Identification of Active Site Residues Essential to 4-Chlorobenzoyl−Coenzyme A Dehalogenase Catalysis by Chemical Modification and Site Directed Mutagenesis†

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ABSTRACT: 4-Chlorobenzoyl−coenzyme A (4-CBA−CoA) dehalogenase catalyzes the hydrolysis of 4-CBA−CoA to 4-hydroxybenzoyl−coenzyme A (4-HBA−CoA) via a nucleophilic aromatic substitution pathway involving the participation of an active site carboxylate side chain in covalent catalysis. In this paper we report on the identification of conserved aspartate, histidine, and tryptophan residues essential to 4-CBA−CoA catalysis using chemical modification and site-directed mutagenesis techniques. Treatment of the dehalogenase with diethyl pyrocarbonate resulted in complete loss of catalytic activity (k_{inact} = 0.17 mM^{-1} min^{-1} at pH 6.5, 25 °C) that was fully regained by subsequent treatment with hydroxylamine. The protection from inactivation afforded by enzyme bound 4-HBA−CoA indicated that the essential histidine residues are located at the active site. Replacement of conserved histidine residues 81, 90, 94, and 208 with glutamine residues resulted in a significant loss of catalytic activity only in the cases of the histidine 81 and 90 mutants. Substrate and product ligand binding studies showed that binding is not significantly inhibited in these mutants. Site directed mutagenesis of a selection of conserved aspartate and glutamate residues, identified aspartate 145 as being essential to dehalogenase catalysis. Ligand binding studies showed that this residue is not required for tight substrate/product binding. Chemical modification of the dehalogenase with *N*-bromosuccinimide resulted in full loss of catalytic activity that was prevented by saturation of the active site with product ligand, providing evidence favoring an essential active site tryptophan. Phenylalanine replacement of conserved tryptophan residues 179 and 137 reduced catalytic activity only in the latter (k_{cat} = 0.03% of wild-type dehalogenase). On the basis of these results and the recently determined X-ray crystal structure of the complex of 4-CBA−CoA dehalogenase and 4-HBA−CoA [Benning, M. M., Taylor, K. L., Liu, R.-Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D., Holden, H. M. (1996) *Biochemistry 35*, 8103−8109] we propose that aspartate 145 functions as the active site nucleophile, that tryptophan 137 serves as a hydrogen bond donor to the aspartate 145 C═O, and that histidine 90 serves to deprotonate the bound H_2O molecule.

4-Chlorobenzoyl−coenzyme A (4-CBA−CoA)[1] dehalogenase catalyzes the hydrolytic dehalogenation of 4-CBA−CoA to 4-hydroxybenzoyl−CoA (4-HBA−CoA) (see Scheme 1) [for a recent review, see Dunaway-Mariano and Babbitt (1994)]. This enzyme functions in the 4-CBA to 4-HBA converting pathway found in certain strains of soil-dwelling bacteria adapted to the use of 4-CBA as an alternate carbon source (Scholten et al., 1991; Chang et al., 1992; Copley et al., 1992; Groenewegen et al., 1992; Adriaens et al., 1989). The mechanism of catalysis by 4-CBA−CoA dehalogenase has been investigated in several laboratories, including our own (Yang et al., 1994; Liu et al., 1995; Taylor et al., 1995; Löffler et al., 1995; Crooks et al., 1995). Emerging from these studies is a chemical pathway in which an active site carboxylate residue adds to the C(4) of the benzoyl ring of the bound substrate to form a Meisenheimer complex as the first reaction intermediate (Scheme 1). Expulsion of the chloride from the Meisenheimer complex with concomitant rearomatization of the benzoyl ring generates an arylated enzyme as the second reaction intermediate. Hydrolysis of the arylated enzyme occurs by addition of a water molecule to the acyl carbonyl carbon to form a tetrahedral intermediate which expels the hydroxybenzoyl group to regenerate the catalytic carboxylate residue and form the 4-HBA−CoA product (Liu et al., 1995).

The goal of the present investigation is the identification of active site residues functioning in catalysis. On the basis of previous single-turnover experiments, carried out in oxygen-18-enriched water, we knew at the outset of this study that an Asp or Glu residue functions in covalent catalysis (Yang et al., 1994). Several failed attempts were made to identify this catalytic residue by isolating (for sequencing) a radiolabeled peptide fragment from protease and cyanogen bromide digests of the arylated enzyme (formed during a single turnover of [^{14}C]4-CBA−CoA). The chemical lability of the acyl linkage thwarted each experimental approach tried (Yang, 1995). We thus turned to scanning mutagenesis of a selected group of conserved Asp/Glu residues to identify this catalytic residue. The roles of

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[1] Abbreviations: 4-CBA, 4-chlorobenzoate; 4-CBA−CoA, 4-chlorobenzoyl−coenzyme A; dATP, deoxyadenosine 5′-triphosphate; dCTP, deoxycytosine 5′-triphosphate; dGTP, deoxyguanosine 5′-triphosphate; dTTP, deoxythymidine 5′-triphosphate; DTT, dithiothreitol; 4-HBA, 4-hydroxybenzoate; 4-HBA−CoA, 4-hydroxybenzoyl−coenzyme A; 4-MeBA−CoA, 4-methylbenzoyl−coenzyme A; 4-FBA−CoA, 4-fluorobenzoyl−coenzyme A; BA−coenzyme A, benzoyl−coenzyme A; DEPC, diethylpyrocarbonate; NBS, *N*-bromosuccinic acid; K^+Hepes, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) potassium salt; K^+Mes, (2-[*N*-morpholino]ethanesulfonic acid) potassium salt; LMP, low melting point; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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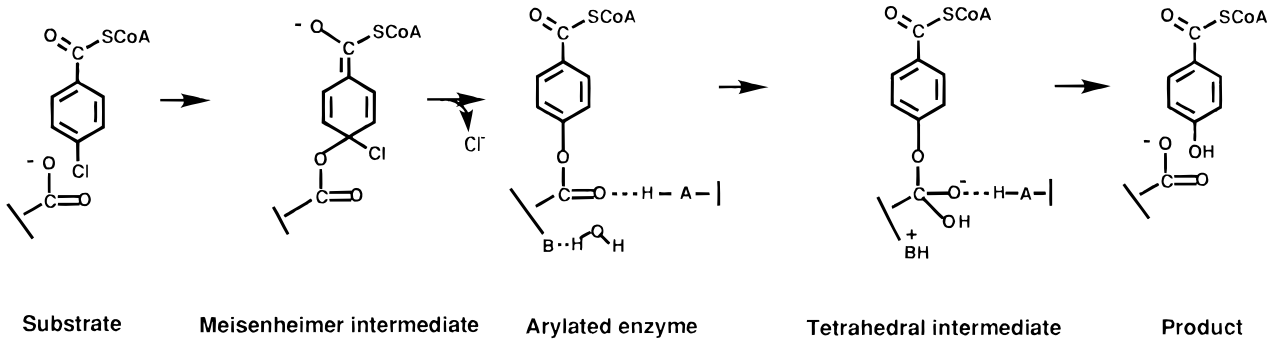
Scheme 1: Proposed Chemical Pathway of the 4-CBA−CoA Dehalogenase-Catalyzed Hydrolysis of 4-CBA−CoA to 4-HBA−CoA (Liu et al., 1995)[a]



[a] "B" represents an active amino acid side chain participating in catalysis as a general base while "AH" represents a general acid. The carboxylate nucleophile shown represents the side chain of an active site glutamate or aspartate residue.

His and Trp residues in catalysis were tested using the group-directed chemical modification reagents DEPC and NBS. Site-directed mutagenesis was then employed to identify specific His and Trp residues. In the text which follows, the results from these investigations are described.

MATERIALS AND METHODS

General. 4-CBA−CoA dehalogenase was purified from *Escherichia coli* K38 cells transformed with a pT7.5 plasmid clone containing the encoding gene as described in Chang et al. (1992). BA−CoA was purchased from Sigma and 4-CBA−CoA, 4-HBA−CoA, 4-FBA−CoA, and 4-MeBA−CoA were prepared in the manner previously described (Liang et al., 1993; Taylor et al., 1995).

Reaction of 4-CBA−CoA Dehalogenase with DEPC. DEPC stock solutions were prepared fresh using cold absolute ethanol. Stock concentrations of DEPC were determined spectrophotometrically by reaction with 10 mM imidazole, pH 7.5. The formation of carboethyl imidazole was measured by the increase in absorbance at 230 nm (ϵ = 3000 M^{-1} cm^{-1}) (Melchior et al., 1970). The rate of decomposition of DEPC was determined by assaying the DEPC concentration in 50 mM K^+Mes, pH 6.5, as a function of time. At specific time intervals aliquots of reaction mixture were removed and quenched in 10 mM imidazole buffer (pH 7.5). The concentration of DEPC remaining was determined spectrophotometrically. A semilog plot of the residual DEPC versus time gave the pseudo-first-order rate constant for hydrolysis of the DEPC: k′ = 0.05 min^{-1}. Enzyme carboethoxylation was carried out with 500 μL reaction mixtures containing 19 μM 4-CBA−CoA dehalogenase with 0.27, 0.44, 0.66, 0.82, and 1.1 mM DEPC in 50 mM K^+Mes, pH 6.5, at 25 °C. The extent of inactivation was determined by measuring the dehalogenase activity contained in a 100 μL aliquot removed from the reaction mixture at various time intervals and added to 1 mL assay solutions of 0.2 mM 4-CBA−CoA in 50 mM K^+Hepes, pH 7.5, at 25 °C. The dehalogenation reaction was monitored spectrophotometrically (300 nm; $\Delta\epsilon$ = 8.2 mM^{-1} cm^{-1}) as described previously (Chang et al., 1992). The apparent rate constant for inactivation (k) was calculated from the fraction of enzyme activity (A/A_0) remaining *vs* incubation time (t) using eq 1

$$\ln(A/A_0) = -(k/k')I^\circ(1 - e^{-k't}) \quad (1)$$

where I° is the initial concentration of DEPC and k' is the pseudo-first-order rate constant for hydrolysis of the DEPC (0.05 min^{-1}) (Gomi & Fujioka, 1982).

Reactivation with Hydroxylamine. Recovery of dehalogenase activity after inactivation with DEPC was tested by adding NH_2OH (pH 7) to a final concentration of 0.1 M to a mixture containing 19 μM 4-CBA−CoA dehalogenase and 1.1 mM DEPC in 50 mM K^+Mes, pH 6.5, at 25 °C. This mixture was then incubated at 25 °C for 16 h. Before the enzyme was assayed for dehalogenase activity, the NH_2OH was removed by extensive dialysis against 50 mM K^+Hepes, pH 7.5, containing 1 mM DTT. A control reaction was treated similarly except the addition of DEPC to the enzyme solution was omitted. The samples were assayed for dehalogenase activity by removing a 100 μL aliquot from the reaction mixture and adding to 1 mL assay solutions of 0.2 mM 4-CBA−CoA in 50 mM K^+Hepes, pH 7.5, at 25 °C. The dehalogenation reaction was monitored spectrophotometrically (300 nm; $\Delta\epsilon$ = 8.2 mM^{-1} cm^{-1}) as described previously (Chang et al., 1992). Enzyme concentrations were determined by the Bradford method (Bradford, 1976).

Reaction of Dehalogenase with N-Bromosuccinimide (NBS). One milliliter reaction solutions containing 1.0 μM dehalogenase, 30 μM NBS, and various concentrations of 4-HBA−CoA (1.0, 2.0, 3.0, 4.0, 6.0, 12, 25 μM) in 50 mM K^+Hepes, 1 mM DTT, pH 7.5, were incubated at 25 °C for 5 min. 4-CBA−CoA (150 μM) was then added, and the rate of dehalogenation measured spectrophotometrically as previously described (Chang et al., 1992). The percent enzyme activity remaining was plotted *vs* the concentration of 4-HBA−CoA present. The binding constant (K_d) for 4-HBA−CoA was calculated by using the computer program KaleidaGraph to fit the rate data to the eq 2

$$v = v_0[L]/(K_d + [L]) \quad (2)$$

in which v = the remaining enzyme activity, v_0 = initial enzyme activity without modification (or the activity under complete protection by ligand binding of 4-HBA−CoA), and [L] = ligand concentration, in this case the concentration of 4-HBA−CoA.

Site-Directed Mutagenesis. Site-directed mutants were prepared from the 4-CBA−CoA gene in plasmid pT7.5 (Scholten et al., 1991) using the methodology described in Erlich (1992). PCR reactions were carried out in a Thermolyne Temp-Tronic PCR using template DNA (prepared using the Wizard PCR Preps DNA purification system supplied by Promega), synthetic primers (supplied by Oligos,

Table 1: Comparison of the Steady-State Kinetic Constants of Wild-Type and W137F Mutant 4-CBA−CoA Dehalogenase Determined at pH 7.5 and 25 °C[a]

enzyme	substrate/ inhibitor	K_m or K_i (μM)	k_{cat} (s^{-1})
wild-type	4-CBA−CoA	3.7 ± 0.3	0.60 ± 0.01
	4-FBA−CoA[b]	40 ± 5	
	BA−CoA[b]	72 ± 8	
W137F	4-CBA−CoA	8.5 ± 0.3	$(1.57 \pm 0.02) \times 10^{-3}$
	4-FBA−CoA[b]	50 ± 4	
	BA−CoA[b]	64 ± 6	

[a] See Materials and Methods for details. [b] 4-Fluorobenzoyl−CoA (4-FBA−CoA) and benzoyl−CoA (BA−CoA) were used as competitive inhibitors *vs* 4-chlorobenzoyl−CoA (4-CBA−CoA).

Etc), and the PCR reagents supplied in the Perkin Elmer Cetus GeneAMP kit. Each 100 mL reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM each of dATP, dCTP, dGTP, and dTTP, 20 pM each of primer, 2.5 units of Taq DNA polymerase, 1.5 mM $MgCl_2$, and 0.5 μg of template DNA. The actual base substitutions, location of primers, and restriction enzymes used are shown in Table 1 of the supporting information. Reaction mixtures were overlaid with 50 mL of sterile silica oil. Denaturation was achieved at 92 °C, annealing at 55 °C, and elongation at 72 °C. The reaction protocol consisted 25 thermocycles, a 2-min dwelling time at 94 °C at the beginning, and a 5-min dwelling period at 72 °C after the cycles. Each cycle consisted three temperature steps: denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongating at 72 °C for 1 min. Each PCR reaction was chromatographed on a 0.8−2.0% LMP agarose gel with TBE buffer (10.8 g of Tris/L, 5.5 g of boric acid/L, 0.74 g of EDTA/L), and the product DNA was extracted using the Geneclean II kit. The DNA mutant fragment and the 4-CBA−CoA/pT7.5 clone were digested with *Mlu*I or *Nru*I and *Bst*BI restriction enzymes and purified by LMP agarose chromatography. Following T4 DNA ligase catalyzed ligation of the mutant fragment to the cut plasmid the clone was transformed into competent *E. coli* JM101 cells (Sambrook et al., 1989). The sequence of the resulting mutated pT7.5 plasmid in the region manipulated was verified by the chain termination methods (Sanger et al., 1977) using the Sequenase DNA sequencing kit from United States Biochemical.

The JM101 cells containing the pT7.5 mutated plasmid were grown to mid-log phase in LB media with 50 μg of ampicillin per mL at 37 °C and then harvested by centrifugation. The mutant plasmid, purified using a Wizard Minipreps DNA purification kit, was transformed into competent *E. coli* K38 cells, which were then spread over the surface of an LB agar plate containing 50 μg/mL ampicillin and kanamycin. Ampicillin and kanamycin resistant colonies were screened, and the DNA insert was identified by restriction analysis (Sambrook et al., 1989).

Purification of Mutant Proteins. 4-CBA−CoA dehalogenase H90Q and W137F were purified by modification of the procedure used by Chang et al. (1992). The DEAE cellulose column fractions were assayed for protein by measuring the absorbance at 280 nm. Those fractions containing high concentrations of protein (where $A_{280} \geq 0.8$ OD) were analyzed by SDS−PAGE. After fractions containing dehalogenase were pooled and concentrated, ammonium sulfate was added to a final concentration of 10%, and the protein solution was applied to a phenyl Sepharose column. The protein was eluted using a gradient of 10−0% ammonium sulfate in 50 mM K^+Hepes, pH 7.5, containing 1 mM DTT. Fractions containing high concentrations of protein (where $A_{280} \geq 0.8$ OD) were analyzed for purity of SDS−PAGE. Fractions containing pure dehalogenase were pooled and concentrated, yielding *ca*. 200 mg of mutant protein for 25 g of wet cell paste. 4-CBA−CoA dehalogenase H81Q and D145A was purified according to the procedure mentioned above, however, after the DEAE cellulose chromatography the fractions containing the H81Q mutant dehalogenase were purified using Pharmacia P-500, LCC-500, and Frac-500 FPLC. Solvent A was 50 mM K^+-Hepes, pH 7.5, containing 1 mM DTT, and solvent B was 50 mM K^+Hepes, 0.5 M KCl, pH 7.5, containing 1 mM DTT. The Mono-Q HR 5/5 column was equilibrated 20% B. A 50-mL loop was used to load the protein solution onto the column at a flow rate of 1 mL/min. The column was washed for 5 min with 20% B, followed by a linear gradient (20 to 50 %) of solvent B in 30 min. The flow rate was 1 mL/min. The protein concentration was monitored using a Pharmacia Single Path Monitor UV-1 at 254 nm. Fractions containing protein were analyzed for purity by SDS−PAGE. Pure fractions were combined and concentrated yielding *ca*. 100 mg H81Q dehalogenase from 25 g of wet cell paste.

Circular Dichroism Spectra. Far-ultraviolet (200−250 nm) circular dichroism spectra of wild-type 4-CBA−CoA dehalogenase, D145A, H81Q, H90Q, and W137F were recorded on a Jasco 500-C spectropolarimeter. The concentration of each enzyme was 0.15 μM in 5 mM potassium phosphate buffer (pH 6.9). Enzyme concentrations were determined by the Bradford method (Bradford, 1976).

Measurement of the Catalytic Activity of Wild-Type and Mutant Dehalogenases. The kinetic parameters K_m and V_{max} of each enzyme were determined from the initial velocity data measured at pH 7.5 as a function of the concentration of 4-CBA−CoA. The increase in the absorbance ($\Delta\epsilon = 8.2$ mM^{-1} cm^{-1} at 300 nm) of a 1-mL assay solution was used to calculate the enzyme activity and the initial velocity data were analyzed using eq 3 and the fortran HYPERL program of Cleland (1979).

$$V_0 = V_{max}[A]/([A] + K_m) \quad (3)$$

In eq 3, V_0 = initial velocity, V_m = maximal velocity, [A] = substrate concentration, and K_m = Michaelis constant. The k_{cat} values were determined from the V_{max} values and total enzyme concentration used in the reaction according to the equation $k_{cat} = V_{max}/[E]$.

The kinetic parameter K_i of wild type and mutants were also determined from the velocity data. The initial velocity of the 4-CBA−CoA dehalogenase-catalyzed reaction was measured as a function of 4-CBA−CoA concentration (5−40 μM) in the absence and in the presence of 4-FBA−CoA (100 μM) or BA−CoA (150 μM). The 4-CBA−CoA dehalogenase concentration used in 1 mL reaction mixtures (50 mM K^+Hepes, pH 7.5; 1 mM DTT) was 0.34 μM. The initial velocity data were analyzed using eq 4 and the Fortran COMPL program of Cleland (1979).

$$V_0 = V_{max}[A]/[K_m(1 + [I]/K_i) + [A]] \quad (4)$$

In eq 4, V_0 = initial velocity, V_m = maximal velocity, [A]

= substrate concentration, K_m = Michaelis constant, [I] = inhibitor concentration, and K_i = the inhibition constant.

Measurement of the Dissociation Constants for Wild-Type and Mutant 4-CBA−CoA Dehalogenase−Ligand Complexes. The dissociation constants (K_d) were determined for H81Q, H90Q, and W137F dehalogenase complexed with 4-HBA−CoA, 4-MeBA−CoA, and 4-CBA−CoA by UV-difference spectroscopy. The titration was carried out using a tandem cuvette starting with 0.5 mL of 20 μM enzyme in one chamber and a variable concentration of ligand (4-HBA−CoA, 4-MeBA−CoA, and 4-CBA−CoA) in 0.5 mL in the other chamber. All solutions were buffered at pH 7.5 using 50 mM K^+Hepes/1 mM DTT at 25 °C. The unmixed solution was used as a reference. After being mixed, the UV-difference spectra was determined and the absorbance at the wavelength corresponding to the maximum absorption difference recorded which for 4-HBA−CoA titration of wild-type, W137F, and D145A dehalogenase is 373 nm and for H81Q and H90Q dehalogenase is 330 nm. For 4-MeBA−CoA and 4-CBA−CoA 306 nm was used. The titration data were fit to quadratic eq 5 described by Anderson et al. (1988), using the KaleidaGraph computer program for nonlinear regression, to obtain the dissociation constant (K_d) of the enzyme·ligand complex.

$$\Delta A = (\Delta A_{max}/[E_0])\{(K_d^{app} + [E_0] + [S_0]) - \sqrt{(K_d^{app} + [E_0] + [S_0])^2 - 4[E_0][S_0]}\}/2 \quad (5)$$

In eq 5, ΔA_{max} = the total change in absorbance, ΔA = the observed change in absorbance, $[E_0]$ = the total enzyme concentration, $[S_0]$ = ligand concentration, and K_d = dissociation constant.

RESULTS

Chemical Modification of Active Site Residues. Chemical modification of the 4-CBA−CoA dehalogenase with DEPC and NBS was carried out to probe for active site His and Trp residues essential for substrate binding and/or catalysis. DEPC reactions were carried out at pH 6.5 and 25 °C. Decomposition of DEPC under these conditions was determined to occur at k = 0.05 min^{-1} (see Materials and Methods). Reaction of 1.1 mM DEPC with 19 μM 4-CBA−CoA dehalogenase (specific activity (SA) = 1.5 units/mg) resulted in the loss of catalytic activity that was linear with time to up to 90% inactivation. Following a 20-min incubation period, at which time no activity remained, hydroxylamine was added to the reaction solution. The liberation of the His residues from the *O*-ethyl-*N*-carbonylated adducts fully restored the enzyme catalytic activity (SA = 1.5 units/mg). Thus, the loss of activity observed with DEPC is attributed to His modification and not to the modification of the other nucleophilic residues present, e.g., Cys and Lys that is not reversed by treatment with hydroxylamine (Miles, 1977). Figure 1A shows the time courses of DEPC inactivation of the dehalogenase measured as a function of DEPC concentration. The apparent rate constants for inactivation derived from the slopes of these plots are replotted *vs* the DEPC concentration in the inset to Figure 1A. From the slope of the replot a $k_{inact} = 0.17 \pm 0.02$ mM^{-1} min^{-1} was calculated. Figure 1B shows the time courses for inactivation of the dehalogenase in the presence and absence of a 12-fold excess of 4-HBA−CoA (K_d = 2.5 μM;

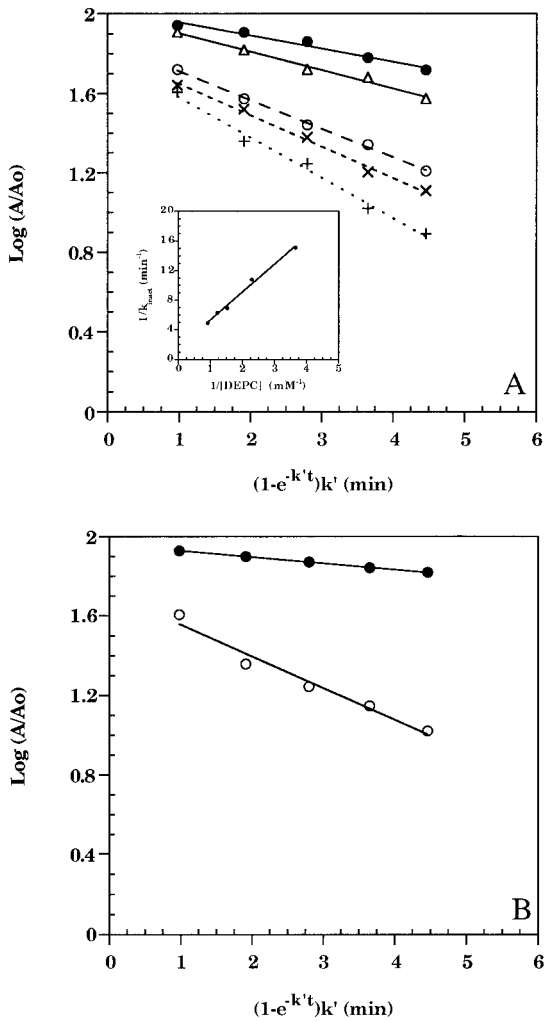


FIGURE 1: (A) Plot of the fraction of catalytic activity of 4-CBA−CoA dehalogenase remaining as a function of the incubation period with DEPC. Reactions were carried out in 50 mM K^+Mes (pH 6.5, 25 °C) with 19 μM enzyme and (●) 0.27 mM, (△) 0.44 mM, (○) 0.66 mM, (×) 0.88 mM, or (+) 1.1 mM DEPC. Aliquots were removed from the reaction and assayed for enzymatic activity as described in Materials and Methods. The lines connecting data points were generated by a computer fit to a linear equation. The inset is a plot of the slopes (k_{app}, min^{-1}) *vs* the concentration of DEPC (mM) used in the inactivation reaction. The line connecting the data points was generated by a computer fit (R = 0.995) to an equation for a straight line to arrive at a Y intercept, k_{inact} = 0.17 mM^{-1} min^{-1}. (B) Same experiment as in A (1.1 mM DEPC) in the (●) presence and (○) absence of 0.13 mM 4-HBA−CoA. The lines connecting the data points were generated by computer fitting the data to an equation for a straight line to arrive at the respective slopes, k_{app} for inactivation equal to 0.02 min^{-1} and 0.2 min^{-1}, for the presence and absence of 4-HBA−CoA, respectively.

Liang et al., 1993). The protection afforded by the product ligand is evident from the 10-fold decrease observed in the rate of DEPC inactivation. Hence, DEPC inactivation of the enzyme results from the modification of one or more His residues whose probable location is the active site.[2]

The dehalogenase was reacted with NBS to modify Trp residues by oxidation of the indole ring to the oxindole adduct. The modification reaction is typically carried out at acidic pH to minimize oxidation of enzyme Tyr residues

[2] Protection by an active site bound ligand against reagent induced modification/activity loss suggests, but does not prove, that the modified residue is located in the active site.

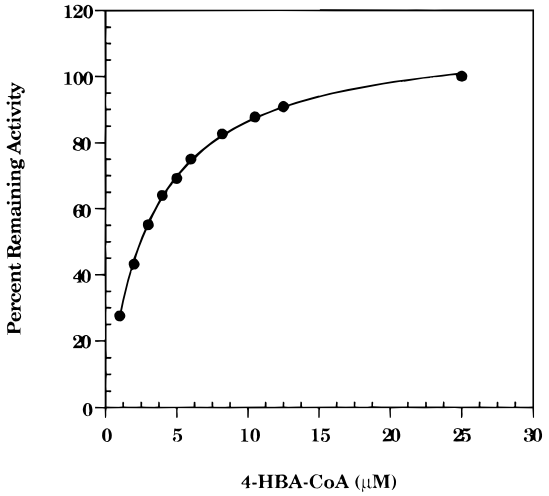


FIGURE 2: Plot of the percent catalytic activity of 4-CBA−CoA dehalogenase (1 μM) remaining following 5 min incubation with 30 μM NBS in 50 mM K^+Hepes (pH 7.5, 25 °C) *vs* the concentration of 4-HBA−CoA present in the reaction mixture. The curve connecting the data points was generated by a computer fit to an equation 2 to give a $K_d = 2.2 \pm 0.06$ μM for 4-HBA−CoA.

(Viswanatha & Lawson, 1961). However, in order to avoid denaturation of the dehalogenase the NBS reaction was carried out at neutral pH. A series of reactions solutions containing dehalogenase (1 μM), 4-HBA−CoA (1−25 μM) and NBS (30 μM) were assayed for enzymatic activity following a 5-min incubation period. A duplicate set of reaction solutions without NBS served as controls. The percent catalytic activity remaining is plotted in Figure 2 against the concentration of 4-HBA−CoA present in the modification reaction. The protection from enzyme activation by NBS afforded by the 4-HBA−CoA could possibly have derived from the destruction of the reagent via reaction with the 4-HBA−CoA present. We ruled this possibility out by measuring the UV absorption spectra of solutions of NBS and 4-HBA−CoA contained in separate chambers of a tandem cell before and after mixing. No change in the absorbance spectrum, which would indicate oxidation of the ligand, was observed. A fit of the data shown in Figure 2 to eq 1, describing a protein−ligand binding curve, gave a K_d for 4-HBA−CoA binding equal to 2.2 ± 0.06 μM. The similarity of this K_d to that derived from the K_i determined for 4-HBA−CoA acting as a competitive inhibitor (2.5 μM; Liang et al., 1994) suggests that the protection from inactivation afforded by the product ligand is the result of its association with the enzyme active site. Thus, the probable location of the essential Trp residue is the active site.[2]

Site-Directed Mutagenesis of Conserved Asp, Glu, His, and Trp Residues. An alignment of the amino acid sequences of the 4-CBA−CoA dehalogenases from *Pseudomonas* sp. strain CBS3 (Babbitt et al., 1992), *Arthrobacter* sp. strain SU (Schmitz et al., 1992), and *Alcaligenes* sp. strain A2 (Lai, 1996) was made in order to identify the residues conserved among the three enzymes. The *Pseudomonas* dehalogenase shares 48% sequence identity with the *Arthrobacter* enzyme and 86% sequence identity with the *Alcaligenes* enzyme. Between the three sequences there are 14 conserved Asp/Glu residues, 10 of which were selected for amino acid replacement. The E43A, D45A, D46A, E68T, D123T, D129T, E163T, and E175D dehalogenase mutants, assayed in cellular extract, displayed catalytic activity comparable to that of the wild-type enzyme. The E34T mutant formed inclusion bodies, and the D145A mutant was catalytically inactive. The D145A mutant was subjected to further characterization as described below. Four out of the six conserved His residues plus the two conserved Trp residues were replaced with Gln and Phe residues, respectively. The H94Q, H208Q, and W179F mutant enzymes, assayed in the cellular extract, were found to have catalytic activity comparable to the wild-type dehalogenase while the H81Q, H90Q, and W137F mutant enzymes did not. These latter mutant enzymes were characterized further as described below.

Physical and Kinetic Properties of the D145A, H81Q, H90Q, and *W137F Mutant Dehalogenases*. The expression level of these mutant enzymes, examined by SDS−PAGE chromatography of the protein contained in the *E. coli* K38 cellular extract, was found to be comparable to that of the wild-type dehalogenase. With the exception of the H81Q and D145A dehalogenase, the mutant enzymes were purified on DEAE-cellulose and phenyl Sepharose columns in the same manner as used with wild-type dehalogenase. Because the H81Q and D145A mutants were found to bind too tightly to the phenyl Sepharose column, they were purified by chromatography on a column of strong anion exchanger. All four mutants displayed essentially the same resistance to proteolysis during purification and storage (as judged by SDS−PAGE analysis) and the same circular dichroism spectral properties (λ_{max} at 210 nm, $\theta = -450\,000$ and at 220 nm, $\theta = -500\,000$) as the wild-type enzyme.

The catalytic activities of the mutant enzymes were tested using the spectrophotometric assay for 4-CBA−CoA conversion to 4-HBA−CoA under steady-state conditions (pH 7.5, 25 °C) (Chang et al., 1992). Low activity was detected with the W137F mutant, and no activity was detected with the H81Q, H90Q, or D145A mutants. Initial velocity kinetic techniques were used to measure K_m and k_{cat} values for wild-type and W137F dehalogenases (see Table 1). The turnover rate for the W137F mutant is 0.3% that of the wild-type enzyme yet the K_m values are similar. This result indicates that Trp137 is important for catalysis but not substrate binding. A confirmation of this result was obtained by measuring the inhibition constants of substrate analogs 4-FBA−CoA and BA−CoA as competitive inhibitors *vs* 4-CBA−CoA. The two sets of K_i values obtained for wild-type and W137F dehalogenase are closely matched (Table 1).

Although catalysis in the H81Q, H90Q, and D145A dehalogenases was too low to measure under steady-state conditions, substrate binding constants could be measured using UV-difference spectral techniques. In previous work we had found that the absorption maximum of the benzoyl moiety in the product, 4-HBA−CoA, and in the substrate analog, 4-MeBA−CoA, undergoes a significant red-shift upon the binding of the ligand to the dehalogenase active site (Taylor et al., 1995). Thus, by measuring the absorbance generated uniquely by the enzyme−ligand complex we were, in the present study, able to determine the binding affinities of these ligands to wild-type and mutant dehalogenase by carrying out titration experiments. The binding affinity of 4-CBA−CoA to the inactive dehalogenase mutants was also measured by spectral titration. The results obtained are reported in Table 2. The differences observed in the binding constants of each ligand with the wild-type enzyme and the

Table 2: Dissociation Constants (K_d) for Wild-Type (WT) and Mutant 4-CBA−CoA Dehalogenase Complexes of 4-Hydroxybenzoyl−CoA (4-HBA−CoA), 4-Methylbenzoyl−CoA (4-MeBA−CoA), and 4-Chlorobenzoyl−CoA (4-CBA−CoA) Determined by UV-Difference Spectroscopy at pH 7.5 and 25 °C[a]

	K_d (μM)				
ligand	WT	H81Q	H90Q	W137F	D145A
4-HBA−CoA	0.5	0.6	0.7	1.8	0.1
4-MBA−CoA	2.8	1.3	2.5	0.6	1.0
4-CBA−CoA	−	2.0	1.0	−	2.0

[a] See Methods for details. The error associated with the K_d values falls within ±10% of the value.

four mutants are small, less than 1 order of magnitude, thus showing that the His81, His90, Asp145, and Trp137 residues (essential for catalysis) do not significantly contribute to the observed 4-CBA−CoA/4-HBA−CoA binding affinity.

DISCUSSION

Identification of 4-CBA−CoA Dehalogenase Active Site Residues Essential to Catalysis. Chemical modification of 4-CBA−CoA dehalogenase with DEPC resulted in complete loss of catalytic activity which was reversed by treatment of the modified enzyme with hydroxyl amine. This finding suggests that a His residue is essential to catalytic functioning. The protection against inactivation afforded by 4-HBA−CoA indicates that this His residue is located in or near the enzyme active site. Amino acid replacement of four His residues conserved among the *Pseudomonas* sp. strain CBS3, *Arthrobacter* sp. strain SU, and *Alcaligenes* sp. strain dehalogenase sequences identified His81 and His90 as necessary for catalytic function. The ability of these mutants to bind substrate, substrate analog and product ligands as tightly as does wild-type enzyme indicates that they do not make a significant contribution to the binding energy. Since the time that these studies were carried out, the X-ray crystal structure of the complex of 4-CBA−CoA dehalogenase and 4-HBA−CoA (at 1.8 Å resolution) has become available (Benning et al., 1996). Now we are able to show (Figure 3) the positioning of the His81 and His90 near or in the active site of the enzyme. As shown in Figure 3 the conserved Asp145, identified by amino acid replacement to be essential for catalysis but not substrate binding, is also located in the dehalogenase active site. The conserved Trp137 shown by NBS chemical modification and site-directed mutagenesis to be important for catalytic turnover is observed to be proximal to Asp145. As is the case with the other residues identified, the Trp137 is not essential for substrate binding.

On the basis of the positioning of the essential Asp145 and the demonstrated participation of a carboxylate residue in covalent catalysis (Scheme 1) we can suggest that it is Asp145 which attacks C(4) of the substrate benzoyl ring to form the Meisenheimer complex as the first reaction intermediate. The side chain C═O of Asp145 must also be the site of hydrolysis as the arylated enzyme intermediate is converted to product (Scheme 1). The indole NH of Trp137 is within H-bonding distance of the Asp145 side chain C═O suggesting a possible role that it might play in activating the acyl moiety of the arylated enzyme for attack by a H_2O molecule. The location of the His90 imidazole ring near the Asp145 suggests a role that it might play in the activation of the attacking H_2O molecule. His81, located close to the mouth of the active site, seemingly out of reach of the substrate ligand, may not directly participate in catalysis but instead play a critical role in maintaining the structure of the active site. Future studies in our laboratory will probe the roles of these and other active site residues in catalysis using transient kinetic techniques to resolve the individual steps of catalytic turnover.

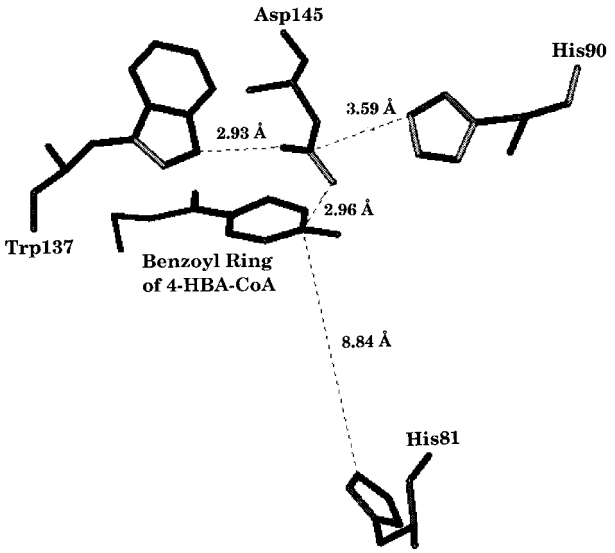


FIGURE 3: Illustration of the disposition of Asp145, His90, His81, and Trp137 relative to the 4-HBA−CoA product bound to 4-CBA−CoA dehalogenase observed in the X-ray crystal structure of the enzyme−4-HBA-CoA complex (Benning et al., 1996). The refined crystallographic coordinates were modeled with the Biosym Technologies software package, InsightII 2.3.

Comparison of the Catalytic Mechanism of 4-CBA−CoA Dehalogenase to That of the Haloalkane Dehalogenase. Despite the absence of structural relatedness between the 4-CBA−CoA dehalogenase (Babbitt et al., 1992) and its (*Xanthobacter autotrophicus* GJ10) haloalkane dehalogenase counterpart (Janssen et al., 1989), the two enzymes do share some aspects of catalysis while they clearly differ in others. For instance, both enzymes use an active site Asp residue to attack the halogen-bearing carbon center of the substrate to form an alkylated enzyme as intermediate (Yang et al., 1994; Pries et al., 1994; Verschueren et al., 1993a,b). In the case of the haloalkane dehalogenase the Asp residue is contained within an Asp260−His289-Asp124 catalytic triad (Verschueren et al., 1993a,b) that is conserved among the family of distantly related hydrolases (Ollis et al., 1992) which includes the mammalian enzyme, epoxide hydrolase (Lacourciere et al., 1993; Lacourciere et al., 1994). The His289 of the catalytic triad of the haloalkane dehalogenase activates the H_2O molecule, which attacks the acyl carbon of the alkylated enzyme intermediate. The His289 is, in turn, activated by the flanking Asp260 contained within the triad. An analogous role is ascribed to His90 of the 4-CBA−CoA dehalogenase although it does not appear to be activated by a nearby Asp. A major deviation in the use of catalytic groups is seen with the essential Trp residues. In the haloalkane dehalogenase, two Trp residues (175 and 125) form a binding pocket for the incipient halide ion (Verschueren et al., 1993c). In 4-CBA−CoA there is only one essential Trp (137) which is distant from the C(4) substituent but close enough to the Asp145 C═O for hydrogen bonding with the indole NH. Thus, the aromatic and the aliphatic dehalogenases display some interesting similarities in their

modes of catalysis, but, on the basis of the total picture of catalysis, it appears unlikely that the two enzymes are the product of convergent evolution.

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SUPPORTING INFORMATION AVAILABLE

Table listing the mutants generated, inside and outside primers, amino acid substitutions, and restriction enzymes (2 pages). Ordering information is given on any current masthead page.

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