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Mechanistic study of the radical SAM-dependent amine dehydrogenation reactions

Xinjian Ji, Wan-Qiu Liu, Shuguang Yuan, Yue Yin, Wei Ding, and Qi Zhang*

Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with a diode array detector equipped with a C18 column (AccucoreXL C18, Thermo Scientific, 4.6 x 260 mm,1.8 um particle size). High resolution mass spectrometry (HR-MS) analysis was performed using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap MassSpectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher). NMR spectra were recorded using Bruker 400 MHz NMR spectrometer at the Nuclear Magnetic Resonance Facility at Fudan University. PCR was performed on a Bio-Rad T100™ Thermal Cycler using PrimeSTAR® HS DNA Polymerase (Takara Biotechnology Co. Ltd, China) or Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd, China).

Chemicals and Biochemicals

All chemical reagents and anhydrous solvents were purchased from commercial sources and used without further purification unless otherwise specified. S-adenosyl-L-methionine (SAM) were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Pyridoxal-5-phosphate (PLP), L-Trp, D-Trp, L-Tyr, $Fe(NH_4)_2(SO_4)_2•6H_2O$ and Na_2S were from Adamas Reagent Co. Ltd (Shanghai, China). [$^2H_7$]-L-Tyr and [$^2H_8$]-L-Trp were from Cambridge Isotope Laboratories, Inc (USA).

Construction of the ThiH expression plasmid

The *thiH* gene was amplified from the genomic DNA of *Escherichia coli BL21 (DE3)* using primers *thiH-F* and *thiH-R* (**Supplementary Table 1**). The PCR amplified product was digested with *EcoRI* and *HindIII*, purified using a Qiagen PCR purification kit, and inserted into the same restriction site of the expression vector pET28a (Novagen). Chemically competent *E. coli* DH5α

cells were transformed with the ligation mixture and plated on LB-agar containing kanamycin (50/μg mL) to screen for positive clones, which were confirmed by DNA sequencing.

Construction of the plasmid for expressing low-specificity amino acid racemase (Alr)

The *alr* gene was amplified from the genomic DNA of *Pseudomonas putida* using primers *alr-F* and *alr-R* (**Supplementary table 1**). The PCR amplified products were purified and inserted into the NdeI/XhoI restriction site of pET28a (Novagen) by homologous recombination using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd). Typically, 20 μL of a mixture containing 4 μL of 5×CE II Buffer, 2 μL of Exnase II, ~50 ng linear plasmid, ~100 ng PCR fragment and ddH_2O was incubated at 37°C for 30 min, and the resulting solution was used to transform chemically competent *E. coli* DH5α cells. Colony PCR and sequencing were carried out to confirm the sequence fidelity of the recombinant plasmid.

Construction of the ThiH-R301K and NosL-R323K expression plasmid

Mutants were constructed using the one-step site-directed mutagenesis method. Briefly, two fragments were amplified from the plasmid ThiH-pET28a using a primer pair *ThiH-F1* and *ThiH-R301K-R*, and a primer pair *ThiH-R301K-F* and *ThiH-R1* (**Supplementary Table 1**), respectively. The resulting PCR products were cloned into pET28a by homologous recombination using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd). 20 μL reaction mixture containing 4 μL 5×CE MultiS Buffer, 2 μL Exnase MultiS, ~50 ng linear pET28a plasmid (pre-digested with NdeI/XhoI), ~20 ng fragment 1 (~ 900 bp), ~10 ng fragment 2 (~ 280 bp) and ddH_2O, was incubated at 37℃ for 30 min. Chemically competent *E. coli* DH5α cells were transformed with the ligation mixture and plated on LB-agar containing kanamycin (50 μg/mL) to screen for positive clones, which were confirmed by DNA sequencing. NosL-R323K expression plasmid was constructed in a way similar to that for ThiH-R301K, using the previously constructed NosL-expressing plasmid[1] as the PCR template.

Protein Expression

E. coli BL21 (DE3) cells were transformed via electroporation with each expression plasmid. A single colony transformant was used to inoculate a 5 mL LB culture supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 1 L of LB medium containing 50 μg/mL kanamycin. Cells were grown at 37 °C and 180 rpm to an OD_{600} 0.6-0.8, and IPTG was then added to a final concentration of 0.2 mM. To express NosL, ThiH and their mutant enzymes, sterilized $Fe(NH_4)_2(SO_4)_2$ solution was added to the culture to a final concentration of 100 μM. After additional 18 h of incubation at 20 °C and 130 rpm, the cells were harvested by centrifugation at 5000 rpm for 15 min at 4 °C. The pellet was used directly for protein purification or stored at −80 °C upon further use.

Purification of Alr

Alr was purified aerobically. The cell pellet collected by centrifugation was re-suspended in 40 ml lysis buffer (50 mM MOPS, 200 mM NaCl, and 10% glycerol, pH 8.0), and was lysed by sonication on ice. Cell debris was removed via centrifugation at 14000 rpm for 30 min at 4 °C. The supernatant was incubated with 4 ml Ni-NTA resin pre-equilibrated with the lysis buffer, and then subjected to affinity purification on a column. The desired elution fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit, and the concentrated protein solution was desalted by using a PD-10 column (GE Healthcare) pre-equilibrated with the elution buffer I (50 mM MOPS, 25 mM NaCl, and 10% (v/v) glycerol in deuterium oxide (～90%), pD 8.0). The protein fraction was collected and concentrated, analyzed by SDS-PAGE (12% Tris-glycine gel), and was used directly for in vitro assay or stored at −80 °C upon further use. Protein concentration was determined using a Bradford Assay Kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Preparation of the reconstituted NosL, ThiH and mutant enzymes

Protein purification and [4Fe-4S] reconstitution were performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 2 ppm of O_2. The cell pellet was resuspended in

20ml of the lysis buffer (50 mM MOPS, 200 mM NaCl, and 10% glycerol, pH 8.0), and was lysed by sonication on ice. Cell debris was removed via centrifugation at 14000 rpm for 30 min at 4 °C. The supernatant was incubated with 4 ml Ni-NTA resin pre-equilibrated with the lysis buffer, and then subjected to affinity purification on a column. The desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit and analyzed by SDS-PAGE (12%Tris-glycine gel). Protein concentration was determined using a Bradford Assay Kit (Bio Rad) using bovine serum albumin (BSA) as a standard.

For reconstitution of the [4Fe-4S] clusters of NosL and ThiH, freshly prepared dithiothreitol (DTT) was added to the purified protein fraction to a final concentration of 5 mM. $Fe(NH_4)_2(SO4)_2$ solution (50 mM) was then added carefully to a final concentration of 500 μM. After 10 min of incubation at the room temperature, Na_2S solution (50 mM) was added in the same way to a final concentration of 500 μM. After further incubation on ice for 5-7 h, the resulting blackish solution was subjected to desalting on a PD-10 column (GE Healthcare) pre-equilibrated with the elution buffer I or elution buffer II (50 mM MOPS, 25 mM NaCl, 10 mM DTT and 10% (v/v) glycerol, pH 8.0). The protein fraction was collected and concentrated, and was used directly for in vitro assay or stored at −80 °C upon further use.

Enzyme assays

A typical assay was carried out by incubating 500 or 200 μM L-Trp or L-Tyr with ～50 μM reconstituted protein, 500 μM SAM and 2 mM of sodium dithionite ($Na_2S_2O_4$) in 50 mM MOPS buffer (pH 8.0). Reaction volumes were typically 200 μL and were maintained at room temperature (～25°C) for 1.5 h prior to quenching. The reactions were quenched by addition of trichloroacetic acid (TCA) to a final concentration of 5% (v/v). After removal of the protein precipitates by centrifugation, the supernatant was subjected to HPLC and/or LC-MS analysis.

To explore the requirement for external reductant, 300μL solution containing 50 μM reconstituted enzyme was supplied with 3 μL 10 mM freshly prepared sodium dithionite. The solution was incubated for 30 min at room temperature and then for 15 h at 10 °C. 10 μL of the reduced enzyme was then added to 90 μL 50 mM Tris buffer (pH 8.0) containing 1 mM DTT, 0.5 mM

SAM and 0.5 mM substrate. As a positive control, a second aliquot of 10 μL protein solution was added to 90 μL of the same solution that also contained 0.4 mM sodium dithionite. Following the addition of the reduced enzyme, 50μL aliquots were filtered to remove protein at 5 min and 5 h, and frozen prior to analysis by LC/MS. This experiment was performed in duplicates.

HPLC analysis was performed using a C18 analytic column (Accucore XL C18, Thermo Scientific, 4.6 x 250 mm). The column was equilibrated with 85% solvent A (H_2O, 0.1% TFA) and 15% solvent B (CH_3CN, 0.1% TFA), and developed at a flow rate of 1 ml/min and UV detection at 254 nm or 298 nm: 0-3 min, constant 98% A / 2% B; 3-20 min, a linear gradient to 80% A / 20% B; 20-23min, a linear gradient to 0% A /100% B; 23-28min, constant 0% A /100% B; 28-30min, a linear gradient to 90% A / 2% B.

Synthesis of D,L-[α-^{2}H]-Trp

200 μL reaction mixture in ～90% D_2O buffer containing 100 μM L-Trp, 1 mM NADH, 1 mM PLP and ～50 μM Alr were incubated at room temperature (～25°C) for 5 h. The reaction was quenched by adding trichloroacetic acid (TCA) to a final concentration of 5% (v/v). After removal of the protein precipitates by centrifugation, the supernatant was analyzed directly by LC-HR-MS/MS.

Molecular Mechanical Calculations

All protein models were prepared in Schrodinger suite software under the OPLS_2005 force field.[2] Hydrogen atoms were added to the repaired crystal structures at physiological pH (7.4) with the PROPKA[3] tool to optimize the hydrogen bond network provided by the Protein Preparation tool in Schrodinger software[4]. Asn, Gln, and His residues were checked for flips automatically in ProPrep in Schrodinger. Constrained energy minimizations were conducted on the full-atomic models, with heavy atom coverage to 0.4 Å.

The docking procedure was performed with Glide[5, 6] (Schrödinger 2015 suite). Each ligand molecule was initially placed in the binding pocket in a pose similar to that observed in the corresponding crystal structure. Cubic boxes centered on the ligand mass center with a radius of

12 Å defined the docking binding regions before flexible ligand docking was executed. Five poses out of 20,000 per ligand were included in post-docking energy minimization. Top scored pose was exported for study.

Fig. S1. Structure characterization of the mono-deuterated Trp produced in NosL-catalyzed reaction in D_2O. (a) Theoretical MS/MS fragmentation of D,L-[α-^{2}H]-Trp.[7] (b) HR-MS/MS spectrum of the mono-deuterated Trp produced in the reaction with NosL and 10 μM D-Trp in D_2O buffer. (c) HR-MS/MS spectrum of the authentic D,L-[α-^{2}H]-Trp synthesized from L-Trp using a low-specificity amino acid racemase (Alr) in D_2O buffer. (d) HR-MS/MS spectrum of the mono-deuterated Trp produced in the reaction with NosL R323K mutant and 10 μM L-Trp in D_2O buffer.

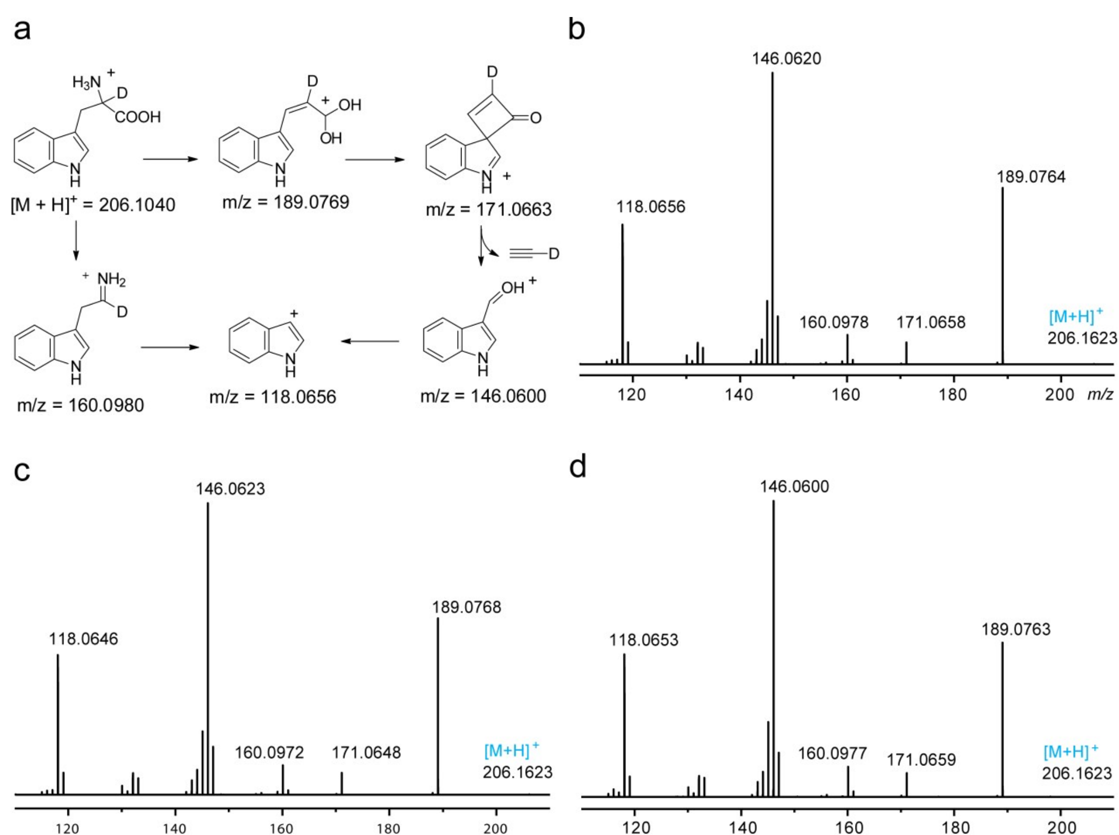


Fig. S2. Incorporation of deuterium atoms into dAdoH and L-Trp. (A) Summed MS spectrum of dAdoH produced in the reaction with the NosL R323K mutant and 500 μM L-Trp in D_2O. (B) Summed MS spectrum of Trp in the reaction with NosL R323K mutant and 10 μM L-Trp in D_2O.

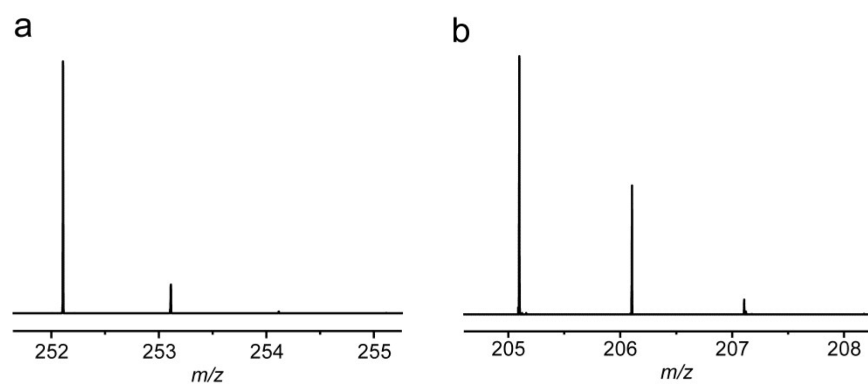


Fig. S3. Redox stoichiometry analysis of L-Trp consumption. (a) HPLC analysis of the reaction mixture containing SAM, L-Trp and the reduced NosL wild type enzyme at (i) 5 min without external dithionite (DTH), (ii) 300 min without DTH, and (iii) 300 min with DTH. (b) LC-MS analysis of the reaction mixture containing SAM, L-Trp, and the reduced NosL R323K mutant, showing the EICs for $[M -H]^-$ = 202.05 (corresponding to indole-3-pyruvate) for the reaction at (i) 5 min without DTH, (ii) 300 min without DTH, and (iii) 300 min with DTH. These analyses demonstrated that production of MIA and 3-methylindole by the NosL wild type enzyme requires external reductant whereas L-Trp amine dehydrogenation catalyzed by NosL R323K mutant does not.

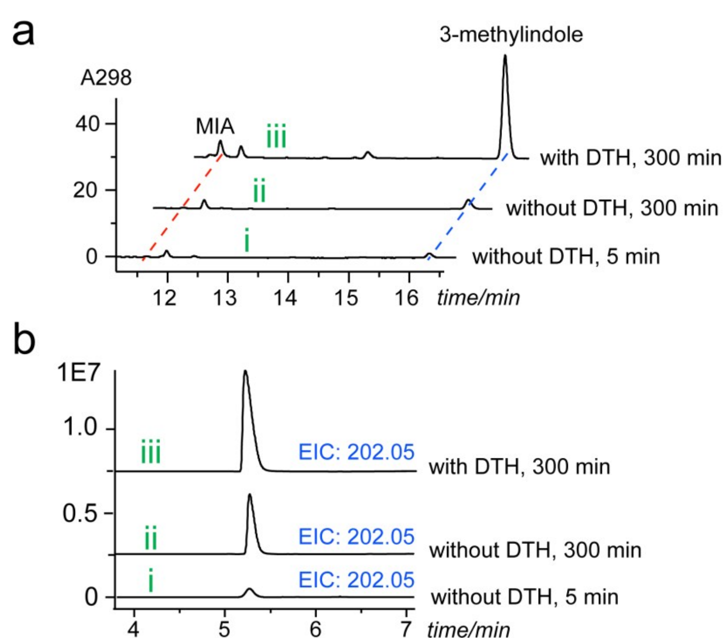


Fig. S4. HPLC analysis of (4-hydroxyphenyl)-3-lactate (HPL) generated by $NaBH_4$ reduction of (4-hydroxyphenyl)-3-pyruvate (HPP), showing the HPLC traces of (i) HPL standard, (ii) the reaction mixture treated with $NaBH_4$, and (iii) the mixture of trace ii co-injected with the authentic HPL standard.

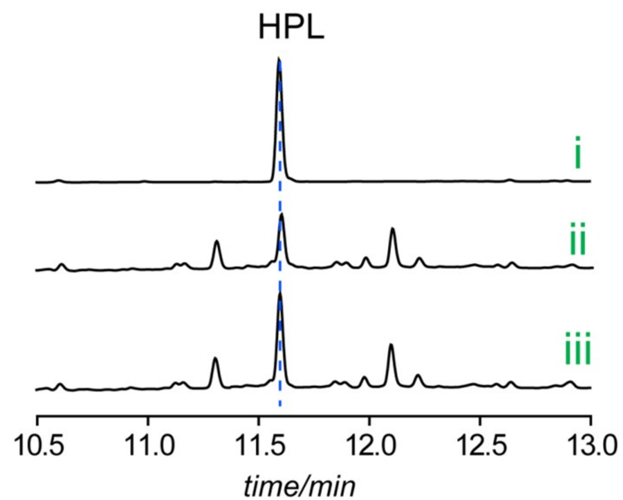
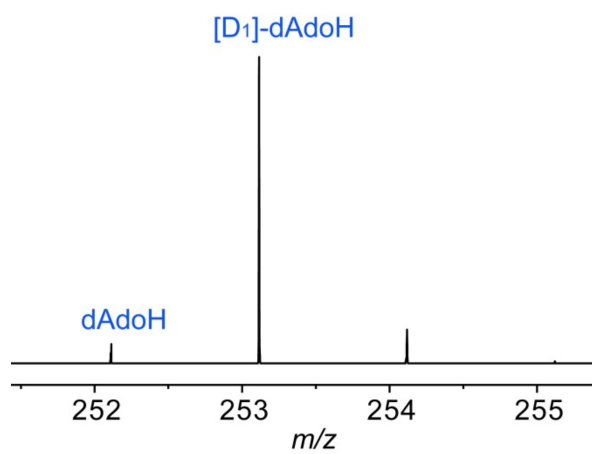


Fig. S5. Summed MS spectrum of dAdoH produced in the reaction with ecThiH R301K mutant and [$^2H_7$]-L-Tyr in H_2O buffer, showing that dAdoH was predominantly mono-deuterated.



Supplementary Table 1. PCR primers used in this study.

Primer	Sequence	Function
thiH-F	AAAAGAATTCATGAAAACCTTCAGCGATCGCTGG	ThiH cloning
thiH-R	AAAAAAGCTTCATGGCCTTTGCGATGGGCGTCC	
alr-F	CCGCGCGGCAGCCATATGATGCAATTTAGCCGTACCCTCCTGGCTGC	Alr cloning
alr-R	GGTGGTGGTGGTGCTCGAGTCAGTCGACGAGTATCTTCGGGTTGGAACTG	
thiH-F1	CCGCGCGGCAGCCATATGATGAAAACCTTCAGCGATCGCT	ThiH-R301K cloning
thiH-R1	GGTGGTGGTGGTGCTCGAGTCATAGTCTTTGCGAGGCGCGTC	
thiH-R301K-F	CCGGAGATTGAACTGTCACTCTCCACGAAAGAATCACCGTGGTTTCGCG	
thiH-R301K-R	GCACCGGAGATTGAACTGTCACTC	
nosL-F1	CCGCGCGGCAGCCATATGATGACGCAGAACTCCCAGG	NosL-R323K cloning
nosL-R1	GGTGGTGGTGGTGCTCGAGTCAGACCGCCCGGGACGCCTC	
nosL-R323K-F	GCGGCTCGTCCTCACCACGAAAGAGCCGCAGGAGTTCCAG	
nosL-R323K-R	CGTGGTGAGGACGAGCCGCTGCTCGGG	

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