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Evidence for the Formation of an Enamine Species during Aldol and Michael-type Addition Reactions Promiscuously Catalyzed by 4-Oxalocrotonate Tautomerase

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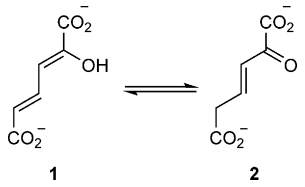
The enzyme 4-oxalocrotonate tautomerase (4-OT), which has a catalytic N-terminal proline residue (Pro1), can promiscuously catalyze various carbon–carbon bond-forming reactions, including aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde, and Michael-type addition of acetaldehyde to a wide variety of nitroalkenes to yield valuable γ-nitroaldehydes. To gain insight into how 4-OT catalyzes these unnatural reactions, we carried out exchange studies in D_2O, and X-ray crystallography studies. The former established that H–D exchange within acetaldehyde is catalyzed by 4-OT and that the Pro1 residue is crucial for this activity. The latter showed that Pro1 of 4-OT had reacted with acetaldehyde to give an enamine species. These results provide evidence of the mechanism of the 4-OT-catalyzed aldol and Michael-type addition reactions in which acetaldehyde is activated for nucleophilic addition by Pro1-dependent formation of an enamine intermediate.

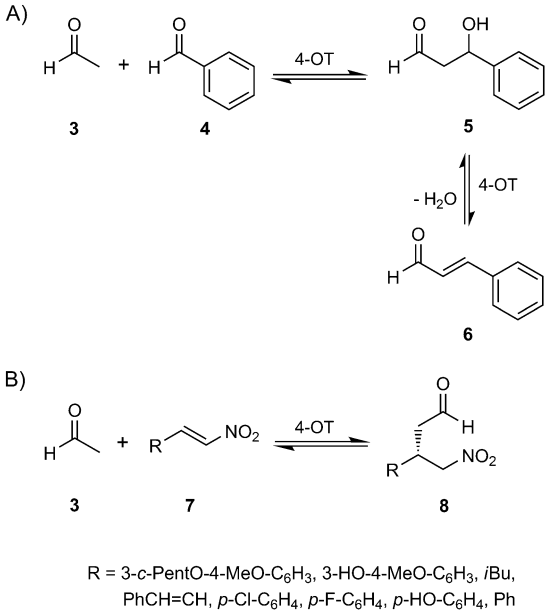
4-Oxalocrotonate tautomerase (4-OT) is a member of the tautomerase superfamily, a group of homologous proteins that share a characteristic β-α-β structural fold and a unique catalytic N-terminal proline (Pro1).[1,2] 4-OT from *Pseudomonas putida* mt-2 catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate (**1**) into 2-oxo-3-hexenedioate (**2**; Scheme 1) as part of a catabolic pathway for aromatic hydrocarbons.[3,4] Pro1 acts as a general base that abstracts the 2-hydroxyl proton of **1** for delivery to the C-5 position to yield **2**. Pro1 can function as a general base because the prolyl nitrogen has a pK_a of ~6.4 and exists largely as an uncharged species at cellular pH.[5]

In addition to its natural tautomerase activity, 4-OT can promiscuously catalyze various carbon–carbon bond-forming reactions, including the aldol condensation of acetaldehyde (**3**) with benzaldehyde (**4**) to yield cinnamaldehyde (**6**), and the



Scheme 1. Native proton-transfer reaction catalyzed by 4-OT.

Michael-type addition of acetaldehyde (**3**) to a variety of nitroalkenes (**7**) to yield chiral γ-nitroaldehydes (**8**; Scheme 2).[6–13] γ-Nitroaldehydes are versatile and practical precursors for chiral γ-aminobutyric acid (GABA) analogues such as the marketed pharmaceuticals Baclofen, Pregabalin, Phenibut, and Rolipram.[14–19]



R = 3-*c*-PentO-4-MeO-C_6H_3, 3-HO-4-MeO-C_6H_3, *i*Bu, PhCH=CH, *p*-Cl-C_6H_4, *p*-F-C_6H_4, *p*-HO-C_6H_4, Ph

Scheme 2. A) Aldol condensation and B) Michael-type addition reactions promiscuously catalyzed by 4-OT.

Site-directed mutagenesis and labeling experiments suggested a key catalytic role for Pro1 in the 4-OT-catalyzed carbon–carbon bond-forming reactions.[6,7,12] Although $NaCNBH_3$ trapping indicated that a Schiff base can form between Pro1 of 4-OT and acetaldehyde, this does not rule out the possibility that Pro1 acts as a catalytic base (as in the natural 4-OT tautomer-

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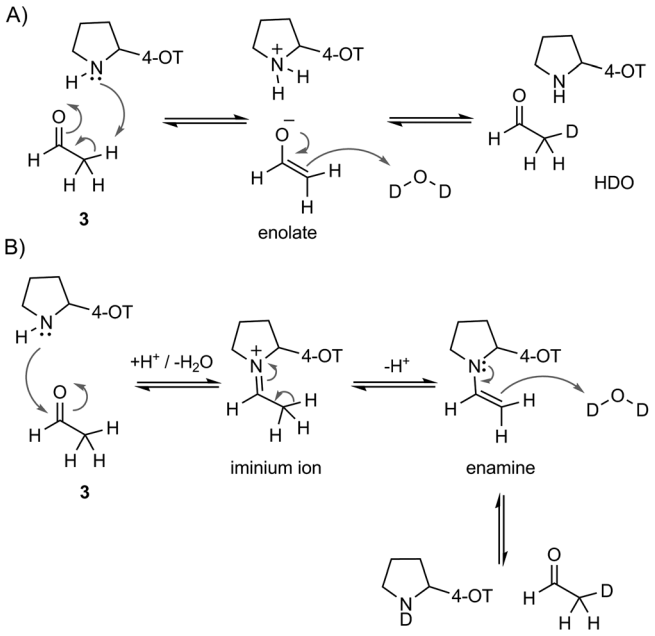
ase activity). Hence, compelling evidence for the precise mechanistic role of Pro1 in the 4-OT-catalyzed carbon–carbon bond-forming reactions is still lacking. To gain further insight into how 4-OT promiscuously catalyzes aldol and Michael-type addition reactions, we carried out exchange studies in D_2O, and X-ray crystallography studies. The former established the Pro1-dependent deprotonation of acetaldehyde; the latter revealed the formation of an enamine species between acetaldehyde and Pro1.

Given that Pro1 has the correct protonation state ($pK_a \sim 6.4$) to act as a base or nucleophile at pH 7.3, we anticipated that 4-OT would initiate catalysis by forming either an enolate or enamine intermediate (Scheme 3). Both mechanisms involve



Scheme 3. Proposed mechanisms for the 4-OT-catalyzed hydrogen–deuterium exchange within **3**, with a catalytic role for Pro1 A) as a base or B) as a nucleophile.

polarization of the carbonyl group of acetaldehyde (thus lowering its pK_a, standard of **3** is ~17 in aqueous solution) and deprotonation at C2 (Scheme 3). To determine whether one (or all three sequentially) of the C2 hydrogens can be removed as a proton by 4-OT during catalysis, we investigated the ability of wild-type 4-OT (WT 4-OT) and a Pro1-to-Ala mutant (4-OT P1A)[20] to catalyze hydrogen–deuterium (H–D) exchange within acetaldehyde. Accordingly, WT 4-OT and 4-OT P1A (0.73 mol% relative to **3**) were incubated with 20 mM **3** in 20 mM NaD_2PO_4 buffer (pD 7.5, which corresponds to pH 7.3), and the progress of the reactions was followed by ^{1}H NMR spectroscopy (Figure 1 and Figure S1 in the Supporting Information). As a control, **3** was incubated in 20 mM NaD_2PO_4 buffer (pD 7.5) in the absence of enzyme. Notably, for each reaction mixture, equilibrium between the hydrated (59%) and unhydrated (41%) forms of **3** was reached in the time between mixing all reaction components and recording the first ^{1}H NMR spectrum. ^{1}H NMR spectroscopic signals of the unhydrated (i.e., acetaldehyde: 2.24 and 9.67 ppm) and hydrated (i.e., [D_2]ethane-1,1-diol: 1.32 and 5.25 ppm) forms of **3** are shown in Figure 1.

Interestingly, for the acidic protons of **3**, which are at the C2 position ("b" in Figure 1), were almost completely exchanged (94%, 24 h) with deuterium when incubated with WT 4-OT (Figure 1, spectrum E; Figure S1). The exchange most likely takes place at C2 of the unhydrated form of **3** (acetaldehyde) and not at C2 of the hydrated form ([D_2]ethane-1,1-diol), as the protons at C2 of the latter are not acidic. However, as the rate for reaching equilibrium between unhydrated and hydrated forms is relatively high compared to the rate of H–D exchange, signals "b" (protons at C2 of the unhydrated form of **3**) and "d" (protons at C2 of the hydrated form of **3**) vanished in equal proportions (spectrum E, Figure 1). A relatively low rate of H–D exchange was found for the control sample (11%, 24 h) and for the sample incubated with 4-OT P1A (19%, 24 h; Figure 1 and S1). These data indicate that the H–D exchange within **3** is enzyme-catalyzed and that Pro1 is essential for catalysis.

The H–D exchange activity indicates that WT 4-OT can indeed deprotonate acetaldehyde, thereby providing evidence for a mechanism for the 4-OT-catalyzed aldol and Michael-type addition reactions in which acetaldehyde is activated for nucleophilic addition by Pro1-dependent formation of an enolate or enamine intermediate. To distinguish between these intermediates, we determined the crystal structures of native 4-OT and 4-OT in complex with **3** (in the absence of $NaCNBH_3$). Homohexameric 4-OT from *P. putida* mt-2 has previously been crystallized in complex with the inhibitor 2-oxo-3-pentynoate, and the structure was solved to 2.4 Å resolution (PDB ID: 1BJP).[21] We crystallized native 4-OT in a new space group ($P2_1$) and solved its structure to a resolution of 1.94 Å (Figure 2A). The *R* factor for the final model was 24.8% (R_{free} = 28.9%, Table S1). The somewhat high values for the *R* factors are most likely due to the presence of ice ring patterns, which were visible after cryo-cooling of the crystal and which partly overlapped with the protein diffraction patterns. Overall, the native 4-OT amino acid residues are well defined in the electron density maps, including those at the active sites. Structure validation further confirmed the reliability of the refined model. Co-crystallization experiments with **3** resulted in a crystal that belongs to space group C2 with 15 chains of 4-OT in the asymmetric unit (2.5 hexamers, solvent content 40%). The structure was solved to a resolution of 1.70 Å (Figure 2B) and refined to *R* and R_{free} values of 19.7% and 22.7%, respectively, with excellent geometry (Table S1).

Analysis of the electron densities of the N-terminal proline residues in the structure of 4-OT complexed with **3** indicates that a covalent modification had taken place. Of the 15 active sites in the asymmetric unit, ten clearly show extra electron density protruding from the amino group of the Pro1 pyrrolidine ring (Figure 2D). This additional electron density was not evident in the structure of native 4-OT (Figure 2C). It is known that secondary amines react with carbonyl compounds to preferentially form enamines.[22] Accordingly, the reaction of acetaldehyde with Pro1 would result in an ethylene moiety bound

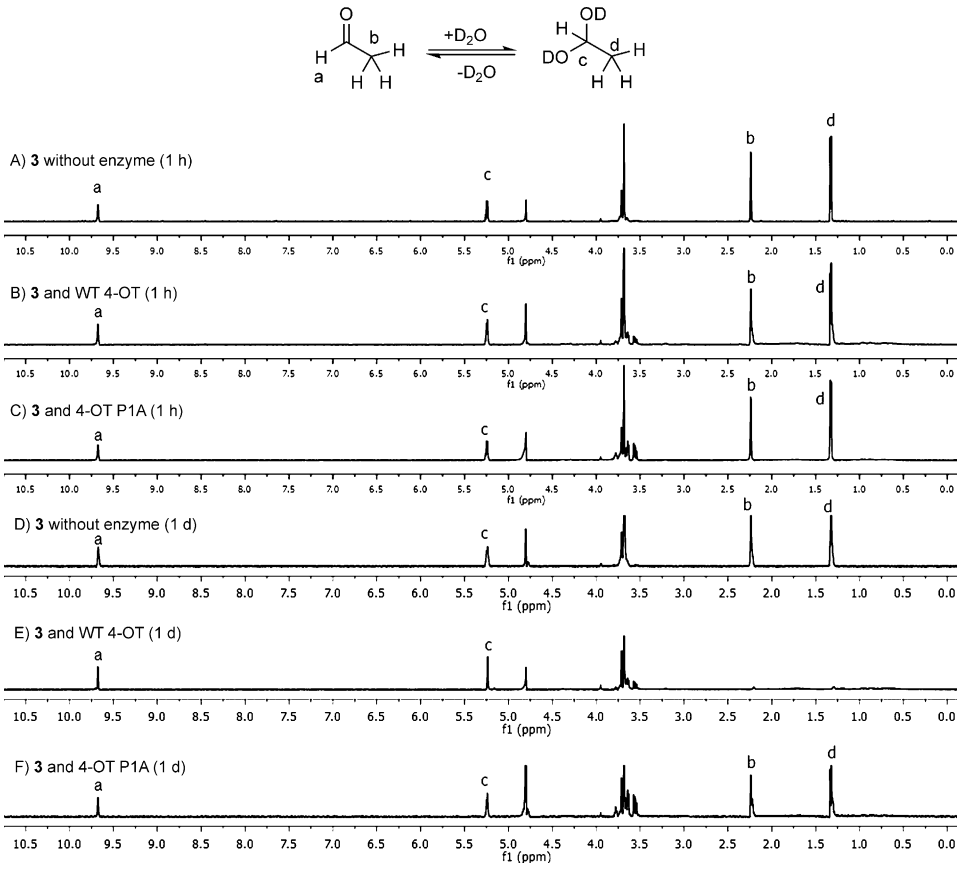


Figure 1. Stack plot of ^{1}H NMR spectra of acetaldehyde (**3**) incubated in 20 mM NaD_2PO_4 (pD 7.5): A) equilibrium is reached after 1 h between unhydrated and hydrated forms of **3** (a, b and c, d respectively); B) incubation with WT 4-OT for 1 h; C) incubation with 4-OT P1A for 1 h; D) incubation for 1 d, E) incubation with WT 4-OT for 1 d (acidic protons of **3** (b and d in spectrum A) are completely exchanged with deuterium); and F) incubation with 4-OT P1A for 1 d.

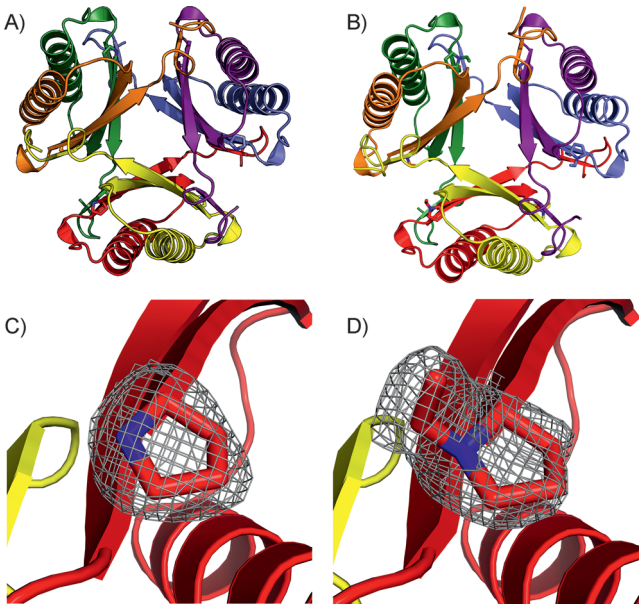


Figure 2. Hexameric structure of A) native 4-OT (1.94 Å) and B) acetaldehyde-bound 4-OT (1.70 Å). Close-up of the N-terminal proline of C) native 4-OT and D) acetaldehyde-bound 4-OT. Individual chains are depicted in different colors. The gray mesh depicts the composite omit $2F_o-F_c$ maps (contoured at 1.0 σ).

to the nitrogen of Pro1. Therefore, ethylene covalently linked to Pro1 in an enamine conformation was used as a model to account for the extra electron density. Subsequent refinements of this model support the presence of this enamine species. It should be noted, though, that the extra electron densities found at the Pro1 residues that had reacted with **3** are not very well defined, most likely because these prolines are not fully modified. This is apparent from the significantly higher B-factors of the N-linked ethylene atoms, as compared to the atoms in the pyrrolidine ring. As a result, the conformation of the enamine adduct cannot be unambiguously defined, especially with respect to the position of the terminal methylene group. It is important to emphasize that noncovalently bound acetaldehyde (or the corresponding enolate anion) was not observed in the structure of 4-OT complexed with acetaldehyde.

Cα-backbone superposition of the structures of native 4-OT with that of 4-OT in complex with **3** resulted in a root-mean-square deviation of only 0.25 Å (Figure 3A and B). Residues lining the Pro1 pocket adopt similar conformations in both the structures; the only exception is Arg11 (from the neighboring chain), which seems to be flexible and favors two alternative conformations (Figure 3C). This shows that modification of Pro1 by acetaldehyde does not result in any significant structural change in the vicinity of this N-terminal residue. To the best of our knowledge, this is the first reported structure of a tautomerase superfamily member with an enamine adduct on the N-terminal proline residue.

In summary, we provide evidence that the 4-OT-catalyzed C–C bond-forming aldol and Michael-type addition reactions proceed through an enamine intermediate. Hence, these reactions are initiated by nucleophilic attack of Pro1 on the carbonyl carbon of **3** to give an iminium ion, which upon deprotonation leads to the formation of an enamine intermediate (Scheme 3B). A reaction between this nucleophilic intermediate and an electrophilic substrate, such as **4** or **7**, results in carbon–carbon bond formation. Although the proposed mechanism mimics that of proline-based organocatalysts,[23,24] it clearly differs from that of class I aldolases.[25] Indeed, class I aldolases use the primary amine of a lysine to form enamines with carbonyl substrates, whereas 4-OT appears to be unique

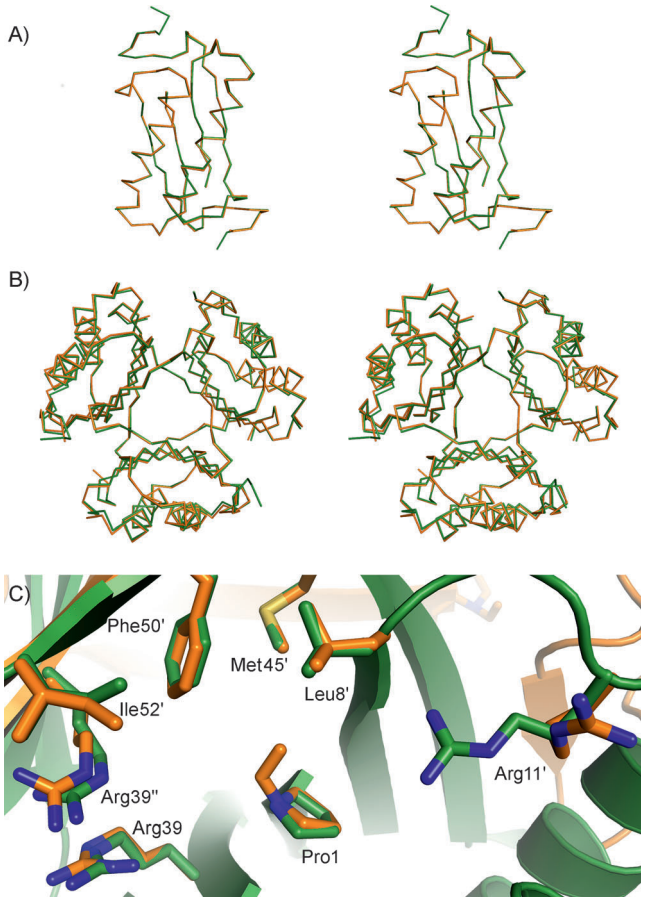


Figure 3. Stereo view of Cα backbone superposition of native and acetaldehyde-bound 4-OT as A) a dimer or B) a hexamer (trimer of dimers). C) Superposition of active-site residues of native 4-OT and acetaldehyde-bound 4-OT. Native 4-OT is depicted in green; acetaldehyde-bound 4-OT is shown in orange; residues are depicted as sticks; apostrophes indentify residues from neighboring chains.

in using the secondary amine of a proline as the nucleophile catalyst to form enamines with carbonyl substrates.

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Keywords: 4-oxalocrotonate tautomerase · aldol reaction · enamine formation · H–D exchange · Michael addition

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