Raman Evidence for Meisenheimer Complex Formation in the Hydrolysis Reactions of 4-Fluorobenzoyl- and 4-Nitrobenzoyl-Coenzyme A Catalyzed by 4-Chlorobenzoyl-Coenzyme A Dehalogenase†

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ABSTRACT: 4-Chlorobenzoyl-coenzyme A (4-CBA-CoA) dehalogenase catalyzes the hydrolytic dehalogenation of 4-CBA-CoA to 4-hydroxybenzoyl-CoA by using an active site Asp145 carboxylate as the nucleophile. Formation of the corresponding Meisenheimer complex (EMc) is followed by chloride ion expulsion to form arylated enzyme (EAr). The EAr is then hydrolyzed to product. In this paper, we report the kinetics for dehalogenase-catalyzed 4-fluorobenzoyl-CoA (4-FBA-CoA) and 4-nitrobenzoyl-CoA (4-NBA-CoA) hydrolysis and provide Raman spectral evidence for the accumulation of EMc in these reactions. The 4-FBA-CoA and 4-NBA-CoA substrate analogues were selected for the poor leaving group ability of their C(4) substituents. Thus, the formation of the EAr from EMc should be hindered, giving rise to a quasi-steady-state equilibrium between EMc and the Michaelis complex. Detailed kinetic studies were carried out to quantitate the composition of the reaction mixtures. Quench experiments demonstrated that significant populations of EAr do not exist in reaction mixtures involving the 4-F- or 4-N-substrates. A kinetic model enabled us to estimate that approximately 10–20% of the enzyme−substrate complexes in the reaction mixtures are present as EMc. Raman difference spectra of 4-NBA-CoA and 4-FBA-CoA bound to WT and H90Q mutant dehalogenase have broad features near 1500 and 1220 cm⁻¹ that are absent in the free ligand. Crucially, these features are also absent in the Raman spectra of the complexes involving the D145A dehalogenase mutant that are unable to form an EMc. Quantum mechanical calculations, at the DFT level, provide strong support for assigning the novel 1500 and 1220 cm⁻¹ features to an EMc.

1 4-Chlorobenzoyl-coenzyme A (4-CBA-CoA) dehalogenase (1−3) mediates the central reaction of the 4-chlorobenzoate (4-CBA) to 4-hydroxybenzoate (4-HBA) pathway found in certain soil-dwelling bacteria (4). Previous studies of substrate activation and turnover occurring within the dehalogenase active site have revealed an elaborate catalytic mechanism (5−15). The individual steps proposed for the reaction are illustrated in Figure 1. According to this mechanism, substrate binding to the dehalogenase active site is followed by attack of the carboxylate side chain of Asp145 at the benzoyl C(4). The Meisenheimer complex formed (henceforth referred to as the EMc intermediate) ultimately expels the Cl⁻ ion, thus forming an arylated enzyme complex (the EAr intermediate). Hydrolysis of the ester function in EAr then occurs, forming the 4-HBA-CoA product, which along with a proton and the Cl⁻ ion is released from the enzyme active site.

The first step of catalysis, substrate activation through thioester C=O polarization, is achieved via H-bonds formed to two active site backbone amide N−Hs, one of which (Gly114) is positioned at the positive pole of an α-helix dipole and the other of which (Phe64) is coupled to the binding of the CoA moiety [backbone C=O to Ade C(6)-NH₂] (see active site pictured in Figure 2) (6). The electron “pull” provided to the C=O is augmented by an electron “push” from the carboxylate of Asp145 poised at C(4) of the substrate benzoyl ring (7−9). The electrostatic interactions are facilitated by the sheath of aromatic side chains (Phe64, Phe82, Trp137, and Trp89) encircling the benzoyl...
moiety. The overall effect of the active site environment on the substrate is dispersion of the ring π electron density away from C(4) and toward the thioester C=O.

The ensuing attack made by the Asp145 at the C(4) of the polarized benzoyl ring has been evidenced through several independent lines of experimental investigation. First, from the X-ray structure of the 4-CBA-CoA dehalogenase—4-HBA-CoA complex (10), the Asp145 carboxylate oxygen Oäs is seen positioned in an “attack conformation” (16, 17), 2.5 Å from C(4) (see Figure 2). Second, within the detection limit of the rate measurement (~1 × 10⁻⁷ s⁻¹), the D145A mutant has been found to be “catalytically inactive” (18). Third, it has been shown that single-turnover reactions carried out in oxygen-18-enriched water produce [C(4)-¹⁶O]4-HBA-CoA as product [i.e., the C(4)OH is derived from an active site carboxylate] (13, 14). Finally, single-turnover reactions carried out with [¹⁴C]4-CBA-CoA in conjunction with rapid quench techniques have demonstrated the formation and decay of a kinetically competent covalent enzyme intermediate (5, 14). These data are consistent with a reaction mechanism that involves the arylation of Asp145 and subsequent hydrolysis of the aryl ester at the Asp145 C=O. The close proximity (2.9 Å) of the Trp137 indole NH to the Asp145 Oäs (Figure 1) indicates that it functions to orient the carboxylate nucleophile and/or activate the Asp145 aryl ester (Figure 2). Likewise, the positioning of the His90 N(3) at 3.6 Å from the Asp145 carboxyl carbon suggests its participation in binding and activating the water nucleophile involved in the hydrolysis of the EAr intermediate. Results from a kinetic analysis of the H90Q mutant support the role of His90 in general base catalysis in EAr hydrolysis (5).

The formation of Meisenheimer complex intermediates in the nucleophilic substitution reactions of electrophilic aromatic rings is well documented (19, 20). Nitro substituents are especially effective in activating the ring for nucleophilic attack because they provide resonance stabilization to the anionic o-complex. The UV/visible absorption spectra of the Meisenheimer intermediates formed from the reaction of nitro-substituted aromatic rings are strongly red-shifted, thus allowing easy detection of the intermediate. A most striking
example of biological Meisenheimer complex formation is seen with the development of an orange—red color in culture media containing Rhodococcus erythropolis and 2,4,6-trinitrobenzene (21). Studies have shown that this color is derived from hydride transfer to the aromatic ring to form the anionic σ-complex (22). Because hydride and nitrite are poor leaving groups, the Meisenheimer complex persists.

Glutathione transferase-catalyzed nucleophilic aromatic substitution reactions have been well characterized. Glutathione transferase catalyzes the formation of the thiolate anion of glutathione and its addition to a variety of carbon electrophiles, including highly activated aromatic rings. The addition of glutathione to activated aromatic rings having poor leaving groups produces relatively stable Meisenheimer complexes (the glutathione anion is also a poor leaving group) which are further stabilized through tight binding by the enzyme active site (23, 24). The X-ray crystal structures of the class μ (25) and class π (26) glutathione transferases bound with 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate anion have been reported, and these structures reveal stabilization through H-bond interactions occurring between the nitro substituents and the enzyme active site.

The aromatic substitution reaction catalyzed by the 4-CBA-CoA dehalogenase differs from that catalyzed by the glutathione transferase in two important ways. First, the aromatic ring of the substrate is only weakly electrophilic, and therefore it must be fully activated for nucleophilic attack. Second, in the dehalogenase, the nucleophile is the carboxylate group, which are further stabilized through tight binding by the anion have been reported, and these structures reveal stabilization through H-bond interactions occurring between the thioester C O (Figure 1) (27). Second, in the dehalogenase, the nucleophile is the carboxylate Asp145 and not a thiolate anion. Unlike the thiolate anion, the carboxylate is an excellent leaving group. Consequently, the reversion of the EMc to E-S is competitive with its partitioning forward to EA. Kinetic studies have indicated that the EMc expels the carboxylate residue at an estimated rate of 2000 s⁻¹ while it expels the chloride ion at a rate of 40 s⁻¹ (5). The EMc intermediate does not accumulate to a significant extent during substrate turnover and is therefore difficult to observe. Through the use of an alternate substrate in which the chloride substituent is replaced by a leaving group having less mobility (fluoride or nitrite), the rate at which the EMc partitions forward can be significantly reduced. This will allow an equilibrium to be established between the E-S and EMc complexes, governed by the relative rates of Asp145 addition to substrate and expulsion from EMc. Based on these rates, we anticipated that ca. 10–20% of the dehalogenase may be populated as the EMc. Raman spectroscopic data, supported strongly by quantum mechanical calculations, provide good evidence that a population of EMcs does indeed exist in reaction mixtures containing the 4-NBA-CoA or 4-FBA-CoA analogues.

**EXPERIMENTAL PROCEDURES**

**General.** 4-CBA-CoA, 4-FBA-CoA, and 4-HBA-CoA were prepared according to ref (15). 4-NBA-CoA was synthesized from coenzyme A and 4-nitrobenzoyl chloride by adaptation of the procedure described in ref (28). 4-CBA-CoA dehalogenase (specific activity = 1.5 units/mg) and the D145A, H90Q mutants were prepared according to the published procedure (14). Enzyme concentration was determined by using the Bradford method (29).

**Ligand Dissociation Constants Measured by Fluorescence Quenching.** A FluoroMax-2 fluorometer was used in protein fluorescence quenching experiments aimed at measuring the binding constants of 4-FBA-CoA and 4-HBA-CoA with 4-CBA-CoA dehalogenase. The fluorescence spectrum of dehalogenase (1 μM) in 50 mM K⁺Hepes (pH 7.5, 25 °C) resulting from 290 nm excitation has an emission wavelength maximum at 337 nm. For a typical titration experiment, 1–10 μL aliquots of ligand were added to a 1 mL solution of 1 μM dehalogenase, and the fluorescence intensity at 337 nm was measured following each addition. The fluorescence data, collected at ligand concentrations ranging from 0.1 to 60 μM for 4-FBA-CoA and from 0.1 to 10 μM for 4-NBA-CoA, were fitted to eq 1 (30) using the Kaleida Graph computer program for nonlinear regression analysis:

\[
\Delta F/F_0 = (\Delta F_{\text{max}}/F_0)[E] \left\{ (K_d + [E] + [S]) \right. - \left. \sqrt{(K_d + [E] + [S]^2 - 4[E][S]/2)} \right) - \left. \frac{1}{2} \right\}
\]

where [S] is the total ligand concentration, [E] is the total enzyme concentration, \( K_d \) is the apparent dissociation constant of the enzyme–ligand complex, \( \Delta F \) is the observed change in fluorescence intensity, \( \Delta F_{\text{max}} \) is the maximum change in fluorescence intensity, and \( F_0 \) is the initial fluorescence intensity.
Ligand Binding Rates Measured by Stopped-Flow Fluorescence. For fluorescence kinetic measurements, a DX.17 MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.) equipped with a 150 W xenon lamp (2 mm slit width) was used. The excitation wavelength was 290 nm, and the emission filter cutoff was 320 nm. Data acquisition and processing was controlled by a 32-bit processor Archimedes workstation, which was also used for subsequent nonlinear regression analysis of the kinetic traces. Stock solutions of ligand and enzyme were diluted to specific concentrations with 50 mM K⁺Hepes buffer (pH 7.5, 25 °C). Data sets were collected in triplicate and averaged. The fluorescence quenching traces were analyzed with a single-exponential equation to obtain the observed rate constant, \(k_{\text{obs}}\). The plot of \(k_{\text{obs}}\) vs ligand concentration was linear for each reaction. These data were fit to eq 2 to obtain \(k_{\text{on}}\) and \(k_{\text{off}}\).

\[
k_{\text{obs}} = k_{\text{on}} + k_{\text{off}}[S]
\]

In eq 2, [S] = substrate concentration, \(k_{\text{obs}}\) = observed rate constant, \(k_{\text{on}}\) = binding rate constant, and \(k_{\text{off}}\) = dissociation rate constant.

Reaction Progress Curves Measured by 4-HBA-CoA Spectrophotometric Detection. (A) Multiple Turnover Reactions. Reaction solutions initially contained 20 \(\mu\)M 4-CBA-CoA dehalogenase and 200 \(\mu\)M 4-NBA-CoA in 50 mM K⁺Hepes (pH 7.5, 25 °C) or contained 44.5 \(\mu\)M 4-CBA-CoA dehalogenase and 300 \(\mu\)M 4-FBA-CoA in 50 mM K⁺Hepes (pH 7.5, 25 °C). After a specified incubation period, 11.0 \(\mu\)L aliquot was removed from the 4-NBA-CoA reaction mixture and mixed with 110 \(\mu\)L of 0.3 M KOH. The absorbance of the resulting solution was measured at 330 nm (ε = 18.2 mM⁻¹ cm⁻¹ for the 4-HBA-CoA phenoxide anion). In the case of the 4-FBA-CoA reaction, 25 \(\mu\L of 2.5 M KOH was added directly to the reaction solution, and the absorbance at 330 nm was measured.

(B) Single-Turnover Reactions. The reaction between 30 \(\mu\L of 250 \muM 4-CBA-CoA dehalogenase in 50 mM K⁺Hepes (pH 7.5, 25 °C) and 32 \(\mu\L of 201 \muM 4-CBA-CoA in 50 mM K⁺Hepes (pH 7.5, 25 °C) was carried out in a KINTEK rapid quench device. After a specified incubation period, the reaction was quenched with 182 \(\mu\L of 0.32 M HCl. Then 40 \(\mu\L of (saturated) 4 M KCl was added to facilitate enzyme precipitation. The mixture was centrifuged in a 0.5 mL NANOSEP MF 0.2 \(\mu\)m centrifugal device (Pall Filtron Corp.). A 220 \(\mu\L aliquot of the supernatant fraction was mixed with 34 \(\mu\L of 2.5 M KOH, and the absorbance of the resulting solution at 330 nm was measured. The enzyme precipitant contained in the centrifugal device was suspended in a 250 \(\mu\L solution containing 0.2 M HCl and 0.56 M KCl and then centrifuged. This cycle was repeated twice more. The washed enzyme precipitant was then dissolved in 230 \(\mu\L of 0.15 M KOH and centrifuged, and the absorbance of the resulting solution at 330 nm was measured. The reaction between the dehalogenase and 4-NBA-CoA was not carried out in the rapid quench device. Instead, after a specified incubation period, the 250 \(\mu\L reaction solution initially containing 70 \(\mu\L of dehalogenase and 60 \(\mu\L of 4-NBA-CoA in 50 mM K⁺Hepes (pH 7.5, 25 °C) was hand-mixed with 10 \(\mu\L of 6 M HCl and 40 \(\mu\L of 4 M KCl. The reaction mixture was separated and analyzed as previously described.

UV/Visible Difference Spectra, Stopped-Flow Absorption Measurements, and Data Analyses. UV/visible difference spectra were measured as described in Taylor et al. (9). The absorption reference spectra were measured by using 1 mL quartz tandem cells containing 0.5 mL of enzyme solution in one compartment and 0.5 mL of 4-FBA-CoA or 4-NBA-CoA solution in the other. All solutions were buffered with 50 mM K⁺Hepes (pH 7.5, 1 mM DTT, 25 °C). The reference absorbance spectrum was recorded and subtracted from the spectrum of the mixed solutions to give the difference spectra of the enzyme—4-FBA-CoA or 4-NBA-CoA complex. For UV/visible absorption kinetic measurements, a DX.17 MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.) equipped with a 150 W xenon lamp (1 mm slit width, 1 cm path length) was used. Stock solutions of ligand and enzyme were diluted to specific concentrations with 50 mM K⁺Hepes buffer (pH 7.5, 25 °C). Reactions were monitored at 430 and 375 nm over 0–200 ms and 0–3600 s time periods. Data sets were collected in triplicate and averaged. The computer program KINSIM (31) was used to simulate curves to the absorption traces using the microscopic rate constants and molar extinction coefficients of the absorbing species as input.

Raman Spectroscopy. The nonresonant Raman spectra were obtained using 647.1 nm laser excitation from an Innova 400 krypton laser system (Coherent, Inc.), a back-illuminated charge-coupled device (CCD) detector (model 1024EH/RB/1, Princeton Instruments, Inc.) operating at 183 K, and a Holospec f/1.4 axial transmission spectrometer (Kaiser Optical Systems, Inc.) employed as a single monochromator, as described in a previous report (7). Enzyme samples contained in cuvettes were 50 \(\mu\L in volume and buffered with 50 mM Tris-HCl at pH 7.5. Enzyme and 4-FBA-CoA or 4-NBA-CoA concentrations used are given in the figure legends. Data were collected immediately after the complex had been made, using a laser power of ~850 mW and CCD exposure times of 5 min. The Raman spectrum of the buffer was subtracted from that of the ligand in buffer (giving the spectrum of free ligand), while the spectrum of the enzyme in buffer was subtracted from that of the enzyme—ligand complex to give the spectrum of the bound ligand.

Calculations. Quantum mechanical computations have been performed to determine the vibrational assignments of the Meisenheimer compounds (Mc) formed by the 4-N and 4-F analogues (Figure 8), and to provide insights into geometry changes occurring in the Mc. For the calculations, the bulky coenzyme A part was truncated to an S-ethyl, and the torsional angle about S-C = S was set at gauche [which is the same as the conformer found in the X-ray crystal data of the final product 4-HBA-CoA (10)]. The Asp145 side chain was represented by an acetate species (\(CH_3 COO^-\)). 4-NBA-CoA and 4-FBA-CoA were also analyzed by substituting S-ethyl for the CoA, to confirm the ring modes of the benzoyl moiety, and the vibrational modes associated with the NO₂ and F substituents.

Using Gaussian 98 software (32), full geometry optimization of the molecules was carried out for the subsequent estimation of harmonic force constants at the density functional theory (DFT) level of B3LYP/6-31+G(d) [i.e., electron correlation effect included Becke’s three-parameter exchange functional using the correlation functional of Lee, Yang, and Parr (33, 34)]. The same Gaussian basis set of...
Table 1: Thermodynamic and Kinetic Constants Measured for 4-CBA-CoA Dehalogenase with 4-CBA-CoA, 4-FBA-CoA, and 4-NBA-CoA (Values Are Defined within 10% Error)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_{on}$ ($\mu$M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (\mu M)$^a$</th>
<th>$k$ (s$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-CBA-CoA$^c$</td>
<td>7</td>
<td>30</td>
<td>4</td>
<td>2.3</td>
</tr>
<tr>
<td>4-NBA-CoA</td>
<td>2.4</td>
<td>1.0</td>
<td>0.5 (0.4)</td>
<td>$1 \times 10^{-3}$</td>
</tr>
<tr>
<td>4-FBA-CoA</td>
<td>1.4</td>
<td>23</td>
<td>16 (16)</td>
<td>$7 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

$^a$ The $K_d$ value not in parentheses was calculated from the binding rate constants while the $K_d$ value in parentheses was determined by titration using fluorescence titration quenching methods. $^b$ The $k$ value was determined by fitting product formation curves for a single turnover measured at saturating ligand concentration. $^c$ Values taken from ref (5).

RESULTS AND DISCUSSION

Kinetic Analysis of Dehalogenase-Catalyzed Hydrolysis of 4-FBA-CoA and 4-NBA-CoA

Prior to carrying out Raman spectroscopic studies of the dehalogenase complexes of 4-FBA-CoA and 4-NBA-CoA, it was necessary to measure binding constants for these complexes and the rate at which they undergo reaction. Also, because the Raman analysis may detect any intermediate present in reaction mixture, it was important to show that the aryalted enzyme does not accumulate in these mixtures.

Binding. The $k_{on}$ and $k_{off}$ values for the binding of 4-FBA-CoA and 4-NBA-CoA to 4-CBA-CoA dehalogenase were measured using stopped-flow fluorescence quenching techniques. The values obtained are listed in Table 1 along with those reported previously for 4-CBA-CoA (5). Based on the $k_{on}$ and $k_{off}$ values for 4-FBA-CoA, a $K_d = 16 \mu$M is calculated. The $K_d$ values for 4-NBA-CoA is 0.5 \mu M. The $K_d$ values for 4-FBA-CoA and 4-NBA-CoA compare with the $K_d = 4 \mu$M calculated from the binding rate constants obtained for 4-CBA-CoA (5).

Owing to slow turnover of the 4-FBA-CoA and 4-NBA-CoA ligands, fluorescence titration curves could be determined. These titration curves reflect the equilibrium between free ligand and the enzyme–ligand complex plus EMc. For 4-FBA-CoA, the apparent $K_d = 16 \mu$M, and for 4-NBA-CoA, the apparent $K_d = 0.4 \mu$M. These values agree with the $K_d$ values calculated from the binding rate constants.

Time Courses for Product Formation. The time course for product formation was examined under multiple-turnover conditions in which the 4-CBA-CoA dehalogenase (20 \mu M) is reacted with a 10-fold excess of 4-NBA-CoA (200 \mu M). The reactions were terminated by adding sufficient KOH solution to raise the pH to 14. Under these conditions, the enzyme is inactive, and the 4-HBA-CoA product exists as the phenoxide anion, which is detected at 330 nm. Figure 4A shows the product formation curve for the first several turnovers of 4-NBA-CoA. The first turnover (corresponding to 20 \mu M 4-HBA-CoA) occurs at the same rate as subsequent turnovers, indicating that a step preceding product release is rate-limiting. The turnover rate derived from the time course is $1 \times 10^{-3}$ s$^{-1}$.

The dehalogenase (44.5 \mu M)-catalyzed reaction of 4-FBA-CoA (300 \mu M) proved to be too slow to monitor over several turnovers. The time course shown in Figure 4B indicates that the enzyme completes only 50% of a turnover at 25 h. From the time course data, a turnover rate of $7 \times 10^{-6}$ s$^{-1}$ is obtained. The poor leaving ability (F$^-$ < NO$_2^-$ < Cl$^-$) in dehalogenase simply implies that breaking of the C4–X bond is rate-limiting in these alternative substrates, a phenomenon of SN Ar reactions that depends on the character of the nucleophile and the leaving group X$^-$ as well as the accessibility of a H-bond donor that may assist the departure of the X$^-$ (20, 35).

A Probe for the Arylated Enzyme Intermediate. Previous studies have shown that during a single turnover of 4-CBA-CoA by the 4-CBA-CoA dehalogenase, both covalent enzyme intermediates accumulate on the enzyme (5). In contrast, we do expect to observe EAr accumulation during dehalogenase turnover of 4-FBA-CoA or 4-NBA-CoA. This is because once formed, the EMc will expel the fluoride or nitrite ion at a rate much slower than the anticipated rate of EAr hydrolysis. To test this hypothesis, we measured EAr accumulation during single-turnover reactions of 4-FBA-CoA and 4-NBA-CoA. To accomplish this, the original single-turnover experiment which employed $[^{14}$C]4-CBA-CoA (along with centrifugal molecular cutoff filtration of enzyme and HPLC separation of $[^{14}$C]4-CBA-CoA and $[^{14}$C]4-HBA-CoA (5) was modified so that it could be carried out with unlabeled reactant.

The new EAr detection method was first demonstrated with 4-CBA-CoA serving as substrate. Reaction mixtures
of dehalogenase (121 μM) and 4-CBA-CoA (104 μM) were quenched with acid after specified incubation periods in a rapid quench device. The precipitated enzyme was separated, and the two fractions were treated with aqueous KOH to convert EAr (from enzyme precipitant) and 4-HBA-CoA (from the supernatant) to the phenoxide anion of the 4-HBA-CoA (detected at 330 nm). The time courses for EAr formation and product formation thus obtained are shown in Figure 5A. EMc present in the reaction solution at the time of the quench may also contribute to the EAr pool through expulsion of chloride ion. The time course reveals the accumulation of EAr over the first 100 ms to a level of 25% [isolation of labeled enzyme by precipitation proved to be more efficient than isolation by repetitive dilution and centrifugal molecular cutoff filtration of solvated enzyme as used previously to detect 16% accumulation; see ref (5)] and then a decline over the next 500 ms to 0%.

The same experiment was carried out with 4-NBA-CoA and 4-FBA-CoA, but owing to the slower reaction rate, the reactions were terminated by hand rather than by rapid quench. Only EAr will be detected in the quenched mixtures because EMc will expel the enzyme carboxylate more efficiently than the fluoride or nitrite ion. In Figure 5B, the time courses for EAr and product formation during the single-turnover reaction of 60 μM 4-NBA-CoA with 70 μM wild-type 4-CBA-CoA dehalogenase are shown. The product formation curve was analyzed with a single-exponential equation to give $k_{obs} = 7 \times 10^{-4}$ s$^{-1}$. The covalent enzyme was not detected. Similarly, aliquots removed from the reaction mixtures of 4-FBA-CoA and 4-CBA-CoA dehalogenase did not contain detectable levels of EAr. These results demonstrate that the reaction intermediate EAr does not accumulate to a significant extent during dehalogenase-catalyzed hydrolyses of 4-FBA-CoA and 4-NBA-CoA. Thus, EAr will not contribute significantly to the Raman spectra measured for mixtures of dehalogenase and 4-FBA-CoA or 4-NBA-CoA.

**Stopped-Flow Absorption Studies of the Single-Turnover Reaction of 4-NBA-CoA.** In the introduction, we speculated that in the dehalogenase-catalyzed reactions of 4-FBA-CoA and 4-NBA-CoA the EMc might accumulate to a level ~10% of the total enzyme present. This estimate is based on a slow rate of fluoride or nitrite expulsion from EMc and on the assumption that the rates of the forward (200 s$^{-1}$) and reverse (2000 s$^{-1}$) directions of the E$^+$ to EMc step are the same in the 4-FBA-CoA and 4-NBA-CoA reactions as they are in the 4-CBA-CoA reaction. Here, the progress of a single-turnover reaction of dehalogenase (70 μM) and 4-NBA-CoA (60 μM) was monitored using stopped-flow absorption techniques. The absorption curves measured at 430 and 375 nm (Figure 6) were fitted using the kinetic model of the 4-CBA-CoA reaction but substituting the experimental values of 4-NBA-CoA $k_{on}$ and $k_{off}$ for $k_{1}$ and $k_{-1}$, and the $k_{obs}$ for $k_{3}$.

The UV/visible absorption spectra of 4-NBA-CoA and 4-HBA-CoA in buffer and the UV/visible absorption difference spectra of the dehalogenase–4-NBA-CoA complex and the dehalogenase–4-HBA-CoA complex are shown in Figure 7. The UV/visible absorption difference spectra of the dehalogenase–ligand complexes were generated by subtracting the absorption spectra of the uncomplexed ligand and
The binding reaction between 4-NBA-CoA and the dehalogenase can be monitored at 430 nm where the dehalogenase–4-NBA-CoA complex absorbs since no other species is expected to contribute at this wavelength. The EMe complex is not expected to absorb at 430 nm wavelength because the nitro substituent, which contributes to the long-wavelength absorption of the benzoyl moiety of the substrate through resonance, does not contribute to the ring π-electron density of the α-complex. Likewise, the absorption by the enzyme–product complex does not extend out to 430 nm.

The 0–200 ms 375 nm absorption curve shown in Figure 6A was fitted with KINSIM (31) using a kinetic model consisting of the substrate binding and EMe forming steps:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E\text{-}Me
\]

where \( k_1 = 2.8 \text{ s}^{-1} \mu\text{M}^{-1} \), \( k_{-1} = 1.0 \text{ s}^{-1} \), \( k_2 = 200 \text{ s}^{-1} \), and \( k_{-2} = 1000 \text{ s}^{-1} \); and at 430 nm, \( E\text{-}S \epsilon = 0.92 \text{ mM}^{-1} \text{ cm}^{-1} \), and for the \( E\text{-}S \) and \( E\text{-}Me \) \( \epsilon = 0.0 \text{ mM}^{-1} \text{ cm}^{-1} \). Like the dehalogenase–product complex, the dehalogenase–4-NBA-CoA complex absorbs at 375 nm. The 0–200 ms 375 nm absorption curve shown in Figure 6A derives from the formation of the dehalogenase–4-NBA-CoA complex, however, and not from the formation of the enzyme–product complex which occurs at longer reaction times (see below). The 375 nm absorption curve was fitted to the kinetic model shown above, using \( E\text{-}S \epsilon = 1.23 \mu\text{M}^{-1} \text{ cm}^{-1} \) and the same rate constants used to fit the 430 nm absorption curve.

At longer reaction periods (0–3600 s), the absorption at 430 nm begins to decrease and the absorption at 375 nm to increase from the respective plateaus reached at the attainment of the quasi-equilibrium between the 4-NBA-CoA, dehalogenase, dehalogenase–4-NBA-CoA complex, and the EMe. The 0–3600 s 430 and 375 nm absorption curves shown in Figure 6B were fitted with KINSIM (31) using a kinetic model consisting of all of the reaction steps:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E\text{-}Me \xrightarrow{k_3} E\text{-}Ar \xrightarrow{k_4} E\text{-}P\text{-}H^+\text{-}NO_2^-
\]

where \( k_1 = 2.8 \text{ s}^{-1} \mu\text{M}^{-1} \), \( k_{-1} = 1.0 \text{ s}^{-1} \), \( k_2 = 200 \text{ s}^{-1} \), \( k_{-2} = 1000 \text{ s}^{-1} \), \( k_3 = 0.005 \text{ s}^{-1} \), \( k_{-3} = 0 \text{ s}^{-1} \), \( k_4 = 25 \text{ s}^{-1} \), \( k_{-4} = 0 \text{ s}^{-1} \), \( k_5 = 1 \text{ s}^{-1} \), \( k_{-5} = 8 \mu\text{M}^{-1} \text{ s}^{-1} \), \( E\text{-}S \epsilon = 1.23 \mu\text{M}^{-1} \text{ cm}^{-1} \) at 375 nm, \( E\text{-}P \epsilon = 10.5 \text{ mM}^{-1} \text{ cm}^{-1} \) at 375 nm, and \( E\text{-}S \epsilon = 0.92 \mu\text{M}^{-1} \text{ cm}^{-1} \) at 430 nm.

The goodness of fit of the simulated curves generated by using the original kinetic model derived for the dehalogenase–4-CBA-CoA reaction (5) with adjustments made for the substrate binding rate constants (experimental \( k_{\text{obs}} \) and \( k_{\text{cat}} \) values for 4-NBA-CoA substituted) and the EMe to EAr rate constant (to reflect the lower mobility of the nitrite leaving group) supports our original hypothesis that the lifetime of the EMe intermediate can be increased by substituting the 4-CBA-CoA with 4-NBA-CoA as substrates. The simulated values for \( k_5 = 200 \text{ s}^{-1} \) and \( k_{-5} = 1000 \text{ s}^{-1} \) reflect the accumulation of EMe derived from 4-NBA-CoA to 20% of the enzyme present. Realistically, the stopped-
flow data cannot be used to define these two rate constants with precision. Nevertheless, the fit is exquisitely sensitive to the ratio $k_3k_{-2}$ and to the value of $k_3$ used as input. Only by increasing the value of $k_3$ can the ratio of $k_3k_{-2}$ be reduced and the goodness of fit preserved. Thus, 20% may be a reasonable estimate of the fraction of enzyme which accumulates as EMc during the course of the Raman spectral experiments described below.

Raman Spectral Analysis of Dehalogenase Reaction Mixtures of 4-FBA-CoA and 4-NBA-CoA

Density Functional Theory Calculations on the Mc. Figure 8 illustrates the structural features of the predicted Meisenheimer compounds obtained by using the ab initio HF method [HF/6-31+G(d)]. Noticeably, the ring C4–F bond is elongated from 1.36 Å in the substrate 4-FBA-CoA to 1.47 Å in the Meisenheimer compound I, and that of the ring C4-nitro bond is elongated from 1.50 Å in the substrates to 1.43 Å in the Mc. The ring bond lengths in II are C1C2 = 1.43 Å; C2C3 = 1.37 Å; C3C4 = 1.46 Å. The ring bond lengths in II are C1C2 = 1.43 Å; C2C3 = 1.35 Å; C3C4 = 1.47 Å.

structure without a negative vibrational frequency, further indicating that the structure represents a truly stable intermediate on the reaction pathway. The DFT frequencies of Meisenheimer compound I are listed in Table 2. The raw frequencies were converted by a single scaling factor of 0.975, mainly to correct for the anharmonicity effect and the use of finite basis set, according to a previous evaluation work (36).

Detection of EMc by Raman Spectroscopy. Detection of the EMc by Raman spectroscopy proved successful. Previous studies of the wild-type and mutant dehalogenase complexes with substrate, substrate analogues, and product have culminated in a large database for the assignments of peaks corresponding to the Meisenheimer compound. i.p., in-plane; o.o.p., out-of-plane.

| Table 2: DFT Predicted Frequencies (cm⁻¹) of Meisenheimer Compound I* |  |
|---|---|---|
| no. | frequency | Raman activity |
| 66 | 1740.0 | 4 | acetoxy ester C=O stretch |
| 65 | 1644.3 | 179 | thioester C=O stretch + |
| 64 | 1595.0 | 118 | 8α-like C=C symmetric stretch + |
| 63 | 1524.9 | 56 | two C=C antisymmetric stretch |
| 59 | 1459.4 | 23 | 8b-like ring quadrant stretch |
| 57 | 1447.7 | 18 | 19α-like ring stretch |
| 53 | 1304.2 | 34 | 19β-like ring stretch |
| 51 | 1277.5 | 17 | 3-like ring stretch |
| 49 | 1258.3 | 14 | 7α-like ring bend + C1–C |
| 48 | 1245.6 | 5 | + acetoxy ester O–C=O bend |
| | | | + 7α-like ring i.p. bend |
| 47 | 1176.4 | 14 | 9α-like ring C–H i.p. bend |
| 46 | 1101.2 | 1 | 15-like ring C–H i.p. bend |
| 41 | 983.5 | 3 | 18α-like ring i.p. bend |
| 40 | 960.9 | 10 | ethyl C–C stretch |
| 39 | 946.9 | 3 | ring C–H o.o.p. bend |
| 38 | 939.3 | 9 | C=O–C bend + 18α |
| 37 | 920.5 | 2 | ring C–H o.o.p. bend |
| 36 | 909.0 | 27 | 13-like ring i.p. bend |
| 35 | 847.4 | 7 | 1-like ring bend + C–S stretch |
| 34 | 797.4 | 1 | ring o.o.p. bend |
| 33 | 734.7 | 4 | ring C–H o.o.p. bend |
| 31 | 724.7 | 13 | ring i.p. bend |
| 30 | 718.3 | 15 | ring C–H o.o.p. bend |
| 29 | 662.2 | 27 | S–C stretch |
| 28 | 653.4 | 15 | acetoxy ester O–C–C bend + ring i.p. bend + C–F stretch |
| 27 | 628.8 | 5 | C–H o.o.p. bend |
| 26 | 610.5 | 9 | 6b-like i.p. bend |
| 25 | 590.9 | 1 | acetoxy ester C=O o.o.p. bend |
| 24 | 573.1 | 4 | 18b-like ring i.p. bend |
| 23 | 537.9 | 2 | ring o.o.p. bend |
| 22 | 504.4 | 3 | ring o.o.p. bend |

* A total of 78 normal modes exist for Meisenheimer compound I. Normal modes associated only with the S-ethyl group and the methyl group from the acetoxy ester are mainly composed of C–H deformation and/or C–C stretching and therefore not listed here. a Wilson notations for benzoid ring modes can roughly be adopted here to describe the ring modes of the Meisenheimer compound. i.p., in-plane; o.o.p., out-of-plane.
dehalogenase in the context of the spectra obtained for these ligands bound to D145A dehalogenase—in which EMc formation cannot occur.

Raman difference spectra of 4-FBA-CoA in solution, bound to wild-type and H90Q dehalogenase, and bound to D145A dehalogenase, are shown in Figure 9. The key Raman modes found in the spectrum of the solvated 4-FBA-CoA are the C=O stretch at 1651 cm\(^{-1}\) and the phenyl ring modes at 1603, 1584, and 1213 cm\(^{-1}\). The feature near 1232 cm\(^{-1}\) is a 7a type ring mode containing a contribution from a C—F stretching motion (Table S1 in Supporting Information lists detailed assignments for vibrational modes of 4-FBA-CoA). Upon binding to wild-type or H90Q enzyme, the C=O is strongly polarized, resulting in a shift from 1651 cm\(^{-1}\) to a shoulder near 1605 cm\(^{-1}\). The ring modes at ca. 1600 cm\(^{-1}\) are only slightly perturbed, showing small changes in frequencies and relative intensities (although this region does contain some contribution from the Mc; see below). Similar effects are seen in the Raman difference spectrum of 4-methylbenzoyl-CoA bound to the dehalogenase active site (8, 12). However, the spectrum of WT dehalogenase-bound 4-FBA-CoA reveals novel features not seen for bound 4-methylbenzoyl-CoA. Specifically, the 4-FBA-CoA complex gives rise to unique features in the form of fairly broad bands near 1222 and between 1480 and 1540 cm\(^{-1}\). Similar broad features are seen for 4-FBA-CoA binding to the H90Q form of dehalogenase. Crucially, however, these “novel” bands are absent for 4-FBA-CoA bound to the D145A mutant enzyme, which cannot form an EMc. We assign the “novel” broad bands from the 4-FBA-CoA complexes with WT and H90Q enzymes to modes from the EMc. The “novel” bands persist over time, consistent with the slow conversion rate of the EMc revealed by the kinetic study. Strong supporting evidence comes from the quantum mechanical calculations and from parallel studies with 4-NBA-CoA–dehalogenase complexes.

Table 2 lists the predicted Raman frequencies for the Mc based on the DFT calculations. The calculations were undertaken for the negatively charged Mc analogue compound I in vacuo, and thus an exact correspondence between the calculated values and those for the Mc in the active site is not expected. However, the calculations do provide good insight into the expected distribution of normal modes. In Table 2, modes 63 and 59 are medium intensity Raman features, associated with ring stretches, and these can account for the broad intensity between 1480 and 1540 cm\(^{-1}\) seen in Figure 9C,D. The increase in intensity and apparent band broadening near 1300 cm\(^{-1}\) in Figure 9C,D upon forming the enzyme complex is explained by the presence of the 19b-like ring stretch for the Mc, mode 53 in Table 2. Some or all of the Mc modes 51, 49, 48 explain the appearance of the broad feature around 1222 cm\(^{-1}\) in Figure 9. Thus, the calculations on the Mc provide a one-to-one correspondence between the most active Raman modes predicted for the Mc between 1100 and 1550 cm\(^{-1}\) and the observed intensity increases for the complex in Figure 9. In addition, the calculations predict that the 1590–1650 cm\(^{-1}\) region will contain significant contributions from Mc ring modes which will overlap with those from the E–S complex. This is borne out by the “double difference” spectrum seen in Figure 9E. These data were obtained by subtracting Figure 9B from Figure 9C, and the most intense Mc modes are indeed in the 1600 cm\(^{-1}\) region. As a result of the multiple data subtractions leading to Figure 9E, minor peaks have to be treated with caution, and the appearance of modes due to protein also is a possibility if conformational change accompanies Mc formation. However, Figure 9E suggests that the Mc has quite a rich Raman spectrum and some of the broad peaks we have commented on are due to multiple features.

Raman difference spectra of 4-NBA-CoA in solution, bound to WT and H90Q dehalogenase, and bound to D145A dehalogenase are compared in Figure 10. In the figure the NO\(_2\) group contributes intensity at 1359 cm\(^{-1}\) (NO\(_2\) symmetric stretch) and 1113 cm\(^{-1}\) (N—C stretch) (see detailed assignments for 4-NBA-CoA in Table S2 of Supporting Information). As in the case of the complexes involving 4-FBA-CoA (Figure 9), the key observations are the presence of broad features near 1530 and 1220 cm\(^{-1}\) for 4-NBA-CoA binding to wild-type and H90Q enzymes (Figure 10C,D) and their absence in the spectrum from the complex with D145A enzyme (Figure 10B). Again, the broad features are assigned to modes from the EMc.

The 4-nitro group is a better leaving group than 4-F, and the 4-NBA-CoA dehalogenase (WT) turns over to the product complex 4-HBA-CoA—dehalogenase at a rate of 7 \(\times 10^{-4}\) s\(^{-1}\). Figure 11 follows this reaction via the Raman difference spectrum, from 1 to 79 min after mixing. At 56 min, the Raman peaks of the substrate at 1586 cm\(^{-1}\) (phenyl ring mode) and 1358 cm\(^{-1}\) (—NO\(_2\), symmetric stretch) have almost disappeared to be replaced by the characteristic modes of the bound product at 1561 and 1523 cm\(^{-1}\) as reported previously for the latter complex (7, 8, 12). By fitting the change in intensity of the 1587 cm\(^{-1}\) band with time, we obtained a single-exponential decay with a rate of 5.4 \(\times 10^{-4}\)
due to intermediate, and its intensity diminution tracking that of bound substrate strongly supports the concept that we are seeing an equilibrium population of E-S ⇄ EMc.

We do not know the absolute Raman intensities (scattering cross sections) associated with the EMc. However, the appearance of novel weak-to-medium intensity bands near 1220 and 1530 cm⁻¹ is consistent with a minor population of EMc. In the E-S and EMc band overlapped region (1600–1570 cm⁻¹), the increase in the relative intensity of the 1580 cm⁻¹ band for the 4-FBA-CoA complex relative to the CoA adenine band near 1330 cm⁻¹ (Figure 9) is about 30% in the WT complex compared to the D145A complex. Again, this is consistent with an EMc population in the ~20–30% range. A similar increase by 30% in the intensity for the band near 1590 cm⁻¹ in the WT vs D145A enzyme occurs for 4-NBA-CoA binding (Figure 10), when using the corresponding CoA band at 1330 cm⁻¹ as a reference.

Summary. Detailed kinetic studies on 4-FBA-CoA or 4-NBA-CoA reacting with dehalogenase are consistent with an equilibrium population of Michaelis and Meisenheimer complexes remaining in reaction mixtures for tens of minutes or longer. For these reaction mixtures, two regions in their Raman difference spectra show “novel” features that are assigned to a population of EMc. The assignment is supported by high level quantum mechanical calculations. This is one of the first reports of the use of Raman spectroscopy to detect transient intermediates in a complex reaction scheme and illustrates the power of a tripartite kinetic, theoretical, and spectroscopic approach.

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SUPPORTING INFORMATION AVAILABLE

Table S1, DFT predicted frequencies (cm⁻¹) of 4-FBA-CoA model compound S-ethyl 4-fluorobenzoate thioester between 2000 and 400 cm⁻¹; Table S2, DFT predicted frequencies (cm⁻¹) of 4-NBA-CoA model compound S-ethyl 4-nitrobenzoate thioester between 2000 and 400 cm⁻¹; and Table S3, complete list of DFT predicted frequencies (cm⁻¹) of Meisenheimer compound I between 2000 and 400 cm⁻¹. This information is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

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