L-Threonine Transaldolase Activity Is Enabled by a Persistent Catalytic Intermediate

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Cite This: ACS Chem. Biol. 2021, 16, 86–95

ABSTRACT: L-Threonine transaldolases (LTTAs) are a poorly characterized class of pyridoxal-5′-phosphate (PLP) dependent enzymes responsible for the biosynthesis of diverse β-hydroxy amino acids. Here, we study the catalytic mechanism of ObiH, an LTTA essential for biosynthesis of the β-lactone natural product obafuorin. Heterologously expressed ObiH purifies as a mixture of chemical states including a catalytically inactive form of the PLP cofactor. Photoexcitation of ObiH promotes the conversion of the inactive state of the enzyme to the active form. UV−vis spectroscopic analysis reveals that ObiH catalyzes the retro-aldol cleavage of L-threonine to form a remarkably persistent glycyl quinonoid intermediate, with a half-life of ∼3 h. Protonation of this intermediate is kinetically disfavored, enabling on-cycle reactivity with aldehydes to form β-hydroxy amino acids. We demonstrate the synthetic potential of ObiH via the single step synthesis of (2S,3R)−β-hydroxyleucine. To further understand the structural features underpinning this desirable reactivity, we determined the crystal structure of ObiH bound to PLP as the Schiff’s base at 1.66 Å resolution. This high-resolution model revealed a unique active site configuration wherein the evolutionarily conserved Asp that traditionally H-bonds to the cofactor is swapped for a neighboring Glu. Molecular dynamics simulations combined with mutagenesis studies indicate that a structural rearrangement is associated with L-threonine entry into the catalytic cycle. Together, these data explain the basis for the unique reactivity of LTTA enzymes and provide a foundation for future engineering and mechanistic analysis.

INTRODUCTION

Nature often employs noncanonical amino acids that bear new and different functional groups to tune the properties of bioactive small molecules.1,2 There are dozens of known modifications to proteogenic amino acids, including a wide variety that bear a hydroxyl group at the β-carbon. Such β-hydroxy amino acids are building blocks of complex natural products with a wide range of biological activities [e.g., sphingofungin (antifungal),3 salinosporamide (anticancer),4 and cyclosporin (immunosuppressant)5] (Figure 1a). β-Hydroxy amino acids are also common synthetic precursors to β-lactams6 and aziridine carboxylic acid derivatives7 in synthetic chemistry and as precursors to chiral bisoxazoline ligands for metal catalysis.8 The medicinal utility of these amino acids range from the treatment of Alzheimer’s disease (Droxidopa)9 to the synthesis of antibiotic analogues (vancomycin and chloramphenicol).10 These diverse applications underlie a broad interest in understanding β-hydroxy amino acid production and utilization in nature.

Mirroring the myriad bioactive molecules that harbor β-hydroxy amino acids, nature has evolved multiple biosynthetic routes to produce these useful compounds. Free amino acids can undergo stereospecific Cβ hydroxylation, as illustrated by...
The enzyme orients the side chain of Thr such that a carbamion is generated. This carbamion is stabilized through resonance, forming an external aldimine. The glycyl quinonoid is subsequently protonated to form the external aldimine of glycine, $E(Aex^{\text{Gly}})$, which is released to complete the catalytic cycle (Figure S1). The LTA reaction can be reversed to run in the synthetic direction in vitro by adding excess glycine and aldehyde. These enzymes have been shown to form diverse β-hydroxy amino acids, setting two stereocenters in the process. However, the reversibility of the reaction and its relatively modest selectivity lead to scrambling of the stereochemistry at the β-position, limiting synthetic utility.

Alternatively, LTTAs cleanly form β-hydroxy amino acids in vivo using the same Thr starting material and PLP cofactor. Although the molecular details have yet to be elucidated, it is believed that these enzymes function by intercepting a highly reactive $E(Q^{2\text{Gly}})$ intermediate (Scheme 1).

**Scheme 1. Catalytic Mechanism of ObiH**

The mechanism of ObiH catalysis with Thr and an aldehyde substrate (outer cycle) along with the disfavored shunt pathway (inner cycle). The PLP cofactor (black) is shown covalently bound to the substrate (outer cycle) along with the disfavored shunt pathway (inner cycle). The UV-vis spectrum of ObiH shows a peak at 485 nm, corresponding to the flavin ring system.

**RESULTS**

Green-Light Irradiation of Purified ObiH Yields Highly Active Catalyst. ObiH was heterologously expressed in E. coli, as was previously described. The enzyme is pink in color, whereas other PLP-dependent enzymes are yellow. The UV–vis spectrum of ObiH shows a peak at 485 nm, corresponding to the flavin ring system.
enzymes. A 2-fold increase in initial turnover rate compared to as-isolated ObiH had between as isolated ObiH (pink) and phototreated ObiH (black). Absorbance spectra of natively purified ObiH forms as a mixture of chemically distinct states that can be photointerconverted and that the species absorbing at 515 nm is not catalytically active. Nevertheless, the phototreatment process afforded us the opportunity to cleanly assay the mechanistic properties of ObiH as a model LTA.

ObiH Forms a Metastable PLP-Glycyl Quinonoid. Previous steady-state kinetic analysis of ObiH established that the enzyme has a relatively high $K_M$ for Thr, 40 mM. We began our detailed mechanistic study by adding 100 mM Thr to ObiH and monitored the reaction by UV–vis spectroscopy. The addition of Thr resulted in an intense absorbance with $\lambda_{\text{max}} = 493$ nm, characteristic of a PLP quinonoid adduct (Figure 3a). We assign this species as E(QGly), which is formed by retroaldol cleavage of a covalently bound PLP-Thr adduct, E(AexThr). We also performed this experiment with as-isolated enzymes (before phototreatment) and observed that the 515 nm peak was unchanged by reaction conditions. Further, as-isolated enzymes formed a less intense E(QGly) band, consistent with a lower population of the catalytically active enzyme (Figure S4).

The E(QGly) absorbance band increased for several minutes before reaching a maximum and slowly decayed over the course of several hours (Figure 3b). To further probe the kinetics of the formation and breakdown of this species, we repeated the experiment with varied concentrations of Thr. Increasing the concentration of Thr above 100 mM resulted in a similarly intense E(QGly) band, and lower concentrations of Thr significantly reduced the population of E(QGly) (Figure S5). We performed a global kinetic analysis of the time course for E(QGly) formation and decay across a range of Thr concentrations using a three-state kinetic model with reversible Thr binding and retroaldol cleavage, followed by an irreversible decay event. However, this simple model decisively failed to fit the data. Hence, additional studies that can substantiate a more sophisticated model will be required to account for the slow formation of E(QGly). Once formed, however, the reactivity of E(QGly) cleanly fit to a single exponential with a half-life of 165 ± 20 min for concentrations ≥100 mM Thr (Figure S5). Given the time scale of this decay, many potential pathways may be responsible for the quenching of this species. We hypothesized that, in analogy to the distinctly related LTA enzymes, E(QGly) reacted through simple protonation to form the glycyl external aldimine, E(AexGly). Consistent with this hypothesis, kinetic analysis at lower pH showed a substantially faster quinonoid decay (Figure S6).

Figure 2. Effect of green light irradiation on ObiH catalytic states. (a) Absorbance spectra of natively purified ObiH (pink) and phototreated ObiH (black). Phototreatment results in the complete loss of the 515 nm species and increase in the E(Ain) peak at 415 nm. Images of ObiH stock solutions before and after phototreatment. (b) Comparison of product formation of (2S,3R)-β-hydroxyleucine between as isolated ObiH (pink) and phototreated ObiH (black). The corresponding reaction is shown on top. Phototreated ObiH had a 2-fold increase in initial turnover rate compared to as-isolated enzymes.

Figure 3. pH-dependent rates of E(QGly) formation and decay. (a) UV–vis analysis revealed complete abolishment of the 515 nm band and an increase at 415 nm. We also observed a decrease in the 515 nm species upon heating of ObiH at 37°C (Figure S3). To minimize the possibility of stochastic protein aggregation at higher temperature, we relied on phototreatment to produce homogeneous ObiH. To further increase reproducibility, purified ObiH was exposed to an 8 W, green LED for 10 min on ice, which led to the rapid and reproducible photoablation of the 515 nm peak and a temporary increase at 340 nm (Figure S3). Over the course of several minutes, this 340 nm band decreased and the 415 nm band increased, indicative of a slow isomerization between the enolimine and ketoenamine forms of the cofactor, respectively. While proton transfers are typically fast, we hypothesized that this apparent tautomerization is coupled to a slower exchange process, such as a conformational or oligomeric change. After isomerization of the 340 and 415 nm species, a small population of 515 nm species reformed. A second round of phototreatment removed the small amount of 515 nm species (Figure 2a). After storage at 4°C overnight, phototreated ObiH samples equilibrated back to a mixture of states including the 515 nm species (Figure S3).

To assess whether the phototreatment increases the concentration of active enzymes and not some other species, we measured the initial velocity of ObiH with isobutyraldehyde before and after phototreatment. Gratifyingly, we observed an approximate 2-fold increase in the rate of product formation with the phototreated enzyme (Figure 2b). These data strongly suggest that heterologously expressed ObiH purifies as a mixture of chemically distinct states that can be photointerconverted and that the species absorbing at 515 nm is not catalytically active. Nevertheless, the phototreatment process afforded us the opportunity to cleanly assay the mechanistic properties of ObiH as a model LTA.
Whereas E(QGly) was slowly depleted, presumably through nm absorbing species that can photoconvert to E(Ain).

The ObiH Quinonoid Rapidly Reacts to Form β-Hydroxy Amino Acids. The native electrophile in the ObiH reaction is p-nitrophenylacetaldehyde, which is generated via a thiamine-dependent decarboxylation from the corresponding α-keto acid. However, due to the inherent instability of aroylacetaldehydes, we sought an alternative electrophile for our mechanistic studies. Recent experiments using a biocatalytic cascade showed ObiH, as well as its downstream enzymes in obafuorin biosynthesis, can react with a range of aliphatic and benzyl aldehydes. While the synthetic utility of ObiH with aromatic aldehydes has been recently reported, we were drawn to the reaction with aliphatic aldehydes as mechanistic probes because they do not have confounding signals in their UV−vis spectra. We probed the on-path reactivity of ObiH via addition of reactive aldehyde to preformed E(QGly).

To further confirm that Gly is formed through an ObiH-mediated process, we added Thr to ObiH to form a large population of E(QGly) and then reductively trapped the decay product as a secondary amine via the addition of NaBH₄. This reaction was monitored both spectrophotometrically and via UPLC-MS. Spectroscopic experiments showed depletion of the absorbing species in the range of 400–420 nm, indicating that the imines present E(AexThr), E(AexGly), or unreacted E(Ain) were rapidly reduced by NaBH₄ (Figure S7).

A new absorbance band at 340 nm appeared, consistent with formation of a reduced, secondary amine adduct. However, the E(QGly) species (493 nm), which is electron-rich, was not immediately reduced by NaBH₄ and decayed at a similar rate to reactions containing only Thr. The products of this reaction were monitored by UPLC-MS analysis and showed clear formation of a reduced glyclyl adduct (Figure S7). Notably, this experiment was performed with protein that had not undergone phototreatment and therefore retained the 515 nm absorbing species that can photoconvert to E(Ain). Whereas E(QGly) was slowly depleted, presumably through an intermediate protonation step, the 515 nm band was completely resistant to reduction with NaBH₄ (Figure S7). While the above data show that E(QGly) is kinetically slow to react, they offer only indirect information on the thermodynamic stability of this intermediate. We therefore probed the effect of saturating Gly (1.0 M) on E(Ain) and observed no evidence of quinonoid formation, indicating that population of E(QGly) is not enabled by thermodynamic stabilization in the enzyme active site (Figure S7). Instead, these data establish that E(QGly) species is a kinetically trapped, high-energy intermediate.

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We next probed the reaction of E(QGly) with different aliphatic aldehydes and measured the resulting steady-state quinonoid population. The addition of propanal resulted in a rapid loss of E(QGly), but the steady-state population was higher than was observed in the acetaldehyde reaction (Figure S10). The addition of isobutyraldehyde differed from the previous two substrates and revealed that isobutyraldehyde reacts in at least two phases. Approximately half of the population of E(QGly) is depleted in the mixing time of the experiment. The remaining fraction reacts more slowly, reaching the steady state over the course of 20 min. Subsequent data establish that this aldehyde does react through a productive catalytic cycle to form a β-hydroxy amino acid product (vide infra). Other effects that occur on slow time scales, such as protonation to form Gly, however, confound further interpretation of these data.

To characterize the full catalytic cycle in action, we monitored product formation over time using isobutyraldehyde. UPLC-MS analysis of reactions with isobutyraldehyde showed a single peak corresponding to β-hydroxy-leucine, indicative of a highly diastereoselective reaction. The reaction proceeded slowly and reached a 71% yield after 24 h. To confirm the identity of the product, we performed an overnight preparative scale reaction on the 5 mmol scale using 0.04 mol % catalyst. Purification of the β-hydroxy-Leu proved to be challenging. β-hydroxy-Leu and Thr had similar retention times by C18 flash chromatography, which necessitated multiple rounds of chromatography to isolate the pure product and resulted in isolation of 141 mg of β-hydroxy-leucine,

Figure 3. Spectroscopic characterization of ObiH catalytic intermediates. (a) Absorbance spectra of phototreated ObiH (black) and after addition of Thr (blue). Addition of Thr results in large peak at 493 nm, E(QGly). (b) Plot of absorbance at 493 nm vs time after addition of Thr. The 493 nm peak increases rapidly before reaching a maximum with subsequent decay over hours. (c) On cycle reactivity of E(QGly) with aliphatic aldehydes. A representative absorbance spectrum (average absorption spectra between three experiments) of ObiH after addition of Thr is shown in blue. Subsequent addition of acetaldehyde (brown), propanal (purple), or isobutyraldehyde (orange) results in substantial reduction of the 493 nm peak.
corresponding to a 38% isolated yield. NMR analysis revealed a >98:2 diastereomeric ratio (dr) of syn/anti products. ObiH, like other fold type-I PLP dependent enzymes, is known to have exquisite selectivity for the 2S configuration. Therefore, we assign this product as (2S,3R)-β-hydroxy-Leu, consistent with other studies of ObiH selectivity. To probe the reactivity of this amino acid with ObiH, we added 25 mM β-hydroxy-Leu and observed a low population of E(QGly) that formed slowly over the course of 30 min. These data demonstrate that the β-hydroxy-Leu product does not readily re-enter the catalytic cycle (Figure S11).

ObiH Crystallizes with an Unanticipated Active Site Conformation. To unravel the structural properties underpinning this unique reactivity, we crystallized N-His-ObiH to capture the enzyme in its internal aldime state, E(Ain). High resolution, 1.66-Å, X-ray diffraction data were collected on crystals that retained the 515 nm species, as the pink color was maintained throughout the process. The structure was solved by molecular replacement with a distantly related serine hydroxymethyltransferase (PDB ID: 4OT8, 28.2% identity).41 The asymmetric unit of the ObiH crystal was comprised of four protamers (two dimers) for a total of four unique subunits and forms a salt bridge with Arg366. Such complexes with a sulfate or phosphate are common among PLP-dependent enzymes and often correspond to the carboxylate-binding motif within the active site.42–45 The pyridine ring of PLP is π-stacked with His131, and the PLP phosphate is buried with an intricate web of hydrogen bonds, including two to Tyr55 and Asn268 from the partner subunit. A multiple sequence alignment reveals that Lys234, Arg366, His131, and Tyr55 are highly conserved across biochemically characterized lTTAs (Figure 4d).

Another highly conserved residue in this family of enzymes is the residue that hydrogen bonds to the pyridine nitrogen of the cofactor, Asp204.40 Ion pairing with the pyridinium moiety enforces protonation of the cofactor, thereby increasing its electrophilicity. However, the universally conserved Asp residue appeared to be tucked under the pyridine ring, and instead, Glu107 is in position to form a salt bridge with the cofactor (Figure 4c). Mutation of the residue that H-bonds to the cofactor of PLP is to Tyr55 and Asn268 from the partner subunit. A multiple sequence alignment reveals that Lys234, Arg366, His131, and Tyr55 are highly conserved across biochemically characterized lTTAs (Figure 4d).

The ObiH active site lies at the dimer interface, with most of the residues contributed from a single subunit. The electron density is consistent with a typical E(Ain) state of the enzyme where the conserved Lys234 forms a Schiff’s base adduct with the PLP cofactor (Figure 4b,c).42 A molecule of sulfate is bound in the active site with the same orientation in all four subunits and forms a salt bridge with Arg366. Such complexes with a sulfate or phosphate are common among PLP-dependent enzymes and often correspond to the carboxylate-binding motif within the active site.42–45 The pyridine ring of PLP is π-stacked with His131, and the PLP phosphate is buried with an intricate web of hydrogen bonds, including two to Tyr55 and Asn268 from the partner subunit. A multiple sequence alignment reveals that Lys234, Arg366, His131, and Tyr55 are highly conserved across biochemically characterized lTTAs (Figure 4d).

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E(AexThr), to assess whether a conformational rearrangement involving Glu107 and Asp204 might be occurring.

Simulations Show Active Site Dynamics. We first conducted 110 ns molecular dynamics (MD) simulations of the ObiH dimer in the E(Ain) state. Trajectories were run in triplicate and were well equilibrated after 10 ns of simulation time. These initial 10 ns were excluded from subsequent analysis. Measurements from both monomers were combined, effectively doubling the active site sampling frequency across the combined 300 ns simulation time. Initial visual inspection of the simulation results revealed that a loop region spanning Tyr56 to Pro71 had moved significantly during the course of the simulation. Per-residue root-mean-square fluctuation (RMSF) analysis of the backbone atoms shows this loop region, which coincides with the putative substrate access channel, is one of the most mobile regions of the protein (Figure S13). These results were not surprising, as this region in the crystal structure was largely void of secondary structure.

To probe the dynamics of the active site, we used H-bonding analysis to assess which residue H-bonded to the cofactor. During the course of the simulations, both Glu107 and Asp204 H-bonded to the cofactor. Analysis revealed that both Glu107 and Asp204 were more than twice as likely to engage the cofactor than the other residues (Table S2). This population distribution revealed that both Glu107 and Asp204 can H-bond to the pyridine nitrogen (PLP-N1) during the course of the simulations. This analysis showed that asp204 was engaged in an H-bond with the cofactor at twice the frequency of Glu107 (Figure S16), strongly suggesting that Asp204 is the residue that engages the cofactor during retroaldol cleavage. There was still a diverse conformational space where each of the potential catalytic His131 and Asp204 interactions were satisfied. To further narrow our search for a catalytic pose, we searched for orientations where the substrate carboxylate was also engaged in a salt bridge with Arg366, as previous structural studies have shown this interaction is conserved across LTAs and SHMTs (Figure 5, Figure S17). This snapshot of ObiH E(AexThr) satisfies each plausible interaction of a catalytically productive pose and may be useful for future studies.

Asp204 Is Essential for Catalysis. To experimentally validate the insights gained from molecular dynamic simulations and bioinformatics analyses, we performed biochemical characterization of His131, Glu107, and Asp204 variants. Rather than the traditional alanine scan approach to probe the “importance” of a residue, we screened a site-saturation mutagenesis library at His131 for retention of function with biphenyl-4-carboxaldehyde, as the products were well-behaved on UPLC-MS. The native ObiH enzyme, bearing His131, possessed the highest activity under the screening conditions (Figure S18). The only residue that supported any catalytic function other than His was Gly, which may allow water to enter the active site and rescue function. This
experiment supports our observation from crystallographic and MD simulation that His131 is the catalytic base. Next, we screened a site-saturation mutagenesis library at Glu107. Similar to His131, the native ObiH enzyme, bearing Glu107, possessed the highest activity. Unlike His131, however, many other variants retained catalytic activity (Figure S18). To further probe the role of Glu107, we expressed and purified the conservative E107Q variant, which has a similar steric profile to the native residue but cannot form an ion pair with the pyridinium nitrogen. This protein was pink in color and behaved similarly to wild type protein upon phototreatment (Figure S19). Spectroscopic analysis affirmed that ObiH–E107Q binds PLP and, upon the addition of Thr, enters the catalytic cycle to form a metastable quinonoid that goes on to react with propanal (Figure 6).

Figure 6. Spectroscopic characterization of ObiH-E107Q. Absorbance spectra of phototreated ObiH-E107Q (black) and after addition of Thr (blue). Addition of Thr results in a large peak at 493 nm, E(Qin)^[20]. Subsequent addition of propanal (purple) results in substantial reduction of the 493 nm peak. These spectral features of ObiH-E107Q are similar to wt-ObiH.

In stark contrast, saturation mutagenesis at the evolutionarily conserved Asp204 position was catastrophic, and only the wild-type enzyme retained activity (Figure S18). We attempted to further probe the contribution of Asp204 through study of the conservative D204N variant. This protein aggregated during purification and was stable only at low concentrations. The purified ObiH-D204N was colorless, and UV–vis analysis revealed evidence of only trace PLP binding (Figure S19). These experiments unambiguously demonstrate that Asp204, not Glu107, is essential for efficient cofactor binding and enforces protonation at PLP-N1 to enable the unique transaldolase activity of this enzyme.

**DISCUSSION**

**ObiH May Form an Internal Quinonoid.** One of the most striking observations about ObiH, by us and others, is the beautiful and uncommon pink color of the heterologously expressed protein. UV–vis analysis indicates the presence of an adduct that absorbs at 515 nm. The λmax of this species is itself highly informative and suggests formation of an extended chromophore with the PLP-cofactor, such as a quinonoid. Indeed, the 515 nm band was originally hypothesized to arise from a small population of tightly bound glycyll quinonoid, E(QGly)^[20]. However, the addition of Thr yields a quinonoid with a distinct unexpected conformation, with Glu107 H-bonded to the pyridinium moiety of the cofactor instead of the evolutionarily conserved Asp204. MD simulations of E(Ain) revealed significant conformational heterogeneity of a loop adjacent to the active site and indicated that a H-bond with either of the two residues was likely, with Glu107 preferred. Hence, even are several lines of evidence that support this assignment. Through an as-yet-unknown process, light abolishes this species and increases the concentration of the E(Ain), establishing that the 515 nm species is a PLP adduct and not some trace contaminant (Figure 2a). Such reactivity is uncommon, and doubtless will inspire further study, but is consistent with a previous report that light can alter the pKₐ of PLP intermediates.^[20] We show that the 515 nm species can interconvert to a catalytically active state through both photochemical and thermal means (Figure S3). However, the 515 nm band was inert to reduction with NaBH₄, indicating that it is electron rich (Figure S7). Last, the structure of ObiH was determined from pink crystals, and despite the high, 1.66Å resolution of the data, no trace of a contaminating chromophore was observed, consistent with an isosteric modification (Figure 4b).

Each of the above lines of evidence support the assignment of the 515 nm band as E(Qin), but none suggest why this species arises in the first place. Detailed pre-steady-state kinetic experiments conclusively established that the native function of ObiH involves formation of E(QGly)^[20] (Figure 3a). No trace of E(Qin)^[20] is formed upon the addition of Gly to solution, indicating that this intermediate is highly basic (Figure S8). However, this species persists for hours in the absence of an electrophile and is therefore kinetically shielded from protonation. We speculate that these same features that underlie the catalytic reactivity of ObiH may also be responsible for stabilization of an E(Qin) state. While this is a parsimonious explanation, many details of E(Qin) formation remain unclear. What is the role of light and temperature in facilitating the apparent protonation of this species? Is this chemistry unique to ObiH, or common among iTTA enzymes? These and other questions leave fertile ground for future study.

**Structure Guided MD Simulations and Mutagenesis Reveal the Role of Active Site Residues.** Crystallographic structures are highly information-rich and often lend to key mechanistic insights. However, they provide a handful of snapshots of the protein in a solid, crystalline environment that does not always reflect behavior in solution. Further, even high-resolution models can only capture states that are <1.0 kcal/mol from the thermodynamic minimum. As described in the Results, we found that N-His-ObiH crystallized in a highly unexpected conformation, with Glu107 H-bonded to the pyridinium moiety of the cofactor instead of the evolutionarily conserved Asp204. MD simulations of E(Ain) revealed significant conformational heterogeneity of a loop adjacent to the active site and indicated that a H-bond with either of the two residues was likely, with Glu107 preferred. Hence, even
the small energetic bias induced by asymmetric packing forces in the crystalline lattice could reasonably alter this equilibrium, resulting in the observed crystallographic state (Figure 4b). In contrast, MD simulation of E(AexThr) revealed that Asp204 is the predominant residue that H-bonds with PLP-N1. These data align with strong literature precedence from related serine hydroxymethyltransferase (SHMT) and tTA enzymes.41–53 For example, the addition of Gly to tTA enzymes results in formation of a thermodynamically stable quinonoid.24 Other enzymes only transiently form quinonoids, and rapid kinetic analysis is needed to observe them.53 ObiH is exceptional, in that it forms a large population of thermodynamically unstable quinonoid. Were protonation and release of Gly to occur, this would be thermodynamically favored in vivo, precluding biosynthesis of new β-hydroxy amino acids. Hence, there is a selective pressure to kinetically shield E(QGly) from protonation. This intermediate rapidly reacts when an aldehyde substrate is added (Figure 3c). This scenario also explains an otherwise perplexing observation made by previous studies of tTTA enzymes that Gly does not effectively enter the catalytic cycle: the E(QGly) is thermodynamically unstable in tTTA active sites.

While the focus of the present work is on structure and mechanism, experiments with the native p-nitrophenylacetaldehyde substrate were hindered by the instability of this compound in water. We therefore sought to probe the ObiH reaction with an α-branched isobutyraldehyde substrate that forms (2S,3R)-β-hydroxy-Leu. Synthesis of this desirable amino acid analog previously required multistep methods, which are vastly simplified with this biocatalytic route.56,57 Notably, initial velocity studies showed ObiH turns over isobutyraldehyde at a rate of 12 min⁻¹ (Figure 2b). Were the competing protonation pathway facile, formation of β-hydroxy-Leu would be severely limited. Hence, the long lifetime of the E(QGly) intermediate enables reactivity with non-native aldehyde substrates.

We envision this mechanistic and structural information will spur future application of ObiH and other tTTA enzymes for preparative scale biocatalysis. Coupled enzyme reactions have shown that ObiH can react with phenylacetaldehydes, as well as a handful of simpler aliphatic aldehydes.37,38 Studies have also revealed that ObiH and its homologues can react with over a dozen aromatic aldehydes.37,38 Unlike the tTAs, several of the resulting phenyl serine analogs can be formed with both high yield and excellent dr. Recently, the ObiH homologue, PsTTA (99% sequence identity), was engineered for improved yield and selectivity en route to (2S,3R)-p-methylsulfonylphenylserine.37 These authors reported a double mutant, N35S/C57N, that increased activity with the corresponding aldehyde by 8-fold. The structure and MD simulations of ObiH reported here reveal that Asn35 is in the active site of the enzyme and that Cys57 is within a highly mobile loop. Although this variant has relatively low conversion with other substrates, the availability of structural data may facilitate targeted engineering approaches to further improve the catalyst. This information, combined with the high expression titer of ObiH and its stability over months at −80 °C, make this enzyme highly attractive for future biocatalytic applications.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00753.

Complete materials and methods as well as supplemental figures and crystallographic information (PDF)

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**Funding**

This work was supported by the Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin—Madison with funding from the Wisconsin Alumni Research Foundation and the NIH DP2-GM137417 to A.R.B.; Morgridge Institute for Research — Metabolism Theme Fellowship to P.K.; NIH Chemistry–Biology Interface Training Grant T32-GM008505 to A.M.; and NIH Biotechnology Training Grant T32-GM008349 to J.M.E. The Bruker AVANCE III-500 NMR spectrometers were supported by the Bender Fund. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

**Notes**

The authors declare no competing financial interest.
**ACKNOWLEDGMENTS**

We acknowledge the invaluable support, assistance, and advice from our colleagues in the Buller lab. We thank R. Trachman for helpful discussion and comments on the manuscript. We are grateful to the Yoon group for the use of their LEDs.

**ABBREVIATIONS**

lTTAs, l-Threonine transaldolas; ncAAs, noncanonical amino acids; NRPS, nonribosomal peptide synthetase; PLP, pyridoxal-5’-phosphate; Thr, l-threonine; FTA, fluorothreonine transaldolase; lTA, Thr aldolase; SHMT, serine hydroxymethyltransferase; Gly, glycine; E(AexTh), Thr external aldolimide; E(Qgly), glycyglycimine; E(AexGly), Gly hydroxymethyltransferase; Gly, glycine; E(AexThr), Thr ex-

**REFERENCES**


https://dx.doi.org/10.1021/acscambio.0c00753