Reaction Mechanisms

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Crystal Structure of Tryptophan Lyase (NosL): Evidence for Radical Formation at the Amino Group of Tryptophan**

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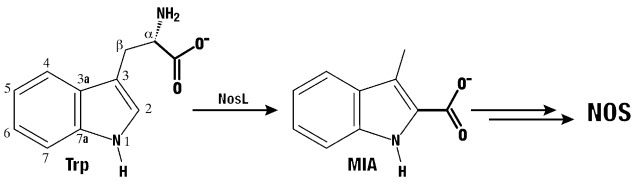
Abstract: *Streptomyces actuosus tryptophan lyase (NosL) is a radical SAM enzyme which catalyzes the synthesis of 3-methyl-2-indolic acid, a precursor in the synthesis of the promising antibiotic nosiheptide. The reaction involves cleavage of the tryptophan Cα–Cβ bond and recombination of the amino-acid-derived -COOH fragment at the indole ring. Reported herein is the 1.8 Å resolution crystal structure of NosL complexed with its substrate. Unexpectedly, only one of the tryptophan amino hydrogen atoms is optimally placed for H abstraction by the SAM-derived 5′-deoxyadenosyl radical. This orientation, in turn, rules out the previously proposed delocalized indole radical as the species which undergoes Cα–Cβ bond cleavage. Instead, stereochemical considerations indicate that the reactive intermediate is a ·NH tryptophanyl radical. A structure-based amino acid sequence comparison of NosL with the tyrosine lyases ThiH and HydG strongly suggests that an equivalent ·NH radical operates in the latter enzymes.*

Nosiheptide (NOS), produced by the actinobacterium *Streptomyces actuosus*, is an antibiotic which belongs to the *e* series of polythiazolyl heterocyclic peptides.[1] It has attracted significant clinical interest because of its potent bactericidal activity against highly resistant strains of pathogens such as *Staphylococcus aureus*, *Streptococcus pneumonia*, and several enterococci.[2] Nosiheptide has a complex structure consisting of a backbone made of a ribosomally synthesized and highly modified 13-residue-long peptide, a central tetrasubstituted pyridine domain, and an appended unusual indolic acid ring (see Figure S1 in the Supporting Information).[3] Zhang et al. have shown that this ring originates from L-tryptophan (tryptophan hereafter), through



Scheme 1. Reaction catalyzed by NosL. The carboxylate fragment in MIA (depicted in bold) originates directly from tryptophan.[4]

a 3-methyl-2-indolic acid (MIA) precursor synthetized by the radical *S*-adenosyl-L-methionine (SAM) enzyme NosL (Scheme 1).[4] These authors have hypothesized that MIA synthesis by this enzyme[4] and the closely related NocL from *Nocardia* sp.[5] starts with H abstraction by the SAM-derived 5′-deoxyadenosyl radical (5-dA·) at the indole nitrogen atom of tryptophan, followed by radical delocalization over the ring moiety. Mechanistic insights into the course of the reaction have been provided by the identification of the "shunt" 3-methylindole (skatole) and glyoxylate[4] as its products. Glyoxylate results from the hydrolysis of either dehydroglycine (DHG) or a glycyl radical.[6] These observations along with calculations led Zhang et al. to favor the cleavage of the tryptophan Cα–Cβ bond by NosL to give 3-methylene indole and a glycyl radical. From additional experimental data, these authors demonstrated the subsequent recombination of the amino-acid-derived COOH fragment at C2 of the indole ring (Scheme 1).[4]

Based on this proposed catalytic function and its amino acid sequence NosL may be classed together with the radical SAM tyrosine lyases ThiH and FeFe-hydrogenase maturase HydG.[4] Indeed, these two enzymes are known to cleave the Cα–Cβ bond of tyrosine.[6a,7] As in the case of NosL, evidence for the Cα–Cβ bond-cleavage reaction comes from in vitro experiments showing that in the presence of tyrosine and an excess of reducing equivalents these enzymes produce *p*-cresol and DHG-derived glyoxylate.[6a,7a,8] In vivo, DHG is subsequently used to either synthesize the thiazole ring of thiamine (ThiH)[6b,9] or the CO/CN^- ligands of the HydA hydrogenase (HydG).[10] In both cases, it is generally accepted that the 5′-dA· radical abstracts the hydrogen atom from the OH group of tyrosine.[6a,7b] However, neither the existence of the postulated delocalized indole radical in NosL[4] nor that of the tyrosyl O· radical in either ThiH or HydG has been demonstrated.[6a,7b]

To structurally characterize the reaction catalyzed by NosL, we anaerobically crystallized the recombinant protein from *S. actuosus* ATCC 25421 both with *S*-adenosyl-L-homocysteine (SAH) and reductively cleaved SAM, and

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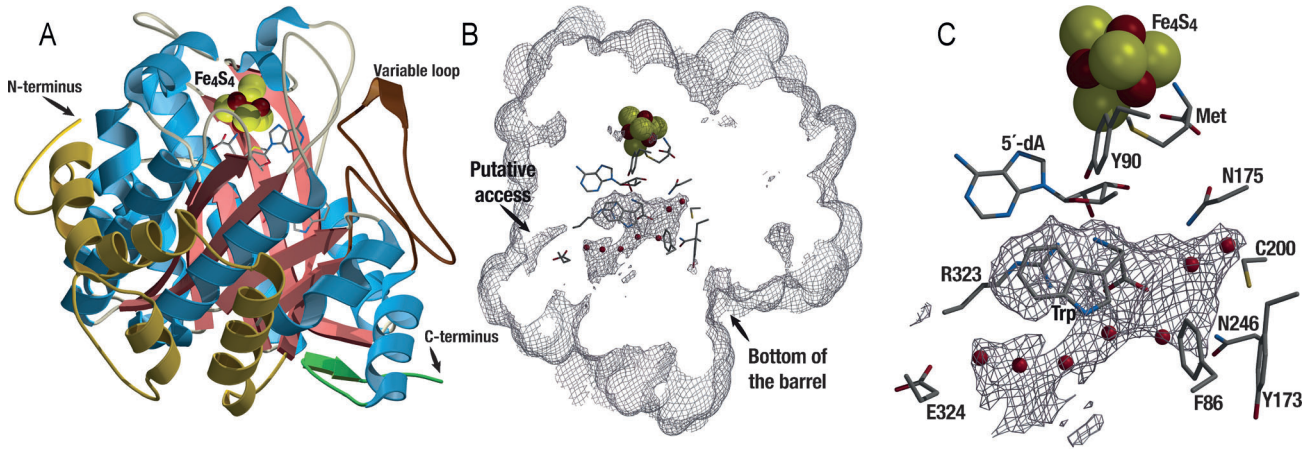

Figure 1. A) Structure of a monomer of NosL. The strands and helices of the TIM barrel domain are depicted in red and light blue, respectively. N- and C-terminal stretches are shown in gold and green, respectively. The variable loop connecting strand *S8* and helix *H8* is depicted in brown. B) Accessibility surface of NosL calculated with a probe radius of 1.0 Å using a protein model with neither water molecules nor tryptophan. C) Close-up of (B) showing the internal cavity and the surrounding residues and solvent.[27]

used the molecular replacement method[11] to solve their 1.8 Å resolution X-ray structures with bound tryptophan (see Table S1). Like other members of the radical SAM family such as BioB,[12] HydE,[13] or PylB,[14] NosL folds into a $(\beta/\alpha)_8$ triose phosphate isomerase (TIM) barrel domain (Figure 1 A). The barrel is preceded by a mostly helical 82-amino-acid-long N-terminal region of which the first 10 residues do not have matching electron density in the $(2F_o-F_c)$ map and must therefore be disordered. This domain bears some topological resemblance to equivalent regions in PylB, HydE, and BioB, although it is significantly longer (see Figure S2 in the Supporting Information). Conversely, the 17-residue-long C-terminal stretch, which is made of a short β strand that interacts with strand *S1* of the TIM barrel domain, has no counterpart in these enzymes (see Figures S2 and S3). Although the protein is a dimer in solution (see the Supporting Information), the dimeric structure is not represented by the two molecules of the asymmetric unit, which although related by approximate twofold noncrystallographic symmetry display a small contact surface and slight differences at both their N and C termini. Instead, an analysis of the interface between symmetry-related molecules using PISA[15] revealed that NosL dimers are generated by crystallographic twofold axes (see Figure S4). The dimer interface is different from the one reported for BioB[12] (see the Supporting Information for details).

In the NosL structure, the canonical Fe_4S_4 cluster is bound as in all the other members of the radical SAM enzyme family.[16] The cleaved SAM products 5′-dA and methionine also bind as previously observed in HydE.[17] Conversely, whereas in HydE SAH binds like SAM, in NosL its sulfur atom is farther away from the Fe_4S_4 cluster and adopts a different conformation. This conformation is stabilized by a hydrogen bond between Gln363 and the SAH thioether, and cannot exist with SAM because of the presence of the extra methyl group (see Figure S5). Consequently, in NosL the bound SAM should adopt the conformation usually observed in related enzymes. It is noteworthy that SAH or 5′dA + methionine binding neither affects the surrounding residues nor tryptophan binding (see Figure S5).

NosL has a smaller internal cavity than HydE and BioB (see Figures 1 B,C and Figure S6). It comprises the site occupied by tryptophan as well as a water channel and an extra pocket, also occupied by water, close to the amino acid fragment of tryptophan and lined by the conserved Cys200. The thiolate group of this residue is the only potentially reactive function found in the active-site region, which is otherwise composed of hydrophobic residues and two asparagines. Consequently, Cys200 may be involved in MIA production. The water channel that connects the active site to the NosL surface may represent a path for either substrate or products trafficking, or may serve as a proton relay channel.

As can be seen in Figure 2 and Figure S5D, there is very well-defined electron density for the added tryptophan substrate. The amino acid establishes close contacts with three protein residues: its ring N atom interacts with the OH and C=O groups of Ser340 away from SAM, its COO^- group binds the guanidinium moiety of Arg323, and its NH_2 group binds both the latter group and the OH function of Tyr90. In addition, in NosL there is a van der Waals contact between the indole ring of tryptophan and Pro88. Phe364, Pro344, Leu140, Phe86, Thr321, and Phe202 are also close to tryptophan, thus defining an exiguous active-site cavity suitable for tight substrate binding. This observation explains the results obtained by Zhang et al. when providing NosL-expressing *Escherichia coli* with tryptophan analogues.[4] Indeed, methyl substitutions at the indole ring, including N methylation, did not result in methylated MIA production. Only added 5- and 6-fluoro tryptophan produced the expected MIA derivatives. Examination of the bound tryptophan in our structure indicates that, among the analogues tested, only these fluorinated substrates can bind NosL without causing severe clashes at the active site cavity.

Quite unexpectedly, only the N atom of the amino group of tryptophan, at about 3.8 Å from the SAH C5′ and at 3.3 Å

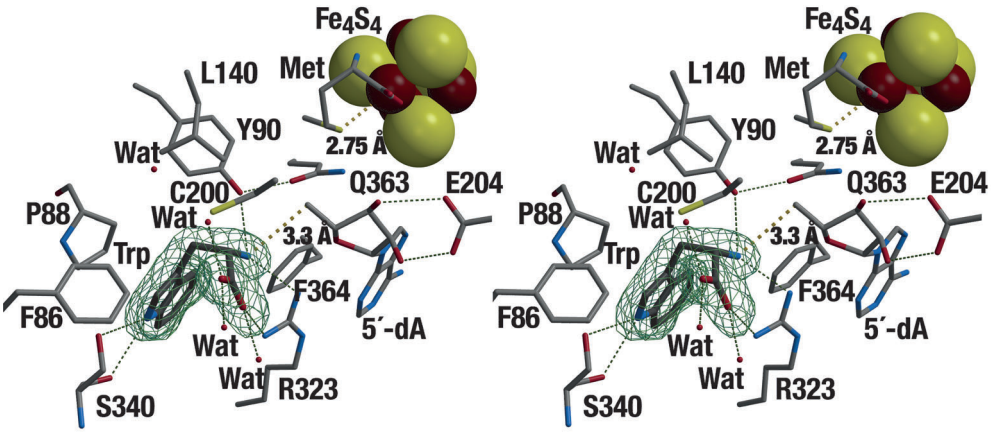

Figure 2. Stereoview of the active site of NosL showing bound tryptophan in the 5′-dA and methionine-containing structure. The F_o-F_c omit map depicted in green mesh is contoured at 3 σ with a cover radius of 4 Å. Only tryptophan was removed from the final model before running one cycle of refinement.

from the 5′-dA-C5′ of the cleaved SAM (Figure 2), is in an orientation and at a distance optimal for H abstraction by the 5′-dA· radical. Thus, the structure strongly suggests that the hydrogen atom is abstracted from this amino group, thereby generating an ·NH radical. To the best of our knowledge, this is the first direct evidence for H-atom abstraction from a noncarbon atom catalyzed by a radical SAM enzyme.

To identify the species that result from the Cα–Cβ bond cleavage of this radical, we performed a series of DFT calculations using the Jaguar program.[18] In our X-ray structure the amino acid fragment of tryptophan binds NosL through NH_2 and COO^- groups. The former accepts one hydrogen bond from Arg323 and donates another to Tyr90, and the latter compensates for its negative charge by interacting with Arg323 (see Figure S7). Consequently, in our calculations we started with both an ·NH amino radical and a protonated carboxylate group to mimic the charge compensation afforded by the protein (see the Supporting Information for details). The highest occupied molecular orbital (HOMO) is not always representative of the radical character of the molecule.[19] Indeed, in our case, its character is contaminated by other nearly degenerate molecular orbitals. In contrast, the lowest unoccupied molecular orbital (LUMO) does not exhibit this problem and therefore allows a clear description of the radical.[20] The LUMO of the ·NH species shows that the radical is localized on the amino nitrogen atom (Figure 3B and see Figure S8). Interestingly, the significant orbital depletion at the Cβ atom already indicates that the Cα–Cβ cleavage will be homolytic, as confirmed by the calculated transition state (Figure 3C), thereby producing a skatole radical and DHG (Figure 3D), with a calculated barrier height of 10.4 kcal mol^{-1}. This value is to be compared with the dissociation energy of a C–C bond of about 90 kcal mol^{-1} in alkanes.[21] The skatole radical is delocalized over Cβ, C2, and N1 (Scheme 1 and Figure 3D).

Our results disagree with the conclusions of Zhang et al.[4] in two central points: first, and with the benefit of structural knowledge, we can exclude the existence of a delocalized indole radical species caused by H abstraction from the N atom of the tryptophan indole ring in NosL by the 5-dA· radical (Figure 2). Second, based on our calculations, we do not favor 3-methylene indole and a glycyl radical as cleavage products of the NosL-catalyzed reaction. Conversely, Zhang et al. reported that thermodynamic calculations strongly favored a $^-$NHC·HCOOH radical with a stabilization energy of 34 kcal mol^{-1} in water (see Figure S9).[4] Because we were intrigued by the large stabilization of a negatively charged glycyl radical we recalculated the free energies of the tryptophan Cα–Cβ bond-cleavage products (see the Supporting Information for details). At odds with their results, we find that in water DHG and the skatole radical are favored by 18.6 kcal mol^{-1} over a negatively charged glycyl radical and a positively charged skatole (**16** and **17** and **18** and **19** in Figure 3 of the paper by Zhang et al.; see Table S2).[4] Using the same DFT method and basis sets, with two different programs, we were able to reproduce their values for all compounds with the exception of the solvation energy calculated for the $^-$NHC·HCOOH species. Their value is surprisingly larger than the one we found (see the Supporting Information for details). Redoing the calculation with the program they used shows that in their case there was

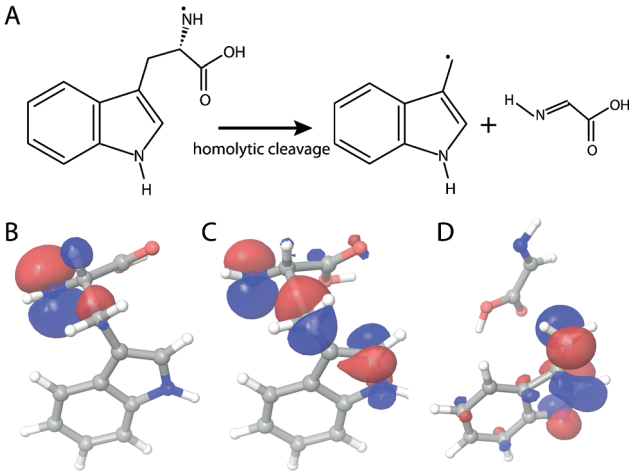

Figure 3. A) Homolytic cleavage of the ·NH tryptophanyl radical Cα–Cβ bond. B) LUMO of ·NH-tryptophanyl radical, C) the transition state for Cα–Cβ cleavage, and D) the products: dehydroglycine and skatole radical. We note that the Cα–Cβ bond in the ·NH-radical species (B) is already long at 1.58 Å compared to 1.54 Å in tryptophan. Surfaces are in red [–] and blue [+] with an isosurface value of 0.05 au.

a problem with this particular molecule (see Table S3). In addition if, as may be expected, the indole nitrogen proton from the positively charged skatole is transferred to $^{-}$NHC•HCOOH to form a neutral NH_2•CHCOOH species we find that DHG and the glycyl radical are equally probable (Table S2). Taken together, these results show that calculations strongly depend on both the protonation state of the studied species and the solvent media used. Furthermore, they confirm the central role the protein matrix plays in controlling the reaction, and makes previous structural knowledge essential. Indeed, if instead of considering the protonation state of the amino acid fragment to be NH_2 and COO^-, which is charge-compensated by Arg323 as suggested from the structure, we optimized the geometry of •NH_2^+- and -COO^- originating from a zwitterionic tryptophan fragment, and we observed spontaneous decarboxylation in both ethanol and water.

Our conclusion that a skatole radical and DHG are the products of tryptophan Cα–Cβ cleavage by NosL (Figure 3D) is also at odds with the assignment of a EPR signal to a glycyl radical reported by Zhang et al. while characterizing NocL reaction products.[5] However, their assignment is not unambiguous. Indeed, the observed changes in the EPR spectrum, resulting from using either L-[$^2H_8$] or L-[1-C^{13}]-labeled tryptophan, cannot be effectively used to discriminate between a glycyl radical, a tryptophanyl radical centered on the amino nitrogen atom, a MIA radical centered on C2, or another unidentified species.

An intriguing feature of the NosL structure is the presence of Tyr90 very close to C5′ ($d_{O\eta\text{-}C5'} = 3.9$ Å) and in direct interaction with the amino group of tryptophan (Figure 2). This configuration raises the possibility of direct H abstraction at the phenolic OH either by 5′-dA• or by the resulting •NH amino radical. Tyrosyl radicals are known to be stable in proteins, even for several days.[22] The location of Tyr90 is puzzling, to say the least. This residue, which is strictly conserved in NosL and the tyrosine lyases, may play a role in the catalytic mechanism during either Cα–Cβ bond cleavage or the termination reaction. As discussed in the Supporting Information (see Figure S10), this situation is somewhat reminiscent of what has been reported for spore photoproduct lyase (Spl), in which a tyrosine residue is proposed to serve as part of a relay to regenerate SAM.[23] In fact, strictly conserved tyrosine residues, able to play an equivalent catalytic role, and located at similar positions, are also observed in the active sites of the radical SAM enzymes LAM (Tyr290),[24] RimO (Tyr227),[25] HydE (Tyr303),[13] and PylB (Tyr66).[14] Although these are all intriguing features of the NosL structure which may be extrapolated to other enzymes, it is too early to propose a comprehensive mechanism for MIA synthesis. Site-directed mutagenesis and spectroscopic studies will be required to shed further light on this process.

Given the amino acid sequence similarities, the related nature of the substrates, and the sameness of the catalyzed reaction, H abstraction from the amino group of tryptophan in NosL may be safely extended to the tyrosine lyases ThiH and HydG. Indeed, the invariant NosL residues Pro88, Tyr90, and Arg323, which interact with tryptophan, are also conserved in ThiH and HydG (see Figure S3). Conversely, NosL Ser340 is found in a region which has significantly less homology with these proteins. Taken together, these amino acid sequence differences help define two zones, one that recognizes the amino acid motif common to tryptophan and tyrosine, and another that is likely to be specific for either indole or phenol rings, thus strongly supporting equivalent modes for substrate recognition between these proteins. It is thus safe to conclude that H abstraction by ThiH and HydG takes place at the amino group of tyrosine and not at its phenolic OH group, as generally proposed but never observed.[6a,7b] From an evolutionary standpoint, this appears to be the most parsimonious solution because it involves a shared binding mode and mechanism, centered at the common amino acid motif.

Our findings will have a significant impact on current models for catalysis by ThiH and HydG.[6a,7b] For example, in our proposed mechanistic model, the 4-oxidobenzyl radical (4-ODB•), for which Kuchenreuther et al. have provided experimental evidence in HydG,[7b] results directly from the homolytic cleavage of the Cα–Cβ bond of the •NH tyrosine radical, without the formation of a tyrosyl radical intermediate. The same rational applies to ThiH because it would also generate 4-ODB• and DHG, the substrate of ThiG.[6a,9] The fate of this radical species, as the radical product in many other radical SAM enzymes has not been thoroughly studied.

From a chemical standpoint H abstraction from the amino nitrogen atom in both tryptophan and tyrosine allows the facile cleavage of their Cα–Cβ bond. The orientation of bound tryptophan in NosL, and presumably of bound tyrosine in ThiH and HydG, positions the indole NH, or phenolic OH, away from the reactive SAM. This positioning makes sense because the formation of a stable radical will not lead to effective Cα–Cβ bond breaking and production of the required products. In addition, aromatic-ring-delocalized radical products should be good leaving groups. This feature is particularly true for both HydG and ThiH because they release *p*-cresol as a by-product.

From our X-ray structure we conclude that neither the delocalized indole radical, as proposed for NosL,[4] nor the tyrosyl O• radical, as postulated for ThiH and HydG,[6a,7b] are plausible candidates to catalyze the Cα–Cβ bond cleavage in these enzymes. Conversely, the existence of a •NH tryptophan radical species, its comparison with tyrosine lyases, and our DFT calculations afford a solid basis for a shared •NH-based catalytic mechanism for Cα–Cβ bond cleavage in NosL, ThiH, and HydG. The common arginine guanidinium moiety fine-tunes the protonation state of the substrate, thus driving the reaction towards Cα–Cβ bond cleavage instead of decarboxylation.

Surprisingly, the amino acid sequence of the radical SAM CofH component of F_0-synthase, which also uses tyrosine as a substrate, does not have residues equivalent to Tyr90, Arg323, or Ser340. Consequently, this enzyme, which transfers the *p*-cresyl fragment of tyrosine to CofG,[26] may have a different way of cleaving its Cα–Cβ bond.

Finally, a thorough understanding of the structural basis for MIA synthesis by NosL will open up the possibility of modifying the enzyme to generate modified versions of the

MIA which, once integrated into the noseheptide framework may lead to novel thiopeptide antibiotics.[1b]

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