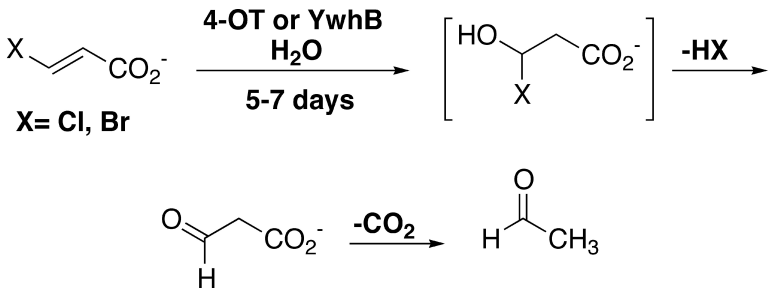
Communication

The 4-Oxalocrotonate Tautomerase- and YwhB-Catalyzed Hydration of 3*E*-Haloacrylates: Implications for the Evolution of New Enzymatic Activities

Susan C. Wang, William H. Johnson, and Christian P. Whitman

J. Am. Chem. Soc., **2003**, 125 (47), 14282-14283• DOI: 10.1021/ja0370948 • Publication Date (Web): 01 November 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 11/01/2003

The 4-Oxalocrotonate Tautomerase- and YwhB-Catalyzed Hydration of 3*E*-Haloacrylates: Implications for the Evolution of New Enzymatic Activities

Susan C. Wang, William H. Johnson, Jr., and Christian P. Whitman*

Division of Medicinal Chemistry, College of Pharmacy, The University of Texas, Austin, Texas 78712-1074

Received July 6, 2003; E-mail: whitman@mail.utexas.edu

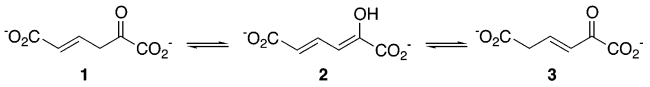
Nature may exploit the catalytic promiscuity observed in some enzymes in order to create new enzymes.[1] A catalytically promiscuous enzyme has one or more low-level activities in addition to its primary, physiological activity.[2] Duplication of the gene, followed by a series of mutations to amplify the desired activity, generates a new enzyme now having the progenitor's low-level activity as the primary activity. We report herein the observation of low-level hydration activities in the bacterial isomerase, 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 and a 4-OT homologue, YwhB, found in *Bacillus subtilis*.[3] In addition to providing very clear examples of catalytically promiscuous enzymes, these observations support the proposed evolutionary link between these enzymes and the recently characterized *trans*-3-chloroacrylic acid dehalogenase (CaaD)[4] and show that 4-OT- and YwhB-like sequences have diverse catalytic capabilities and may have served as templates for the creation of new enzymatic activities.

4-OT, found in a pathway that degrades aromatic hydrocarbons, catalyzes the conversion of 2-oxo-4*E*-hexenedioate (**1**; Scheme 1) to 2-oxo-3*E*-hexenedioate (**3**) through 2-hydroxy-2,4*E*-hexadienedioate (**2**).[5] Among the key catalytic residues are Pro-1, which abstracts the C-3 proton of **1**, and Arg-11, which participates in both substrate binding (at C-6) and in catalysis.[6] 4-OT is the title enzyme of the 4-OT family, whose structurally homologous members are constructed from short monomers (61−81 amino acids), which conserve a signature $\beta-\alpha-\beta$ structural motif as well as Pro-1.[7] YwhB shares 36% pairwise sequence identity with 4-OT and retains both Pro-1 and Arg-11.

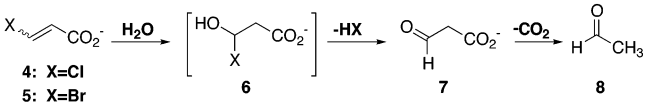
Sequence analysis first suggested that the heterohexameric CaaD, responsible for the hydrolytic dehalogenation of 3*E*-chloro- and 3*E*-bromoacrylate [(*E*)-**4** and (*E*)-**5**, respectively; Scheme 2], might be a 4-OT family member.[4a] The pairwise sequence identities observed between YwhB and the α-subunit of CaaD (35%) and 4-OT and the β-subunit (25%) are low, but mutagenesis indicated that β-Pro-1 and α−Arg-11 are critical for activity, further substantiating a relationship between CaaD and the 4-OT family.[4a]

In view of these observations, we examined whether 4-OT and YwhB catalyzed the hydration of 3*E*-haloacrylates, resulting in their dehalogenation. Accordingly, each protein was incubated with (*E*)-**4**, and the reactions were monitored by ^{1}H NMR spectroscopy.[8] The ^{1}H NMR spectrum of (*E*)-**4** in Na_2HPO_4 buffer (pH 6.8) shows two doublets (6.09 and 6.89 ppm), which correspond to the C-2 and C-3 protons, respectively. After incubation of (*E*)-**4** with 4-OT (0.6 mg) for 136 h, the intensity of these two signals diminishes and four new signals appear. Two signals (2.04 and 9.48 ppm) correspond to acetaldehyde (**8**), whereas the other two signals (1.13 and 5.05 ppm) correspond to its hydrate (**9**).[4a,9] Integration of the signals indicates that ∼74% of (*E*)-**4** has been converted to a mixture of **8** and **9**. Incubation of YwhB with (*E*)-**4** results in the same products, although the reaction is not as efficient.[10] Although less YwhB (0.3 mg) was used, a longer incubation period (171 h) was required to convert ∼30% of (*E*)-**4** to a mixture of **8** and **9**. Both enzymes will process (*E*)-**5**: ∼92% of (*E*)-**5** is converted to **8** and **9** after 209 h by 4-OT and ∼93% of (*E*)-**5** is converted to a mixture of **8** and **9** after 616 h by YwhB.

Scheme 1



Scheme 2



A control experiment demonstrates that the hydration of (*E*)-**4** is an enzyme-catalyzed process. An ^{1}H NMR spectrum of (*E*)-**4** incubated in Na_2HPO_4 buffer (pH 6.8) for 192 h (8 days) showed that only (*E*)-**4** was present, ruling out a nonenzymatic hydration. The nonenzymatic hydration of (*E*)-**4** requires much harsher conditions: only ∼10% of the chloride is removed after 24 h in 0.5 M aqueous NaOH at 60 °C.[11]

The results show that the incubation of (*E*)-**4** and (*E*)-**5** with 4-OT or YwhB generates **8**, which is readily hydrated to yield **9**. A likely scenario for the formation of **8** from these compounds involves the initial enzymatic hydration of the 3*E*-haloacrylates to produce an unstable halohydrin species (**6**, Scheme 2), which decomposes to malonate semialdehyde (**7**).[4b] Nonenzymatic decarboxylation of **7** yields **8**. Compound **7** is not sufficiently stable to accumulate in quantities detectable by ^{1}H NMR spectroscopy during the lengthy incubation periods.

The preparations of 4-OT and YwhB used in these experiments were highly purified, but it remained possible that a contaminating enzyme could be responsible for the observed activity. To eliminate this concern, two control experiments were performed. First, a partially purified protein sample from cells harboring an "empty" pET-24a(+) vector (i.e., the genes encoding 4-OT or YwhB are absent) was examined.[12] Neither (*E*)-**4** nor (*E*)-**5** was converted to product after 10.5 days using 2.6 mg of protein. In addition, incubation of synthetically prepared 4-OT (likely to be free of contaminating cellular enzymes) with (*E*)-**4** for 208 h led to the same product mixture in comparable amounts.

The isomer specificities of 4-OT and YwhB were also investigated. An ^{1}H NMR spectrum of a reaction mixture containing (*Z*)-**4** and 4-OT showed that ∼3−4% of the *Z*-isomer was converted to **8** and **9** after lengthy incubation (139 h using 0.64 mg). In contrast, YwhB showed no detectable activity with (*Z*)-**4** after prolonged incubation (209 h using 0.51 mg). The enzymes show a clear preference for the *E*-isomer, which is consistent with the structural resemblance of (*E*)-**4** to the acrylate portion of 4*E*-**1** and **2**. Moreover, this observation implicates the active sites of the two enzymes in the low-level activity.[13]

10.1021/ja0370948 CCC: $25.00

Table 1. Kinetic Parameters for 4-OT, YwhB, and Mutants

enzyme	sub.	k_{cat}, (s^{-1})	k_{cat}/K_m, ($M^{-1}s^{-1}$)
4-OT	**2**[6c]	3500 ± 500	$(2.0 \pm 0.3) \times 10^7$
4-OT	(*E*)-**4**	$(8.3 \pm 0.5) \times 10^{-4}$	$(2.6 \pm 0.4) \times 10^{-2}$
P1A-4-OT	(*E*)-**4**	–	$(2.0 \pm 0.3) \times 10^{-4}$
R11A-4-OT	(*E*)-**4**	–	$(1.6 \pm 0.1) \times 10^{-1}$
YwhB	**2**	26 ± 1.4	$(2.8 \pm 0.1) \times 10^4$
YwhB	(*E*)-**4**	–	$(4.4 \pm 1.0) \times 10^{-2}$
R11A-YwhB	(*E*)-**4**	–	$(1.9 \pm 0.2) \times 10^{-3}$
CaaD	(*E*)-**4**[4b]	3.8 ± 0.1	$(1.2 \pm 0.1) \times 10^5$

Having established a low-level CaaD activity for both enzymes, kinetic parameters were determined (Table 1).[14] Saturation with (*E*)-**4** was not achieved for YwhB. A comparison of the k_{cat}/K_m values shows that 4-OT and YwhB are 4.6×10^6-fold and 2.7×10^6-fold, respectively, less efficient than CaaD.[15] For 4-OT, binding (K_m) and turnover (k_{cat}) are adversely and comparably impacted such that a 10^3-fold decrease in K_m coupled with a 10^3-fold increase in k_{cat} will produce CaaD activity levels. With regard to the conversion of **2** to **3**, the CaaD activities of 4-OT and YwhB are 7.7×10^8-fold and 6.4×10^5-fold less efficient, respectively.

To gauge the importance of Pro-1 and Arg-11 to the activity, k_{cat}/K_m values were also determined for three mutants, as saturation could not be achieved with these mutants (Table 1).[16] The k_{cat}/K_m value for the P1A-4-OT mutant is 108-fold less than that of the wild type, whereas the k_{cat}/K_m value for the R11A-4-OT mutant is slightly greater (6-fold). The k_{cat}/K_m value for the R11A-YwhB mutant is 23-fold less than that of YwhB, whereas there is no detectable activity for the P1A-YwhB mutant. These results provide further evidence indicating that 4-OT and YwhB are responsible for the observed activities and that Pro-1 is critical for activity in both enzymes, whereas Arg-11 is only essential for the CaaD activity of YwhB.

Two mechanisms might explain the observed hydratase activity. In both mechanisms, a positively charged residue (e.g., Arg-11) may interact with the C-1 carboxylate group of (*E*)-**4** or (*E*)-**5** and draw electron density away from the C-3 position to form an enolic intermediate. The partial positive charge at C-3 is now susceptible to a Michael addition of water. 4-OT has two active-site arginines, Arg-11 and Arg-39, whereas YwhB has only Arg-11. Thus, mutation of Arg-11 in 4-OT might not lead to a loss of activity because (*E*)-**4** may interact with Arg-39. In one mechanism, Pro-1 may function as a general base and activate the water molecule for addition to C-3. Subsequently, Pro-1, now functioning as a general acid, would deliver the proton to C-2 to complete the addition of water. In a second mechanism, water may add to C-3 as a result of the partial positive charge, and Pro-1 might act as a general acid catalyst and deliver a proton to the C-2 position of (*E*)-**4** or (*E*)-**5** upon ketonization of the enediolate intermediate.

The proposed role for Pro-1 in the latter mechanism is suggested by recent findings implicating the β-Pro-1 as a general acid catalyst in the CaaD-catalyzed reaction.[17] The β-Pro-1 of CaaD has a pK_a of $\sim$9.2, enabling it to function as a general acid catalyst.[17] In contrast, the catalytic Pro-1 in 4-OT has a pK_a of $\sim$6.4 due to its presence in the hydrophobic active site.[6a] This lowered pK_a value enables it to function as a general base catalyst in its physiological activity at cellular pH. Under the conditions of the kinetic experiments (pH 7.8), very little 4-OT is present with Pro-1 in the correct protonation state to function as a general acid catalyst. Both mechanisms offer an interesting evolutionary route for the production of a more efficient dehalogenase. One or more mutations could decrease the hydrophobic environment of 4-OT's active site, making it more hydrophilic. The resulting active site would now be more amenable to a hydrolytic reaction, thereby raising the pK_a of Pro-1 and increasing the concentration of enzyme in the reactive form. This possibility is being explored.

Acknowledgment. We thank Dr. Michael C. Fitzgerald (Department of Chemistry, Duke University) for synthetic 4-OT. This research was supported by the National Institutes of Health Grant GM-41239 and the Robert A. Welch Foundation (F-1334). S.C.W. is a Fellow of the American Foundation for Pharmaceutical Education.

References

(1) (a) Jensen, R. A. *Annu. Rev. Microbiol*. **1976**, *30*, 409−425. (b) Hughes, A. L. *Proc. R. Soc. London, Ser. B* **1994**, *256*, 119−124. (c) Palmer, D. R. J.; Garrett, J. B.; Sharma, V.; Meganathan, R.; Babbitt, P. C.; Gerlt, J. A. *Biochemistry* **1999**, *38*, 4252−4258. (d) Copley, S. D. *Trends Biochem. Sci*. **2000**, *25*, 261−265.

(2) (a) O'Brien, P. J.; Herschlag, D. *Chem. Biol*. **1999**, *6*, R91−R105. (b) O'Brien, P. J.; Herschlag, D. *Biochemistry* **2001**, *40*, 5691−5699.

(3) Whitman, C. P. *Arch. Biochem. Biophys.* **2002**, *402*, 1−13.

(4) (a) Poelarends, G. J.; Saunier, R.; Janssen, D. B. *J. Bacteriol.* **2001**, *183*, 4269−4277. (b) Wang, S. C.; Person, M. D.; Johnson, W. H., Jr.; Whitman, C. P. *Biochemistry* **2003**, *42*, 8762−8773.

(5) Whitman, C. P.; Aird, B. A.; Gillespie, W. R.; Stolowich, N. J. *J. Am. Chem. Soc*. **1991**, *113*, 3154−3162.

(6) (a) Stivers, J. T.; Abeygunawardana, C.; Mildvan, A. S.; Hajipour, G.; Whitman, C. P. *Biochemistry* **1996**, *35*, 814−823. (b) Czerwinski, R. M.; Johnson, W. H., Jr.; Whitman, C. P.; Harris, T. K.; Abeygunawardana, C.; Mildvan, A. S. *Biochemistry* **1997**, *36*, 14551−14560. (c) Harris, T. K.; Czerwinski, R. M.; Johnson, W. H., Jr.; Legler, P. M.; Abeygunawardana, C.; Massiah, M. A.; Stivers, J. T.; Whitman, C. P.; Mildvan, A. S. *Biochemistry* **1999**, *38*, 12343−12357.

(7) Almrud, J. J.; Kern, A. D.; Wang, S. C.; Czerwinski, R. M.; Johnson, W. H., Jr.; Murzin, A. G.; Hackert, M. L.; Whitman, C. P. *Biochemistry* **2002**, *41*, 12010−12024.

(8) The ^{1}H NMR spectra were recorded in 100% H_2O on a Varian Unity INOVA-500 spectrometer.[4b] The NMR tube contained 100 mM Na_2HPO_4 buffer (0.6 mL, pH 9.2) and (*E*)-**4** (4 mg, 0.04 mmol) dissolved in DMSO-d_6 (30 μL). Adding (*E*)-**4** adjusted the pH to 6.8. Subsequently, a quantity of enzyme (in 20 mM NaH_2PO_4 buffer, pH 7.3) was added to the NMR tube. The purification of 4-OT has been reported.[6b] The enzymes do not lose significant activity in the time frame of these experiments.

(9) Gil, A. M.; Duarte, I. F.; Delgadillo, I.; Colquhoun, I. J.; Casuscelli, F.; Humpfer, E.; Spraul, M. *J. Agric. Food Chem.* **2000**, *48*, 1524−1536.

(10) The purification and characterization of YwhB has been reported. YwhB converts **2** to **1** as well as **2** to **3** but does not catalyze the latter reaction as efficiently as 4-OT (Table 1). Wang, S. C.; Johnson, W. H., Jr.; Czerwinski, R. M.; Whitman, C. P. *Biochemistry*, in press.

(11) Braddon, S. A.; Dence, C. W. *Tappi* **1968**, *51*, 249−256.

(12) An aliquot of pET-24a(+) (0.5 μL) was transformed into electrocompetent *Escherichia coli* strain BL21-Gold(DE3)pLysS cells. Cells carrying the pET-24a(+) plasmid were grown and induced for protein expression as described.[6b] Cells were lysed by sonication, the cell lysate was centrifuged, and the resulting supernatant was made 2 M in $(NH_4)_2SO_4$. After being stirred for 1 h (4 °C), the solution was centrifuged, and the supernatant was transferred to a Millipore Ultrafree 15 (MWCO 5000 Da) centrifugal concentrator and exchanged into 20 mM NaH_2PO_4 buffer, pH 7.3, to give a final volume of 0.6 mL.

(13) The low-level *cis*-CaaD activity of 4-OT suggests that a 4-OT-like sequence could have been the template for the evolution of a known *cis*-CaaD: van Hylckama Vlieg, J. E. T.; Janssen, D. B. *Biodegradation* **1992**, *2*, 139−150. *cis*-CaaD has recently been identified as a tautomerase superfamily member. Poelarends, G. J.; Serrano, H.; Person, M. D.; Johnson, W. H., Jr.; Murzin, A. G.; Whitman, C. P. *Biochemistry*, in press.

(14) Assay mixtures (1-mL) contained 100 mM K_2HPO_4 buffer, pH 7.8, NAD^+ (0.27 mg), aldehyde dehydrogenase (0.7 mg), β-mercaptoethanol (4 μg), substrate (4-OT: 0.94−79.4 mM; YwhB: 23.5−70.4 mM), and enzyme (4-OT: 0.5 mg; YwhB: 0.05 mg). Assays were performed at 23 °C following the conversion of NAD^+ to NADH.

(15) The k_{cat} for 4-OT using (*E*)-**5** is $(3.2 \pm 0.4) \times 10^{-3}$ s^{-1}, and the k_{cat}/K_m is 0.52 ± 0.12 M^{-1} s^{-1}, which are 3.8-fold and 2-fold greater than those measured for 4-OT using (*E*)-**4**.

(16) The preparation of the 4-OT mutants is described elsewhere.[6b,c] YwhB mutants were constructed by overlap extension PCR (Ho, S. N.; Hunt, H. D.; Horton, R. M.; Pullen, J. K.; Pease, L. R. *Gene* **1989**, *77*, 51−59) using the protocol described for the P1A- and R11A-4-OT mutants with the YwhB gene as template.[6b,c]

(17) Azurmendi, H. F.; Wang, S. C.; Massiah, M. A.; Whitman, C. P.; Mildvan, A. S. *Biochemistry* **2003**, *42*, 8619; *Chem. Abstr.* **2003**, *42*, 113.

JA0370948