Site-selective deuteration of amino acids through dual protein catalysis

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ABSTRACT: Deuterated amino acids have been recognized for their utility in drug development, for facilitating NMR analysis, and as probes for enzyme mechanism. Small molecule-based methods for the site-selective synthesis of deuterated amino acids typically involve *de novo* synthesis of the compound from deuterated precursors. In comparison, enzymatic methods for introducing deuterium offer improved efficiency, operating directly on free amino acids to achieve hydrogen-deuterium (H/D) exchange. However, site-selectivity remains a significant challenge for enzyme-mediated deuteration, limiting access to desirable deuteration motifs. Here we use enzyme-catalyzed deuteration, combined with steady-state kinetic analysis and UV-vis spectroscopy to probe the mechanism of a two-protein system responsible for the biosynthesis of L-*allo*-Ile. We show an aminotransferase (DsaD) can pair with a small partner protein (DsaE) to catalyze Cα and Cβ H/D exchange of amino acids, while reactions without DsaE lead exclusively to Cα-deuteration. With conditions for improved catalysis, we evaluate the substrate scope for Cα/Cβ-deuteration and demonstrate the utility of this system for preparative-scale, selective labeling of amino acids.

INTRODUCTION

Deuterated compounds have received significant attention owing to their unique physical and chemical properties.[1] For example, the deuteration of drug molecules can alter their pharmacokinetic properties by slowing oxidative metabolism of the compound *in vivo*.[2–4] This change can extend the lifetime of the active pharmaceutical agent and enable lower dosing to achieve the same physiological effects.[2–4] As a result, deuterium isotopologs of several known pharmacocores (Figure 1A) are currently in clinical trials (such as d_3-L-DOPA (**1**), and d_9-L-868417 (**2**)) or have been fully approved (deutetrabenazine, **3**).[3] Other deuterated molecules, such as amino acids, are particularly useful in biochemistry and have been used in evaluating enzyme mechanisms, tracking metabolites through biosynthesis and for improving signal in NMR analysis.[5–8] Control over the site of the modification (Cα or Cβ deuteration) of amino acids is particularly important in protein NMR, enabling the attenuation of specific signals to improve resolution.[8] These applications have spurred strong demand for methods to generate selectively deuterated α-amino acids. However, there are significant synthetic challenges for efficiently accessing isotopologs in a site- and stereoselective manner.

A few general approaches have been developed to access Cα and Cβ deuterated α-amino acids including *de novo* synthesis from deuterated building blocks or by pre-activation of the amine, followed by hydrogen/deuterium (H/D) exchange under basic conditions.[9–11] Small molecule-based methods that avoid pre-functionalization of amino acids are rare and typically involve catalytic hydrogenation (Pd/C or Pt/C) in D_2O.[12,13] This approach has been generally limited to the synthesis of Phe or Tyr isotopologs.[12,13] Amino acids exclusively labelled at Cβ are useful isotopologs for NMR studies and have been used to probe enzyme mechanism.[14,15] However, the synthesis of selectively Cβ-deuterated amino acids is particularly challenging, and has only been accomplished by multi-step synthesis from selectively deuterated building blocks or by radical deuteration under gamma irradiation conditions.[12,13,16–18] These approaches are not general for amino acid substrates, as *de novo* amino acid synthesis requires unique synthetic routes for each desired product. Direct functionalization of amino acids using radical chemistry has been demonstrated, but site-selectivity is highly substrate-dependent, reducing the appeal of this approach.[17]

The search for techniques to directly and selectively deuterate amino acids has led to the development of several enzymatic and chemoenzymatic processes.[9,19] The three-dimensional architecture of an enzyme active site can provide tight control over the site- and stereoselectivity of reactions. Enzymes also operate directly on free amino acids, avoiding the need for protecting or directing group strategies and streamlining synthetic routes. Previous chemoenzymatic strategies for amino acid deuteration at Cα and Cβ have proceeded through enzyme-catalyzed deuteride delivery (via NAD(P)D) to achieve reductive amination or through transamination of deuterated α-keto acids.[20–22] Such approaches require the *in situ* regeneration of deuterated reducing equivalents or pre-functionalization of ketone substrates, which present additional challenges to reaction design.[20–22] Enzymes that catalyze simple H/D exchange avoid these requirements and efficiently access isotopologs from their proteo-

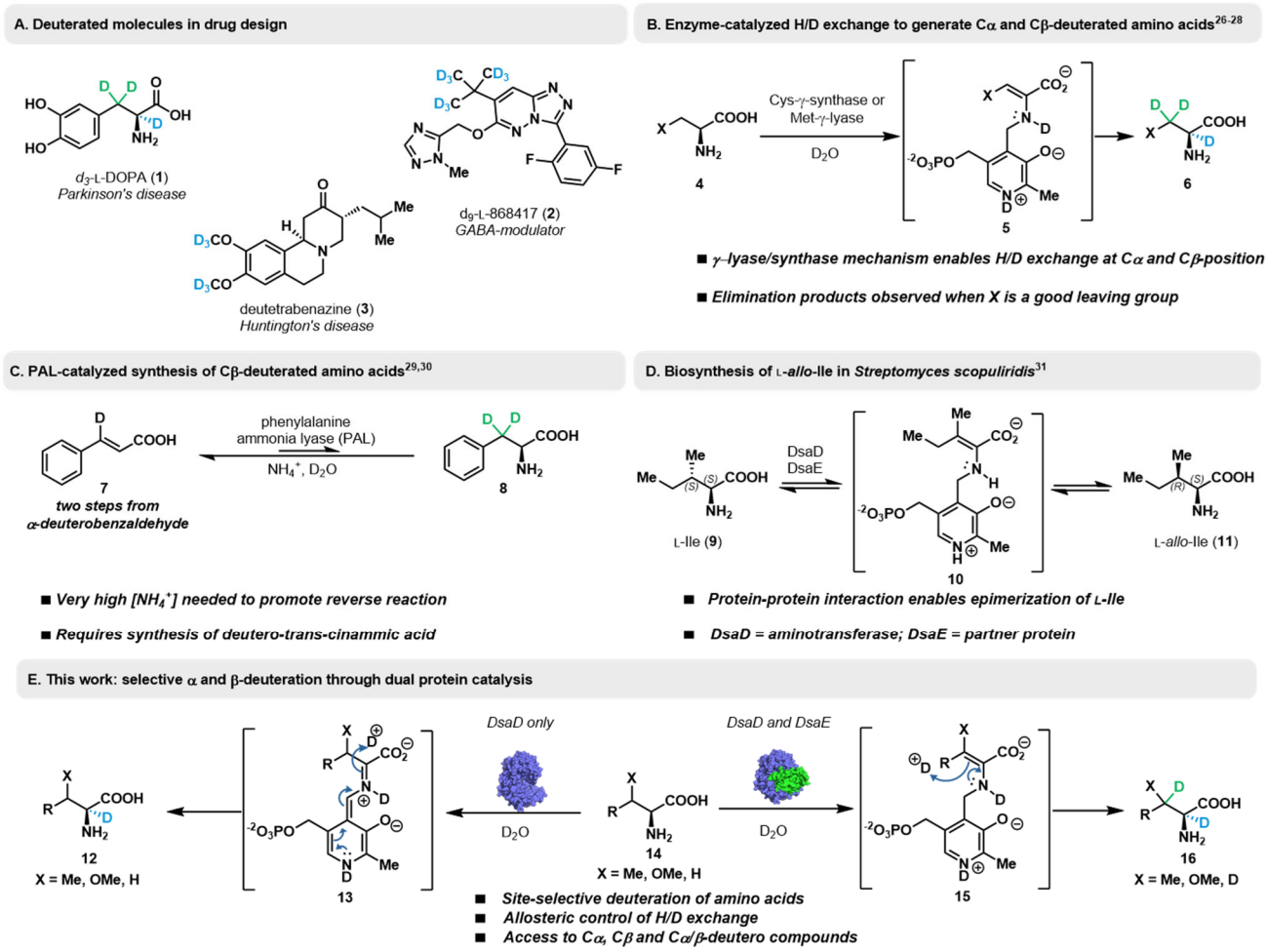

Figure 1. A. Representative examples of deuterated pharmaceuticals. B. Enzyme-catalyzed hydrogen/deuterium (H/D) exchange to produce Cα and Cβ-deuterated amino acids. C. PAL-catalyzed synthesis of Cβ-deuterated amino acids from deutero-*trans*-cinnamic acid. D. Biosynthesis of L-*allo*-Ile by two-protein catalyzed epimerization of L-Ile. E. This work: leveraging the Ile epimerization system for selective deuteration of amino acids at Cα and Cβ through dual protein catalysis.

precursors using inexpensive D_2O as the heavy label source. For example, PLP-dependent enzymes that catalyze Cα-deprotonation have been used to generate Cα-deuterated amino acids and esters.[23–25] In similar fashion, enzymes that catalyze Cα and Cβ deprotonation (such as methionine-γ-lyase and cystathionine-γ-synthase) can generate Cα/Cβ-deuterated products when reactions are performed in D_2O (Figure 1B).[26–28] These enzymes provide efficient access to isotopologs, but label both Cα and Cβ indiscriminately and have relatively narrow substrate scopes.[26,27] Site-selective Cβ-deuteration remains a challenging pattern to access, and has only been accomplished on aromatic amino acids by the reverse action of phenylalanine ammonia lyase in D_2O (Figure 1C).[29,30] We envisioned that an operationally-simple enzymatic route to selectively deuterated materials would be attractive to the synthetic community. In particular, we anticipated that the ability to tune the site-selectivity of an H/D exchange reaction would enable efficient synthesis of isotopologs with the desired labelling pattern, precluding the need for amino acid pre-functionalization steps.

Recently, Li et al. elucidated the biosynthetic origins of L-*allo*-Ile, a non-standard amino acid (nsAA) found in several bacterial peptide natural products.[31] Two biosynthetic proteins were shown to work in tandem to catalyze the epimerization of canonical (2*S,* 3*S*)-Ile (**9**) to (2*S*, *3R*)-Ile (L-*allo*-Ile, **11**) in *Streptomyces scopuliridis*: (1) DsaD, originally annotated as a PLP-dependent branched chain aminotransferase (BCAT) and (2) DsaE, a small partner protein, that shares very little sequence identity with other known protein families (Figure 1D).[31] In the absence of either protein, the epimerization reaction was not observed, indicating that epimerization proceeds through a unique, two protein-dependent mechanism. In addition, when DsaD was incubated with α-ketoglutarate and Ile, no aminotransferase activity was observed, indicating an unusual catalytic role for this protein.[31] The epimerization reaction observed by Li et al. was proposed to occur through binding of L-Ile to the PLP cofactor, followed by Cα-deprotonation of L-Ile to form an iminium ion (**13**).[31] A second deprotonation was proposed to occur at Cβ to form an achiral enamine intermediate (**10**, Figure 1D).[31] Subsequent reprotonation of Cβ on the opposite face would lead to the observed epimerization and facially selective reprotonation at Cα would deliver L-*allo*-Ile (**11**) as the product.[31]

Although a mechanism for the L-Ile (Ile) epimerization reaction was previously proposed, little is known about

the role of each protein in this transformation. The practical limitations of studying the Ile epimerization reaction (i.e. the efficient chromatographic separation of diastereomers) present significant roadblocks to a detailed analysis of kinetics and mechanism. In addition, using epimerization as a readout for enzyme activity provides no information about the contributions of each protein to individual steps in the catalytic cycle. For example, must DsaD and DsaE be in complex for substrate binding to occur? Can non-branched amino acids productively enter a catalytic cycle? In the absence of a second stereocenter, any reaction would simply return the starting material and provide no readout of activity. Here, we show that epimerization reactions performed in D_2O lead to H/D exchange at Cα and Cβ of Ile, providing a simple, mass spectrometry-based readout of enzyme activity (Figure 1E). We utilized this assay to probe key features of DsaD/E catalysis and leverage these insights to prepare selectively deuterated amino acids, providing a unique biocatalytic platform to access these important materials.

RESULTS AND DISCUSSION

To answer outstanding mechanistic questions about the two protein-dependent epimerization of Ile, we sought a simple, efficient, and reproducible assay for measuring enzyme activity. We envisioned that running the Ile epimerization reaction in D_2O would lead to hydrogen-deuterium (H/D) exchange, which would be used resolve distinct proton transfer steps in the mechanism. According to the mechanism of Li et al.,[31] reactions of the DsaD/E complex with L-Ile in D_2O would deliver a mixture of Cα and Cβ-deuterated d_2-2,3-L-Ile and d_2-2,3-L-*allo*-Ile (**23**). To ease chromatographic challenges with highly polar amino acids, reactions were quenched and the crude reaction was treated with Marfey's reagent (L-FDAA).[32] Reactions were analyzed by mass spectrometry after reverse phase chromatography (see Supporting Information for detailed procedure). Initial test reactions were performed using conditions described by Li et al. for Ile epimerization, except in D_2O instead of H_2O. In our reaction, 0.05 mol% purified DsaD and DsaE (1:1) were combined in D_2O with 50 mM sodium phosphate (pD 8.4), 0.1 mol% PLP and 1 mM Ile. Reagents were prepared in D_2O to reduce ^{1}H-water contamination to <1%. After an 8 h incubation with DsaD and DsaE at 37 °C, a 1:1 mixture of d_2-2,3-L-Ile and d_2-2,3-L-*allo*-Ile was observed as the major product. No appreciable deuterium exchange (< 3%) was observed in reactions without protein.

To begin probing the independent roles of the enzymes in this complex, we conducted H/D exchange reactions with just DsaD (excluding partner protein DsaE). We observed no transaminase activity under these conditions, in accordance with previous studies of the DsaD/E system, which would otherwise confound kinetic analysis.[31] However, L-Ile still appeared to bind DsaD, which catalyzed a single H/D exchange event.

Steady-state kinetic analysis of H/D exchange reactions. With a reproducible assay in hand for kinetic analysis of DsaD/E-catalyzed reactions, we sought to untangle the nature of the DsaD/E complex by assessing how changes in relative protein stoichiometry affect the activity. With one equivalent of partner protein DsaE, the Cβ-deuteration reaction proceeds with a k_{cat} of 0.07 ± 0.007 s^{-1} and a K_M value of 2.4 ± 0.68 mM (Figures 2A and 2D). The addition of 5 equiv. of DsaE (5:1 DsaE:DsaD) did not significantly change k_{cat}, but we did observe an 11-fold decrease in the observed K_M, to 0.2 ± 0.01 mM. Increasing partner protein stoichiometry further to 50 equiv. (50:1 DsaE:DsaD) led to a nearly 4-fold increase in k_{cat} with a similar K_M value (0.3 ± 0.09 mM). To quantitate the strength of the DsaD/E interaction, we fixed the concentration of Ile and measured the initial rate of Cβ-deuteration. The reaction rate increased with additional equivalents of DsaE until reaching a plateau around 0.3 mM DsaE, corresponding to a 100:1 ratio of the two proteins (Figure 2B). As the system is under steady state, not equilibrium conditions, we fit these data to the Michaelis-Menten equation, from which we calculated a K_M of 40 ± 5 μM for formation of the active DsaD/DsaE complex. This is a notably weak interaction when compared to other PLP-dependent enzymes that form protein complexes, such as the tryptophan synthases.[33,34] We next sought to probe how complexation affects the earlier steps in the reaction.

We measured the initial rates of Cα deuteration of Ile and fitting to the Michaelis-Menten equation (Figure 2C) showed that DsaD alone catalyzes Cα exchange with a k_{cat} of 1.04 ± 0.04 s^{-1} and K_M of 0.7 ± 0.1 mM (Figure 2D). Hence, the two reactions are not well-coupled, with Cα-exchange being much faster than the Cβ exchange reaction of the full complex under similar conditions. To assess how far into the mechanism DsaD can progress in the absence of DsaE, we performed a steady-state UV-visible spectroscopic analysis. In the absence of substrate, DsaD exists as a classic internal aldimine (**17**) with λ_{max} of 423 nm (Figure 2E). Upon addition of saturating L-Ile, the internal aldmine peak disappears concomitant with the appearance of a new absorbance band at 328 nm, consistent with a ketimine adduct (**21**) with a protonated C4'.[35,36] Because DsaD has minimal BCAT activity, the iminium present in the ketimine adduct must be kinetically shielded from hydrolysis, which affords time for DsaE to bind and enable deprotonation at Cβ. We observed DsaE binding lowers the apparent K_M for Ile (0.12 ± 0.02 mM) and, curiously, decreases the k_{cat} of Cα-deuteration (0.75 ± 0.01 s^{-1}). Further increasing the concentration of DsaE to 150 μM (50:1 DsaE:DsaD, above the K_M) did not significantly impact the observed k_{cat} or K_M for Cα-deuteration (Figure 2D). Combined, these data indicate that Ile binds DsaD and forms a reversible ketimine adduct that can undergo multiple Cα exchange events. Upon DsaE binding, changes in the active site decrease the K_M for Ile, slowing the rate of Cα deuteration, which we suggest increases the lifetime of bound Ile, providing time for the slower Cβ-epimerization reaction to occur.

The DsaD/E system catalyzes H/D exchange with a variety of amino acid substrates. Our analysis of the kinetic parameters of Cα and Cβ deuteration revealed core characteristics of the Ile epimerization system. However, it was still not known if the enzyme complex could productively engage amino acids other than Ile, as unbranched amino acids have no additional stereocenter to epimerize. To evaluate if the DsaD/E system could

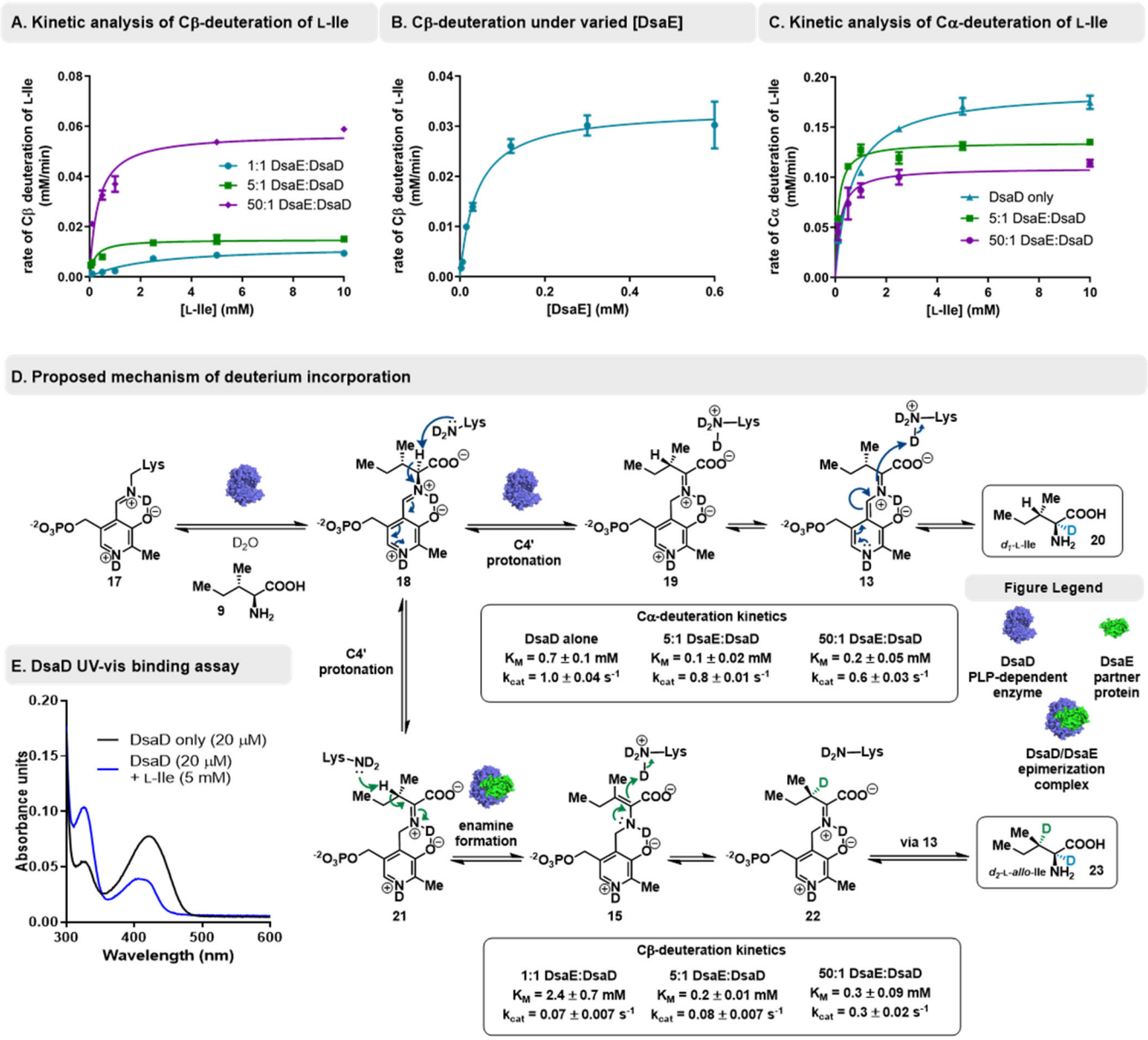

Figure 2. Steady-state kinetic analysis of H/D exchange by the Ile epimerization system. A. Steady-state kinetic analysis describing Cβ-deuteration[a] of L-Ile under varied equivalents of partner enzyme DsaE. B. Steady-state kinetic analysis of Cβ-deuteration rates[a] at a constant 1 mM L-Ile measured against increasing equivalents of DsaE. C. Steady-state kinetic analysis of Cα-deuteration[a] of L-Ile in the presence and absence of partner protein DsaE. D. Proposed mechanism of selective deuterium incorporation at Cα and Cβ catalyzed by dual protein catalysis. E. UV-visible spectrum of DsaD in the absence and presence of L-Ile. [a]Measurement of initial rates was performed in duplicate at 24 °C. Conditions: 0.1-10 mM L-Ile, 3 μM DsaD, 50 mM sodium phosphate (pD 8.4), 0.1 mM PLP in D_2O (99.9%). Proteins were exchanged into a 50 mM sodium phosphate-D_2O (pD 8.4) solution prior to reaction initiation to minimize proton contamination (< 1% H_2O). Following quench with MeCN, crude reaction products were subjected to functionalization with Marfey's reagent (L-FDAA) to enable analysis by reverse-phase chromatography and quantification of isotope incorporation by mass spectrometry.

operate on other substrates, we subjected a small set of amino acids to Cα/β H/D exchange conditions (see Figure 3A). We initially chose three amino acids that bear structural similarity to the native L-Ile: L-Leu, L-Val and L-Phe. Reaction conditions used a 1:1 mixture of DsaD and DsaE (both at 0.05 mol% catalyst). Interestingly, these reactions (Figure 3A) delivered high conversion to the Cα-deuterated isotopologs (94-99% at Cα), showing that DsaD retains the ability to bind diverse substrates, similar to BCAT homologs.[37] We also observed modest incorporation of deuterium at the Cβ-position (30-62% at Cβ), indicating the Cβ-exchange reaction promoted by DsaE is robust to modest changes in substrate structure (Figure 3A). Although successful deuteration of non-native substrates suggests the possibility of a biocatalytic platform for site-selective deuteration of amino acids, the deuterium incorporation at Cβ would need to be increased to produce a practical system for scalable amino acid labelling.

The kinetic characterization of DsaD/E-catalyzed deuteration of Ile suggested that maximal rates of Cβ-deuteration could be achieved by increasing the

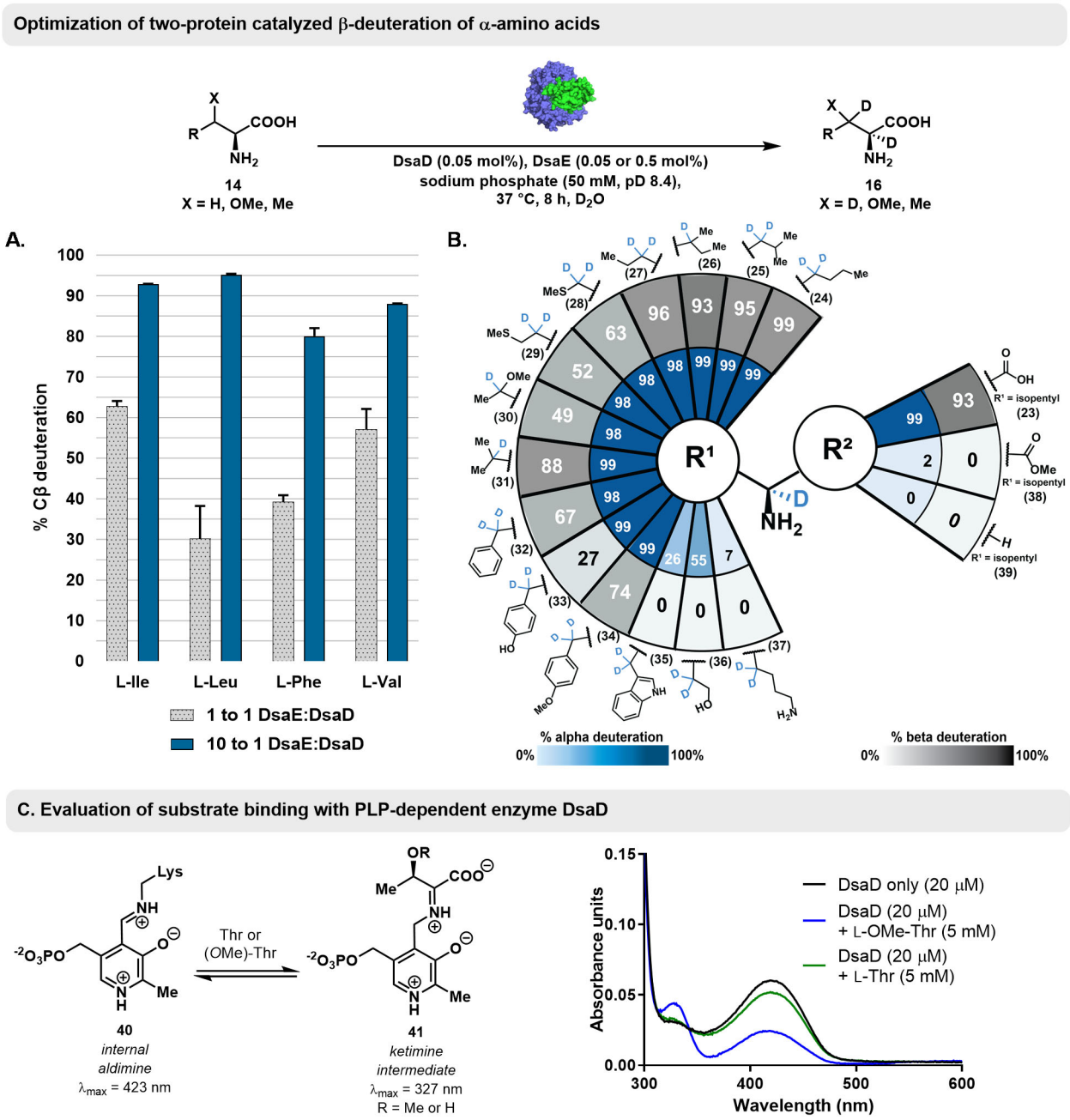

Figure 3. Optimization of H/D exchange reactions and evaluation of the substrate scope. A. Optimization of Cβ-deuteration by increasing equivalents of partner protein DsaD. B. Evaluation of substrate scope of dual protein catalyzed Cα/Cβ deuteration under optimized conditions. C. UV-visible spectroscopy used to evaluate non-native substrate binding to DsaD. Reaction conditions: 10 mM amino acid substrate, 50 μM DsaE, 5 μM DsaD (10:1), 50 mM sodium phosphate (pD 8.4), 0.1 mM PLP, D_2O, 37 °C, 8 h. Reactions performed in duplicate and percent isotope incorporation is reported as the average of the replicates.

concentration of the partner protein DsaE (Figure 2B). We therefore increased the concentration of DsaE to 50 μM (10:1 DsaE:DsaD) which we hypothesized would bring the degree of labelling up to a synthetically useful level while keeping the overall catalyst loading within a reasonable range. Satisfyingly, a 10-fold increase (to 0.5 mol%) in DsaE loading improved Cβ-deuteration for the amino acids tested, delivering moderate to high levels of Cβ-exchange (Figure 3A, 80-94%). With these conditions in hand, we sought to perform a more thorough evaluation of the substrate scope of Cα/Cβ-deuteration using the DsaD/E system.

We performed analytical scale Cα/Cβ deuteration reactions on a variety of standard and non-standard amino acids (Figure 3B, **23**-**39**). Reactions were performed in duplicate under the optimized conditions for deuteration of Ile (0.05 mol% DsaD and 0.5 mol% DsaE). Aliphatic amino acids underwent successful H/D exchange, showing high Cα and Cβ *D*-incorporation (88-99%) for Nle (**24**), Leu (**25**), Ile (**26**), Nva (**27**) and Val (**31**). Thioether-containing amino acids, such as *S*-Me-Cys (**28**) and Met (**29**)

demonstrated high levels of exchange at Cα, but moderate levels of *D*-incorporation at Cβ. Aromatic amino acid Phe (**32**) showed high Cα deuteration, but moderate deuterium incorporation at Cβ (67%). In comparison, Tyr (**33**) underwent Cα-deuteration (99%), but low incorporation at Cβ (27%), presumably due to unfavorable interactions with the polar phenolic group. To test this hypothesis, we subjected the protected (OMe)-L-Tyr (**34**) to Cα/β deuteration conditions. To our delight, **34** underwent succesful H/D exchange, with high *D*-incorporation at Cα (99%) and improved conversion at Cβ (74%). Interestingly, Trp (**35**) underwent Cα-deuteration, but no Cβ-deuteration. We observe a similar pattern with alcohol (homoserine, **36**) and amine-containing (Lys, **37**) substrates. Amino acids with hydroxyl moieties at Cβ (such as Thr and Ser) did not undergo any deuteration. However, protection of Thr as the methyl ether (**30**) enabled productive catalysis with the DsaD/E complex, with high levels of deuteration observed at Cα and moderate deuterium incorporation and scrambling of configuration at Cβ. These results indicate that DsaD is able to engage polar substrates, albeit with diminished efficiency, but that Cβ-deprotonation is not achieved unless the substrate is modified to reduce polar interactions.

To assess whether catalyis with polar molecules is diminished because substrates cannot bind DsaD, or if the subsequent catalysis by the DsaD/E complex is perturbed, we leveraged steady-state UV-vis spectroscopy to monitor amino acid binding to DsaD. Following incubation of DsaD with unmodified Thr, no binding was observed, consistent with the results of the deuteration reaction screen (Figure 3C). However, incubation of DsaD with the Thr methyl ether (L-(*O*Me)-Thr, **30**) enables productive binding of this substrate and formation of the ketimine-species (**41**) (Figure 3C). Based on these results, we conclude that the inability of DsaD to bind unprotected Cβ-hydroxy amino acids prevents productive catalysis by the Ile epimerization complex. This behavior is consistent with the preference for non-polar amino acids exhibited by related BCATs.[36] Notably, capping the polar group as an ether restores both DsaD binding and deuteration activity. Finally, we tested substrates lacking the α-carboxylate moiety, including the methyl ester of Ile (**38**) and isopentylamine (**39**). Neither of these substrates underwent deuteration by the DsaD/E system, demonstrating the importance of an α-carboxylate motif for achieving a catalytically productive pose in the active site.

In the original report by Li et al describing DsaE, a homologous enzyme with 42% sequence identity, MfnH, was disclosed.[31] This homolog could operate with DsaD to catalyze Cβ-epimerization of Ile. Here, we test the ability of MfnH to productively catalyze H/D exchange reactions. We performed reactions with purified MfnH, DsaD (1:10 DsaD:MfnH) and L-Leu under the conditions described for DsaD/E-catalyzed reactions (Figure S42). We found that, L-Leu underwent efficient exchange at Cα (93%) and moderate deuterium labelling at Cβ (58%). Although the extent of deuteration using the DsaD-MfnH protein pair is diminished when compared to the native DsaD-DsaE pair under the same conditions, these experiments demonstrate the unique ability of these partner proteins to react with enzymes from outside their biosynthetic pathway. We also attempted isolation of MfnO, the native BCAT partner of MfnH, but produced only *apo*-enzyme.

Overall, the substrate screen used here showcases the broad tolerance of the DsaD/E system to changes in side chain structure, which would be challenging to assess without a robust assay to differentiate these distinct reactivities. Given the broad utility of deuterated amino acids, we envisioned that this unique dual protein system could be leveraged for preparative-scale synthesis.

The Ile epimerization system catalyzes site- and enantioselective deuteration of amino acids. The reactions on analytical scale demonstrated that the DsaD/DsaE catalytic system could achieve productive catalysis with a variety of amino acids. However, the development of a scalable biocatalytic method requires overcoming additional challenges. Operational simplicity is critical and demands facile access to the biocatalysts, particularly as high enzyme loadings for DsaE were required to achieve satisfactory H/D exchange at the Cβ-position. Use of clarified cell lysates would obviate costly protein purification and enable mmol-scale exchange reactions. Initial test reactions with L-Leu were carried out using lysates at an equivalent concentration of 1.2 mg wet cell mass/mL reaction for each biocatalyst. Because DsaE expresses similarly to DsaD, but has a lower molecular weight, these conditions provide a modest stoichiometric excess of the partner protein. These conditions limit the overall concentration of ^{1}H-water in reactions to 5%, setting the maximum achievable *D*-incorporation to 95%. After 16 h, reactions were quenched and purified by reverse phase chromatography. ^{1}H NMR analysis (see Figure 4) confirmed production of the Cα and Cβ-deuterated isotopolog, L-Leu-2,3,3-d_3 (**25**) with high deuterium incorporation levels (95% for Cα and 86% for Cβ). UPLC analysis of isolated material following treatment of product with Marfey's reagent demonstrated that stereoconfiguration at the Cα-position was retained under the reaction conditions (>99% ee), demonstrating that DsaD catalyzed an enantioselective H/D exchange. The level of *D*-incorporation in this system can also be controlled by modifying the concentration of ^{1}H-water in the reaction. To increase labelling, we pre-dialyzed DsaD and DsaE lysates into a D_2O-Na_3PO_4 buffer (pD 8.4) for 2 h, then ran the H/D exchange reaction. Following this simple procedure, a reaction of L-Leu (Figure S40) led to very high *D*-incorporation at Cα (>99%) and Cβ (98%), demonstrating that nearly quantitative labelling can be achieved (see Supplemental Information for details).

Inspired by the potential utility of the clarified cell lysate system to achieve site-selective deuteration, we envisioned that the addition of DsaD alone would catalyze scalable, selective H/D exchange at the Cα-position, including back-exchange of L-Leu-2,3,3-d_3 (**25**) to access L-Leu-3,3-d_2 (Figure 4, **44**). We treated L-Leu with DsaD in D_2O, leading to the site- and enantio-selective formation of Cα-deuterated L-Leu-2-*d* (**42**, 95% *D* incorporation, >99% *ee*), as determined by ^{1}H NMR and UPLC analyses (Figure 4). To further expand the scope of selective deuteration accessible using the DsaD/E system, we performed a two-step biocatalytic reaction sequence to access Cβ-deuterated products.

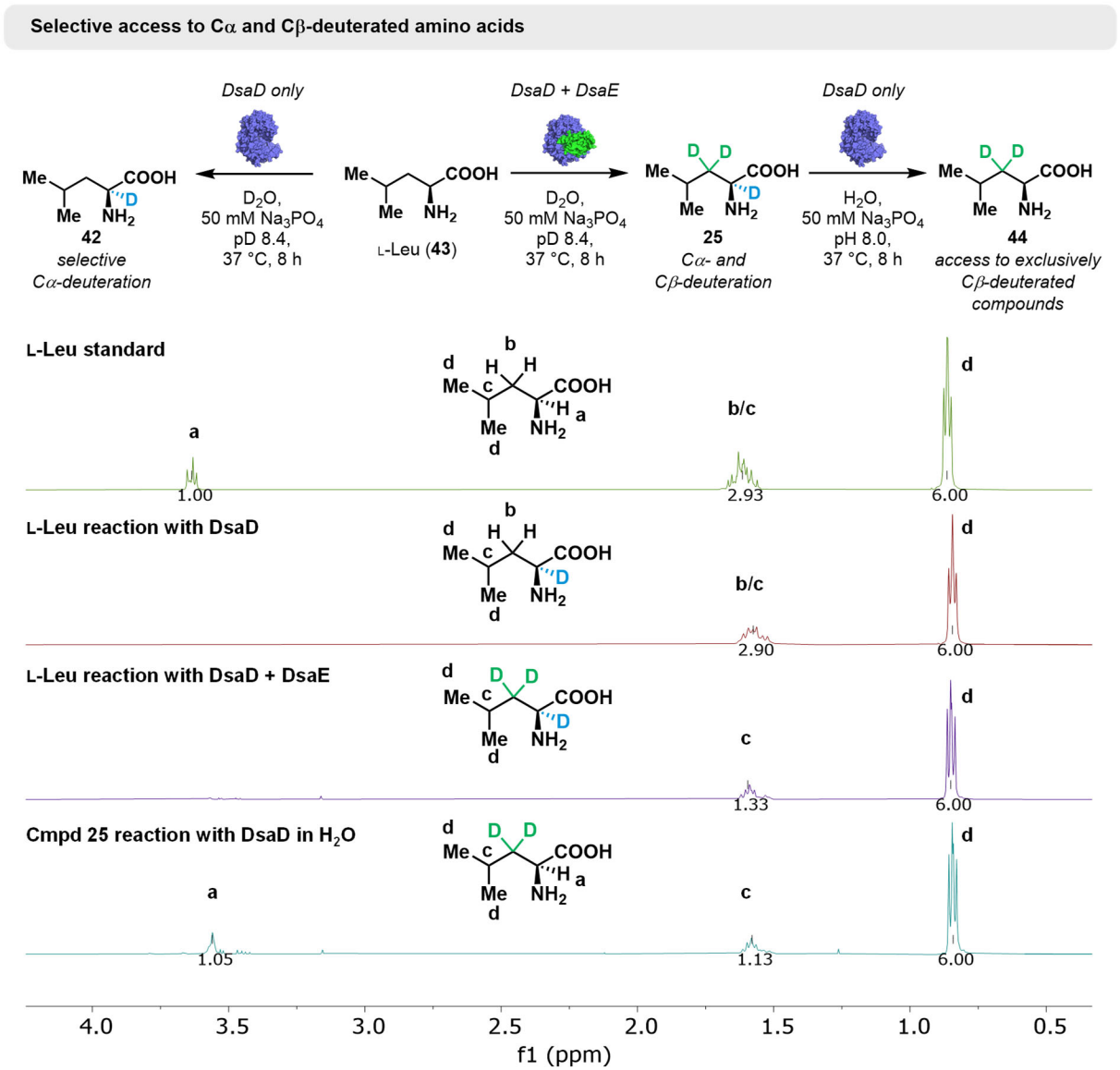

Figure 4. ^{1}H NMR analysis of site-selective deuteration of L-Leu. Reaction conditions: 20 mM L-Leu 2.5% v/v DsaD clarified lysate, 2.5% v/v DsaE clarified lysate (when needed), 50 mM sodium phosphate (pD 8.4), 0.1 mM PLP, D_2O (99.9% *D*).

An initial reaction was performed with DsaD and DsaE to produce Cα/Cβ-deuterated L-Leu-2,3,3-*d*$_3$ (**25**). Following reaction quench with acetone, centrifugation to remove protein products, and removal of acetone and D_2O via rotary evaporation, the dry crude product mixture was subjected to standard reaction conditions with DsaD in water. This reaction led to washout of the Cα deuterium, providing exclusively Cβ-deuterated L-Leu-3,3-*d*$_2$ (**44**) with high levels of deuterium incorporation at Cβ (Figure 4, 86% *D* incorporation and 98% *ee*). The site of H/D exchange in these reactions is dictated by the presence or absence of DsaE from the reaction conditions, enabling tight control of amino acid deuteration patterns.

Achieving site-selective Cα and Cβ deuteration in biocatalytic H/D exchange systems was an outstanding challenge, as enzymes that catalyze Cβ-exchange (such as PLP-dependent γ-synthases and γ-lyases) initially proceed through Cα-deprotonation, leading to concomitant H/D exchange at Cα. Therefore, catalyst-controlled site-selectivity provides a novel route by which the desired deuteration pattern can be achieved. The clarified cell lysate system used here serves as an efficient and inexpensive method for preparing the H/D exchange biocatalyst. For example, in an average 0.5 L expression of DsaE in *E. coli*, ~16 grams of cells are isolated, providing enough cell lysate from a single protein expression to perform H/D exchange on ~37 grams of L-Leu under the standard conditions developed here.

Because DsaE has been observed to operate with a variety of BCAT enzymes, we questioned whether DsaE, in just the presence of *E. coli* BCATs, could effect a Cα/Cβ-exchange without DsaD. We performed an analytical scale H/D exchange reaction on L-Leu (**43**) using 5% v/v DsaE lysate (Figure S41). This reaction resulted in high labelling at Cα (>95%) and moderate deuterium incorporation at Cβ (70%), demonstrating that DsaE can utilize native BCATs present at biological concentrations (without overexpression) to perform H/D exchange. However, the level of *D*-incorporation at Cβ was not high enough to merit a change in our deuteration protocol and we opted to use the DsaD/E system for the remaining preparative-scale studies.

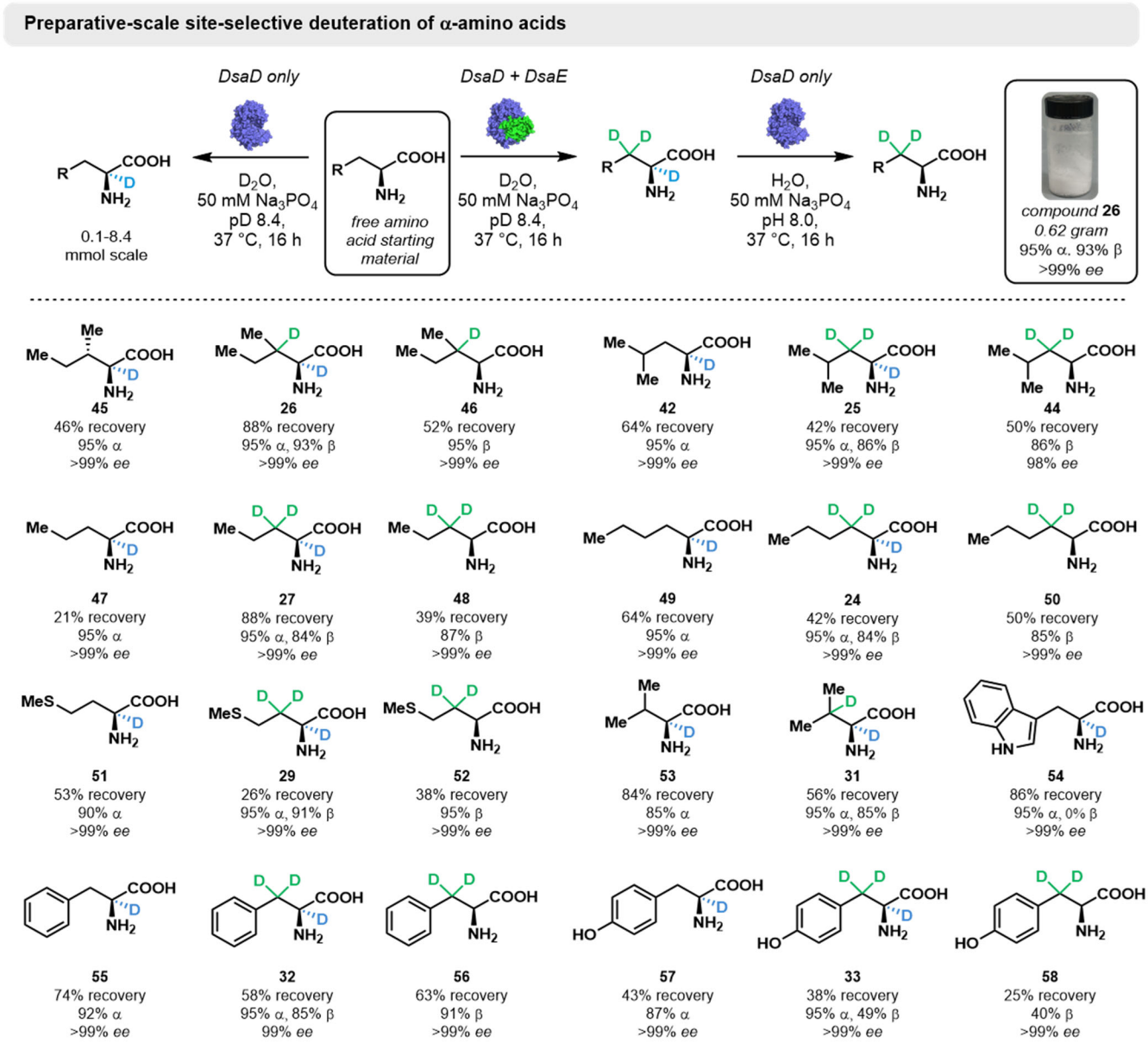

Figure 5. Preparative-scale and site-selective deuteration of amino acids. Conditions: 20 mM L-Leu, 2.5% v/v DsaD clarified lysate, 2.5% v/v DsaE clarified lysate, 50 mM sodium phosphate (pD 8.4), 0.1 mM PLP, D_2O (99.9%).

After demonstrating the site- and enantioselectivity provided by the DsaD/E system on a single substrate, we pursued the scalable, site-selective deuteration of a variety of aliphatic and aromatic amino acids. We subjected both standard and non-standard amino acids to preparative-scale deuteration conditions. Deuterium incorporation levels were determined by UPLC-MS analysis and site-selectivity was confirmed by ^{1}H NMR. In reactions with only DsaD, both aliphatic and aromatic amino acids (Figure 5) demonstrated high deuterium incorporation at Cα (85-95%), with excellent retention of configuration (>99% *ee*). We also performed 0.2–0.5 mmol scale reactions with DsaD and DsaE to catalyze H/D exchange at both Cα and Cβ. Aliphatic amino acids were successfully deuterated, with high incorporation levels at Cα (95%) and Cβ (84-93%) and >99% *ee*. As a further demonstration of scalability, Ile was deuterated on >600 mg scale, delivering high levels of deuteration at Cα (95%) and Cβ (93%). Aromatic and thioether-containing amino acids proved slightly more challenging and reactions were run at lower substrate loading (10 mM) to produce higher deuterium incorporation levels. Under these conditions, L-Phe-2,3,3-d_3 (**32**) was produced with high deuterium incorporation at Cα (95%) and Cβ (85%). However, the Cβ-deuteration of L-Tyr was less efficient, leading to moderate Cβ deuterium incorporation (49%). Cα deuterium incorporation was still high for this reaction (95%), suggesting that the catalytic limitations of the DsaD/E complex are different than observed with DsaD as a standalone enzyme. These observations are in agreement with analytical scale

experiments, which showed that unprotected polar functional groups led to poor incorporation of deuterium at Cβ. We also note that clarified cell lysate reactions led to improved deuterium incorporation with poor substrates when compared to purified enzyme reactions. This improvement is likely due to relatively high protein titers in clarified lysates and demonstrates that lysate-based approaches can contend with use of costly purified enzymes.

As there are few site-selective methods for accessing Cβ-deuterated amino acids, we last sought to demonstrate the utility of the DsaD/E enzymatic platform for accessing this challenging pattern of isotope labelling. We performed Cα/Cβ deuteration on a panel of amino acids, quenched the reactions and resubjected the crude product to Cα deuterium washout with DsaD in H_2O. Following this sequence, aliphatic amino acids were labelled with high deuterium incorporation at Cβ (84%-95%) and excellent retention of configuration (98-99% *ee*). Reactions with aromatic and thioether-containing amino acids were again performed at lower substrate loading (10 mM), leading to high deuterium incorporation at Cβ for L-Phe (91%) and moderate incorporation for L-Tyr (40%). We note that even incomplete deuteration can provide useful material, as mixtures of isotopologs can be deployed for powerful mechanistic experiments, such as isotopic labelling and elucidation of kinetic isotope effects.[2,5]

The relatively wide scope of this native enzyme system, along with its slow rate of reaction, contrasts with other PLP-dependent enzymes. Because L-allo-Ile is only required in small amounts for secondary metabolism, and L-Ile is essential for protein synthesis, there is a clear selective pressure for this complex to only operate at a slow rate. In contrast, in the absence of D_2O, the activity of the DsaD/E complex is totally masked for substrates lacking a Cβ-branch. Consequently, there is no selective pressure for the system to discriminate against any standard amino acid other than Thr. Our data here show this selectivity is achieved on the simple basis of hydrophobicity, which leaves open the wide chemical space that reacts in the H/D-exchange disclosed here.

CONCLUSIONS

We have characterized the two protein-dependent Ile epimerization system and demonstrated the synthetic utility of this system for the scalable and selective deuteration of several α-amino acids. H/D exchange was initially used as a convenient proxy for epimerase activity. Kinetic experiments illustrated that rates of Cβ-deuteration are highly dependent on the concentration of partner protein, DsaE, with a comparatively weak K_M for their association, 40 uM. These observations were used to improve Cα and Cβ deuterium incorporation in analytical experiments. Substrate screening efforts identified numerous amino acids that could undergo productive H/D exchange reactions, including a variety of aliphatic and aromatic amino acids. Furthermore, a preparative-scale biocatalytic reaction platform was established which enabled access to selectively deuterated materials with Cα, Cα/Cβ, and the challenging Cβ-only deuteration patterns. This operationally simple and inexpensive reaction system delivers the desired deuteration pattern without the need for protein purification or multi-step substrate deuteration procedures. These data provide a foundation for future study of the intriguing DsaD/E protein complex, as well as demonstrate this system can be leveraged to efficiently access a variety of amino acid isotopologs.

ASSOCIATED CONTENT

Supporting Information. Full experimental details, including ^1H NMR spectra for all compounds, LC/MS spectra, supporting figures and methods. This Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

H/D: hydrogen/deuterium, NAD(P)D: nicotinamide adenine dinucleotide (phosphate) deuteride, *ee*: enantiomeric excess

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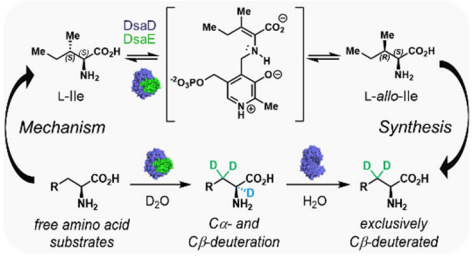
DsaD
DsaE
L-Ile
L-allo-Ile
Mechanism
Synthesis
D2O
H2O
free amino acid substrates
Cα- and Cβ-deuteration
exclusively Cβ-deuterated