free ligand in solution showed only very weak NOEs, some of which were positive and some negative (Figure 2a). No long range NOEs were observed. In the 300 ms mixing time ROESY spectrum of the free substrate (data not shown), a very weak long range NOE between the adenine H8 proton and one of the diastereotopic methyl groups located on the C2″ carbon (either the 10″ or the 11″ methyl group) was observed; this weak NOE may imply that the free substrate, although flexible, has some propensity to adopt a bent conformation in solution. This NOE was not observed in the ROESY spectrum with a 75 ms mixing time recorded under the same conditions in which the TRROE experiment was performed.

Overall Conformation of the Bound Ligand. The NOESY spectra of HD-CoA recorded in the presence of enzyme (i.e. TRNOE spectra) (Figure 2b) showed many NOE cross-peaks in contrast to the NOESY spectrum of the free ligand (Figure 2a). Among the TRNOE peaks, four long range NOEs were observed from the adenine H8 proton (i) to one of the C2″ diastereotopic methyl groups (10″ or 11″ methyl), (ii) to the $1''_A$ proton, (iii) to the N1″ amide proton, and (iv) to the 3″ proton (visible at lower contour levels). This region of the TRNOE spectrum is displayed in Figure 3. TRROE experiments conducted in 100% D_2O and in 92% H_2O using a 75 ms mixing time clearly show that these observed long range NOEs are direct NOEs and are not due to two-step spin−spin diffusion. The signs of the cross-peaks in the TRROE spectra were opposite to that of the diagonal peaks. These long range NOEs indicated that the HD-CoA binds to enoyl-CoA hydratase in a bent conformation. A survey of the structures of CoA derivatives bound to proteins shows, in many cases, that the CoA moiety adopts a bent conformation in which the direction of the molecule roughly reverses in the region of the pyrophosphate group. Thus, a bent conformation is observed for CoA derivatives bound to 4-(chlorobenzoyl)-CoA dehalogenase (1NZY),[3] acyl-CoA binding protein (1ACA),[3] citrate synthase (1CSR, 1CSH, 2CTS, and 3CSC),[3] butyryl-CoA dehydrogenase (1BUC),[3] medium-chain acyl-CoA dehydrogenase (3MDE),[3] and dihydrolipoyl transacetylase (1EAB, 1EAC, and 1EAD).[3]

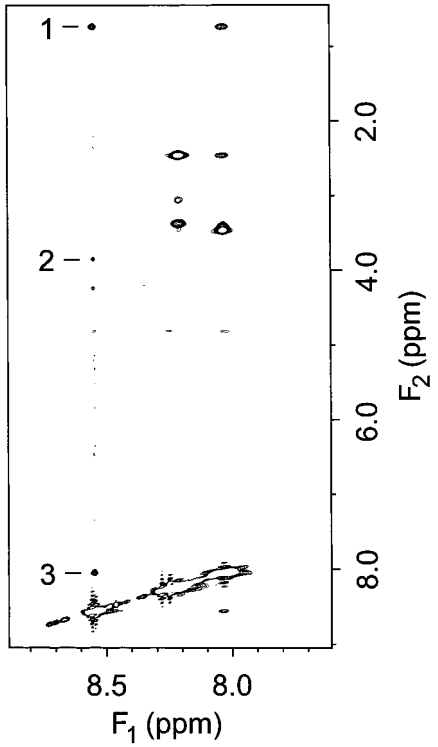


FIGURE 3: Portion of the 2D TRNOE spectrum (75 ms mixing time) of HD-CoA in 92% H_2O/8% D_2O at 298 K and pH 7.0 showing NOE cross-peaks from the adenine H8 proton to (1) one of the diastereotopic methyl groups (10" or 11″), (2) the upfield $1''_A$ proton, and (3) to the N1″ amide proton.

[2] The lower case *s* stands for "single bond", and the *cis* and *trans* refer to the orientation of the double bonds. Formally, the rotamers about a single bond connecting two double bonds may be defined by the dihedral angle determined by the four atoms contained in the two double bonds. If this dihedral is 0°, the conformation is described as *s-cis*, while if the dihedral angle is 180°, the conformation is referred to as *s-trans*.

[3] The coordinates for each structure were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977), and the PDB file names are given in parentheses.

Although this appears to be a common feature in many of the structures, there is considerable variability in the exact conformation. In the recently determined structure of 4-(hydroxybenzoyl)-CoA bound to 4-(chlorobenzoyl)-CoA dehalogenase (Benning et al., 1996), a hydrogen bond is observed between the pantetheine N1″ amide proton and the adenine N7. This intramolecular hydrogen bond brings the N1″ amide proton and the adenine H8 proton close in space. The distance between the adenine H8 proton and the N1″ amide proton in this structure ranges from 2.5 Å to 2.9 Å (from the three crystallographically distinct subunits). This intramolecular hydrogen bond and the close approach of the H8 proton and the N1″ amide proton are unique among the known bound CoA structures. In all of the other known structures, this distance is at least 5.2 Å. In this study, the intensity of the NOE cross-peak between the adenine H8 proton and the N1″ amide proton of HD-CoA corresponds to an approximate interproton distance of 2.9 Å. This strongly suggests that the amide proton of N1″ is close to N7 of the adenine ring, and thus, this part of the bound HD-CoA adopts a conformation similar to that of 4-(hydroxybenzoyl)-CoA bound to 4-(chlorobenzoyl)-CoA dehalogenase but different from all of the other CoA structures examined.

Conformation about the Glycosidic Bond. In order to distinguish between the two possible conformations about the adenine−ribose glycosidic bond (*anti* or *syn*), we measured distances between the adenine H8 proton and ribose ring protons. Due to the overlap of the H2′ and H3′ protons with the HOD peak at 298 K, distances could not be measured for these two protons at this temperature. However, at 277 K, the H2′ and H3′ peaks are well separated from the HOD peak, although they slightly overlap with each other, and NOEs can be observed between the adenine H8 proton and the H2′ and H1′ protons. The significantly larger NOE between the H8 proton and H2′ proton, compared to the NOE between the H8 and H1′ protons, suggests that the glycosidic bond is in the *anti* conformation. Additional NOEs from H8 to other sugar protons also indicate that the glycosidic bond is *anti*. The approximate distances derived from the TRNOE data collected at 298 K are 3.7 Å from the H8 proton to the H1′ proton, 4.9 Å from the H8 to the H4′ proton, and 3.7 Å from the H8 proton to the pseudoatom corresponding to the H5′ protons. The NOE from the H8 proton to the H5′ protons is particularly noteworthy and indicates an *anti* conformation. These constraints limit the allowed range of the glycosidic dihedral to between −110 and −160°. By comparison, 4-(hydroxybenzoyl)-CoA is bound to the dehalogenase in an *anti* conformation where the glycosidic dihedral is between −137 and −139° in each of the three subunits. The distance between the H8 proton and the H1′ proton is 3.9 Å in each of the subunits. The H8 to H4′ proton distance ranges from 4.5 to 4.7 Å in the three subunits, while the distance between the H8 proton and the H5′ methylene group ranges from 3.8 to 4.1 Å.

Conformation of the Pantetheine Portion. The pantetheine portion of several enzyme-bound CoA structures (1ACA,[4] 1CSR, 1CSH, and 3CSC) adopts a curled conformation. In this type of structure, the pantetheine curls such that the 9″ methylene protons are close to the 10″ or 11″ methyl groups with the closest interproton distances ranging from 1.8 to 3 Å. In contrast, the pantetheine region of 4-(hydroxybenzoyl)-CoA bound to 4-(chlorobenzoyl)-CoA dehalogenase adopts a more extended conformation in which the closest interproton distance from the 9″ methylene protons to the two C2″ methyl groups (10″ or 11″) is 7.7 and 6.7 Å. The presence or absence of a medium to strong NOE from the 9″ methylene protons to the 10″ or 11″ methyl groups thus provides a simple means of distinguishing between these two alternative conformations. NOEs of this size should be easily detectable, and indeed, NOEs corresponding to distances of at least 3.9 Å are readily observed in the enoyl-CoA hydratase−HD-CoA TRNOE spectra. However, no NOEs were observed between the 9″ methylene protons at 3.05 ppm and the C2″ methyl groups (10″ and 11″ methyls) at 0.89 or at 0.76 ppm (Figure 4). The absence of these long range NOEs indicates that the pantetheine region of the bound HD-CoA adopts a more extended conformation instead of a curled structure.

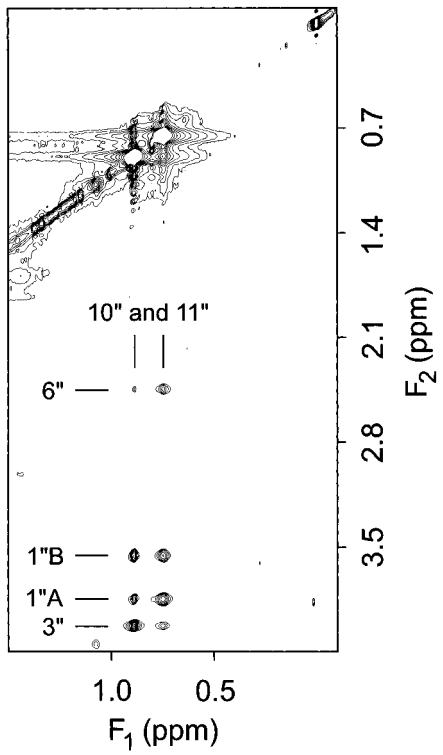


FIGURE 4: Portion of the 2D TRNOE spectrum (75 ms mixing time) of HD-CoA recorded in 100% D_2O at 298 K and pD 7.4. NOE crosspeaks from the 6″ methylene protons to the 10″ methyl and to the 11″ methyl group are visible. Cross-peaks corresponding to NOEs from the H3″ proton to the 10″ methyl group and to the 11″ methyl group are also indicated. Only positive contour levels are shown.

Having established that the overall conformation of the HD-CoA pantetheine is extended, we now discuss local conformational preferences in the pantetheine moiety. Analysis of the TRNOE spectra of the HD-CoA−enoyl-CoA hydratase complex indicates that there is a small kink in the pantetheine portion near the 4″ carbon. A similar conformation is found in the structure of 4-(hydroxybenzoyl)-CoA bound to the dehalogenase. In the dehalogenase structure the pantetheine group is in an extended conformation with a small kink roughly centered at the 4″ carbon due to both the 2″−3″−4″−N1″ and 4″−N1″−5″−6″ dihedral angles being gauche. In the dehalogenase structure, methyl groups

[4] The acyl-CoA binding protein (1ACA) structure was determined by NMR; 18 of the 20 deposited structures fall within this range, and two do not.

on the C2″ carbon (the 10″ and 11″ methyl groups) are noticeably closer to the 6″ methylene protons than to the 5″ methylene protons. The measured interproton distance (pseudoatom to pseudoatom) between the 6″ methylene protons and the more distant of the two methyl groups ranges from 4.9 to 5.2 Å for the three different subunits, while the distance between the pseudo atom representing the 6″ methylene group and a pseudoatom corresponding to the other methyl group ranges from 3.6 to 4.1 Å in the three subunits. In contrast, the pseudoatom to pseudoatom distances between the 5″ methylene group and the more distant methyl group fall within the range of 5.8−6.0 Å. The distance between the pseudoatom corresponding to the 5″ methylene group and the closer of the two methyl groups ranges from 5.0 to 5.2 Å. If HD-CoA adopted a similar structure, NOEs of unequal intensity would be expected between the 6″ methylene protons and the two C2″ methyls and only weak NOEs would be expected between the 5″ methylene protons and the C2″ methyls. This is precisely what is observed. Sizable NOEs of unequal intensity between the 6″ methylene group and the two C2″ methyl groups (10″ and 11″ methyls) are clearly visible in the TRNOE spectra of the HD-CoA−enoyl-CoA hydratase complex (Figure 4). Only weak NOEs between the 5″ methylene protons and the 10″ and 11″ methyl groups were observed (visible at contour levels lower than that used in Figure 4). Other experimentally determined distances were also consistent with those found in the 4-(hydroxybenzoyl)-CoA−4-(chlorobenzoyl)-CoA dehalogenase complex. The experimentally derived distances from the N1″ proton to the two C2″ methyl groups were 4.6 and 3.2 Å. The calculated distance between the N1″ proton and the H3″ proton was 3.6 Å. For comparison, the corresponding distances in the 4-(hydroxybenzoyl)-CoA−4-(chlorobenzoyl)-CoA dehalogenase complex are 4.8−5.1, 3.3−3.5, and 3.6−3.7 Å, respectively. These observations reinforce the belief that CoA adopts a very similar conformation in both the HD-CoA−enoyl-CoA hydratase and the 4-(hydroxybenzoyl)-CoA−4-(chlorobenzoyl)-CoA dehalogenase complexes.

It has been suggested that the geminal dimethyls on the C2″ carbon may play an important role in determining the conformational properties and/or dynamics of the pantetheine portion of CoA (Milstein & Cohen, 1972; Borchardt & Cohen, 1972; D'Ordine et al., 1995). Three separate noneclipsed rotamers are possible for the torsion angle corresponding to rotation about the C2″−C3″ bond. In the presence of enoyl-CoA hydratase, NOEs of differing intensity are observed from the H3″ proton of HD-CoA to the geminal methyl groups (Figure 4), indicating that one of the methyl groups is closer to H3″. The approximate distances derived from the TRNOE measurements from H3″ to the two methyl groups are 3.0 and 3.7 Å. The observation of unequal NOEs from H3″ to the diastereotopic methyls eliminates one of the three possible rotamers [rotamer-2 in the notation of D'Ordine et al. (1995)] since roughly equal intensity NOEs would be expected for that conformation. In the absence of diastereotopic assignments, it is not possible to distinguish between the two remaining rotamers. These distances are, however, consistent with the known structures of other CoA derivatives. For example, in the X-ray structure of 4-(hydroxylbenzoyl)-CoA bound to 4-(chlorobenzoyl)-CoA dehalogenase, the distances from the H3″ proton to pseudoatoms corresponding to the two methyl groups are 2.8−3.0 and 3.8−3.9 Å.

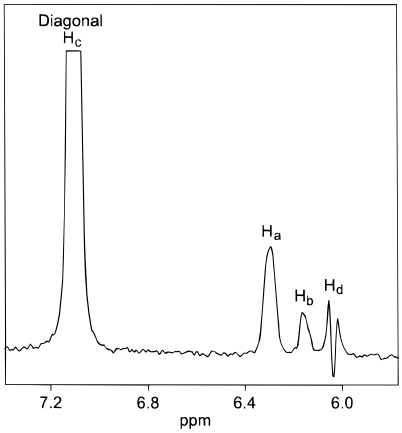


FIGURE 5: Slice through the Hc proton from a 2D TRNOE spectrum (75 ms mixing time) of HD-CoA at 277 K. Cross-peaks due to the a, b, and d protons of the hexadienoyl group are indicated. The distorted line shape of the cross-peak due to proton d arises from zero quantum effects.

Conformation of the Hexadienoyl Region. A major goal of this study was to determine the conformation of the hexadienoyl region of the substrate. HD-CoA binds to enoyl-CoA hydratase but is not hydrated because the additional conjugation of C4═C5 stabilizes the enoyl thioester (Wakil & Hübscher, 1960; Cuebas & Schulz, 1982). Since HD-CoA is not significantly hydrated, it has been extensively used as a spectroscopic probe of the electronic nature of the enzyme active site. The available UV−visible absorption, Raman, and ^{13}C NMR experiments all indicate that binding to the enzyme results in a localized polarization of the hexadienoyl C═O and C2═C3 bonds (Tonge et al., 1995). The polarization arises from interaction of the HD molecule with the enzyme's electrostatic field and specifically, at least in part, from the interaction of the HD carbonyl group with an amino acid residue situated at the N terminus of an α-helix (see below). However, the question remains as to why the polarization is limited to the C3═C2−C═O portion of the HD molecule, especially as this is the part of the substrate wherein the chemistry of hydration occurs. In principle, the selective polarization of the C═O and C2═C3 bonds could arise due to the relative orientation of the enzyme's electrostatic field with the HD molecule or due to twisting about the C3−C4 single bond, which would break the planarity of the conjugated system. In order to examine the latter possibility, the conformation about the C3−C4 bond was determined by measuring the relative intensities of the NOEs between the hexadienoyl Hc−Hb and Hc−Ha proton pairs (Figure 1). When the two double bonds are in an *s-cis* configuration, the expected distance between Hc and Hb (D_{cb}) is 2.6 Å and that between Hc and Ha (D_{ca}) is 3.7 Å. In this conformation, the expected ratio of the NOE intensity is 8.3/1 for these two proton pairs (Hc−Hb/Hc−Ha). On the other hand, when these two double bonds are in an *s-trans* conformation, the expected distances are 3.2 Å for D_{cb} and 2.5 Å for D_{ca}, which gives an expected NOE ratio of 0.23/1 (Hc−Hb/Hc−Ha). Due to a slight overlap of the Ha and Hb resonances at room temperature, the exact NOE ratio could not be determined. Fortunately, the peaks are resolved at 277 K, and the intensities can be accurately measured. A slice through the Hc proton diagonal peak of the 75 ms spectrum collected at 277 K is shown in Figure 5. The much

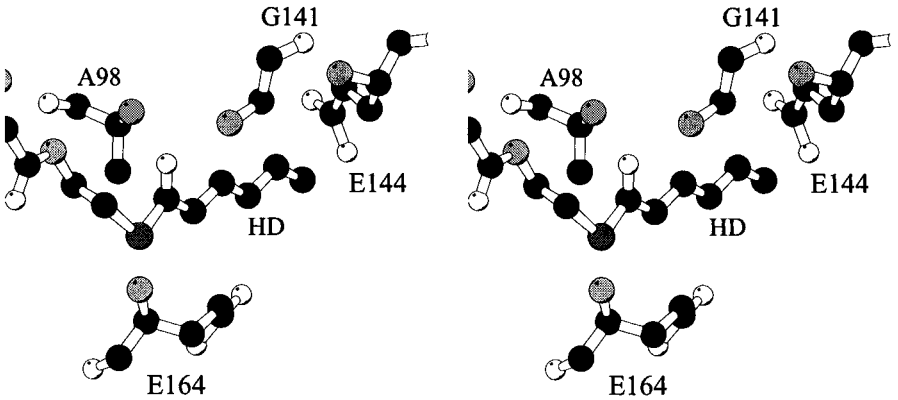


FIGURE 6: Stereofigure showing a model of the enoyl-CoA hydratase active site. The numbering system is based upon the enoyl-CoA hydratase sequence. The structure shown was generated from the dehalogenase structure by replacing the 4-hydroxybenzoyl group with the hexadienoyl (HD) group. Atom colors used are as follows: carbon, black; sulfur, dark gray; nitrogen, light gray; and oxygen, white. Trp 137 in the dehalogenase was replaced with a Glu, and the position was renumbered (E164) to reflect the numbering of the hydratase sequence. Similarly, Gly 117 was replaced with Glu 144 (E144), Phe 64 with Ala 98 (A98), and Gly 114 with Gly 141 (G141). The HD carbonyl oxygen is 2.9 Å from the backbone nitrogen of A98 and 2.7 Å from the backbone nitrogen of G141. The conformations of the E144 and E164 side chains have been adjusted to position the carboxyl groups as close as possible to the HD group, while also ensuring that the side chains adopt one of the known conformations for glutamate residues in proteins (Dunbrack & Karplus, 1993). Thus, the HD C3 carbon is 4.1 Å from one of the E144 carboxyl oxygens ($\chi_1 = 60°$, and $\chi_2 = 180°$), and the HD C2 carbon is 3.6 Å from one of the E164 carboxyl oxygens ($\chi_1 = 300°$, and $\chi_2 = 300°$). The figure was created using Molscript (Kraulis, 1991).

larger NOE from Hc to Ha than to Hb qualitatively excludes the *s-cis* conformation and supports an *s-trans* conformation.

Quantitative analysis of the NOE buildup curves supports this conclusion. TRNOE spectra with mixing times of 30, 50, 75, and 175 ms were collected. There is clear evidence for spin diffusion in the longer mixing time data set, and the signal to noise of the 30 ms data set is somewhat limited; nevertheless, the data clearly exclude an *s-cis* conformation. More importantly, the data also exclude a significant deviation from planarity and strongly favor a planar *s-trans* conformation. The largest deviation from planarity that is consistent with the data is less than 30°. The measured ratio of the NOEs is 4.6 for the 30 ms data set and 3.7 for the 50 ms data set, corresponding to a calculated dihedral angle between 180 and 158°. These results do not support the notion that the localized polarization of the hexadienoyl C═O and C2═C3 bonds is due to twisting about the C3−C4 single bond.

Structure of 4-(Chlorobenzoyl)-CoA Dehalogenase and Its Relevance to Enoyl-CoA Hydratase. Recently, Holden and co-workers solved the X-ray crystal structure of 4-(chlorobenzoyl)-CoA dehalogenase from *Pseudomonas* sp. strain CBS-3 with 4-(hydroxybenzoyl)-CoA bound in the active site (Benning et al., 1996). Besides the previously identified sequence homology (Babbitt et al., 1992), several other lines of evidence suggest that enoyl-CoA hydratase and 4-(chlorobenzoyl)-CoA dehalogenase are structurally homologous. Firstly, both enzymes bind substrates that are thioesters of CoA, and both enzymes are expected to stabilize negative charge buildup on the substrate's carbonyl oxygen in the transition state. Secondly, on the basis of Raman spectroscopic studies (Tonge et al., 1995; Taylor et al., 1995; D'Ordine et al., 1994a), the dehalogenase and hydratase enzymes both activate their substrates via π-electron polarization. Thirdly, on the basis of sequence homology with enoyl-CoA isomerase, site-directed mutagenesis has been used to show that Glu 164 in enoyl-CoA hydratase is a key catalytic group (D'Ordine et al., 1994b; Müller-Newen et al., 1995). In the sequence alignment shown in Table 1, the dehalogenase residue corresponding to Glu 164 is Trp 137. In the dehalogenase crystal structure, the indole of Trp 137 is positioned 3.5 Å from C2 of the CoA thioester's benzoyl group. Thus, replacement of Trp 137 with Glu positions the Glu carboxylate side chain so that it can function as the proton donor during the hydration reaction, consistent with the site-directed mutagenesis studies (see below).

In this study, the TRNOE experiments have identified over 25 NOEs for the CoA portion of HD-CoA bound to enoyl-CoA hydratase. All the observed NOEs are consistent with the CoA molecule being bound to the hydratase in a conformation highly similar to that of CoA in the 4-(hydroxybenzoyl)-CoA−4-(chlorobenzoyl)-CoA dehalogenase complex. These data, taken together with the sequence alignment and site-directed mutagenesis studies described above, provide strong evidence that the dehalogenase crystal structure can be used to identify catalytically important interactions in the enoyl-CoA hydratase active site.

Mechanistic Predictions. Both the dehalogenase and hydratase polarize the π system that is conjugated with the carbonyl of the substrate CoA thioester. In the dehalogenase, the substrate polarization arises in part from two hydrogen bonds formed between the thioester carbonyl oxygen and the backbone amide NH groups of Gly 114 and Phe 64. As Gly 114 is positioned at the N terminus of an α-helix formed by residues 114−121, the dipole of this α-helix may also contribute to the observed polarization. Since the conformation of CoA bound to the hydratase as determined by this study is similar to that of CoA in the dehalogenase crystal structure, we propose that the adenine and pantetheine moieties occupy similar positions in the two different enzymes so that the thioester carbonyl will be oriented toward the same polarizing functionalities, i.e. two backbone amides and the amino terminus of an α-helix. Gly 114 is part of a GGG tripeptide that is conserved amongt all of the enzymes in the low-homology enoyl-CoA hydratase/isomerase family (Table 1). In the dehalogenase, the residues that form the α-helix are 114-GGLGISLA-121. The equivalent residues in the hydratase are 141-GGCELAMM-148, and secondary structure prediction suggests that these residues are also capable of forming an α-helix. Finally, Phe 64 in the dehalogenase is replaced by Ala 98 in the hydratase.

Table 1: Alignment of the low homology enoyl-CoA hydratase/isomerase superfamily.[a]

Protein (Swiss-Prot or NCBI sequence identifier)	Sequence	Last aa	Reference
Identified hydratases	········X·····················X·····························		
ECHM_RAT	GYALGGGCELAMMCDIIYAGEKAQF--GQPEILLGTIPGAGGTQRLTRAVGKSLAMEMVL	193	Minami-Ishii et al., (1989)
ECHM_HUMAN	GYPFGGGCELAMMCDIIYAGEKAQF--AQPEILIGTIPGAGGTQRLTRAVGKSLELEMVL	193	Kanazawa et al., (1993)
1055218	GFALGGGCEIAMSCDIRIASSNARF--GQPEVGLGITPGFGGTQRLSRLVGMGMAKQLIF	163	Boynton et al., (1996)
ECHM_CAEEL	GFALGGGNELAMMCDIIYAGEKARF--GQPEINIGTIPGAGGTQRWARAAGKSFAMEVCL	191	Wilson et al., (1994)
ECH_RhMeliloti	GFALGGGCELAMMCDFIIASETAKF--GLPEITLGVIPGMGGSQRLTRAVGKAKAMDLIL	160	Margolin et al., (1995)
CAID_EColi	GYAFGGAFELALAADFIVCADNASF--ALPEAKLGIVPDSGGVLRLPKILPPAIVNEMVM	196	Eichler et al., (1994)
AUH_HUMAN	GLALGGGLELALACDIRVAASSAKM--GLVETKLAIIPGGGGTQRLPRAIGMSLAKELIF	238	Nakagawa et al., (1995)
ECHP_HUMAN	GMAFGGGLELALGCHYRIAHADAQV--GLPEVTLGLLPGARGTQLLPRLTGVPAALDLIT	153	Chen et al., (1991)
ECHP_RAT	GVALGGGLELALGCHYRIANAKARV--GLPEVTLGILPGARGTQLLPRVVGVPVALDLIT	152	Osumi et al., (1985)
FABB_ECOLI	GYALGGGCECVLATDYRLATPDLRI--GLPETKLGIMPGFGGSVRMPRMLGADSALEIIA	168	Yang et al., (1991)
1363338	GMALGGGLELSLGCHYRIAHAEARI--GFPEVTLGILPGARGTQLLPRLIGVPAALDLIT	155	Caira et al., (1995)
493657	GSCVGGGLEVAISCQYRIATKDRKTVLGTPEVLLGALPGAGGTQRLPKMVGVPAALDMML	202	Kamijo et al., (1994)
Gastrin binding protein			
433066	GSCLGGGLELAISCQYRIATKDRKTVLGAPEVLLGILPGAGGTQRLPKMVGVPAVFDMML	262	Zhang & Baldwin (1994)
Naphthoate synthases			
MENB_HIN	GYAIGGGHVLHMLCDLTIAAENAIFGQTGPKV--GSFDGGWGASYMARLVGQKKAREIWF	185	Tatusov et al., (1996)
MENB_BACSU	GYAIGGGHVLHIVCDLTIAADNAIFGQTGPKV--GSFDAGYGSGYLARIVGHKKAREIWY	161	Driscoll & Taber (1992)
MENB_ECOLI	GYSIGGGHVLHMMCDLTIAADNAIFGQTGPKV--GSFDGGWGASYMARIVGQKKAREIWF	185	Sharma et al., (1992)
MENB_HPH	GYAVGGGHVLHMMCDLTIAADNAKFGQTGPKV--GSFDGGWGASYMARIVGQKKAREIWF	185	Lubys et al., (1996)
Enoyl-CoA Δ3Δ2 isomerases			
D3D2_RAT	GASPAGGCLMALTCDYRIMADNSKYTIGLNESLLGIVAPFWLKDNYVNTIGHRAAERALQ	254	Müller-Newen & Stoffel (1991)
human	GACPAGGCLVALTCDYRILADNPRYCIGLNETQLGIIAPFWLKDTLENTIGHRAAESAPE	231	Kilponen et al., (1994)
mouse	GASPAGGCLLALCCDYRVMADNPKYTIGLNESLLGIVAPFWFKDMYVNTIGHREADGALQ	254	Stoffel et al., (1993)
4-Chlorobenzoyl-CoA dehalogenases			
419529	GVAAGGGLGISLASDMAICADSAKF--VCAWHTIGIGNDTATSYSLARIVGMRRAMELML	226	Babbitt et al., (1992)
477389	GPAVGGGLGMSLACDLAVCTDRATF--LPAWMSIGIANDASSSFYLPRIVGYRRAMEWLL	229	Schmitz et al., (1992)
Unidentified function			
216461	GYAFGGAFELALAADFIVCADNASF--ALPEAKLGIVPDSGGVLRLPKILPPAIVNEMVM	196	Yura et al., 1992
551444	GLALGGGLEVAMACHARISTPTAQL--GLPELQLGIIPGFGGTQRLPRLVGLSKALEMML	76	Preisig-Muller et al., (1994)
ECH_CLODI	GFAL-GGCELAMACDIRIASAKAKF--GQPEVTLGITPGYGGTQRLTRLVGMAKAKELIF	68	Mullany et al., (1994)
478983	GGCIGGGVDLISACDIRYCTQDAFF--QVKEVDVGLAADVGTLQRLPKVIGNRSLVNELT	225	FitzPatrick et al., (1995)
1255852	GYCLGAALDIATACDVRVATKDAVL--SVKEVDIGMAADVGTLNRLPKIVGNHSWIKDIS	178	Wilson et al., (1994)
564065	GGCIGGGVDLVTACDIRYCAQDAFF--QVKEVDVGLAADVGTLERLPKVIGNQSLVNELA	228	FitzPatrick et al., (1995)
1314045	GPAIGAGLQLAMQCDLRVVAPDAFF--QFPTSKYGLALDNWSIRRLSSLVGHGRARAMLL	152	Philipp et al., (1996)
528993	GHGIGGGFVMGLFADIVILSRESVY--TANFMKYGFTPGMGATFIVPKKLGFSLAQEILL	160	Albertini et al., (1995)
882448	GSVWGGAFEMIMSSDLIIAASTSTF--SMTPVNLGVPYNLVGIHNLTRDAGFHIVKELIF	176	Plunkett (1995)
1226272	GFAVAGGLELSLMADLRVSSPSAKF--GVFCRRVGVPLIDGGTVRLPRVIGLGRALDMIL	164	Wilson et al., (1994)
45984	GAAAGAGANLALAADVVIAAQSAAF--MQAFTRIGLMPDAGGTWWLPRQVGMARAMGMAL	160	Beckman and Kranz (1991)

[a] The region aligned begins with the defining GGG tripeptide that is at the amino terminus of an α-helix. The first grouping is of sequences where the protein has been identified as having hydratase activity. The next three groupings are proteins that have other identified physiological functions, although the alignment suggests that the gastrin binding protein will have enoyl-CoA hydratase activity. The final grouping includes all homologs identified by other sequencing efforts where the function of the expressed protein is not known. The highly conserved residues of the superfamily are boxed. Glu 144 and Glu 164 are identified with an X, indicating that they are present at the active site as determined by homology with 4-(chlorobenzoyl)-CoA dehalogenase, as discussed in this work, and by site-directed mutagenesis (D'Ordine et al., 1994; Müller-Newen et al., 1995), respectively.

If the carbonyl thioesters are positioned similarly in the two active sites, the C1−C2 bond of HD-CoA will be oriented in the same direction as the single bond between the carbonyl and benzene ring of benzoyl-CoA. To model the conformation of the C1−C2 bond in HD-CoA, additional stereochemical information is required. The identification of Glu 164 in the hydratase as the active site functional group required for proton abstraction provides the necessary assistance. This functional group must protonate C2 on the *re* face to donate the 2-*pro*-*R* proton of the hydrated product. As shown in Figure 6, substitution of Glu 164 for Trp 137 positions the Glu carboxylate side chain on the *re* face of the carbonyl carbon.

Given the placement of the proton donor, Glu 164, the C1−C2 bond of the substrate must be *s*-*cis*, in order for the proton to be donated to the C2 *re* face of C═C. This arrangement is consistent with the addition of water to the *si* face of the double bond at C3 in a *syn* addition to generate (3*S*)-3-(hydroxyacyl)-CoA. However, if the thioester is fixed, and the *s*-*trans* arrangement of the C1−C2 bond assumed, Glu 164 would donate the proton to the *si* face at C2 of C═C and would require an *anti* addition of water to generate the correct stereochemical product, (3*S*)-3-(hydroxyacyl)-CoA. Consequently, the establishment of similar conformations for the CoA thioester and the implied orientation of the active site general acid serve to define the required active conformation about the C1−C2 bond.

The hydration reaction requires at least two separate proton transfers during the course of the reaction; the water molecule must be deprotonated, and C2 of the substrate must be protonated. It remains to be determined whether there is a single general acid/base that catalyzes both transfers. This has been suggested as a possible rationale for the development of *syn* reactions (Hanson & Rose, 1975) and has been proposed as a possible explanation of the observed isotope effects. Examination of the dehalogenase crystal structure and the location of potential ionizable groups in the homologous hydratase sequence has identified one additional residue that has the potential of contributing a general acid/base to the active site, Glu 144 (the corresponding residue in the dehalogenase sequence is Gly 117). If Glu 144 were to function as the general acid/base functional group activating the nucleophilic water (or protonating the 3-hydroxyl in the reverse reaction), it would be expected to be conserved in the hydratase members of the superfamily but be variable in those members, such as the isomerase and dehalogenase, that do not require this functional group. In the homology alignment (Table 1), Glu 144 fits these requirements as it is present in all of the sequences with known hydratase activity but is missing from both the enoyl-CoA isomerase and dehalogenase where the substitutions are Leu 143 and Gly 117, respectively. In a model-building exercise using the dehalogenase structure, conversion of Gly 117 to Glu positions a carboxylate side chain within 4.1 Å of the 4-(hydroxybenzoyl)-CoA C3 atom on what would be the *si* face of an enoyl-CoA substrate. Preliminary data indicate that a mutant in which Glu 144 is changed to Gln shows at least a 10000-fold decrease in activity, which supports the hypothesized role of this residue (P. J. Tonge, unpublished results).

CONCLUSIONS

The TRNOE studies on the enoyl-CoA hydratase−HD-CoA complex have revealed a number of important structural features for the bound ligand. In the CoA portion of bound HD-CoA, the adenosine glycosidic bond is *anti*, the pantetheine segment is in an extended conformation, and the overall structure of the CoA is bent. In addition, the hexadienoyl moiety of the bound HD-CoA is planar with *s*-*trans* geometry about the C3−C4 bond. The structure of the CoA in the HD-CoA−enzyme complex appears very similar to that of CoA in the X-ray crystal structure of 4-(hydroxybenzoyl)-CoA bound to 4-(chlorobenzoyl)-CoA dehalogenase. This validates the modeling of the enoyl-CoA hydratase structure with the dehalogenase backbone. On the basis of the modeling studies, we hypothesize that enoyl-CoA substrates bind to the hydratase with *s*-*cis* geometry about the enoyl C1−C2 bond. The modeling studies are consistent with the identification of Glu 164 as a catalytic residue and position the side chain of this residue so that it can protonate and/or deprotonate the substrate's C2 atom. Finally, we also propose that Glu 144 functions as a second acid/base in the catalytic mechanism of the enzyme.

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