Evidence for Electrophilic Catalysis in the 4-Chlorobenzoyl-CoA Dehalogenase Reaction: UV, Raman, and ^{13}C-NMR Spectral Studies of Dehalogenase Complexes of Benzoyl-CoA Adducts†

Kimberly L. Taylor, Rui-Qin Liu, Po-Huang Liang, Jack Price, and Debra Dunaway-Mariano*

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

Peter J. Tonge

The Picower Institute for Medical Research, Manhasset, New York 11030

John Clarkson and Paul R. Carey

Department of Biochemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

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ABSTRACT: This paper reports on the mechanism of substrate activation by the enzyme 4-chlorobenzoyl coenzyme A dehalogenase. This enzyme catalyzes the hydrolytic dehalogenation of 4-chlorobenzoyl coenzyme A (4-CBA-CoA) to form 4-hydroxybenzoyl coenzyme A (4-HBA-CoA). The mechanism of this reaction is known to involve attack of an active site carboxylate (Asp or Glu side chain) at C(4) of the substrate benzoyl ring to form a Meisenheimer complex. Loss of chloride ion from this intermediate results in the formation of an arylated enzyme intermediate. The arylated enzyme is hydrolyzed to free enzyme plus 4-HBA-CoA by the addition of water at the acyl carbon [Yang, G., Liang, P.-H., & Dunaway-Mariano, D. (1994) *Biochemistry 33*, 8527]. The present studies have focused on the activation of the 4-CBA-CoA for nucleophilic attack by the active site carboxylate group. UV−visible, ^{13}C-NMR, and Raman spectroscopic techniques were used to monitor changes in the distribution of the π electrons of the benzoyl moiety of benzoyl-CoA adducts [substituted at C(4) with methyl (4-MeBA-CoA), methoxy (4-MeOBA-CoA), or hydroxyl (4-HBA-CoA) groups or at C(2) or C(3) with a hydroxyl group (2-HBA-CoA and 3-HBA-CoA)] resulting from the binding of these ligands to the dehalogenase active site. The UV−visible spectra measured for 4-HBA-CoA in aqueous buffer at pH 7.5 and in the dehalogenase active site revealed that a large red shift (from 292 to 373 nm) in the λ_{max} of the benzoyl moiety occurs upon binding. The UV−visible spectra of 4-MeBA-CoA and 4-MeOBA-CoA in aqueous buffer show λ_{max} values for the benzoyl chromophore at ca. 260 nm (ϵ = 4 mM^{-1} cm^{-1}) and at 292 nm (ϵ = 11 mM^{-1} cm^{-1}), respectively, which are shifted to 302 nm (ϵ = 6 mM^{-1} cm^{-1}) and to 323 nm (ϵ = 10 mM^{-1} cm^{-1}) upon enzyme complexation. In contrast, the other benzoyl-CoA adducts displayed essentially the same spectral properties on and off the enzyme. The ^{13}C-NMR spectrum was measured for [^{13}C=O]-4-HBA-CoA (enriched with the carbon-13 isotope at the thioester C=O) in aqueous buffer and in the enzyme active site. A 2.8 ppm downfield shift of the ^{13}C=O resonance was observed upon binding to the enzyme. The Raman spectra of 4-HBA-CoA and 4-MeBA-CoA bound to the dehalogenase active site provide evidence for polarization of the benzoyl π electrons. Specifically, the thioester C=O stretch observed at 1646 cm^{-1} and the in-plane phenyl C−H stretches observed at 1221 and 1173 cm^{-1} in the spectrum of 4-HBA-CoA in aqueous buffer are not discernible in the spectrum of the enzyme-bound 4-HBA-CoA, and the benzene ring modes 8a and 8b observed at 1603 and 1589 cm^{-1} are replaced by features at 1560 and 1525 cm^{-1}, respectively. The aromatic ring-stretching modes observed for 4-MeBA-CoA at 1609 and 1581 cm^{-1} in buffer undergo shifts to 1596 and 1570 cm^{-1} upon enzyme complexation, while the thioester C=O stretch band observed at 1651 cm^{-1} (buffer) is shifted to 1610 cm^{-1} (enzyme). The UV−visible absorption, ^{13}C-NMR, and Raman spectral data are interpreted as evidence for substrate activation via polarization of the benzoyl π electrons away from C(4) and onto the thioester carbonyl oxygen.

Chlorobenzoic acids are formed as byproducts from microbial biodegradation of polychlorinated biphenyls (Higson, 1992; Abramowicz, 1990; Commandeur & Parsons, 1990). Dehalogenation of the chlorobenzoates allows for their complete degradation via the aromatic oxidative pathways. 4-Chlorobenzoyl coenzyme A dehalogenase (4-CBA-CoA[1] dehalogenase) is an enzyme produced in some 4-chlorobenzoate (4-CBA)-degrading bacteria to function in a reaction pathway which converts 4-CBA to 4-HBA [for a recent review, see Dunaway-Mariano and Babbitt (1994)]. The first step of this pathway, conversion of 4-CBA to 4-CBA-CoA, is catalyzed by the enzyme 4-CBA:CoA ligase.

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* To whom communications should be sent.
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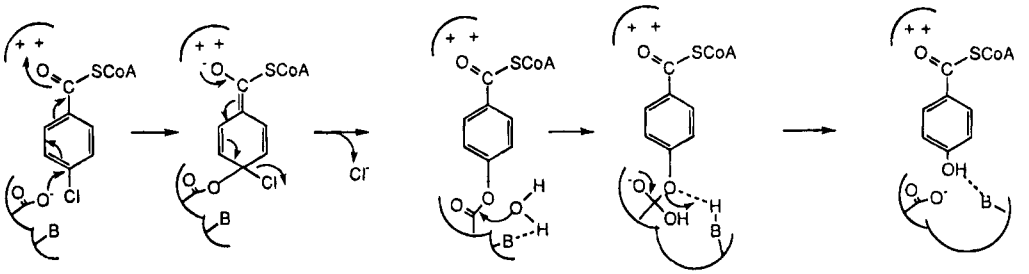
Scheme 1: Chemical Pathway of the 4-CBA-CoA Dehalogenase-Catalyzed Dechlorination of 4-CBA-CoA as Proposed in Yang et al. (1994)[a]



[a] B represents an active site general base, while BH represents an active site general acid. The carboxylate nucleophile represents the side chain of an active site aspartate or glutamate residue.

The 4-CBA-CoA thus formed is the substrate of the dehalogenase which catalyzes its conversion to 4-hydroxybenzoyl-CoA (4-HBA-CoA). The final step of the pathway is the hydrolysis of the 4-HBA-CoA to 4-HBA catalyzed by 4-HBA-CoA thioesterase. The 4-HBA produced is then metabolized through the *ortho* cleavage pathway (Ornston, 1990), thus allowing the bacterium to take advantage of a new carbon source in its environment. This adaptation was made possible through the recruitment of the dehalogenase, ligase, and thioesterase from a preexisting pathway(s) (Babbitt et al., 1992; Dunaway-Mariano & Babbitt, 1994). Through the study of the structure and catalytic mechanism of these three enzymes, we hope to uncover the path of their evolution.

The present study is concerned with the mechanism of catalysis employed by the *Pseudomonas* sp. strain CBS3 4-CBA-CoA dehalogenase to effect the hydrolytic displacement of the chloride from the benzoyl ring of the 4-CBA-CoA. Previous studies of the chemical pathway of this reaction have provided evidence for a novel form of covalent catalysis in which an active site carboxylate adds to the C(4) of the benzoyl ring to form a Meisenheimer complex as the first reaction intermediate (Scheme 1) (Yang et al., 1994). Loss of chloride from the Meisenheimer complex generates the arylated enzyme intermediate, which in the ensuing step(s) is hydrolyzed at the acyl carbon, liberating the enzyme carboxylate and 4-HBA-CoA. The use of its own active site carboxylate (in place of water) as the nucleophile accounts in part for the efficiency with which the dehalogenase catalyzes the displacement of the chloro substituent from the aromatic ring (Liu et al., 1995).

Because the benzoyl ring is not activated for nucleophilic attack by ring (electron-withdrawing) substituents other than the CoA thioester, the dehalogenase must function in this capacity. *A priori*, the C(4) of the 4-CBA-CoA could be activated for nucleophilic attack by interaction of the substrate thioester carbonyl oxygen with an electropositive group (or dipole) in the enzyme active site. Such an interaction would polarize the benzoyl ring by drawing the π electrons to the thioester substituent through resonance. In the text which follows, the results from UV−visible, Raman, and ^{13}C-NMR spectral studies of benzoyl-CoA adducts, bound in the 4-CBA-CoA dehalogenase active site, are presented. The results obtained indicate that a significant redistribution of the benzoyl ring π electrons does occur in the substrate ligand when it binds to the enzyme. This finding is interpreted as evidence for electrophilic catalysis.

EXPERIMENTAL PROCEDURES

Materials. [^{13}C=O]-4-Hydroxybenzoic acid was purchased from Isotec Inc. [thioester ^{13}C=O]-4-HBA-CoA was synthesized from this compound by modifying the procedure reported by Mieyal et al. (1974) and Merkel et al. (1989) as described in Liang et al. (1993). The compounds 4-hydroxy-, 3-hydroxy-, and 2-hydroxybenzoyl-CoA were synthesized according to Liang et al. (1993). The same general procedure was used for the synthesis of 4-methoxybenzoyl-CoA, 4-methylbenzoyl-CoA, 4-HBA-ethyl thioester, and 4-HBA-*N*-acetylcysteinyl thioester. The synthetic procedures and the spectral data for these compounds are given in the supporting information. 4-CBA-CoA dehalogenase (SA = 1.5 units/mg) was prepared acording to the procedure of Chang et al. (1992) as modified in Liang et al. (1993). Dehalogenase concentrations were determined by using the Bradford method (Bradford, 1976) and a subunit molecular mass of 30 kDa (and are reported as active site concentrations). HPLC separations were carried out with a Beckman HPLC instrument equipped with an ultrasphere 4.6 mm × 25 cm ODS-C_{18} reversed-phase column monitored at 260 nm and eluted (1.0 mL/min) with an acetonitrile/ammonium acetate gradient. Enzyme spectrophotometric assays were carried out as previously described (Change et al., 1992; Liang et al., 1993).

UV−Visible Absorption Difference Spectra of Enzyme-Bound Benzoyl-CoA Adducts. These spectra were measured using 1 mL quartz tandem cells containing 0.5 mL of enzyme solution (50 μM) in one compartment and 0.5 mL of ligand (400 μM 3-HBA-CoA, 1.0 mM 2-HBA-CoA, 1.6 mM BA-CoA, 800 μM 4-MeOBA-CoA, 100 μM 4-MeBA-CoA, or 200 μM 4-HBA-CoA) solution in the other. All solutions were buffered in 50 mM K^+Hepes, pH 7.5, at 25 °C. The absorbance spectrum was recorded and subtracted from the spectrum of the mixed solutions to give the difference spectra of the enzyme−ligand complex.

^{13}C-NMR Spectral Measurements. NMR samples were prepared as follows. Twenty-five milliliters of dehalogenase (OD_{280} = 3.7) in 50 mM K^+Hepes and 1 mM DTT, pH 7.5, was concentrated to 2 mL using an Amicon protein concentrator (YM10 membrane). The volume of the protein

[1] Abbreviations: CBA, chlorobenzoate; HBA, hydroxybenzoate; 4-CBA-CoA, 4-chlorobenzoyl coenzyme A; 4-HBA-CoA, 4-hydroxybenzoyl coenzyme A; BA-CoA, benzoyl coenzyme A; 4-MeBA-CoA, 4-methylbenzoyl coenzyme A; 4-MeOBA-CoA, 4-methoxybenzoyl coenzyme A; 4-HBA-NALC, 4-hydroxybenzoyl-*N*-acetylcysteinyl thioester; CoA, coenzyme A; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

solution was measured, and the protein concentration was determined by the Bradford method (Bradford, 1976). Enough [thioester ^{13}C=O]-4-HBA-CoA was added to equal two-thirds the concentration of enzyme. Ten milliliters of 5 mM K^+Hepes and 0.5 mM DTT in D_2O, pD 7.5, was added, and the enzyme was further concentrated with the ligand present to 2 mL. The addition of buffer and concentration of the protein solution were repeated three times. The enzyme solution was concentrated to a final concentration of 1.5 mM, without any significant loss of ligand. Enzyme samples containing either 4-HBA-CoA at natural isotope abundance or not containing 4-HBA-CoA were prepared in a similar fashion. The pD of all the NMR samples was adjusted to 7.5 by the addition of KOD, and the final concentration of the buffer was approximately 5 mM K^+Hepes and 0.5 mM DTT (90−95% D_2O). The NMR spectra were measured at 125.77 MHz on a Bruker AM 500 MHz NMR spectrometer using the general protocal given in Jaffe and Markham (1987). The probe temperature was 25 °C. Chemical shifts are reported with respect to an external reference dioxane, which was assigned a chemical shift of 66.5 ppm. All protein and protein−ligand spectra were acquired in the ^{13}C−proton-decoupled mode with a 45° pulse angle and a relaxation delay of 0.5 s. Data were acquired every 1.5 s. The spectra are a result of 24 000 transients each. The NMR solutions (0.5−0.6 mL in 5 mm quartz NMR tubes) typically contained 1.5 mM 4-CBA-CoA dehalogenase in 5 mM Hepes and 0.5 mM DTT, pD 7.5 ± 0.1 (90−95% D_2O), and varying concentrations of [thioester ^{13}C=O]-4-HBA-CoA. The sample enzyme activity and pD were checked before and after each experiment. The spectra of free ligand and denatured enzyme were acquired using similar conditions; however, only 1000 transients were collected. The spectra of 4-HBA-NALC in 5 mM K^+Hepes and 0.5 mM DTT, pD 7.5, and in DMSO were the result of 8000 transients each (see the supporting information for the spectrum).

Determination of Inhibition Constants. 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-HBA-CoA (3.4 and 6.7 μM), 3-HBA-CoA (31, 52, and 82 μM), 2-HBA-CoA (80 and 200 μM), 4-MeBA-CoA (20 μM), and 4-MeOBA-CoA (14 μM). The 1 mL reaction solutions contained 30−40 nM dehalogenase in 50 mM K^+Hepes, pH 7.5, and 1 mM DTT. The initial velocity data were analyzed using eq 1 and the FORTRAN COMPL program of Cleland (1979). In eq 1, V_o = the initial velocity, V_{max} = the maximum velocity, [A] = the substrate concentration, [I] = the inhibitor concentration, K_m = the Michealis constant, and K_i = the inhibition constant.

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

Raman Spectral Measurements. Acquisition of the spectra using a Spex 0.5 m single monochrometer equipped with a CCD detector and a supernotch filter (Kim et al., 1993) enabled data collection to be carried out under Raman (i.e., nonresonance) conditions. Raman spectra were obtained using 350 mW of 647.1 nm irradiation from a krypton ion laser and 90° scattering geometry. Samples contained in quartz cuvettes were typically 0.2 mL in volume and were buffered with 50 mM K^+Hepes (pH 7.5) at ambient temperature. The concentrations of ligand and enzyme used are provided in the figure legends along with the scan number and exposure time. The Raman spectrum of the buffer was subtracted from that of the ligand in buffer (providing the spectrum of the unbound ligand), while the spectrum of the enzyme in buffer was subtracted from the spectrum of the enzyme and ligand in buffer (providing the spectrum of the enzyme-bound ligand). The subtraction of data sets was accomplished using Spectracalc software from Galactic Industries, Salem, NH.

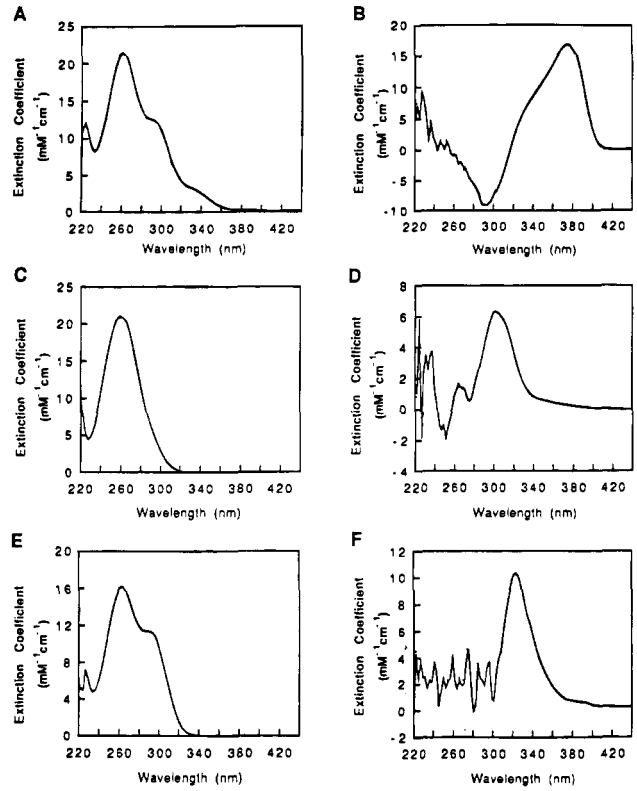


FIGURE 1: UV−visible absorbance spectrum of (A) 4-HBA-CoA (40 μM), (C) 4-MeBA-CoA (30 μM), and (E) 4-MeOBA-CoA (70 μM) in 50 mM K^+Hepes (pH 7.5) and UV−visible difference spectrum of (B) 4-HBA-CoA (100 μM) and dehalogenase (22 μM), (D) 4-MeBA-CoA (100 μM) and dehalogenase (22 μM), and (F) 4-MeOBA-CoA (200 μM) and dehalogenase (22 μM) in 50 mM K^+Hepes (pH 7.5).

RESULTS AND DISCUSSION

The spectral studies described below were carried out for the purpose of determining the mechanism of substrate activation by 4-CBA-CoA dehalogenase. Evidence for electrophilic catalysis was sought by measuring the extent to which the enzyme active site environment polarizes the π electrons of the benzoyl ring of 4-HBA-CoA (the reaction product), 4-MeBA-CoA (a substrate analog), and other benzoyl-CoA adducts.

UV−Visible Spectral Studies of Benzoyl-CoA Adducts Complexed to the Active Site of 4-CBA-CoA Dehalogenase. The UV−visible spectrum of 4-HBA-CoA in 50 mM K^+Hepes buffer (pH 7.5) is shown in Figure 1A. The CoA moiety (λ_{max} at 259.5 nm, ϵ = 16.8 mM^{-1} cm^{-1}) contributes to the absorbance maximum seen at 262 nm (ϵ = 21 mM^{-1} cm^{-1}), while the 4-hydoxybenzoyl moiety gives rise to the absorbance peaks at 292 nm (ϵ = 13 mM^{-1} cm^{-1}) and 335

nm (ϵ = 3 mM^{-1} cm^{-1}). The changes which take place in the absorbance properties of the 4-HBA-CoA when it is moved from aqueous buffer to the 4-CBA-CoA dehalogenase active site are represented by the difference spectrum shown in Figure 1B. From this spectrum, it is apparent that, while the absorbance peak from the CoA moiety is not shifted, that from the 4-hydroxybenzoyl unit is shifted from 292 to 373 nm (ϵ = 17 mM^{-1} cm^{-1}).

The 81 nm red shift observed in the 4-HBA-CoA benzoyl λ_{max} indicates that the dehalogenase active site environment is different from that of the water solvent. For the purpose of testing whether the active site environment is simply more hydrophobic in nature, the spectral properties of the 4-hydroxybenzoyl moiety were measured in an aprotic, organic solvent. This was accomplished using the 4-HBA-*N*-acetylcysteine thioester, which is soluble in acetonitrile as well as in water, in place of the 4-HBA-CoA (which is insoluble in acetonitrile). The absorbance maximum observed for aqueous 4-HBA-*N*-acetylcysteine (pH 7.5) at 292 nm (ϵ = 8.7 mM^{-1} cm^{-1}) and shoulder at 325 nm (ϵ = 3.6 mM^{-1} cm^{-1}) was seen as a single absorbance maximum at 287 nm (ϵ = 9.7 mM^{-1} cm^{-1}) when the measurement was made with the compound dissolved in 100% acetonitrile (the spectra are shown in the supporting information). The displacement of the 325 nm shoulder under the 292 nm peak was also observed for 4-HBA-CoA dissolved in 50% aqueous acetronitrile (λ_{max} 263 nm = 19.3 mM^{-1} cm^{-1}; λ_{max} 292 nm = 14.1 mM^{-1} cm^{-1}). On the basis of these spectral data, we conclude that simple desolvation of the 4-HBA-CoA cannot account for the UV−visible spectral perturbation (viz. the 81 nm red shift of the 292 nm absorbance peak) that is observed with the enzyme complex.

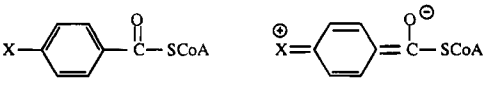
We also considered the possibility that a change in the ionization state of the C(4)−OH group may accompany the binding of the 4-HBA-CoA to the dehalogenase active site and that this could be the source of the red shift. Indeed, ionization of the ring hydroxyl group of 4-HBA-CoA (pK_a = 8.6) results in a red shift in the 292 nm absorbance peak to 330 nm (ϵ = 24 mM^{-1} cm^{-1}) (Mieyal et al., 1974), and although the UV−visible spectrum of enzyme-bound 4-HBA-CoA (Figure 1) was measured at a pH (7.5) well below the 4-HBA-CoA pK_a, the environment of the dehalogenase active site could perturb the pK_a to a lower value. To test this possibility, we measured the binding affinity of the 4-HBA-CoA toward the enzyme as a function of the solution pH. If the enzyme binds the ionized form, the apparent association constant of 4-HBA-CoA should increase with increasing pH near the pK_a. This was not observed, and instead, the binding affinity (which was pH independent below pH 8) decreased as the solution pH was increased above pH 8 (the pH binding profile is shown in the supporting information). This result suggests that the 4-HBA-CoA binds to the enzyme in the phenolic form but leaves open the possibility that the C(4)−OH is engaged in a hydrogen bond with an active site base.

At this juncture, the polarization of the benzoyl ring π electrons through interaction (electrostatic) with active site side chains became viewed as the most probable cause of the large UV−visible spectral shift observed with enzyme-bound 4-HBA-CoA (Figure 1B). The generality of this "electronic effect" was tested by measuring the UV−visible difference spectra of the dehalogenase complexes formed with other benzoyl-CoA adducts (viz., 2-HBA-CoA, 3-HBA-CoA, BA-CoA, 4-MeOBA-CoA, and 4-MeBA-CoA). The adducts 3-HBA-CoA, 4-MeOBA-CoA, and 4-MeBA-CoA were observed to bind most tightly to the active site (K_i = 10 ± 1, 17 ± 2, and 4.2 ± 0.5 μM, respectively), while 2-HBA-CoA and BA-CoA bind less tightly (K_i = 100 ± 20 and 72 ± 8 μM, respectively). For comparison to these values, we report that the K_i value of 4-HBA-CoA measured in the same manner is 2.5 ± 0.2 μM while the K_m for the substrate 4-CBA-CoA is 4 μM (Chang et al., 1992; Liang et al., 1993).

Of this group of benzoyl-CoA adducts, 4-MeBA-CoA is judged to be the closest structural analog of the substrate 4-CBA-CoA. The small K_i (4 μM) measured for 4-MeBA-CoA (vs 70 μM for BA-CoA) suggests that the methyl substituent is indeed filling the region in the active site normally occupied by the chloro substituent of 4-CBA-CoA and that the 4-MeBA-CoA complex binds to the active site in the same orientation as the substrate. As shown in Figure 1C, the absorbance peak of the benzoyl moiety in 4-MeBA-CoA is coincident with that of the CoA group. Subtraction of the absorbance contribution from the CoA unit (ϵ = 16 mM^{-1} cm^{-1}) from the total absorbance at the 260 nm λ_{max} yields a ϵ = 4 mM^{-1} cm^{-1} for the benzoyl chromophore. The λ_{max} of the benzoyl moiety in the enzyme complex of 4-MeBA-CoA is observed at 302 nm (ϵ = 6 mM^{-1} cm^{-1}), signifying that a 42 nm red shift in λ_{max} occurs upon binding.

The benzoyl-CoA adduct 4-MeOBA-CoA, which binds to the active site only a little less tightly than does the 4-MeBA-CoA (K_i = 17 vs 4 μM), displays a 31 nm red shift upon binding [from 292 nm (ϵ = 11 mM^{-1} cm^{-1}) to 323 nm (ϵ = 10 mM^{-1} cm^{-1})] (Figure 1E,F). In contrast, the difference spectra measured for the dehalogenase complexes of 2-HBA-CoA, 3-HBA-CoA, and BA-CoA (Figure 3 of the supporting information) do not reveal a shift in the benzoyl chromophore λ_{max}, suggesting that the C(4) substituent is important for productive binding and perturbation of the benzoyl chromophore. The comparatively large red shift observed for 4-HBA-CoA (81 nm) indicates that a more extensive redistribution of the benzoyl ring electrons is occurring in this particular adduct which, in turn, may be the result of the formation of a hydrogen bond between an active site base and the C(4) hydroxyl group (see below).

The origins of the observed red shifts in the λ_{max} values of the 4-HBA-CoA, 4-MeBA-CoA, and 4-MeOBA-CoA ligands lie in the polarization of the benzoyl ring π electron clouds which, in turn, generates a dipole. In valence bond terms, polarization is represented by increasing contributions from resonance (cononical) form **2** (Carey et al., 1978; Carey & Storer, 1984). Hence, while the solvated ligand may resemble resonance form **1**, the active site-bound ligand may by comparison more closely resemble resonance form **2**.

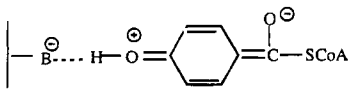


1 **2**

Stabilization of resonance form **2** (the quinonoid form) can occur in two ways. First, it can occur by an intramolecular effect, e.g., by making X, the C(4) substituent, a strong electron donor. This effect can be seen in the shift in λ_{max} for solvated 4-HBA-CoA in the phenol form [the Hammett σ_p constant is −0.38 (March, 1992) and the λ_{max} is 292 nm] vs the phenolate form [σ_p = −0.81 (March, 1992)

and λ_{max} = 330 nm (Mieyal et al., 1974)]. Second, stabilization can occur by an intermolecular effect e.g., by the placement of positively charged groups in the active site (or dipoles with net positive charge) next to the thioester C=O oxygen and/or net negative charges near the C(4) of the benzoyl ring. The largest red shifts would result when both effects occur synergistically, i.e., "electron push" near the C(4) and "electron pull" near the C=O.[2]

The 42 and 31 nm red shifts observed for 4-MeBA-CoA and 4-MeOBA-CoA indicate that significant polarization of the benzoyl rings is taking place upon active site binding. The comparatively large red shift (81 nm) observed for 4-HBA-CoA upon binding is suggestive of increased electron donation to the benzoyl ring through hydrogen bonding of the ring hydroxyl with electronegative active site residue (see below).[3] This interaction would in effect stabilize the quinonoid form of 4-HBA-CoA (**3**) but not those of 4-MeBA-CoA or 4-MeOBA-CoA.



3

Determination of the Raman Spectrum of 4-HBA-CoA and 4-MeBA-CoA Bound to the Active Site of 4-CBA-CoA Dehalogenase. The UV−visible spectral data described above indicate that the π electrons of the benzoyl moiety of the dehalogenase substrate and product are distributed differently in the water-solvated state (**1**) than in the enzyme-bound state (**2**). Raman spectra of 4-HBA-CoA and 4-MeBA-CoA in aqueous buffer and in the dehalogenase active site were measured to more closely define the bonding differences existing between these two states. The Raman data have the added advantage that peak positions are a property of the ground state exclusively, and thus, unlike absorption spectroscopy, ground state effects can be clearly distinguished from excited state effects. The Raman difference spectra, in which the contributions from buffer or buffer plus enzyme are subtracted, are compared in Figure 2. The Raman spectrum measured for the *S*-ethylthioester of 4-HBA in acetonitrile (Figure 4 of the supporting information) allowed us to distinguish stretch bands of the benzoyl moiety from those of the CoA moiety.[4]

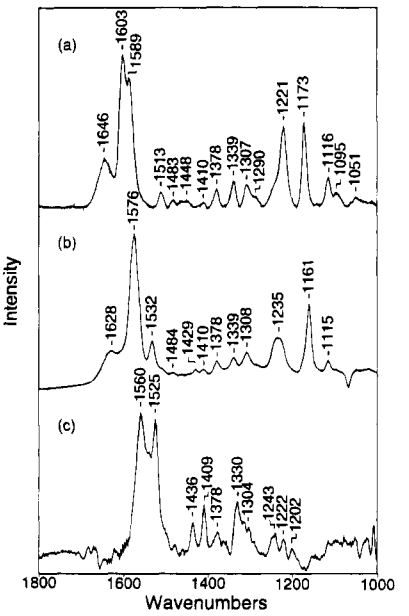


FIGURE 2: Raman difference spectra of (a) 5 mM 4-HBA-CoA in 50 mM K^+Hepes (pH 7.5), buffer spectrum subtracted; (b) 10 mM 4-HBA-CoA in 50 mM Na_2CO_3 (pH 10.0), buffer spectrum subtracted; and (c) 1 mM 4-HBA-CoA and 1 mM 4-CBA-CoA dehalogenase in 50 mM K^+Hepes/1 mM DTT (pH 7.5), enzyme/buffer spectrum subtracted. Conditions were 647.1 nm Kr laser excitation, 400 mW, 20 accumulations of 20 s each.

The main regions of interest in the spectrum of solvated 4-HBA-CoA at neutral pH (Figure 2a) are 1646 cm^{-1} (thioester C=O stretch), 1603 and 1589 cm^{-1} [benzene stretch modes 8a and 8b as discussed in Varsanyi (1969) and Harada and Takeuchi (1986)], and 1221 and 1173 cm^{-1} (in-plane benzene−hydrogen-stretching modes). The Raman difference spectrum of the enzyme-bound 4-HBA-CoA (Figure 2c) reveals a major reorganization of the normal modes. The benzene ring modes 8a and 8b dissappear and are replaced by features at 1560 and 1525 cm^{-1} which are outside of the range for 8a and 8b bands (Varsanyi, 1969). Moreover, the in-plane benzene−hydrogen modes and the thioester C=O stretch are missing from the spectrum of the bound ligand. Together, these spectral changes indicate that a major redistribution of π electrons within the benzoyl moiety of the 4-HBA-CoA occurs as it leaves the environment of the solvent water and enters the active site of the enzyme. It is noteworthy that the ionized form of the unbound 4-HBA-CoA still gives rise to a recognizable benzene-type signature for its ring modes (Figure 2b). This reinforces the conclusion drawn from the UV−visible absorption data that active site interactions at the thioester C=O oxygen, in addition to electron release at the C(4) position, are needed to bring about the phenyl (**1**) to quinonoid (**2**) transformation. In Raman difference spectra, there is always the possibility of the appearance of protein modes due to the ligand perturbing the structure of the protein upon binding. Then the protein features do not subtract

[2] Precedent for this type of interaction is found in the published studies of the spectral properties of 4-(*N*,*N*-dimethylamino)cinnamaldehyde complexes with the enzymes alcohol dehydrogenase (Dahl & Dunn, 1984; Jagodzinski et al., 1982; Callender et al., 1988), aldehyde dehydrogenase (Dunn & Buckley, 1985), and crotonase (D'Ordine et al., 1994). Each of these enzymes withdraws π electron density from the bound π-conjugated ligand through interaction with the carbonyl oxygen, thereby shifting the λ_{max} of the ligand to a longer wavelength.

[3] The spectral properties of the dehalogenase-bound 4-HBA-CoA agree well with those predicted by the 4-methylene-2,5-cyclohexadienone model. The absorbance maximum for 4-methylene-2,5-cyclohexadienone, which occurs at 282 nm (ϵ = 35.4 mM^{-1} cm^{-1}; permitted $\pi-\pi^*$ transition), is shifted to longer wavelength by substituents replacing hydrogen at the 4-methylene carbon atom (Pospisek et al., 1974). For instance, the replacement of one methylene hydrogen with a methoxy (MeO) group is reported to increase the λ_{max} by ca. 40 nm (Pospisek et al., 1975). Substitution of the two methylene hydrogens with a hydroxyl (HO) and a thiol (CoAS) substituent would therefore be expected to increase the λ_{max} by ca. 80 nm. The λ_{max} observed for 4-HBA-CoA bound to the dehalogenase active site (373 nm λ_{max}; 81 nm red shift) is thus within range of the value predicted for the quinoid structure based on the 4-methylene-2,5-cyclohexadienone model.

[4] Spectral interpretation is based on the spectral data presented in this paper, on spectral data measured for isotopically labeled 4-HBA-CoA, on spectral data measured for 4-HBA-CoA structural analogs, and on literature assignments. The complete set of data and their interpretation will be published separately.

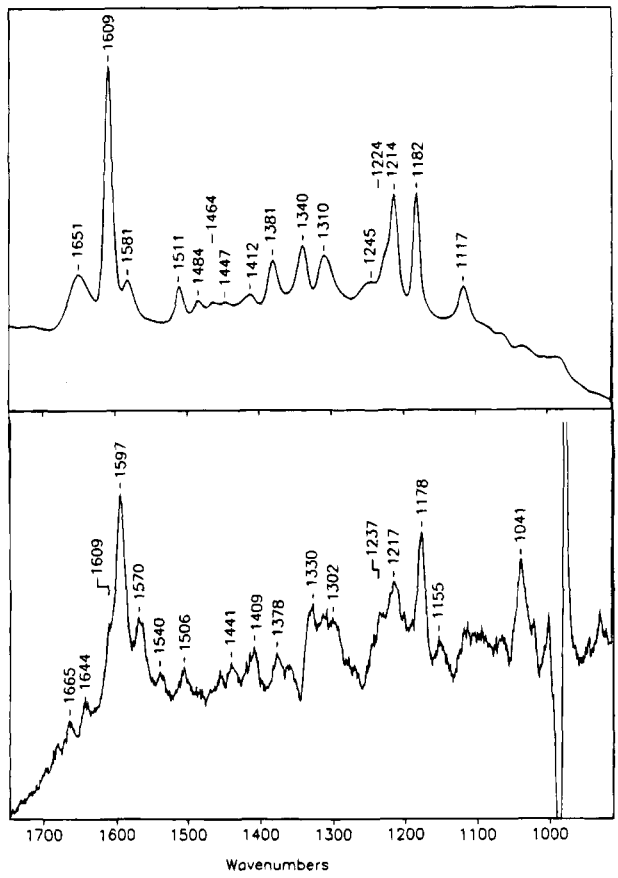


FIGURE 3: Raman difference spectra of (top) 5 mM 4-MeBA-CoA in 50 mM K$^+$Hepes (pH 7.5), buffer spectrum subtracted and (bottom) 1 mM 4-MeBA-CoA and 1 mM 4-CBA-CoA dehalogenase in 50 mM K+Hepes (pH 7.5)/0.5 mM DTT, enzyme/buffer spectrum subtracted. Conditions were 647.1 nm Kr laser excitation, 750 mW, 40 accumulations of 10 s each.

exactly to zero in the operation [spectrum of protein·ligand complex] − [spectrum of protein]. However, in the present work, we discuss only the relatively intense features which are dominated by ligand modes; in Figure 2, the bands at 1560 and 1525 cm^{-1} are due to ligand modes since they shift upon $^{13}C{=}O$ or $C{=}^{18}O$ substitution in the thioester group (unpublished work in this laboratory). In Figure 3, the intense or medium intensity bands at 1597, 1570, 1217, and 1178 cm^{-1} are ligand benzene ring modes by comparison with the data for the unbound ligand (Figure 3, top).

The Raman difference spectra of 4-MeBA-CoA in aqueous buffer and in the dehalogenase active site shown in Figure 3 also reflect changes occurring in π bonding in the benzoyl moiety upon enzyme complexation. The aromatic ring-stretching modes (8a and 8b) observed for 4-MeBA-CoA at 1609 and 1581 cm^{-1} in buffer are shifted to 1596 and 1570 cm^{-1} upon enzyme complexation, while the phenyl C−H bands seen at 1213 and 1182 cm^{-1} have been moved to 1215 and 1278 cm^{-1}. The thioester C=O stretch band observed at 1651 cm^{-1} (buffer) is shifted to 1610 cm^{-1} (enzyme). These spectral data indicate that the environment of the enzyme active site induces a significant redistribution of benzoyl π electrons in the substrate but not the massive change observed with the product ligand. Strong electron polarization is more localized at the thioester C=O oxygen, whereas the ring retains an essentially benzenoid character.

Determination of the Chemical Shift of the ^{13}C-NMR Signal from the Thioester Carbonyl Carbon of 4-HBA-CoA Bound to the Dehalogenase Active Site. Our investigations up to this point indicated that the dehalogenase stabilizes the 4-HBA-CoA in its quinoid resonance form (**3**). In principle, this could be accomplished by interaction of an active site electropositive group with the thioester C=O. Polarization of the C=O was tested by using ^{13}C-NMR techniques to access shielding of the C=O carbon nucleus for 4-HBA-CoA in aqueous solution and in the dehalogenase active site. In the aqueous solvent, the thioester C=O is engaged in hydrogen bonding with neighboring water molecules. If binding 4-HBA-CoA to the active site simply desolvates the thioester C=O, we expect, on the basis of our model studies carried out with 4-HBA-*N*-acetylcystiene, that the ^{13}C resonance of the C=O will be shifted upfield. The chemical shift of the $^{13}C{=}O$ resonance from 4-HBA-*N*-acetylcysteine measured in 5 mM K$^+$Hepes and 0.5 mM DTT at pD 7.5 is 193.1 ppm, while that measured in DMSO is 189.5 ppm (3.6 ppm upfield shift resulting from change from protic solvent to aprotic solvent) (Figure 5 of the supporting information). If, on the other hand, the active site increases the polarization of the 4-HBA-CoA thioester C=O (relative to the polarization experienced in the water solvent), the carbon nucleus may experience electron deshielding. This deshielding would effect the chemical shift of the ^{13}C resonance of the thioester carbonyl carbon, causing it to move downfield of the resonance measured in the absence of the enzyme.

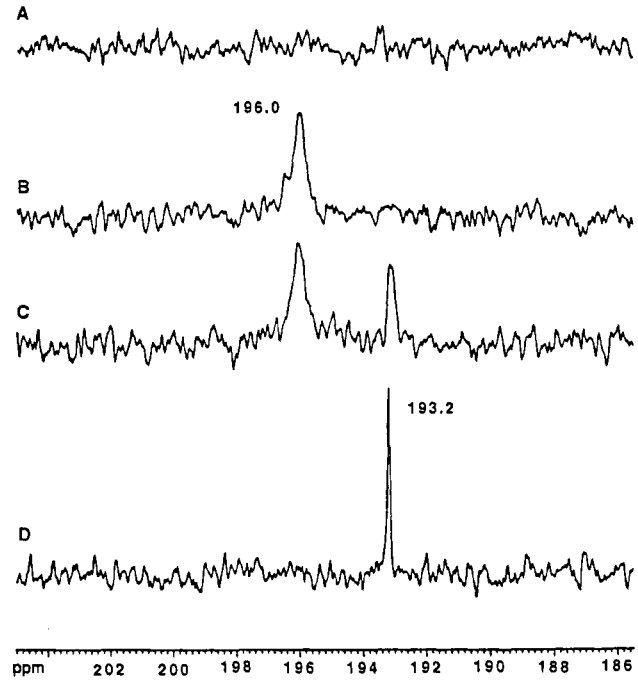
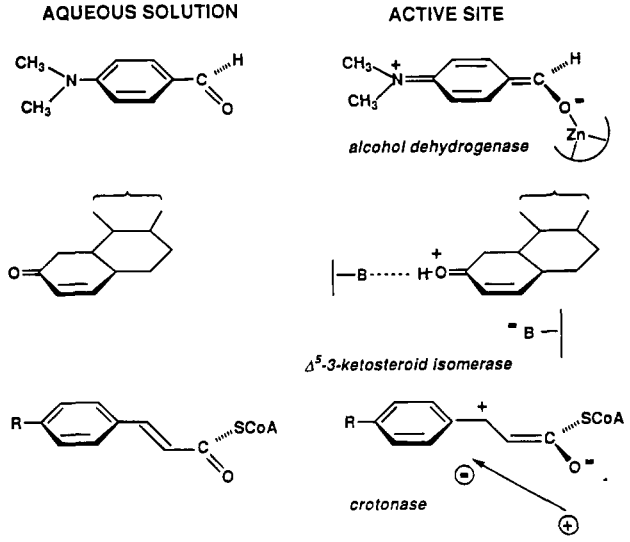


FIGURE 4: ^{13}C-NMR (125.77 MHz) spectrum of (A) 1.5 mM 4-CBA-CoA in 5 mM K$^+$Hepes/0.5 mM DTT buffer (pH 7.5) (control), (B) 1.2 mM [thioester $^{13}C{=}O$]-4-HBA-CoA and 1.5 mM dehalogenase in 5 mM K$^+$Hepes/0.5 mM DTT buffer (pH 7.5), (C) 2.0 mM [thioester $^{13}C{=}O$]-4-HBA-CoA and 1.5 mM dehalogenase in 5 mM K$^+$Hepes/0.5 mM DTT buffer (pH 7.5), and (D) 1.0 mM [thioester $^{13}C{=}O$]-4-HBA-CoA in 5 mM K$^+$Hepes/0.5 mM DTT buffer (pH 7.5).

Figure 4 shows the ^{13}C-NMR spectra measured for [thioester $^{13}C{=}O$]-4-HBA-CoA (dissolved in 5 mM K$^+$Hepes and 1 mM DTT, pD 7.5, 90−95% D_2O) in the presence and absence of 4-CBA-CoA dehalogenase. In the presence of excess enzyme, essentially all of the [thioester $^{13}C{=}O$]-4-HBA-CoA is complexed and the ^{13}C-NMR signal observed under these conditions is centered at 196.0 ppm (Figure 4B).

Scheme 2: Proposed Resonance Representations of the Alcohol Dehydrogenase Ligand DABA (Jagodzinski et al., 1982; Callender et al., 1988), the Crotonase Ligand Cinnamoyl-CoA (D'Ordine et al., 1994), and the Δ^5-3-Ketosteroid Isomerase Ligand 19-Nortestosterone (Austin et al., 1992) in Aqueous Solution and in the Enzyme Active Site



The chemical shift of the uncomplexed ligand (Figure 4C,D) is measured at 193.2 ppm. Thus, the ^{13}C-NMR resonance of the 4-HBA-CoA thioester C=O shifts 2.8 ppm downfield upon ligand binding to the dehalogenase, suggesting that the enzyme active site polarizes the C=O more strongly than does water. At the present time, the exact nature of the ligand–enzyme interaction which results in the C=O chemical shift is not known.

Mechanistic Implications of 4-CBA-CoA Dehalogenase-Induced Substrate Polarization. Active site stabilization of the quinonoid form (**3**) of 4-HBA-CoA could, in theory, be accomplished through the utilization of two active site residues: one to serve as a hydrogen bond acceptor to the C(4) ring OH and the other to serve as a hydrogen bond donor to the O=C of the CoA thioester substituent as is illustrated in Scheme 1. By working backward through the dehalogenation steps, the manner in which these two active site residues might contribute to catalysis can be seen. The interaction between the active site residue and the thioester C=O represented in Scheme 1 would activate the C(4) of the 4-CBA-CoA for nucleophilic attack, providing electrophilic catalysis.

Substrate Activation in Related Enzymatic Reactions; Origins of the Dehalogenase Active Site (Scheme 2). UV–visible and Raman spectral techniques have been previously used to examine substrate activation by enzymes catalyzing enolization of, or nucleophilic addition to, unsaturated carbonyl compounds. The most clear-cut example of substrate polarization is found in alcohol dehydrogenase, an enzyme which catalyzes the transfer of hydride from NADH to a substrate carbonyl. The carbonyl carbon is activated for this nucleophilic addition by coordination of the Zn^{2+} cofactor to the carbonyl oxygen atom where it functions as an "electron sink". The effects of polarization of the substrate carbonyl moiety by the Zn^{2+} are clearly apparent in the UV–visible spectra (λ_{max} of DABA shifted from 352 to 380 nm) and in the Raman spectra (shift of C=O stretch mode) of the slow substrate, *p*-(dimethylamino)benzaldehyde (DABA) bound in the (equine) alcohol dehydrogenase active site (Jagodzinski et al., 1982; Callender et al., 1988). Similar spectral changes were observed with Zn^{2+} alone, suggesting that the coordination of the Zn^{2+} to the DABA C=O is solely responsible for the stabilization of the quinoid resonance form. The dehalogenase does not utilize a divalent cofactor in catalysis (Chang et al., 1992; Liang, 1994), and therefore, interaction with an electropositive amino acid side chain(s) (and/or dipoles) must serve in the place of a metal ion cofactor.

Crotonase (D'Ordine et al., 1994) and Δ^5-3-ketosteroid isomerase (Austin et al., 1992) also induce the characteristic changes in the UV–visible and Raman spectra of their bound ligand indicative of the polarization of π electrons within the conjugated chromophore (cinnamoyl-CoA and 19-nortestosterone, respectively). Neither enzyme uses a metal cofactor for substrate polarization. In the case of the Δ^5-3-ketosteroid isomerase, the polarization of the α,β-unsaturated ketone ligand (19-nortestosterone) has been attributed to a H bond formed between active site tyrosine and the carbonyl oxygen of the ligand and the electrostatic interaction between an active site carboxylate and the ligand C=C.

The mechanism of substrate activation used by crotonase is of particular relevance to that employed by the dehalogenase because the two enzymes share structure homology (30% global identity) (Babbitt et al., 1992) and catalyze very similar reactions (hydration of C=C conjugated to a CoA thioester). D'Ordine et al. (1994) reported, on the basis of UV–visible, Raman, and ^{13}C-NMR spectral data, that the binding of the substrate analog cinnamoyl-CoA to the crotonase active site causes a major redistribution of the π electrons of the acryloyl moiety of the ligand whereas the π electron distribution in the phenyl moiety is not noticeably perturbed. A model, which we have depicted in Scheme 2, was proposed for crotonase catalysis in which an electropositive group positioned at the C=O oxygen and an electronegative group at the C(3) carbon of the cinnamoyl moiety would create an electric field which would serve to move electron density from C(3) to the carbonyl oxygen. One can imagine a similar electric field set up in the dehalogenase active site. In this case, the catalytic carboxylate would be aligned with the C(4) of 4-CBA-CoA, poised for nucleophilic attack (Scheme 1), while the electropositive group(s) would be aligned with the thioester C=O, serving as an electron sink. Efforts to solve the X-ray structures of crotonase (Vern Anderson, personal communication) and 4-CBA-CoA dehalogenase are in progress. From these structures, the origin of the dehalogenase may become apparent.

SUPPORTING INFORMATION AVAILABLE

Procedures for the synthesis of and data for ethyl 4-hydroxybenzoyl thioester, 4-methoxybenzoyl-CoA, 4-methylbenzoyl-CoA, and 4-HBA-*N*-acetyl-L-cysteine, UV–visible spectra of various solutions, pH dependence of the absorbance of E·4-HBA-CoA, UV–visible absorption spectra of enzyme-bound ligand, and Raman spectrum of ethyl 4-hydroxybenzoyl thioester (12 pages). Ordering information is given on any current masthead page.

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