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COMMUNICATION

Reactivity of the nitrogen-centered tryptophanyl radical in the catalysis of the radical SAM enzyme NosL

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The radical SAM tryptophan (Trp) lyase NosL invovled in nosiheptide biosynthesis catalyzes two parallel reactions, converting L-Trp to 3-methyl-2-indolic acid (MIA) and to dehydroglycine and 3-methylindole, respectively. The two parallel reactions diverge from a nitrogen-centered tryptophanyl radical intermediate. Here we report investigation on the intrinsic reactivity of the tryptophanyl radical by chemical model study and by DFT calculations. The kinetics in the formation and fragmentation of this nitrogen-centered radical in NosL catalysis were also studied in detail. Our analysis explains the intriguing catalytic promiscuity of NosL and highlights the remarkable role this enzyme plays in achieving a energetically highly unfavorable transformation.

The tryptophan (Trp) lyase NosL cleaves the Cα-C bond of L-Trp and converts it to 3-methyl-2-indolic acid (MIA) (Fig. 1), which is an intermediate involved in the biosynthesis of a clinically interesting thiopeptide antibiotic nosiheptide.[1, 2] NosL belongs to the radical S-adenosylmethionine (SAM) superfamily, a large and rapidly growing enzyme superfamily currently containing over 165, 000 predicted members.[3-5] Enzymes of the radical SAM superfamily share a common mechanism in radical generation, utilizing a [4Fe-4S] cluster to bind SAM and reductively cleave its carbon-sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical, which then initiates remarkably diverse reactions.[3-5] Structural and biochemical studies have demonstrated that the NosL-catalyzed reaction is initiated by the dAdo radical-mediated hydrogen abstraction from the L-Trp amino group,[6-10] which results in a nitrogen-centered tryptophanyl radical (**1**) (Fig. 1). MIA biosynthesis then proceeds via cleavage of the Cα-C bond of **1** and subsequent attachment of the carboxylate radical onto the indole C2 to produce an indolyl radical **2**.[11] Upon Cα-Cβ cleavage and a further isomerization process, **2** is converted to the final product MIA (Fig. 1).

Similar to the NosL-catalyzed reaction, the nitrogen-centered radicals are also produced by other radical SAM enzymes, including the hydrogenase-maturating enzyme HydG,[12-15] thiamine biosynthesis protein ThiH,[16, 17] and the F_{420} biosynthesis protein CofH.[18, 19] However, all these enzymes cleave the Cα-Cβ bond of L-Tyr, not the Cα-C bond as found for NosL. Interestingly, NosL also cleaves the Cα-Cβ bond of L-Trp with an efficiency comparable to that of the Cα-C bond cleavage, resulting in the production of dehydroglycine (DG) and 3-methylindole (MI) as shunt-pathway products (Fig. 1).[7, 8] The fact that a wild type enzyme catalyzes two parallel reactions on its natural substrate is a highly unusual observation in enzymology,[20] raising intriguing questions regarding the reactivity of **1** and the energetic role that enzyme plays in the catalysis.

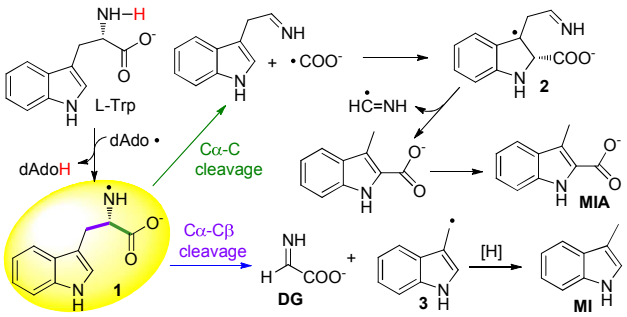

Fig. 1. Two parallel reactions catalyzed by NosL. The nitrogen-centered tryptophanyl radical **1** is shown in a yellow eclipse, and the two fragmentation patterns of **1** are shown in green and purple.

To investigate the intrinsic reactivity of **1**, we used a method similar to that reported by Lin and Li in studying the reactivity of thymine radicals.[21] In the crystal structure of NosL, the guanidinium moiety of Arg323 binds the carboxyl group of L-Trp and compensates its negative charge.[6] Because the protonation state of a carboxylate could vary and complicate the analysis, **5** (the methyl ester form of **1**, Fig. 2a) was used as a chemical model for investigation. Indeed, density functional theory (DFT) calculations showed that **5** and **1** exhibit similar energetic profiles in fragmentation (Fig.S1, ESI† and vide infra). We therefore synthesized compound **4** and cleaved its N-S bond by photolysis (Fig. 2a). Liquid chromatography (LC) with high-resolution (HR) mass spectrometry (MS) analysis of the reaction mixture showed the production of two compounds X1

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and X2, which exhibit the same protonated molecular ions at m/z = 501.160 (Fig. 2b), and UV-vis spectra that are very similar to that of compound **4** (Fig.S2, ESI†). X1 and X2 also exhibit the same set of fragment ions in HR-MS/MS analysis, including several ions that are also observed in the MS/MS spectrum of **4**, and a unique fragment ions at m/z = 283.054 (Fig. S3-S4, ESI†). These results suggest that X1 and X2 are two regioisomers resulting from addition of the 3-methylindole radical (**3**) to the nitrophenyl moiety of **4**, and apparently, **3** is resulted from homolytic cleavage of the Cα-Cβ bond of the nitrogen-centered radical **5** (Fig. 2a). DFT calculations and orbital analysis showed that addition of **3** to **4** occurs preferentially at the two positions ortho to the nitro group (Fig. S5, ESI†), suggesting that the 3-methylindole moiety in X1 (major product) and X2 (minor product) are at the nitro ortho- and meta-position, respectively (Fig. 2a). We also observed a series of peaks (Y1, Y2, and Y3) that exhibit the same protonated molecular ions at m/z = 525.090, which are resulted from addition of the thyil radical **6** to **4** (Fig. 2). However, distinct from the methylindole radical **3** that is an electron-rich radical, **6** is much more electrophilic and the radical addition reactions preferentially occur on the indole ring of **4**, as revealed by DFT calculation and orbital analysis (Fig. S6, ESI†); this proposal is supported by comparative HR-MS/MS analysis (Fig. S7, ESI†).

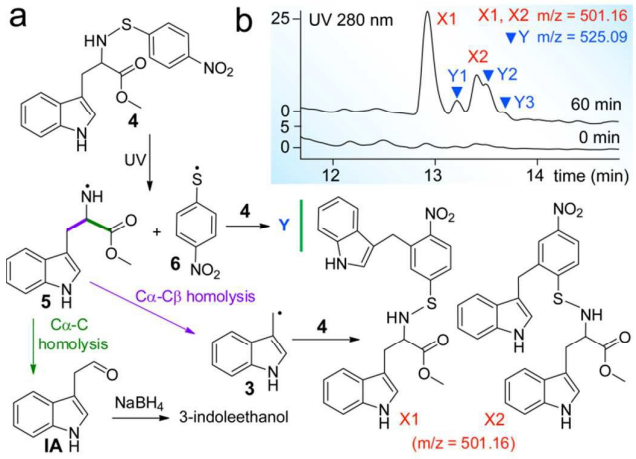

Fig. 2. Model study on the intrinsic reactivity of the nitrogen-centered radical. (a) Photoreaction of the compound **4**, a precursor of the nitrogen-centered radical **5**. (b) LC-HRMS analysis of the photoreaction of **4**. The reaction was carried out under 254 nm UV light for 60 min; the unreacted solution of **4** was also analyzed for comparison.

In contrast to the products originated from the Cα-Cβ bond cleavage of **5**, 3-indoleacetaldehyde (IA), the product of the Cα-C cleavage of **5**, was barely observable in HPLC analysis. The reaction mixture was treated with an excess amount of $NaBH_4$ to reduce any IA, and the resulting 3-indoleethanol was concentrated and carefully quantified with the standard. This analysis showed that the yield of IA is 15-fold lower than the total amount of X1 and X2 (Fig. S8, ESI†). IA is the direct product of the Cα-C cleavage of **5** (Fig. 2a), whereas production of X1 and X2 requires successful addition of **3** to **4** and a subsequent oxidative aromatization process (Fig. S5, ESI†), and **3** can also be converted to other products such as 3-methylindole (Fig. S8, ESI†). As a result, the ratio of Cα-C to Cα-Cβ fragmentation of **5** is lower than 5%, which is in contrast to the ratios of **1** fragmentation in NosL catalysis (ranging from 30% to 75% in different reaction conditions).[2, 8] This analysis provides direct evidence that, although both Cα-C and Cα-Cβ bond cleavages of the nitrogen-centered radical are possible, the latter process is more favorable than the former.

To further investigate the reactivity of the nitrogen-centered tryptophanyl radical **1**, we carried out DFT calculation on the possible reaction pathways. We performed calculations using both B3LYP and M06 hybrid functionals in parallel and the SMD solvation model[22] in both water and ethanol, and all calculations show similar results (Fig. 3 and Fig. S9, ESI†). Because the L-Trp carboxylate interacts with Arg323,[6] it was treated as a protonated carboxyl group in our calculation. These analyses show that cleavage of **1** Cα-Cβ bond has a relatively low activation barrier (36.3 kJ/mol, hereafter values are from calculations at the B3LYP/6-311+G(2d,p)/SMD(water) level) (Fig. 3), which is similar to that reported by Nicolet *et al*.[6] Cleavage of the Cα-C bond of **1**, however, is much more demanding, with an activation barrier of 86.8 kJ/mol, 52.5 kJ/mol higher than that of the Cα-Cβ bond cleavage (Fig. 3). These results are consistent with the chemical model study discussed above and indicate that the Cα-Cβ bond of **1** is intrinsically prone to homolytic cleavage, whereas the Cα-C bond cleavage of **1** is not a favorable process.

Further DFT calculations showed that the addition of the carboxyl radical to the indole C2 is a strong exothermic process (ΔG = –64.3 kJ/mol), with an activation barrier of only 30.2 kJ/mol. Remarkably, cleavage of the Cα-Cβ bond of the radical intermediate **2** is energetically highly demanding, with an activation barrier of 162.0 kJ/mol. Such a high energetic barrier suggests that **2** is a stable intermediate in catalysis, and this is consistent with the work by Nicolet *et al*. in identification of **2** by electron paramagnetic resonance (EPR) analysis.[11] Together, our analysis demonstrates the MIA production is a highly unfavorable process, indicating that enzyme should play a remarkable role in tuning the reactivity of the radical intermediates to achieve MIA production.

Previous biochemical studies on NosL have shown that multiple deuterium was incorporated into dAdoH when the reactions were performed in D_2O, suggesting that the dAdo radical-mediated hydrogen abstraction that produce **1** is a reversible process.[7, 8, 23] To study the rates for **1** formation and cleavage, we ran NosL reaction in highly enriched D_2O buffer (D_2O > 98%, pD 8.0) and carried out a time course analysis. LC-HRMS analysis of the reaction mixture showed that the sum of di- and tri-deuterated dAdoH accounts for more than 75% of the resulting dAdoH, and the ratios of multiply deuterated dAdoH species remain roughly constant at different time points (Fig. 4a). We noted that even for the reaction that was rapidly quenched within 3 seconds, the resulting dAdoH is still mainly tri-deuterated (Fig. 4a).

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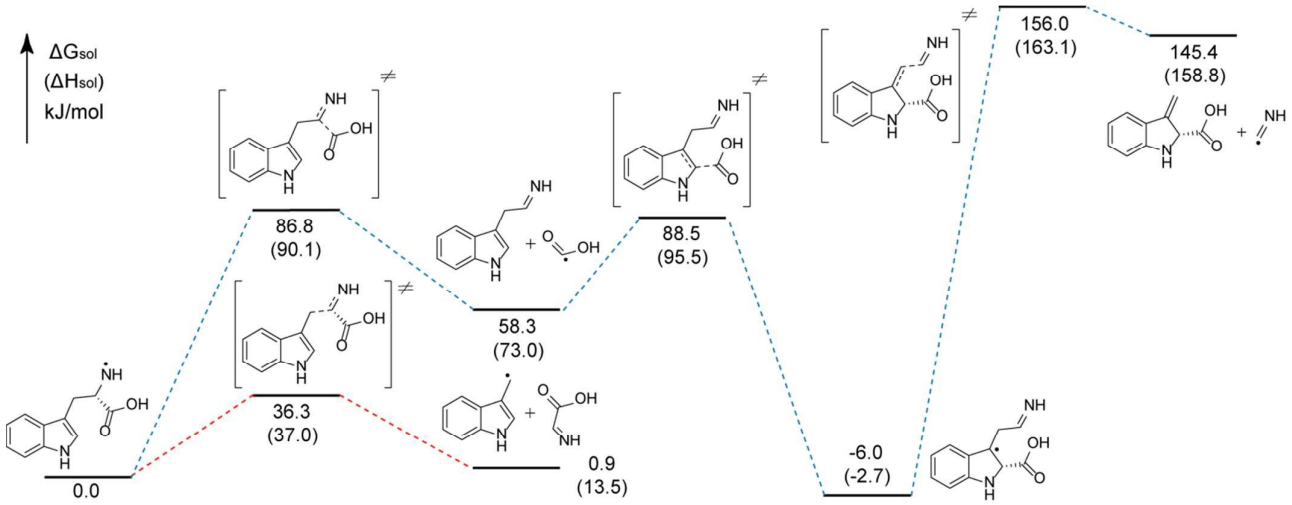

Fig. 3. DFT-calculated potential energy profile in NosL catalysis. Energies were calculated at the B3LYP/6-311+G(2d,p)/SMD(water) level of theory with geometry optimized at the B3LYP/6-31+G(d,p)/SMD(water) level. The potential energy profiles calculated by using M06 hybrid functionals and SMD solvation model in water and ethanol are shown in Fig S9, ESI†. All the transition states (shown in brackets) have been validated by intrinsic reaction coordinate (IRC) calculation in both forward and reverse directions (Fig. S10, ESI†). ΔG_{sol}, free energy of activation; ΔH_{sol}, enthalpy of activation.

We proposed a reaction model for the NosL-catalyzed hydrogen abstraction in D_2O (Fig. 4b, a detailed model is shown in Fig. S11, ESI†). Briefly, the dAdo radical abstracts a deuterium from the L-Trp amino group to produce **1**, which could be converted to the reaction products upon C-C bond cleavage (k_{frag}) (Fig. 4b). On the other hand, **1** can abstract a protium or deuterium from dAdoH to produce dAdo radical and L-Trp again, and protium abstraction is kinetically more favorable (see below). The resulting protium on the L-Trp amino group is then replaced by a deuterium via a rapid solvent exchange process (k_{sol}), and thereby each hydrogen abstraction/rebound cycle leads to accumulation of a deuterium into dAdoH (Fig. 4b). The observation that the multiply deuterated dAdoH accounts for more than 75% of the total dAdoH suggests that k_{frag} is significantly smaller than k_2.

We next estimated the rate constants of the dAdo radical-mediated hydrogen abstraction by DFT and statistical mechanics analysis using the canonical transition state theory with Wigner tunneling correction (TSTW),[24] which takes quantum tunneling effect[25, 26] into consideration. Because the transition state for **1** formation involves hydrogen transfer between two radical-like centers, it appears to be insensitive to the changes away from the reaction center.[27] Therefore, in our analysis dAdo radical is modeled as an ethyl radical and L-Trp is modeled as methylamine, in a way similar to a recent study on the radical SAM enzyme HydE.[27] By using such small quantum models, we were able to carry out high accuracy calculation with large basis set and avoid local minima in the potential energy surface, which could have severe consequences on the energetics. Calculations were performed for all the model hydrogen abstraction reactions to address the effect of H/D exchange (Fig. S12, ESI†), showing that the rate constants for the dAdo radical-based protium transfer is generally 10～12 folds higher than those of deuterium transfer (Table S1, ESI†), and this is consistent with a recent stop flow study on the adenosylcobalamin-dependent ethanolamine ammonia lyase.[28] Based on these results, the reaction rates for the generation and fragmentation of **1** can be roughly inferred (Fig. 4b, see Fig. S11, ESI† and the legend for a detailed discussion).

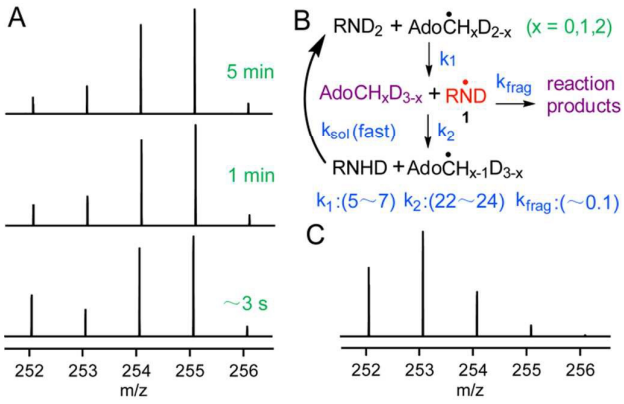

Fig 4. Kinetic study of NosL catalysis. (A) Summed MS spectra of dAdoH produced in highly enriched (98%) D_2O buffer at different time points. (B) A model for NosL reaction in D_2O. For a detailed model and discussion on NosL kinetics, see Fig. S11, ESI†. The tryptophanyl radical **1** is shown in red, and the estimated rate constants are shown in s^{-1}. (C) Summed MS spectrum of dAdoH produced by the NosL Y90F mutant in 98% D_2O at 5 min.

The observation that a significant proportion of dAdoH was triply deuteriated in the rapid quench experiment suggests

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that the L-Trp amino group undergoes a rapid solvent exchange during catalysis. In NosL crystal structure, the L-Trp amino group forms a hydrogen bond with Tyr90, which could possibly mediate hydrogen exchange between solvent and the L-Trp amino (Fig. S13, ESI†). To test this hypothesis, we replaced Tyr90 with a Phe, and the resulting Y90F mutant was used to run a reaction in highly enriched D_2O buffer same to that used for the wild type enzyme. LC-HRMS analysis of the reaction mixture showed that, in stark contrast to the wild type NosL (Fig. 4a), dAdoH produced by the mutant enzyme was predominately singly deuterated (Fig. 4c). The catalytic efficiency of the Y90F mutant is only about 2-fold lower than that of the wild type enzyme (Fig. S14, ESI†), suggesting that the mutant folds in the same way as the wild type enzyme. These results demonstrate that the solvent exchange mechanism is significantly, if not completely, abolished in the Y90F mutant, supporting the essential role of Tyr90 in mediating the hydrogen exchange between solvent and the L-Trp amino group. In NosL active site, no water molecule was found around the phenol moiety of Tyr90 and L-Trp amino group.[6] Our analysis thus suggests that generation of **1** involves multiple rounds of solvent molecules move in and out of the enzyme active site. It remains to be seen whether the dynamic solvent exchange process contributes to the reaction thermodynamics.

In summary, by using a combination of model chemical study and DFT calculations, this investigation clearly demonstrates that cleavage of the Cα-Cβ of the nitrogen-centered tryptophanyl radical **1** is thermodynamically more favorable than that of the Cα-C cleavage. We also estimated the rate constants for the formation and fragmentation of **1** in NosL catalysis, and showed that a key Tyr residue mediates rapid hydrogen exchange between solvent and the L-Trp amino group. Our study demonstrates the remarkable role that NosL plays in modulating the reactivity of radical intermediates, indicating that the unusual catalytic promiscuity of NosL is likely a compromise to achieve an energetically highly demanding reaction. Similar scenarios of catalytic promiscuity likely apply to other radical-mediated enzymatic reactions.

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