JOURNAL OF RAMAN SPECTROSCOPY
J. Raman Spectrosc. 2005; **36**: 320–325
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jrs.1325

JRS

Raman evidence for product binding to the enzyme W137F 4-chlorobenzoyl-CoA dehalogenase in two conformational states

Jian Dong,[1] Lusong Luo,[2] Debra Dunaway-Mariano[2] and Paul R. Carey[1]*

[1] Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, USA
[2] Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, USA

Received 17 November 2004; Accepted 27 January 2005

The enzyme 4-chlorobenzoyl-CoA dehalogenase catalyzes the hydrolytic dehalogenation of 4-chlorobenzoyl-CoA (4-CBA-CoA) to yield 4-hydroxybenzoyl-CoA (4-HBA-CoA). Part of the catalytic mechanism involves interaction between an active site side-chain, from Asp145, with the 4-position of the benzoyl moiety. For the complex consisting of product 4-HBA-CoA in the active site the interaction between Asp145 and the 4-OH group helps produce a major rearrangement in the benzoyl's π-electron system with the system becoming highly polarized. This leads to a large red shift in the benzoyl λ_{max}, from 260 to 370 nm, and radical changes in the Raman spectrum. In addition to the Asp145-4-OH interaction, the aspartate side-chain is held in place by a hydrogen bond to the indole side-chain of Trp137. We explored the consequences of removing this hydrogen bonding interaction by using protein engineering techniques to replace Trp137 by Phe, Ala or His. For 4-HBA-CoA binding to Trp137Phe dehalogenase there is spectroscopic evidence for two conformational populations for bound product. One species (highly polarized) has a λ_{max} near 375 nm and intense Raman bands at 1550, 1520 and 658 cm^{-1}, whereas the second species (moderately polarized) has a λ_{max} near 330 nm and an intense Raman mode at 1575 cm^{-1}. Raman data collected between 6 and 34 °C demonstrate population changes with the highly polarized species having the highest thermodynamic stability.

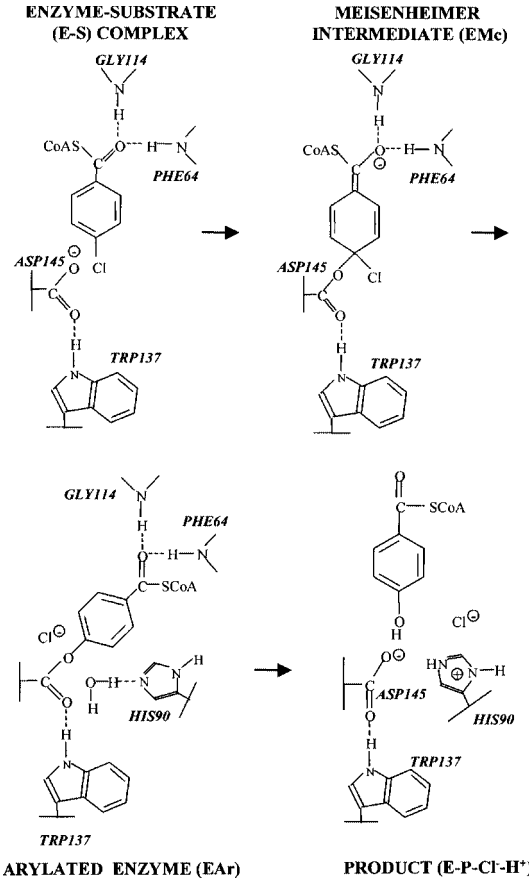
KEYWORDS: Raman difference spectroscopy; dehalogenase enzyme; product complex; conformers; hysteresis

INTRODUCTION

4-Chlorobenzoyl-coenzymeA dehalogenase catalyzes the hydrolytic dehalogenation of 4-chlorobenzoyl-coenzyme A (4-CBA-CoA) to 4-hydroxybenzoyl-CoA (4-HBA-CoA). This enzyme has been discovered in a number of soil-dwelling bacterial strains where it functions along with 4-CBA-CoA ligase and 4-HBA-CoA thioesterase to convert *p*-chlorobenzoic acid to *p*-hydroxybenzoic acid.[1] Studies of the *Pseudomonous* sp. strain CBS3 4-CBA-CoA dehalogenase have shown that it utilizes a unique form of catalysis in which an active site carboxylate bonds to the C-4 of the benzoyl ring of the bound substrate to form a Meisenheimer complex,[2,3] which then proceeds to product through chloride ion expulsion, the formation of an arylated intermediate and hydrolysis as shown in Scheme 1.[4]

*Correspondence to: Paul R. Carey, Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, USA.
E-mail: paul.carey@case.edu
Contract/grant sponsor: NIH; Contract/grant numbers: GM-28688; GM-54072.

The nature of the active site forces which increase the electrophilic character of the C-4 position is of interest since the formation of a Meisenheimer-like complex in the reaction is not chemically facile. An indication that strong electrostatic forces are present in the active site came from absorption, Raman and NMR spectroscopic studies of enzyme complexes involving the product and, separately, a substrate analog.[5] The spectroscopic data showed that electron polarizing forces can be brought to bear in the active site, which, in the case of the bound product, cause a major rearrangement of the electrons in the benzoyl moiety. Interest in these findings has been heightened by the publication of the structure of the dehalogenase–4-HBA-CoA complex derived by x-ray crystallography.[6] An analysis of the polarizing forces in the active site has been published, combining Raman spectroscopic data on the bound product, including results on ^{13}C- and ^{18}O-substituted isotopomers of the product, with structural conclusions culled from the x-ray crystallographic studies.[7] The spectral characteristics of strong polarization seen for the bound product are a large shift in λ_{max}, from



Scheme 1. The position of CoA bound to the enzyme is invariant throughout the reaction; it is shown in different orientations here to facilitate the layout of the scheme.

292 to 370 nm, and a dramatic rearrangement of the Raman normal modes of the benzoyl ring.[5,7] The conclusions from the combined Raman and X-ray data were as follows:

1. Strong electron pull is exerted on the benzoyl carbonyl group by hydrogen bonds from the peptide NHs of Gly114 and Phe64 (these interactions are shown for the substrate in Scheme 1; they are also present, although not shown, for the product complex, bottom right). This carbonyl polarization effect is accentuated by the electrostatic field due to the α-helix which has its N-terminus at Gly114. The total polarizing effects of the hydrogen bonds and the α-helix are equivalent to an effective hydrogen bonding strength of 57 kJ mol^{-1}.
2. For product binding in the active site of the wild-type (WT) enzyme a major rearrangement of the benzoyl π-electrons occurs. The entire π-system becomes highly polarized, with an incremental positive charge near the 4-position and an incremental negative charge on the benzoyl carbonyl oxygen. This complex interaction between the 4-OH and the side-chain of Asp145 provides strong 'electron push' into the benzoyl moiety.
3. Polarization effects throughout the benzoyl's π-electrons are assisted by the active site environment about the benzoyl group. The benzoyl group is encased in a sheath of aromatic amino acid side-chains. Hence, the benzoyl is akin to a π-electron wire surrounded by a low dielectric insulator.

Undoubtedly, the above polarizing effects are 'designed into' the active site in order to facilitate the difficult chemistry of the aromatic substitution of the 4-Cl group by the 4-OH group.

As already noted, binding of the product to dehalogenase's active site results in large perturbations to absorption and Raman spectral properties. The x-ray structure of the complex revealed that the side-chain of Asp145 is close to the 4-OH group (2.4 Å).[6] It is likely that the formally negative Asp —COO$^-$ and neutral product 4-OH groups share the protium atom in an, as yet, ill-defined manner. However, it is certain that the interaction provides electron release at the 4-position. A further aspect emerging from the x-ray structure is that a hydrogen bond between the side-chains of Trp137 and Asp145 (Scheme 1) serves to help position Asp145 for its interaction with the product's 4-OH group. In the present work, we explored the consequences of removing the Trp–Asp hydrogen bond by replacing Trp137 with another amino acid residue. Remarkably, we find that for the variant Trp137Phe the product 4-HBA-CoA binds in two conformational states in the active site. In these two forms the 4-hydroxybenzoyl moiety is experiencing modest and very strong electron polarizing environments. Moreover, the two forms may be interconverted by varying the temperature.

EXPERIMENTAL

Materials

4-CBA-CoA, 4-MBA-CoA and 4-HBA-CoA were prepared according to Liang *et al*.[8] 4-CBA-CoA dehalogenase was prepared according to the procedure of Chang *et al*.[9] as modified by Liang *et al*.[8] Dehalogenase concentrations were determined by using the Bradford method[10] and a subunit molecular mass of 30 kDa (and are reported as active site concentrations). The W137F and W137A mutants were generated using the clone of the WT gene[9] in conjunction with polymerase chain reaction (PCR) techniques. Both mutants behaved similarly to the wild-type dehalogenase during purification steps and were obtained in a homogeneous state as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The purification of the W137F, W137H and W137A mutant dehalogenase was carried out in essentially the same manner as described by Yang *et al*.[4]

Raman spectroscopy

The non-resonant Raman spectra were obtained using 647.1 nm laser excitation from an Innova 400 krypton

JRS

laser system (Coherent), a back-illuminated charge-coupled device (CCD) detector (Model 1024EHRB/1, Princeton Instruments) operating at 183 K, and a Holospec $f/1.4$ axial transmission spectrometer (Kaiser Optical Systems) employed as a single monochromator, as described in a previous report.[11] Enzyme samples contained in cuvettes were 50 µl in volume and buffered with 50 mM Tris–HCl at pH 7.5. Enzyme and 4-HBA-CoA or 4-CBA-CoA concentrations used are given in the figure captions. 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), and the spectrum of the enzyme in buffer was subtracted from that of the enzyme-ligand complex to give the spectrum of the bound ligand.

UV–visible absorption difference spectral analysis of the formation of ligand–enzyme complexes

UV–visible absorption difference spectra were measured with a Beckman DU 7400 diode-array spectrometer. In a 1 ml quartz tandem cell, one compartment contains 0.5 ml of enzyme solution and the other 0.5 ml of ligand solution (all solutions were buffered with 50 mM K^+ HEPES, pH 7.5, at 25 °C). After mixing these two solutions, the absorbance spectrum was recorded and subtracted from the spectrum of the unmixed solutions to give the difference spectrum of the enzyme–ligand complex. The dissociation constants (K_d) of enzyme–4-MBA-CoA and enzyme–4-HBA-CoA complexes were measured by spectral titration as described previously.[4,12]

RESULTS AND DISCUSSION

Binding of the substrate analog 4-methylbenzoyl-CoA to dehalogenase

This analog binds to the active site but can experience only two out of the three 'polarizing effects' listed in the Introduction since the 4-methyl group cannot undergo a strong interaction with Asp145. As a consequence, λ_{max} for the benzoyl chromophore shows only a modest red shift upon binding to the WT, from 260 to 304 nm (ε = 6.5 mM^{-1} cm^{-1}).[12] Similar shifts are seen upon binding 4-MBA-CoA to W137F (ε = 6.5 mM^{-1} cm^{-1}), W137A (ε = 14.1 mM^{-1} cm^{-1}) and W137H (ε = 7.1 mM^{-1} cm^{-1}). The Raman difference spectra for 4-MBA-CoA binding to WT and various Trp137 mutants are shown in Fig. 1. The spectra are very similar, with the benzoyl C=O stretch showing a large shift to lower wavenumbers, compared with that for the free ligand in aqueous solution. Hence the C=O stretch in aqueous solution is near 1650 cm^{-1} [Fig. 1(A)] and appears as a shoulder near 1612 cm^{-1} for the ligand bound to various forms of the enzyme [Fig. 1(B)–(E)]. However, this strong polarization is localized at the C=O group since the benzene ring modes 8a and 8b move down by only about

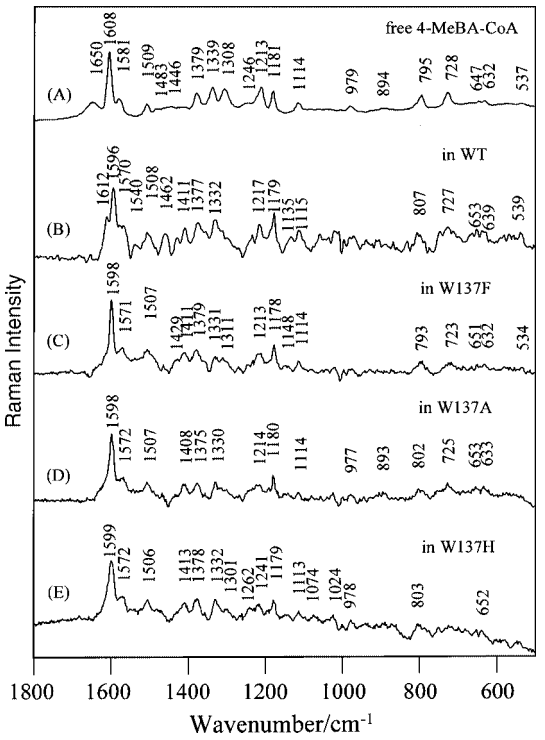
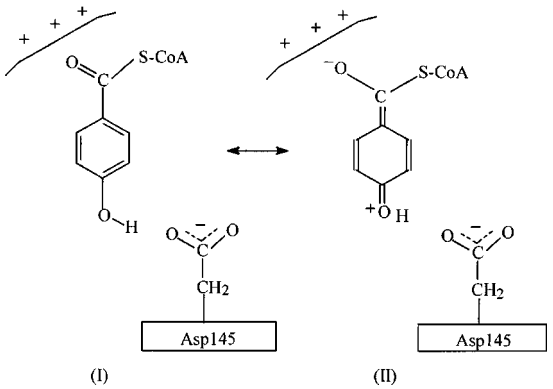


Figure 1. Raman spectra of 4-MBA-CoA in various states: (A) free 4-MBA-CoA (4.2 mM), in Tris–HCl buffer (50 mM); (B) 4-MBA-CoA bound to WT (350 µM); (C) bound to W137F (193 µM); (D) bound to W137A (251 µM); (E) bound to W137H (272 µM); all at pH 7.5, 50 mM Tris–HCl buffer (25 °C). Enzyme : ligand ratios for all complexes are 1 : 1.

10 cm^{-1} upon binding. Again, this is due to the lack of strong electron 'push' near the 4-position reinforcing the 'pull' on the C=O oxygen. No detectable differences are seen among the W137F, W137A and W137H variants.

Binding product, 4-HBA-CoA, to dehalogenase and its W137 variants

On binding to WT dehalogenase, the absorption maximum of 4-HBA-CoA shifts from 292 to 373 nm (ε = 17.0 mM^{-1} cm^{-1}).[12] A shoulder is observed at 325 nm, suggesting a minor population of a second conformer. For 4-HBA-CoA bound to either dehalogenase W137A (λ_{max} 325 nm, ε = 21.9 mM^{-1} cm^{-1}; λ_{max} 375 nm, ε = 3.4 mM^{-1} cm^{-1}) or W137H (λ_{max} 325 nm, ε = 17.6 mM^{-1} cm^{-1}; λ_{max} 375 nm, ε = 4.2 mM^{-1} cm^{-1}), the major absorption peak is centered near 325 nm and the 375 nm peak is comparatively small.[13] This suggests slightly more polarization compared with 4-MBA-CoA binding to these variants (λ_{max} 302 nm). The additional polarization is due to the superior electron-releasing property of the 4-OH over the 4-CH_3 group, which have Hammett σ_p parameters of −0.38 and −0.14, respectively.[14] The additional electron release leads to an increased contribution from valence bond structure II to the true structure (Scheme 2).



Scheme 2

The absorption spectrum of 4-HBA-CoA bound to dehalogenase W137F is shown in Ref. 12. In contrast to the other mutants listed above, it clearly has two maxima in the near-UV region near 375 and 330 nm. Taking into account the above discussion and the published absorption data for ligand–dehalogenase binding,[12] these findings strongly suggest the presence of two major populations for 4-HBA-CoA, one with a strongly polarized benzoyl group giving rise to the 375 nm band and the other with an 'intermediate' polarized benzoyl group giving rise to the 330 nm band. The findings and conclusions from the absorption data are confirmed and extended by the Raman analysis.

Raman difference spectroscopic data for 4-HBA-CoA binding to the WT, W137F, W137A and W137H forms of dehalogenase are compared in Fig. 2. The WT complex has been discussed extensively in earlier publications[5,7] and its structure has been solved by x-ray crystallography.[6] Some details are given in the Introduction but essentially the benzoyl moiety is present predominantly as a highly polarized conformer in the active site, with major contributions for valence bond form II (Scheme 2) to the true structure. For the W137A and W137H complexes the C=O stretch feature

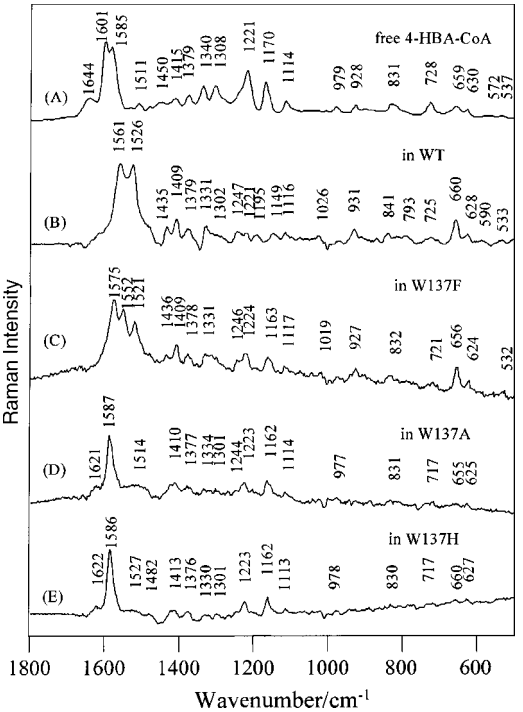


Figure 2. Raman difference spectra of (A) free 4-HBA-CoA (6.1 mM) in Tris–HCl buffer (50 mM); (B) 4-HBA-CoA bound to WT (444 μM); (C) bound to W137F (193 μM); (D) bound to W137A (251 μM); (E) bound to W137H (272 μM); all at pH 7.5, 50 mM Tris–HCl buffer (25 °C). Enzyme : ligand ratios for all complexes are 1 : 1.

appears near 1620 cm^{-1}, resembling the corresponding 4-MBA-CoA complexes. Now, however, the 'center of gravity' of the 8a and 8b modes is near 1586 cm^{-1}, suggesting some additional polarization in the benzoyl group compared with the 4-MBA-CoA complexes. Hence we see a correspondence between the absorption and Raman data in the double bond stretching region (see Table 1). In Table 1, the degree of

Table 1. Characteristic Raman modes and UV absorption bands of 4-HBA-CoA and 4-MBA-CoA at pH 7.5 in aqueous solution ('free ligand') and in enzyme complexes

Species	Raman modes: $\tilde{\nu}/cm^{-1}$	UV absorption: λ_{max}/nm	UV band shift: $\Delta\lambda_{max}/nm$	extinction coefficient: $\varepsilon/mM^{-1}\ cm^{-1}$
Free ligand 4-HBA-CoA	1601, 1585	292 (unpolarized)	—	13.0
W137A–or W137H-4-HBA-CoA complex	1586	325 (intermediate polarized)	33	21.9
W137F-4-HBA-CoA complex	1575, 1552, 1521	330 (intermediate polarized), 370 (strongly polarized)	38, 78	10.5, 10.2
WT–4-HBA-CoA complex	1561, 1526	373 (strongly polarized)	81	17.0
Free ligand 4-MBA-CoA	1608	260 (unpolarized)	—	4.0
W137A–, W137H–or W137F-4-MBA-CoA complex	1598	302 (slightly polarized)	42	6.5
WT-4-MBA-CoA complex	1596	302 (slightly polarized)	42	6.5

polarization refers to the benzoyl group. The features in the Raman spectra at 1580–1610 cm^{-1} are best described as benzene-like 8a and 8b modes. However, for the WT–4-HBA-CoA complex it is likely that the peaks at 1561 and 1526 cm^{-1} are due to highly delocalized double bond modes.[7]

The most striking result in Fig. 2 is the Raman spectrum of 4-HBA-CoA bound to W137F. Consonant with the absorption data discussed above, the Raman spectrum shows clear evidence for two species. The two peaks at 1552 and 1521 cm^{-1}, together with the feature at 656 cm^{-1}, strongly resemble the signature of the highly polarized species seen in Fig. 2(B) for the WT complex. However, the band at 1575 cm^{-1} suggests that a second 'less polarized' conformation is present and that this corresponds to the species giving rise to the 330 nm band in the electronic absorption spectrum,[12] whereas the 1552/1521/656 cm^{-1} peaks are associated with the species giving rise to the 375 nm band.[12] Confirmation comes from Raman data acquired at different temperatures.

At every temperature point from 6.7 to 33.9 °C, a new W137F–4-HBA-CoA complex (1 : 1 ratio) was made, and the Raman spectrum was recorded in a 5 min exposure time immediately after mixing. The Raman spectra of the W137F enzyme alone were also recorded at each temperature point, and taken as subtrahends to generate the Raman difference spectra of 4-HBA-CoA bound to W137F. Figure 3(A)–(C) show Raman difference spectra of 4-HBA-CoA at three temperature points. The population of the 'less polarized' form, providing the 1576 cm^{-1} band, increases with increasing temperature, suggesting that this form has less thermodynamic stability than the highly polarized conformer.

Using the x-ray structure of the WT–4HBA-CoA complex as a starting point, a model describing the two conformational states can be put forward. Figure 4 shows the HBA group in the active site of WT dehalogenase. Note how the H-bond between the side-chains of Trp137 and Asp145 positions the aspartate side-chain with respect to the hydroxyl group at the 4-position of the benzoyl moiety. It is the latter interaction that gives rise to electron 'push' at the *para* position and optimal electron polarization. For the W137F form of dehalogenase, a substantial proportion of the bound benzoyl groups retain this interaction, even in the absence of the tryptophan side-chain. However, a second population exists where the Asp145 side-chain no longer interacts with the 4-hydroxy group. Now electron push is missing at the *para* position and the second population acquires intermediate polarization owing to electron 'pull' at the C═O oxygen and the inherent electron releasing ability of the 4-OH group. For the forms W137A and W137H, the highly polarized conformer is absent (Fig. 2), demonstrating the absence of an interaction between Asp145 and the 4-OH group.

For W137F, the two populations show evidence for thermal irreversibility or hysteresis. When the sample is formed at 33.9 °C and then cooled to 6.7 °C (Fig. 3), the

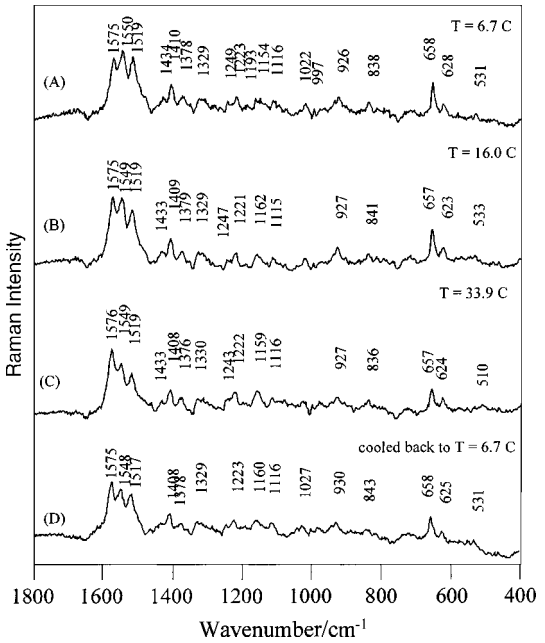


Figure 3. Temperature-variable Raman difference spectra of 4-HBA-CoA in W137F enzyme (169 μM), 1 : 1 complex at (A) 6.7 °C; (B) 16.0 °C; (C) 33.9 °C; (D) cooled back to 6.7 °C in 50 mM Tris–HCl buffer (pH 7.5). The spectra of the complexes were referenced to those of the pure enzyme at each corresponding temperature. The data were collected for the first 5 min after the complex was made.

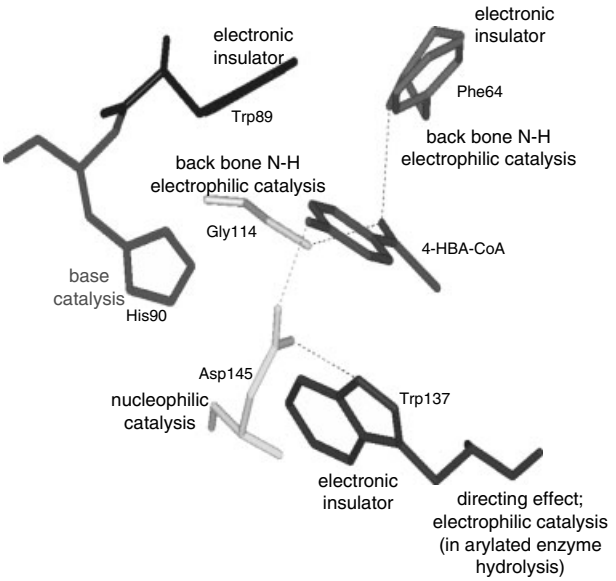


Figure 4. Detail from x-ray structure of 4-HBA-CoA bound to 4-CBA-CoA dehalogenase, showing interactions at the benzoyl moiety of 4-HBA-CoA.

population ratio is not the same as that for the sample formed at 6.7 °C. Such behavior is an indication of a very interesting property of the conformational space involving

elements of kinetic and thermodynamic control. This will be the subject of a future publication from our laboratories.

Acknowledgment

The authors gratefully acknowledge support from the NIH: GM-28688 (to D.D.-M.) and GM-54072 (to P.R.C.).

REFERENCES

1. Dunaway-Mariano D, Babbitt PC. *Biodegradation* 1994; **5**: 259.
2. Dong J, Carey PR, Wei Y, Luo L, Lu X, Liu R-Q, Dunaway-Mariano D. *Biochemistry* 2002; **41**: 7453.
3. Dong J, Lu X, Wei Y, Luo L, Dunaway-Mariano D, Carey PR. *Biochemistry* 2003; **42**: 9482.
4. Yang G, Liu R-Q, Taylor KL, Xiang H, Price J, Dunaway-Mariano D. *Biochemistry* 1996; **35**: 10 879.
5. Taylor KL, Liu R-Q, Liang P-H, Price J, Dunaway-Mariano D, Tonge PJ, Clarkson J, Carey PR. *Biochemistry* 1995; **34**: 13 881.
6. Benning MM, Taylor KT, Liu R-Q, Yang G, Xiang H, Wesenberg G, Dunaway-Mariano D, Holden HM. *Biochemistry* 1996; **35**: 8103.
7. Clarkson J, Tonge PJ, Taylor KL, Dunaway-Mariano D, Carey PR. *Biochemistry* 1997; **36**: 10 192.
8. Liang P-H, Yang G, Dunaway-Mariano D. *Biochemistry* 1993; **32**: 12 245.
9. Chang K-H, Liang P-H, Beck W, Scholten JD, Dunaway-Mariano D. *Biochemistry* 1992; **31**: 5605.
10. Bradford M. *Anal. Biochem.* 1976; **72**: 248.
11. Dong J, Dinankarpandian D, Carey PR. *Appl.Spectrosc.* 1998; **52**: 1117.
12. Taylor KL, Xiang H, Liu R-Q, Yang G, Dunaway-Mariano D. *Biochemistry* 1997; **36**: 1349.
13. Luo L. Ph.D. Thesis, University of New Mexico, 2000.
14. Smith MB, March J. *March's Advanced Organic Chemistry* (5th edn). Wiley: New York, 2001; 370.