Reactions of 4-Oxalocrotonate Tautomerase and YwhB with 3-Halopropiolates: Analysis and Implications†

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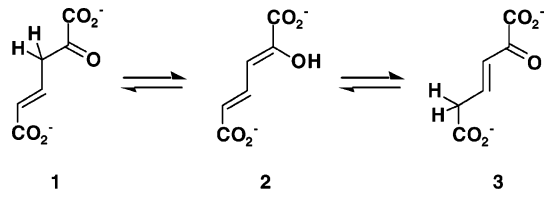
ABSTRACT: 4-Oxalocrotonate tautomerase (4-OT) and YwhB, a 4-OT homologue found in *Bacillus subtilis*, exhibit a low level hydratase activity that converts *trans*-3-haloacrylates to acetaldehyde, presumably through a malonate semialdehyde intermediate. The mechanism for the initial transformation of the 3-haloacrylate to malonate semialdehyde involves Pro-1 as well as an arginine, two residues that play critical roles in the 4-OT-catalyzed isomerization reaction and the YwhB-catalyzed tautomerization reaction. These residues are also critical for the *trans*-3-chloroacrylic acid dehalogenase (CaaD)-catalyzed conversion of *trans*-3-haloacrylates to malonate semialdehyde. Recently, 3-bromo- and 3-chloropropiolate, the acetylene analogues of 3-haloacrylates, were characterized as potent irreversible inhibitors of CaaD due to the covalent modification of the catalytic proline. In view of these observations, an investigation of the behavior of 4-OT and YwhB with the 3-halopropiolates was undertaken. The results show that these compounds are potent irreversible inhibitors of 4-OT and YwhB with Pro-1 being the sole site of covalent modification by 3-bromopropiolate. The inactivation process could involve the enzyme-catalyzed addition of water to the 3-halopropiolate yielding an acyl halide, which would inactivate the enzyme or be initiated by the nucleophilic attack of Pro-1 at the C-3 position of the 3-halopropiolate in a Michael type reaction. The presence of the halogen along with Arg-11 could facilitate both reactions with the latter causing the polarization of the α,β-unsaturated acids. The 3-halopropiolates are the first identified inhibitors of YwhB and confirm the importance of Pro-1 in its mechanism. In addition, the results set the stage for the use of these compounds as mechanistic probes of the primary as well as low level activities of 4-OT and YwhB.

4-Oxalocrotonate tautomerase (4-OT, EC 5.3.2),[1] a bacterial isomerase found on the TOL plasmid in *Pseudomonas putida* mt-2, catalyzes the conversion of 2-oxo-4-hexenedioate (**1**; Scheme 1) to 2-oxo-3-hexenedioate (**3**) through a dienol intermediate known commonly as 2-hydroxymuconate (**2**) (*1*, *2*). The presence of the TOL plasmid enables this soil bacterium to use aromatic hydrocarbons such as benzene, toluene, and xylenes as its sole sources of carbon and energy (*1*). The simplicity of the reaction, coupled with the small monomer size (62 amino acids) of the 4-OT hexamer (*3*, *4*), has resulted in an intense investigation of the enzyme's mechanism by a battery of techniques over the past 15 years (*5*−*12*).

Scheme 1



1 **2** **3**

These efforts have established the basic elements of the mechanism. The amino terminal proline functions as a general base catalyst. Arg-39 and an ordered water molecule provide hydrogen bonds to the carbonyl oxygen of **1**, thereby facilitating deprotonation at C-3 (*10*). Arg-11 has a binding and a catalytic role: it interacts with the C-6 carboxylate group and functions as an electron sink to draw electron density to the C-5 position for protonation (*13*). Phe-50 plays a structural role in maintaining the hydrophobic active site environment, which assists in catalysis and contributes to the lowered pK_a of Pro-1 (*12*).

Despite these extensive mechanistic studies, it was only with the recent discovery of CaaD that the structural and mechanistic versatility of the β−α−β structural motif encoded by the 4-OT monomer was realized (*14*). CaaD transforms the *trans*-isomers of 3-bromo- and 3-chloroacrylates (**4** and **5**, respectively; Scheme 2) to malonate semialdehyde (**7**), presumably through an unstable halohydrin intermediate (**6**). CaaD is found in a degradative pathway for *trans*-1,3-dichloropropene, which is used as a nematocide

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[1] Abbreviations: CaaD, *trans*-3-chloroacrylic acid dehalogenase; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactoside; Kn, kanamycin; LB, Luria−Bertani medium; MALDI−PSD, matrix-assisted laser desorption−ionization postsource decay; MALDI-TOF, matrix-assisted laser desorption−ionization time-of-flight; 4-OT, 4-oxalocrotonate tautomerase; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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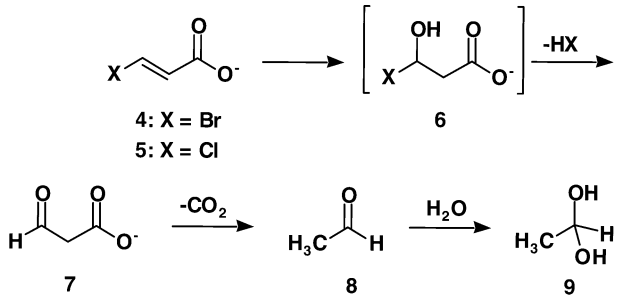
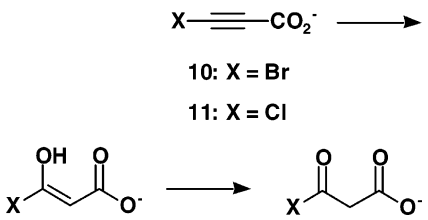
Scheme 2



Scheme 3



(*15*). The enzyme is a heterohexamer comprised of three α-subunits and three *β*-subunits.

Sequence analysis and mutagenesis identified CaaD as a member of the 4-OT family of the tautomerase superfamily (*14*). The α-subunit of CaaD shares 35% sequence identity with YwhB, a 4-OT homologue found in *Bacillus subtilis*, while the *β*-subunit has 25% sequence identity with 4-OT (*16*). Both subunits have an amino terminal proline, one of which, the *β*-Pro-1, is a critical mechanistic residue that functions as a general acid catalyst to protonate C-2 of substrate (*17*). Arginine-11 in the α-subunit is also important in the mechanism—it is one of the residues that interacts with the carboxylate group (*14*).

In view of the sequence and mechanistic similarities, we examined 4-OT and YwhB for low level dehalogenase activity and found that both exhibited the activity, converting either **4** or **5** to a mixture of acetaldehyde (**8**) and its hydrate (**9**) (*18*, *19*). The proposed mechanism involves the enzymatic conversion of the haloacrylates to **7**, which most likely undergoes a nonenzymatic decarboxylation to afford **8**. The presence of this activity further supported a relationship between CaaD and the 4-OT family members, 4-OT and YwhB. It also prompted us to examine the behavior of 4-OT and YwhB with 3-bromo- and 3-chloropropiolate (**10** and **11**; Scheme 3). CaaD converts the 3-halopropiolates into highly reactive acyl halides, which inactivate the enzyme by covalent modification at the *β*-Pro-1 (*20*).

Incubation of the two enzymes with either 3-halopropiolate led to their irreversible inactivation. Both enzymes are inactivated by 3-bromopropiolate due to the covalent modification of Pro-1, which could occur by one of two mechanisms. In the first mechanism, the low level hydratase activity converts the 3-halopropiolates into acyl halides, in an analogous fashion to the inactivation process observed for CaaD. In a second mechanism, Pro-1 (from either 4-OT or YwhB) and the 3-halopropiolates undergo a Michael reaction, resulting in alkylation of the proline. The interaction of Arg-11 with the C-1 carboxylate group of the 3-halopropiolates, in addition to the halogen substituent, would make the C-3 position more susceptible to nucleophilic attack by water (in the first mechanism) or Pro-1 (in the second mechanism). Characterization of the inactivation process for both enzymes and the previously established mechanism for inactivation of 4-OT by another acetylene compound, 2-oxo-3-pentynoate, suggest the Michael reaction as the primary mechanism for inactivation.

Although irreversible inhibitors of 4-OT have previously been reported (*6*, *9*), **10** and **11** are the first such inhibitors reported for YwhB. Moreover, inactivation of YwhB by the modification of Pro-1 confirms the importance of this residue in catalysis. These compounds may be useful ligands in crystallographic studies to further delineate the roles of active site residues in the 4-OT- and YwhB-catalyzed reactions. Such studies may also shed light on the structural factors responsible for the presumed different mode of inactivation observed for 4-OT and YwhB vs that observed for CaaD by the 3-halopropiolates.

MATERIALS AND METHODS

Materials. All reagents, buffers, and solvents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Spectrum Laboratory Products, Inc. (New Brunswick, NJ), or EM Science (Cincinnati, OH), unless noted otherwise. Literature procedures were used for the syntheses of the 3-bromo- and 3-chloropropiolates (**10** and **11**) (*21*, *22*). Tryptone, yeast extract, and agar were obtained from Becton, Dickerson, and Company (Franklin Lakes, NJ). The Ultrafree DA centrifugal filter units, the YM-3 ultrafiltration membranes, and the Amicon stirred concentrators were obtained from Millipore Corp. (Bedford, MA). 4-OT was purified by a previously described procedure (*8*). The expression vector pET-24a-(+) was obtained from Novagen, Inc. (Madison, WI). Restriction enzymes, T4 DNA ligase, agarose, the Wizard PCR Preps DNA purification kit, and the Wizard Plus Minipreps DNA Purification System were obtained from Promega Corp. (Madison, WI). The Geneclean II kit was purchased from Bio 101, Inc. (La Jolla, CA). The PCR was carried out with reagents from the GeneAmp kit obtained from Perkin-Elmer Cetus (Norwalk, CT). Oligonucleotides for DNA sequencing, amplification, or mutant construction were synthesized by Oligos Etc. (Wilsonville, OR). Genomic DNA from *B. subtilis* was prepared by a previously published procedure (*23*). Thin-walled PCR tubes were obtained from Ambion, Inc. (Austin, TX).

Strains. B. subtilis strain 168 was obtained from the *Bacillus* Genetic Stock Center (Department of Biochemistry, Ohio State University, Columbus, Ohio). *Escherichia coli* strain JM109 (Promega) was used for cloning and isolation of plasmids. *E. coli* strains BL21(DE3)pLysS from Novagen or BL21-Gold(DE3)pLysS from Stratagene (La Jolla, CA) were used for recombinant protein expression.

Methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere (*24*). The composition of LB is described elsewhere (*24*). DNA sequencing was done at the University of Texas (Austin) Sequencing Facility. Kinetic data were obtained on an Agilent 8453 Diode Array spectrophotometer (Agilent Technologies, Palo Alto, CA). The solutions in the cuvettes were mixed using a stir/add cuvette mixer (Bel-Art Products, Pequannock, NJ). The kinetic data were fitted by nonlinear

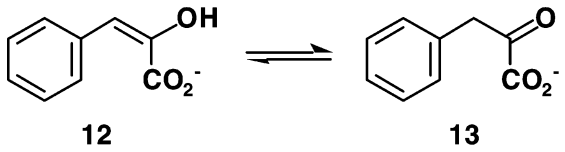
regression data analysis using the Grafit program (Erithacus Software Ltd., Horley, U.K.) obtained from Sigma Aldrich Chemical Co. HPLC was performed on a Beckman System Gold HPLC (Fullerton, CA) using a TSKgel Phenyl-5PW hydrophobic column (Tosoh Bioscience, Montgomeryville, PA). Protein was analyzed by Tris glycine SDS−PAGE under denaturing conditions on 17.5% gels using either the Mini-Protean II or III vertical gel electrophoresis apparatus obtained from Bio-Rad (Hercules, CA) (*25*). Protein concentrations were determined using the method of Waddell (*26*).

Construction of the Expression Vector for ywhB from B. subtilis. Two oligonucleotides, 5′-GAAGGAGATATA-*CATATG*CCATACGTAACTGTCAAA-3′ and 5′-GAGCT-CGAATTC*GGATCC*CTATTATTCCATATCGTCCAG-3′, were synthesized for the amplification of the *ywhB* gene from *B. subtilis*. The first primer contains a *Nde*I restriction site (underlined) followed by 18 bases corresponding to the coding sequence of the *ywhB* gene. The second primer contains a *BamH*I restriction site (underlined) followed by 21 bases corresponding to the complementary sequence of the *ywhB* gene. The additional 12 bases preceding the restriction sites in both primers facilitate cleavage of the DNA by the restriction enzymes. Amplification of the *ywhB* gene was carried out by the PCR in a Perkin-Elmer 480 DNA thermal cycler using the two synthetic primers, genomic DNA from *B. subtilis* as the template (∼2000 μg), and the PCR reagents following a protocol described elsewhere (*8*). The resulting gel-purified PCR product and the pET-24a-(+) vector were treated with *Nde*I and *BamH*I restriction enzymes, purified, and ligated using T4 DNA ligase following a previously described protocol (*8*). Aliquots of the resulting mixture were transformed into competent *E. coli* JM109 cells and grown on LB/Kn (100 μg/mL) agar plates at 37 °C. Single colonies were chosen at random and grown in liquid LB/Kn media (50−100 μg/mL) at 37 °C. The newly constructed plasmid (designated pET-24a-ywhB) was isolated and sequenced. Subsequently, the plasmid containing the *ywhB* gene was transformed as described elsewhere into *E. coli* strain BL21(DE3)pLysS for protein expression (*8*).

Overexpression, Purification, and Characterization of the YwhB Product. The product of the *ywhB* gene was overexpressed using a previously described protocol (*12*). Typically, 4.5 L of culture grown under these conditions yields ∼14 g of cells. The enzyme was purified to near homogeneity (>99% as assessed by the presence of a single band in SDS−PAGE) using both the Tosoh Phenyl 5-PW hydrophobic column and the Sephadex G-75 gel filtration column (2.5 cm × 100 cm) as described elsewhere (*8*). Typically, the yield of purified protein per liter of culture was ∼50 mg. The native molecular mass of the protein was estimated by size exclusion chromatography on a Superose 12 column (Amersham Biosciences, Piscataway, NJ). The enzyme was chromatographed in 100 μL portions (∼6.2 mg/mL) on the column equilibrated with 20 mM NaH_2PO_4 buffer, pH 7.3, at a flow rate of 0.2 mL/min. The protein was monitored at 280 nm and typically eluted at ∼74 min.

Mass Spectral Analysis of YwhB. The monomeric molecular mass of the YwhB product was determined by ESI-MS using a LCQ Finngan octapole electrospray mass spectrometer. Samples for ESI-MS were prepared as previously described (*10*). The observed molecular mass (MH^+) for the YwhB product was 7015.0 Da (calcd, 7014.0 Da).

Enzyme Assays. The activity of 4-OT was determined by a previously described assay using **2** (*2*), following the increase in absorbance at 236 nm due to the formation of **3**. The activity of YwhB was determined using the enol isomer of phenylpyruvate (**12**). Ketonization of **12** to generate the keto isomer, **13**, was followed by the decay at 288 nm (*27*).



12 **13**

*Irreversible Inhibition of 4-OT by **10** and **11**.* The inactivation of 4-OT by **10** or **11** was determined by incubating various concentrations of inhibitor (**10**, 50−600 μM; and **11**, 50−750 μM) with 4-OT in 20 mM sodium phosphate buffer (pH 6.0) at 30 °C. At each inhibitor concentration, three runs were performed. Stock solutions of **10** and **11** (50 mM) were made in 100 mM Na_2HPO_4 buffer and had a final pH ∼7. The 50 mM stock solutions were diluted with a sufficient volume of the 100 mM NaH_2PO_4 buffer (pH 7.3) to give 5 mM solutions of **10** or **11**. A quantity of 4-OT (4 μL of a 21.5 mg/mL solution) was diluted 2000-fold into 20 mM sodium phosphate buffer (pH 6.0), resulting in a final concentration of 1.58 μM. The diluted enzyme was stored at 4 °C. Subsequently, the enzyme was divided into 105 μL aliquots and equilibrated at 30 °C for ∼1 h prior to use. The time zero point (i.e., 100% activity) was determined at each inhibitor concentration by removing and assaying an aliquot (5 μL) of enzyme just before the addition of inhibitor. After the addition of inhibitor, aliquots (5 μL) were removed at 7 s intervals (for a total 56 s), diluted into 1 mL of 20 mM NaH_2PO_4 buffer (pH 7.3), and assayed for residual 4-OT activity. The assay was initiated by the addition of **2** to give a final concentration of 500 μM. The volume of inhibitor added did not exceed 7.5% of the total volume of the incubation mix.

*Protection of 4-OT from Inactivation by **10** Using **1**−**3**.* The protection studies of 4-OT from inactivation by **10** were carried out using the equilibrium mixture of **1**−**3** as described elsewhere (*6*), with the following modifications. A stock solution of **2** (83.5 mM) was made in 100 mM Na_2HPO_4 buffer (pH 9.2), resulting in a final pH of ∼6.8. The stock solution was diluted 10-fold into 100 mM NaH_2PO_4 buffer (pH 7.3). Quantities of 4-OT (105 μL) were prepared as described above. Aliquots of **2** (0−5 μL from the 8.35 mM solution resulting in concentrations ranging from 0−1.04 mM) were added to the enzyme and incubated at 30 °C for ∼5 min, thereby allowing the mixture of **1**−**3** to reach equilibrium. Subsequently, an aliquot of **10** (2.5 μL from a 5 mM solution) was added, resulting in a final concentration of 125 μM. Aliquots (5 μL) were removed at 7 s intervals (for a total of 56 s), diluted 200-fold into 20 mM sodium phosphate buffer (1 mL, pH 7.3), and assayed for residual activity as described above.

Irreversibility of the Inactivation of 4-OT. 4-OT (1.58 μM based on the molecular weight of the monomer) was incubated with an excess of **10** or **11** (50 μM) in 4 mL of 20 mM NaH_2PO_4 buffer (pH 7.3) for 2 h at 29 °C. The final

pH of the solution was ~7. In a separate control reaction, an identical quantity of 4-OT was incubated without inhibitor under identical conditions. The treated samples had no activity after 2 h. The three samples were dialyzed against 20 mM NaH_2PO_4 buffer, pH 7.3, at 4 °C. After 2 weeks, the control sample lost no detectable activity, while the treated samples did not regain any detectable activity.

*ESI-MS Analysis of 4-OT and 4-OT Modified by **10** or **11***. Each sample contained ~1 mg of 4-OT (47 μL of a 21.5 mg/mL solution) in a final volume of 1 mL of 20 mM sodium phosphate buffer (pH 7.3). The modified samples were generated by the incubation of 4-OT with either **10** or **11** (53 μL of a 50 mM stock solution to give a final concentration of 2.65 mM). The control samples were treated with an equivalent amount of 100 mM sodium phosphate buffer (pH 7.3). Samples were incubated for ~18 h at 4 °C and assayed for activity. The control sample retained activity while the treated samples had no detectable activity. Subsequently, the samples were passed through separate PD-10 Sephadex columns equilibrated in deionized water. Fractions (~0.5 mL) were collected, and protein was identified by the Waddell method (*26*). Samples were loaded as 50% infusions with methanol (8−15 μL) or through a peptide trap for ESI-MS analysis.

*Proteolytic Digestion of 4-OT and 4-OT Modified by **10***. Two samples were made up for mass spectral analysis. Each sample contained ~1 mg of 4-OT (0.5 mL of a 21.5 mg/mL solution) in a sufficient quantity of 20 mM sodium phosphate buffer, pH 7.3, to give a final volume of 2.5 mL. One sample was treated with **10** (50 μL from a 50 mM stock solution of **10** in 100 mM Na_2HPO_4 buffer). After the samples were incubated at 4 °C for ~12 h, the individual samples were exchanged (~99.8%) into 40 mM ammonium acetate buffer, pH 4.0, using an Amicon equipped with a YM-3 membrane. The treated 4-OT gave a yellow color, while the untreated sample remained colorless. Subsequently, both samples were treated with sequencing grade endoproteinase Glu-C from *Staphylococcus aureus* (protease V8) using a modification of a literature procedure (*28*). Accordingly, two vials of endoproteinase Glu-C (25 μg) were each reconstituted in 40 mM ammonium acetate buffer (50 μL), pH 4.0. A quantity (50 μL) of the reconstituted protease was added to each sample, and the two samples were incubated at 37 °C overnight. The mixtures were subjected to MALDI mass spectral analysis.

*Irreversible Inhibition of YwhB by **10** and **11***. The inhibition studies of YwhB with **10** and **11** were carried out as described above for 4-OT with the following modifications. A quantity of YwhB (100 μL of a 25.6 mg/mL solution) was diluted 100-fold into 20 mM sodium phosphate buffer (pH 6.0) to give a final concentration of 36.5 μM. The enzyme was stored at 4 °C. The diluted enzyme was divided into 220 μL portions and allowed to equilibrate to 30 °C for ~1 h just prior to use. The inactivation of YwhB by **10** or **11** was determined by the incubation of inhibitor (**10**, 50−1000 μM; and **11**, 50−750 μM) with enzyme (220 μL quantities) in 20 mM sodium phosphate buffer (pH 6.0) at 30 °C. The volume of inhibitor added did not exceed 4% of the total volume of the inhibition mix. Aliquots (20 μL) were removed at various time points (for **10**, 10 s intervals for 50−150 μM and 7 s intervals for 200−1000 μM; and for **11**, 10 s intervals for 50−100 μM and 7 s intervals for 125−750 μM), diluted into 1 mL of 20 mM sodium phosphate buffer (pH 7.3), and assayed for residual activity. The assay was initiated by the addition of **12** to give a final concentration of 150 μM.

*pH Dependence of the Inactivation of YwhB Using **11***. Inactivation experiments were carried out as described above at five different pH values (pH 5.2, 6, 7, 8, and 9) using a quantity of YwhB (10 μL of a 25.6 mg/mL solution) diluted 100-fold into 20 mM sodium phosphate buffer at the indicated pH values. The diluted enzyme solutions were incubated at 4 °C overnight. The concentration of **11** tested at each pH was 250 μM. Time points were determined at 7 s intervals.

*Protection of YwhB from Inactivation by **10** Using **1**−**3***. The protection of YwhB from inactivation by **10** was carried out using **1**−**3** as described above in the 4-OT protection studies with the following modifications. Quantities of YwhB (220 μL portions made up as described above) were placed in 1.5 mL eppendorf tubes. Aliquots (0−6.6 μL) of **2** were added to the enzyme, and the resulting solution was equilibrated for ~5 min. The concentrations of **2** in the eppendorf tubes ranged from 0−2.5 mM. Subsequently, an aliquot of **10** (1.2 μL from a 50 mM solution) was added to the mixture, resulting in a final concentration of 300 μM. Aliquots (20 μL) were removed from the mixtures at 7 s intervals, diluted 50-fold into 20 mM sodium phosphate buffer (1 mL, pH 7.3), and assayed for residual activity as described above.

Irreversibility of the Inactivation of YwhB. The irreversibility of the reaction was established using both **10** and **11**. YwhB (142 μM based on the monomer molecular weight) was incubated with an excess of **10** or **11** (3 mM) in 1 mL of 20 mM NaH_2PO_4 buffer (pH 7.3) for 2 h at 29 °C. The final pH of the solution was ~7. In a separate control reaction, the same quantity of enzyme was incubated without inhibitor under similar conditions. The samples treated with inhibitors had no activity after 2 h. The three samples were dialyzed against 20 mM NaH_2PO_4 buffer, pH 7.3, at 4 °C. After 8 days, the control sample lost ~6% of its original activity, while the treated samples did not regain any activity.

*ESI-MS Analysis of YwhB and YwhB Modified by **10** or **11***. Each sample contained ~1 mg of YwhB (40 μL of a 25.6 mg/mL solution) in a final volume of 1 mL of 20 mM sodium phosphate buffer (pH 7.3). The modified samples were generated by the incubation of YwhB with either **10** or **11** (60 μL from a 50 mM stock solution to give a final concentration of 3 mM). The control samples contained an equivalent amount of 100 mM sodium phosphate buffer (pH 7.3). Samples were incubated for ~14 h at 4 °C and assayed for activity. The control samples retained activity while the treated samples had no detectable activity. Subsequently, the samples were passed through separate PD-10 Sephadex columns equilibrated in deionized water. Fractions (~0.5 mL) were collected, and protein was identified by the Waddell method (*26*). Samples (5−10 μL) were loaded through a peptide trap for ESI-MS analysis.

*Proteolytic Digestion of YwhB and YwhB Modified by **10***. To determine the site of covalent modification, it was necessary to treat YwhB and YwhB modified by **10** with protease V8 and trypsin (in separate reactions). For the proteolytic digestion using protease V8, each sample contained ~0.5 mg of YwhB (20 μL of a 25.6 mg/mL solution)

in a sufficient quantity of 20 mM sodium phosphate buffer (pH 7.3) to give a final volume of ~0.5 mL. One sample was treated with **10** (30 μL from a 50 mM stock solution in 100 mM Na_2HPO_4 buffer). The samples were incubated at 4 °C for ~20 h. Subsequently, each sample was treated using the protocol described above and subjected to MALDI mass spectral analysis. For the proteolytic digestion using trypsin, the YwhB samples were prepared as described above and incubated at 4 °C for ~14 h. Subsequently, they were passed through separate PD-10 Sephadex columns equilibrated in 100 mM $(NH_4)_2CO_3$ buffer, pH 8. Fractions (~0.5 mL) were collected, and protein was identified by the Waddell method (*26*). Both samples were treated with sequencing grade trypsin according to literature procedures (*29*) and subjected to MALDI mass spectral analysis.

Mass Spectrometry of the Intact and Digested Proteins. The intact samples (4-OT, 4-OT modified by **10** or **11**, YwhB, and YwhB modified by **10** or **11**) were analyzed using an LCQ electrospray ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) as described previously (*20*). The protease-digested control and treated samples (using trypsin or protease V8) were analyzed on the delayed extraction Voyager-DE PRO MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using a previously described protocol (*20*). The ions in the trypsin-digested treated YwhB modified by **10** were subjected to MALDI−PSD analysis using a protocol described elsewhere (*20*, *30*).

RESULTS

Production, Expression, and Kinetic Characterization of YwhB. The *ywhB* gene was amplified from genomic DNA, cloned into a pET-24a(+) vector, and expressed in *E. coli* strain BL21(DE3)pLys. The DNA sequence of the cloned gene was confirmed by sequence analysis. Subsequently, the gene product was purified to homogeneity (as assessed by SDS−PAGE) using two chromatographic steps (hydrophobic interaction and gel filtration) (*8*). Mass spectral analysis confirmed the expected subunit molecular mass of the YwhB product, further establishing that no mutations had been introduced into the gene sequence during the PCR and demonstrating that Pro-1 is not blocked by the initiating methionine. On a size exclusion column, YwhB elutes comparably to 4-OT, indicating that YwhB is a homohexamer. This observation is consistent with the crystal structure of YwhB, where a hexamer is observed (*19*).

Several enol and dienol substrates have been examined as potential substrates for YwhB. Although a physiological function for YwhB has not been established, it is able to catalyze a 1,3-keto-enol tautomerization reaction (*19*). In the inhibition studies reported here, the ketonization of the enol isomer of phenylpyruvate (**12**) to the keto isomer, **13**, was monitored as a measure of activity (following the decay at 288 nm).

*Time-Dependent Inactivation of 4-OT by **10** and **11***. The initial inactivation experiments of 4-OT by **10** and **11** were carried out at pH 7.3 (data not shown). The data indicated that it was not possible to saturate the enzyme with inhibitor, which prompted us to examine the behavior of 4-OT with these inhibitors under different conditions. In view of the observation that the inactivation of YwhB by **11** is pH-dependent (vide infra) and the enzyme approached saturation kinetics at lower pH values, the 4-OT inhibition studies were carried out at pH 6.0.

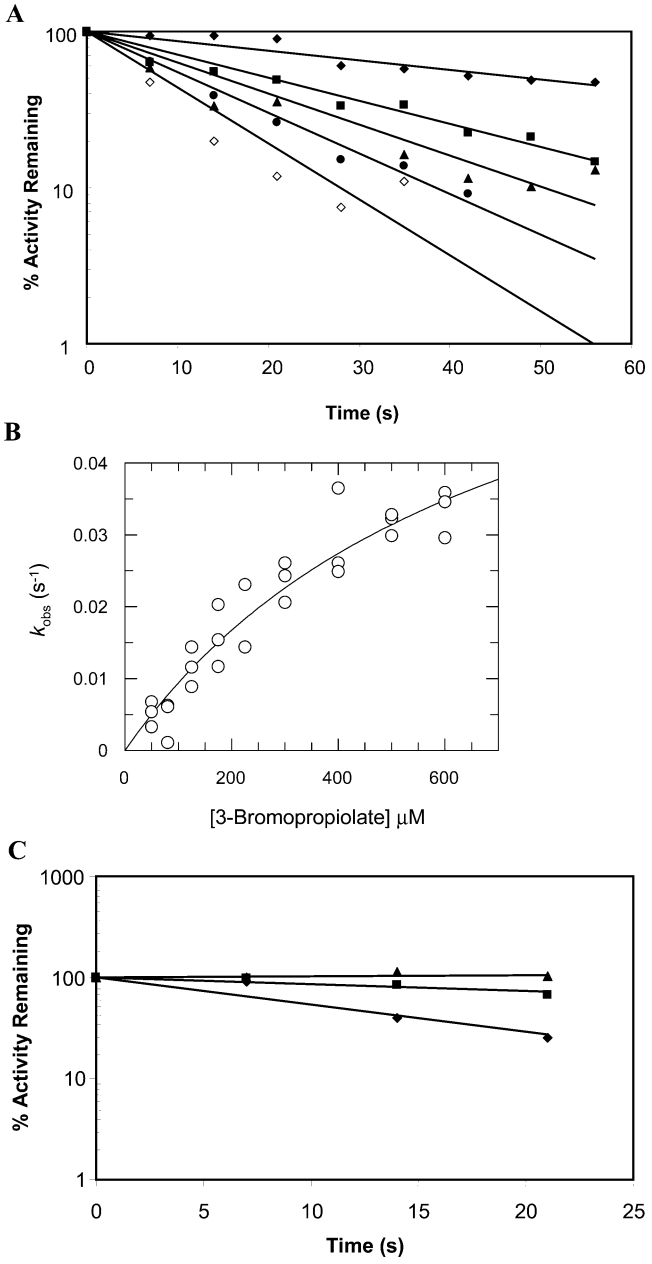


FIGURE 1: Kinetics of the inactivation of 4-OT by **10** at pH 6.0 and protection using **1**−**3**. (A) A logarithmic plot of the percent of activity remaining as a function of time with varying amounts of **10** (filled diamonds, 80 μM; filled squares, 175 μM; filled triangles, 225 μM; filled circles, 400 μM; and open diamonds, 500 μM). (B) A plot of k_{obs} for inactivation vs the concentration of **10**. (C) Protection of 4-OT by an equilibrium mixture of **1**−**3**. 4-OT was preincubated with varying amounts of **1**−**3** (filled diamonds, 0 μM; filled squares, 0.41 mM; and filled triangles, 1.04 mM). The concentration of **10** was maintained at 125 μM.

Accordingly, incubation of 4-OT with either **10** or **11** results in a rapid time-dependent, irreversible inactivation of the enzyme at pH 6.0 (Figures 1A and 2A). For **10**, the k_{obsd} values measured in nine experiments were plotted vs the inhibitor concentration and fit to a rectangular hyperbola (Figure 1B). The values of k_{inact} and K_I obtained from this plot are 0.08 ± 0.02 s^{-1} and 0.71 ± 0.23 mM, respectively. Treating the k_{obsd} values measured in seven experiments for **11** (Figure 2A,B) similarly yielded k_{inact} and K_I values of

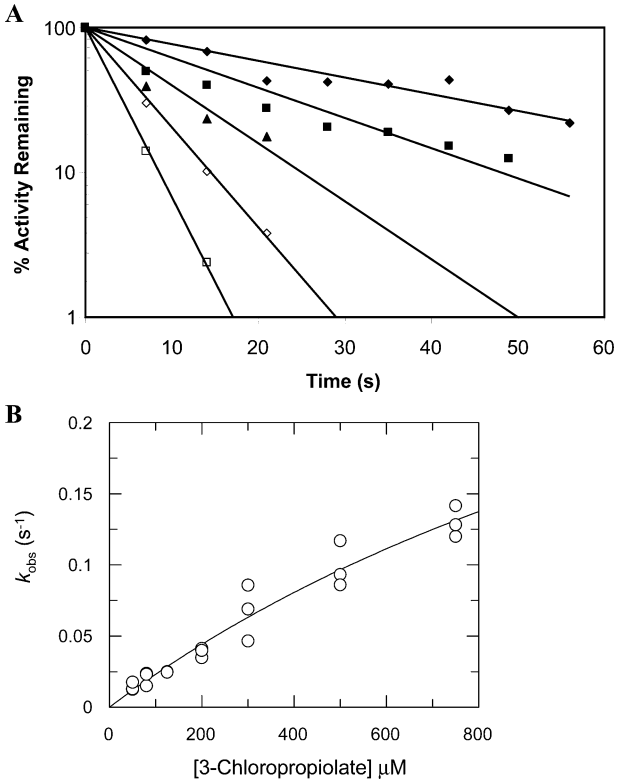


FIGURE 2: Kinetics of the inactivation of 4-OT by **11** at pH 6.0. (A) A logarithmic plot of the percent of activity remaining as a function of time with varying amounts of **11** (filled diamonds, 50 μM; filled squares, 80 μM; filled triangles, 200 μM; open diamonds, 300 μM; and open squares, 500 μM). (B) A plot of k_{obs} for inactivation vs the concentration of **11**.

0.47 ± 0.16 s^{-1} and 1.91 ± 0.85 mM, respectively. The rapid loss of activity at higher concentrations of **10** (>600 μM) or **11** (>750 μM) did not allow us to collect sufficiently precise data to obtain more accurate inactivation rates, thereby accounting for the large errors in the values of k_{inact} and K_I. Nonetheless, inactivation with both inhibitors nears saturation, indicative that a dissociable complex forms between enzyme and **10** (or **11**) before inactivation. This supposition is supported by the observation that the enzyme could be protected from inactivation (by **10**) when incubated with the equilibrium mixture of **1**−**3** (Figure 1C). At ~1 mM, 4-OT is completely protected from inactivation by **10** (125 μM). In the absence of **1**−**3**, the enzyme loses ~70% of its activity within ~21 s. Exhaustive dialysis of 4-OT (inactivated by **10** or **11**) does not result in the reactivation of 4-OT, which indicates that a covalent bond has formed between 4-OT and **10** (or **11**) or a species derived from **10** (or **11**).

*ESI-MS Analysis of 4-OT and 4-OT Modified by **10** or **11***. To determine whether the enzyme had been covalently modified by **10** (or **11**), 4-OT was incubated with both inhibitors in separate reactions, and the inactivated protein was isolated and analyzed by ESI-MS. The molecular mass of 4-OT increases from 6810.0 (±3 Da) to 6896.0 (±3 Da) after incubation with **10**, and it increases to 6895.0 (±3 Da) after incubation with **11**. Hence, both treated proteins are covalently modified by a species having a mass of 85 (±3 Da). In addition, the spectra displayed another signal at 6852 (±3 Da), which is due to modification of 4-OT by species having a mass of 42 (±3 Da).

Table 1: Mass Spectral Analysis of V8 Digest of 4-OT

peptide fragment	calcd mass[a]	observed mass (control)	observed mass (treated)
1−9	1034.24	1033.59, 1055.69,[b] 1071.64[b]	1077.73, 1097.62,[b] 1113. 68[b]
10−17	948.96	948.44	948.47
18−22	631.75	631.38	631.40
23−44	2357.70	2356.22	2356.33
45−62	1915.27	1913.98	1914.07

[a] These values were calculated using the average molecular mass (Da). [b] The +22 and +38 peaks are sodium and potassium adducts, respectively.

Identification of the Modified Amino Acid Residue in 4-OT by Mass Spectrometry. To identify the covalently modified residue, 4-OT was inactivated by **10** (designated the treated sample) and incubated overnight with protease V8, and the mixture was analyzed by MALDI-TOF mass spectrometry. A control sample was made up similarly, but **10** was excluded from the mixture. The site of covalent attachment was determined by comparing the two spectra. In the MALDI-MS spectrum of the control sample, there is a prominent peak at m/z 1033.59 ± 0.6 Da, which corresponds to the calculated mass of the N-terminal sequence (PIAQI-HILE) (Table 1). Two smaller peaks at 1055.69 and 1071.64 Da are also present and represent the sodium and potassium adducts of this fragment, respectively. In the MALDI spectrum of the treated sample, these peaks are not present. However, there are three new peaks at 1075.73, 1097.71, and 1113.68 Da. These new peaks are consistent with covalent modification of the N-terminal fragment by a species having a mass of 42 Da.[2] The two potential sites for covalent modification in this fragment are Pro-1 and His-6. It can be reasonably concluded that Pro-1 is the site of attachment because the kinetic studies indicate that **10** binds in the active site, and His-6 is not in the active site. Finally, a comparison of the MALDI spectra indicates that no other fragments are modified, which is consistent with the results of the ESI-MS analysis showing a single site of attachment.

*Time-Dependent Inactivation of YwhB by **10** and **11***. When the inactivation experiments (using **10** and **11**) were carried out at pH 7.3, YwhB was rapidly inactivated, which precluded the collection of accurate data for the determination of k_{obsd} values. Hence, the pH dependence of the inactivation process was examined as a function of pH. Because the process slowed at lower pH values, the inactivation studies of YwhB were carried out at pH 6.0. Like 4-OT, the incubation of YwhB with either **10** or **11** results in a rapid time-dependent, irreversible inactivation of the enzyme (Figures 3A and 4A). For **10**, the k_{obsd} values measured in nine experiments were plotted vs the inhibitor concentration and fit to a rectangular hyperbola (Figure 3B). The values of k_{inact} and K_I values obtained from this plot are 0.08 ± 0.02 s^{-1} and 1.60 ± 0.47 mM, respectively. Treating k_{obsd} values measured in 12 experiments for **11** similarly yielded

[2] ESI-MS analyses of the intact, modified 4-OT and YwhB proteins show increases of +85 and +42 Da, corresponding to the malonyl adduct and the acetyl adduct, respectively. However, the mass spectra of the digested proteins (inactivated by **10**) show only mass shifts of +42 Da, reflecting the presence of the acetyl adduct. This discrepancy is likely due to the complete decarboxylation of the malonyl adduct during proteolysis or in the MALDI deposition or ionization process.

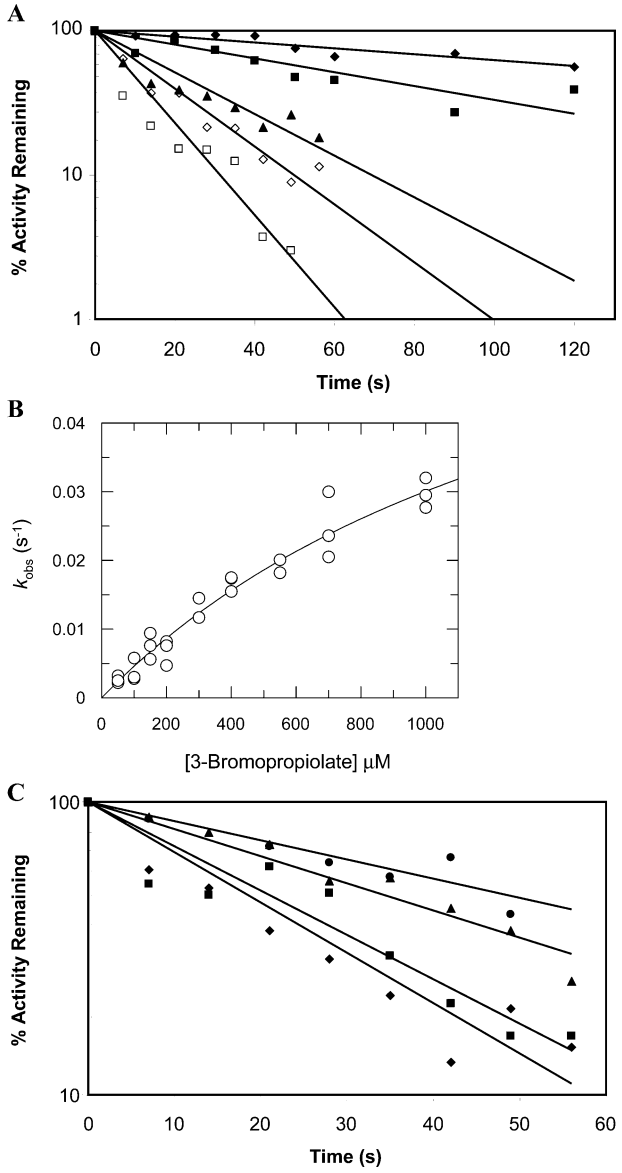


FIGURE 3: Kinetics of the inactivation of YwhB by **10** at pH 6.0 and protection using **1**−**3**. (A) A logarithmic plot of the percent of activity remaining as a function of time with varying amounts of **10** (filled diamonds, 50 μM; filled squares, 100 μM; filled triangles, 300 μM; open diamonds, 550 μM; and open squares, 1000 μM). (B) A plot of k_{obs} for inactivation vs the concentration of **10**. (C) Protection of YwhB by an equilibrium mixture of **1**−**3**. YwhB was preincubated with varying amounts of **1**−**3** (filled diamonds, 0 μM; filled squares, 0.83 mM; filled triangles, 1.67 mM; and filled circles, 2.5 mM). The concentration of **10** was maintained at 300 μM.

k_{inact} and K_I values of 0.11 ± 0.02 s^{-1} and 0.88 ± 0.18 mM, respectively (Figure 4B). Inactivation with both inhibitors approaches saturation, indicative that a dissociable complex forms between enzyme and **10** (or **11**) before inactivation. The results of the protection studies (using a mixture of **1**−**3**) further support binding at the active site (Figure 3C). At ~2.5 mM, YwhB is partially protected from inactivation by **10** (300 μM). In the absence of **1**−**3**, the enzyme loses ~85% of its activity within 1 min. In the presence of **1**−**3** (2.5 mM), the enzyme only loses ~60% of its activity within 1 min. Exhaustive dialysis of YwhB (inactivated by **10** or **11**) does not result in the reactivation of YwhB, which indicates that a covalent bond has formed between YwhB and **10** (or **11**) or a species derived from **10** (or **11**).

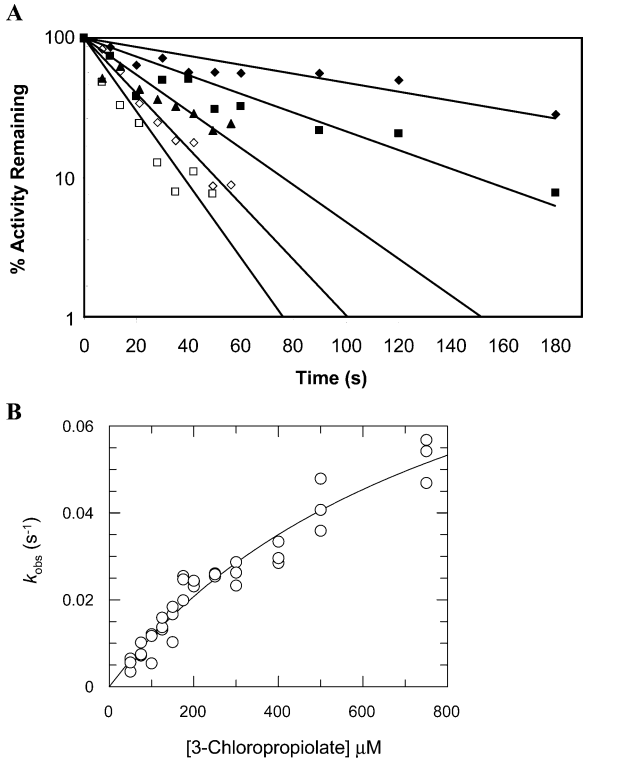


FIGURE 4: Kinetics of the inactivation of YwhB by **11** at pH 6.0. (A) A logarithmic plot of the percent of activity remaining as a function of time with varying amounts of **11** (filled diamonds, 50 μM; filled squares, 75 μM; filled triangles, 125 μM; open diamonds, 175 μM; and open squares, 300 μM). (B) A plot of k_{obs} for inactivation vs the concentration of **11**.

Table 2: Mass Spectral Analysis of the V8 Digest of YwhB

peptide fragment	calcd mass[a]	observed mass (control)	observed mass (treated)
1−9	1080.33	1079.57	1121.58 (weak)
1−25	2964.40	2962.49	3004.58
10−25	1903.10	1901.95	1901.98
30−44	1652.84	1651.80 (weak)	1651.84
36−44	1105.29	1105.60	1105.61
36−61	3095.60	3093.59	3093.51
37−43	976.10	976.58	976.57
44−61	2136.43	2135.98	2136.02
45−61	2008.32	2006.95	2006.97

[a] These values were calculated using the average molecular mass (Da).

*ESI-MS Analysis of YwhB and YwhB Modified by **10** or **11***. ESI-MS analysis demonstrated that the incubation of YwhB with **10** or **11** resulted in an increase in its molecular mass from 7013.0 (±3 Da) to 7099.0 (±3 Da). This observation is consistent with the covalent attachment of a species to YwhB with a mass of 86 (±3 Da). ESI-MS analysis also showed another peak at 7055 (±3 Da), which is consistent with the covalent modification of YwhB by a species having a molecular mass of 42 (±3 Da).

Identification of the Modified Amino Acid Residue in YwhB by Mass Spectrometry. To determine the residue modified by **10**, it was necessary to carry out two separate proteolytic digestions using protease V8 and trypsin. The mixtures were analyzed by MALDI-TOF mass spectrometry. The protease V8 digestion of the YwhB and YwhB modified by **10** generated nine fragments (Table 2). A molecular mass (1079.84 Da) corresponding to the N-terminal fragment

Table 3: Mass Spectral Analysis of the Tryptic Digest of YwhB

peptide fragment	calcd mass[a]	observed mass (control)	observed mass (treated)
1−6	706.86	not detected	748.38 (weak)
7−11	605.73	605.30	605.24
18−37	2163.38	2163.17	not detected
23−37	1579.70	not detected	1579.73 (weak)
38−46	1136.40	1135.61	1135.55
38−47	1263.56	1263.71	1263.65
38−55	2106.49	2105.15	2105.05
38−56	2262.68	2261.24	2261.13

[a] These values were calculated using the average molecular mass (Da).

Table 4: PSD Analysis of the N-Terminal Fragment (Residues 1−6) of Treated YwhB

ion	calcd mass[a]	observed mass
parent	748.4	748.4
b_1	140.1	140.0
b_2	303.1	303.0
b_3	402.2	402.6
b_4	503.2	503.3
b_5	602.3	not detected

[a] These values were calculated using the monoisotopic molecular mass (Da).

(residues 1−9) was observed in the control sample and was not present in the treated sample. However, a signal appeared at 1121.6 Da, which corresponds to the N-terminal fragment modified by a species with a molecular mass of 42 Da. Unfortunately, the intensity of this signal placed it just above the noise, so that a definitive conclusion could not be made. A second signal appeared in the control sample at 2962.5 Da, corresponding to residues 1−25. This signal was not present in the YwhB sample treated with **10** (and digested by V8), but a new signal appeared at 3004.6, which corresponds to the N-terminal fragment (residues 1−25) modified by a species with a molecular mass of 42 Da. A third signal appeared in the control sample at 1901.95 Da, which corresponds to residues 10−25. The same signal also appears in the sample treated with **10**. These observations localize the site of modification of YwhB by **10** to the first nine amino acids of YwhB (PYVTVKMLE). The other fragments were not modified.

To identify the modified amino acid within this fragment, YwhB and YwhB modified by **10** were subjected to proteolysis by trypsin (Table 3). The digestion generated eight fragments with the most prominent signal being displayed at 1135.61 Da, corresponding to residues 38−46. This signal was also present in the sample treated with **10**, indicating that these residues had not been modified, consistent with the results obtained for the V8 digestion. A molecular mass (calcd, 706.86 Da) corresponding to one expected product of the trypsin digest, the N-terminal fragment (residues 1−6), was not observed in the control sample. However, a signal appeared at 748.38 Da in the treated sample, which corresponds to residues 1−6 modified by a species with a molecular mass of 42 Da.[2] A MALDI−PSD spectrum of this peptide shows a series of b ions, which have a charge on the N-terminal side of the fragmented peptide (Table 4 and Figure 5). Each ion is modified by a mass of +42 Da (except the b_5 ion, which is not detected), indicating that the modification is on the N-terminal proline.

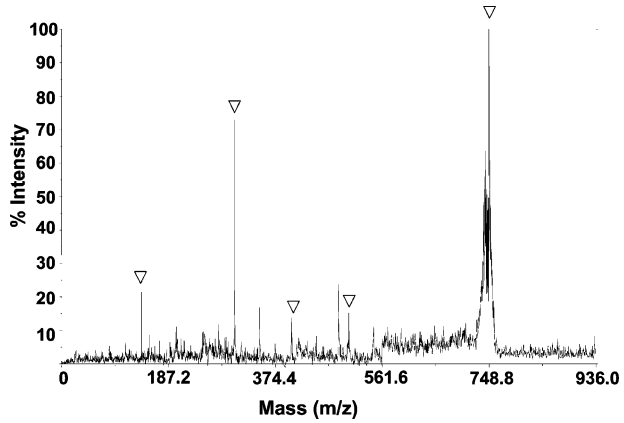


FIGURE 5: MALDI−PSD fragmentation spectra of the peak at 748.38 Da in the trypsin digest, corresponding to the PYVTVK (1−6) fragment of YwhB modified by a covalent adduct of +42 Da. The parent signal and the b_1−b_4 ions, shifted by +42 Da, are identified by open triangles.

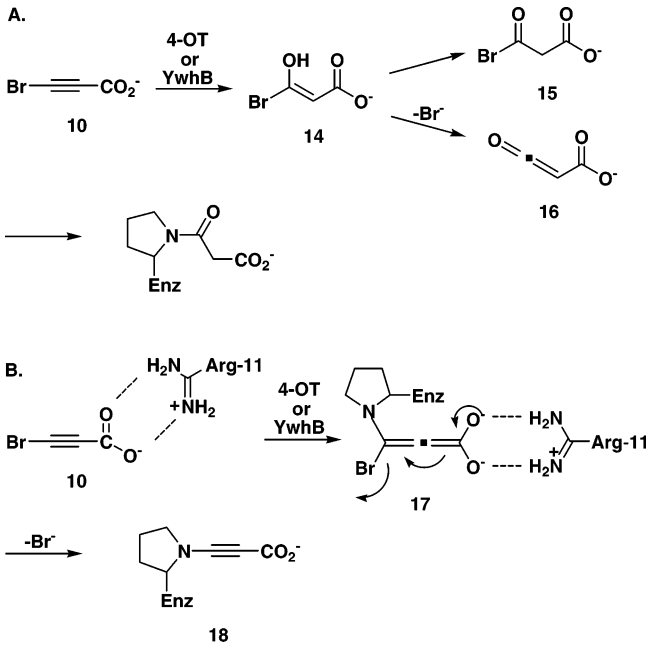
DISCUSSION

The question of how new enzymatic activities arise in nature is an intriguing one, which has been studied over the past several years (*31*−*36*). The catalytic promiscuity of enzymes, that is, the capability of some enzymes to carry out one or more low level activities in addition to a primary activity, plays an important role in the creation of new activities (*35*, *36*). We have recently reported that 4-OT and YwhB have a low level dehalogenase activity, transforming **4** and **5** to acetaldehyde (**8**) (*18*, *19*). We found this to be a particularly interesting observation because of the striking differences between the primary activities of these enzymes (i.e., isomerization and tautomerization) and the low level activity (hydration resulting in dehalogenation).

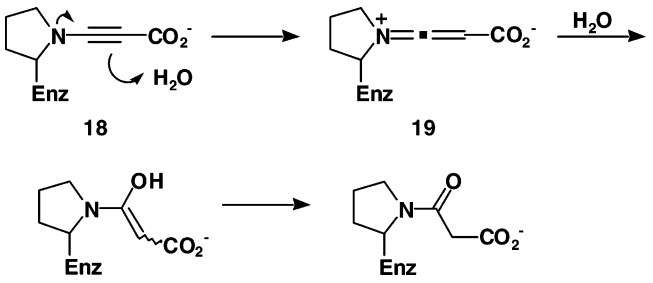
Two mechanisms were postulated for this dehalogenation reaction that rely on Pro-1 as well as a positively charged arginine (Arg-11 or Arg-39 in 4-OT or Arg-11 in YwhB). In both mechanisms, arginine interacts with the carboxylate group of either **4** or **5** and through this interaction polarizes the α,β-unsaturated acids to form an enolic intermediate. This role is analogous to that proposed for Arg-11 in the 4-OT-catalyzed conversion of **1** to **3**, in which the interaction between Arg-11 and the substrate's carboxylate group draws electron density to the C-5 position to facilitate protonation (*13*). The presence of the positive charge at C-3 (of either **4** or **5**) would facilitate the attack of water at C-3. (*13*). In the first mechanism, Pro-1 may function as a general base to activate the water molecule for attack at 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 the second mechanism, the water molecule could be activated by another residue (or not activated at all) and Pro-1 would function as a general acid catalyst and deliver a proton to the C-2 position upon ketonization of the enolic intermediate.

To further explore these mechanisms, we examined the behavior of 4-OT and YwhB with 3-bromo- and 3-chloropropiolate (**10** and **11**). Incubation of 4-OT or YwhB with **10** or **11** results in their irreversible inactivation by the modification of a single residue. Further analysis of the inactivation process using **10** identified Pro-1 as the site of modification in both enzymes.

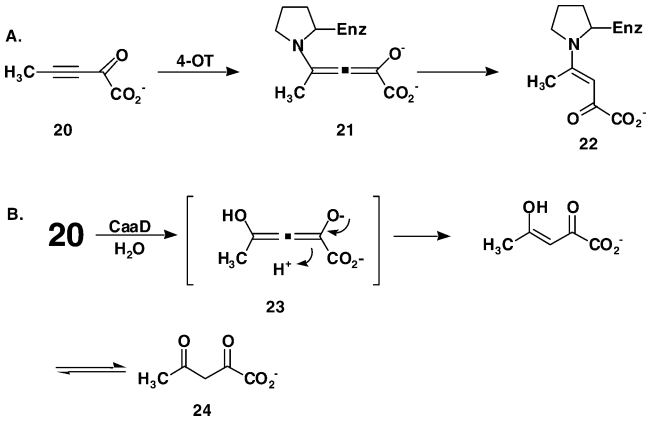
Scheme 4



Scheme 5



Scheme 6



A priori, the modification of Pro-1 can occur by a mechanism-based route (involving hydration and rearrangement to the highly reactive acyl halide species) or by a Michael addition of Pro-1 to C-3 of **10** or **11** (Scheme 4) (*20*). In the first mechanism (Scheme 4A), the enzyme-catalyzed hydration of **10** (or **11**) affords **14**. The presence of an arginine residue (i.e., Arg-11 or Arg-39 in 4-OT or Arg-11 in YwhB) would make the C-3 position more susceptible to nucleophilic attack by water by polarizing the α,β-unsaturated acids (*37*), in addition to the activating effect caused by the halogen substituent. The unstable **14** will readily rearrange to form either the acyl halide (**15**) or possibly a ketene (**16**) (*20*). Formation of the acyl halide seems more probable because the proton transfer reaction is likely faster. Nonetheless, either species would result in the alkylation of a nearby nucleophile and cause enzyme inactivation. In the second mechanism (Scheme 4B), a nucleophilic Pro-1 could attack the C-3 position of **10** (or **11**) in a Michael type reaction. While a conjugate addition to an α,β-unsaturated carboxylic acid is not normally favorable (*37*), the inhibitor can be activated by an interaction between the carboxylate group of **10** and an arginine residue on the enzyme (i.e., Arg-11 or Arg-39 in 4-OT or Arg-11 in YwhB). Such an interaction could draw electron density away from the C-3 position of the 3-halopropiolates, making them more vulnerable to a Michael reaction. The C-3 position would again be further activated by the presence of halogen substituent. Rearrangement of the enolate intermediate, **17**, to reform the triple bond would expel the bromide and complete the covalent attachment of the propargyl moiety to the enzyme to form **18**.

The covalent adduct on Pro-1 in both 4-OT and YwhB has a molecular mass of 85 Da, which is consistent with the attachment of a 3-oxoacetate moiety (*20*). Decarboxylation, presumably an artifact of the mass spectrometry analysis,[2] generates an acetyl adduct and accounts for the observation that the Pro-1 is modified by a covalent adduct having a mass of 42 Da. While these observations seemingly implicate the mechanism-based route of inactivation, a rearrangement of the propargyl moiety, as shown in Scheme 5, could also produce the 3-oxoacetate moiety. In one scenario, the propargyl moiety could rearrange to an electrophilic allene type compound (**19**), which will be readily attacked by a water molecule. Ketonization of the resulting enol generates the observed 3-oxoacetate moiety.

The transformation of **18** to **19** could be an enzymatic or nonenzymatic process. In the enzyme-catalyzed route, an interaction between the carboxylate group of the propargyl moiety and an arginine residue of the enzyme could facilitate the formation of **19** by polarization of the α,β-unsaturated moiety. In the nonenzymatic reaction, water could act as a proton source at C-2 (as shown in Scheme 5) to produce **19**. While mass spectral evidence for the presence of a propargyl group has never been observed (+70 Da), the mass spectral analysis is carried out after a 14 (YwhB) or 18 h (4-OT) incubation period followed by a column chromatography purification step. Under these conditions, the propargyl group is apparently not stable.

The preceding discussion indicates that the presence of the 3-oxoacetate group (or the acetyl group) is consistent with either proposed route of inactivation. However, previous work with 2-oxo-3-pentynoate (**20**; Scheme 6), an irreversible inhibitor of 4-OT (*9*) and a substrate for CaaD with kinetic constants nearing those of the physiological substrate (*20*), suggests that 4-OT and YwhB are inactivated by the 3-halopropiolates primarily by a Michael type reaction. Mass spectral and crystallographic studies showed that incubation of 4-OT with **20** results in the production of the modified Pro-1 species, **22** (Scheme 6A). The most likely route leading to formation of **22** involves the nucleophilic attack of Pro-1 at C-4 of **20** to form the allenic species **21**. Ketonization of **21** produces **22**. In contrast, incubation of **20** with CaaD generates acetopyruvate (**24**; Scheme 6B) through the unstable enolate, **23** (*20*). On the basis of these observations,

the primary nucleophile available to react with the activated acetylene compounds in the hydrophobic active sites of 4-OT and YwhB is Pro-1 whereas in the more hydrophilic active site of CaaD, it is water. For this reason, a Michael reaction between the 3-halopropiolates and 4-OT and YwhB is the favored route for inactivation.

The inactivation studies using **10** and **11** were carried out at pH 6.0. Pro-1 in 4-OT has a pK_a of ~6.4, as measured by direct NMR titration as well as pH rate profiles (*6*, *7*). Thus, under the conditions of the experiment, about 28% of the enzyme is in the correct state to function as a nucleophile. However, the reaction of Pro-1 with **10** (or **11**) will shift the equilibrium and place more of the enzyme in a nucleophilic state to react with the carbonyl group. In this fashion, Pro-1 can be completely alkylated. The faster rates of inactivation for these enzymes, observed at higher pH, are consistent with this analysis.

This investigation confirms the importance of Pro-1 in the YwhB-catalyzed reaction and suggests that this proline, like Pro-1 in 4-OT, also has a low pK_a value. If experimentally verified, the low pK_a value would be another trait shared between 4-OT and YwhB. It has previously been shown that the crystal structures of the two enzymes are nearly superimposable with positionally conserved active site residues (Pro-1, Arg-11, and Val-39 in place of Arg-39 and Tyr-50 in place of Phe-50) (*19*). It can be further surmised based on the structural similarities along with the presumed low pK_a value of Pro-1 that the active site of YwhB is largely hydrophobic.

On the basis of a previously reported sequence analysis, the YwhB gene product from *B. subtilis* was classified as a member of the 4-OT family (*16*), sharing 36% sequence identity with 4-OT. The 61 amino acid YwhB sequence retains the catalytic amino terminal proline as well as Arg-11 and replaces Phe-50 with a tyrosine. The only "missing" catalytic residue in YwhB is Arg-39, which is replaced with a valine (*19*). Yet, despite the many similarities between YwhB and 4-OT, YwhB functions efficiently as a 1,3-keto-enol tautomerase and only poorly as a 1,5-keto-enol tautomerase (i.e., converting **2** to **3**) (*19*). Crystal structures of the inactivated YwhB with **10** or **11** may be useful in the elucidation of the structural basis for this difference in function between 4-OT and YwhB as well as in the delineation of the role of these active site residues in the mechanism of the YwhB-catalyzed reaction.

Proton transfer reactions play a central role in numerous enzyme-catalyzed reactions. 4-OT was initially chosen as a model system to investigate these reactions because its small size and the absence of cofactors made it amenable to study by a variety of techniques (*5*−*12*). While 4-OT still remains an ideal system to study proton transfer reactions, it may also turn out to be a very useful system for the study of other reactions as well as the role of catalytic promiscuity in the evolution of new enzyme activities.

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