Published in final edited form as:

Biochemistry. 2018 June 05; 57(22): 3130–3133. doi:10.1021/acs.biochem.8b00110.

Mechanistic Implications of the Deamination of TDP-4-amino-4-deoxy-D-fucose Catalyzed by the Radical SAM Enzyme DesII

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Abstract

DesII is a radical SAM lyase that catalyzes a deamination reaction during the biosynthesis of desosamine in *Streptomyces venezuelae*. Competing mechanistic hypotheses for this radical-mediated reaction are differentiated according to whether or not a 1, 2-migration takes place and the timing of proton abstraction following generation of a substrate α -hydroxyalkyl radical intermediate. In the present study, the deuterated C4-epimer of the natural substrate, TDP-4-amino-4-deoxy-D-[3-²H]fucose, was prepared and shown to be a substrate for DesII undergoing deamination along with a specific activity that is only marginally reduced (ca. 3-fold) with respect to deamination of the natural substrate. Furthermore, pH titration of the deamination reaction implicates the presence of a hydron acceptor that facilitates catalysis but does not appear to be necessary. Based on these as well as previously reported results, a mechanism is proposed involving direct elimination of ammonium concerted with proton transfer to the nucleofuge from the adjacent α -hydroxyalkyl radical.

Graphical TOC Entry

DesII is a radical *S*-adenosyl-L-methionine (SAM) enzyme that catalyzes the deamination of TDP-4-amino-4,6-dideoxy-D-glucose (**1**) during the biosynthesis of TDP-desosamine (**3**) in *Streptomyces venezuelae* (see Figure 1).^{1,2} The mechanism of this deamination reaction is

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not completely understood; however, there are three principle hypotheses to explain elimination of ammonia from the substrate radical intermediate **9** following reductive homolysis of SAM and initial H-atom abstraction from the C3 position of **1** (see Figure 2).³ The first involves deprotonation of the C3 α -hydroxyalkyl radical (**9**) to generate a ketyl radical intermediate (**10**) prior to elimination of ammonia and formation of an enol radical (**10** $\rightarrow$ **11**). Alternatively, elimination of ammonium may take place in concert with proton transfer from the α -hydroxyalkyl radical (**9** $\rightarrow$ **12** $\rightarrow$ **11**). Finally, the reaction may proceed via 1, 2-migration of NH₃ to form a carbinolamine radical (**9** $\rightarrow$ **13** $\rightarrow$ **14**) prior to reduction and elimination (**14** $\rightarrow$ **15** $\rightarrow$ **2**). Previous computational and experimental investigations of the B₁₂-dependent ethanolamine ammonia lyase^{4–6} and diol dehydratase⁷ enzyme systems have generally favored the 1, 2-migration mechanism. However, investigation of the DesII reaction using a deuterated C3-fluorinated substrate analog (**8**) as well as TDP-D-quinovose (**4**)^{3,8} have been more suggestive of general base catalysis and less consistent with a 1, 2-migration.

DesII is also converted from a lyase to a dehydrogenase (see **4** $\rightarrow$ **5**, Figure 1) with minor changes in the steady-state parameters when the C4-amine is converted to a hydroxyl in TDP-D-quinovose (**4**).^{2,8} Lyase activity with TDP-D-quinovose can be partially restored when the stereochemistry at C4 is inverted to form TDP-D-fucose (**6**) such that dehydration competes roughly 1 : 1 with dehydrogenation, though the majority of the reaction flux proceeds via C3-epimerization and with an overall 2000-fold reduction in specific activity (k_{cat}).⁹ Given known examples of enzyme-catalyzed, radical-mediated dehydration reactions,^{7,10–12} this observation suggests that the partitioning between lyase and dehydrogenase activity in DesII is sensitive to the relative geometry of the partially-filled *p*-orbital at C3 versus the adjacent C–N/O bond at C4 in the substrate radical intermediate.¹³ Moreover, this hypothesis is consistent with recent structural and computation studies on the the radical SAM diol dehydratase AprD4.¹⁰

Based on these observations it was hypothesized that the C4-epimer of the amino substrate **1** (i.e., TDP-4-amino-4-deoxy-D-fucose, **7**) would also undergo deamination in the presence of DesII. Furthermore, if a 1, 2-migration were to be operant, then DesII should be nearly as effective at deaminating **7** as it is with dehydrating **6** based on observations of B₁₂-dependent diol dehydratase activity, which is believed to proceed via 1, 2-migration of a hydroxyl.⁷ However, if the rate of DesII turnover with **7** under near-saturating conditions, where the effects of substrate binding are eliminated, is similar to that of **1** (i.e., much faster than dehydration of **6**), then it would imply that the enzyme active site is configured specifically for the elimination of ammonia rather than water. Such an observation would thus tend to further discredit a 1, 2-migration reaction as the mechanism of lyase activity.

To test this hypothesis, TDP-4-amino-4-deoxy-D-[3-²H]fucose (**7D**) was prepared following previously reported procedures as shown in Figure 3.^{1,14} The C3-deuterated isotopolog was designed for these studies as an internal control for H-atom abstraction (see below). The deuterium was introduced at C3 via the oxidation of diisopropylidenglucofuranose (**16**) to **17** followed by reduction with NaBD₄ to yield **18**. Stereochemical inversion at C3 was achieved via sequential tosylation and nucleophilic substitution by benzoate (**18** $\rightarrow$ **20**). The

protecting group at the C3-hydroxyl was switched to benzyl, and the 5, 6-isopropylidene was hydrolyzed prior to selective tosylation at C6-OH (**20** → **23**). The pyranose form of the hexose was then generated via reduction of the C6-tosyl group with LiAlH₄ and hydrolysis of the isopropylidene prior to disiloxanyl protection (**23** → **24**). This permitted triflation of the C4-hydroxyl and subsequent exchange for an axial azido group thereby introducing nitrogen at C4 with the proper stereochemistry (**24** → **25**).¹⁵ Deprotection and acetylation of the diol facilitated selective hydrolysis of the C1-acetate using N₂H₄ · HOAc and C1-phosphorylation (**25** → **28**). Global hydrogenation, deacetylation, and final coupling with thymidine monophosphate (TMP) yielded **7D**.¹⁶

The consumption of both **7D** and SAM was observed upon incubation of 10 μM DesII with 0.5 mM **7D** and 0.5 mM SAM in the presence of 0.5 mM Na₂S₂O₄ (see Figure 4 and Supporting Information for experimental details). During the 6 h incubation period, 5'-deoxyadenosine (5'-dAdo) was produced and found to be monodeuterated based on mass spectroscopic analysis (calc. for C₁₀H₁₃DO₃N₅⁺: 253.1154 *m/z*, found: 253.1162 *m/z*). This observation indicated that **7D** was indeed able to bind into the DesII active site and undergo C3 H-atom abstraction implying substrate radical generation despite the altered stereochemistry at C4. In addition to 5'-dAdo, a second product was noted by HPLC at a retention time of 25.4 min. This new product co-eluted with the deamination product **2** obtained from the DesII reaction with **1**, indicating that DesII can catalyze the deamination of **7D** (trace **d** in Figure 4). This assignment was further supported by high resolution MS analysis of the product following isolation, where-upon a signal at 529.0672 *m/z* was observed in negative ion mode consistent with the [M - H]⁻ ion adduct of **2** (calc. for C₁₆H₂₃O₁₄N₂P₂⁻: 529.0630 *m/z*).

Although no other TDP-sugar product was observed by HPLC, dehydrogenase activity could not be completely ruled out, because the dehydrogenation product, if formed, is expected to be unstable.¹⁷ Therefore, in order to test for the possible formation of the dehydrogenation product, the DesII reaction was treated with NaBH₄. If the dehydrogenation reaction takes place, then the corresponding product should be reduced by NaBH₄ to regenerate the undeuterated starting material **7**. However, there was no significant change in the deuterium content of the residual substrate as determined by MS analysis following a 6 h incubation of **7D** with DesII in the presence of 5 mM NaBH₄. Similarly, reactions treated with 100 mM NaBH₄ following a 1 h incubation did not show any evidence of deuterium depletion in the residual starting material. Hence, DesII does not appear to exhibit any significant dehydrogenase activity with respect to **7D**.

The observed *k_{cat}* for DesII-catalyzed deamination of **7D** at pH 8.0 was 3.4 ± 0.1 fold lower compared to that measured for deamination of the natural substrate (**1**) with the same enzyme preparation. This is a relatively modest reduction in *k_{cat}* that may be explained at least in part by a primary deuterium kinetic isotope effect on H-atom abstraction. Furthermore, after more than 40% conversion of **7D** to **2** over a 6 h reaction, no change in the deuterium content of the residual substrate was observed following isolation and MS analysis. This contrasts with the observation that the fluoro-analog **8** rapidly exchanges deuterium with SAM unlike the C3-deuterated natural substrate.³ These results imply that

C4-epimerization of **1** does not strongly impact the ability of DesII to catalyze deamination once the ternary Michaelis complex has formed between DesII, SAM and **7**.

Production of 5'-deoxyadenosyl-5'-sulfinate was also observed (see Figure 4, peak D). This species is derived from trapping of the 5'-deoxyadenosyl radical (or SAM directly) by $\text{Na}_2\text{S}_2\text{O}_4$ (or a derivative thereof) as previously reported in the reactions of DesII with the C4-epimer (**6**) of the dehydrogenation substrate TDP-D-quinovose (**4**).⁹ Consequently, C4-epimerization of the natural substrate **1** does result in some perturbation or destabilization of the active site during the radical generation process leaving the 5'-deoxyadenosyl radical (or SAM) more susceptible to radical-mediated side reactions. As a similar phenomenon is observed with TDP-D-fucose (**6**)—but not with **1** or TDP-quinovose (**4**)—it appears that the 3, 4-*cis* configuration of the substrate is primarily responsible for destabilization of the radical generating process regardless of whether a hydroxyl or amine functionality is installed at C4.

The pH-dependence of the DesII-catalyzed deamination of **1** was also investigated by measuring initial rate curves in the pH range of 6.7 to 10.0 using a discontinuous assay (see Supporting Information for details). This assay provides an additional test of the mechanistic hypotheses, because the 1, 2-migration of NH_3 is not expected to require formal acid-base catalysis but rather a “push-pull” interaction between complementary H-bond acceptors and donors.^{19,20} In contrast, an active site base is expected to be necessary for ketyl radical formation.¹⁰ While the initial rate data was too poorly conditioned to provide reliable estimates of the dependence of V/K on pH, a reduction in V_{max} for the deamination of **1** with decreasing pH was observed (see Figure 5). While the uncertainty in the observations precludes a definitive analysis, the data was modeled best by assuming a transition between two non-zero maximum rates of turnover (8 ± 3 fold different) with a $\text{p}K_a$ of 8.5 ± 0.2 . This $\text{p}K_a$ is similar to that measured for the dehydrogenation reaction⁸ and is consistent with titration of the same basic site. In contrast to the dehydrogenation reaction, however, the sigmoidal pattern of the pH-dependence of deamination suggests that the titratable base facilitates deamination but may not be critical arguing against the formation of a discrete ketyl radical intermediate.

The results with **7D** tend to argue against the hypothesis that deamination proceeds via a 1, 2-migration, because they suggest that the active site is configured to support elimination of ammonia/ammonium in preference to hydroxide/water. Were a 1, 2-migration to be operant, then DesII should be nearly as effective at dehydrating **6** as it is with deaminating **7** based on observations of diol dehydratase activity and α -hydroxyalkyl radicals generated via pulse radiolysis.^{7,21,22} A similar conclusion was drawn given the inability of DesII to catalyze deamination of the C3-fluorinated analog **8**,³ and is consistent with recent studies of the radical SAM catalyzed dehydration of paromamine by AprD4.¹⁰ However, the observed partial dependence of the deamination reaction on base catalysis favors a mechanism involving a H-bond acceptor capable of hydron-exchange with solvent that may not necessarily be critical for the deamination reaction, which is not entirely consistent with the formation of a ketyl radical intermediate (e.g., **9** $\rightarrow$ **10**). Therefore, based on the evidence collected to date, the mechanism of DesII-catalyzed deamination is proposed to involve direct elimination of NH_3 from C4 concerted with proton transfer from the α -hydroxy alkyl

radical to yield an enol radical (**9** → **12** → **11**) prior to reduction and protonation (**11** → **2**). In this case, a H-bond acceptor is expected to facilitate the proton transfer during deamination of **1** while effecting complete deprotonation of the α -hydroxyalkyl radical during dehydrogenation of TDP-D-quinovose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (NIH GM035906).

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Figure 1.
Substrate analogs and reactions catalyzed by DesII.

Figure 2.
Mechanistic hypotheses for the DesII-catalyzed deamination of **1** following formation of the substrate radical intermediate **9**.

Figure 3.

Synthesis of the deuterated C4-epimer **7D** of the DesII deamination substrate (**1**). See text and Supporting Information for details.

Figure 4.

HPLC traces demonstrating the consumption of TDP-4-amino-4-deoxy-D-[3-²H]fucose (**7D**) and SAM in the presence of DesII and Na₂S₂O₄. Trace **a** corresponds to the complete reaction mixture with **7D** following a 2 h incubation without DesII. Traces **b** & **c** correspond to the complete reaction mixture with **7D** following 2 and 6 h incubations in the presence of DesII, respectively. Trace **d** represents the reaction mixture analyzed in trace **b** coinjected with a similar DesII reaction mixture containing TDP-4-amino-4,6-dideoxy-D-glucose (**1**) instead of **7D**, this latter reaction being shown in trace **e**. The retention times in traces **b–e** have been successively shifted right by 1.5 min for presentation. Peak A is the methylthioadenosine decomposition product of SAM,¹⁸ peak B is thymidine monophosphate (TMP) due to hydrolysis of **7D**, peak C is a contaminant in the SAM reagent and peak D is 5'-deoxyadenosyl-5'-sulfinate.⁹

Figure 5.

Maximum velocity (V) of DesII-catalyzed deamination of **1** as a function of pH. The observed values of V obtained from initial rate curves have been scaled with respect to the minimum value observed (V_0) for presentation. Error bars represent ± 1 standard error following propagation through the base-10 logarithm. The solid line corresponds to a two-state model for the pH-dependence of V -max fit to the observed values using weighted nonlinear least squares. See Supporting Information for further detail.