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Mechanistic Studies of the Radical *S*-Adenosyl-L-methionine Enzyme DesII: EPR Characterization of a Radical Intermediate Generated During Its Catalyzed Dehydrogenation of TDP-D-Quinovose

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Supporting Information

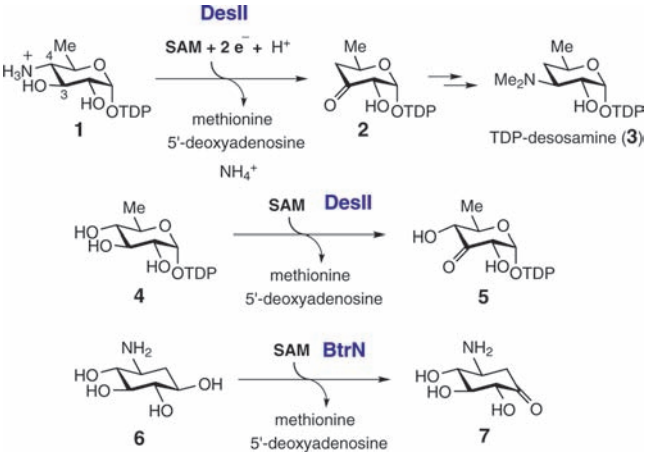
ABSTRACT: DesII, a radical *S*-adenosyl-L-methionine (SAM) enzyme from *Streptomyces venezuelae*, catalyzes the deamination of TDP-4-amino-4,6-dideoxy-D-glucose to TDP-3-keto-4,6-dideoxy-D-glucose in the desosamine biosynthetic pathway. DesII can also catalyze the dehydrogenation of TDP-D-quinovose to the corresponding 3-keto sugar. Similar to other radical SAM enzymes, DesII catalysis has been proposed to proceed via a radical mechanism. This hypothesis is now confirmed by EPR spectroscopy with the detection of a TDP-D-quinovose radical intermediate having a *g*-value of 2.0025 with hyperfine coupling to two spin 1/2 nuclei, each with a splitting constant of 33.6 G. A significant decrease in the EPR line width is observed when the radical is generated in reactions conducted in D_2O versus H_2O. These results are consistent with a C3 α-hydroxyalkyl radical in which the *p*-orbital harboring the unpaired electron spin at C3 is periplanar with the C−H bonds at both C2 and C4.

The radical *S*-adenosyl-L-methionine (SAM) enzymes are an important class of biocatalysts that are widespread in Nature. While their catalytic functions are remarkably diverse, a reductive homolytic cleavage of the C5′−S bond of SAM facilitated by a $[4Fe-4S]^{1+}$ cluster in the active site is a common step en route to products for these enzymes.[1−3] The resulting 5′-deoxyadenosyl radical is used to abstract a hydrogen atom, commonly from an unactivated C−H moiety, of the substrate in a manner reminiscent of B_{12}-dependent enzymes[4,5] to initiate the chemical reactions. The substrate radical intermediates so produced then undergo further transformations distinctive for each enzyme to generate the final products. Characterization of the proposed substrate-derived radical intermediates is critical for functional verification and may also lead to useful mechanistic insight regarding the target enzyme. However, direct detection of these radical intermediates is experimentally challenging due to the transient nature of the radical species. Thus far, only a few radical SAM enzymes for which successful detection of the substrate radical intermediates has occurred have been reported. These include lysine-2,3-aminomutase, HemN, and BtrN.[6−9]

In our study of the biosynthesis of TDP-desosamine (**3**)[10] in *Streptomyces venezuelae* we have identified a key enzyme, DesII, that catalyzes the deamination of TDP-4-amino-4,6-dideoxy-D-glucose (**1**) to TDP-3-keto-4,6-dideoxy-D-glucose (**2**) (see Scheme 1).[11,12] This enzyme has been characterized as a radical SAM enzyme on the basis of the presence of a **CxxxCxxC** motif in its sequence, the requirement of a reduced $[4Fe-4S]^{1+}$ cluster and SAM for its activity, and the transfer of a hydrogen atom from the C3 position of **1** to C5′ of 5′-deoxyadenosine during catalysis.[13] Interestingly, when the enzyme is incubated with TDP-D-quinovose (**4**) instead of the natural substrate (**1**), analogous elimination of the C4 hydroxyl is not observed; rather, the C3 hydroxyl is oxidized to the corresponding ketone (**5**) with electron transfer back to the oxidized $[4Fe-4S]^{2+}$ cluster to regenerate the reduced $[4Fe-4S]^{1+}$ state.[13,14] This chemistry resembles the dehydrogenation of 2-deoxy-*scyllo*-inosamine (**6**) to 3-amino-2,3-dideoxy-*scyllo*-inosose (**7**) catalyzed by BtrN.[15] While DesII is an established member of the radical SAM enzyme family, experimental verification of the expected radical intermediates during turnover has yet to be provided. In this communication, we report the detection and characterization of a substrate radical intermediate formed in the DesII-catalyzed dehydrogenation of TDP-D-quinovose (**4**) to **5** by electron paramagnetic resonance (EPR) spectroscopy.

When DesII is incubated with **4** (3:1 substrate/enzyme, manually freeze-quenched at ∼11 s) an EPR signal is observed

Scheme 1. Deamination and Dehydrogenation Reactions of DesII



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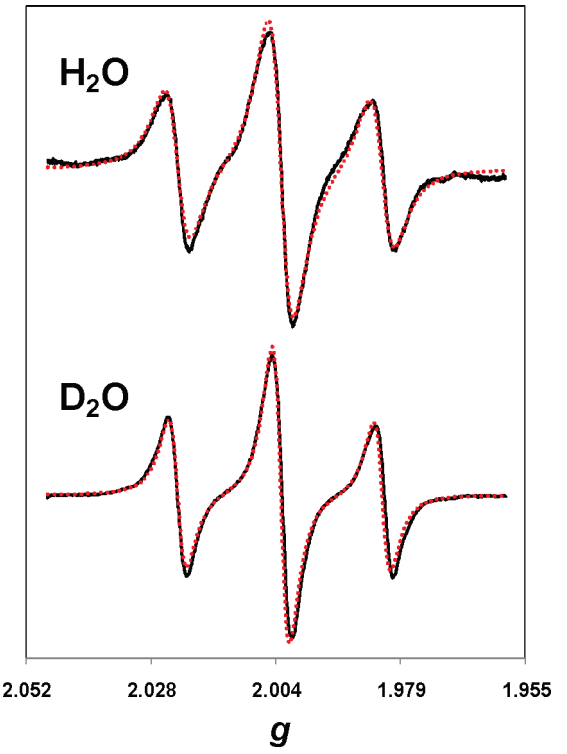

Figure 1. X-band EPR spectra of DesII (90 μM) in the presence of 250 μM TDP-D-quinovose (**4**), 560 μM SAM, and 700 μM $Na_2S_2O_4$ in H_2O and D_2O (25 mM EPPS, 1 mM DTT, H_2O pH 8.0). Reactions were frozen using precooled isopentane approximately 11 s after mixing. Overlayed red broken lines denote simulated spectra. EPR acquisition parameters: microwave frequency, 9.46 GHz (H_2O), 9.44 GHz (D_2O); microwave power, 2.0 mW; receiver modulation frequency, 100 kHz; receiver modulation amplitude, 5.0 G; signal channel time constant, 5.12 ms; sweep time, 121 s; number of scans, 32 (H_2O), 16 (D_2O).

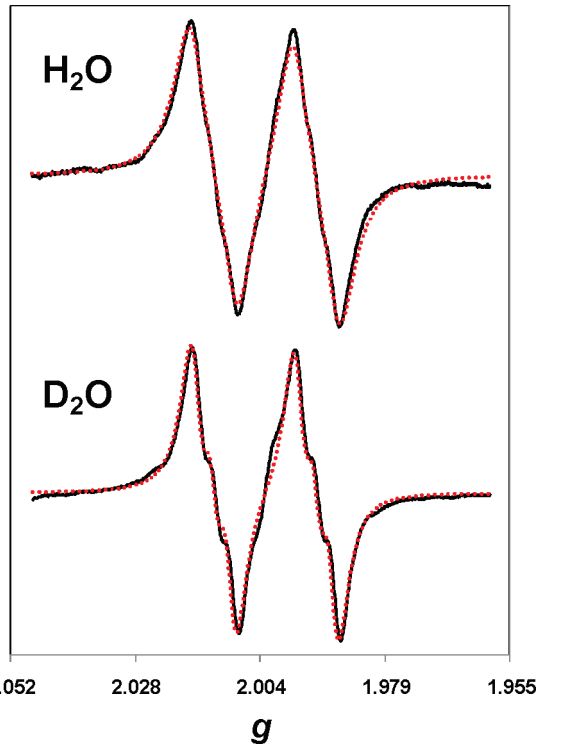

Figure 2. X-band EPR spectra of DesII (90 μM) in the presence of 250 μM TDP-D-[4-^{2}H]quinovose (**4D**), 560 μM SAM, and 700 μM $Na_2S_2O_4$ in H_2O and D_2O (25 mM EPPS, 1 mM DTT, H_2O pH 8.0). Reactions were frozen using precooled isopentane approximately 11 s after mixing. Overlayed red broken lines denote simulated spectra. EPR acquisition parameters: microwave frequency, 9.45 GHz (H_2O), 9.44 GHz (D_2O); microwave power, 2.0 mW; receiver modulation frequency, 100 kHz; receiver modulation amplitude, 5.0 G; signal channel time constant, 5.12 ms; sweep time, 121 s; number of scans, 32 (H_2O), 16 (D_2O).

as shown in Figure 1 (in H_2O). The EPR spectrum can be well simulated using an isotropic *g*-factor of 2.0025 with the inclusion of isotropic hyperfine interactions with two equivalent spin $^1/_2$ nuclei having splitting constants of 33.6 G (red broken line in Figure 1). In line with previous experiments demonstrating abstraction of a hydrogen atom from C3 of **1** during turnover,[13] this doublet of doublets splitting pattern is consistent with a C3 radical of **4** with hyperfine couplings to the hydrogen atoms at C2 and C4. To validate this assignment, the C4-deuterated isotopologue, TDP-D-[4-^{2}H]quinovose (**4D**), was prepared by treatment of TDP-4-keto-6-deoxy-D-glucose with $NaBD_4$, followed by HPLC separation from the minor epimeric byproduct, TDP-D-[4-^{2}H]fucose. As shown in Figure 2 (in H_2O), when **4D** is reacted with DesII, the EPR signal collapses from a doublet of doublets to a broadened doublet. EPR line shape simulations utilizing the same *g*-factor (2.0025) and line width (7.2 G) revealed that the doublet is due to coupling to a spin $^1/_2$ nucleus with a splitting constant of 33.6 G, whereas the broadening arises from hyperfine coupling to a spin 1 nucleus with a splitting constant of 5.2 G (red broken line in Figure 2). Such a reduction in the magnitude of the splitting constant is consistent with that expected from the ratio of the magnetogyric ratios (g_H/g_D),[16] which is equal to the observed value of 6.5.

Control experiments where either substrate or enzyme was excluded showed only a small, nondescript EPR signal due to the cavity (see Supporting Information). This background signal has been subtracted from the spectra in Figures 1 and 2. Unfortunately, attempts to capture a radical intermediate in the deamination of **1** by DesII were unsuccessful even when the reaction was freeze-quenched within 100 ms of mixing enzyme with substrate. The inability to trap such a radical during steady state turnover may be related to the different redox reactions comprising the catalytic cycles associated with the deamination of **1** versus the dehydrogenation of **4**.[14]

The above observation confirms the formation of a substrate-derived radical intermediate in the DesII-catalyzed dehydrogenation reaction. Judging from the equivalence of the hyperfine splitting constants due to the C2 and C4 H-nuclei, the π-system of the C3 centered radical is expected to have an equivalent orientation with respect to the C−H bonds at C2 and C4. The corresponding dihedral angle, χ, can be estimated from the Heller−McConnell relation,[17]

$$a(\chi) = \rho(A_1 + A_2 \cos^2 \chi)$$

with empirical values of 0.92 and 42.6 G for A_1 and A_2, respectively.[18] The Mulliken spin density, ρ, at C3 was computed to be 0.83 by density functional theory (DFT) calculations on the corresponding D-quinovose radical (see Supporting Information). On the basis of the observed splitting constants of 33.6 G each, the dihedral angle, χ, between the *p*-orbital at the *sp*^{2}-hybridized C3 center and the adjacent C2−H and C4−H

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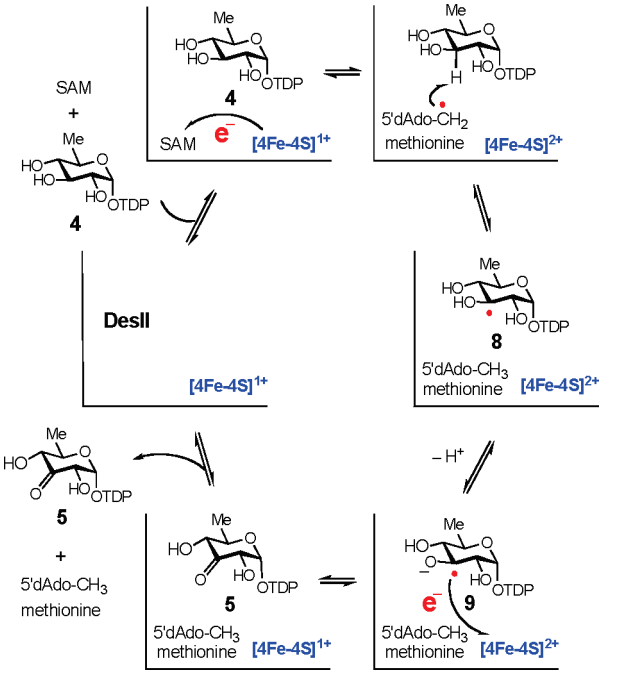
Scheme 2. Proposed Mechanism for Dehydrogenation by DesII



bonds is thus predicted to be ~15.0°. Were all the spin density to be localized at C3, i.e., $\rho = 1$, the dihedral angle would remain no greater than 30°. These observations are consistent with a chair conformation of the TDP-D-quinovose radical intermediate in which the *p*-orbital at C3 is nearly eclipsed by the C−H bonds at the two adjacent carbon centers. A comparable geometry has also been reported for the BtrN substrate radical intermediate.[8]

To gain more information about the ionization state of the TDP-D-quinovose radical, enzymatic incubations in D_2O were also carried out. The corresponding EPR spectra of the reaction mixtures are presented in Figures 1 (D_2O) and 2 (D_2O) along with results from the simulation of the EPR line shapes (red broken lines). The radicals derived from either isotopologue (**4** and **4D**) of TDP-D-quinovose exhibit a marked narrowing of the line widths of their EPR signals from 7.2 to 5.0 G when H_2O is exchanged for D_2O. In the case of **4D**, this narrowing permits clear visualization of the hyperfine splitting of the EPR signal due to the C4 deuterium nucleus. The narrowing of the line widths strongly suggests that the C3 hydroxyl group of the observed radical is not deprotonated. Thus, by replacing •C3−OH with •C3−OD, the larger splitting from H is reduced to that of D leading to an apparent reduction in inhomogenous broadening of the EPR signal. Interestingly, DFT calculations on the D-quinovose ketyl radical, obtained by deprotonation of the C3 hydroxyl group, yields a ρ value of 0.63. This value is inconsistent with the large magnitude of the experimental hyperfine splitting constants and, thus, supports the assigned ionization state of the •C3−OH radical.

Although, the radical detected in these experiments is an α-hydroxyalkyl radical (•C3−OH), deprotonation of the hydroxyl group to form the corresponding ketyl radical (•C3−O^-) is believed to be important for DesII catalysis. One hypothesis posits that the C3 radical (**8**), after formation, can readily be deprotonated by an active site base, facilitated by an expected ~5 unit decrease in pK_a of the C3 hydroxyl group to form the ketyl radical **9** (Scheme 2).[19] For the dehydrogenation of **4**, deprotonation of the hydroxyl group may drop the redox potential of the resulting ketyl radical[19] to a value sufficiently negative to facilitate electron transfer from **9** back to the $[4Fe-4S]^{2+}$ cluster to complete the reaction. The shift of the reaction flux to electron transfer instead of C4 elimination, as might be expected considering the deamination of **1** and the precedence set by the dioldehydratases,[5] may in part be explained by the observed geometry that places the C4 hydrogen, as opposed to the C4 hydroxyl group, periplanar to the singly filled *p*-orbital of the adjacent α-hydroxyalkyl radical. Consequently, the different catalytic outcome of deamination in the case of **1** may result from some combination of differences in nucleofugality of ammonia versus hydroxide and a potentially different binding conformation that is more conducive to the elimination of ammonia from C4.

In summary, the intermediacy of a C3 radical in the DesII-catalyzed dehydrogenation of **4** has been firmly established by EPR spectroscopy. This finding, in conjunction with previous results, confirms that DesII catalysis follows radical SAM chemistry. The characteristics of the radical are consistent with an α-hydroxyalkyl radical at the sp^2-hybridized C3 position, where the *p*-orbital harboring the unpaired electron spin is periplanar with the C−H bonds at both C2 and C4. This conformation would disfavor elimination of hydroxide at C4, while subsequent deprotonation to a C3 ketyl radical is expected to promote electron transfer to the $[4Fe-4S]^{2+}$ cluster. Though similar chemistry is observed with BtrN, for which dehydrogenation is its primary role *in vivo*, BtrN has recently been shown to utilize a second [4Fe−4S] cluster to effect the electron transfer.[20] In contrast, DesII (having only four cysteine residues) only possesses one [4Fe−4S] complex,[13] such that dehydrogenation of TDP-quinovose may proceed via direct electron transfer back to the oxidized cluster. How this might relate to deamination and the relative timing of electron transfer versus deprotonation during dehydrogenation remains unanswered and is currently being pursued in the study of this interesting example of radical SAM chemistry.

■ ASSOCIATED CONTENT

Supporting Information. Details regarding experimental procedures, NMR characterization of TDP-D-[4-^{2}H]quinovose, EPR line shape simulation, and DFT calculations as well as a list of abbreviations. This material is available free of charge via the Internet at http://pubs.acs.org.

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