Product Catalyzes the Deamidation of D145N Dehalogenase To Produce the Wild-Type Enzyme†

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ABSTRACT: Aspartate 145 plays an essential role in the active site of 4-chlorobenzoyl-CoA dehalogenase, forming a transient covalent link at the 4-position of the benzoate during the conversion of the substrate to 4-hydroxybenzoyl-CoA. Replacement of Asp 145 by residues such as alanine or serine results in total inactivation, and stable complexes can be formed with either substrate or product. The Raman spectroscopic characterization of some of the latter is described in the preceding publication (Dong et al.). The present work investigates complexes formed by D145N dehalogenase and substrate or product. Time-resolved absorption and Raman difference spectroscopic data show that these systems evolve rapidly with time. For the substrate complex, initially the absorption and Raman spectra show the signatures of the substrate bound in the active site of the asparagine 145 form of the enzyme but these signatures are accompanied by those for the ionized product. After several minutes these signatures disappear to be replaced with those closely resembling the un-ionized product in the active site of wild-type dehalogenase. Similarly, for the product complex, the absorption and Raman spectra initially show evidence for ionized product in the active site of D145N, but these are rapidly replaced by signatures closely resembling the un-ionized product bound to wild-type enzyme. It is proposed that product bound to the active site of asparagine 145 dehalogenase catalyzes the deamidation of the asparagine side chain to produce the wild-type aspartate 145. For the complexes involving substrate, the asparagine 145 enzyme population contains a small amount of the WT enzyme, formed by spontaneous deamidation, that produces product. In turn, these product molecules catalyze the deamidation of Asn 145 in the major enzyme population. Thus, conversions of substrate to product and of D145N to D145D dehalogenase go on simultaneously. The spontaneous deamidation of asparagine 145 has been characterized by allowing the enzyme to stand at RT in Hepes buffer at pH 7.5. Under these conditions deamidation occurs with a rate constant of 0.0024 h^{-1}. The rate of product-catalyzed deamidation in Hepes buffer at 22 °C was measured by stopped-flow kinetics to be 0.024 s^{-1}, 36000 times faster than the spontaneous process. A feature near 1570 cm^{-1} could be observed in the early Raman spectra of both substrate and product−enzyme complexes. This band is not associated with either substrate or product and is tentatively assigned to an ester-like species formed by the attack of the product's 4-O^- group on the carbonyl of asparagine's side chain and the subsequent release of ammonia. A reaction scheme is proposed, incorporating these observations.

While there are many examples of enzymes modifying inhibitors that subsequently react to irreversibly block the active site, examples of substrates or products that chemically modify side chain residues in the active site are less common. In this paper we present spectroscopic evidence that product in the active site of 4-chlorobenzoyl-CoA dehalogenase, that has been modified to have an essential active site residue, aspartate145, replaced by asparagine, catalyzes the deamidation of the Asn 145 to aspartate. Thus, the product catalyzes the reversion of the protein-engineered D145N to the wild-type enzyme.

This paper is the second of two articles. The preceding paper explores the effects of mutations D145A, D145S, and D145E on the chemistry of the bound product of dehalogenase, 4-hydroxybenzoyl-CoA (*1*). The technique used in these studies, Raman difference spectroscopy (*2*, *3*), reveals a wealth of molecular detail regarding the interactions occurring in the active site. There, we show that the product, 4-hydroxybenzoyl-CoA, binds in the active sites of the D145A and D145S forms of the enzyme as the ionized, 4-O^-, ligand, above neutral pH (*1*). Binding substrate, 4-CBA-CoA, to the D145S and D145A variants of dehalogenase results in the formation of stable complexes (*4*), since these forms of the enzyme are totally inactive as catalysts. Although we expected to form stable complexes between substrate and D145N dehalogenase, quite rapid changes were seen in the absorption spectral properties of the complex, suggesting that reactions are occurring. These observations are fully supported by Raman data that demonstrate that one of the transient species formed involves the ionized product. Similar absorption and Raman observations were made when product, 4-hydroxybenzoyl-CoA, was added to D145N dehalo-

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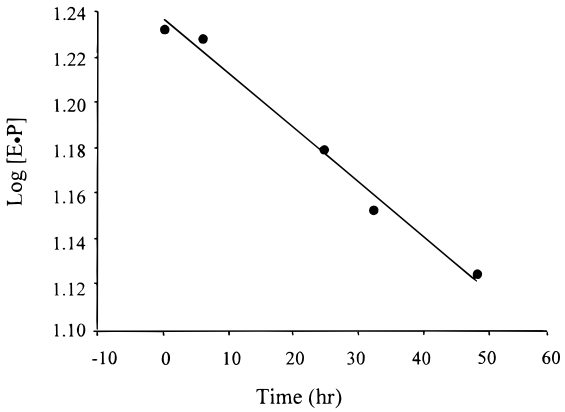


FIGURE 1: Time course of the "spontaneous" deamidation of D145N (19 μM) in 50 mM K^+Hepes, pH 7.5, at room temperature in the absence of 4-HBA-CoA.

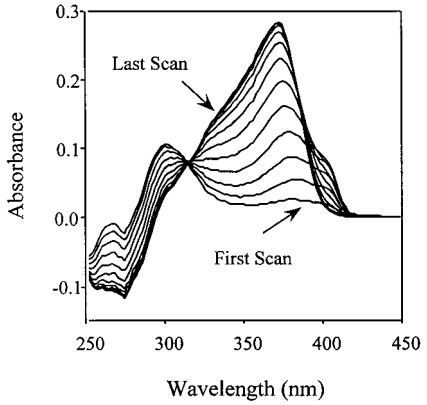


FIGURE 2: Time-resolved UV difference spectra of D145N (25 μM) with 4-CBA-CoA (25 μM) in 50 mM K^+Hepes (pH 7.5, 25 °C): 6 s per scan with 30 s delay between scans.

genase. The proposed reaction scheme encompassing the spectroscopic data emphasizes the role of the product in catalyzing the deamidation of the side chain of asparagine 145 to form the wild-type aspartate 145.

The Raman measurements on the reaction mixtures demonstrate that, with recent improvements in spectral sensitivity (*5*), it is now possible to detect transients in 30 s of spectral accumulation time, at approximately 100 μM concentrations, under normal, nonresonance, conditions.

EXPERIMENTAL SECTION

Materials and Methods. The 4-CBA-CoA dehalogenase mutant D145N was expressed and purified using the methods of Yang et al. (*6*) and Taylor et al. (*4*) as described in Xiang (*7*). 4-CBA-CoA and 4-HBA-CoA were prepared according to the method of Liang et al. (*8*).

Spontaneous deamidation of the D145N dehalogenase, in the absence of ligand, was measured by titrating aliquots of the enzyme with product, 4-HBA-CoA. D145N (38 μM) dehalogenase was incubated in 50 mM K^+Hepes buffer (1 mM DTT, pH 7.5) at room temperature. The 405 nm absorbance of D145N−4-HBA-CoA complex was used to assay the percentage of D145N present in the enzyme solution by comparing the ratio of absorbance at 405 and 378 nm. For fresh enzyme containing >99% D145N, A_{405}/A_{378} is 1.326. This ratio was measured at different time intervals, and the time course of the amount of D145N remaining (log [E·P]) was fit to a first-order rate equation to yield a deamidation rate of 0.0024 h^{-1} (Figure 1).

The time-resolved absorbance difference spectra were obtained by mixing 0.5 mL of 50 μM dehalogenase with 0.5 mL of 50 μM ligand in a tandem cell, with unmixed solutions serving as the reference in a second cell. All solutions were buffered in 50 mM K^+Hepes, pH 7.5, at 25 °C.

Instrumentation. Stopped-flow absorbance data were obtained with an Applied Physics DX.17MV sequential stopped-flow spectrophotometer. The reaction between D145N dehalogenase and 4-HBA-CoA was followed by observing the decrease in absorbance at 405 nm. Data were analyzed with a single-exponential equation. For enzyme at 4 μM the rate was found to be invariant for 4-HBA-CoA concentrations between 4 and 28 μM.

The Raman spectra were obtained using 647.1 nm laser excitation from an Innova 400 krypton laser system (Coherent, Inc.) and a back-illuminated CCD detector (Model 1024EHRB/1, Princeton Instruments, Inc.) operating at 183 K. Details of the spectrometer have been described previously (*5*). Enzyme samples contained in cuvettes were 60 μL in volume and buffered with 50 mM Tris-HCl at pH 7.5. 4-CBA-CoA or 4-HBA-CoA was added in a concentration equal to or less than that of the active sites of enzyme. The concentration of the "free" enzyme was made identical to the enzyme concentration in the complex to circumvent the introduction of a subtraction factor. The data were collected immediately after the complex had been incubated for 1 min, under a laser power of ~850 mW with CCD exposure times specified in the figure captions. 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 in buffer (giving the spectrum of the enzyme-bound ligand). Sharply well-defined peaks have accurate positions reproducible within ±1 cm^{-1}; therefore changes in Raman band positions greater than 2 cm^{-1} are believed to be significant.

RESULTS AND DISCUSSION

Binding Substrate (4-CBA-CoA) to D145N Dehalogenase. Aspartate 145 has been shown to be an essential residue in the reaction mechanism for dehalogenase, and replacing it by any residue other than glutamic acid leads to total enzyme inactivation. The D145A and D145S variants of dehalogenase form stable complexes with both product and substrate (*1*, *4*). Thus, it is a reasonable prediction that the substrate, 4-CBA-CoA, will bind to the asparagine 145 enzyme variant to form a stable 1:1 complex. However, both the absorption (Figure 2) and Raman data (Figure 3) for the complex show that complicated changes occur over a period of 5−10 min. To interpret these changes, one must review the spectroscopic results for substrate and product binding to the D145A and D145S forms of the enzyme. First, the λ_{max} for a substrate binding in the active site occurs near 302 nm (*4*). The λ_{max} for product, 4-HBA-CoA, binding to WT dehalogenase is 370 nm (*1*). While also in ref *1*, we demonstrate that product binding to D145A or D145S shows evidence for a population that is ionized (i.e., with 4-O^-) near and above neutral pH and that the ionized product in the active site has a λ_{max} near 400 nm.

The preceding absorption data suggest an interpretation of the main features in Figure 2. Namely, at early times,

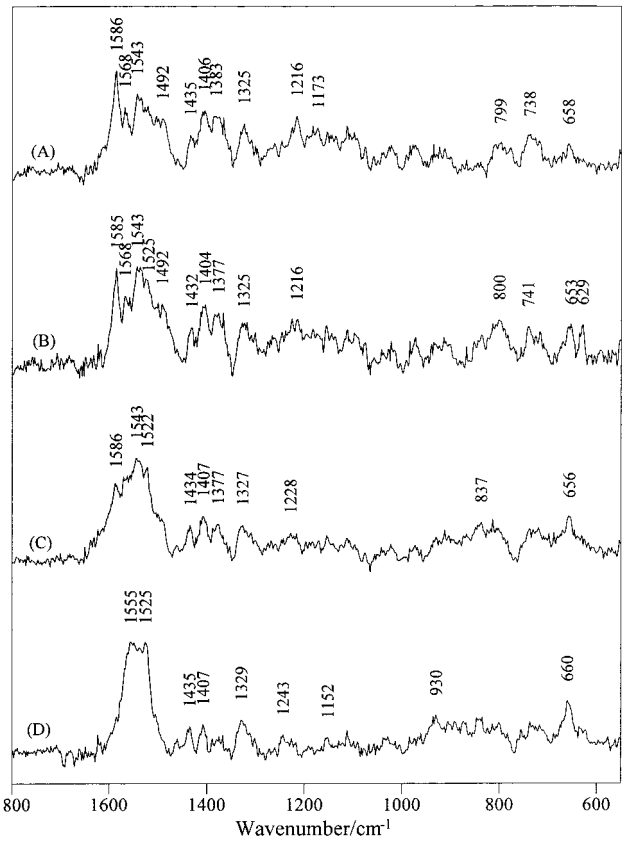


FIGURE 3: Raman difference spectra of 4-CBA-CoA (197 μM) bound to D145N (197 uM) in 50 mM Tris buffer, pH 7.5. Each spectrum was collected for 30 s at a reaction time *t*. Displayed here are (A) $t = 1.0\sim1.5$ min; (B) $t = 1.5\sim2.0$ min; (C) $t = 2.0\sim2.5$ min; and (D) $t = 4.5\sim5.0$ min.

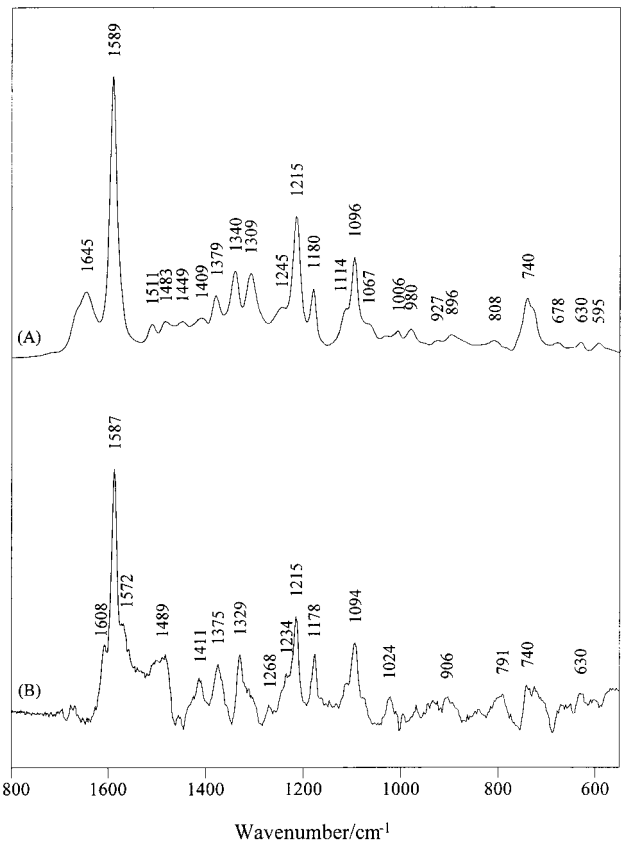


FIGURE 4: Raman spectra of the substrate 4-CBA-CoA, generated by difference spectroscopy: (A) free state (7.9 mM); (B) bound to the D145A (392 μM). Both are in 50 mM Tris buffer, pH 7.5.

approximately $t = 0-3$ min, the sequential scans indicate the presence of substrate bound to enzyme and giving rise to the observed λ_{max} near 300 nm. This feature disappears with increasing time connoting decreasing substrate. The early doublet near 390 and 405 nm suggests the presence of ionized product in the active site; this population appears to increase in quantity over about 2 min and then disappear. Finally, there is a steady increase of the absorption band near 373 nm, at the signature position for product bound to the WT enzyme. Thus, to summarize, initially the evidence is for substrate bound to the enzyme, with a small amount of ionized product that must be bound to D145N, since ionized product does not occur in the WT enzyme (*1*). The final trace suggests that all of the substrate has been hydrolyzed and that all of enzyme has been converted to WT, since the product binding to D145N would keep λ_{max} values near 400 nm due to ionized product at pH 7.5 used to record the data in Figure 2.

To interpret the time-resolved Raman data, we need to consider one spectrum that has not been published heretofore. This is the Raman spectrum for substrate bound to an inactive form of dehalogenase. Figure 4 exemplifies such data by comparing the Raman spectrum of free substrate, 4-CBA-CoA, to that for the substrate bound in the stable complex with the D145A variant. In the present context, the key finding in Figure 4 is that the intense ring modes near 1590 cm^{-1} are essentially unchanged upon binding (detailed assignments of the Raman peaks of the substrate by using a density functional method are listed as Table 1 in Supporting Information). Thus, a band in this region can be taken as a marker for substrate in the active site.

The time-resolved Raman spectra, seen in Figure 3, covering the time span from 1 to 6 min from mixing, support the conclusions drawn from the absorption spectroscopic data. The accumulation time for each Raman spectrum is 30 s; the first trace was accumulated after a 60 s period had elapsed to incubate the sample and stabilize it. For the spectrum recorded between 60 and 90 s (Figure 3A), the relatively intense feature at 1586 cm^{-1} that decreases rapidly in Figure 3B−D is evidence for the substrate being bound to the asparagine 145 form of the enzyme. This is the position at which the benzene ring modes (8a and 8b) occur for the substrate binding to the D145A form of the enzyme, as shown in Figure 4. In Figure 3A−C (reaction times $t = 1.0\sim2.5$ min), strong and broad bands at 1216, 1098, 796, and 737 cm^{-1} disappear quickly with increasing time. These bands have their counterparts in the Raman spectra of the substrate bound to D145A (Figure 4) and thus are characteristic of the bound substrate. In addition, in Figure 3A−C there is evidence for a peak near 1543 cm^{-1} and a shoulder near 1490 cm^{-1}, both of which decrease with time. These are positions where markers for the ionized form (4-O^-) of the product occur for product bound to D145S (Figure 5 in ref *1*) and D145A (Figure 6, ref *1*). This suggests that some product has been formed and is binding to the asparagine mutant in the form with the 4-hydroxy-group deprotonated (recall that the product binds to WT only in its protonated, 4-OH form).

With increasing time, $t = 3.0$ min onward, there is little evidence for populations of the substrate or the ionized

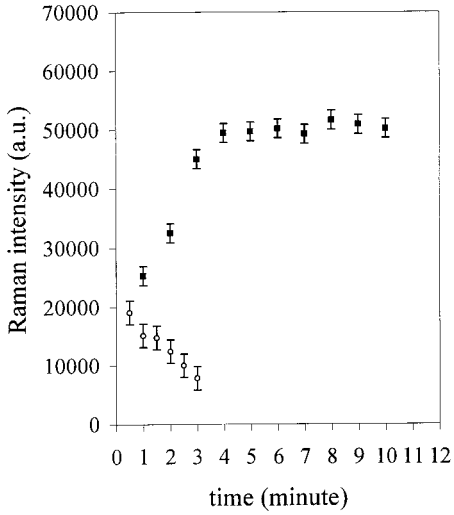


FIGURE 5: Time-dependent changes of the Raman intensities of the substrate band at 1586 cm^{-1} (hollow circle) and those of the product band at 1525 cm^{-1} (solid square) for the complex detailed in Figure 3.

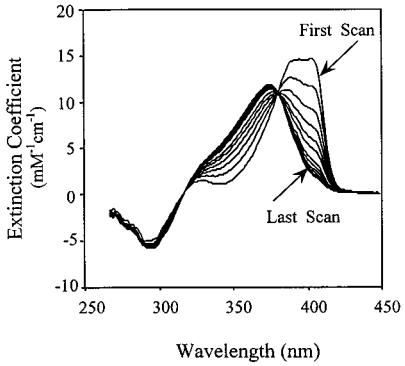


FIGURE 6: Time-resolved UV difference spectra of D145N (25 μM) with 4-HBA-CoA (25 μM) in 50 mM K^+Hepes (pH 7.5, 25 °C): 6 s per scan with 60 s delay between scans.

product, and the Raman spectra increasingly resemble the spectrum of product added to WT enzyme (see Figure 3 in ref *1*). The rise of the 1524 cm^{-1} peak that corresponds to protonated product bound to WT enzyme is shown in Figure 5; it plateaus in the 4−5 min region, in good accord with the time dependency seen in the absorption spectral data of Figure 2. The decrease of intensity near 1586 cm^{-1} is also given in Figure 5; beyond 3 min a separate feature cannot be detected. It is most likely that the decrease of the 1586 cm^{-1} intensity seen in Figure 5 corresponds to a decrease in population of both substrate and ionized product, since the latter has a medium intensity band in that region (*1*).

There is one feature in the Raman spectra in Figure 3 that cannot be assigned on the basis of comparison to previously identified species. This is the band near 1568 cm^{-1} seen in Figure 3A,B and as a shoulder for the next two minutes. The 1568 cm^{-1} is reproducible, being detected in each of three separate experiments where the substrate was added to D145N dehalogenase. We shall speculate on the origin of this band when we propose a mechanism for the observed spectral changes in the final section. After $t = 4.5$ min, the Raman spectrum bears close resemblance to the spectrum of product added to WT enzyme, except that Figure 3D exhibits a band at 1555 cm^{-1} which is ∼5 cm^{-1} lower than that of the WT−4-HBA-CoA complex (Figure 3 in ref *1*).

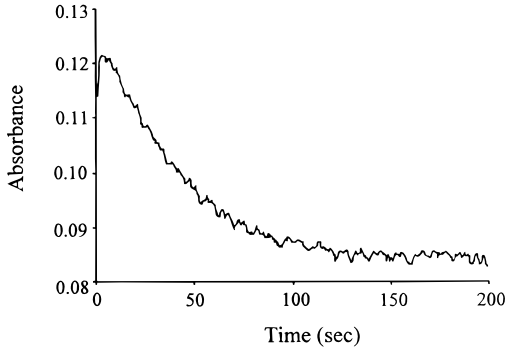


FIGURE 7: Stopped-flow trace of D145N (4 μM) mixing with 4-HBA-CoA (8 μM) in 50 mM K^+Hepes (pH 7.5, 22 °C). The trace was monitored at 405 nm.

Binding Product, 4-HBA-CoA, to D145N Dehalogenase. For the binding of product to the D145N enzyme, the time-dependent UV absorption spectra seen in Figure 6 can be interpreted along the lines set out for the substrate complex in the foregoing section. The bands seen near 405 and 400 nm near $t = 0$ are due to ionized product in the active site of D145N. These decay quickly over a period of 1∼2 min to yield a band at 370 nm, characteristic of the protonated product in the active site of WT. These two sets of absorption peaks share an apparent isobestic point near 378 nm. Furthermore, there is evidence for a less intense absorption feature near 320 nm in the early traces. To quantitate the temporal changes seen in Figure 6, we used stopped-flow techniques (with a dead time of 2 ms) to monitor the binding reaction. Figure 7 shows the stopped-flow trace for D145N mixing with 4-HBA-CoA monitored at 405 nm. The trace shows that the population of ionized product, which forms rapidly in 4∼6 s, essentially disappears in 2.0 min. The apparent rate constant of absorbance decrease at 405 nm was obtained using the single-exponential equation:

$$A_t = A_{max} + P\exp(-k_{obs}t)$$

where A_t = absorption at time t, A_{max} = maximum absorption, P = amplitude, k_{obs} = apparent rate constant, and t = time. The observed rate constant was independent of the concentration of 4-HBA-CoA and was determined to be 0.024 s^{-1} at 22 °C, pH 7.5, in 50 mM K^+Hepes buffer (*7*).

Since spectral changes occur for the product−D145N complex more rapidly than for the corresponding substrate complex, the most informative Raman data for the former are in the first trace in Figure 8, collected in the time frame 1−2 min after mixing. In keeping with the absorption data, in Figure 8A, the feature at 1543 cm^{-1} and the shoulder near 1490 cm^{-1} are evidence for a population of ionized product. The shoulder in Figure 8A at 1525 cm^{-1} that becomes more apparent in subsequent collections demonstrates the presence of protonated product in the WT active site. The band seen in all traces near 657 cm^{-1} is also characteristic of the protonated product population in the WT active site. As in the case of the substrate−enzyme complex, the Raman data show consistent evidence for an early spectral feature in the 1565−1570 cm^{-1} region that has not been characterized in other studies. This will be discussed in the next section. Also, after $t = 6.5$ min, the Raman spectrum of D145N−4-HBA-CoA complex becomes identical to the final spectrum of the D145N−4-CBA-CoA complex and closely resembles that

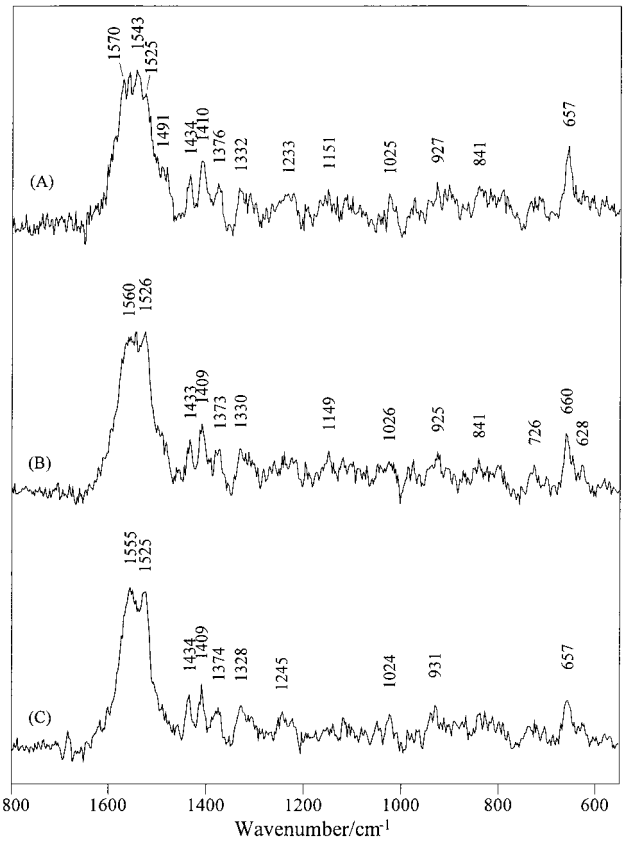
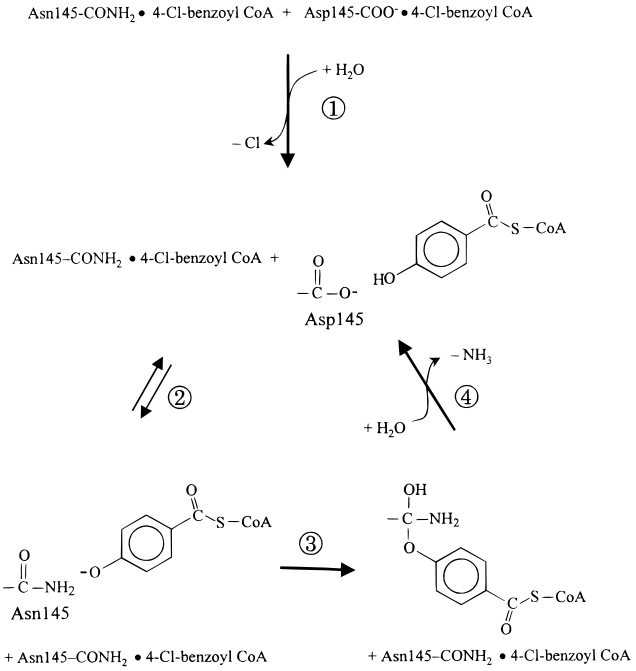


FIGURE 8: Raman difference spectra of 4-HBA-CoA (202 μM) bound to D145N (202 uM) in 50 mM Tris buffer (pH 7.5). Each spectrum was collected for 60 s at a reaction time *t*. Displayed are (A) $t = 1.0\sim2.0$ min; (B) $t = 2.0\sim3.0$ min; and (C) $t = 6.0\sim7.0$ min.

of product added directly into WT, except that the strongest band at 1555 cm^{-1} in the D145N−4-HBA-CoA spectrum is $\sim$5 cm^{-1} lower than the WT−4-HBA-CoA complex.

A Proposed Mechanism for the Product-Catalyzed Deamidation of D145N Dehalogenase. Deamidation of asparagine occurs in many enzymes during aging, generally leading to a decrease or loss of activity. Usually, deamidation proceeds by nucleophilic attack on the amide carbon by the peptide bond nitrogen that is located at the C-terminal side of the asparagine to yield a cyclic imide peptide, which is subsequently hydrolyzed to aspartate or isoaspartate (*9*−*11*). The D145N mutant dehalogenase showed increasing activity with storage, suggesting that slow deamidation is occurring. This process was measured in the absence of substrate or product using the protocol outlined under Experimental Procedures. The time course, at room temperature and pH 7.5, is set out in Figure 1. The rate was determined to be 0.0024 h^{-1}, 36000-fold slower than deamidation in the presence of 4-HBA-CoA. It is interesting to note that Pries et al. (*12*) observed the spontaneous deamidation of an asparagine side chain to regenerate the WT enzyme from the D124N mutant of haloalkane dehalogenase. This spontaneous hydrolysis occurred over several hundred hours at 4 °C and near neutral pH. Returning to 4-chlorobenzoyl-CoA dehalogenase and recalling that the observed rate constant in the presence of 4-HBA-CoA is independent of the concentration of 4-HBA-CoA (in the concentrations ranging from 1 to 7 times larger than the concentration of the enzyme active site (Materials and Methods)), one may

Scheme 1: Catalytic Cycle of Asp145Asn Dehalogenase Mutant[a]



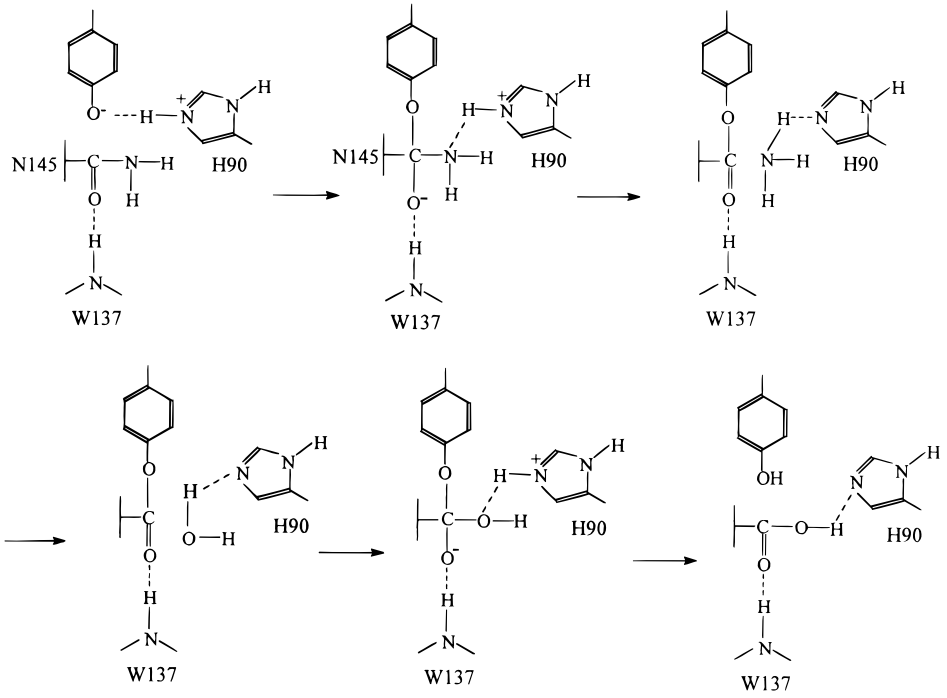
[a] Step 1: the bound substrate 4-chlorobenzoate CoA is hydrolyzed by a small amount of coexisting Asp145 to the product 4-hydroxybenzoate CoA. Step 2: the generated product exchanges to Asn145 site as ionized form. Step 3: the bound product attacks Asn145 by nucleophilic addition. Step 4: the adduct breaks down to acetyl-oxybenzoyl-CoA, through removal of NH_3, and is subsequently hydrolyzed to the 4-hydroxy-benzoyl-CoA and WT enzyme.

conclude that 4-HBA-CoA actually plays a role of catalyst rather than that of a reactant in the transformation of Asn145.

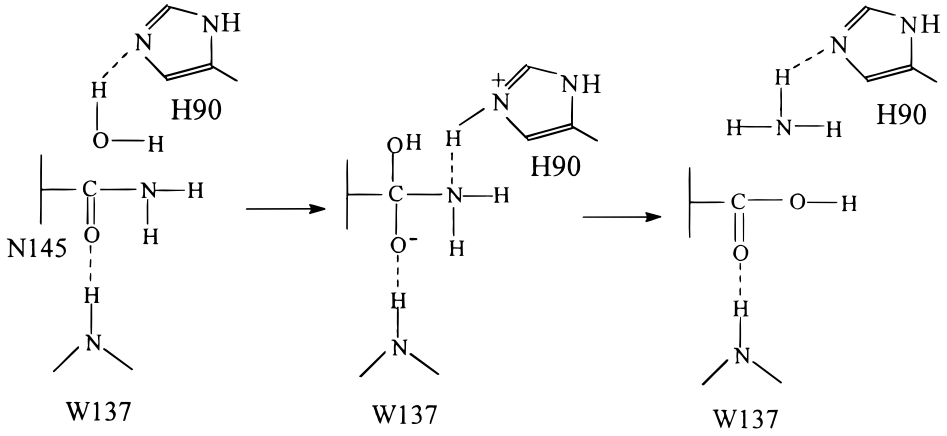
The rapid deamidation of D145N dehalogenase by 4-HBA-CoA, outlined above, taken with the very similar Raman spectra of WT plus product (*1*) and D145N plus 4-HBA-CoA after several minutes (Figure 8), suggests that asparagine 145 is being converted to aspartate rather than isoaspartate. This conclusion is supported by kinetic measurements on the regenerated (Asn → Asp) WT enzyme that show that its activity is very close to that of the original WT material (*7*). Moreover, the conversion of 4-CBA-CoA plus D145N to a species strongly resembling 4-HBA-CoA plus WT suggests that initially a small amount of WT enzyme, formed by spontaneous deamidation during storage or purification, catalyzes the hydrolysis of substrate. The resultant product then catalyzes the deamidation of the major enzyme population from D145N to WT. The spectroscopic changes seen in Figures 2 and 3 are fully consonant with this conclusion. A possible reaction scheme setting out these conclusions is given in Scheme 1.

Deamidation by product is postulated to occur via nucleophilic attack by the ionized form of product (4-O^-) at the asparagine side chain C═O. Certainly, there is good Raman and absorption evidence for the presence of the ionized product at early times in the deamidation sequence (Figures 2, 3, 6, and 8). A possible reaction scheme for the deamidation is shown in Scheme 2. Here the asparagine side chain is hydrogen bonded to tryptophan 137 (identified by X-ray studies (*13*)), and the ionized form of the 4-hydroxy ligand is hydrogen bonded to the protonated side chain of histidine 90. By invoking protonation of His 90, a means of

Scheme 2



Scheme 3



the NH_2 group leaving as ammonia is found. This removes the difficulty of the NH_2 leaving as $^{-}NH_2$, which would be energetically expensive.

The mechanism of the "spontaneous" (in the absence of product or substrate) deamidation of D145N probably involves His 90-assisted nucleophilic attack by water on the asparagine side chain C═O as shown in Scheme 3.

A similar mechanism was proposed by Pries and co-workers (*12*) to explain the "spontaneous" (i.e., in the absence of any ligand) hydrolytic deamidation of the Asn 124 mutant of haloalkane dehalogenase. For the D145N form of 4-CBA-CoA dehalogenase the 36000-fold increase in the rate of deamidation in the presence of product, 4-HBA-CoA, can be ascribed to effects such as the higher nucleophilicity of the phenoxide ion over water and the possible superior positioning of the phenoxide for attack on the asparagine side chain.

The postulated covalent intermediate between the product and the asparagine side chain seen in Schemes 1 and 2 suggests an identity for spectral features as yet unassigned. We hypothesize that the Raman bands seen in Figures 3 and 8 near 1570 cm^{-1}, as well as the absorption band seen in Figure 6 near 320, nm are due to the tetrahedral species, or the subsequent ester species, formed after NH_3 leaves, depicted in the bottom right-hand corner of Scheme 1. More evidence is needed to validate this claim, but the positions of the Raman peak and the λ_{max} are where we expect the spectral signatures for such a species on the basis of the preceding paper in the present studies. The tetrahedral intermediate, or ester, formed by addition of 4-HBA-CoA to amide C═O group, is predicted to undergo a lesser degree of polarization in the active site, compared to ionized product bound to the enzyme (based on ref *1* and unpublished work, this laboratory).

The Raman spectra of the final reaction product bound to D145N that has converted back to aspartate 145 show a strong peak at 1555 cm^{-1}, which is ~5 cm^{-1} lower than the direct WT−product adduct. This seems to suggest that the overall conformation around the active site is slightly perturbed after the reaction. This perturbation may arise from a minor change in the hydrophobic nature of the active site, which is caused by a very small change in the orientation of

the four surrounding aromatic residues (Trp137, Trp89, Phe64, and Phe82). Alternatively, it could arise from a small change in the relative position of Asp145 with respect to 4-HBA-CoA.

SUPPORTING INFORMATION AVAILABLE

Density functional theory predicted normal modes for *S*-ethyl 4-chlorobenzoate thioester. This material is available free of charge via the Internet at http://pubs.acs.org.

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