Kinetic and Stereochemical Analysis of YwhB, a 4-Oxalocrotonate Tautomerase Homologue in *Bacillus subtilis*: Mechanistic Implications for the YwhB- and 4-Oxalocrotonate Tautomerase-Catalyzed Reactions[†]

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ABSTRACT: YwhB, a 4-oxalocrotonate tautomerase (4-OT) homologue in *Bacillus subtilis*, has no known biological role, and the gene has no apparent genomic context. The kinetic and stereochemical properties of YwhB have been examined using available enol and dienol compounds. The kinetic analysis shows that YwhB has a relatively nonspecific 1,3- and 1,5-keto−enol tautomerase activity, with the former activity prevailing. Replacement of Pro-1 or Arg-11 with an alanine significantly reduces or abolishes these activities, implicating both residues as critical ones for the activities. In D_2O, ketonization of two monoacid substrates (2-hydroxy-2,4-pentadienoate and phenylenolpyruvate) produces a mixture of stereoisomers {2-keto-3-[^2H]-4-pentenoate and 3-[^2H]-phenylpyruvate}, where the (3*R*)-isomers predominate. Ketonization of 2-hydroxy-2,4-hexadienedioate, a diacid, in D_2O affords mostly the opposite enantiomer, (3*S*)-2-oxo-[3-^2H]-4-hexenedioate. The mono- and diacids apparently bind in different orientations in the active site of YwhB, but the highly stereoselective nature of the YwhB reaction using a diacid suggests that the biological substrate for YwhB may be a diacid. Moreover, of the three dienols examined, 1,3- and 1,5-keto−enol tautomerization reactions are only observed for 2-hydroxy-2,4-hexadienedioate, indicating that the C-3 and C-5 positions are accessible for protonation in this compound. Incubation of 4-OT with 2-hydroxy-2,4-hexadienedioate in D_2O results in a racemic mixture of 2-oxo-[3-^2H]-4-hexenedioate, suggesting that 4-OT may not catalyze a 1,3-keto−enol tautomerization reaction using this dienol. It has previously been shown that 4-OT catalyzes the near stereospecific conversion of 2-hydroxy-2,4-hexadienedioate to (5*S*)-[5-^2H]-2-oxo-3-hexenedioate in D_2O. Taken together, these observations suggest that 4-OT might function as a 1,5-keto−enol tautomerase using 2-hydroxy-2,4-hexadienedioate.

4-Oxalocrotonate tautomerase (4-OT[1]) is encoded by the TOL plasmid pWW0, which is present in various soil bacteria including *Pseudomonas putida* mt-2 (*1*, *2*). The enzyme is part of the meta-fission pathway, which is a catabolic route for the conversion of simple aromatic hydrocarbons to Krebs cycle intermediates (*3*). The hexameric enzyme, composed of 62 amino acid monomers, purportedly catalyzes the conversion of 2-oxo-4-hexenedioate (**1**, Scheme 1) to 2-oxo-3-hexenedioate (**3**) via the intermediate 2-hydroxy-2,4-hexadienedioate, known commonly as 2-hydroxymuconate (**2**) (*4*−*7*). This reaction, a 1,3-allylic rearrangement, was proposed on the basis of indirect kinetic and stereochemical studies using **2** and other mono- and diacid dienols (*4*, *6*, *7*). The conversion of **1** to **3** cannot be observed directly because

Scheme 1

1: R = CO_2^-　**2**: R = CO_2^-　**3**: R = CO_2^-
4: R = H　　**5**: R = H　　**6**: R = H
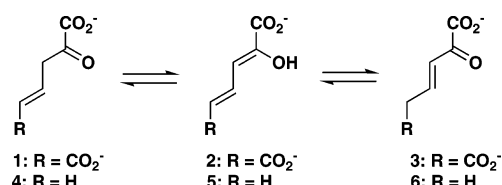
1 is not isolable and exists only in rapid equilibrium with **2** (*4*).

Pro-1, Arg-11, Arg-39, and Phe-50 have been identified as critical residues for 4-OT activity (*8*−*15*). In the context of the reaction shown in Scheme 1, Pro-1 functions as the general base and is responsible for removing a proton at C-3 (of **1**) and placing the proton at C-5 (of **3**) (*8*−*11*). Arg-11 and Arg-39 interact respectively with the C-6 and C-1 carboxylate groups of the substrate, playing roles in both binding and catalysis (*8*, *12*−*14*). Phe-50 plays a role in maintaining a hydrophobic environment near Pro-1, and this environment is partially responsible for the low pK_a of Pro-1 (∼6.4) (*15*).

YwhB is a closely related 4-OT homologue (∼36% sequence identity and 46% similarity) found in *B. subtilis* (*16*). The biological role of YwhB is not known, and there
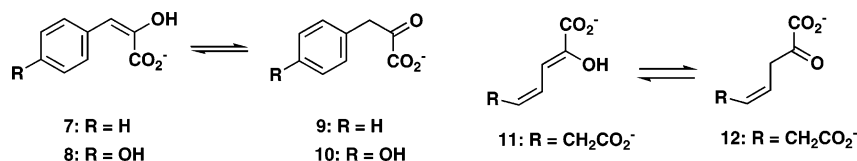
Scheme 2

7: R = H
8: R = OH

9: R = H
10: R = OH

11: R = CH$_2$CO$_2^-$

12: R = CH$_2$CO$_2^-$

is no apparent genomic context to suggest one. However, YwhB functions as an effective 1,3-keto−enol tautomerase, converting 2-hydroxy-2,4-pentadienoate (**5**, Scheme 1) to 2-oxo-4-pentenoate (**4**), and phenylenolpyruvate (**7**, Scheme 2) to phenylpyruvate (**9**) (*17−19*). YwhB is also a hexamer, consisting of 61 amino acid monomers (*16*). The YwhB sequence retains Pro-1 and Arg-11, replaces Phe-50 with a tyrosine, and more significantly, has a valine in place of Arg-39. An inhibition study showed that covalent modification of Pro-1 by 3-bromopropiolate inactivates YwhB, implicating Pro-1 as a critical catalytic residue in the conversion of **7** to **9** (*18*).

As part of our effort to identify a physiological role for YwhB and define a mechanism, we examined three additional compounds [**2**, Scheme 1, (*p*-hydroxyphenyl)-enolpyruvate, **8**, and 2-hydroxy-2,4-heptadiene-1,7-dioate, **11**, Scheme 2] as potential substrates and carried out a mutagenesis and stereochemical analysis of three YwhB-catalyzed tautomerization reactions.[2] The results indicate that YwhB is a more efficient 1,3-keto−enol tautomerase than it is a 1,5-keto−enol tautomerase, and that Pro-1 and Arg-11 are critical residues for these two activities. The stereochemical analysis shows different consequences for the monoacids (**5** and **7**) and the diacid, **2**, possibly reflecting different binding modes in the active site of YwhB. In D$_2$O, YwhB processes **5** and **7** to a mixture of the 3*R*- and 3*S*-isomers of [3-^{2}H]**4** and [3-^{2}H]**9**, respectively, with the 3*R* isomers predominating. In contrast, YwhB converts **2** to the 3*S* isomer of [3-^{2}H]**1** in a highly stereoselective manner. A racemic mixture of [3-^{2}H]**1** is generated from **2** in the presence of 4-OT under the same conditions. The sum of these observations indicates that the biological reaction of YwhB may involve a 1,3-keto−enol tautomerization of a diacid substrate while 4-OT might function almost exclusively as a 1,5-keto−enol tautomerase in the meta-fission pathway.

EXPERIMENTAL PROCEDURES

Materials. All reagents, buffers, and solvents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific Inc. (Pittsburgh, PA). The syntheses of 2-hydroxymuconate (**2**), 2-hydroxy-2,4-pentadienoate (**5**), and 2-hydroxy-2,4-heptadiene-1,7-dioate (**11**) are described elsewhere (*4, 7, 20*). Techniques for restriction enzyme digestion, ligation, transformation, and other standard mo-

lecular biology manipulations were based on methods described elsewhere (*21*). The sources for the components of Luria−Bertani (LB) media as well as the enzymes and reagents used in the molecular biology procedures are reported elsewhere (*18, 21*).
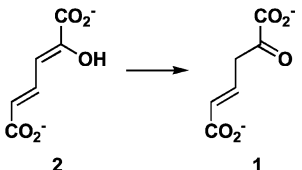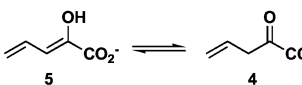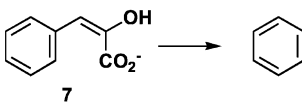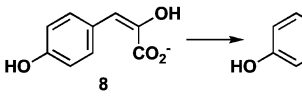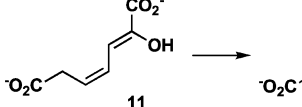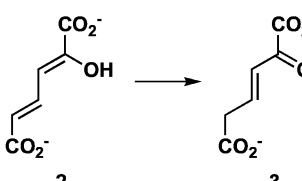
General Methods. Kinetic data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer or an Agilent 8453 UV−visible spectrophotometer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. Proteins were analyzed by tricine sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under denaturing conditions on 16−17.5% gels on a vertical gel electrophoresis apparatus obtained from Bio-Rad (Hercules, CA) (*22*). Protein concentrations were determined using the method of Waddell (*23*). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian UNITY-plus 300 MHz spectrometer or a Varian Unity INOVA-500 spectrometer. Chemical shifts were referenced as noted below.

Construction, Purification, and Characterization of the YwhB Mutants. The experimental procedures used for the construction, expression, overproduction, purification, and mass spectral analysis of the P1A- and R11A-YwhB mutants are provided in the Supporting Information.

Kinetic Characterization of YwhB and Mutants. Kinetic runs were performed in 20 mM NaH$_2$PO$_4$ buffer, pH 7.3, at 23 °C. Stock solutions (50 mM) of **2**, **7**, and **8** were initially made up in ethanol, and diluted (with ethanol) to generate 5 mM and/or 10 mM solutions. Solutions of **5** were made by diluting a stock solution (153 mM) stored in ethanol at −20 °C, to make 3.05 mM and 30.5 mM solutions. The tautomerization activities of YwhB were measured by monitoring the ketonization of the following substrates at the reported λ_{max} values using the corresponding ϵ values, within the indicated concentration ranges, as follows: **2** (294 nm, ϵ = 19,400 M^{-1} cm^{-1}, 20−150 μM), **5** (266 nm, ϵ = 12,100 M^{-1} cm^{-1}, 3−150 μM), **7** (288 nm, ϵ = 18,000 M^{-1} cm^{-1}, 10−150 μM), **8** (292 nm, ϵ = 20,800 M^{-1} cm^{-1}, 10−150 μM), **11** (276 nm, ϵ = 12,300 M^{-1} cm^{-1}, 15−150 μM), and as described elsewhere (*4, 7, 18, 20, 24*). The ketonization of **2** to **3** was monitored at 236 nm (ϵ = 6580 M^{-1} cm^{-1}) using a substrate concentration ranging from 30 to 600 μM, and YwhB (100 μL of a 10.9 mg/mL solution) that had been equilibrated in buffer (50 mL) for at least 1 h prior to use (*4*). For experiments with **7** and **11**, a quantity of YwhB (4 μL of a 26 mg/mL solution for **7** and 120 μL for **11**) was equilibrated in buffer (40 mL) for at least 1 h prior to use. For experiments with **2**, **5**, and **8**, a quantity of YwhB (12.5 μL of a 10.9 mg/mL solution, 2.5 μL of a 29.7 mg/mL solution, 100 μL of a 10.9 mg/mL solution, respectively) was equilibrated in buffer (50 mL) for at least an hour prior to use. For all experiments, aliquots (1 mL) were withdrawn and assayed for activity. Reactions were

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[2] These compounds were examined as potential substrates for three reasons: they were synthetically available, their nonenzymatic properties and reactions have been well characterized in our laboratory, and the presence of key active site groups (except Arg-39) in YwhB known to be critical for 4-OT activity suggested that they would be substrates. The absence of Arg-39 in the active site suggests that dienols with a neutral group at C-1 (e.g., a C-1 aldehyde or ester) might also be potential substrates. However, it will be necessary to synthesize and characterize the nonenzymatic properties of these compounds before such studies can be carried out.

Table 1: Kinetic Parameters for YwhB Using Enol and Dienol Substrates[a]

reaction	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
2 → **1**	10 ± 1	76 ± 17	$1.3 \pm 0.3 \times 10^5$
5 ⇌ **4**	112 ± 14	243 ± 42	$4.6 \pm 0.8 \times 10^5$
7 → **9**	125 ± 18	300 ± 60	$4.2 \pm 0.8 \times 10^5$
8 → **10**	4.1 ± 0.4	160 ± 22	$2.6 \pm 0.4 \times 10^4$
11 → **12**	3.0 ± 0.4	335 ± 55	$9.0 \pm 1.5 \times 10^3$
2 → **3**	26.0 ± 1.4	940 ± 70	$2.8 \pm 0.2 \times 10^4$

[a] The steady-state kinetic parameters were determined in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) at 23 °C. Errors are standard deviations.
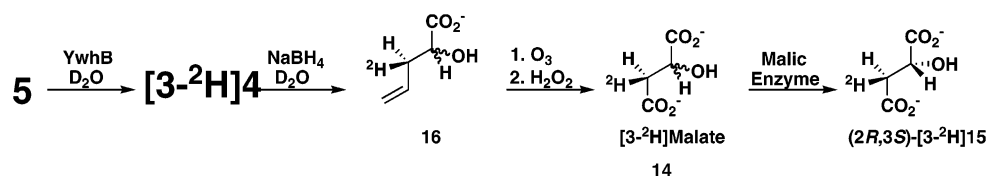
Scheme 3
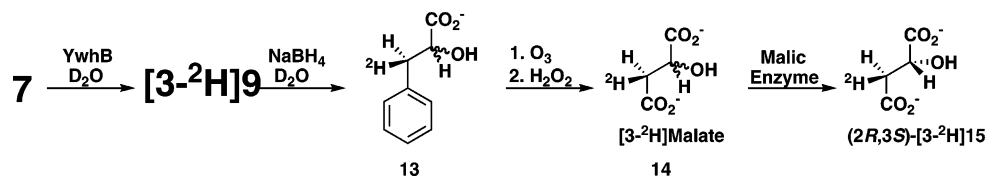
initiated by the addition of substrate. It was not possible to achieve saturation for some substrates due to the high ϵ value coupled with the quantity of substrate required. In these cases, the k_{cat} value reported in Table 1 is an estimate obtained from the Grafit program.

The YwhB-Catalyzed Conversion of 7 to [3-²H]9 in D₂O and Conversion of [3-²H]9 to [3-²H]13. The stereochemical analysis of [3-²H]9 was carried out at room temperature by the series of reactions shown in Scheme 3 using protocols described elsewhere (24). A solution of **7** (4 mg, 24 μmol)

dissolved in DMSO-d_6 (30 μL) was added to 100 mM Na$_2$-[²H]PO$_4$ (0.6 mL, pD 9.3) buffer made up in D$_2$O. Twenty individual reaction mixtures were made up. The addition of **7** (as the free acid) lowered the pH to ∼6.8. Enzyme (15 μL of a 1.5 mg/mL solution) was added to the individual reaction mixtures and the reactions were allowed to proceed for 1.5 min before they were quenched with NaBH$_4$. The product, [3-²H]**13**, was isolated and purified as described (24) to give 77.5 mg of the compound. The ^{1}H NMR spectrum corresponded to that previously reported (24).

Scheme 4

Scheme 5
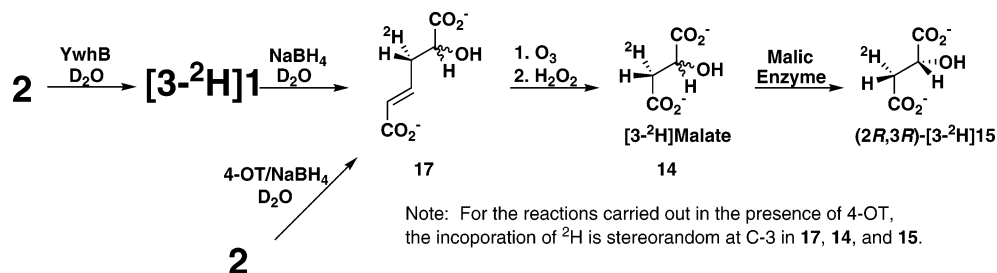
Note: For the reactions carried out in the presence of 4-OT,
the incoporation of ^2H is stereorandom at C-3 in **17**, **14**, and **15**.

Conversion of [3-^2H]13 to (2R,3S)-[3-^2H]Malate (15). The conversion of [3-^2H]**13** to **14** (Scheme 3) was carried out by the ozonolysis of [3-^2H]**13** (and treatment of the product with H_2O_2) to generate **14**, which was purified by anion-exchange chromatography to afford 22.3 mg of the compound. The (2S)-isomer was removed by treating the diastereomeric mixture with malic enzyme as described (*24*). Subsequently, the (2R)-isomer was purified by anion-exchange chromatography, yielding 8.7 mg of (2R,3S)- and (2R,3R)-[3-^2H]-malate (**15**). The ^1H NMR spectrum corresponded to that previously reported (*24*) and indicated that the (2R,3S)-isomer of [3-^2H]malate (**15**) predominated by a ratio of ~3:1.

Conversion of 5 to [3-^2H]4 in D$_2$O and Processing to (2R,-3S)-[3-^2H]Malate (15). The stereochemical analysis of [3-^2H]-**4**, generated by the incubation of YwhB and **5** in D$_2$O, was carried out by a modification of a literature procedure (*24*). The individual reactions (20 total) contained **5** (4 mg dissolved in 30 μL of DMSO-d_6, 35 μmol) and YwhB (1 μL of a 3.4 mg/mL solution) in 100 mM Na$_2$[^2H]PO$_4$ (0.6 mL, pD 9.3) buffer made up in D$_2$O. The reaction mixtures were allowed to proceed for 1.75 min before they were quenched with NaBH$_4$, combined, and purified to yield 26 mg of [3-^2H]**16** (Scheme 4). Subsequently, [3-^2H]**16** was processed to [3-D]**14** as described elsewhere to yield 9.8 mg of product (*24*). Treatment with malic enzyme yielded ~1 mg of (2R,3S)- and (2R,3R)-[3-^2H]malate (**15**). The ^1H NMR spectrum corresponded to that previously reported (*24*) and indicated that the (2R,3S)-isomer of [3-^2H]malate (**15**) predominated by a ratio of ~4.4:1.

YwhB-Catalyzed Conversion of 2 to [3-^2H]1 in D$_2$O and Conversion of [3-^2H]1 to [3-^2H]17. A solution of **2** (4 mg, 25 μmol) dissolved in DMSO-d_6 (30 μL) was added to 100 mM Na$_2$[^2H]PO$_4$ (0.6 mL, pD 9.3) buffer made up in D$_2$O. Ten individual reaction mixtures were made up similarly. The addition of **2** (as the free acid) adjusted the pH of the individual reaction mixtures to ~6.8. Compound **2** had been previously dissolved in CD$_3$OD and evaporated to dryness (3×) in order to exchange the acidic protons for deuterons. The enzyme (25 μL of a 20.7 mg/mL solution) was added to the individual reaction mixtures, and the reactions were allowed to proceed for 1 min before they were quenched with NaBH$_4$ (20 μL of a 300 mg/mL solution in 100 mM Na$_2$[^2H]PO$_4$ buffer, pH ~9.3). ^1H NMR analysis of an

individual reaction mixture indicated that the highest concentration of **1** was observed after 1 min. The reaction mixtures were combined after a 1 h incubation period with NaBH$_4$, and the pH was adjusted to 6 with H$_3$PO$_4$ (8.5%) in order to destroy the excess NaBH$_4$. Subsequently, the pH of the reaction mixture was adjusted to ~8 (using aliquots of a 1 M NaOH solution), and loaded onto an anion-exchange column (formate), as described elsewhere (*24*). The product (37 mg) was isolated and purified as described (*24*), and identified as a mixture of [3-^2H]**17** (~26 mg as estimated by ^1H NMR spectroscopy) and the reduced product of **3** (~11 mg). The ^1H NMR spectrum corresponded to that previously reported (*24*).

Conversion of [3-^2H]17 to (2R,3R)-[3-^2H]Malate (15). The conversion of [3-^2H]**17** to **15** (Scheme 5) was carried out using ozonolysis (and subsequent treatment of the product with H$_2$O$_2$) to generate [3-^2H]**14**, which was purified by anion-exchange chromatography, resulting in the isolation of 4.2 mg of the compound. The (2S)-isomer was removed by treating the diastereomeric mixture with malic enzyme as described (*24*). Subsequently, the (2R)-isomer was purified by anion-exchange chromatography, yielding 0.7 mg of (2R,3R)- and (2R,3S)-[3-^2H]malate (**15**). The ^1H NMR spectrum corresponded to that previously reported (*24*) and indicated that the (2R,3R)-isomer of [3-^2H]malate (**15**) predominated by a ratio of ~8:1.

The Conversion of 2 to [3-^2H]1 in the Presence of 4-OT in D$_2$O and Conversion of [3-^2H]1 to (2R,3R)- and (2R,-3S)-[3-^2H]Malate (15). Twenty individual reaction mixtures were made up as follows. Each reaction mixture contained a solution of **2** (4 mg, 25 μmol) dissolved in DMSO-d_6 (30 μL) and added to 100 mM Na$_2$[^2H]PO$_4$ (0.6 mL, pD 9.3) buffer made up in D$_2$O. The addition of **2** (as the free acid) lowered the pH to ~6.8. The crystalline **2** had previously been dissolved in CD$_3$OD and evaporated to dryness repeatedly in order to exchange the acidic protons for deuterons. Subsequently, an aliquot (100 μL) of a solution composed of 4-OT (120 μL of a 3 mg/mL solution in 20 mM Na[$^2H_2$]-PO$_4$ buffer, pD ~7.0) and NaBH$_4$ (160 mg dissolved in 1.88 mL of 100 mM Na$_2$[^2H]PO$_4$ buffer, pH ~9.3) was added to each individual reaction mixture. The reaction mixtures were allowed to stand at room temperature overnight, combined, and processed according to the procedure described above

for the YwhB reaction (using **2**) to yield a mixture (74 mg) of [3-^{2}H]**17** and the reduced product of **3** (in a 1:2 ratio as assessed by ^{1}H NMR spectroscopy). Subsequently, the solid material was dissolved in ~100 μL of CH$_3$OH, and subjected to ozonolysis, as described above for YwhB. Purification by anion-exchange chromatography resulted in the recovery of 18.2 mg of [3-^{2}H]**14**. The addition of CH$_3$OH improved the product yield of the ozonolysis step. The diastereomeric mixture was then treated with malic enzyme, and the resulting (2*R*)-isomer was purified by anion-exchange chromatography, yielding 5.8 mg of (2*R*,3*R*)- and (2*R*,3*S*)-[3-^{2}H]malate (**15**). The ^{1}H NMR spectrum corresponded to that previously reported (*24*) and indicated that both the (2*R*,3*R*)- and (2*R*,3*S*)-isomers of [3-^{2}H]malate (**15**) had been obtained in comparable amounts. Repeating the experiment gave the same result.

RESULTS

Construction, Expression, Overproduction, and Characterization of the YwhB Mutants. Two mutants of YwhB (P1A and R11A) were constructed by overlap extension PCR (*25*), overproduced in *Escherichia coli* strain BL21(DE3)pLysS, and purified to >95% homogeneity (as assessed by SDS−PAGE), using the protocol described for the wild type enzyme (*17, 18*). The purification protocol resulted in large quantities of both mutants (20−40 mg/L culture). The DNA sequence encoding each mutant was confirmed by DNA sequencing. In addition, each purified mutant generates one major peak in a mass spectrum, which corresponds to the expected mass. These observations provide additional evidence that only the intended mutations had been introduced into the proteins and confirm that Pro-1 is not blocked by the initiating methionine.

Kinetic Properties of YwhB. The kinetic properties of YwhB were investigated using the monocarboxylated dienol, **5**, two enols (**7** and **8**), and two dicarboxylated dienols (**2** and **11**). For the three dienol substrates, the rates for the formation of the corresponding β,γ-unsaturated ketones were measured. It was also possible to determine a rate for the formation of **3**, the α,β-unsaturated ketone, from **2**. However, the YwhB-catalyzed rates for the formation of the corresponding α,β-isomers for **5** and **11** could not be accurately measured because they were similar to the nonenzymatic rates. The kinetic data are summarized in Table 1.

Several trends emerge from the data. First, YwhB catalyzes a 1,3-keto−enol tautomerization reaction using all of these substrates although with different catalytic efficiencies (k_{cat}/K_m values ranging from 9.0×10^3 to 4.6×10^5 M^{-1} s^{-1}). Second, the highest catalytic efficiencies are observed for the monocarboxylated compounds **5** and **7**, followed by the dicarboxylated compound **2** (showing a 3.5-fold decrease in k_{cat}/K_m). Replacing the vinyl group of **5** with a phenyl group (i.e., **7**) has little effect on k_{cat} and K_m. The addition of a second carboxylate group to **5** (i.e., **2**) results in an 11.2-fold decrease in k_{cat}, as well as a 3.2-fold decrease in K_m. The decrease in catalytic efficiency observed for **2** is primarily a reflection of the decrease in k_{cat}. Third, the addition of the hydroxyl group to **7** (i.e., **8**) results in a 30-fold decrease in k_{cat} as well as a 1.9-fold decrease in K_m. The overall effect is a 16-fold decrease in the value of k_{cat}/K_m. Finally, the addition of a carboxymethyl group to **5** (i.e.,

Table 2: Kinetic Parameters for YwhB and Mutants: Conversion of **5** to **4**[a]
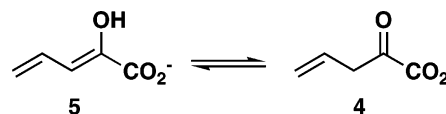
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}	relative k_{cat}/K_m
YwhB (wt)	112 ± 14	243 ± 42	$4.6 \pm 0.8 \times 10^5$	1	1
P1A	2.5 ± 0.5	378 ± 100	6600 ± 1750	0.02	0.01
R11A	0.06 ± 0.01	134 ± 22	450 ± 75	5.4×10^{-4}	0.001

[a] The steady-state kinetic parameters were determined in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) at 23 °C. Errors are standard deviations.

Table 3: Kinetic Parameters for YwhB and Mutants: Conversion of **7** to **9**[a]
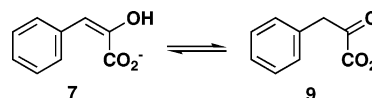
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}	relative k_{cat}/K_m
YwhB (wt)	125 ± 18	300 ± 60	$4.2 \pm 0.8 \times 10^5$	1	1
P1A	1.2 ± 0.1	210 ± 24	$5.7 \pm 0.6 \times 10^3$	~0.01	~0.01
R11A	0.055 ± 0.003	80 ± 10	690 ± 86	4.4×10^{-4}	0.002

[a] The steady-state kinetic parameters were determined in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) at 23 °C. Errors are standard deviations.

11) results in a 37-fold decrease in k_{cat}, a 1.4-fold increase in K_m, and an overall 51-fold decrease in k_{cat}/K_m.

The data in Table 1 also show that YwhB catalyzes a 1,5-keto−enol tautomerization reaction using **2**, although not as efficiently as 4-OT. 4-OT catalyzes the conversion of **2** to **3** with a $k_{cat} = 3500$ s^{-1} and a K_m of 180 μM, resulting in an overall k_{cat}/K_m of 1.9×10^7 M^{-1} s^{-1} (*13*). In comparison to 4-OT, YwhB shows a 135-fold decrease in k_{cat}, a 5.2 increase in K_m, and a 680-fold decrease in k_{cat}/K_m. Interestingly, the K_m value is 12-fold higher than that measured for the 1,3-keto−enol tautomerization reaction. The difference may reflect less than optimal binding for **2** when it undergoes the 1,5-keto−enol tautomerization reaction.

Kinetic Properties of the P1A and R11A Mutants of YwhB. The P1A and R11A mutants of YwhB were constructed to determine the importance of these residues in the 1,3- and 1,5-keto−enol tautomerization reactions catalyzed by YwhB. The mutants were analyzed using **2** (determining the rate for the conversion to **1** and **3**), **5**, and **7**, and the data are summarized in Tables 2 and 3. For **2**, there was no detectable formation of **1** or **3** using either mutant. For **5**, the P1A mutant showed a 45-fold decrease in k_{cat} and a 1.6-fold decrease in K_m, resulting in a ~70-fold decrease in k_{cat}/K_m (Table 2). Replacing Arg-11 with an alanine has a profound effect on the activity. The R11A showed a ~1900-fold decrease in k_{cat} and a 1.8-fold decrease in K_m, producing a 1022-fold decrease in k_{cat}/K_m (Table 2). Similar results are observed for both mutants using **7** (Table 3). The P1A mutant showed a 104-fold decrease in k_{cat} and a 1.4-fold decrease in K_m. This results in a ~74-fold decrease in k_{cat}/K_m. The R11A showed a ~2300-fold decrease in k_{cat} and a 3.7-fold decrease in K_m, producing a ~610-fold decrease in k_{cat}/K_m.
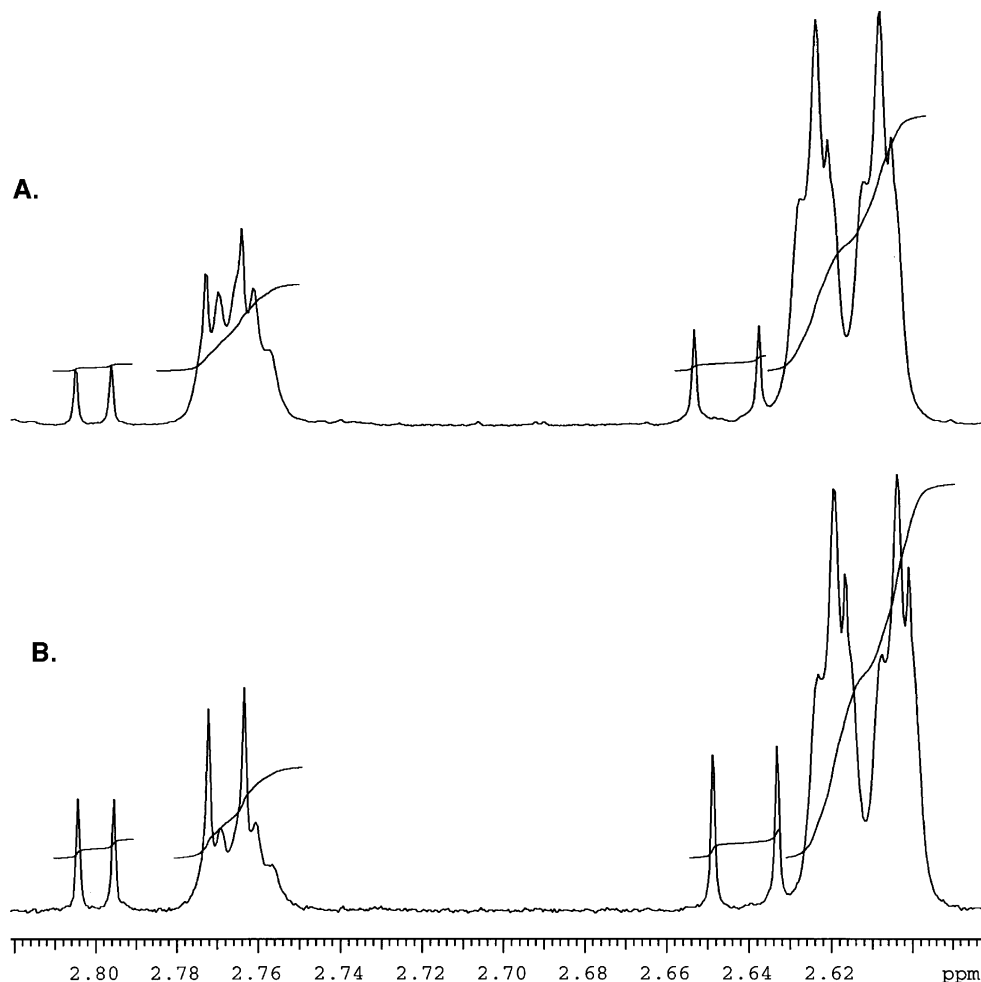
FIGURE 1: Partial ^{1}H NMR spectra (500 MHz, CD$_3$OD) of the $(2R)$-[3-^{2}H]malate isolated in the stereochemical analysis of the YwhB-catalyzed reactions using (A) **7** as substrate and (B) **5** as substrate. In both spectra, the broadened doublet of triplets (centered at ~2.62) is the predominant signal. The smaller and sharper doublets (2.64 and 2.80 ppm) correspond to the fully protio malate.

Stereochemical Analysis of the YwhB-Catalyzed Formation of [3-^{2}H]9 from 7 in D$_2$O. The stereochemical analysis of [3-^{2}H]**9** was carried out using a previously described protocol, which involves the chemical and enzymatic conversion of [3-^{2}H]**9** to the diastereomeric [3-^{2}H]malate (**14**) (Scheme 3). The addition of YwhB to a buffered solution made up in D$_2$O and containing **7** results in the ketonization of **7** to [3-^{2}H]**9**. The subsequent addition of NaBH$_4$ reduces [3-^{2}H]**9** to [3-^{2}H]**13**, which is not susceptible to epimerization at the C-3 position. The conversion of [3-^{2}H]**13** to [3-^{2}H]**14** is carried out by the ozonolysis of [3-^{2}H]**13**, followed by treatment of the ozonide with hydrogen peroxide (*24*). These reactions result in the formation of the (2*R*)- and (2*S*)-isomers of [3-^{2}H]**14** because the NaBH$_4$ reduction of [3-^{2}H]**9** is stereorandom. Incubation of [3-^{2}H]**14** with malic enzyme yields the (2*R*)-isomer, which is analyzed by ^{1}H NMR spectroscopy.

Each diastereotopic proton at C-3 of the fully protio malate appears as a doublet of doublets at 2.33 and 2.63 ppm in D$_2$O (*26*). (In CD$_3$OD, the signals are shifted downfield and appear at 2.62 and 2.78 ppm.) The stereospecific incorporation of a deuteron at C-3 results in the loss of one signal and the collapse of the remaining signal into a broadened doublet (*27*). The resonances for (2*R*)-[3-^{2}H]malate have been assigned by the reaction of maleate with maleate hydratase in D$_2$O (*27*). The product of this reaction is (2*R*,3*R*)-[3-^{2}H]-malate, and the resulting ^{1}H NMR spectrum shows the loss of the upfield signal (2.33 ppm) and the presence of a downfield doublet (2.66 ppm).

For the YwhB-catalyzed reaction (in D$_2$O) using **7**, the ^{1}H NMR spectrum (recorded in CD$_3$OD) shows two major signals, one at ~2.62 (a broadened doublet of triplets) and one at ~2.77 ppm (a broadened multiplet) (Figure 1A).[3] (The smaller coupling constant for the signal at 2.77 ppm and the presence of protio malate does not permit resolution of the multiplet.) The signal at 2.62 ppm corresponds to (2*R*,3*S*)-[3-^{2}H]malate (**15**), and the signal at 2.77 ppm corresponds to (2*R*,3*R*)-[3-^{2}H]malate. The value of the integral assigned to the (2*R*,3*S*) isomer is about 3-fold greater than that of the corresponding integral for the (2*R*,3*R*) isomer. Thus, the reaction is stereoselective and the (2*R*,3*S*) isomer predominates. The *S* configuration at C-3 of **15** indicates that the stereochemistry at C-3 of **13** is *R* because a phenyl group replaces the carboxylate group at C-4. On the basis of this assignment, YwhB catalyzes the ketonization of **7** to yield (3*R*)-[3-^{2}H]**9**, favoring the *R*-isomer by a ratio of ~3:1.

Stereochemical Analysis of the YwhB-Catalyzed Formation of [3-^{2}H]4 from 5. The stereochemical analysis of [3-^{2}H]**4**, generated by the YwhB-catalyzed ketonization of **5** in D$_2$O, was carried out as described above and elsewhere (*24*). Accordingly, [3-^{2}H]**4** was converted by the series of reactions shown in Scheme 4 to the (2*R*)-isomer of [3-^{2}H]malate,
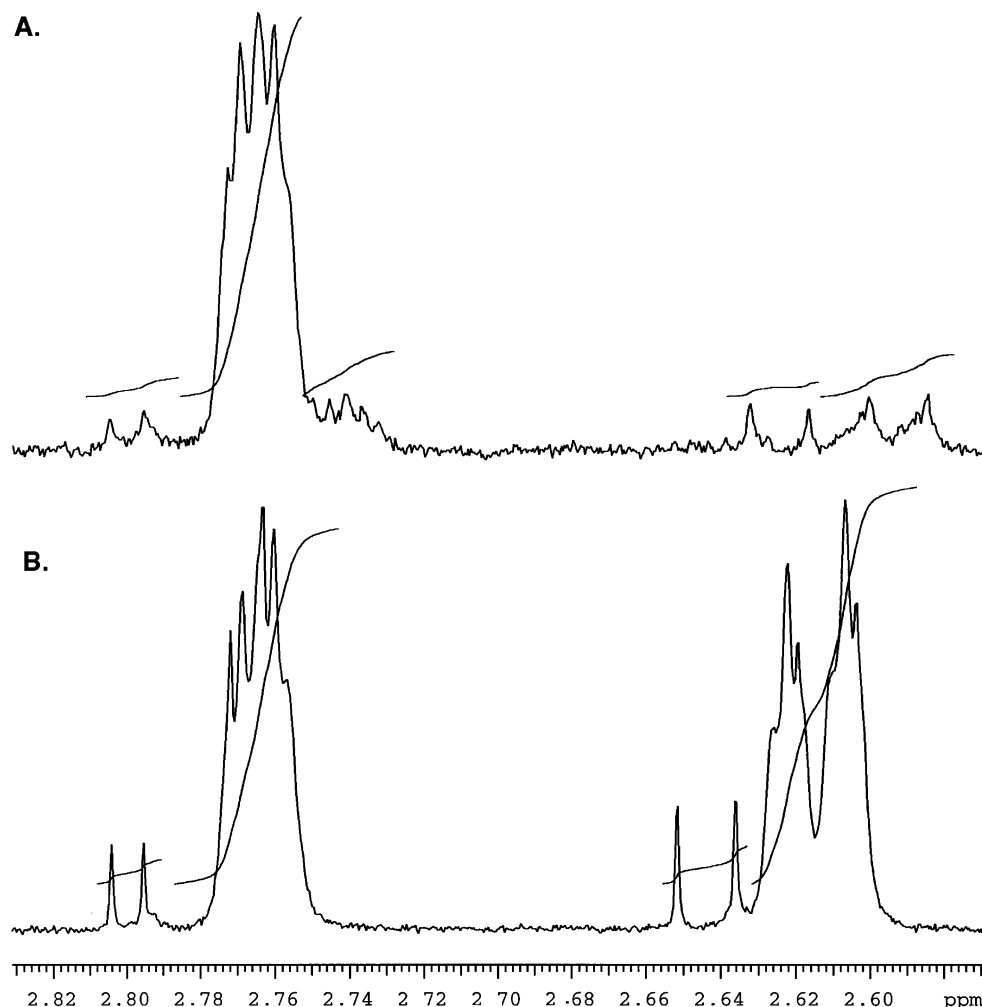
FIGURE 2: Partial ^{1}H NMR spectra (500 MHz, CD$_3$OD) of the (2R)-[3-^{2}H]malate isolated from the YwhB-catalyzed reaction using **2** and the incubation mixture containing **2** and 4-OT. (A) The broadened multiplet is the major signal present and corresponds to (2R,3R)-[3-^{2}H]malate. (B) The broadened multiplet (2.76 ppm) and a broadened doublet of triplets (2.61 ppm) are the major signals present. Integration of the signals indicates that (2R,3R)- and (2R,3S)-[3-^{2}H]malate are present in comparable amounts. Signals corresponding to the fully protio malate are also present at 2.64 and 2.80 ppm.

which was analyzed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum (recorded in CD$_3$OD) shows the two characteristic signals at 2.61 (corresponding to (2R,3S)-[3-^{2}H]**15**) and 2.77 ppm (corresponding to (2R,3R)-[3-^{2}H]**15**) (Figure 1B).[3] The value of the integral assigned to the (2R,3S) isomer is about 4.4-fold greater than that of the corresponding integral for the (2R,3R) isomer. Again, the reaction is stereoselective and the (2R,3S) isomer predominates. The S configuration at C-3 of **15** indicates that the stereochemistry at C-3 of **16** is R because a double bond replaces the carboxylate group at C-4. On the basis of this assignment, YwhB catalyzes the ketonization of **5** to produce (3R)-[3-^{2}H]**4**, favoring the R-isomer by a ratio of ~4.4:1.

Stereochemical Analysis of the YwhB-Catalyzed Formation of [3-^{2}H]1 from 2. A stereochemical analysis was also carried out on [3-^{2}H]**1**, generated from the YwhB-catalyzed ketonization of the diacid **2**, using the series of reactions described elsewhere as shown in Scheme 5 (*24*). The ^{1}H NMR spectrum (recorded in CD$_3$OD) of the resulting (2R)-isomer of [3-^{2}H]malate shows major signals at ~2.59 and ~2.77 ppm, but the broadened multiplet at 2.77 ppm is clearly the predominant one (Figure 2A). This signal corresponds to (2R,3R)-[3-^{2}H]malate, and indicates that (2R,3R)-[3-^{2}H]**15**

is the predominant product of the reaction. The R configuration at C-3 of **15** indicates that the stereochemistry at C-3 of **17** is S because a double bond replaces the carboxylate group at C-4. On the basis of this assignment, YwhB catalyzes the ketonization of **2** to (3S)-[3-^{2}H]**1**, favoring the S-isomer by a ratio of ~8:1.

Stereochemical Analysis of [3-^{2}H]1 Generated from 2 in the Presence of 4-OT. A stereochemical analysis of [3-^{2}H]-**1**, generated in an incubation mixture containing 4-OT and **2** in D$_2$O, was also carried out. Using the protocol described above and elsewhere (*24*), the (2R)-isomer of [3-^{2}H]malate was generated and analyzed by ^{1}H NMR spectroscopy (in CD$_3$OD). Integration of the doublet of triplets at 2.62 and the broadened multiplet at 2.78 ppm showed that both the (3R)- and (3S)-isomers were present in comparable amounts (within experimental error) indicating that the conversion of **2** to [3-^{2}H]**1**, in D$_2$O, in the presence of 4-OT, is stereorandom (Figure 2B).[3] Our ability to obtain highly stereoselectively labeled [3-^{2}H]malate using YwhB and **2** under

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[3] The smaller doublets at 2.64 and 2.80 ppm correspond to the fully protio malate, which results from the presence of H$_2$O in the reaction mixture.

otherwise identical conditions suggests that this observation is not an artifact of the protocol. A repeat of the stereochemical analysis gave the same result.

DISCUSSION

The tautomerase superfamily is a group of structurally homologous enzymes sharing a characteristic $\beta-\alpha-\beta$ building block and a catalytic Pro-1 (*16*, *28–30*). The 4-OT family, named for the first characterized enzyme in the family, is one of the five known families in this superfamily. 4-OT family members, including YwhB, are typically constructed from short sequences (61–84 amino acids) and are found throughout the microbial community (*29*). Only a handful of these family members have assigned functions (*29*, *30*). The inability to assign functions for the majority of the family members precludes an understanding of this niche in microbial metabolism. Defining parameters for potential substrates may ultimately assist in the determination of biological functions.

Our initial interest in YwhB stemmed from two observations: it shared modest sequence identity, yet significant structural homology with 4-OT, and appeared to be "missing" only Arg-39, which is one of four residues known to be critical for 4-OT activity (*16*, *29*). In YwhB, a valine residue occupies this position. Nonetheless, the similarities suggested two mechanistic possibilities. In one, YwhB functions as an isomerase/tautomerase using a mechanism that largely parallels that of 4-OT where another group takes the place of Arg-39.[4] Alternatively, YwhB acts analogously to the R39 mutants of 4-OT (e.g., R39A or R39Q) (*13*). In either mechanism, Pro-1 serves as the catalytic base and Arg-11 interacts with a substrate carboxylate group and plays a role in binding and perhaps catalysis. This proposed mechanism is analogous to that of 4-OT where Pro-1 is the catalytic base and Arg-11 binds substrate through its interaction with the C-6 carboxylate group of **1** (*12*, *13*, *31*). This interaction also facilitates protonation at C-5 (to form **3**) because it draws electron density to the C-5 position by resonance and inductive effects. Arg-39 interacts with the C-1 carboxylate group (of **1**, in the context of Scheme 1) as well as the 2-keto group (*12*, *13*). The latter interaction facilitates catalysis by polarizing the carbonyl group.

The kinetic assessment of YwhB using **2** shows that it behaves like the R39 mutants of 4-OT. YwhB catalyzes the conversion of **2** to **3** with a k_{cat}/K_m of 2.8×10^4 M^{-1} s^{-1}, similar to the values measured for R39A-4-OT (9.7×10^4 M^{-1} s^{-1}) and R39Q-4-OT (1.9×10^4 M^{-1} s^{-1}) (*13*). These studies also showed that YwhB processed **2** to **1** preferentially before a slower formation of **3**. Further kinetic characterization using **5**, **7**, **8**, and **11** showed that YwhB is a highly efficient 1,3-keto–enol tautomerase, but clearly not a 1,5-keto–enol tautomerase using these four dienols.[5] A possible explanation for the different reactions is different binding modes. In one scenario, the C-6 carboxylate group of **2** may interact with Arg-11 and the C-1 carboxylate group may interact with an unknown group. Alternatively, the C-1

carboxylate groups of **5**, **7**, **8**, and **11** may interact with Arg-11 in a reverse binding mode.

The mutagenesis results implicate Pro-1 and Arg-11 as critical residues in the reactions using **2**, **5**, and **7**. Replacing either residue with alanine eliminates the ability of YwhB to carry out a 1,3- or 1,5-keto–enol tautomerization reaction using **2** and diminishes its ability to carry out a 1,3-keto–enol tautomerization reaction using **5** or **7**. Previous work on P1 mutants in the tautomerase superfamily suggested that the P1A-mutant of YwhB would retain some activity because it still has an α-amino group that can function as a catalytic base (*11*, *32*). For example, the P1A mutant of 4-OT catalyzes the conversion of **2** to **3** with only a ~22-fold decrease in k_{cat}/K_m from that observed for the wild type reaction (*11*). The decreased activity of the 4-OT mutant was attributed to the lowered basicity of the primary amine and the increased flexibility of the alanyl nitrogen, which results in suboptimal positioning of the base. The reduced activity of P1A-YwhB using **5** and **7** can likewise be explained by the lowered basicity and increased flexibility of the alanyl nitrogen. The complete loss of activity for P1A-YwhB using **2** suggests that the two reactions (i.e., the 1,3- or 1,5-keto–enol tautomerization reactions) are more sensitive to the base change, and it may be that Ala-1 is no longer able to access C-3 or C-5.
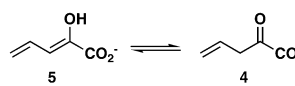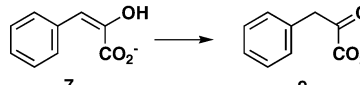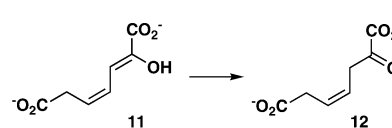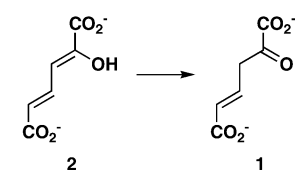
In contrast, the R11A-YwhB reactions are completely inactive or severely compromised. The absence of activity using **2** and the significantly decreased k_{cat} values and relatively unchanged K_m values for **5** and **7** suggest that Arg-11 plays primarily a catalytic role in these reactions. For the 1,5-keto–enol tautomerization reaction using **2**, the absence of Arg-11 may no longer favor the attraction of electron density to C-5 (*13*). For protonation at C-3, the absence of the positively charged arginine may destabilize the developing carbanionic character of the intermediate after deprotonation of the 2-hydroxyl group. Alternatively, in all three reactions, changing Arg-11 could affect the pK_a of Pro-1 or the positioning of another catalytic group.

The stereochemical analysis of YwhB (using **2**, **5**, and **7**) suggests that the mono- and diacids bind in different orientations in the active site. The YwhB-catalyzed reactions using the monoacids **5** and **7** are stereoselective with the 3R-isomers predominating (Table 4). In contrast, the YwhB-catalyzed conversion of diacid **2** to **1** is highly stereoselective, with the 3S-isomer predominating by a ratio of ~8:1. The stereochemical results could be governed by the interactions of the substrate carboxylate groups, as follows. For the monoacids, the C-1 carboxylate groups of **5** and **7** might interact with Arg-11 such that the proton is delivered primarily to the *re* face to produce the 3R-isomers. For the diacid, the C-6 carboxylate group of **2** could also interact with Arg-11, but this interaction would now result in the proton being delivered to the *si* face to produce the 3S-isomer. This binding mode may also allow YwhB to carry out both a 1,3- and a 1,5-keto–enol tautomerization reaction, which is only observed with **2**. Moreover, the high degree of stereoselectivity observed for the YwhB-catalyzed reaction using **2** suggests that the biological activity may involve a 1,3-keto–enol tautomerization reaction on a substrate with two carboxylate groups. This proposal is supported by the high k_{cat}/K_m values observed for the 1,3-keto–enol tautomerization reactions (~10^5 M^{-1} s^{-1}).

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[4] The unliganded crystal structure does not suggest an obvious candidate to take the place of Arg-39 (*16*).

[5] It is not known if R39A- and R39Q-4-OT behave similarly, but the possibility is currently being examined.

Table 4: Stereochemical Consequences of Reactions in the Presence of YwhB, 4-OT, and R11A-4-OT in D_2O

Reaction	YwhB	4-OT	R11A-4-OT
5 ⇌ 4	R/S (4.4:1)	R/S[a] (1.7:1)	R/S[b] (5:1)
7 → 9	R/S (3:1)	-	-
11 → 12	-	R/S[c] (2:1)	-
2 → 1	S/R (8:1)	Racemic	-

[a] This result is obtained from ref 7. [b] This result is obtained from ref 13. [c] This result is obtained from ref 31.

The stereochemical analysis of YwhB provides an explanation for previous stereochemical studies of 4-OT using mono- and diacid substrates where two arginine residues (Arg-11 and Arg-39) are present. 4-OT processes 5 to [3-^{2}H]-4 in ^2H_2O, but produces only a slight excess of the (3R)-isomer (1.7:1) (Table 4) (7). The R11A mutant of 4-OT produces the (3R)-isomer of [3-^{2}H]4 (from 5 in D_2O), but in a much higher ratio (5:1) (13), and one that is similar to that observed for the YwhB reaction (~4.4:1). Taken together, these observations suggest that 5 binds in two orientations in 4-OT. In one orientation, the C-1 carboxylate group might interact with Arg-11 while in the other orientation it might interact with Arg-39. The 1.7:1 excess of the 3R-isomer suggests that one of these orientations is only slightly favored, but removing Arg-11 significantly increases the preference for one orientation. The stereochemical results observed for the 4-OT-catalyzed conversion of 11 to 12 (in D_2O) are consistent with this analysis in that this reaction results in a 2:1 mixture with the 3R-isomer predominating (31). The additional carboxymethyl group at C-5 in 11 may favor one binding mode over the other due to the bulk and charge of the carboxymethyl group.

The highly stereoselective ketonization of 2 to 1 (in D_2O) by YwhB has a major implication for the 4-OT-catalyzed reaction. Kinetic and stereochemical studies using 2, 5, and 11 led to the proposed 4-OT reaction shown in Scheme 1. In the kinetic studies, it was found that 4-OT produced 3 faster when using a mixture of 1 and 2 (as substrate) as opposed to using 2 alone as substrate (4). The stereochemical studies showed that the conversion of 2 to 3 in D_2O was highly stereoselective, producing (5S)-5[^{2}H]3 (6), whereas the conversion of the monoacid 5 to 4 was stereoselective (preferring the 3R-isomer by a ratio of 1.7 to 1) (7). It was inferred from this latter observation that the 4-OT-catalyzed conversion of the diacid 2 to 1 (in D_2O) would also generate the 3R-isomer {i.e., (3R)-[3-^{2}H]1} with a high degree of stereoselectivity because the two carboxylate groups would firmly anchor the substrate at the active site (7). The combined stereochemical observations for 2 to 3 and 5 to 4 implied that 4-OT catalyzed a suprafacial, 1,3-allylic rearrangement of 1 to 3. The overall stereochemical course is consistent with a so-called one base mechanism where Pro-1 was eventually identified as the single base (33).

It has now been determined that a racemic mixture of [3-^{2}H]1 results when 4-OT is incubated with 2 in D_2O. Such a result has been previously observed but was thought to be an artifact due to an inability to trap [3-^{2}H]1 before it underwent a rapid chemical and enzymatic ketonization to

Scheme 6
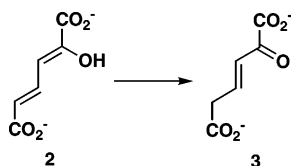
[3-^{2}H]**2** (*7*, *31*). The work with YwhB indicates that the labeled species can be trapped and the stereochemistry assigned. Since stereospecificity is characteristic of an enzyme-catalyzed reaction, the resulting racemic mixture of [3-^{2}H]**1** suggests that the conversion of **1** and **2** may not be enzyme-catalyzed (*34*). This observation could further suggest that 4-OT does not catalyze a 1,3-allylic rearrangement of **1** to **3**, but instead, may simply catalyze the conversion of **2** to **3** (Scheme 6) (*3*, *35*).

The proposed 4-OT-catalyzed 1,3-allylic rearrangement of **1** to **3** could still be "salvaged" if it can be demonstrated unequivocally that 4-OT enhances the rate of formation of **1** from **2**. If a rate acceleration is observed, then one possible explanation for the stereochemical results is that 4-OT binds **2** in either of two orientations. The 1,3-enol−keto tautomerization occurs in both orientations such that Pro-1 transfers the enol proton (or deuteron) of **2** to the *re* or *si* face of C-3 and generates a racemic mixture (in D$_2$O). In contrast, the 1,5-enol−keto tautomerization and 1,3-allylic rearrangement may only occur in one of these orientations. As a result, in D$_2$O, a deuteron is incorporated with a high degree of stereoselectivity at C-5. Although the appropriate kinetic experiments will be pursued, they are complicated by the fact that 4-OT converts **2** to **3** near the diffusion control limit (*10*, *13*).

In lieu of these results, it is interesting to note that 4-OT functions somewhat promiscuously, and accelerates the partitioning of various dienols (e.g., **5** and **11**) to the β,γ-unsaturated ketones, stereoselectively, before a slower conversion to their α,β-unsaturated isomers. There are two explanations for this behavior. 4-OT likely encounters different dienols in the degradation of various aromatic compounds and the promiscuous binding enables their accommodation. It is also possible that the 1,3-keto−enol tautomerase of 4-OT is a vestigial activity and/or plays a "rescue" role by converting β,γ-unsaturated ketones (e.g., **4**) to dienols (e.g., **5**) if the dienol undergoes a nonenzymatic ketonization to **4**. The YwhB studies have provided valuable clues into its biological role and new insights into the 4-OT mechanism. The future delineation of similarities and differences will ultimately shed light on the structural basis for the 1,3- and the 1,5-keto−enol tautomerization reactions and perhaps a biological rationale.

SUPPORTING INFORMATION AVAILABLE

The experimental procedures used for the construction, expression, overproduction, purification, and mass spectral analysis of the P1A- and R11A-YwhB mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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