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Characterization and Mechanistic Studies of DesII: A Radical *S*-Adenosyl-L-methionine Enzyme Involved in the Biosynthesis of TDP-D-Desosamine

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Abstract: D-Desosamine (**1**) is a 3-(*N*,*N*-dimethylamino)-3,4,6-trideoxyhexose found in a number of macrolide antibiotics including methymycin (**2**), neomethymycin (**3**), pikromycin (**4**), and narbomycin (**5**) produced by *Streptomyces venezuelae*. It plays an essential role in conferring biological activities to its parent aglycones. Previous genetic and biochemical studies of the biosynthesis of desosamine in *S. venezuelae* showed that the conversion of TDP-4-amino-4,6-dideoxy-D-glucose (**8**) to TDP-3-keto-4,6-dideoxy-D-glucose (**9**) is catalyzed by DesII, which is a member of the radical *S*-adenosyl-L-methionine (SAM) enzyme superfamily. Here, we report the purification and reconstitution of His_6-tagged DesII, characterization of its [4Fe-4S] cluster using UV−vis and EPR spectroscopies, and the capability of flavodoxin, flavodoxin reductase, and NADPH to reduce the $[4Fe\text{-}4S]^{2+}$ cluster. Also included are a steady-state kinetic analysis of DesII-catalyzed reaction and an investigation of the substrate flexibility of DesII. Studies of deuterium incorporation into SAM using TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose as the substrate provides strong evidence for direct hydrogen atom transfer to a 5′-deoxyadenosyl radical in the catalytic cycle. The fact that hydrogen atom abstraction occurs at C-3 also sheds light on the mechanism of this intriguing deamination reaction.

D-Desosamine (**1**) is a 3-(*N*,*N*-dimethylamino)-3,4,6-trideoxyhexose found in a number of macrolide antibiotics including methymycin (**2**), neomethymycin (**3**), narbomycin (**4**), and pikromycin (**5**) produced by *Streptomyces venezuelae*. Both biochemical and structural studies have shown that desosamine is an essential structural component crucial to the biological activity of the parent aglycones (e.g., **12**, **13**).[1] Desosamine is biosynthetically derived from thymidine diphosphate (TDP)-D-glucose (**6**), and a key step in its formation is the removal of the C-4 hydroxyl group of the hexose ring. Early studies of desosamine biosynthesis in *S. venezuelae* revealed that the C-4 deoxygenation is unique among biological deoxygenation processes[2] and the reaction proceeds in two stages: the conversion of TDP-4-keto-6-deoxy-D-glucose (**7**) to the corresponding 4-amino sugar intermediate (**8**), and the deamination of **8** to afford TDP-3-keto-4,6-dideoxy-D-glucose as the final product (**9**, Scheme 1). The former reaction is catalyzed by DesI, a pyridoxal-5′-phosphate (PLP)-dependent aminotransferase, whereas the latter reaction is catalyzed by DesII, a radical *S*-adenosyl-L-methionine (SAM)-dependent enzyme.[3] Subsequent C-3 aminotransfer (**9** → **10**) by DesV followed by *N*,*N*-dimethylation (**10** → **11**) by DesVI complete the desosamine biosynthesis.[4] All enzymes in the pathway have been biochemically characterized, but the details of the catalytic properties of DesII and the mechanism of its catalysis remain obscure.

Thus far, more than 2800 proteins have been identified, mainly based on sequence alignment, as members of the radical SAM enzyme superfamily.[5] These enzymes catalyze a variety of reactions, such as isomerization, protein radical formation, sulfur insertion, ring formation, anaerobic oxidation, and unusual methylations, and are involved in the biosynthesis of DNA precursors, cofactors, vitamins, many types of secondary metabolites, and various biodegradation pathways.[6] Enzymes of this superfamily possess a characteristic conserved sequence motif, **C**XXX**C**XX**C**, which coordinates a [4Fe-4S] cluster.[7] Four different oxidation states of the iron−sulfur cluster have been observed. The intact iron−sulfur cluster exists mainly as $[4Fe\text{-}4S]^{2+}$ in the purified enzyme, but $[4Fe\text{-}4S]^{3+}$ is found as

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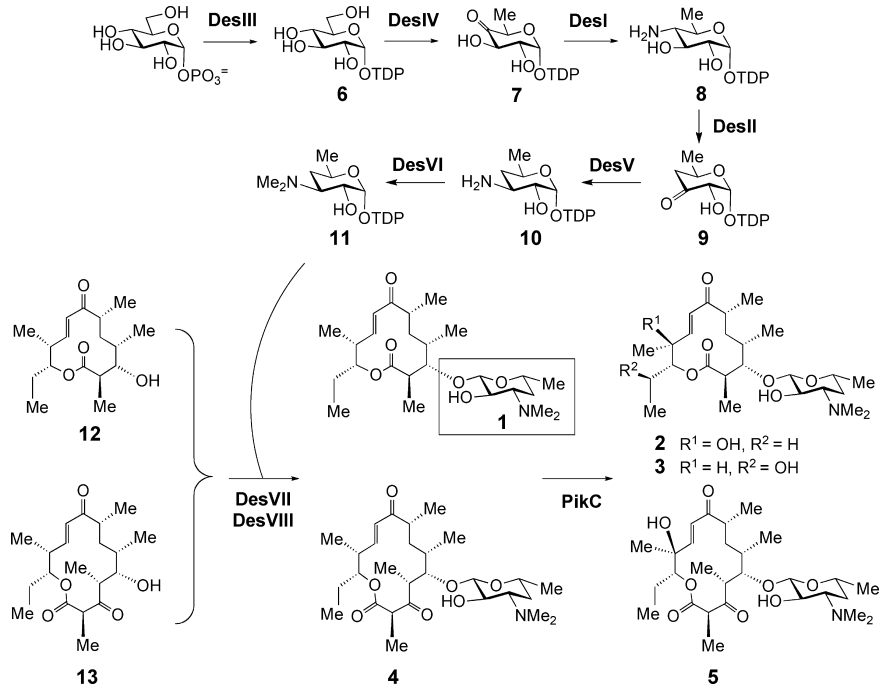
Scheme 1



a minor component and readily converts to $[3Fe\text{-}4S]^{1+}$ via air oxidation. Upon treatment with dithionite in the presence of SAM, the iron−sulfur cluster is reduced to $[4Fe\text{-}4S]^{1+}$, which is the catalytically active form of all radical SAM enzymes.[8] Catalysis by this class of enzymes is always initiated by one electron transfer from the $[4Fe\text{-}4S]^{1+}$ cluster to SAM. This induces the homolytic cleavage of the $C_{5'}$−S bond of SAM to generate methionine and a 5′-deoxyadenosyl radical (**22**, see Schemes 5 and 6).[6] The subsequent abstraction of a hydrogen atom from the substrate by the reactive 5′-deoxyadenosyl radical triggers the chemical transformations during turnover.[6,9]

Studies of this class of enzymes are difficult because reconstitution of the iron−sulfur cluster is technically challenging and the reduced $[4Fe\text{-}4S]^{1+}$ cluster is highly oxygen sensitive. Thus far, only a handful of radical SAM enzymes have been investigated. Better known examples include lysine 2,3-aminomutase,[7f,10] pyruvate formate-lyase activase,[7a,8a,11] anaerobic ribonucleotide reductase activase,[7b,8c,12] biotin synthase,[7d,13] lipoyl synthase,[14] spore photoproduct lyase,[15] and BtrN,[16] a radical SAM dehydrogenase involved in the biosynthesis of butirosin. More recently, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase (ThiC) in the thiamine pyrimidine biosynthetic pathway has been characterized as a previously unrecognized radical SAM enzyme using SAM as a cosubstrate and producing HMP and 5′-deoxyadenosine as the products.[17]

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DesII, which also contains the CXXXCXXC iron−sulfur cluster binding motif, has been identified as a member of the radical SAM enzyme superfamily.[5b] Previous studies have established the dependence on a [4Fe-4S] cluster and SAM for DesII activity.[3] In this contribution, we report a full account of the characterization of the [4Fe-4S] cluster in DesII, a steady-state kinetic analysis of the DesII-catalyzed reaction, an investigation of the substrate flexibility of DesII, and the demonstration of a biological reducing system, flavodoxin, flavodoxin reductase, and NADPH, capable of reducing the $[4Fe\text{-}4S]^{2+}$ cluster. Moreover, studies of deuterium incorporation into SAM using TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**, see Scheme 4) provide insight into the mechanism of this intriguing deamination reaction.

Experimental Procedures

General. Protein concentrations were determined by the method of Bradford[18] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.[19] The native molecular mass of DesII was determined by the gel filtration method of Andrews.[20] ^{1}H,^{13}C, ^{31}P NMR spectra were recorded using either a Varian Unity Plus 300, 500, or 600 MHz spectrometer. Mass spectra were recorded by the MS facility at the Department of Chemistry and Biochemistry of the University of Texas at Austin. DNA sequencing and amino-terminal sequencing of the purified enzyme were performed by the Core Facilities of the Institute of Cellular and Molecular Biology at the University of Texas at Austin. Electron paramagnetic resonance (EPR) spectra were recorded at liquid helium temperature on a Bruker EMX EPR spectrometer at the University of Texas at Houston Medical School. Analytical thin layer chromatography (TLC) was carried out on precoated TLC glass plates, and the spots on TLC plates were visualized first under UV light and then by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% in EtOH). Flash column chromatography was performed on silica gel by elution with the specified solvents. CarboPac PA1 HPLC columns (4 mm × 250 mm and 9 mm × 250 mm) were obtained from Dionex (Sunnyvale, CA). The fast protein liquid chromatography (FPLC) Mono-Q H/R 16/10 column was purchased from Pharmacia (Uppsala, Sweden). Kinetic data were analyzed by nonlinear regression using Grafit5 (Erithacus Software, UK). All anaerobic experiments were performed in a glovebox (Coy Laboratory Products, Grass Lake, MI) under an atmosphere consisting of 95% N_2 and 5% H_2 at less than 5 ppm O_2. The catalyst used to remove the oxygen was regenerated each month by heating at 120 °C for 2 h.

Materials. All chemicals were analytical grade or the highest quality commercially available and were used without further purification unless otherwise noted. Enzymes used in the cloning experiment were obtained from Invitrogen (Carlsbad, CA), Amersham (Arlington Heights, IL), Gibco BRL (Grand Island, NY) or Promega (Madison, WI). Culture medium ingredients were purchased from Difco (Detroit, MI). DNA minipreps were performed using the Wizard DNA purification kit from Promega. All oligonucleotide primers for polymerase chain reaction (PCR) amplification of the desired inserts were prepared by Gibco BRL. All electrophoresis materials were purchased from Gibco BRL or Bio-Rad (Hercules, CA). The plasmid pET28b containing the *desI* gene and the plasmid pET24b containing the *desII* gene were previously constructed.[3] Enzymes DesI, RfbB (a DesIV equivalent from *Salmonella typhi*, which catalyzes the conversion of **6** to **7**), and DesV were purified from the corresponding recombinant *Escherichia coli* strains using published procedures.[3b,21] TDP-D-glucose and the products of DesII and DesV were synthesized by literature procedures.[21a,22]

Purification of DesII from *Escherichia coli* BL21 Star (DE3)-*desII*/pET24 Cells. An overnight culture of *E. coli* BL21 Star (DE3)-*desII*/pET24b grown at 37 °C in Luria−Bertani (LB) medium containing kanamycin (50 μg/mL) was used, in a 200-fold dilution, to inoculate 6 L of the same medium. When the OD_{600} reached 0.4−0.6, the incubation temperature was lowered to 18 °C and isopropyl-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce gene expression. $Fe(NH_4)_2(SO_4)_2$ was also added to a final concentration of 0.2 mM to provide additional iron for iron−sulfur cluster biosynthesis. After incubation for an additional 18 h at 18 °C, cells were harvested by centrifugation (4000*g*, 10 min) at 4 °C, washed with 50 mM potassium phosphate buffer (pH 7.5), collected again by centrifugation (4000*g*, 10 min), and stored at −80 °C. The typical yield was 6 g of wet cells per liter of culture. Subsequent purification was carried out at 4 °C, and all buffers were degassed and saturated with nitrogen before use.

Thawed cell pellets were resuspended in 120 mL of lysis buffer (50 mM sodium phosphate, 10% glycerol, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the cells were disrupted by sonication. Cell debris was removed by centrifugation (12000*g*, 30 min), and the supernatant was mixed by slow agitation with 10 mL of packed Ni-NTA resin (Qiagen, Valencia, CA) for 1 h at 4 °C. The slurry was poured into a capped column and washed with 100 mL of wash buffer (50 mM sodium phosphate, 10% glycerol, 300 mM NaCl, 20 mM imidazole, pH 8.0). The brownish protein was eluted with elution buffer (50 mM sodium phosphate, 10% glycerol, 300 mM NaCl, 250 mM imidazole, pH 8.0). The desired fractions, as detected by SDS-PAGE were pooled, dialyzed three times against 1 L of 20 mM tris(hydroxymethyl)aminomethane (Tris)·HCl buffer (pH 7.5) containing 15% glycerol, and concentrated by ultrafiltration (YM-10 membrane). The collected protein was further purified by FPLC on a MonoQ HR (16/10) column using buffer A (20 mM Tris·HCl, pH 7.5) and buffer B (Buffer A plus 0.5 M NaCl). The column was run with a linear gradient of 0 to 50% B in 25 min, followed by 50 to 70% B in 2 min. Finally, the column was washed with 100% B for 5 min. The flow rate was 3 mL/min, and the detector was set at 280 nm. The DesII protein eluted at 55% B. It was then dialyzed against 20 mM Tris·HCl buffer (pH 7.5) and concentrated by ultrafiltration (YM-10 membrane) prior to storage at −80 °C.

Construction of the Expression Plasmids for Flavodoxin (*fld*) and Flavodoxin Reductase (*fpr*). The *fld* gene from *E. coli* was PCR amplified and cloned into the pET24b(+) vector (Novagen, Madison, WI) at the *Nde*I and *Xho*I restriction sites. The primers used were: 5′-TCGCCATATGAACAGCTGCGTAT-TAATAA-3′ (forward, with the *Nde*I site underlined) and 5′-GCAACTCGAGTTAATAAAAACGTGGACA-3′ (reverse, with the *Xho*I site underlined). Similarly, the *fpr* gene from *E. coli* was amplified using 5′-AAAACCATGGCTGATTGGGTAAC-3′ (containing an *Nco*I restriction site), and 5′-AAGTCTCGAGCCAG-TAATGCTCCGCTGT-3′ (containing an *Xho*I restriction site) as the forward and reverse primers, respectively, and cloned into the pET28b(+) vector (Novagen) at the *Nco*I and *Xho*I restriction sites. The resulting plasmids were used to separately transform *E. coli* BL21 Star (DE3) cells for gene expression. The general methods and protocols for recombinant DNA manipulations followed those described by Sambrook et al.[23]

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Expression and Purification of *E. coli* Flavodoxin (FLD) and Flavodoxin Reductase (FPR). Overnight cultures of *E. coli* BL21(DE3)-*fld*/pET24b(+) and *fpr*/pET28b(+) separately grown at 37 °C in LB medium containing kanamycin (50 μg/mL) were each used, in a 100-fold dilution, to inoculate 6 L of the same medium. These cultures were incubated at 37 °C until the OD_{600} reached 0.5−0.6. Protein expression was then induced by the addition of IPTG to a final concentration of 0.1 mM. After incubation at 37 °C for an additional 6 h, the cells were harvested by centrifugation (4000*g*, 10 min) at 4 °C, washed with 50 mM potassium phosphate buffer (pH 7.5), and stored at −80 °C. The typical yield was 6 g of wet cells per liter of culture. Procedures for the subsequent protein purification using Ni-NTA affinity chromatography were identical to those used for DesII purification.

Iron Content Determination. DesII samples (1 mL each) for iron analysis[24] were mixed with 500 μL of reagent A (1:1 of 4.5% $KMnO_4$:1.2 N HCl) and incubated at 60 °C for 2 h. To these samples, 100 μL of reagent B (8.8 g of ascorbic acid, 9.7 g of ammonium acetate, 80 mg of ferrozine, 80 mg of neocuproine, and ddH_2O to 25 mL total volume) was added with immediate vortexing. The absorbance at 562 nm was recorded after the samples had incubated for 1 h at room temperature. The iron concentration was determined by comparing the reading to a standard curve prepared using $Fe(NH_4)_2(SO_4)_2$.

Sulfur Content Determination. The sulfur content of DesII was determined following a literature procedure.[25] All glassware used in this experiment was acid-washed and all solutions were prepared using metal-free distilled H_2O. Approximately 75 μg of DesII (1.5 nmol, in duplicate) was diluted with water or 20 mM Tris·HCl buffer (pH 7.6) to a total volume of 200 μL. A freshly prepared deoxygenated 1% zinc acetate solution (600 μL) was added followed immediately by the addition of 30 μL of 12% NaOH. The mixture was stirred until the color became homogeneous. After the reaction was incubated at room temperature for 1 h, 150 μL of DMPD solution (0.1% *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride in 5 N HCl) was added. The resulting solution was gently stirred until only the top 2-mm layer retained undissolved zinc hydroxide as indicated by a pink color. A 30 μL $FeCl_3$ (23 mM $FeCl_3$ in 1.2 N HCl) solution was added and mixed rapidly to generate a colorless solution. The mixture was incubated for 30 min and the color of the solution changed to blue. This blue solution was centrifuged to remove precipitated protein, and the absorbance at 670 nm (ε = 34,500 M^{-1} cm^{-1}) was recorded. A blank (in the absence of DesII) and a series of Na_2S standards (in 0.03% NaOH aqueous solution) were measured in parallel with the DesII sample. The sulfur content was determined by comparison to the standard curve derived from the Na_2S standards.

Reconstitution of the [4Fe-4S] Cluster of DesII in vitro. All procedures including the apoprotein preparation, reconstitution, reduction of the iron−sulfur cluster, and activity assay were performed anaerobically inside a glovebox. All solutions were deoxygenated prior to use by repeated freeze−vacuum−thaw cycles under argon, and all solids were deoxygenated prior to use by vacuum and argon cycles.

Step 1: Preparation of Apo-DesII. Protein-bound iron was removed by incubating the as-isolated DesII in the presence of 100 mM EDTA and 2 mM sodium dithionite in 100 mM Tris·HCl buffer, pH 8, for 1 h at room temperature. The solutions became colorless after 30 min of reduction with sodium dithionite. The resulting protein was loaded onto a Sephadex G-25 column (50 mL) pre-equilibrated and eluted with 100 mM Tris·HCl buffer, pH 8, and concentrated to approximately 8 mg/mL by ultrafiltration through a YM10 membrane (Amicon) (80% recovery).

Step 2: Reconstitution of [4Fe-4S] Center. The above apoprotein was incubated with a 10-fold molar excess of Na_2S and $Fe(NH_4)_2(SO_4)_2$ in 1 mL of 100 mM Tris·HCl buffer (pH 8, with 5 mM dithiothreitol [DTT]) for 6 h at room temperature. A deaerated solution of ethylenediaminetetraacetic acid (EDTA) (2 mM final concentration) was then added to the protein. The resulting solution was incubated for 30 min. Protein was loaded onto a 50 mL Sephadex G-25 column equilibrated and eluted with 100 mM Tris·HCl buffer, pH 8. Green-gray fractions were collected and concentrated to approximately 10 mg/mL by ultrafiltration through a YM10 membrane (Amicon) (75−80% recovery). This preparation was then analyzed for iron and sulfur content, and the iron−sulfur cluster was characterized by UV−vis and EPR spectroscopy.

Step 3: Reduction of [4Fe-4S] Center. Reduction of the [4Fe-4S] center of the reconstituted protein was monitored by UV−vis spectroscopy. The reducing agent was sodium dithionite, which was prepared freshly in 100 mM Tris·HCl buffer, pH 8, prior to the reduction procedure. The protein solution was reduced with a 6-fold molar excess of sodium dithionite over 1 h. Reduction was monitored by the decrease of the absorption at 420 nm.

EPR Spectroscopy. All samples were freshly prepared anaerobically inside a glovebox. Each EPR sample (500 μL) contained 2 mM dithionite, 1.5 mM SAM, and 200 μM reconstituted DesII in 100 mM Tris·HCl buffer (pH 8.0, including 1 mM DTT). The solution was mixed, transferred to an EPR tube, and frozen by immersing the tube in cold isopentane (approximately −140 °C) within 30 s after mixing. The dithionite was omitted in the control sample. These EPR samples were stored in liquid nitrogen until analysis. EPR spectra were recorded at liquid helium temperature on a Bruker EMX EPR spectrometer. A GFS600 transfer line and an ITC503 temperature controller were used to maintain the temperature. An Oxford ESR900 cryostat was used to accommodate the sample. Data analysis was conducted using WinEPR provided by Bruker.

DesII Activity Assay Using Dithionite as the Reducing Agent. The DesII protein was reduced with a 6-fold molar excess of sodium dithionite over 1 h. A typical activity assay contained 0.5 mM TDP-4-amino-4,6-dideoxy-D-glucose (**8**), 0.1 mM dithionite-reduced DesII, and 0.1 mM SAM in 1 mL of 100 mM Tris·HCl buffer (pH 8, including 2 mM DTT). The reaction mixture was incubated at 25 °C for 3 h, and stopped by filtration through a YM10 membrane (Amicon) to remove the enzyme. The reaction mixture was analyzed by HPLC on a Dionex CarboPac PA1 column (4 × 250 mm). A linear gradient from 20% to 35% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 40 min gave satisfactory separation between substrate **8** (with a retention time of 5.9 min) and the product, TDP-3-keto-4,6-dideoxy-D-glucose (**9**) (with a retention time of 28.2 min). The flow rate was 0.6 mL/min, and the detector was set at 267 nm. The product peak was collected and its identity was verified by high-resolution mass spectrometry analysis.

Coupled DesII and DesV Assay. The assay was carried out in 1 mL of 100 mM Tris·HCl buffer (pH 8, including 2 mM DTT) containing 0.5 mM TDP-4-amino-4,6-dideoxy-D-glucose (**8**), 0.1 mM dithionite-reduced DesII, and 0.1 mM SAM. After incubation at 25 °C for 3 h, 10 mM of L-glutamate, 0.8 mM of PLP, and 0.1 mM DesV enzyme were added to the assay mixture. The reaction was incubated at 25 °C for another 30 min and then was stopped by removing the enzymes by ultrafiltration through a YM10 membrane (Amicon). The reaction solution was analyzed by HPLC as described above. The new product, TDP-3-amino-3,4,6-trideoxy-D-glucose (**10**), which eluted at 3.7 min, was collected and subjected to high-resolution mass spectrometry verification.

DesII Activity Assay Using Flavodoxin and Flavodoxin Reductase as the Reducing System. A typical activity assay contained 0.5 mM TDP-4-amino-4,6-dideoxy-D-glucose (**8**), 0.1 mM reconstituted DesII, 0.1 mM SAM, 1 mM NADPH, 12 μM flavodoxin (FLD), and 6 μM flavodoxin reductase (FPR) in 1 mL of 100 mM Tris·HCl buffer (pH 8) with 2 mM DTT. The reaction mixture was incubated at 25 °C for 1 h and stopped by ultrafiltration through a YM10 membrane (Amicon) to remove the enzymes. The reaction mixture was analyzed by HPLC on a Dionex CarboPac

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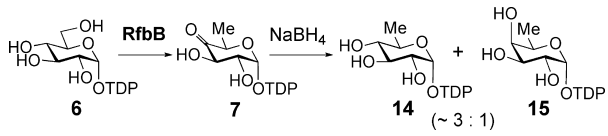
Scheme 2



PA1 column (4 × 250 mm) with a two-stage linear gradient: first from 2.5% to 10% of 1 M ammonium acetate, pH 7.0 versus ddH_2O over 20 min, followed by a second gradient from 10% to 30% 1 M ammonium acetate, pH 7.0 versus ddH_2O over 20 min (1 mL/min). The retention times are 18.3 and 37.2 min for **8** and the product, TDP-3-keto-4,6-dideoxy-D-glucose (**9**), respectively. The product peak was collected, and its identity was verified by high-resolution mass spectrometry analysis.

Determination of Kinetic Parameters for the DesII-Catalyzed Reaction. The steady-state kinetic parameters of DesII-catalyzed reaction were determined by the activity assay as described above[3a] using 3 μM dithionite-reduced DesII, 0.1 mM SAM, and varied amounts of TDP-4-amino-4,6-dideoxy-D-glucose (**8**, 3 μM to 1 mM) in 100 mM Tris·HCl buffer (pH 8.0). The reaction volume was 0.3 mL, but larger volumes (0.6 mL) were used for the lower substrate concentrations to facilitate HPLC analysis. The reaction was incubated at 25 °C, and aliquots were taken at appropriate time points from each sample and analyzed by HPLC. Since the initial substrate concentration in each sample was known, the percent conversion as determined by HPLC could be used to calculate the amount of product formed for each time point. The amounts of product formed for a given substrate concentration at different time points were plotted against time, and the initial rate was then plotted versus substrate concentration. The resulting data were fit to the Michaelis−Menten equation by nonlinear regression using Grafit 5 to determine the k_{cat} and K_M values.

Preparation of TDP-D-quinovose (14) and TDP-D-fucose (15). TDP-D-quinovose (**14**) and TDP-D-fucose (**15**) were prepared following a literature procedure[26] (Scheme 2). First, TDP-4-keto-6-deoxy-D-glucose (**7**) was prepared by incubating TDP-D-glucose (**6**, 46.2 mg, 8.2 mM) and RfbB (28 μM) in 10 mL of 50 mM potassium phosphate buffer, pH 7.5, at 37 °C for 1 h. The enzyme was removed by filtration through an Amicon ultrafiltration unit (YM10 membrane). To the filtrate was added $NaBH_4$ (10 μM), and the reaction was allowed to proceed at room temperature for 1 h. The reaction mixture was desalted on a Sephadex G-10 column equilibrated with water. After lyophilization, the reaction mixture was analyzed by HPLC using a Dionex CarboPac PA1 column (4 mm × 250 mm). The flow rate was 1.0 mL/min, and the detector was set at 267 nm. A linear gradient from 2.5% to 10% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min, followed by a second linear gradient from 10% to 30% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min gave satisfactory separation between TDP-D-quinovose (**14**) and TDP-D-fucose (**15**) (the ratio of **14**:**15** = 3:1). The retention times are 18.9 and 13.5 min for TDP-D-quinovose (**14**) and TDP-D-fucose (**15**), respectively. Both compounds were collected, lyophilized to dryness, and confirmed by NMR spectroscopy.

Activity Assay Using TDP-D-quinovose (14) as the Substrate. To determine whether TDP-D-quinovose (**14**) can be recognized and processed by DesII, 0.5 mM of **14** was incubated with 0.1 mM dithionite-reduced DesII, 0.1 mM SAM in 1 mL of 100 mM Tris·HCl buffer (pH 8) containing 2 mM DTT. The reaction mixture was incubated at 25 °C for 3 h, and stopped by ultrafiltration through a YM10 membrane to remove the enzymes. The reaction mixture was analyzed by HPLC on a CarboPac PA1 anion-exchange column (4 × 250 mm). The flow rate was 1.0 mL/min, and the detector was set at 267 nm. A linear gradient from 2.5% to 15% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min, followed by a second linear gradient from 15% to 20% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min was used to separate the substrate **14** (with a retention time of 18.9 min) and the resulting product (with a retention time of 27.8 min).

Characterization of the DesII and DesII/DesV Products from TDP-D-quinovose (14). A 4.3 mL solution of 5.25 mM TDP-D-quinovose (**14**) containing an excess of SAM (7.8 mM) and sodium dithionite (10 mM) was incubated anaerobically in the 2 mM DTT, 100 mM Tris·HCl buffer, pH 8.0, with 2 μM DesII at room temperature. Following a 7 h incubation the reaction was judged >95% complete by analytical HPLC as previously described. At this point, the reaction was divided into two equal fractions.

The first fraction was filtered to remove protein, and the new product was isolated using a Dionex CarboPac PA1 semiprep column with a flow rate of 3 mL/min and a two-stage linear gradient: 100% ddH_2O to 75% 300 mM NH_4HCO_3 in 20 min followed by 75 to 100% 300 mM NH_4HCO_3 in 20 min. The new product was collected at 24.8 min, lyophilized to dryness, and characterized by electrospray ionization (ESI) mass spectroscopy.

The second fraction was made 1 mM in PLP, 25 mM in glutamate, and approximately 2 mg DesV was added. Following a 30 min incubation at room temperature, the reaction was judged >95% complete by analytical HPLC. The mixture was then filtered to remove protein, and the DesII/DesV product was isolated using the Dionex CarboPac PA1 semiprep HPLC methodology described above. The product eluted at 28 min and was confirmed by reinjections on the analytical HPLC. The isolated material was lyophilized to dryness and characterized by ESI mass spectrometry, 1D and 2D ^{1}H NMR spectroscopy.

Activity Assay Using TDP-D-fucose (15) as the Substrate. Assays were carried out in 1 mL of 100 mM Tris·HCl buffer (pH 8, with 2 mM DTT) in the presence of 0.5 mM TDP-D-fucose (**15**), 0.1 mM dithionite-reduced DesII, and 0.1 mM SAM. The reaction mixture was incubated at 25 °C for 24 h and was stopped by removing the enzymes by ultrafiltration through a YM10 membrane. Analysis of the reaction mixture by HPLC followed the same protocol as described above for the DesII activity assay using TDP-D-quinovose (**14**).

Activity Assay Using TDP-3-amino-3,6-dideoxy-D-glucose (17) as the Substrate. TDP-3-amino-3,6-dideoxy-D-glucose (**17**), which is the product of the TylB reaction,[27] was prepared according to a published procedure[28] (Scheme 3). The assay mixture contained 0.5 mM of **17**, 50 μM dithionite-reduced DesII, and 50 μM SAM in 1 mL of 100 mM Tris·HCl buffer (pH 8 with 2 mM DTT). The reaction was incubated at 25 °C for 3 h and quenched by removing the enzyme (YM10 membrane, Amicon). The reaction was subjected to HPLC analysis as described above using a CarboPac PA1 column and a two-stage linear gradient: first from 2.5% to 10% of 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min, followed by a second gradient from 10% to 30% 1 M ammonium acetate, pH 7.0, versus ddH_2O over 20 min (1 mL/min). The retention times are 18.1 and 37.2 min for **17** and the resulting product, respectively. The product was verified via HPLC co-injection with the DesII products from both TDP-4-amino-4,6-dideoxy-D-glucose (**8**) and TDP-D-quinovose (**14**).

Coupled DesII and DesV Assay Using TDP-3-amino-3,6-dideoxy-D-glucose (17) as the Substrate. After incubation of the DesII assay mixture (0.5 mM of **17**, 0.1 mM dithionite-reduced DesII, and 0.1 mM SAM in 1 mL of 100 mM Tris·HCl buffer, pH 8, with 2 mM DTT) at 25 °C for 3 h, 10 mM of L-glutamate, 0.8 mM of PLP, and 0.1 mM DesV enzyme were added to the assay

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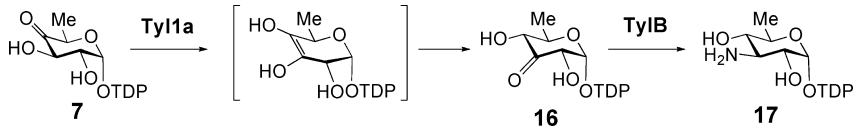
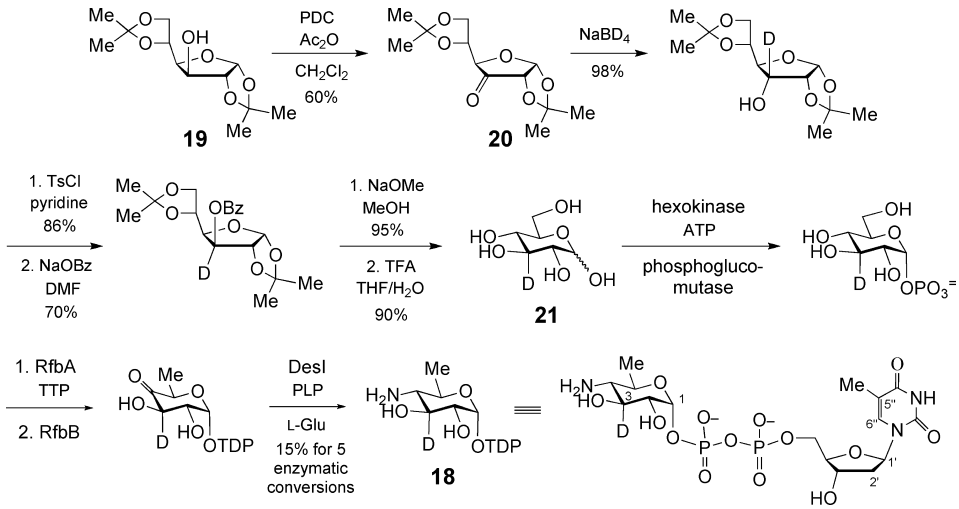
Scheme 3



Scheme 4



mixture, and the reaction was incubated at 25 °C for 30 min. The reaction was stopped by removing the enzymes through a YM10 membrane (Amicon ultrafiltration). Subsequent HPLC analysis was the same as described above.

Synthesis of TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (18). The overall strategy for making TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**) is depicted in Scheme 4. The synthesis of [3-^{2}H]-α-D-glucose (**21**), starting from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**19**), was performed according to previously reported procedures.[29] The conversion of **21** to TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**) followed the same procedure used to prepare TDP-4-amino-4,6-dideoxy-D-glucose (**8**) as described before.[5] The overall yield of the five enzymatic conversions was 15%.

Deuterium Incorporation into *S*-Adenosyl-L-methionine (SAM). The incorporation of deuterium from TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**) into SAM was determined following a literature procedure[14b,16a,30] with some modifications. Briefly, the assay contained 500 μM **18**, 100 μM SAM, and 100 μM dithionite-reduced DesII in 1 mL of 100 mM Tris·HCl buffer (pH 8.0, with 1 mM DTT). The reaction was initiated by the addition of SAM and reconstituted DesII after incubation of the other components of the assay mixture at 25 °C for 5 min. After incubation at 25 °C for 3 h, the reaction was quenched by ultrafiltration through a YM10 membrane (Amicon) to remove the enzyme. The reaction mixture was analyzed by HPLC on an analytical Microsorb-MV C_{18} column (4.5 × 250 mm, 5 μm, Varian) with the detector set at 260 nm. The column was pre-equilibrated with 0.1% trifluoroacetic acid (TFA) in ddH_2O and subsequently eluted with a 20-min linear gradient of 0 to 45% acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min. *S*-adenosylmethionine (SAM), which was eluted at 5.1 min, was collected, concentrated, and analyzed by ESI mass spectrometry.

Deuterium Incorporation into 5′-Deoxyadenosine. A similar procedure was followed to determine the incorporation of the deuterium atom from TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**) into 5′-deoxyadenosine. The assay contained 100 μM **18**, 700 μM SAM, and 50 μM dithionite-reduced DesII in 100 mM Tris·HCl buffer (pH 8.0, with 1 mM DTT). The final volume was 540 μL. The reaction was initiated by the addition of SAM and reconstituted DesII. After incubation at 25 °C for 3 h, the reaction was quenched by ultrafiltration through a YM10 membrane (Amicon) to remove the enzyme. The reaction mixture was analyzed by HPLC on an analytical Microsorb-MV C_{18} column as described above. 5′-Deoxyadenosine, which was eluted at 10.6 min, was collected, concentrated, and subjected to analysis by ESI mass spectrometry.

Results

Purification and Characterization of DesII Protein. The *C*-terminal His_6-tagged DesII was purified aerobically to near homogeneity using a Ni-NTA column followed by FPLC on a MonoQ column. The desired fractions were selected based on SDS-PAGE results (Figure 1a). A 6-L culture yielded 15 mg of homogeneous DesII protein, which is slightly brown in color. The *N*-terminal peptide sequencing analysis confirmed that the first 10 amino acid residues of the purified DesII are identical to those of the translated DesII sequence except for the initiating methionine residue, which must have been removed during protein synthesis in *E. coli.* A molecular mass of 48 kDa for DesII as estimated by gel filtration chromatography suggests that His-tag-DesII, which has a calculated molecular mass of 54,265 Da (including the His_6 tag), is a monomer in solution. It is not clear that the observed monomeric state is due to the presence of the His-tag.

UV−vis Spectral Analysis of Purified DesII. The UV−vis spectrum of the purified protein exhibits a broad shoulder between 400 and 500 nm (Figure 1b), which is characteristic for iron−sulfur cluster containing proteins, such as the anaerobic *E. coli* ribonucleotide reductase,[31] spore photoproduct lyase,[15d] BtrN in the biosynthesis of butirosin,[16a] and ThiC in thiamine

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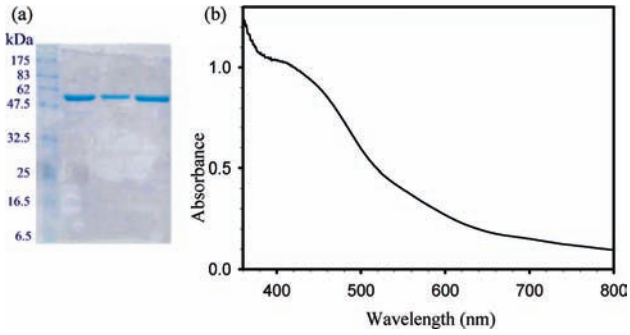

Figure 1. (a) SDS-PAGE of purified DesII isolated from *E. coli* BL21 Star (DE3): lane 1, the molecular mass standards: MBP-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin A (25 kDa), lysozyme (16.5 kDa), and aprotinin (6.5 kDa), lanes 2−4, fractions of DesII purified using a Ni-NTA column followed by FPLC on a MonoQ HR (16/10) column. (b) UV−vis absorption spectrum of aerobically purified DesII in 50 mM Tris·HCl buffer, pH 7.5. The protein concentration is 0.35 mM.

pyrimidine biosynthesis.[17a] The extinction coefficients determined are $\varepsilon_{420} = 4000\ M^{-1}\cdot cm^{-1}$ for DesII and $\varepsilon_{420} = 3700\ M^{-1}\cdot cm^{-1}$ for the anaerobic *E. coli* ribonucleotide reductase. Since the [4Fe-4S] cluster is oxygen sensitive and is known to degrade to $[3Fe\text{-}4S]^{1+}$ and/or $[2Fe\text{-}2S]^{2+}$ clusters upon exposure to oxygen,[32] the UV−vis spectrum of the as-isolated DesII is not likely due to an intact $[4Fe\text{-}4S]^{2+}$ cluster.

Reconstitution and Reduction of the Iron Sulfur Center of DesII. The purified His-tag-DesII was anaerobically converted to the colorless apoprotein by incubating with EDTA in the presence of 2 mM sodium dithionite. Subsequent reconstitution of the iron−sulfur cluster was achieved by incubating the apo-DesII anaerobically with an excess of ferrous iron and sodium sulfide in the presence of DTT for 6 h at 25 °C. After reconstitution, the color of DesII changed to green-gray. The reconstituted DesII exhibits featureless absorption across the entire UV−vis range with a broad absorption hump at 420 nm ($\varepsilon_{420} = 9200\ M^{-1}\cdot cm^{-1}$) (Figure 2), which is typical for a $[4Fe\text{-}4S]^{2+}$ cluster. Similar spectra have also been observed for other $[4Fe\text{-}4S]^{2+}$ containing enzymes.[7a,32] The catalytically active form, $[4Fe\text{-}4S]^{1+}$, of the iron−sulfur cluster was generated by anaerobic reduction of the reconstituted DesII with sodium dithionite. The reduction was monitored by following the decrease in absorbance at 420 nm, which is associated with the reduction of a $[4Fe\text{-}4S]^{2+}$ cluster to the $[4Fe\text{-}4S]^{1+}$ state[33] (Figure 2).

The Iron and Sulfur Content in DesII. The iron titration results showed that the as-isolated His-tag-DesII contained approximately 0.6 iron equivalents per monomer of protein, which clearly indicated iron depletion during aerobic purification. In contrast, the fully reconstituted His-tag-DesII contained approximately 4 iron equivalents per monomer. The labile sulfur content of DesII was determined by quantitating the released sulfide after protein denaturation using guanidinium hydrochloride in the presence of dithiothreitol.[25] The released inorganic sulfide was first absorbed onto zinc as zinc sulfide, and then freed by acid treatment to react with *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride to give methylene blue, which could be detected at 670 nm. It was determined that the as-isolated DesII contained approximately 0.5 sulfur equivalents per monomer of protein, but the fully reconstituted DesII contained nearly 4 sulfur equivalents per monomer. These data are consistent with reconstituted DesII containing a [4Fe-4S] cluster as suggested by its UV−vis spectrum (Figure 2).

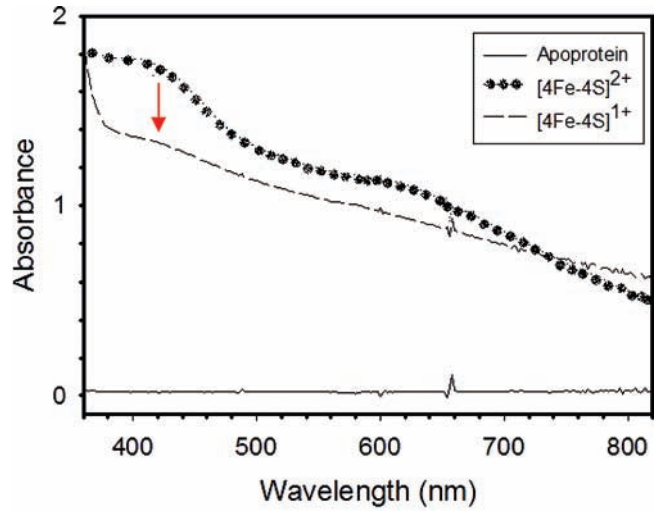

Figure 2. UV−vis absorption spectra of apo-DesII (solid line), reconstituted DesII before (dotted line) and after (dashed line) reduction with 1.14 mM sodium dithionite. The protein concentration is 0.19 mM in 100 mM Tris·HCl buffer, pH 8.0.

Characterization of the $[3Fe\text{-}4S]^{1+}$ Cluster in the Aerobically Purified DesII by EPR Spectroscopy. The aerobically purified DesII exhibits a strong EPR signal at 10 K with a *g* value centered at 2.010 (Figure 3a). This EPR signal is characteristic for a $[3Fe\text{-}4S]^{1+}$ cluster (spin state (S) = 1/2), which has been observed in several aerobically purified radical SAM enzymes. For example, the aerobically purified spore photoproduct lyase exhibits a similar EPR signal arising from a $[3Fe\text{-}4S]^{1+}$ cluster with a *g* value centered at 2.02.[15d] Likewise, the anaerobic ribonucleotide reductase activase exhibits an EPR signal for a $[3Fe\text{-}4S]^{1+}$ cluster with a *g* value centered at 2.017 upon exposure to air.[34] The $[3Fe\text{-}4S]^{1+}$ cluster is thought to result from air oxidation of the $[4Fe\text{-}4S]^{3+}$ cluster and renders the parent enzymes catalytically inactive. However, the majority of aerobically purified DesII appears to be in the apoprotein form with less than 10−20% of the monomers containing residual iron as part of a $[3Fe\text{-}4S]^{1+}$ cluster. This is based on the observation of approximately 0.6 iron equivalents and 0.5 labile sulfur equivalents per protein monomer. Interestingly, no EPR signal was observed for the as-isolated DesII protein in the presence of dithionite recorded under the same conditions. This observation suggests that its iron−sulfur cluster is in the EPR-silent $[3Fe\text{-}4S]^{0}$ form.

Characterization of the $[4Fe\text{-}4S]^{1+}$ Cluster in the Anaerobically Reconstituted DesII by EPR Spectroscopy. The reconstituted DesII, reduced by dithionite in the presence of SAM, exhibits a rhombic EPR signal with *g* values at 2.01, 1.96, and 1.87 ($g_{av} = 1.95$) (Figure 3b), which is similar to that of the $[4Fe\text{-}4S]^{1+}$ cluster in the anaerobic ribonucleotide reductase activase (ARR activase) when reduced by dithionite in the presence of SAM with *g* values at 2.00, 1.92, and 1.86 (g_{av} =

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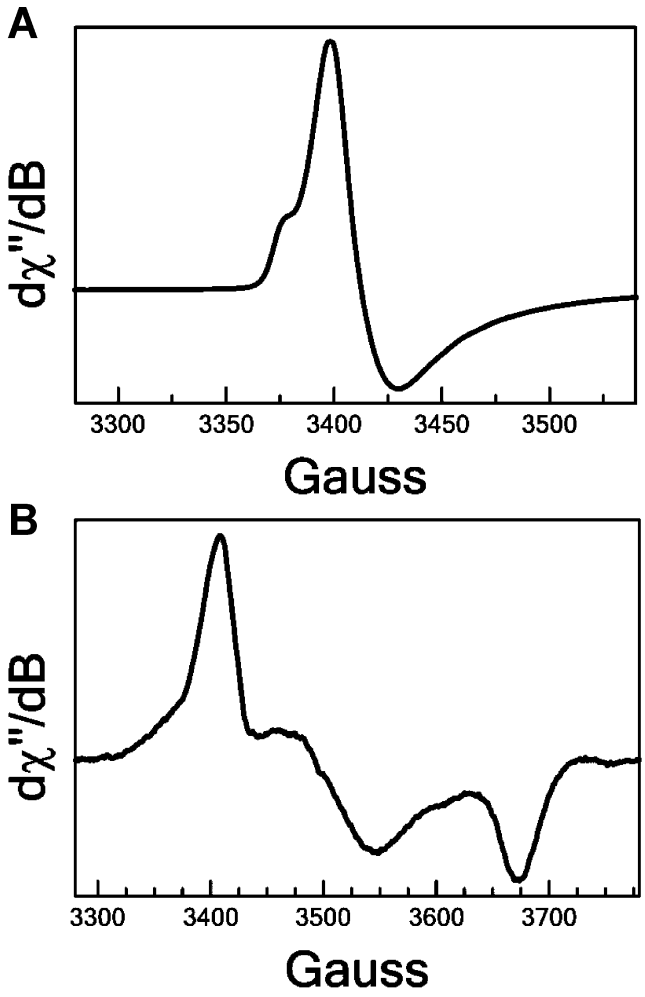

Figure 3. (a) EPR spectrum of as-purified DesII containing a $[3Fe\text{-}4S]^{1+}$ cluster with a g value centered at 2.010. The protein concentration is 0.2 mM in 100 mM Tris·HCl buffer, pH 8.0. The spectrum was recoded at 10 K with a modulation amplitude of 10 G, a microwave power of 1 mW, and a microwave frequency of 9.60 GHz. (b) EPR spectrum of reconstituted DesII containing a $[4Fe\text{-}4S]^{1+}$ cluster with g values at 2.01, 1.96, and 1.87 ($g_{av} = 1.95$). The protein concentration is 0.2 mM in 100 mM Tris·HCl buffer, pH 8.0. The spectrum was recoded at 10 K with a modulation amplitude of 10 G, a microwave power of 10 mW, and a microwave frequency of 9.60 GHz.

1.93).[12c] Unlike ARR activase whose EPR signal is more axial than rhombic in the absence of SAM, DesII is EPR silent in the absence of SAM. It is likely that the $[4Fe\text{-}4S]^{2+/1+}$ cluster of DesII is unstable without SAM binding to its open iron site and can easily lose one iron to become a $[3Fe\text{-}4S]^{1+}$ cluster. The one-electron reduced form of the latter cluster, $[3Fe\text{-}4S]^{0}$, is EPR-silent. Another possibility is that the $[4Fe\text{-}4S]^{2+}$ cluster of DesII is more difficult to reduce in the absence of SAM. Precedence for such a phenomenon has been set by lysine 2,3-aminomutase, where SAM binding elevates the reduction potential of its $[4Fe\text{-}4S]^{2+}$ cluster by 50−86 mV.[35] No EPR signal was observed in the reconstituted DesII protein in the absence of dithionite recorded under the same conditions, suggesting the iron−sulfur cluster is in its EPR-silent $[4Fe\text{-}4S]^{2+}$ state.

Activity Assay of the DesII-Catalyzed Reaction. When the fully reconstituted and dithionite reduced DesII was incubated with TDP-4-amino-4,6-dideoxy-D-glucose (**8**) in the presence of SAM, a product was formed. The retention time of 28.2 min for this product (**9**) under the HPLC conditions is identical to that of the chemoenzymatically synthesized standard. The high-resolution fast atom bombardment (FAB) MS data (calcd for $C_{16}H_{24}N_2O_{14}P_2$ $[M\text{-}H]^-$ 529.0625, found 529.0620) is consistent with the composition of the assigned product (**9**). To gain additional evidence for the formation of **9** in the DesII reaction, the above reaction mixture was incubated with DesV, PLP and L-glutamate to convert **9** to TDP-3-amino-3,4,6-trideoxy-D-glucose (**10**). A new HPLC peak with a retention time of 3.7 min was observed. This new product coeluted with a standard of **10** (Figure 4a). The identity of **10** was further validated by high-resolution FAB-MS analysis (calcd for $C_{16}H_{27}N_3O_{13}P_2$ $[M\text{-}H]^-$ 530.0941, found 530.0949). These results unequivocally established that the product of DesII reaction is TDP-3-keto-4,6-dideoxy-D-glucose (**9**).

Determination of the Kinetic Parameters for DesII-Catalyzed Reaction. The discontinuous HPLC assay was used to determine the steady state kinetic parameters for His-tag-DesII-catalyzed conversion of **8** to **9**. The reconstituted enzyme (3 μM) was activated by dithionite reduction of its $[4Fe\text{-}4S]^{2+}$ center to $[4Fe\text{-}4S]^{+}$. The plot of v_0 (the initial velocity) versus [S] (3 μM to 1 mM) (Figure 5) was fit to the Michaelis−Menten equation by nonlinear regression to yield a k_{cat} of 1.0 ± 0.1 min^{-1} and a K_m of 50 ± 2 μM for **8**. These parameters correspond to the TDP-4-amino-4,6-dideoxy-D-glucose substrate in the presence of a constant concentration of 0.1 mM SAM.

Purification and Characterization of *E. coli* Flavodoxin (FLD) and Flavodoxin Reductase (FPR). The *C*-terminal His_6-tagged flavodoxin (FLD) and flavodoxin reductase (FPR) were purified to near homogeneity using Ni-NTA affinity chromatography. A 6-L culture yielded 90−100 mg of the desired proteins. Judging by SDS-PAGE, the molecular masses of FLD and FPR were estimated to be 22 kDa and 29 kDa, respectively, which correlates well with the corresponding calculated molecular mass of 22,765 Da and 29,765 Da (including the His_6 tag). The UV−vis spectrum of the purified FPR exhibits peaks at 360 and 460 nm as well as a shoulder near 500 nm, revealing the presence of a flavin mononucleotide (FMN) cofactor. The observed UV−vis spectrum of FPR, which exhibits absorption maxima at 400 and 465 nm, and a shoulder at 480 nm, is characteristic for a flavin adenine dinucleotide (FAD) in its active-site.

Reduction of DesII Mediated by Flavodoxin (FLD) and Flavodoxin Reductase (FPR). Similar to the results of using dithionite-reduced DesII in the assay, anaerobic incubation of substrate (**8**), the reconstituted DesII, SAM, NADPH, flavodoxin, and flavodoxin reductase also led to the production of TDP-3-keto-4,6-dideoxy-D-glucose (**9**). In this experiment, the $[4Fe\text{-}4S]^{2+}$ of DesII is reduced to $[4Fe\text{-}4S]^{1+}$ by stepwise electron transfer from NADPH through flavodoxin and flavodoxin reductase. Accordingly, a system consisting of flavodoxin/flavodoxin reductase-NADPH or its equivalent from the cellular pool may serve as the *in vivo* reducing system for the DesII-catalyzed reaction.

Characterization of TDP-D-quinovose (14) and TDP-D-fucose (15). These two compounds were synthesized from TDP-4-keto-6-deoxy-D-glucose (**7**) by reducing the C-4 keto group using $NaBH_4$ (Scheme 2). The yields of **14** and **15** were 40% and 13%, respectively. The spectral data for TDP-D-quinovose (**14**): ^1H NMR (300 MHz, D_2O) δ 0.80 (3H, d, J = 5.7 Hz, 5-Me), 1.52 (3H, s, 5″-Me), 1.98−2.08 (2H, m, 2′-Hs), 2.84

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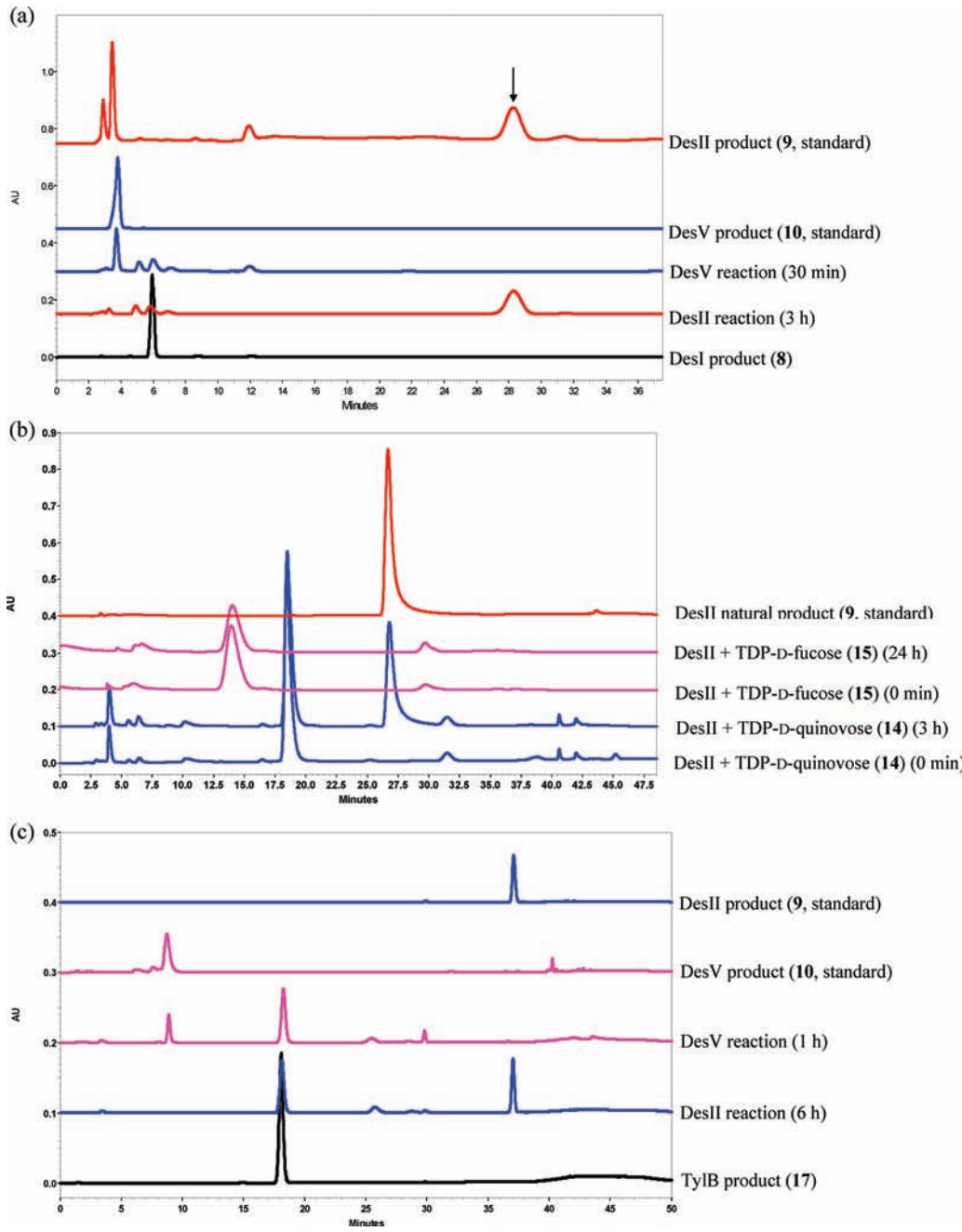


Figure 4. (a) HPLC traces demonstrating DesII activity using dithionite as the reducing agent. (b) HPLC traces demonstrating DesII substrate specificity using TDP-D-quinovose (**14**) and TDP-D-fucose (**15**) in the incubation. (c) HPLC traces demonstrating DesII substrate specificity using TylB product (**17**) in the incubation. See Experimental Procedures for details.

(1H, dd, $J_{3,4} = J_{4,5} = 9.9$ Hz, 4-H), 3.22−3.31 (1H, m, 2-H), 3.28 (1H, dd, $J_{2,3} = 9.3$, $J_{3,4} = 9.9$ Hz, 3-H), 3.53 (1H, m, 5-H), 3.75−3.88 (3H, m, 4′-H, 5′-Hs), 4.18−4.22 (1H, m, 3′-H), 5.15 (1H, dd, $J_{1,p} = 6.8$, $J_{1,2} = 3.1$ Hz, 1-H), 5.94 (1H, t, $J = 6.7$ Hz, 1′-H), 7.29 (1H, s, 6″-H); ^{31}P NMR (121 MHz, D_2O) δ −11.79 (d, $J = 18.3$ Hz), −10.34 (d, $J = 18.3$ Hz). The spectral data for TDP-D-fucose (**15**): ^{1}H NMR (300 MHz, D_2O) δ 1.21 (3H, d, $J = 6.6$ Hz, 5-Me), 1.93 (3H, s, 5”-Me), 2.36 (2H, m, 2′-Hs), 3.74 (1H, dt, $J = 10.5$, 3.2 Hz, 2-H), 3.81 (1H, $J = 3.2$ Hz, 4-H), 3.91 (1H, dd, $J = 10.5$, 3.2 Hz, 3-H), 4.17 (3H, m, 4′-H and 5′-Hs), 4.28 (1H, $J = 6.6$ Hz, 5-H), 4.62 (1H, m, 3′-H), 5.56 (1H, dd, $J = 6.8$, 3.6 Hz, 1-H), 6.34 (1H, t, $J = 7.0$ Hz, 1′-H), 7.74 (1H, s, 6″-H); ^{31}P NMR (121 MHz, D_2O) δ −11.75 (d, $J = 18.3$ Hz), −10.39 (d, $J = 18.3$ Hz). These spectral data are in agreement with those reported in the literature.[26]

Testing the Competence of TDP-D-quinovose (14), TDP-D-fucose (15) and TDP-3-amino-3,6-dideoxy-D-glucose (17) as Substrates for DesII. Compounds **14**, **15**, and **17** were used to assess the ability of DesII to accept different substrate analogues (HPLC traces in b and c of Figure 4). Anaerobic incubation of TDP-D-quinovose (**14**) with SAM and the reconstituted and reduced DesII led to the formation of TDP-3-keto-6-deoxy-D-glucose (**16**). Assignment is based on ESI MS. In the negative ion mode, peaks identified at 544.93 and 567.13 *m/z* are consistent with the $(M - H^+)^-$ and $(MNa^+ - 2H^+)^-$ ions of

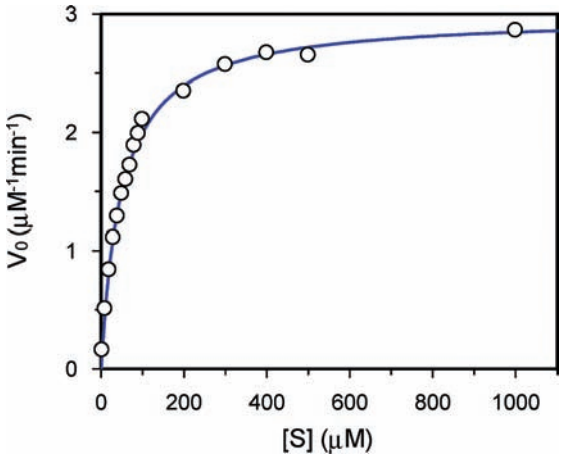


Figure 5. Plot of v_o versus [S] (**8**) determined by an HPLC assay from which the steady state kinetic constants for the DesII-catalyzed reaction were determined. See Experimental Procedures for details.

16, which has a neutral monoisotopic mass of 546. In the positive ion mode, peaks at 568.87, 590.80, and 612.87 *m/z* are consistent with the MNa^+, MNa_2^+, and MNa_3^+ ions of **16**. In both spectra, no peaks were observed corresponding to ions of either **9**, neutral mass 530, or its hydrate, neutral mass 548. Consistent with this assignment, the DesII product from **14** is indistinguishable by analytical HPLC from the Tyl1a product (**16**, see Scheme 3) where co-injection yielded an inseparable composite peak but was distinctly separable on co-injection with **9**. Unfortunately, product **16** itself is relatively unstable, and the ^{1}H NMR spectra obtained from the isolated material contained a number of contaminating species, making peak assignments difficult.

Subsequent reaction of DesV with the DesII product (**16**) from TDP-D-quinovose yielded TDP-3-amino-3,6-dideoxy-D-glucose (**17**), consistent with the known ability of DesV to replace TylB *in vivo*.[21b] Positive ion ESI MS of the isolated material demonstrated peaks at 547.93, 569.80, and 591.67 *m/z* consistent with the MH^+, MNa^+, and MNa_2^+ ions of **17**, which has a neutral monoisotopic mass of 547. No peaks corresponding to ions of **10** where observed (neutral mass 531). In the negative ion ESI MS a signal at 546.13 is present, consistent with the $(M - H^+)^-$ anion of **17**, while no peaks corresponding to ions of **10** were detected.

In contrast to the DesII product from **14**, the coupled DesII/DesV product (**17**) is significantly more stable such that a much cleaner ^{1}H NMR spectrum was obtained. This spectrum is consistent with the assignment of structure **17**: ^{1}H NMR (600 MHz, D_2O) δ 1.18 (3H, d, $J = 6.3$ Hz, 5-Me), 1.82 (3H, s, 5″-Me), 2.26 (2H, m, 2′-Hs), 3.25 (1H, dd, $J_{4,5} = J_{4,3} = 9.6$ Hz, 4-H), 3.33 (1H, dd, $J_{3,4} = J_{3,2} = 10.5$ Hz, 3-H), 3.68 (1H, ddd, $J_{2,1} = J_{2,P} = 3.6$ Hz, $J_{2,3} = 10.2$ Hz, 2-H), 3.91 (1H, dq, $J_{5,4} = 9.6$ Hz, $J_{5,6} = 6.6$ Hz, 5-H), 4.07 (3H, m, 4′-H and 5′-Hs), 4.51 (1H, m, 3′-H), 5.48 (1H, dd, $J_{1,2} = 3.6$ Hz, $J_{1,P} = 7.2$ Hz, 1-H), 6.23 (1H, dd, $J_{1',2'R} = J_{1',2'S} = 6.6$ Hz, 1′-H), 7.62 (1H, s, 6″-H). Cross-coupling assignments were confirmed by ^{1}H COSY NMR. On analytical HPLC, co-injection of the DesII/DesV product from **14** is indistinguishable from the TylB product (**17**) but is separable from **10**.

Interestingly, TDP-3-amino-3,6-dideoxy-D-glucose (**17**) is itself a substrate for DesII (Figure 4c). The product was identified as TDP-3-keto-6-deoxy-D-glucose (**16**) as it is indistinguishable from the DesII product from **14**, although clearly distinct from **9** upon corresponding HPLC co-injections. When TDP-D-fucose (**15**) was tested, no turnover was observed (Figure 4b).

Synthesis of TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (18). Compound **18** was prepared from 1,2:5,6-di-*O*-isopropylidene-β-D-glucofuranose (**19**) as shown in Scheme 4. The deuterium was incorporated into 1,2:5,6-di-*O*-isopropylidene-β-D-*ribo*-hexofuranos-3-ulose (**20**) by the reduction of the C3-keto group using $NaBD_4$. Subsequent inversion of the C-3 configuration followed by acid hydrolysis gave [3-^{2}H]-glucose (**21**). The conversion of **21** to **18** was carried out enzymatically, where hexokinase, phosphoglucomutase, RfbA, RfbB, and DesI were employed to afford TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**).[3a] Spectral data of **18**: ^{1}H NMR (500 MHz, D_2O) δ 0.80 (3H, d, $J = 5.7$ Hz, 5-Me), 1.52 (3H, s, 5′-Me), 1.98−2.03 (2H, m, 2′-Hs), 2.84 (1H, d, $J = 9.9$ Hz, 4-H), 3.28 (1H, d, $J = 3.6$ Hz, 2-H), 3.53 (1H, m, 5-H), 3.75−3.88 (3H, m, 4′-H, 5′-Hs), 4.18−4.22 (1H, m, 3′-H), 5.48 (1H, dd, $J = 6.8$, 3.1 Hz, 1-H), 5.94 (1H, t, $J = 6.7$ Hz, 1′-H), 7.29 (1H, s, 6′-H); ^{13}C NMR (125 MHz, D_2O) δ 12.8, 17.6, 39.7, 55.9, 66.6 (d, $J = 6.0$ Hz), 70.1 (t, $J = 20$ Hz), 70.6 (d, $J = 8.4$ Hz), 72.1, 73.3, 86.2, 86.4 (d, $J = 9.2$ Hz), 95.6 (d, $J = 6.1$ Hz), 112.8, 138.5, 153.0, 167.9; ^{31}P NMR (202 MHz, D_2O) δ −13.5 (d, $J = 20.7$ Hz), −11.8 (d, $J = 20.7$ Hz); ESI-HRMS calcd for $C_{16}H_{25}DN_3O_{14}P_2$ $[M - H]^-$ 547.0958, found 547.0951.

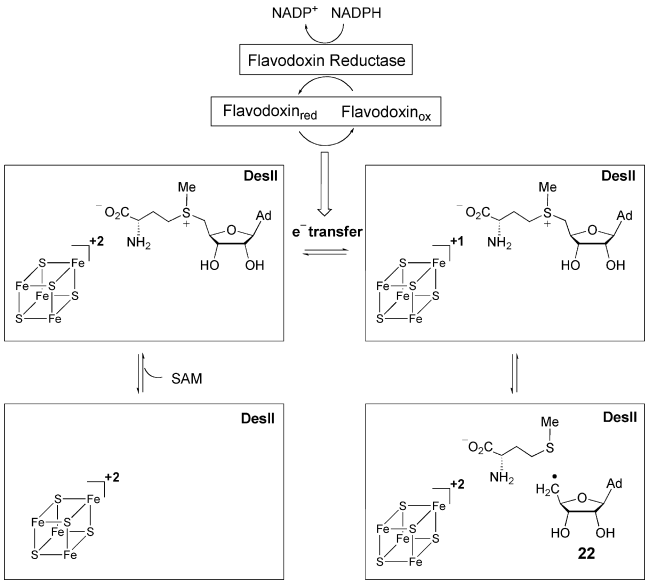
Deuterium Incorporation into *S*-Adenosyl-L-methionine (SAM) from TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (18). In a similar manner, the reaction mixture obtained after anaerobic incubation of the deuterium-labeled substrate (**18**), was analyzed by HPLC on an analytical C_{18} column. SAM, which was eluted at 5.1 min, was collected, concentrated, and subjected to ESI-MS analysis (ESI-HRMS calcd for $C_{15}H_{21}{}^2H_2N_6O_5S$ $[M + H]^+$ 401.1571, found 401.1562). A control using the nonlabeled substrate was also carried out in parallel (ESI-MS analysis of the isolated SAM: ESI-HRMS calcd for $C_{15}H_{23}N_6O_5S$ $[M + H]^+$ 399.1450, found 399.1452). The increase of two mass units of SAM derived from the labeled sample clearly indicated the incorporation of two deuterium atoms.

Deuterium Incorporation into 5′-Deoxyadenosine from TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (18). After anaerobic incubation of the deuterium-labeled substrate (**18**), the reaction mixture was analyzed by HPLC on an analytical C_{18} column. The 5′-deoxyadenosine, which was eluted at 10.6 min, was collected, concentrated, and subjected to ESI-MS analysis (ESI-HRMS calcd for $C_{10}H_{13}{}^2HN_5O_3$ $[M + H]^+$ 253.1152, found 253.1134). A control using the nonlabeled substrate was carried out in parallel (ESI-MS analysis of the isolated 5′-deoxyadenosine: ESI-HRMS calcd for $C_{10}H_{14}N_5O_3$ $[M + H]^+$ 252.1132, found 252.1091). The increase of one mass unit of 5′-deoxyadenosine derived from the labeled sample revealed the incorporation of one deuterium atom.

Discussion

At the time when the function of DesII from *S. venezuelae* was established in 2005,[3a] DesII was one of the first radical SAM enzymes involved in secondary metabolite biosynthesis to be characterized at the enzymatic level. Previous biochemical studies had shown that DesII together with DesI carries out the C-4 deoxygenation of TDP-4-keto-6-deoxy-D-glucose (**7**) to give TDP-3-keto-4,6-dideoxy-D-glucose (**9**) as the product. The function of DesII is to convert the TDP-4-amino-4,6-dideoxy-D-glucose (**8**) intermediate generated by DesI to the final product **9**.

Scheme 5



Although chemical reductants such as dithionite and 5-deazaflavin are commonly used to reduce the [4Fe-4S]$^{2+}$ cluster in enzymes, flavodoxin, flavodoxin reductase, and NADPH have been demonstrated to be an effective reducing system for several radical SAM enzymes.[9] The reducing equivalents are transferred from NADPH to the [4Fe-4S]$^{2+}$ cluster by relay through flavodoxin and flavodoxin reductase (Scheme 5). Not surprisingly, similar results were also noted for DesII catalysis. The *in vivo* effectiveness of this reducing system for DesII activity has already been demonstrated by studies of TDP-D-desosamine biosynthesis in a 6-deoxyerythromycin D-producing recombinant *E. coli* strain, where coexpression of flavodoxin and flavodoxin reductase from *E. coli* increased DesI/DesII activity.[36]

A k_{cat} of 1.0 ± 0.1 min^{-1} and a K_m of 50 ± 2 μM with respect to the sugar substrate **8** were determined for the DesII-catalyzed reaction with SAM constant at 0.1 mM. Kinetic parameters are also known for lysine 2,3-aminomutase,[37] pyruvate formate-lyase activase,[38] anaerobic ribonucleotide reductase activase,[39] biotin synthase,[40] lipoyl synthase,[14a] and BtrN,[16a] with the k_{cat} values ranging from 27 s^{-1} (lysine 2,3-aminomutase) to 0.175 min^{-1} (lipoyl synthase), and the K_m values ranging from 1.4 μM (pyruvate formate lyase-activase) to 8.0 mM (lysine 2,3-aminomutase). Clearly, radical SAM enzymes demonstrate a large range of turnover rates, and the reaction catalyzed by DesII appears to be more sluggish than many others. However, it cannot be ruled out that the relatively low rate of DesII turnover may be due to the presence of the *C*-terminal His_6 tag used in this study.

Two possible mechanisms have been proposed for the DesII reaction.[3a] As depicted in Scheme 6, generation of a 5′-deoxyadenosyl radical (**22**) is expected to be the first part of the reaction facilitated by the reduced [4Fe-4S]$^{1+}$ cluster as found in other radical SAM-dependent enzymes.[6] The chemical conversion is triggered by hydrogen atom abstraction from **8** by **22**. Abstraction likely occurs at C-3 to give **23** as an intermediate. The mechanism for the subsequent transformation is less obvious, but may parallel the reaction catalyzed by the coenzyme B_{12}-dependent ethanolamine ammonia-lyase, which converts ethanolamine to ammonia and acetaldehyde.[41] As shown in Scheme 6 path A, the key step may be a radical-induced 1,2-amino shift (**23** → **24/25** → **26**), to form an aminol radical **26**. Reclaiming a hydrogen atom from 5′-deoxyadenosine (**27**) would then result in the formation of **28** and regeneration of **22**, which could recombine with methionine to reform the [4Fe-4S]$^{1+}$-SAM complex.[9,10a] Elimination of an ammonium ion from **28** would afford the final product **9**. The reaction may also proceed by deprotonation of the 3-hydroxyl group of **23** to yield a ketyl radical anion **29**, whose resonance form **30** facilitates the β-elimination of the 4-amino group (Scheme 6, path B). The reaction catalyzed by (*R*)-2-hydroxyacyl-CoA dehydratase[42] in the fermentation of β-amino acids by anaerobic bacteria provides such a precedent.

To investigate the proposed mechanisms, we first considered hydrogen atom abstraction by the 5′-deoxyadenosyl radical (**22**). When TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose (**18**) was used as substrate, deuterium incorporation into SAM and 5′-deoxyadenosine were observed. This provides strong evidence for cleavage of the C−H bond at C-3 (**8** → **23**) during turnover. The mechanistic implications of these findings are at least 2-fold. First, they provide compelling evidence supporting the intermediacy of a sugar C-3 radical (**23**) in DesII catalysis. Second, they support the hypothesis that SAM serves catalytically as a cofactor that is regenerated with each turnover of the TDP-sugar substrate. As depicted in Scheme 6, the outcome is consistent with the formation of [5′-^{2}H]-5′-deoxyadenosine ([5′-^{2}H]-**27**) as a result of ^{2}H-atom abstraction from **18** by the 5′-deoxyadenosyl radical (**22**). Owing to the primary deuterium isotope effect, a H-atom rather than the ^{2}H-atom from the methyl group of [5′-^{2}H]-**27** will be preferentially reclaimed by the aminol radical intermediate (**26**) in path A or the enol radical intermediate (**31/32**) in path B to yield a [5′-^{2}H]-5′-deoxyadenosyl radical ([5′-^{2}H]-**22**), from which the [4Fe-4S]$^{1+}$−[5′-^{2}H]SAM complex reforms to complete the catalytic cycle. A second-round of turnover with another molecule of deuterated substrate **18** would then lead to [5′-$^2H_2$]SAM as was detected by mass spectrometry. The precedence is set by the reactions of lysine 2,3-aminomutase[9] and spore photoproduct lyase,[15] in which SAM functions catalytically as a temporary oxidant and is regenerated at the end of each catalytic cycle.

However, these results do not rule out stoichiometric turnover of SAM suggested by the observation of the formation of [5′-^{2}H]-5′-deoxyadenosine when **18** was used in the reaction. Under this scenario, the consumption of one molecule of SAM should be accompanied by the production of one molecule of [5′-^{2}H]-5′-deoxyadenosine after each catalytic cycle. The formation of

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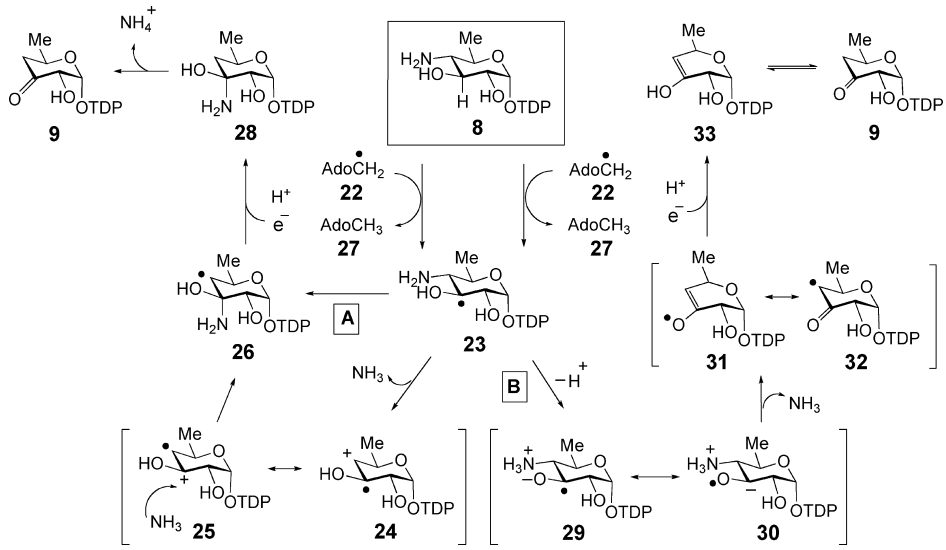
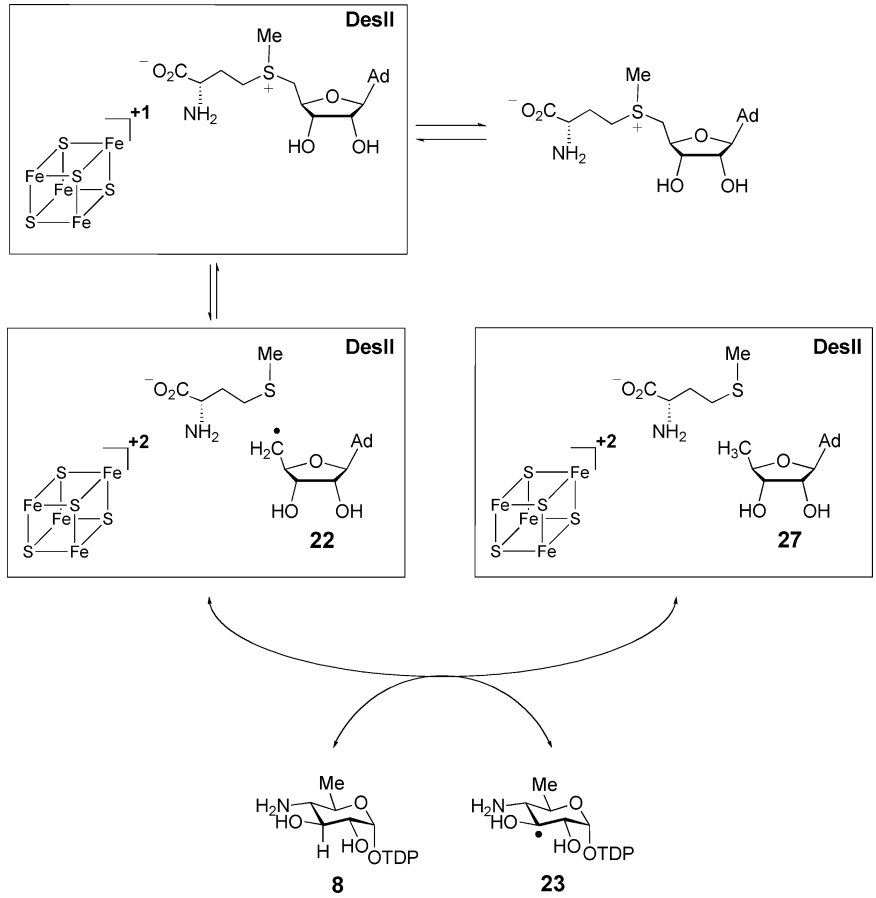
Scheme 6



Scheme 7



$[5'\text{-}^2H_2]$SAM in this case may be ascribed to the reversible formation of the substrate radical (**23**) via direct H-atom interchange with 5′-deoxyadenosine (**27**). As depicted in Scheme 7, H-atom return from [5′-^{2}H]-**27** to the substrate radical (**23**) could eventually lead to a Michaelis complex with $[5'\text{-}^2H_2]$SAM, which will then be released. Evidence for such reversible H-atom interchange between 5′-deoxyadenosine and a substrate radical has been gathered for the radical SAM dehydrogenase BtrN.[16] Overall, a catalytic role for SAM in DesII-catalyzed reaction is attractive and consistent with the observation of $[5'\text{-}^2H_2]$SAM in the reaction with TDP-[3-^{2}H]-4-amino-4,6-dideoxy-D-glucose. However, the possibility that SAM may not be used in a catalytic fashion is implicated by the observation of monodeuterated 5′-deoxyadenosine produced in the DesII reaction and the near unit concentration ratios of substrates to enzyme employed in the present studies. Efforts are currently underway in our laboratory to resolve these mechanistic ambiguities.

In exploring the substrate specificity of DesII, it was discovered that DesII catalyzes oxidation of TDP-D-quinovose (**14**) and TDP-3-amino-3,6-dideoxy-D-glucose (**17**) to form in

both cases TDP-3-keto-6-deoxy-D-glucose (**16**). The latter substrate is presumably first dehydrogenated to a Schiff base that subsequently hydrolyzes to give the ketone. An analogous reaction is reported for BtrN, which catalyzes the oxidation of the C-3 hydroxyl group of 2-deoxy-*scyllo*-inosamine to yield the corresponding C-3 ketone.[16] In contrast, TDP-D-fucose (**15**), which contains an axial hydroxyl group at C-4, is not a substrate for DesII. Therefore, the stereochemical orientation of the substituent at the C-4 position appears to be important for substrate recognition. These results demonstrate that DesII tolerates variation in substituents at both the C-3 and C-4 positions; however, while an amino group at C-4 leads to a redox neutral deamination reaction, the presence of a hydroxyl group at C-4 leads to dehydrogenation at C-3.

In conclusion, DesII has been fully established as a radical SAM-dependent deaminase involved in C-4 deoxygenation in TDP-D-desosamine biosynthesis. The fact that DesII is responsible for a radical-mediated deamination reaction further illustrates the catalytic versatility of the radical SAM enzyme superfamily. Although the details of its catalytic mechanism remain elusive, participation of DesII with the transaminase DesI provides another unique method by which C−O cleavage can proceed in biological systems. Mechanistic studies of this intriguing enzyme are in progress.

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