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Mechanistic Studies of the Spore Photoproduct Lyase via a Single Cysteine Mutation

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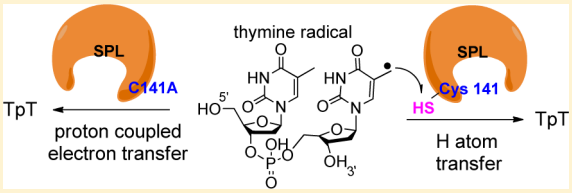
Supporting Information

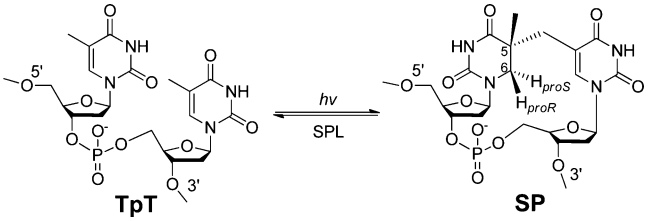
ABSTRACT: 5-Thyminyl-5,6-dihydrothymine (also called spore photoproduct or SP) is the exclusive DNA photodamage product in bacterial endospores. It is repaired by a radical SAM (*S*-adenosylmethionine) enzyme, the spore photoproduct lyase (SPL), at the bacterial early germination phase. Our previous studies proved that SPL utilizes the 5′-dA• generated by the SAM cleavage reaction to abstract the H_{6proR} atom to initiate the SP repair process. The resulting thymine allylic radical was suggested to take an H atom from an unknown protein source, most likely cysteine 141. Here we show that C141 can be readily alkylated in the native SPL by an iodoacetamide treatment, suggesting that it is accessible to the TpT radical. SP repair by the SPL C141A mutant yields $TpTSO_2^-$ and TpT simultaneously from the very beginning of the reaction; no lag phase is observed for $TpTSO_2^-$ formation. Should any other protein residue serve as the H donor, its presence would result in TpT being the major product at least for the first enzyme turnover. These observations provide strong evidence to support C141 as the direct H atom donor. Moreover, because of the lack of this intrinsic H donor, the C141A mutant produces TpT via an unprecedented thymine cation radical reduction (proton-coupled electron transfer) process, contrasting to the H atom transfer mechanism in the wild-type (WT) SPL reaction. The C141A mutant repairs SP at a rate that is ∼3-fold slower than that of the WT enzyme. Formation of $TpTSO_2^-$ and TpT exhibits a V_{max} deuterium kinetic isotope effect (KIE) of 1.7 ± 0.2, which is smaller than the $^{D}V_{max}$ KIE of 2.8 ± 0.3 determined for the WT SPL reaction. These findings suggest that removing the intrinsic H atom donor disturbs the rate-limiting process during enzyme catalysis. As expected, the prereduced C141A mutant supports only ∼0.4 turnover, which is in sharp contrast to the >5 turnovers exhibited by the WT SPL reaction, suggesting that the enzyme catalytic cycle (SAM regeneration) is disrupted by this single mutation.



Because of its high energy and efficient absorption by biological macromolecules, UV light is considered to be immediately lethal to most microorganisms. However, this method fails to kill endospores because of their unique DNA photochemistry. The spore genomic DNA is bound by a group of DNA binding proteins named small acid soluble proteins (SASPs). In the resulting protein−DNA complex, the DNA adopts an A-like conformation, which facilitates formation of a thymine dimer, 5-thyminyl-5,6-dihydrothymine (Scheme 1), also called spore photoproduct or SP, as the exclusive UV damage product.[1−7] SP is rapidly repaired by a metalloenzyme, spore photoproduct lyase (SPL), when spores start germinating,[1,8−16] thus posing little threat to their survival. However, despite the fact that SP was discovered nearly half a century ago[17] and the strong interest of the scientific community to understand its formation and repair,[1] neither process is well understood at this point.

Our laboratory is interested in understanding both aspects of SP biochemistry. We recently employed deuterium-labeled dinucleotide TpT and demonstrated that SP is formed via an intramolecular H atom transfer process between two thymine residues,[18] answering a key question in SP photoformation.[1,17] By using a neutral formacetal linker instead of a negatively charged phosphate between the two thymine residues, we successfully obtained the crystal structure of SP 50 years after its discovery.[19] Analyzing the structure not only confirms SP to

Scheme 1



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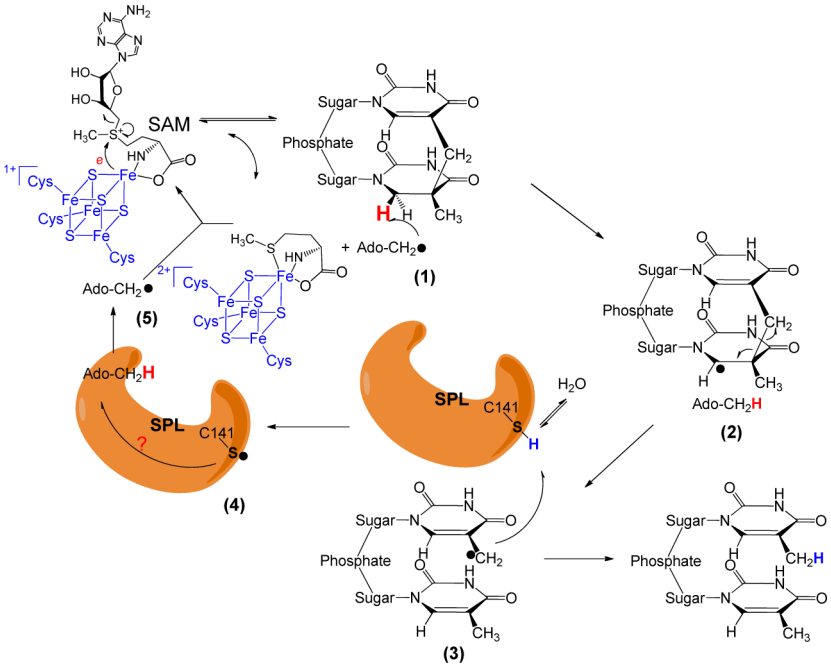
Scheme 2



have a chiral center with an *R* configuration but also reveals that both 2-deoxyriboses adopt the C_3'-endo conformation, which is typically found in A-form DNA. Although SP formation needs the genomic DNA to adopt an A-like conformation,[3,4,6] once formed, the methylene bridge between the thymine bases becomes dominant, forcing the two 2-deoxyriboses to maintain the A-like conformation even though the DNA binding proteins are absent.

SP is repaired by the radical SAM enzyme SPL at the bacterial early germination phase.[1,20,21] All radical SAM enzymes use a tricysteinate motif to coordinate to three irons in the [4Fe-4S] cluster, and *S*-adenosylmethionine (SAM) to the fourth one (Scheme 2).[20−38] The cluster donates an electron to SAM to cleave its C−S bond, generating a 5′-deoxyadenosyl radical (5′-dA●). Utilizing dinucleotide SP TpTs with the two H6 atoms selectively labeled by deuterium, we proved that it is the H_{6proR} atom that is taken by the 5′-dA●, confirming that the SPL reaction is highly stereoselective.[39] However, in a manner different from the prediction by the previously hypothesized mechanism,[10,11] the resulting TpT radical does not take the H atom back from 5′-dA, but from an unknown protein residue, which is able to exchange a proton with the aqueous solution via acid−base chemistry, to produce the repaired TpT.[39]

Which protein residue serves as the direct H atom donor to the TpT radical? The research to date suggests that a conserved cysteine residue, C141, in *Bacillus subtilis* SPL is the most likely candidate. An in vivo study found that a C → A mutation totally abolished the enzyme activity.[40] An in vitro assay found that the C141A mutant produced a $TpTSO_2^-$ species as the major product from the SP repair reaction, with the SO_2^- group originating from the dithionite added as the reductant to the SAM $[4Fe\text{-}4S]^{2+}$ cluster.[14] These results suggest that the C141 residue plays a key role in enzyme catalysis.

The crystal structures of the WT SPL as well several SPL C140 mutants from *Geobacillus thermodenitrificans* were determined at high resolution very recently.[41] The C140 mutants were found to have the same structures as the WT SPL, suggesting that the conserved cysteine has no structural role. The enzyme contains a dinucleoside SP. As shown in Figure 1, the bridging methylene carbon in SP was found to be

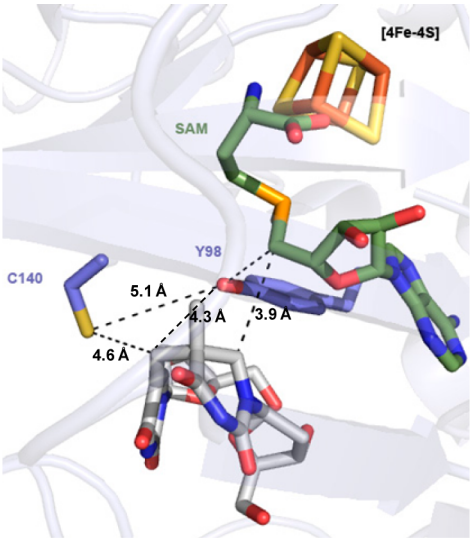


Figure 1. Crystal structure of the WT SPL from *G. thermodenitrificans* that contains a SAM and a dinucleoside SP in the enzyme active site. The distance between the SP methylene carbon and the conserved cysteine (C140) was found to be 4.6 Å, and that between the methylene carbon and a conserved tyrosine (Y98) was found to be 4.3 Å.

4.6 Å from C140 (equivalent to C141 in *B. subtilis* SPL) and 4.3 Å from an active-site tyrosine (Y98). It was suggested that after the methylene bridge breaks during the repair process, the resulting thymine allylic radical moves toward C140, making C140 perfectly positioned as the intrinsic H atom donor. To support this assumption, the activity of the C140A mutant was studied and the correspondent SO_2^- adduct to DNA was found by matrix-assisted laser desorption ionization spectroscopy. The

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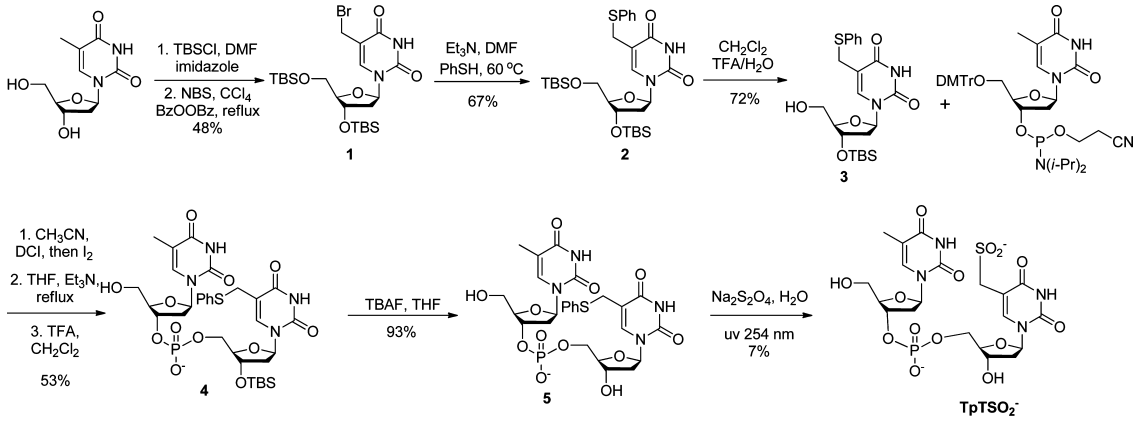
Scheme 3



yield of the SO_2^- adduct, however, appeared to be low. Furthermore, the <0.5 turnover observed also makes the mechanistic analysis complicated. In contrast, when dinucleotide SP was used as substrate for the *B. subtilis* C141A mutant, multiple turnovers were observed and >70% of the repaired SPs yielded the $TpTSO_2^-$ adduct, with TpT a minor species.[14]

Although the structural data suggest that the conserved cysteine is likely the H atom donor, the possibility that a conserved tyrosine (Y98 in *G. thermodenitrificans* SPL and Y99 in *B. subtilis* SPL) is the donor cannot be excluded. This Y98 is closer to the SP methylene carbon in the SPL structure; it may be able to donate the H atom to the thymine allylic radical, which could explain why TpT is also formed in the C141A mutant reaction.[14] To address this problem, the *B. subtilis* C141A-catalyzed SP TpT repair reaction was certainly worthy of revisiting to reveal if $TpTSO_2^-$ and TpT form at the same time. Such information is significant because if Y99 in *B. subtilis* SPL, but not C141, is the direct H atom donor, it is still perfectly positioned to donate the H atom in the C141A mutant, allowing the formation of TpT.

Here, we report the enzyme kinetic studies of the SPL C141A mutant. Our data confirm that C141 is the direct H atom donor to the TpT radical. Moreover, after this putative H atom donor had been removed from SPL, the SPL C141A mutant produces TpT likely via an unprecedented thymine radical cation reduction (proton-coupled electron transfer) mechanism.

EXPERIMENTAL PROCEDURES

Materials and Methods. Unless otherwise stated, all solvents and chemicals used were of commercially available analytical grade and used without further purification. Purification of reaction products was conducted by flash chromatography using silica gel (Dynamic Adsorbents Inc., 32−63 μm). The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker 500 MHz NMR Fourier transform spectrometer. UV−visible spectra were recorded using a UV-Mini 1240 spectrophotometer and the associated data manager software package. The photoreaction was conducted using a Spectroline germicidal UV lamp (Dual-tube, 15 W, intensity of 1550 $\mu W/cm^2$) with samples ~5 cm from the lamp. The protein purification and the enzyme reactions were conducted under an inert atmosphere using a Coylab (Grass Lake, MI) anaerobic chamber with ~3% H_2. DNA sequencing was performed by GENEWIZ Inc. (South Plainfield, NJ).

All DNA-modifying enzymes and reagents were purchased from Fermentas Life Sciences (Glen Burnie, MD). *B. subtilis* strain 168 chromosomal DNA was purchased from the ATCC (ATCC 23857D). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). *Escherichia coli* BL21(DE3) and expression vector pET-28a were purchased from Novagen (Madison, WI). The construct containing the SPL gene was coexpressed with plasmid pDB1282, which was a generous gift from S. Booker (The Pennsylvania State University, State College, PA). Five Prime Perfectpro* nickel nitrilotriacetic acid (Ni-NTA) resin was purchased from Fisher Scientific, and the SP Sepharose fast flow ion-exchange resin was purchased from the GE Healthcare Bio-Sciences Corp.

Construction of the SPL C141A Expression Vector. The *splB* gene was cloned from the *B. subtilis* chromosomal DNA (strain 168) into the in pET-28a vector with an N-terminal His_6 tag as previously described.[39] A site-directed mutagenesis was performed to change the cysteine 141 to an alanine using the synthetic oligonucleotide primers 5′-CAAGGTTCGAAGCATCAGCTACGTCAGACATTG-3′ and 5′-CAATGTCTGACGTAGCTGATGCTTCGAACCTTG-3′ with the QuickChange site-directed mutagenesis kits from Stratagene following the manufacturer's instructions. The construct was transformed into *E. coli 10 G* chemically competent cells purchased from Lucigen Corp. (Middleton, WI) for isolation and amplification of the resulting plasmid DNA. The resulting vector was named SPL C141A-pET28 and cotransformed with a pDB 1282 vector into *E. coli* BL21(DE3) cells obtained from Stratagene (La Jolla, CA) for protein overexpression as described previously.[39]

Expression and Purification of the SPL C141A Mutant. Both the WT SPL and the C141A mutant were expressed in LB medium containing the appropriate antibiotics as previously described.[39] The proteins were purified via Ni-NTA chromatography followed by ion-exchange chromatography using the SP Sepharose fast flow ion-exchange resin (GE Healthcare Life Sciences, Piscataway, NJ). The bound protein was washed using a buffer consisting of 25 mM Tris, 250 mM NaCl, and 10% glycerol (pH 7.0) for 10 column volumes. The protein was then eluted using the same buffer containing 500 mM NaCl instead. The resulting protein was diluted 2-fold to reduce the salt concentration to 250 mM and saved for activity studies. In a separate purification process, phosphate buffer containing 25 mM sodium phosphate, 250 mM NaCl, and 10% glycerol (pH 7.0) was used for protein purification.

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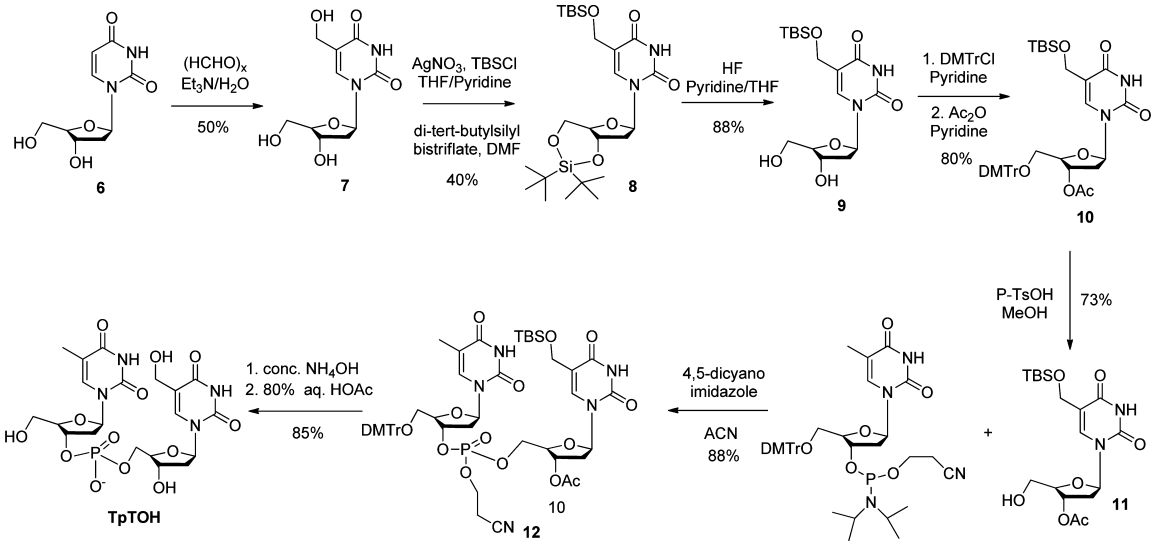
Scheme 4



Protein, Iron, and Sulfide Assays. Routine determinations of protein concentration were conducted by the Bradford method,[42] using bovine gamma globulin as the protein standard. Protein concentrations were calibrated on the basis of the absorption of aromatic residues at 280 nm in the presence of 6 M guanidine hydrochloride using the method of Gill and von Hippel.[43] Iron content was determined using *o*-bathophenanthroline (OBP) under reductive conditions after protein digestion in 0.8% $KMnO_4$ and 1.2 M HCl as described by Fish.[44] Iron standards were prepared from commercially available ferric chloride. Sulfide assays were conducted using the method described by Beinert.[45]

Preparation of SP TpT and d_4-SP TpT. These compounds were prepared via either chemical synthesis or solid state TpT photolysis as described previously.[39]

Synthesis of $TpTSO_2^-$. $TpTSO_2^-$ was prepared via a modified procedure as previously described.[14] As shown in Scheme 3, the synthesis took advantage of the weak strength of the C−S bond connected at the thymine methyl moiety. Photocleavage of this bond readily generates a thymine allylic radical, which subsequently reacts with dithionite to yield $TpTSO_2^-$. Although the yield for the photosubstitution is only 7%, the unreacted compound **5** can be readily recovered and reused for the next round of photoreaction. Care has been taken to remove the dissolved O_2 by purging with argon. The photosynthesis was then conducted in the Coy anaerobic chamber. Experimental details about the synthesis are available in the Supporting Information (SI).

Synthesis of TpTOH. The synthesis of TpTOH was conducted via the reactions shown in Scheme 4. The hydroxyl group was introduced first to the methyl moiety of the thymine residue, which was subsequently protected before being cross-linked to another thymine residue via standard phosphoramidite chemistry. Detailed information regarding the synthesis is available in the SI.

SPL C141A Activity Assay. In a typical set of experiments, the reaction mixture contained 30 μM SPL C141A, 0.6 mM SP TpT substrate, and 100 μM SAM in a final volume of 500 μL of buffer consisting of 25 mM Tris, 250 mM NaCl, and 10% glycerol (pH 7.0). Freshly made sodium dithionite (final concentration of 1 mM) was added as the reductant to initiate the enzyme reaction. The reaction was conducted as described previously and analyzed by high-performance liquid chromatography (HPLC).[39] In a separate set of experiments, the C141A mutant reaction was examined in phosphate buffer (containing 25 mM sodium phosphate instead of 25 mM Tris) to exclude the involvement of Tris in enzyme catalysis.

Enzyme Activity with a Limited Amount of SAM. To study the impact of SAM on the SP repair reaction, the WT SPL and the C141A mutant reactions were repeated using 1, 2, 5, and 10 equiv of SAM (relative to protein). A typical reaction mixture contained 15 μM enzyme, 0.4 mM SP TpT substrate, and differing amounts of SAM in a final volume of 800 μL of buffer consisting of 25 mM Tris, 250 mM NaCl, and 10% glycerol (pH 7.0). Freshly made sodium dithionite (final concentration of 1 mM) was added as a reductant to initiate the reaction. At certain reaction times, 100 μL of the reaction solution was taken out, quenched by 3 μL of 3 M HCl, and analyzed by HPLC.

Enzyme Activity in the Absence of an External Reductant. The C141A mutant protein was reduced with 1 mM freshly prepared sodium dithionite for 30 min. Excess inorganic ions were removed via a Thermo Scientific Zeba Spin Desalting Column. SP TpT and SAM were then added to final concentrations of 0.3 mM and 50 μM (~3-fold of SPL), respectively, to initiate the SP repair reaction. In another set of experiments, 1 or 10 mM DTT was added together with SP TpT and SAM. At different time points, 100 μL of the solution was removed, quenched by HCl, and analyzed by HPLC.

SPL C141A Activity with a Limited Amount of Dithionite. To understand the effect of dithionite on the C141A mutant reaction, the protein was prereduced and desalted as described above. Dithionite or DTT was then resupplemented to the desalted enzyme. The reactions were quenched by HCl after 1 h.

Examination of the TpTSPh (5) Photoreaction. To reveal the reaction mechanism of TpT formation, the TpTSPh photoreaction (final concentration of 1 mM) was studied in dd H_2O or in a Tris buffer consisting of 25 mM Tris, 250 mM NaCl, and 10% glycerol (pH 7.0) in the presence and absence of varying amounts of sodium dithionite under an inert atmosphere. The reaction was allowed to proceed under 254 nm UV light for 10 min, and the products were analyzed by liquid chromatography and mass spectrometry (LC−MS). In

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another set of experiments, the reaction mixtures described above were supplemented with DTT to a final concentration of 1 or 10 mM.

HPLC Assay for Product Analysis. HPLC was performed at room temperature with a Waters (Milford, MA) breeze HPLC system with a 2489 UV−visible detector at 268 nm. An Agilent Zorbax reverse-phase C-18 column (3.5 μM, 4.6 mm × 50 mm) was equilibrated in 50 mM triethylammonium acetate (pH 6.5) (buffer A), and compounds were eluted with an ascending gradient (5 to 20%) of buffer B over 15 min, which is composed of 50% buffer A and 50% acetonitrile, at a flow rate of 1 mL/min. With this gradient, SP TpT was eluted at 5.4 min, 5′-dA at 8.9 min, $TpTSO_2^-$ at 9.8 min, TpTOH at 12.9 min, and TpT at 14.1 min. The identity of the products was confirmed by co-injection of respective authentic samples as well as by LC−MS. The area of the product peak was determined after subtraction of the baseline from the t = 0 chromatograph, and the amounts of 5′-dA, $TpTSO_2^-$, and TpT formed were determined by reference to standard curves constructed with authentic samples.

The SAM analysis was conducted according to a modified literature procedure.[46] Briefly, An Agilent Zorbax reverse-phase C-18 column (3.5 μM, 4.6 mm × 75 mm) was equilibrated in 0.1% formic acid supplemented with 1 mM heptanesulfonic acid (buffer A), and compounds were eluted from the HPLC column with an ascending gradient (from 0 to 30%) of buffer B over 14 min, which consists of 50% buffer A and 50% acetonitrile at a flow rate of 1 mL/min. With this gradient, SP TpT was eluted at 3.3 min, $TpTSO_2^-$ and TpT were eluted at 6.5 min under a single peak, 5′-dA was eluted at 9.8 min, SAH (*S*-adenosyl-L-homocysteine) was eluted at 10.2 min, SAM was eluted at 11.4 min, and MTA (methylthioadenosine) was eluted at 12.5 min.[47]

LC−MS Assay for Product Analysis. LC−MS analyses were conducted via an Agilent 6130 Quadrupole LC−MS spectrometer coupled to an Agilent 1100 series chromatography system using a Waters X-bridge OST C18 column (2.5 μM, 4.6 mm × 50 mm). The column was equilibrated in solvent A [5 mM ammonium acetate (pH 6.5)], and compounds were eluted with an ascending gradient (from 0 to 17%) of solvent B (5 mM ammonium acetate in a 1:1 acetonitrile/methanol mixture) at a flow rate of 1 mL/min over 12 min. With this gradient, $TpTSO_2^-$ was eluted at 4.8 min, TpTOH at 7.6 min, and TpT at 8.2 min. The mass signals were monitored under positive and negative ion mode.

The photoreaction of TpTSPh was analyzed with an Agilent Eclipse XDB reverse-phase C-18 column (5 μM, 4.6 mm × 150 mm). Buffers A and B are the same as those described above. The compounds were eluted with an ascending gradient (from 1 to 15%) over 20 min followed by another gradient (from 15 to 95%) over 10 min. With this program, $TpTSO_2^-$ was eluted at 9.3 min, TpTOH at 16.3 min, TpT at 17.7 min, and TpTSPh at 25.2 min.

Alkylation of Cysteine 141 and Analyses. Cysteine alkylation of WT SPL or the C141A mutant was conducted in 200 μL of sodium phosphate buffer (pH 7.0) containing 250 mM NaCl and 10% glycerol. The protein (5 μM) was treated with 0.5 mM iodoacetamide in the dark for 1 h. The reaction was conducted in an anaerobic chamber, and the protein was kept on ice through the treatment to minimize protein denaturation. The solution was then diluted by 2-fold using dd water, and 0.1 μL of the resulting solution was injected into the Agilent 6520 Accurate-Mass Q-TOF LC−MS spectrometer. The data were acquired via Agilent MassHunter Workstation Data Acquisition (B.03.00) and analyzed via Qualitative Analysis of MassHunter Acquisition Data (B.03.00).

The remaining protein was digested overnight by α-chymotrypsin and trypsin in a 37 °C incubator. The resulting peptide fragments were injected into the Agilent 6520 Accurate-Mass Q-TOF LC−MS spectrometer and analyzed as described above. The activity of the alkylated protein was also studied via the standard procedure using 5-fold SAM and 10-fold SP and analyzed by HPLC.

EPR Experiments. Continuous wave (CW) EPR spectra were recorded on a modified Varian spectrometer at 35 GHz ("Q"-band) and 2 K.[48] The as-isolated SPL mutant (300 μM, 3.0 irons/protein) was reduced with 2 mM dithionite inside the anaerobic chamber for 60 min, placed into the EPR tube, and immediately frozen in liquid N_2. The EPR spectra were recorded as described previously.[39] EPR simulations were performed using QPOW,[49] as modified by J. Telser.

RESULTS

SPL C141A Expression, Purification, and Characterization. The C141A-pET28 vector contains a His_6 tag, which allows the protein to be purified to up to ∼90% purity via Ni-NTA chromatography as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).[47] As SPL protein is positively charged at pH 7 (pI = 8.5), it binds tightly to the SP Sepharose ion-exchange resin in the presence of 150 mM NaCl while most of the *E. coli* proteins do not bind. In our hands, ion-exchange chromatography is absolutely needed. *The protein purified only by Ni-NTA chromatography was found to be contaminated by an unknown enzyme, which consumes the 5′-dA generated from the SP repair process.* This enzyme can be removed by a thorough washing process from the ion-exchange column (a minimum of 5 column volumes of washing is needed). After elution from the ion-exchange column, the SPL C141A mutant was obtained in >99% purity.[47]

The purified protein possesses a dark-brown color, suggesting the presence of the [4Fe-4S] cluster. The presence of such a cluster is supported by the characteristic UV absorption band at 420 nm.[47] The iron−sulfur content analysis of the as-isolated protein found that each protein molecule contains 3.2 Fe and 3.0 S atoms. Upon dithionite reduction, the cluster exhibits an $S = {}^1/_2$ signal (Figure S3 of the SI) that is essentially indistinguishable from the EPR signal from the WT SPL cluster.[47] Simulation yields $\mathbf{g}$ = [2.026(5), 1.928(5), 1.890(5)], which is equivalent to the g values reported for this center by other workers.[11,12,50] These observations agree with the previous studies of this mutant enzyme,[14] namely that the C141 residue is not involved in coordinating with the radical SAM FeS cluster.

Iodoacetamide Treatment. As described by our previous work, 5′-dA is not the direct H atom donor to the thymine allylic radical.[39] The TpT radical takes an H atom from a source that is able to exchange proton with the buffer, making cysteine 141 in *B. subtilis* SPL the most likely H donor. Such an assumption is further supported by an in vivo study,[40] which showed that spores carrying the SPL C141A mutant are highly sensitive to UV irradiation as well as an in vitro study by Foncecave[12] that showed that TpT is no longer the major SP repair product in the C141A mutant reaction. The *G. thermodenitrificans* SPL structure further suggests that the cysteine is close to the SP substrate,[41] although a conserved tyrosine cannot be excluded as the H donor. If C141 is the

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direct H donor, we expect that (1) the C141 is solvent exposable so that the TpT radical can have a chance to accept the H atom and (2) no other protein residues are involved in this H transfer process.

We used the iodoacetamide treatment assay to test if C141 is solvent exposable. Iodoacetamide treatment is widely used to label the cysteine residues in proteins in either the native form or the denatured form.[51,52] It was used to alkylate the free cysteine residues in iron−sulfur proteins such as APS reductase[53] and aconitase.[54] Were cysteine 141 in *B. subtilis* SPL to be the direct H atom donor to the thymine allylic radical,[39] it would be accessible from the aqueous solution and readily alkylated by the iodoacetamide treatment. As shown in Figure 2A, after treatment with 100-fold iodoacetamide under

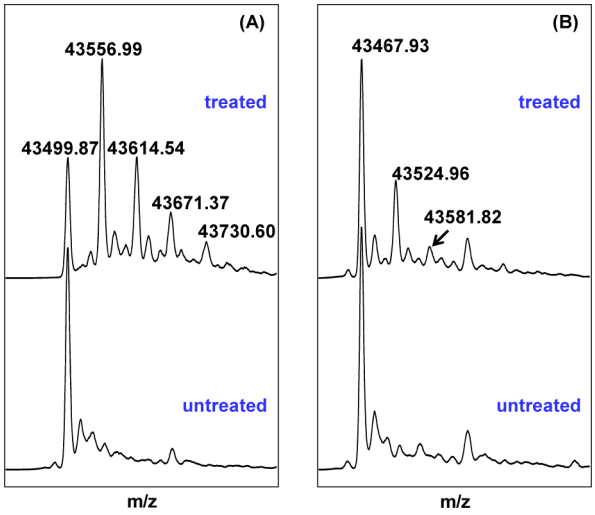

Figure 2. (A) Deconvoluted ESI-MS spectrum of the WT SPL enzyme treated with excess iodoacetamide under native conditions. Compared with the untreated protein, the major species in the treated enzyme exhibits a mass gain of 57.13. The intensity of this peak is much stronger than those corresponding to proteins that carry two, three, or four labels. This observation suggests that one of the four cysteines in WT SPL is prone to alkylation. (B) Deconvoluted ESI-MS spectrum of the SPL C141A mutant treated with excess iodoacetamide under an identical condition. The monolabeled species in the treated C141A mutant exhibited a peak whose intensity is comparable with that of the treated WT enzyme carrying two alkyl labels, but much weaker than that corresponding to the monoalkylated WT enzyme, suggesting that the alkylation site is at one of the three cluster cysteines. The different behavior toward the iodoacetamide treatment between these two proteins suggests that C141 in the WT SPL can be accessed from the aqueous solution.

the native condition, ∼50% of the resulting enzyme molecules carry one alkyl label and the rest carry zero, two, three, or four labels. In contrast, ∼80% of the C141A mutant remains unlabeled after the reaction. Among the labeled C141A species, the intensity of the monoalkylated protein is comparable to that of the dialkylated peak in the treated WT enzyme, indicating that the alkylation site is at one of the three cluster cysteines. The trypsin digestion experiment further proves that the C141 residue of the WT SPL carried the acetamide label. Such a label was missing in the same peptide fragment from the SPL C141A mutant, suggesting that C141 in WT SPL is prone to the iodoacetamide treatment.

As shown by Cravatt et al., a fully exposed cysteine in a native protein can be readily labeled by a stoichiometic amount of iodoacetamide.[52] Even with 100-fold iodoacetamide, only 50% of the WT enzymes have their C141 alkylated, suggesting that although it can be accessed from the aqueous buffer, C141 is relatively well protected in the enzyme binding pocket. Prolonged incubation resulted in the alkylation of all cysteine residues in the WT SPL as well as the C141A mutant.[47] The fact that the three cysteines in the radical SAM motif were alkylated is surprising as the cysteinate residues are usually protected once they are chelated by the iron−sulfur cluster as revealed in iron−sulfur enzymes such as the APS reductase[53] and aconitase.[54] On the other hand, the radical SAM motif typically resides on a flexible loop at the protein surface,[24] making the three cysteine residues less likely to be effectively protected. Such a secondary structure may cause the cysteines to be attacked by the labeling reagent, as indicated by our observations.

To exclude the possibility that the alkylation of the four cysteine residues in WT SPL is due to the SPL denaturation during the treatment, we tested the activity of the alkylated WT enzyme. In contrast to the WT enzyme, which yields the dinucleotide TpT as the SP repair product, the alkylated SPL should behave like the C141A mutant, producing $TpTSO_2^-$ as the major product.[14] Under the hypothesis that the monoalkylated species shown in Figure 2A had only the C141 residue modified, the alkylated protein and unmodified WT SPL were thus present in a ∼5:3 ratio. As described in the following section, the WT enzyme repairs SP at a rate that is ∼3-fold faster than that of the C141A mutant. Therefore, TpT and $TpTSO_2^-$ should be produced in a ∼ 2: 1 ratio. Indeed, the isolated TpT and $TpTSO_2^-$ exhibited a mole ratio of 2.5:1,[47] suggesting that the SPL carrying alkylated cysteine 141 is still in its native state; C141 in the WT SPL enzyme can be accessed from the aqueous buffer.

Reaction of SPL C141A with Regular SP TpT. The iodoacetamide treatment proves that C141 can be accessed by the TpT radical, thus fulfilling the prerequisite to be the H atom donor. Next, we ask whether other protein residues are involved in this H atom transfer process. Were a protein residue other than C141, for instance, Y99 in *B. subtilis* SPL as suggested by the structure shown in Figure 1, to serve as the H donor, the residue would still be present in the C141A mutant, which is expected to produce TpT as the SP repair product at least for the first enzyme turnover. A lag phase in $TpTSO_2^-$ formation is thus expected. In our kinetic studies of the SPL C141A reaction, we found that in the presence of 1 mM sodium dithionite and in the absence of DTT, $TpTSO_2^-$ was isolated as the major reaction product and TpT as the minor one. The third product, TpTOH, was also detected, although its yield was extremely low (<0.1%). This is consistent with the earlier findings from Fontecave et al. More importantly, using the calibration curves constructed with the authentic TpT and $TpTSO_2^-$, their formation rates were calculated and are listed in Table 1. The rate of formation of TpTOH cannot be determined because of its low reaction yield. Our studies revealed that the $TpTSO_2^-$ and TpT formed under constant reaction rates from the very beginning of the reaction, and no lag phase was observed for $TpTSO_2^-$ formation (Figure 3). These findings strongly suggest that no other protein residue is located between the TpT radical and C141 in the WT SPL reaction pathway.

TpT Formation in the SPL C141A Reaction. Our data suggest that C141 is the most likely intrinsic H atom donor in SPL. However, the C141A mutant still produces TpT during

Table 1. Rates of Formation (min^{-1}) of $TpTSO_2^-$ and TpT in the C141A Mutant Reaction with 1 mM Sodium Dithionite, 0.1 mM SAM, and Varying Concentrations of DTT as the H Atom Donor

	no DTT	1 mM DTT	10 mM DTT
$TpTSO_2^-$	0.11 ± 0.01	0.11 ± 0.01	0.05 ± 0.005
TpT	0.014 ± 0.002	0.019 ± 0.002	0.041 ± 0.005
$TpTSO_2^-$ and TpT	0.12 ± 0.01	0.13 ± 0.01	0.09 ± 0.01

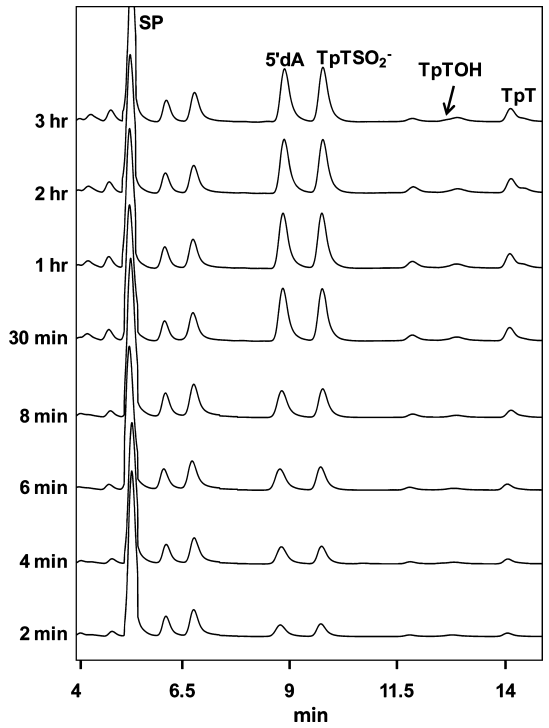

Figure 3. HPLC chromatograph of the SP TpT repair process mediated by the *B. subtilis* SPL C141A mutant with 30 μM enzyme, 150 μM SAM, and 1 mM dithionite. Under the HPLC program, the SP TpT was eluted at 5.4 min, 5′-dA at 8.9 min, $TpTSO_2^-$ at 9.8 min, TpTOH at 12.9 min, and TpT at 14.1 min. Linear formations of $TpTSO_2^-$ and TpT were observed in the first 30 min of the reaction. The TpTOH peak overlapped with an uncharacterized compound in our HPLC chromatograph. The yield of TpTOH was too low to be determined.

the SP repair reaction, which is puzzling because it does not contain such a H donor any more. One possibility is that the TpT radical obtains an H atom from the small molecules in the reaction buffer. We first excluded the possibility of Tris being the H atom donor, as the same reaction pattern was obtained when the C141A reaction was conducted in the phosphate buffer. As radical SAM reaction mixtures are usually supplemented with thiol compounds, we next examined the SPL C141A reaction in the presence of various concentrations of DTT. As shown in Table 1, with equal amounts of dithionite and DTT (1 mM), little difference was observed between TpT and $TpTSO_2^-$ formation. In contrast, the presence of 10 mM DTT increased the rate of TpT formation by 3-fold; however, the formation of $TpTSO_2^-$ and the overall SP repair rate were found to be slower. Other small thiol compounds such as β-mercaptoethanol and methanethiol also facilitate TpT formation; however, they are not as effective as DTT probably because of the stronger S−H bond in these compounds.

The dithionite reduces the $[4Fe\text{-}4S]^{2+}$ cluster via an outer-sphere electron transfer process; the excess DTT may block the enzyme binding pocket, preventing the dithionite from accessing the cluster and resulting in a partial inhibition of the SP repair reaction. A similar inhibition by concentrated DTT was also observed in the WT SPL reaction. Despite the inhibitory effect, the improved yield suggests that the presence of an H atom donor could facilitate TpT formation. However, this enhancement requires a large amount of DTT. Considering that no small molecule is present at a concentration of 10 mM in our enzyme reaction mixture, the donation of an H atom from a small molecule can be ruled out.

SPL C141A Reaction with Varying Amounts of Dithionite. A second possibility of formation of TpT in the C141A mutant is that TpT radical obtains an H atom from the residues on the protein such as Y99. We hence studied the enzyme reaction at varying dithionite concentrations. Dithionite possesses dual functions in the SPL C141A reaction: (1) reducing the $[4Fe\text{-}4S]^{2+}$ cluster to initiate the SAM cleavage reaction and (2) quenching the thymine radical to yield $TpTSO_2^-$. Were the protein to provide the needed H atom, the availability of this H atom would be constant. With a low dithionite supply, $TpTSO_2^-$ formation would be disfavored because of the lack of reactant; the level of production of TpT should be subsequently increased.

To focus on the latter function, we prereduced the cluster using a large access of dithionite and removed the unreacted dithionite by desalting columns. We then added varying amounts of dithionite or 10 mM DTT as the radical quenching reagent, together with SP and SAM, to reveal the quenching effect of dithionite on the SP repair reaction.

Surprisingly, at low dithionite concentrations, $TpTSO_2^-$ becomes an even more dominating product (Table 2). Increasing the dithionite concentration, however, favors the formation of TpT, as indicated by the ascending $TpTSO_2^-$:TpT ratio found in Table 2. At 10 mM dithionite, almost comparable amounts of $TpTSO_2^-$ and TpT were obtained. Were the H atom donor from the protein to be responsible for TpT formation, an opposite trend for $TpTSO_2^-$ and TpT versus dithionite concentration would be expected.

Table 2. $TpTSO_2^-$:TpT Ratios Found in (A) the SP Repair Reaction Mediated by a Prereduced C141A Mutant (10 μM) Supplemented with Varying Amounts of Dithionite or DTT and (B) the Photoreaction of TpTSPh (1 mM) Supplemented with Varying Amounts of Dithionite

Dithionite (mM)	0.02	0.05	0.2	1	10	10
(A)$TpTSO_2^-$/TpT	7.1 ± 1	5.8 ± 0.6	3.2 ± 0.3	2.8 ± 0.3	1.8 ± 0.3	<0.1
(B)$TpTSO_2^-$/TpT[a]	0.08 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.21 ± 0.02	0.44 ± 0.03	0.22 ± 0.02[b]

[a]The yield of the TpTSPh photoreaction is ~7%, making the concentration of the TpT radical generated from the photoreaction <7 μM. This concentration should be comparable to the TpT radical concentration produced by the SPL C141A mutant reaction. [b]The reaction was conducted in the presence of both 10 mM dithionite and 10 mM DTT.

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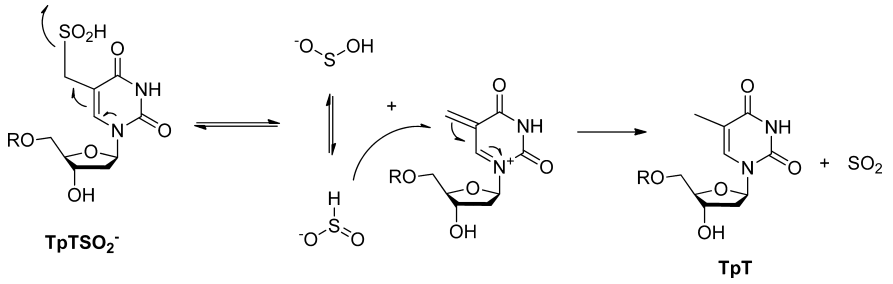
Scheme 5



We thus conclude that dithionite is directly responsible for TpT formation; TpT found in the mutant reaction mixture is unlikely generated via a direct H atom transfer process as that in the WT SPL reaction.[39] Although the addition of 10 mM DTT to the prereduced mutant resulted in an almost exclusive formation of TpT (Table 2),[47] the TpT here is clearly formed via a mechanism different from that in the regular SPL C141A reaction.

TpT Is Not Produced via $TpTSO_2^-$ Decomposition. A third possibility of TpT formation in the C141A mutant is via a $TpTSO_2^-$ degradation reaction as indicated in Scheme 5. This reaction resembles the reduction of the pyridinium salts by dithionite in the coenzyme NAD^+.[55−57] To test this hypothesis, we incubated $TpTSO_2^-$ with the enzyme in a pH 7.0 buffer for 1 h. No TpT was found as analyzed by HPLC. Moreover, we repeated the experiments in pH 7.0 and 9.0 Tris buffer in the presence and absence of UV light, respectively, as the neutral or mildly basic condition is suggested to favor SO_2 elimination.[55−57] Analysis of the reaction products revealed that $TpTSO_2^-$ is stable under these conditions and no TpT was observed. These observations suggest that the TpT found in the C141A reaction mixture originates directly from the TpT radical; it is not a secondary product resulting from the $TpTSO_2^-$ degradation process.

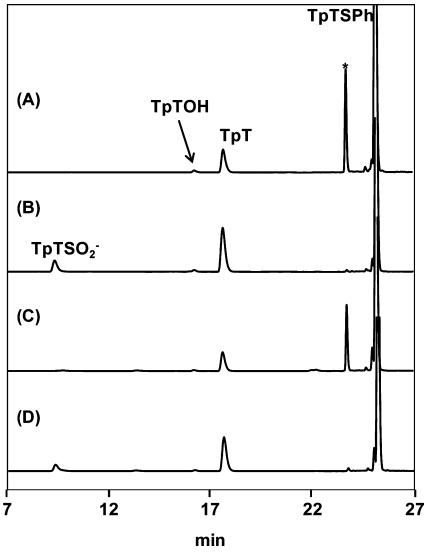


Figure 4. HPLC chromatograph of the TpTSPh (**5**) photoreaction conducted under 254 nm UV light in the anaerobic chamber. (A) Photoreaction in H_2O with 1 mM **5**. (B) Reaction A with 1 mM sodium dithionite. (C) Reaction A with 1 mM DTT. (D) Reaction A with 1 mM sodium dithionite and 1 mM DTT. The peak marked with an asterisk is likely due to the addition of TpT cation to **5** as indicated by ESI-MS, the nature of which is characterized in ref 74.

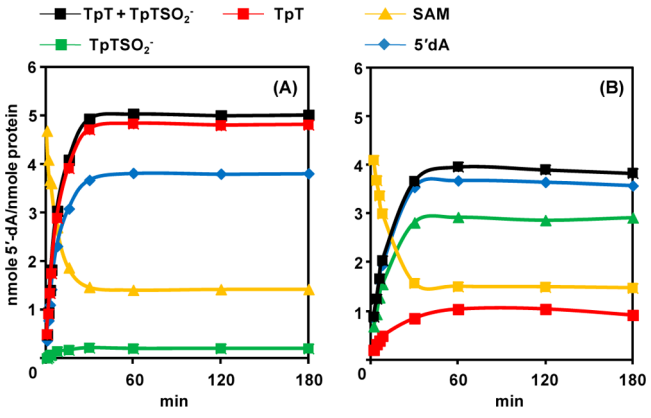


Figure 5. (A) Formation of TpT, $TpTSO_2^-$, and 5′-dA as well as the consumption of SAM in the WT SPL reaction. Five equivalents of SAM was added to initiate the reaction. A background level of $TpTSO_2^-$ was observed along with the formation of TpT. The ratio between the SP repair products (TpT and $TpTSO_2^-$) and 5′-dA is found to be 1.5:1 for the WT SPL reaction, suggesting that SAM is partially catalytic here. (B) Formation of TpT, $TpTSO_2^-$, and 5′-dA as well as the consumption of SAM in the SPL C141A reaction with 5 equiv of SAM supplemented. The ratio between the SP repair products (TpT and $TpTSO_2^-$) and 5′-dA was found to be 1.08:1 for the SPL C141A reaction, suggesting that SAM plays a noncatalytic role in the C141A reaction and is a cosubstrate. With the 5 equiv of SAM, the rate of repair of SP TpT by WT SPL was determined to be 0.41 ± 0.03 min^{-1} and that by SPL C141A mutant to be 0.14 ± 0.02 min^{-1}.

Formation of TpT in the TpTSPh (5) Photoreaction. The formation of TpT in the SPL C141A mutant reaction is neither due to the quenching of the TpT radical from the small molecules in the solution or from the residues on the protein nor due to $TpTSO_2^-$ degradation. To reveal the mechanism of TpT formation, we examined the TpTSPh photoreaction shown in Scheme 3 under an inert atmosphere using conditions roughly identical to those of the enzyme reaction. Surprisingly, $TpTSO_2^-$ becomes a minor species even when the dithionite concentration approaches 10 mM. Additionally, as shown in Table 2, as the dithionite concentration increased from 0.02 to 10 mM, the $TpTSO_2^-$:TpT ratio also increased, which is totally opposite from those observed in the SPL C141A reaction. As discussed below, $TpTSO_2^-$ formation likely occurs via a radical quenching mechanism, and it should be fairly favorable for the TpT radical to recombine with $\bullet SO_2^-$, yielding $TpTSO_2^-$. If TpT forms via an H atom abstraction process, the reaction is relatively unfavorable unless a very weak X−H bond is available from the environment. The absence of such an X−H bond thus

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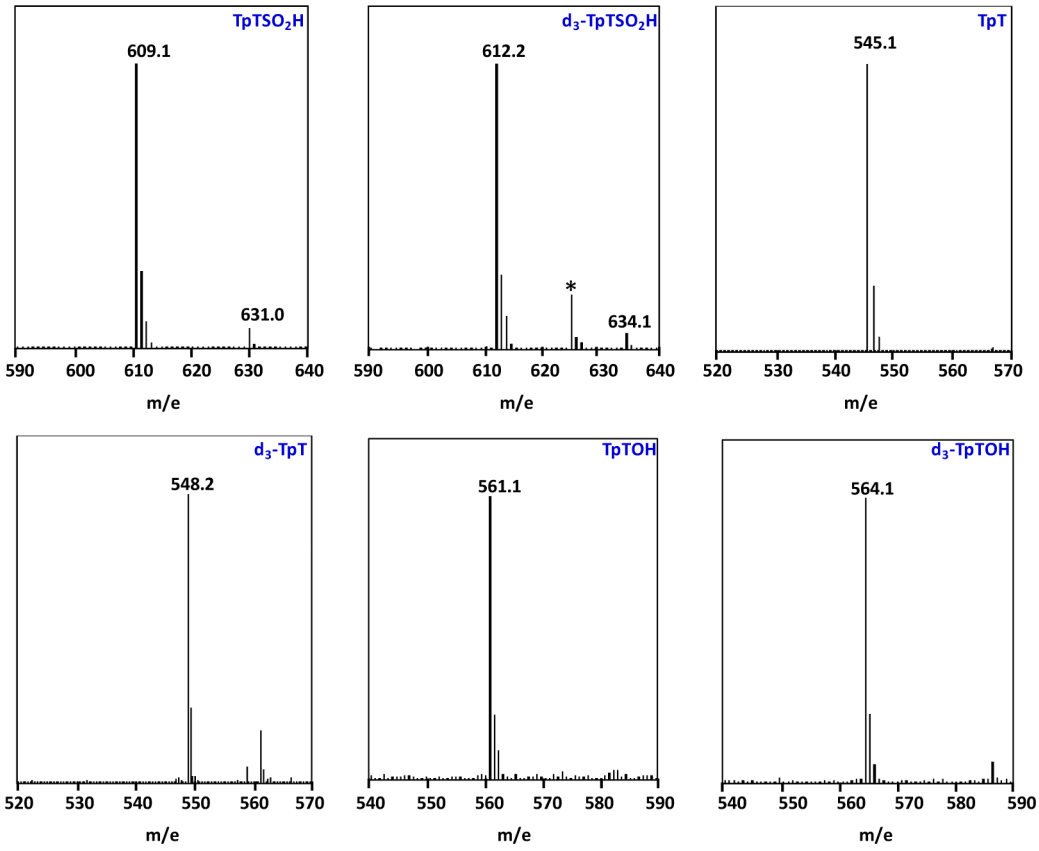

Figure 6. $[M - H]^-$ signals of TpT, $TpTSO_2^-$, and TpTOH isolated from the SP TpT and d_4-SP TpT repair reactions mediated by the SPL C141A mutant. The deuterium abstracted from the d_4-SP TpT during the repair process is not returned to the products, and the corresponding d_3 species were isolated for all three products. The asterisk denotes an impurity in the ESI-MS spectrum of d_3-$TpTSO_2^-$.

implies that TpT must form via a mechanism other than the radical-mediated H atom abstraction reaction.

Further analysis of the TpTSPh (**5**) photoreaction revealed that in the absence of dithionite, irradiating 1 mM **5** in H_2O still produces TpT (Figure 4). Addition of dithionite as a reductant improves the TpT yield by ~3-fold, suggesting that TpT forms via a radical reduction process. Because of the strong bond dissociation energy (BDE) for the O−H bond in water, it is highly unlikely that TpT formation proceeds via an H atom abstraction mechanism. Moreover, neither thiophenol nor diphenol disulfide was observed in the reaction, suggesting that they are unlikely to be involved in the reaction here. Thus, the reaction route in which the thiophenol first exchanges a proton with the solvent followed by S−H bond homolytic cleavage to donate the H atom to the TpT radical is unlikely to be involved here. The presence of an H atom donor (1 mM DTT) has little impact on the reaction, as reflected by the similar reaction patterns exhibited by panels A and C of Figure 4, further supporting this conclusion.

Consumption of SAM and Formation of 5′-dA. After elucidating the role of C141 in SPL catalysis and the mechanism behind TpT formation in the SPL C141A mutant reaction, we next attempted to further characterize the SPL C141A reaction. In radical SAM enzyme reactions, the ratio between 5′-dA and product is significant. A 1:1 ratio suggests that SAM is a cosubstrate, being consumed after each catalytic turnover. A 1:X (X > 1) ratio suggests that SAM is a cofactor, being regenerated after each catalytic cycle.[58] As shown in Figure 5B (also in the SI), SAM was consumed as the reaction progressed; the ratio between the consumed SAM (formed 5′-dA) and reacted SP was determined to be 1.08 ± 0.1, suggesting that in the SPL C141A reaction, SAM serves as a cosubstrate. As a control, analyzing the amounts of 5′-dA and TpT formed by the reaction using the carefully purified WT enzyme resulted in a ratio of 1.5 ± 0.2, which equals the ratio shown in the first several minutes of the WT SPL reaction in our previous report.[39] As shown in Figure 5, when plotted together, the differences between the C141A and WT SPL reactions are obvious, suggesting that SAM plays a partially catalytic role in the WT SPL reaction but a noncatalytic role in the C141A mutant.

As mentioned earlier, the protein purified from the Ni-NTA column contains some uncharacterized enzyme(s), which is very difficult to remove without a thorough washing on the ion-exchange column, even though SDS−PAGE suggests that the isolated protein is >99% pure. Different from the well-characterized *S*-adenosylhomocysteine nucleosidase (SAH nucleosidase) that is known to hydrolyze the 5′-dA to adenine,[59−62] the contaminant protein not only hydrolyzes the 5′-dA to adenine but also further degrades the adenine to an unknown product, which cannot be detected by the HPLC UV detector at 260 nm. The presence of this contaminant protein drastically disturbs the SPL activity studies, as the 5′-dA can no longer be used as a marker to reflect the SAM regeneration process. *Therefore, the disappearance of the 5′-dA in our previous report is not due to the SAM regeneration, but due to the presence of this contaminant enzyme. Figure 9B in our previous report is in error.*[39]

Carefully analyzing the amount of SAM left in the solution, we found that the amount of SAM could be slightly increased

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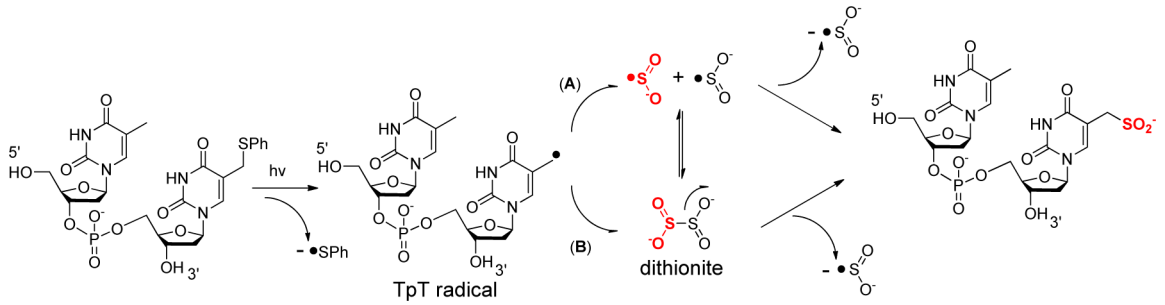
Table 3. Rates of Formation (min^{-1}) of $TpTSO_2^-$ and TpT and V_{max} KIEs in the SPL C141A Reaction

	without DTT			with 1 mM DTT		
substrate	SP TpT	d_4-SP TpT	V_{max} KIE	SP TpT	d_4-SP TpT	V_{max} KIE
$TpTSO_2^-$	0.1 ± 0.01	0.055 ± 0.005	1.78	0.1 ± 0.01	0.055 ± 0.005	1.70
TpT	0.02 ± 0.002	0.012 ± 0.001	1.67	0.026 ± 0.003	0.015 ± 0.002	1.73
$TpTSO_2^-$ and TpT	0.12 ± 0.01	0.067 ± 0.006	1.76	0.126 ± 0.013	0.07 ± 0.007	1.71

Scheme 6



by 0−0.2 equiv as the SP repair reaction slowed,[47] which may indicate that a small portion of SAM was regenerated in the WT SPL reaction. No increase in the amount of SAM is found in the SPL C141A mutant reaction. However, we are hesitant to draw any conclusion here as the SAM increase can be observed in only ∼50% of the WT SPL reactions we conducted (7 of 13 experiments). Despite the inconsistency, our findings could still be useful to other researchers as few experiments that have characterized the stoichiometry of SAM involved in a radical SAM reaction have been reported.

SPL C141A Reaction in the Absence of Excess Reductant. Being able to reliably measure the amount of yielded 5′-dA provides us a marker for accurately determining the reaction turnover numbers. In our previous report, after using the desalting column to remove the excess dithionite, the prereduced cluster of WT SPL was found to support 12 turnovers under ∼3 equiv of SAM.[39] Using our latest calibration curve, the number is now determined to be 5.4. This number still suggests that the WT SPL reaction has a closed catalytic cycle and is consistent with our latest results.

We performed a similar experiment with the C141A mutant to test if the C → A mutation disrupts such a cycle. After prereduction with dithionite for 30 min, the resulting $[4Fe\text{-}4S]^+$ cluster in the SPL C141A mutant supported only ∼0.4 turnover, with TpT being the dominant product when 10 mM DTT was supplemented. No turnover was observed in the absence of DTT, suggesting that being able to quench the TpT radical is the prerequisite for the occurrence of the SP repair reaction. Other thiol compounds can also rescue the SPL C141A reaction, but the yield of TpT would be even lower. The low concentration of DTT (<1 mM) has little effect in stimulating the reaction, suggesting that the thymine radical is well protected by the enzyme against external H donors. This finding is consistent with the result obtained in the C141 labeling studies described above that C141 appears to be protected in the enzyme binding pocket. As participants for the key H atom transfer process during the SP repair process, both the TpT radical and C141 are protected by the SPL enzyme.

The 0.4 turnover could be due to the incomplete reduction of the radical SAM cluster upon the 30 min dithionite treatment. As shown by our previous EPR experiment, a 30 min reduction led to only ∼40% SPL cluster reduction; a 1 h reduction resulted in ∼70% of the cluster being converted to the +1 oxidation state.[39] The observation is in line with the 20% $[4Fe\text{-}4S]^+$ cluster reduction observed by Broderick[63] and the 40% cluster reduction by Knappe[64] in PFL-AE studies upon prolonged reduction. More importantly, the difficulty in the externally added thiol being correctly positioned to quench the TpT radical may play a major role in the low yield of TpT as under an identical condition, >5 turnovers were observed for the WT SPL reaction.[39] Nevertheless, the <1 turnover in the C141A mutant suggests that the C141A mutant no longer possesses a closed catalytic cycle.

SPL C141A Reaction with d_4-SP TpT as the Substrate. As shown in Scheme 2, the WT SPL enzyme takes the H_{6proR} atom to initiate the SP repair reaction; the abstracted H atom, however, is not returned to the TpT product. In the aqueous buffer, the repair of d_4-SP TpT by WT SPL leads to the formation of d_3-TpT. As shown in Figure 6, all three products, $TpTSO_2^-$, TpT, and TpTOH, from the d_4-SP TpT repair reaction mediated by the C141A mutant contain only three deuteriums, suggesting that the deuterium abstracted by the 5′-dA• was not returned as expected. The C → A mutation disturbs the H atom back-donation step and the steps after it but likely leaves the previous steps intact.

Linear formations against reaction time can be obtained for $TpTSO_2^-$ and TpT species at a saturated substrate concentration; comparing the corresponding rates using d_0- and d_4-SP TpT as substrates reveals the $^DV_{max}$ KIE to be 1.7 ± 0.2 for both $TpTSO_2^-$ and TpT (Table 3). The identical KIEs for TpT and $TpTSO_2^-$ suggest that these species share the same rate-determining step. Thus, the $^DV_{max}$ KIE for the C141A mutant reaction can be reported as 1.7 ± 0.2, which is smaller than the KIE of 2.8 ± 0.3 exhibited by the WT enzyme.[39]

DISCUSSION

Photosynthesis of $TpTSO_2^-$. As shown in Scheme 3, the synthesis of $TpTSO_2^-$ took advantage of the weak C−S bond that is readily cleaved upon photoexcitation. The dithionite dianion has a small dissociation constant in water with a K_d of $\approx 10^{-6}$ mM;[65−68] autodissociation of $S_2O_4^{2-}$ results in the formation of $2•SO_2^-$. The $•SO_2^-$ radical readily recombines with the thymine allylic radical after photocleavage of the C−S bond in TpTSPh to yield $TpTSO_2^-$ (Scheme 6A). The $•SO_2^-$

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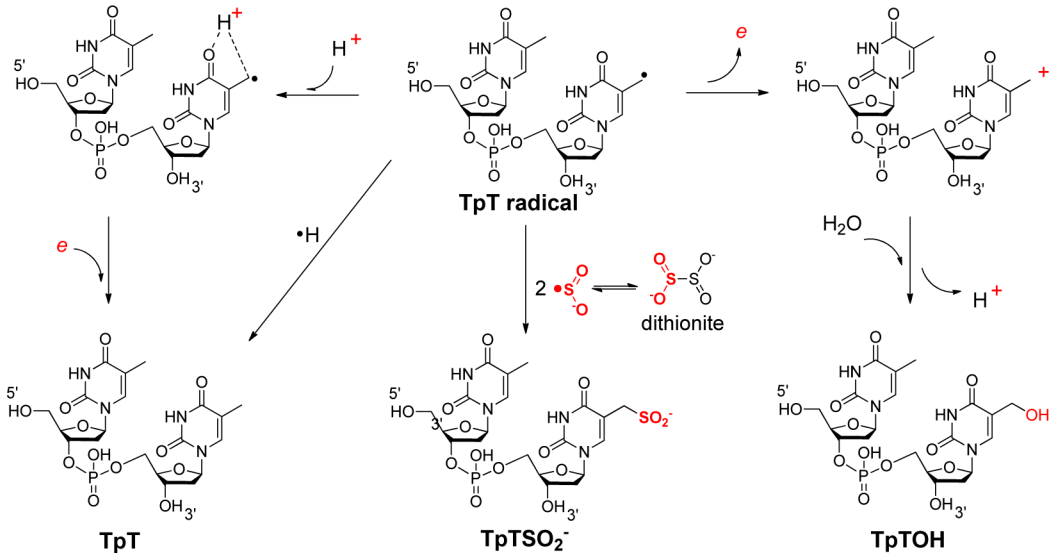
Scheme 7



produced by the dithionite autodissociation reaction was used to activate the initiator and trigger the radical polymerization of the vinyl chloride,[69] further supporting the mechanism in Scheme 6A.

Although the radical recombination is the most plausible mechanism, the radical-induced S−S bond replacement reaction as shown in Scheme 6B cannot be excluded. The thymine allylic radical attacks the disulfide bond in dithionite, replacing a SO_2^- moiety of the dithionite to form $TpTSO_2^-$. Thiyl radical-based S−S bond replacement is a common reaction encountered in biochemistry;[70] other organic radicals are implied to be able to induce the homolytic cleavage of the S−S bond as well.[71] Although the vast majority of the $TpTSO_2^-$ is believed to form via the radical recombination reaction, considering the millimolar dithionite concentration used in our synthesis, we cannot exclude the involvement of the radical replacement mechanism to produce a very minor amount of $TpTSO_2^-$.

It is worth pointing out in $TpTSO_2^-$ synthesis that the reaction should be conducted under an inert atmosphere. Were the photoreaction to be conducted in air, the product would contain ~30% $TpTSO_3^-$, the two-electron oxidation product of $TpTSO_2^-$. As $TpTSO_2^-$ is reasonably stable and exposing its aqueous solution to air for 24 h did not result in obvious oxidation (as monitored by LC−MS), we conclude that the oxidation likely occurred during the photosynthesis, where the UV light excited the O_2, which then oxidized the sulfinic acid to yield the sulfonate product $TpTSO_3^-$.

C141 Is Likely the H Atom Donor. To donate the H atom directly to the thymine allylic radical, the C141 SH moiety must be accessible to the substrate. Our observation that the C141 residue can be alkylated by iodoacetamide under the native condition suggests that this cysteine is solvent accessible and should be readily available to the thymine radical. Such a conclusion is also supported by the crystal structures of the *G. thermodenitrificans* SPL.[41] The similar behaviors of WT SPL and the SPL C141A mutant under the iodoacetamide treatment suggest that the C → A mutation is unlikely to change the protein structure, which is also supported by the structural data. Were C141 not to be the direct H atom donor but pass the H atom to another protein residue (Y99, for instance) during the course of the catalysis, the residue would also possess a weak X−H bond, which is perfectly positioned for the transfer of the H atom to the TpT radical. Different from the class I ribonucleotide reductase, where the radical species has to be passed to the substrate binding pocket before the ribonucleotide modification reaction is initiated,[72,73] the radical chain in the SPL reaction is initiated from the substrate. Given the weak BDE for the protein residues that are known to be involved in the radical relay process, were a protein residue other than C141 (for instance, Y99) to serve as the direct H atom donor, an H atom would be readily available to the TpT radical, producing TpT during the first turnover. Such a hypothesis conflicts with our observations as the formations of TpT and $TpTSO_2^-$ are linear from the very beginning of the SP repair reaction, suggesting that both species form at the same time.

Furthermore, were the putative residue to be responsible for H atom donation, an X• radical would be left behind on this residue. Consequently, the enzyme is likely to be modified (formation of a SO_2^- adduct, for instance) to quench this X• as the electron transfer chain is broken after the C → A mutation. However, the C141A protein is not modified after the reaction, as proven by the ESI-MS analysis.[47] Thus, even though our data tell us nothing about the proximity between C141 and the TpT radical, it is extremely unlikely that a residue other than C141 would directly donate an H atom to the TpT radical.

Formation of Three SP Repair Products. Among the three products, $TpTSO_2^-$, TpT, and TpTOH, formed in the SPL C141A reaction, $TpTSO_2^-$ likely forms via a radical recombination reaction as discussed above. The radical replacement mechanism may also play a very minor role in its formation.

The formation of TpT is more interesting. Although TpT is produced in both SPL WT and C141A mutant reactions, it likely forms via different mechanisms. In the WT SPL reaction, the SH moiety on C141 likely donates an H atom to the thymine radical to form TpT. Such a low-energy H donor is no longer present in the C141A mutant unless the reaction solution is supplemented with a large amount of thiols. As revealed by our thymine allylic radical activity studies, such a radical readily undergoes disproportionation and reduction reactions, with reaction rates likely comparable to that of the

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radical recombination reaction.[74] Actually, the fact that the presence of a large excess of thiol compounds has little impact on TpT formation further suggests that the H atom abstraction is an unfavorable reaction because of the relatively slow reaction rate.[74] A reduction of thymine radical is the most likely thymine formation mechanism here, which is likely involved in TpT formation in the C141A reaction as well.

However, a direct reduction of the thymine allylic radical is unfavored as the resulting thymine methyl anion is extremely unstable, which makes the thymine radical cation reduction the most favorable mechanism.[74] Because the reaction is conducted in water, the thymine C4═O bond must readily associate with a proton through the hydrogen bonding interaction with the aqueous solvent. Reducing the resulting TpT radical cation would generate TpT (Scheme 7). Such a radical cation reduction mechanism (proton-coupled electron transfer) has not received much attention in DNA biochemical studies.[75−78] Our data here argue that the thymine cation radical reduction pathway may play an important role in thymine damage repair, which has been largely overlooked in the past.

As shown in Table 2, in the SPL C141A mutant reaction, $TpTSO_2^-$ is the dominating product. Contrastingly, TpT prevailed during the photoreaction of TpTSPh. The trend of $TpTSO_2^-$:TpT ratio versus dithionite concentration is also different in these two reactions. We believe that the protein network plays a major role here in changing the reaction pattern. In the TpTSPh photoreaction, all reactants can freely interact in the solution. The cation radical reduction to produce TpT is relatively unaffected when the dithionite concentration increases, while the formation of $TpTSO_2^-$ becomes more competitive because of the availability of more reactants. Contrastingly, the TpT radical is well protected in the C141A binding pocket, as suggested by the observation that a large amount of externally added DTT (10 mM) is needed to quench the TpT radical. As shown by the CPD photolyase structure, the thymine C4═O bond is recognized by a protein residue via the hydrogen bonding interaction.[79] A similar recognition pattern is expected here. The preorganized enzyme−substrate complex likely hinders a proton from the solvent (or protein) to adopt the right position to associate with the allylic radical as shown in Scheme 7, thus slowing TpT formation and making $TpTSO_2^-$ formation the favorable reaction.

Similar to that in the TpTSPh photoreaction, the dithionite is likely to serve as the electron donor in the SPL C141A mutant reaction to facilitate the formation of TpT as well. However, we cannot exclude the possibility that the protein-harbored $[4Fe\text{-}4S]^+$ cluster donates the needed electron. Nevertheless, the TpT likely forms via a proton-coupled electron transfer process in the SPL C141A mutant, which is different from the H atom transfer mechanism in the WT SPL reaction.

TpTOH was isolated in previous thymine oxidation studies; its formation is typically induced by the oxidation of the TpT allylic radical by either addition of O_2[80] or radical recombination with a •OH radical.[81] Our latest data suggest that it can also be produced via a thymine radical disproportionation reaction.[74] As the enzyme reaction was conducted anaerobically, O_2 can be ruled out, making the radical disproportionation the most likely mechanism. The thymine cation then reacts with water to produce TpTOH (Scheme 6). Under the reducing conditions adopted in the enzyme reaction, the thymine cation must be reductively quenched. Consequently, only a tiny amount of TpTOH was produced.

Enzyme Activity and Kinetic Isotope Effect. The bond dissociation energy (BDE) of the cysteine S−H bond is suggested to be ∼3−4 kcal/mol lower than that of the allylic C−H bond.[82−85] Assuming that the SPL configuration is such that C141 is in the vicinity of the thymine allylic radical, the H atom back transfer step must be heavily favored and should not affect the overall SP repair rate. In contrast, the C → A mutation removes this natural H atom transfer process; the TpT allylic radical either has to be released before it is quenched or has to wait until a quenching reagent diffuses into the enzyme binding pocket to react with the radical and generate a stable product. Such a quenching step must be slow, resulting in the ∼3-fold rate reduction exhibited by the C141A mutant relative to that of the WT enzyme (Figure 5).

Theoretical calculations suggest that the two steps involved in the H atom exchange with 5′-dA possess the highest energy barriers.[82,83,86,87] Of these two steps, the H atom back-donation step has the higher barrier because of the difference in energy between the allylic radical and the C−H bond associated with the methyl moiety of 5′-dA. Although our data suggest that it is the SH moiety of C141, not the CH_3 group of 5′-dA, that serves as the H atom donor for the thymine allylic radical, the resulting protein radicals are likely to be stable as well. The regeneration of the 5′-dA• is expected to be slow.[72,88] Thus, it is reasonable to hypothesize that the rate-determining step in the SPL reaction is the regeneration of 5′-dA•; all steps prior to that, including the H atom abstraction by the 5′-dA• from C6 of SP to obtain the SP radical, belong to the "rate-determining zone".[89] The rate constants from all steps in this zone will contribute to the V_{max} KIE.[89−91] The intrinsic isotope effect caused by the reaction between the 5′-dA• and the deuterated SP substrate is likely to be "diluted" by other steps in this zone, resulting in a moderate V_{max} KIE of 2.8 for the WT SPL reaction.[39]

The rationale described above assumes that SAM is regenerated after every catalytic cycle. However, the observed TpT:5′-dA ratio of 1.5 suggests that the roughly two-thirds of the formed 5′-dA and methionine molecules exchange with SAM from the environment during the SPL catalysis. Currently, no data are available to help us determine how fast this exchange step is. However, it is clear that the reaction rates we observed here seem to be the average of two reactions: one results in SAM regeneration, and the other results in the exchange of 5′-dA and methionine with SAM. Therefore, the V_{max} KIE here is unlikely to represent the true V_{max} KIE occurring in vivo. To determine the V_{max} KIE for the in vivo reaction, a method has to be found to prevent the SAM/5′-dA-methionine exchange from occurring.

Such a moderate V_{max} KIE is further reduced in the C141A mutant reaction because of the slower thymine radical quenching process. Although changing the SP TpT to deuterated d_4-SP TpT slows the SP repair reaction, the repair rate was reduced to a lesser extent, resulting in a V_{max} KIE of 1.7 ± 0.2 for both $TpTSO_2^-$ and TpT formations. The almost identical KIE exhibited by these two products indicates that despite the different quenching mechanism, the reaction rates for this step must be similar. In comparison with that of the WT SPL,[39] the reduced V_{max} KIE in the mutant reaction suggests that the H abstraction step by 5′-dA• becomes even less rate-determining.[92] The radical quenching step likely becomes the new rate-limiting step, as the SAM regeneration

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process is no longer possible. However, in the C141A reaction, SAM exchanges with the formed 5′-dA and methionine at the end of each catalytic cycle. Were this step to be rate-determining, a slower TpT radical quenching step would still lead to the reduced V_{max} KIE as observed here.

SAM Regeneration. Recycling SAM after each turnover is probably the most efficient choice for the germinated spores. UV irradiation can convert as much as 8% of the total thymine in bacterial genomic DNA to SP;[4,93] these SPs must be repaired by SPL for the bacteria to survive.[7,94,95] Germinated spores have many urgent needs in resuming their normal life cycle with limited resources; making good use of every SAM molecule must be important. The in vitro reactions under a large excess of SAM reported here are unlikely to happen in vivo.

Despite the importance of SAM regeneration for the in vivo reaction, such a process is not fully established by in vitro experiments. Previous efforts to establish SAM regeneration were conducted via a tritium labeling experiment in SPL and lysine-2,3-aminomutase (LAM).[10,20,37] In both cases, the percentage of label transferred from the substrate into SAM was thought to be very low.[39,96,97] Recent proposals by Eguchi in the studies of BtrN[98] and by Liu in the studies of DesII,[27] both of which are radical SAM enzymes, suggested that SAM cleavage, as well as the subsequent H atom abstraction by the 5′-dA• from enzyme substrates, may be reversible. Such reversible processes could explain the incorporation of tritium into SAM as previously observed in the SPL reaction.[10]

SAM becomes a cosubstrate in the C141A reaction. This is reasonable considering that C141 is a part of the catalytic pathway; disrupting this pathway makes the closed catalytic cycle open. In the WT SPL reaction, the TpT:5′-dA ratio of 1.5 ± 0.1 suggests that only one-third of SAM behaves like a cofactor. We would like to observe SAM regeneration on a more significant scale; however, this seems unlikely using the dinucleotide SP TpT as the enzyme substrate.

The small regeneration scale predicted here highlights the difficulty in directly detecting the SAM regeneration process. In B-12 enzymes, where no excess B-12 cofactor is needed, the 5′-dA recycling process can be readily observed. In contrast, the binding of SAM to the radical SAM enzyme is weak; to achieve enough enzyme activity, 2−3 equiv of SAM is needed.[10,39] This then determines that there are always excess SAM molecules available in the reaction buffer, which can readily exchange with the yielded 5′-dA and methionine, making the SAM recyclization unnecessary. To study the SAM regeneration process, a limited supply of SAM is required. In addition, the enzyme has to bind SAM (5′-dA and methionine) tightly. Such a condition may be achievable by using a tightly bound SP substrate. As shown in Broderick's experiments, using a SP containing plasmid DNA, 1 equiv of SAM can support >500 turnovers.[10] How tighter substrate binding enhances the SAM recyclization is currently unclear.

How is SAM regenerated in the WT enzyme? Cysteine-based thiyl radical is known to abstract an H atom from 5′-dA in class II ribonucleotide reductase (RNR) to produce the 5′-dA• before it recombines with cobalamin to regenerate the adenosylcobalamin at the end of each catalytic cycle.[99] However, in class II RNR, the 5′-dA• interacts with only the active-site cysteine and does not associate with the substrate at any point of the catalytic cycle. In contrast, in SPL, the 5′-dA• abstracts the H_{6proR} atom of the 5′-T of SP to initiate the repair reaction,[39] while C141 likely provides the needed H atom to the methyl radical at the 3′-T to generate the repaired TpT. Were the thiyl radical on C141 to be directly responsible for H atom abstraction from 5′-dA to regenerate SAM, the TpT would be released first, followed by a major protein conformational change before the thiyl radical can interact with the 5′-dA. On the other hand, it is reasonable for the thiyl radical to oxidize a neighboring protein residue before the reaction with 5′-dA occurs. In that case, SPL must harbor an electron transfer pathway with C141 as a key element. As shown in the *G. thermodenitrificans* SPL structure, a conserved tyrosine is found between the conserved cysteine and SAM.[41] Although protein conformational change is expected after the methylene bridge in SP and the SAM C−S bond are cleaved, the conserved tyrosine is still likely to be involved in SPL catalysis and SAM regeneration. The role of this tyrosine residue in SPL reaction is to be investigated in the future.

Alternative Route for SAM Regeneration? Although SAM regeneration is the most reasonable hypothesis, we cannot rule out other possibilities for the SPL-catalyzed SP repair reaction. For instance, what if the 5′-dA generated from the SAM reductive reaction is responsible only for the first catalytic cycle and the protein radical generated takes over and catalyzes the rest of the turnovers? Under in vitro conditions, the presence of excess SAM and reductant determines that both catalytic routes may occur.

The mechanistic studies of SPL thus far suggest that the enzyme reaction can be divided into two half-reactions: (1) SP repair with TpT formation and (2) SAM regeneration. These two half-reactions are tightly coupled at a low SAM concentration in vivo but are uncoupled with sufficient SAM. Progress has been made in elucidating the half-reaction involved in SP repair and TpT formation; however, the other half, SAM regeneration, remains largely unclear. Future work should focus on the second half-reaction before a clear mechanistic understanding of SPL catalysis can be achieved.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses and characterizations of $TpTSO_2^-$ and TpTOH, protein purification and characterization, and enzyme activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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