Catalytic promiscuity of a proline-based tautomerase
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CHAPTER 2

EVIDENCE FOR FORMATION OF AN ENAMINE SPECIES DURING ALDOL AND MICHAEL-TYPE ADDITION REACTIONS PROMISCUOUSLY CATALYZED BY 4-OXALOCROTONATE TAUTOMERASE

The enzyme 4-oxalocrotonate tautomerase (4-OT), which has a catalytic N-terminal proline residue (Pro-1), can promiscuously catalyze various carbon-carbon bond-forming reactions, including the aldol condensation of acetaldehyde with benzaldehyde to yield cinnamaldehyde and the Michael-type addition of acetaldehyde to a wide variety of nitroolefins to yield valuable γ-nitroaldehydes. To gain insight into how 4-OT catalyzes these unnatural reactions, we carried out both exchange studies in D₂O and X-ray crystallography studies. The former establishes that H-D exchange within acetaldehyde is 4-OT-catalyzed and that the Pro-1 residue is crucial for this activity. The latter shows that Pro-1 of 4-OT has reacted with acetaldehyde to give an enamine species. These results provide evidence for a mechanism of the 4-OT-catalyzed aldol and Michael-type addition reactions in which acetaldehyde is activated for nucleophilic addition via Pro-1 dependent formation of an enamine intermediate.

2.1 Introduction

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 (Pro-1).[1,2] 4-OT catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate (1) to 2-oxo-3-hexenedioate (2) (Scheme 2.1) as part of a catabolic pathway for aromatic hydrocarbons in Pseudomonas putida mt-2.[3,4] The Pro-1 residue acts as a general base that abstracts the 2-hydroxyl proton of 1 for delivery to the C-5 position to yield 2. Pro-1 can function as a general base because the prolyl nitrogen has a pKₐ of ~6.4 and exists largely as the 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 Michael-type addition of acetaldehyde (3) to a variety of nitroalkenes (7) to yield chiral γ-nitroaldehydes (8) (Scheme 2.2).[6–13] γ-Nitroaldehydes are versatile and practical precursors for chiral γ-aminobutyric acid (GABA) analogues such as marketed pharmaceuticals Baclofen, Pregabalin, Phenibut and Rolipram.[14–19]
2.2. Results and discussion

Site-directed mutagenesis and labeling experiments suggested a key catalytic role for Pro-1 in the 4-OT-catalyzed carbon-carbon bond-forming reactions.\[6,7,12\] Although NaCNBH\textsubscript{3} trapping suggested that a Schiff base can form between 4-OT’s Pro-1 residue and acetaldehyde, this observation does not rule out the possibility that Pro-1 acts as a catalytic base (like in 4-OT’s natural tautomerase activity). Hence, compelling evidence for the precise mechanistic role of Pro-1 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 both exchange studies in D\textsubscript{2}O and X-ray crystallography studies. The former establishes Pro-1 dependent deprotonation of acetaldehyde; the latter reveals formation of an enamine species between acetaldehyde and Pro-1.

\[
\text{Scheme 2.2: Aldol condensation (A) and Michael-type addition (B) reactions promiscuously catalyzed by 4-OT.}
\]

2.2 Results and discussion

We anticipated that 4-OT would initiate catalysis by formation of either an enolate or enamine intermediate, which in both cases involves deprotonation at C2 of acetaldehyde (Scheme 2.3). To evaluate 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 the Pro-1-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% compared to \[3\]) were incubated with 20 mM acetaldehyde in 20 mM NaD\textsubscript{2}PO\textsubscript{4} buffer (pD 7.5, which corresponds to pH 7.3), and the progress of the reactions was followed by \[1\]H NMR spectroscopy (Figures 2.1 and Supp. Inf. 2.S1). A control experiment, in which \[3\] was incubated in 20 mM NaD\textsubscript{2}PO\textsubscript{4} buffer (pD 7.5) in the absence of enzyme, was also performed. Notably, for each reaction mixture, an equilibrium between the hydrated (59%) and unhydrated (41%) form 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., ethane-1,1-diol-d2: 1.32 and 5.25 ppm) form of 3 are shown in Figure 2.1.

Interestingly, the acidic protons of substrate 3, which are located at the C2 position (marked with b in Figure 2.1), were almost completely exchanged (94%, 24 h) with deuterium in the reaction mixture incubated with WT 4-OT (Figure 2.1, spectrum E; Supp. Inf. Figure 2.S1). The exchange most likely takes place at C2 of the unhydrated form of 3 (i.e., acetaldehyde) and not at C2 of the hydrated form (i.e., ethane-1,1-diol-d2) since the protons at C2 of the latter are not acidic. However, since the rate for reaching equilibrium between unhydrated and hydrated form is relatively high compared to the rate of H-D exchange, the vanishing of signals b (protons at C2 of unhydrated form of 3) and d (protons at C2 of hydrated form of 3) was witnessed in equal proportion (spectrum E, Figure 2.1). A relatively low rate of H-D exchange was found for the control sample without enzyme (11%, 24 h) and the sample incubated with 4-OT P1A (19%, 24 h) (Figures 2.1 and Supp. Inf. Figure 2.S1). These data indicate that the H-D exchange within 3 is enzyme-catalyzed and that the Pro-1 residue 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 via Pro-1 dependent formation of an enolate or enamine intermediate. To distinguish between these two possible intermediates, we determined the crystal structures
2.2. Results and discussion

Figure 2.1: Stack plot of $^1$H NMR spectra. A) $^1$H NMR spectrum of acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 for 1 h. Equilibrium is reached between unhydrated and hydrated forms of 3. Their signals are marked with a,b and c,d respectively; B) Acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 with WT 4-OT for 1 h; C) Acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 with 4-OT P1A mutant for 1 h; D) Acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 for 1 d; E) Acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 with WT 4-OT for 1 d. Acidic protons of 3 (marked with b and d in spectrum A) are completely exchanged with deuterium; F) Acetaldehyde (3) incubated in 20 mM NaD$_2$PO$_4$ buffer at pH 7.5 with 4-OT P1A for 1 d.
Chapter 2. Evidence for Formation of an Enamine Species during Aldol and Michael-type Addition Reactions Promiscuously Catalyzed by 4-Oxalocrotonate Tautomerase

of native 4-OT and 4-OT in complex with 3 (in the absence of NaCNBH$_3$). Homohexameric 4-OT from _Pseudomonas putida_ mt-2 has been crystallized before, in complex with the inhibitor 2-oxo-3-pentynoate, and the structure was solved to 2.4 Å resolution (PDB code 1BJP).[21] We have crystallized native 4-OT in a new space group (P21) and solved its structure to a resolution of 1.94 Å (Figure 2.2A). The crystallographic R-factor for the final model is 24.8% (Rfree = 28.9%, Supp. Inf. Table 2.S1). The somewhat high values for the R-factors are most probably due to problems with the data from ice-ring interference. Overall, the native 4-OT amino acid residues are well defined in the electron density maps, including those located at the active sites. Structure validation further confirmed the reliability of the refined model. Co-crystallization experiments with substrate 3 resulted in a crystal which belonged 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 2.2B) and refined to R and Rfree values of 19.7% and 22.7%, respectively, with excellent geometry (Supp. Inf. Table 2.S1).

Analysis of the electron densities of the N-terminal proline residues in the structure of 4-OT complexed with 3 evidently indicates that a covalent modification has taken place. Of the 15 active sites present in the asymmetric unit, 10 clearly show extra electron density protruding from the amino group of the Pro-1 pyrrolidine ring (Figure 2.2D). This additional electron density was not visible in the structure of native 4-OT (Figure 2.2C). It is known that secondary amines react with carbonyl compounds to preferably form enamines.[22] Accordingly, reaction of acetaldehyde with Pro-1 of 4-OT would result in an ethylene moiety bound to the nitrogen atom of Pro-1. Therefore, ethylene covalently linked to Pro-1 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 Pro-1 residues that have reacted with 3 are not very well defined, most likely because these prolines are not fully modified. This is apparent from the significant 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 non-covalently bound acetaldehyde (or the corresponding enolate anion) was not observed in the structure of 4-OT complexed with acetaldehyde.
Figure 2.2: 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 colours. The grey mesh depicts the composite omit 2Fo - Fc maps (contoured at $1.0 \sigma$).
Chapter 2. Evidence for Formation of an Enamine Species during Aldol and Michael-type Addition Reactions Promiscuously Catalyzed by 4-Oxalocrotonate Tautomerase

Cα-backbone superposition of the structure 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 2.3A,B). Residues lining the Pro-1 pocket adopt similar conformations in both the structures with the only exception of Arg-11 from the neighboring chain which seems to be flexible and favors two alternative conformations (Figure 2.3C). This shows that modification of Pro-1 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 from a tautomerase superfamily member with an enamine adduct on the N-terminal proline residue.
Figure 2.3: Stereo view of Cα backbone superposition of native and acetaldehyde-bound 4-OT as A) dimer or B) hexamer (which is a trimer of dimers). C) Superposition of active-site residues of native 4-OT and acetaldehyde-bound 4-OT. The structure of native 4-OT is depicted in green whereas the acetaldehyde-bound 4-OT structure is shown in orange. The residues are depicted as sticks and the apostrophes indicate that the residues are from the neighboring chains.
2.3 Conclusion

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 Pro-1 on the carbonyl carbon of 3 to give an iminium ion, which upon deprotonation leads to the formation of an enamine intermediate (Scheme 2.3B). A reaction between this nucleophilic intermediate and an electrophilic substrate such as 4 or 7 results in carbon-carbon bond formation. While the proposed mechanism of the reaction mimicks that used by proline-based organocatalysts,[23,24] it clearly differs from that used by 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 in using the secondary amine of a proline as the nucleophile catalyst to form enamines with carbonyl substrates.
References


Chapter 2. Evidence for Formation of an Enamine Species during Aldol and Michael-type Addition Reactions Promiscuously Catalyzed by 4-Oxalocrotonate Tautomerase


[20] Before applying the 4-OT P1A mutant, which has essentially no H-D exchange activity, we first confirmed that purified 4-OT P1A was catalytically active by measuring its promiscuous oxaloacetate decarboxylase activity; see: A. Brik, L. J. D’Souza, E. Keinan, F. Grynszpan, P. E. Dawson, *ChemBioChem* 2002, 3, 845-851.


2.A Supplementary Information

2.A.1 General methods

Protein was analyzed by polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) gels containing polyacrylamide (10%). Coomassie brilliant blue was used to stain the gels. Protein concentrations were determined using the Waddell method.\cite{1} Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). \textsuperscript{1}H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale and are referenced to H\textsubscript{2}O (\(\delta = 4.80\)). Dynamic light scattering (DLS) experiments were performed using a DynaPro MS800TC instrument (Wyatt Technology Corporation, Santa Barbara, CA) at 20 \({}^\circ\)C. DLS data were processed and analyzed with Dynamics software (Wyatt Technology Corporation).

2.A.2 Expression and purification of WT 4-OT and 4-OT P1A

The enzymes WT 4-OT and 4-OT P1A were produced in \textit{E. coli} BL21(DE3) as native proteins without His-tag using the pET20b(+) expression system as described before.\cite{2} The construction of the expression vectors and the purification procedure for WT 4-OT and 4-OT P1A were reported previously.\cite{2}

2.A.3 Crystallization of WT 4-OT and 4-OT in complex with acetaldehyde

Purified WT 4-OT was concentrated to 8 mg/mL in 20 mM Tris-HCl buffer (pH 7.5), containing 200 mM NaCl. DLS experiments indicated that the enzyme exists as a hexamer in solution. Attempts to crystallize native 4-OT by using the crystallization condition previously determined for 4-OT inactivated by 2-oxo-3-pentynoate were unsuccessful.\cite{3} New crystallization conditions were determined by vapour-diffusion sitting-drop experiments in MRC 96-well crystallization plates (Molecular Dimensions), using a Mosquito crystallization robot (TTP Labtech) for automatic drop-dispensing. Protein drops were prepared by mixing 150 nL protein solution with an equal volume of precipitant reservoir solution. Initial crystallization leads were observed in the Pact Premier screen (Molecular Dimensions), and were optimized manually using the vapour-diffusion sitting-drop method. The final crystallization condition was as follows: 0.1 M Bis-Tris propane (pH 8.5), 0.2 M potassium thiocyanate, 20 mM hexaammine cobalt chloride, 20-22% polyethylene glycol 3350 (PEG 3350). Thin rod-shaped crystals appeared within 16 h and grew within 4 d to a maximum size of 250 \(\times\) 75 \(\times\) 50 \(\mu\)m\textsuperscript{3}. WT 4-OT was also co-crystallized in the presence of 50 mM acetaldehyde to get the structure of 4-OT in which Pro-1 is covalently modified by acetaldehyde (4-OT-enamine).
Prior to X-ray diffraction data collection, the crystals were transferred to a drop containing the mother liquor supplied with 15% glycerol, and subsequently flash-frozen in liquid nitrogen. The datasets for WT 4-OT and 4-OT-enamine were collected at the ID29 and ID23-2 beamlines, respectively, at the European Synchrotron Radiation Facility (Grenoble, France). Diffraction data were processed, scaled and merged using the programs XDS\cite{4} and SCALA.\cite{5} The native WT 4-OT crystal belonged to space group P21 whereas the structure of 4-OT-enamine belonged to space group C2. A summary of the data collection and model refinement statistics is given in Supplementary Table 2.S1.

Phaser\cite{6} was used to calculate initial phases by the molecular replacement method using the published structure of 4-OT inactivated by 2-oxo-3-pentynoate (PDB code 1BJP) as a search model. Iterative cycles of refinement in Refmac5, together with manual model building in COOT, were used to improve the structures. The models were completed by performing final cycles of refinement using the program phenix.refine from the Phenix software suite.\cite{7} The coordinates and topology for the enamine moiety (Pro-1 modified by acetaldehyde) were generated using the Prodrg server.\cite{8}

Molprobity was used to validate the stereochemical quality of the models.\cite{9} Superpositions and calculation of Cα-backbone rmsd values were performed using the protein structure comparison service Fold at the European Bioinformatics Institute (PDBeFold).\cite{10} PyMOL\cite{11} (Schrödinger) was used for structure analysis and figure preparations. Coordinates for the structures of native 4-OT and 4-OT modified by acetaldehyde have been deposited with the Protein Data Bank (accession codes 4X19 and 4X1C, respectively).

NaH$_2$PO$_4$ buffer (20 mL, 20 mM; pH 7.3) was lyophilized. Subsequently, the residue was dissolved in D$_2$O (2 mL), and stirred for 60 min. This solution was again lyophilized, dissolved in D$_2$O (2 mL), and stirred for 60 min. The resulting mixture was lyophilized once more, after which the residue was dissolved in D$_2$O (20 mL) yielding a stock solution of NaD$_2$PO$_4$ (20 mM; pD 7.5).

A VIVASPIN concentrator (from Sartorius Stedim Goettingen, Germany) with a cut-off filter of 5000 Da was washed four times with H$_2$O by centrifugation (4000 rpm, 20 min). Subsequently, the concentrator was charged with a solution of 4-OT (either WT or mutant; 300 µL with a concentration of 10 mg/mL in 20 mM NaH$_2$PO$_4$ buffer, pH 7.3) and
centrifuged (4000 rpm, 30 min). The enzyme was retained on the filter and redissolved in NaD$_2$PO$_4$ (200 µL, 20 mM; pD 7.5) and centrifuged (4000 rpm, 30 min). Once more, the remaining enzyme on the filter was redissolved in NaD$_2$PO$_4$ (300 µL, 20 mM; pD 7.5) after which the final enzyme concentration was determined.

2.A.8  $^1$H NMR spectroscopy assay for H-D exchange within acetaldehyde (3)

In separate experiments, WT 4-OT and 4-OT P1A (145 µM each) were incubated with 3 (20 mM) and 18-crown-6 ether (internal standard; 2.5 mM) at room temperature in NaD$_2$PO$_4$ buffer (20 mM; pD 7.5, final volume of 650 µL in an NMR tube). A control sample was prepared containing all the reaction components except for enzyme. The progress of the reactions was followed by $^1$H NMR spectroscopy.

Internal standard 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecaan)

![Image of 18-crown-6](image)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.5): δ 3.68 (s, 24H)

Acetaldehyde (3)

![Image of Acetaldehyde](image)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.5): δ 9.68 (q, J=3.0 Hz, 1 H), 2.24 (d, J=3.0 Hz, 3H).

Ethane-1,1-diol-d2

![Image of Ethane-1,1-diol-d2](image)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.5): δ 5.25 (q, J=4.8 Hz, 1 H), 1.32 (d, J=4.8 Hz, 3 H).
Table 2.S1: Crystallographic data collection and refinement statistics

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Figure 2.S1: The progress curve of the H-D exchange within 3 in control (□), WT 4-OT (●) and 4-OT P1A samples (▼).
References


