Transient State Kinetic Analysis of the Chemical Intermediates Formed in the Enzymatic Dehalogenation of 4-Chlorobenzoyl Coenzyme A

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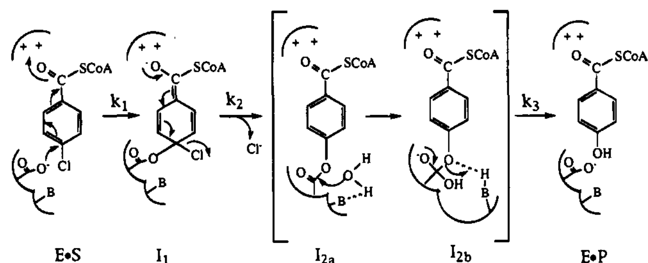
Halogenated aromatics rank high among our most problematic pollutants. Bioremediation offers the most promising approach to their removal from contaminated soils and water.[1] In recent years bacteria that are able to metabolize halogenated aromatics by first removing the halogen from the ring have been isolated and characterized. The 4-chlorobenzoate (4-CBA)[2] degrading bacteria,[3] for example, are known to possess a dehalogenation pathway (mediated by three enzymes: 4-CBA:CoA ligase, 4-CBA-CoA dehalogenase, and 4-HBA-CoA thioesterase) which transforms 4-CBA to the metabolite 4-hydroxybenzoate (4-HBA).[4,5] The dehalogenation reaction occurs on the aromatic ring of 4-CBA-CoA, generated from 4-CBA in the first step of the pathway. Given that the CoA thioester substituent is a relatively weak ring activator,[6] it is amazing that the dehalogenase is able to dechlorinate the 4-CBA-CoA with H_2O via a S_NAr pathway as efficiently as it does ($k_{cat} = 2\ s^{-1}$ at pH 7.5 and 25 °C).[7] Previous studies of the *Pseudomonas* sp. strain CBS3 4-CBA-CoA dehalogenase[8] have shown that it utilizes a unique form of covalent catalysis (Scheme 1) wherein an active site carboxylate adds to the benzoyl ring C(4) of 4-CBA-CoA, ultimately displacing the halide and forming an arylated enzyme.[9] Hydrolysis of the arylated enzyme occurs at the acyl carbon, liberating 4-HBA-CoA and the native enzyme.

In the present study, stopped-flow absorbance methods were used to kinetically resolve and identify the intermediates of the 4-CBA-CoA dehalogenase catalyzed reaction. Accordingly, 50 μL of 80 μM 4-CBA-CoA dehalogenase in 50 mM K^+Hepes (pH 7.5) was mixed with 50 μL of 80 μM 4-CBA-CoA (forming *ca.* 80% enzyme•4-CBA-CoA complex)[7] in a stopped-flow spectrophotometer.[10] The UV spectrum (280–400 nm) of the reaction solution was measured periodically throughout the course of a single turnover on the enzyme (Figure 1). The first scan at 10 ms reflects the enzyme•4-CBA-CoA complex (E•S), and the final scan at 1590 ms reflects the enzyme•4-HBA-CoA complex (E•P).[11] The wavelength at which the absorbance curves of the E•S (unbound) and E•P complexes intersect (316 nm; Figure 1) was used in ensuing experiments to monitor the rates of appearance and disappearance of reaction intermediates. Following the rapid increase in solution absorbance at 316 nm associated with substrate binding (k_0 of Figure 2a),[12] two slower phases of absorbance increase were noted (k_1 and k_2 of Figure 2). These were followed by an even slower absorbance decrease (k_3 of Figure 2b). The rate of the absorbance decrease correlates well with the rate of absorbance increase observed at 375 nm (Figure 2b), which is associated with the formation of the E•P complex. Analysis of the data using equations describing single- or double-exponential decay processes yielded apparent rate constants for substrate binding ($k_0 = 600\ s^{-1}$) and for formation of the first intermediate ($k_1 = 67\ s^{-1}$), the second intermediate ($k_2 = 3.8\ s^{-1}$), and the E•P complex ($k_3 = 2.1\ s^{-1}$) (Table 1).

As a working model, k_1 was assigned to the formation of the Meisenheimer adduct I_1 of Scheme 1 and k_2 was assigned to

Scheme 1. Chemical Pathway Proposed for the 4-CBA-CoA Dehalogenase Catalyzed Dechlorination of 4-CBA-CoA as Adapted from Ref 9[a]



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

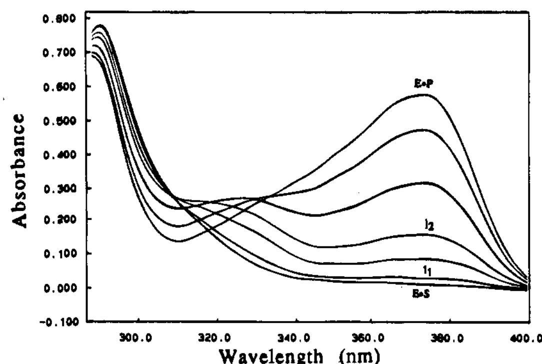


Figure 1. The UV absorbance spectra of the reaction solution of 40 μM 4-CBA-CoA and 40 μM 4-CBA-CoA dehalogenase in 25 mM K^+-Hepes (pH 7.5, 25 °C) measured at varying conversion. Absorbance spectra were measured at 10 ms (E•S), 75 ms (I_1), 165 ms, 370 ms (I_2), 575 ms, 800 ms, and 1590 ms (E•P).

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(1) Higson, F. K. *Adv. Appl. Microbiol.* **1992**, *37*, 135. Abramowicz, D. A. *Crit. Rev. Biotechnol.* **1990**, *10*, 241. Commandeur, L. C. M.; Parsons, J. R. *Biodegradation* **1990**, *1*, 207.

(2) Abbreviations: CBA, chlorobenzoate; HBA, hydroxybenzoate; 4-CBA-CoA, 4-chlorobenzoyl coenzyme A; 4-HBA-CoA, 4-hydroxybenzoyl coenzyme A; 4-BBA-CoA, 4-bromobenzoyl coenzyme A; 4-IBA-CoA, 4-iodobenzoyl coenzyme A; 4-FBA-CoA, 4-fluorobenzoyl coenzyme A; BA-CoA, benzoyl coenzyme A.

(3) See the following recent review: Dunaway-Mariano, D.; Babbitt, P. C. *Biodegradation* **1994**, *5*, 259.

(4) Ornston, L. N. In *Proceedings of the Symposium on Genetics of Industrial Microorganisms*; Heslot, H., Davies, J., Florent, J., Bibichon, L., Duran, G., Penasse, L., Eds.; Societe Francaise de Microbiologie: Strasbourg, 1990; Vol. 2, p 1061.

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(6) For a complete review on the reactivities of arenes toward nucleophilic aromatic substitution, see: Bunnett, J. F.; Zahler, R. E. *Chem. Rev.* **1951**, *49*, 273.

(7) Liang, P.-H.; Yang, G.; Dunaway-Mariano, D. *Biochemistry* **1993**, *32*, 12245.

(8) Chang, K.-H.; Liang, P.-H.; Beck, W.; Scholten, J. D.; Dunaway-Mariano, D. *Biochemistry* **1992**, *31*, 5605. Loffler, F.; Muller, R.; Lingens, F. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 1106.

(9) Yang, G.; Liang, P.-H.; Dunaway-Mariano, D. *Biochemistry* **1994**, *33*, 8527.

(10) Experiments were performed with an Applied Photophysics DX.17MV sequential stopped-flow spectrophotometer.

(11) For Cl^- K_D > 10 mM while for 4-HBA-CoA K_D = 1 μM (unpublished data). Thus, the E•4-HBA-CoA complex will predominate in the final mixture.

(12) A plot of the apparent rate constant for this fast step vs [4-CBA-CoA] (10–140 μM) measurement at 25 μM 4-CBA-CoA dehalogenase, 25 mM K^+Hepes (pH 7.5, 25 °C), is hyperbolic, reflecting a two-step substrate binding process (i.e., fast encounter followed by slower isomerization). The maximum apparent rate constant was determined to be 730 s^{-1}, which, at 25 μM enzyme, yields $k_{on} = 30\ \mu M^{-1}\ s^{-1}$.

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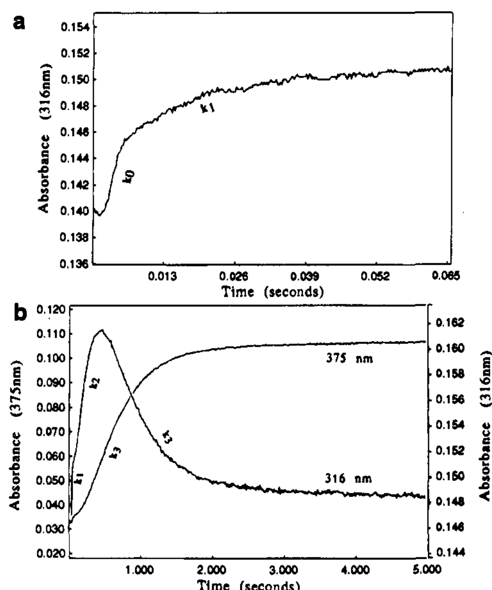

Figure 2. The UV absorbance of the reaction solution of 40 μM 4-CBA-CoA and 40 μM 4-CBA-CoA dehalogenase in 25 mM K^+-Hepes (pH 7.5, 25 °C): (a) monitored at 316 nm for the first 65 ms of the reaction and reflecting the rate of absorbance increase associated with E·S (k_0) and I_1 (k_1) formation; (b) monitored at 316 and 375 nm for the entire single-turnover (5 s) reaction and reflecting the rate of change in absorbance associated with I_1 (k_1), I_2 (k_2), and E·P (k_3) formation.

Table 1. Apparent Rate Constants for Reaction of 40 μM 4-CBA-CoA Dehalogenase (Specific Activity = 2.5 units/mg) with Substrate in 25 mM K^+Hepes (pH 7.5, 25 °C)[a]

substrate (concn, μM)	C(4) subst	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})
4-CBA-CoA (40)	Cl	67 ± 3	3.8 ± 0.4	2.1 ± 0.2
4-BBA-CoA (40)	Br	58 ± 4	5.2 ± 0.5	2.9 ± 0.2
4-IBA-CoA (40)	I	25 ± 3	7.5 ± 0.5	3.2 ± 0.2
4-FBA-CoA (290)	F	75 ± 5	$(8.6 \pm 0.5) \times 10^{-4}$	
4-BA-CoA (620)	H	72 ± 3		

[a] The measurements of the apparent rate constants for formation of I_1 (k_1), I_2 (k_2), and E·P (k_3) (see Scheme 1) from each of the substrates listed, in reaction with pure 4-CBA-CoA dehalogenase,[17] were shown to be reproducible. The K_D values (μM) derived from Michaelis constants or competitive inhibition constants; ref 7) are 4-CBA-CoA (3.7 ± 0.3), 4-BBA-CoA (4.2 ± 0.3), 4-IBA-CoA (6.5 ± 3), 4-FBA-CoA (40 ± 5), and 4-BA-CoA (72 ± 8).

the formation of the arylated enzyme I_{2a} or its hydrate I_{2b}. The spectral profiles observed for I_1 and I_2 (see Figure 1) were not helpful in our evaluation of these assignments;[13] however, the structure/activity relationships that were measured did prove to be useful in this regard. Specifically, the rates of formation and decay of I_1 and I_2 were determined as a function of the nature of the leaving-group substituent at C(4) of the benzoyl ring. On the basis of reactivity scales derived for substituted arynes undergoing S_NAr reactions in solution, we might expect that the inductive properties of the C(4) substituent would govern the $\Delta G^\ddagger$ for formation of the Meisenheimer intermediate I_1,[14] while the leaving-group abilities of the C(4) substituent would influence the $\Delta G^\ddagger$ for conversion of I_1 to the arylated enzyme intermediate I_{2a}.[14]

The apparent rate constants[15] determined for the formation of I_1, I_2, and E·P from the reactions of dehalogenase with the 4-CBA-CoA analogs benzoyl-CoA (BA-CoA), 4-bromobenzoyl-CoA (4-BBA-CoA), 4-iodobenzoyl-CoA (4-IBA-CoA), and 4-fluorobenzoyl-CoA (4-FBA-CoA) are listed in Table 1.[16] Reaction of the dehalogenase with α-phenylacetyl-CoA (a substrate analog lacking thiocarbonyl−arene ring conjugation) was also tested.[17] This analog was observed to bind to the dehalogenase, but not to undergo reaction. The kinetic data of Table 1 show that the rates of formation of I_2 correlate with the leaving-group ability of the C(4) substituent on the substrate benzoyl ring (I > Br > Cl >> F >>> H). The most dramatic substituent effects are seen with benzoyl-CoA and 4-FBA−CoA. The reaction of benzoyl-CoA does not proceed beyond I_1 owing to the high energy barrier to hydride formation. On the other hand, a slow release of fluoride ion from I_1, formed from 4-FBA-CoA, is evidenced by product formation which was observed to occur at a rate of 1×10^{-4} s^{-1}.[18] The correlation observed between the rate at which I_2 is formed from I_1 and the intrinsic leaving-group ability of the C(4) substituent strongly supports the assignment of I_2 as the arylated enzyme intermediate.

If we are correct in assuming that the conversion of E·S to arylated enzyme (I_2) proceeds via a S_NAr reaction pathway, then it follows that I_1 is the Meisenheimer intermediate. Consistent with this assignment is the observation that the dehalogenase can catalyze the formation of I_1 from all of the reactants listed in Table 1 (including 4-BA-CoA) but not from α-phenylacetyl-CoA, the substrate analog lacking thiocarbonyl−arene ring conjugation. Contrary to our original tenet (*vide infra*), the rate of formation of I_1 does not seem to vary significantly with the inductive properties of the benzoyl C(4) substituent (Table 1). The similarity observed in the rates of formation of I_1 from BA-CoA and its 4-halogenated derivatives might be due to a slow conformational change which follows substrate binding and precedes the chemical step leading to I_1. More probable, however, is the overriding effect that the enzyme active site environment has on the energy of the transition state leading to I_1. The electrophilic catalysis (i.e., proton transfer to, or electropositive interaction with, the benzoyl C=O) offered by the enzyme might mask the inductive effect of the benzoyl ring C(4) substituent if the transition state is indeed neutral.

Enzymes, by design, use binding energy to lower the energy of reaction intermediates and their transition states.[19] Nonetheless, it is remarkable that the dehalogenase is so proficient ($k_1 = 67$ s^{-1}; Table 1) in forming the Meisenheimer complex from 4-CBA-CoA. Studies directed at determining the identity of the groups in the dehalogenase active site that are responsible for the stabilization of the Meisenheimer complex are now in progress.

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(13) Shown in Figure 1 are the respective scans deriving from I_1 and I_2. The difference spectrum of E·S and I_1 reveals a trough at 240−255 nm and a peak at 260−380 nm. An accurate comparison between the spectral properties of the enzyme-bound reactant/intermediates and those of chemical models (e.g., methylbenzene (λ_{max} 260 nm) vs 1-methylene-2,5-cyclohexadiene (λ_{max} 240 nm; Plieninger, H.; Maier-Borst, W. *Angew. Chem.* **1963**, *75*, 1177)) cannot be easily made because of the overlapping absorbancies of the benzoyl ring and CoA moieties and because of the large and unpredictable spectral perturbations caused by the active site environment (as exemplified by the observed shift in the benzoyl ring λ_{max} from 290 to 375 nm upon binding 4-HBA-CoA to the enzyme).

(14) Miller, J. In *Reaction Mechanisms in Organic Chemistry*; Eaborn, C., Chapman, N. B., Eds.; Monograph 8; Elsevier: New York, 1968.

(15) The apparent rate constants reported in Table 1 represent the sum of the forward and reverse rate constants for the step in question. Thus, as one reviewer pointed out, changes observed in the apparent rate constants could derive from changes in the rate constants for the forward or reverse steps, or both.

(16) Benzoyl-CoA and phenylacetyl-CoA were purchased from Sigma Chemical Co., and 4-CBA-CoA, 4-BBA-CoA, 4-IBA-CoA, and 4-FBA-CoA were prepared according to ref 7.

(17) In this stopped-flow experiment, 30 μM dehalogenase was reacted with 700 μM phenylacetyl-CoA (K_D = 110 μM).

(18) In ref 7 we reported the observation of a thioesterase activity associated with the dehalogenase. Careful chromatography separated the thioesterase from the dehalogenase, allowing us to measure the rate of defluorination of 4-FBA-CoA (8.6×10^{-4} s^{-1} in this study) in the absence of the competing side reaction.

(19) Jencks, W. P. *Adv. Enzymol.* **1979**, *43*, 219.