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A Radical Transfer Pathway in Spore Photoproduct Lyase

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Abstract

Spore photoproduct lyase (SPL) repairs a covalent UV-induced thymine dimer, spore photoproduct (SP), in germinating endospores and is responsible for endospores' strong UV resistance. SPL is a radical SAM enzyme, which uses a [4Fe-4S]¹⁺ cluster to reduce the *S*-adenosyl-L-methionine (SAM), generating a catalytic 5'-deoxyadenosyl radical (5'-dA•). This in turn abstracts an H atom from SP, generating an SP radical that undergoes β scission to form a repaired 5'-thymine and a 3'-thymine allylic radical. Recent biochemical and structural data suggest that a conserved cysteine donates an H atom to the thymine radical, resulting in a putative thiyl radical. Here we present structural and biochemical data which suggest that two conserved tyrosines are also critical in enzyme catalysis. One (Y99_(BS)) in *Bacillus subtilis* SPL) is downstream of the cysteine, suggesting that SPL uses a novel hydrogen atom transfer (HAT) pathway with a pair of cysteine-tyrosine residues to regenerate SAM. The other tyrosine (Y97_(BS)) has a structural role to facilitate SAM binding; it may also contribute to the SAM regeneration process by interacting with the putative •Y99_(BS) and/or 5'-dA• intermediates to lower the energy barrier for the second H-abstraction step. Our results indicate that SPL is the first member of the radical SAM superfamily (comprising more than 44,000 members) to bear a catalytically operating HAT chain.

Introduction

5-Thymine-5,6-dihydrothymine, commonly called spore photoproduct or SP, is the exclusive DNA photo-lesion identified in bacterial endospores (1–3). SP accumulates in the bacterial sporulation phase and is repaired rapidly at the early germination phase, thus posing little threat to bacterial survival. The key enzyme responsible for SP repair is spore photoproduct lyase (SPL) (1–3). SPL is a radical SAM enzyme which utilizes the classic tri-cysteinate CX₃CX₂C motif to bind a [4Fe-4S] cluster. At the 1+ oxidation state, the cluster

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Supporting Information available

Experimental details and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

donates an electron to *S*-adenosyl-L-methionine (SAM), to cleave the C5'-S bond associated with the sulfonium ion and generate a 5'-deoxyadenosyl radical (5'-dA•) and methionine (4–15). This 5'-dA• abstracts an H atom from SP to initiate the radical cascade reaction to restore TpT (Figure 1) (6, 16).

Yang *et al.* recently showed that the 5'-dA• stereoselectively abstracts the H_{6proR} atom of SP (Figure 1) (13); the resulting SP radical then fragments to generate a 3'-thymine allylic radical. This radical abstracts an H atom from a protein residue to yield the repaired thymine pair, TpT. The source of this H atom has recently been proposed to be the C141 residue in *Bacillus subtilis* (*Bs*) SPL (5, 12–14), rather than the 5'-dA as proposed previously (6, 7). The new mechanism is strongly supported by crystal structures of wild-type (WT) and mutant SPL from *bacterium Geobacillus thermodenitrificans* (*Gt*) solved by Benjdia *et al.* (17). The structure shows that the distance between the methylene bridge of the dinucleoside SP and the conserved cysteine is 4.5 Å (Figure 2), which is close enough for the cysteine being the direct H atom donor (17). SPL_(Gt) shares ~ 77% sequence identity with SPL_(Bs), but exhibits a -1 sequence shift for the conserved amino acids.

Previous work showed that SP repair by the SPL C141A_(Bs) mutant results in the formation of TpTSO₂⁻ as the major product and TpT as the minor one (12). Since neither the equivalent mutation (C140A_(Gt)) nor the isosteric C140S_(Gt) mutation changes the *Gt* SPL structure (17), Benjdia *et al.* concluded that that removal of the cysteine disturbs the H atom back-donation process in SPL catalysis (17). Enzyme kinetic studies of the SPL C141A_(Bs) mutant further demonstrate that both products form simultaneously, indicating that the conserved cysteine must be the intrinsic H atom donor to the substrate radical (14). Such an H atom transfer (HAT) process leaves a thiyl radical on this cysteine. As SAM is suggested to be regenerated at the end of each catalytic cycle (6, 13), the thiyl radical must be involved in this regeneration process. Here we present experimental evidence to suggest that besides the cysteine, a couple of tyrosines are also essential for catalysis.

Materials and Methods

General

The DNA-modifying enzymes were purchased from Fermentas Life Sciences (Glen Burnie, MD). Oligonucleotide primers were obtained from either Integrated DNA Technologies (Coralville, IA) or Eurofins MWG operon. *E. coli* BL21 (DE3) and expression vector pET-28a were purchased from Novagen (Madison, WI). The construct containing the SPL_(Bs) gene was co-expressed with plasmid pDB1282, which was a generous gift from Prof. Squire Booker at the Pennsylvania State University. 5'-deoxyadenosine (5'-dA) and *S*-adenosylmethionine (SAM) were purchased from Aldrich and used without further purification. All other buffers and chemicals were of the highest grade available.

Construction of the SPL mutant expression vectors

The *spIB* gene was cloned from the *Bs* chromosomal DNA (strain 168) into the in pET-28a vector with a N-terminal His₆-tag as previously described (13). Site-directed mutagenesis was performed to obtain the desired mutations with the QuickChange site-directed mutagenesis kits from Stratagene under the manufacturer's instruction. The synthetic oligonucleotide primers used are listed in Table S1. The constructs were transformed into *E. coli* 10 *G* chemically competent cells purchased from Lucigen Corporation (Middleton, WI) for isolation and amplification of the resulting plasmid DNA. The resulting vectors were then co-transformed with the pDB1282 vector into *E. coli* BL21 (DE3) for protein over-expression as described before (13). The Y98F_(Gt) mutant used for the crystal structure derives from the gene *GTNG_2348* coding for SPL from *G. thermodenitrificans*. Site-

directed mutagenesis was performed by QuickChange® PCR mutagenesis using the plasmid pETM11-His-tag-SPL previously described (17), and the primers listed in Table S1.

Expression and purification of SPL mutants

Both the WT enzyme and the tyrosine mutants for SPL_(Bs) were expressed in lysogeny broth (LB) medium containing the appropriate antibiotics as previously described (13). The proteins were purified via Ni-NTA chromatography followed by an 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 containing 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 by 2-fold to reduce the salt concentration to 250 mM and saved for activity studies.

The expression and purification conditions used for the SPL Y98F_(Gt) mutant protein were similar to the WT enzyme (17). The harvested cells were re-suspended in buffer A (50 mM Tris-HCl pH 8, 500 mM NaCl, 10 mM MgCl₂, 10% glycerol) supplemented by 5 mM 2-mercaptoethanol, 1 tablet of protease inhibitor cocktail (Complete, EDTA-free, Roche), 4 µg.ml⁻¹ of DNase I (Roche), 4 µg.ml⁻¹ of RNase (Roche) and 0.1 mg.ml⁻¹ lysozyme (Sigma-Aldrich) and disrupted by sonication. After ultracentrifugation, the supernatant was loaded on a Ni-NTA (Qiagen) gel column equilibrated with buffer A containing 20 mM imidazole (Sigma-Aldrich). The mutant protein was eluted with 500 mM imidazole which was then removed by a desalting column (Sephadex G-25, GE healthcare). The SPL Y98F_(Gt) protein was further purified by heparin affinity column chromatography (GE Healthcare) using 50 mM Tris-HCl at pH 8, 300 mM NaCl, 1% glycerol and 3 mM dithiothreitol as initial buffer.

Protein, iron, and sulfide assays

Routine determinations of protein concentration were conducted by the Bradford method (18), 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 (19). Iron content was determined using *o*-bathophenanthroline (OBP) under reductive conditions after protein digestion in 0.8% KMnO₄ and 1.2 M HCl as described by Fish (20). Sulfide assays were carried out using the method described by Beinert (21).

Enzyme activity assay

Typically, a reaction mixture contained 30 µM SPL_(Bs) enzyme (or tyrosine mutant), 1 mM correspondent SP TpT substrate, and 150 µM SAM in a final volume of 400 µL of buffer containing 25 mM Tris-HCl, 300 mM NaCl and 10% glycerol at pH 7.0. Sodium dithionite (final concentration 1 mM) was added as a reductant to initiate the enzyme reaction. The reactions were carried out under anaerobic conditions at ambient temperature for various periods of time. Under the conditions of the assay, the formation of TpT was linear with time for up to 15 min. At each time point, 90 µL of the solution was taken out to an Eppendorf tube and quenched by 10 µL of 3 M HCl. After removing the protein residues via centrifugation at 15,000 rpm for 20 min, the resulting supernatants were loaded onto HPLC, separated and analyzed via the previous procedure (13).

Deuterium Kinetic Isotope Effects (KIEs)

The apparent (^D V) KIEs for the WT SPL_(Bs) and C141A_(Bs) mutant were reported in our previous publications (13, 14). The apparent KIEs for both Y/F mutants were determined in

a similar manner by direct comparison of the initial rates with 1 mM of SP TpT and d_4 -SP TpT respectively (22). As pointed out by Cleland, this is the only method for apparent KIE determination in enzyme reaction (23).

There are three possible procedures to determine the competitive ($D(V/K)$) KIE, comparison of reciprocal plots with labeled and unlabeled substrates, internal competition, and equilibrium perturbation (23, 24). As shown previously, we were unable to obtain a meaningful reciprocal plot due to the extremely low enzyme activity using the dinucleotide SP TpT at a low substrate concentration (13). The perturbation experiment only works for reactions that are readily reversible (23, 24); such a requirement is obviously not fulfilled in our SP repair reaction. Therefore, we adopted the internal competition approach, measuring the competitive KIE with an equimolar mixture of SP TpT and d_4 -SP TpT (1 mM total concentration). The reaction was quenched by addition of HCl at different time points, the amount of TpT formed was determined by HPLC, and the deuterium content of the resulting TpT was subsequently determined by ESI-MS spectrometry using the Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer. The data was acquired via Agilent MassHunter Workstation Data Acquisition (B.03.00) and analyzed via Qualitative Analysis of MassHunter Acquisition Data (B.03.00) software.

At the steady state, the reaction rate can be described below (25):

$$v = (k_{\text{cat}}/K)[E][S] \quad \text{Thus, for competing substrates, } \frac{v_{\text{SP}}}{v_{d_4\text{-SP}}} = \frac{(k_{\text{cat}}/K)_{\text{SP}}[E][\text{SP}]}{(k_{\text{cat}}/K)_{d_4\text{-SP}}[E][d_4\text{-SP}]}$$

$$\text{As } \left(\frac{[\text{SP}]}{[d_4\text{-SP}]} = 1\right), \quad D(V/K) = \frac{v_{\text{SP}}}{v_{d_4\text{-SP}}}$$

Therefore, the competitive KIE is reflected by the ratio between the two reaction rates when the concentrations of SP TpT and d_4 -SP TpT are equal ($t = 0$). Due to the KIE, as the reaction progresses, the SP TpT and d_4 -SP TpT concentrations are no longer the same. In our experiments the KIEs at varying time points were measured at relatively low extents of reaction of between 1% and 10%. Under these conditions the isotopic composition of the SPs varies approximately linearly with the extent of reaction. The competitive KIE was therefore calculated by linear extrapolation of the measured KIEs to zero extent of reaction when the concentrations of SP TpT and d_4 -SP TpT were equal (Figure S6) (26).

One potential problem with such a treatment is the assumption that the enzyme reaction is at the steady state. Both the Y99F_(BS) and Y97F_(BS) mutants exhibit very short linear repair activities (1–2 turnovers with unlabeled substrate), which may bring uncertainty that the steady state hypothesis used above may not be valid. We therefore used a more general approach as defined by Cleland to reexamine the competitive KIEs from these internal competition reactions (24). During the internal competition experiments, the $D(V/K)$ can be calculated via the following formula:

$$D(V/K) = \log [1 - f / (X_H + X_D R_p / R_0)] / \log [1 - f / (X_H + X_D R_0 / R_p)]$$

where f = fraction of reaction for unlabeled SP TpT (d_4 -SP TpT is treated as an inhibitor); R_0 = mass ratio of starting substrates (SP TpT/ d_4 -SP TpT) ≈ 1 ; R_p = mass ratio of product at fraction of reaction; X_H and X_D are initial mole fractions of unlabeled and deuterium labeled substrates. In our experiments, both X_H and $X_D = 0.5$.

The f was determined by comparing the amount of TpT product obtained with the initial amount of unlabeled SP TpT substrate added. The R_p can be readily determined by product

analysis via ESI-MS spectrometry. We thus re-calculated the competitive KIEs and listed them in Table S3–S5 in SI. These KIEs agree reasonably well with those determined under steady state assumption. We therefore utilized the values determined via steady state kinetics for our discussions in this report.

EPR experiments for *BS* SPL and mutants

Continuous wave (CW) EPR spectra were recorded on a modified Varian spectrometer at 35 GHz (“Q”-band) and 2 K for SPL Y97A-Y99A_(BS) mutant (27). The as-isolated mutant (300 μ M, 3.0 iron/protein) was reduced with 2 mM sodium dithionite inside the anaerobic chamber for 0.5 hr, transferred to the EPR tube, and immediately frozen in liquid N₂. The EPR spectra were recorded as described previously (13). The EPR simulations performed using the program QPOW (28) as modified by J. Telser. The EPR samples for WT SPL_(BS), Y97F_(BS) and Y99F_(BS) mutant were prepared in the anaerobic chamber by loading 200 μ L of protein (~200 μ M), which was reduced with 2 mM sodium dithionite for 0.5 hr, into 4mm o.d. quartz tubes (Wilmad). The samples were frozen in liquid N₂ and shipped with a cryoshipper. EPR spectra were recorded on a Bruker E580 spectrometer using an ER4116DM dual-mode resonator in perpendicular mode, resonating at 9.633 GHz (X-band). The spectra were acquired at 10K using an in-cavity liquid helium flow cooling system (Oxford ESR900 cryostat, Oxford ITC503S temperature controller). The microwave power was 1 mW, well below the estimated half-saturation power of 10 mW (data not shown). Further experimental parameters: modulation amplitude 1 mT, modulation frequency 100 kHz, sweep rate 1.2 mT/s, number of scans 11 (WT) and 7 (Y99F_(BS) and Y97F_(BS)).

Crystallization and structure determination of the SPL Y98F_(Gt) mutant

The purified SPL Y98F_(Gt) protein was concentrated (30,000-MWCO spin concentrator, Amicon, Millipore). The iron-sulfur cluster was reconstituted under anaerobic conditions in a glove box (Belle), after which, the mutant protein was applied on a desalting column. As for the WT SPL_(Gt), crystals of the substrate-free Y98F_(Gt) mutant grew in a hanging-drop vapor diffusion setup using 70 mM octanoyl-N-hydroxyethylglucamide (Hampton Research), 200 mM lithium sulfate, 100 mM Tris-HCl at pH 9 and 19–27% (wt/vol) PEG 8000. Single crystals were harvested into a cryoprotectant containing 15% (v/v) ethylene glycol before flash cooling in liquid N₂ inside the glove box. Diffraction data were collected at the PX-II beam line of the Swiss Light Source. Data collection statistics are given in Table S3. The WT SPL structure was used to phase the Y98F_(Gt) mutant structure by molecular replacement. Data were processed with XDS and refinement of the Y98F_(Gt) mutant was performed using REFMAC. The structure superposition was carried out with Coot using the SSM program and including all residues.

Results and Discussion

As shown in Figure 2, a conserved tyrosine (Y98_(Gt)) is located between the C140_(Gt) and the bound SAM in the *Gt* SPL active site, suggesting that it is likely involved in SPL catalysis (17). Furthermore, most radical SAM enzymes possess an aromatic residue Φ located around the third cysteine in the radical SAM motif CXXXCX Φ C, suggested to maintain the position of SAM in the enzyme binding pocket through several interactions (17). The conserved Φ residue (Y96_(Gt)) is indeed involved in a weak hydrogen bonding interaction with SAM (Figure 2). We hence hypothesize that one or both of these tyrosine residues may act as an H atom relay between the thiyl radical and 5'-dA, and are thus involved in the SAM regeneration process.

To test this hypothesis, we mutated both tyrosines to alanines in SPL_(BS). The resulting Y97A-Y99A_(BS) double mutant still contains an intact [4Fe-4S] cluster, as demonstrated by

the characteristic UV absorption at 420 nm and the iron-sulfur content analysis (22). After reduction by dithionite, the resulting $[4\text{Fe-4S}]^{1+}$ cluster exhibited an EPR signal similar to the wild-type (WT) enzyme (22). Thus, the Y→A double mutation did not affect the stability of the iron-sulfur cluster. However, incubating the Y/A double mutant with excess SAM and dinucleotide SP TpT under reducing conditions did not yield any TpT product during a 3-hr reaction, long enough for multiple turnovers by active enzyme (13, 14). This inactivity suggests that removing these tyrosines disrupts the interaction between the phenol ring of the Y97_(Bs) and the adenine ring of SAM, subsequently affecting SAM binding and/or reductive cleavage. Since the ring-ring interaction is conserved in the Y→F mutation, the SP repair reaction should not be affected in the Y97F-Y99F_(Bs) double mutant. However, compared to the WT SPL_(Bs) reaction, the Y/F double mutant reaction is at least 100 fold slower (Table 1) (13).

Compared to tyrosine, phenylalanine retains the aromatic ring, but does not support the radical propagation reaction due to the loss of the OH moiety (29, 30). Should any tyrosine be involved in the radical reaction, the SPL catalysis/SAM regeneration process would be altered by the Y→F mutation. We thus constructed the Y97F_(Bs) and Y99F_(Bs) single mutations respectively in SPL_(Bs) and investigated their impacts on the SP repair reaction. The $[4\text{Fe-4S}]$ cluster remains intact in these Y→F mutants, as proved by iron-sulfur content analysis and EPR spectroscopy (22).

The resulting Y→F single mutants are able to repair SP in the presence of excess dithionite and SAM (Figure 3 and Table 1). However, the reaction rates are 3-fold and 7-fold lower for the Y97F_(Bs) and Y99F_(Bs) mutants, respectively, than for the WT enzyme (13, 14), but comparable to that of the C141A_(Bs) mutant (14). Under saturating substrate conditions (30-fold of enzyme concentration), linear repair activity was observed during the first 2~5 turnovers for the WT SPL_(Bs), C141A_(Bs), and Y97F_(Bs) mutants; afterwards the linear response is lost, likely due to enzyme inactivation. No burst kinetics was observed for these enzymes. For the Y99F_(Bs) mutant, the first turnover results in the fastest reaction and the reaction slows down after that (22). However, given the low activity of this mutant, it is difficult to conclude whether the observed rate reduction is due to enzyme inactivation or pre-steady state kinetics. All reactions slow down when d_4 -SP TpT, which has the H_{6proR} position deuterated (Figure 1), is used as a substrate. Comparing the initial reaction rates using the unlabeled SP and d_4 -SP TpT as substrate respectively gives us the traditional $^D V$ KIE (23). Given the very short “steady state” for our enzyme reactions, all $^D V$ KIEs were determined within the first two enzyme turnovers except that for the Y99F_(Bs) mutant, which was determined within the first turnover, thus we term the derived $^D V$ KIEs apparent KIEs. Analyzing the ratio of TpT and d_3 -TpT (the abstracted deuterium is washed out during catalysis (13)) via mass spectroscopy results in the $^D(V/K)$ KIE, which is termed competitive KIE in our discussion.

Theoretical calculations suggest that in the WT SPL reaction, both H atom abstraction steps associated with 5'-dA (5'-dA formation and 5'-dA• regeneration) have high energy barriers (31, 32). Between them, it is more difficult to take an H-atom from 5'-dA, indicating that 5'-dA• regeneration is likely the rate limiting (irreversible) step (31). All previous steps, including the H atom abstraction from SP to form 5'-dA, belong to the “rate determining zone” (33) and contribute to the rate determining process (34). Thus, the intrinsic deuterium isotope effect associated with H-abstraction from the d_4 -SP TpT could be weakened by the subsequent catalytic steps. These computations consider SAM to be regenerated after each catalytic cycle (31, 32), as indicated by a previous experimental finding that using SP containing plasmid DNA, one molecule of SAM was suggested to support > 500 turnovers (6). However, with the dinucleotide SP TpT as substrate, one SAM molecule is found to support only 1.5 turnovers in our WT SPL_(Bs) reaction probably due to the weak affinity of

the substrate to the enzyme (14). This result suggests that roughly two thirds of the formed 5'-dA and methionine molecules exchange with the excess of SAM from the environment during SPL catalysis. Although no data is available on how fast this exchange step is, the fact that the competitive KIE (3.4 ± 0.3) is slightly higher than the apparent KIE (2.8 ± 0.3) for the WT SPL_(BS) indicates that the SAM binding or product releasing steps are also involved in the rate limiting process (34). As the reaction rates we observed are the average of two reactions, with one involving SAM regeneration and the other including SAM exchange with 5'-dA, these numbers are unlikely to represent the true $^D V$ and $^D(V/K)$ KIEs resulted from SP containing oligomeric DNA. Therefore, although important mechanistic insight can still be obtained from our KIE studies, a systematic data analysis using mathematical models is difficult to perform due to the very complicated reaction process in our system.

Similar results were obtained in the C141A_(BS) mutant reaction. Comparing with the WT SPL reaction, the apparent KIE is reduced to 1.7 ± 0.2 . Furthermore, the competitive KIE is determined to be 3.0 ± 0.2 , which is also smaller than that by WT SPL_(BS) (14). We tentatively ascribe the reduced KIEs to the slower radical quenching step due to the removal of the intrinsic H-atom donor on the protein (14). Additionally, disruption of the putative catalytic chain indicates that SAM regeneration no longer occurs; all formed 5'-dA has to be exchanged by SAM at the end of a turnover, which contrasts to the two thirds exchange ratio observed in WT enzyme. Therefore, if the exchange process between 5'-dA and SAM is slow enough to change the reaction energy profile, it will also contribute to the reduced KIEs exhibited by the C141A_(BS) mutant. On the other hand, the competitive KIEs for the WT SPL_(BS) and C141A_(BS) mutant are both larger than their respective apparent KIEs, suggesting that the SAM binding/5'-dA releasing processes are similar in these two proteins.

As indicated by Figure 2, the [4Fe-4S] cluster in SPL restricts Y96_(Gt) (Y97_(BS)) from directly participating in radical transfer processes via ligation to C98, the third cysteine in the radical SAM motif. The SPL crystal structure identified Y97_(BS) as the Φ residue in the radical SAM motif (35), likely playing a structural role to ensure the correct orientation of SAM as well as subsequent 5'-dA/5'-dA• intermediates for an efficient catalysis. Such a function can be readily fulfilled by a phenylalanine residue. Indeed, Y, F, H, and W have all been found to serve as the Φ residue in other radical SAM enzymes (35, 36). Thus, the >3-fold rate reduction exhibited by the Y97F_(BS) mutant (Table 1) is surprising. Moreover, the apparent KIE is changed to 16 ± 1.5 and the competitive KIE to 11.5 ± 1.5 , which are much larger than the respective KIEs exhibited by the WT SPL. These observations may be explained by altered interaction of residue Y97_(BS) with SAM after the Y→F mutation. A specific possibility is the loss of the interaction between the -OH moiety in Y97F_(BS) and the 2'-OH at the SAM ribose. Alternatively, these results suggest that although the crystal structure indicates that Y97_(BS) is not a direct component of the HAT chain, it may not only be involved in SAM binding, but potentially possesses other roles in enzyme catalysis (also see discussions below).

The SPL structure indicates that Y98_(Gt) (equivalent of Y99_(BS)) may be a direct component of the HAT chain, which is also confirmed by our kinetic data. Mutating Y99_(BS) in SPL to a phenylalanine residue results in a 7-fold reduced catalytic rate. Additionally the Y99F_(BS) mutant exhibits an apparent KIE of 10.5 ± 1 and a competitive KIE of 9 ± 1 , both of which are similar to the KIEs shown by the Y97F_(BS) mutant, but much higher than those of the WT SPL_(BS) and C141A_(BS) mutant. These high KIEs also suggest that hydrogen tunneling may occur in this HAT step (37, 38). Moreover, the competitive KIEs are lower than the apparent KIEs in both Y97F_(BS) and Y99F_(BS) reactions, which is in contrast to the trend

exhibited by WT SPL_(Bs) and C141A_(Bs) mutant, indicating that the SAM binding/5'-dA releasing steps have been disturbed by these Y→F mutations.

The high KIEs exhibited by these Y/F mutants are accompanied by much enhanced uncoupled SAM cleavage reactions. As shown in Figure 4, when *d*₄-SP was used as substrate, both WT SPL_(Bs) and C141A_(Bs) mutant led to ~10% of uncoupled SAM cleavage, as reflected by the small amount of unlabeled 5'-dA observed. In contrast, production of the unlabeled 5'-dA was enhanced by ~5-fold in either Y97F_(Bs) or Y99F_(Bs) mutant reaction, with a yield comparable to that of *d*₁-5'-dA – product of the H atom abstraction from *d*₄-SP. This suggests that deuterium abstraction by 5'-dA• becomes unfavorable, which is in line with the high isotope effect observed in both mutants. As a consequence, ~50% of the 5'-dA• is quenched by reductants from the environment. The enhanced uncoupled SAM cleavage reaction in these Y/F mutants implies that the protein local conformation is essential for an efficient SPL catalysis. Cleavage of the strong C5'-S bond in SAM is assisted by the protein network to rapidly quench the resulting 5'-dA•, enabling a quick and efficient SP repair reaction in a synergistic manner.

Such a rationale also suggests that the “rate determining zone” (or the first irreversible step) in Y97F_(Bs) and Y99F_(Bs) mutants may be different from that in the WT SPL_(Bs) enzyme. As shown in radical SAM enzymes BtrN and DesII, the hydrogen atom abstraction step between the 5'-dA radical and the enzyme substrate is reversible as indicated by the multiple deuterium transfers from the labeled substrate to 5'-dA/SAM (39, 40). Although a similar multiple deuterium transfer was not observed in SPL catalysis probably due to the poor binding affinity for the dinucleotide SP TpT substrate, the low extent of uncoupled SAM cleavage reaction in WT SPL_(Bs) suggests that this step is facilitated by the protein network and thus could very well be reversible. Therefore, in WT SPL_(Bs) enzyme, the “rate determining zone” likely ends at the H-abstraction step to produce the 5'-dA radical for SAM regeneration. In contrast, the enhanced uncoupled cleavage in the two Y/F mutants suggests that the first H-abstraction associated with SP is no longer a favorable step, making it highly unlikely to be reversible. Therefore, it is possible that the “rate determining zone” may only include SAM/SP TpT binding, SAM reductive cleavage, and the H-abstraction step for these Y/F mutants. As the consequence, much larger apparent and competitive KIEs have been observed.

To exclude any effect of the Y99F_(Bs) mutation on the protein structure, we solved the crystal structure of the corresponding Y98F_(Gt) mutant in *Gt* SPL to 2.3 Å resolution. Comparison of the substrate-free Y98F_(Gt) mutant with the structure of the WT enzyme confirms the structural integrity of the mutant protein and shows a conserved conformation with overall r.m.s.d of 0.15 Å for the superimposed protein backbone (Figure 5). A closer view of the Y98F_(Gt) mutant active site shows that its overall architecture as well as the interactions between the protein and SAM are highly similar to the WT enzyme (17). In particular, the phenylalanine backbone superposes well with the WT tyrosine. The WT SPL_(Gt) and Y98F_(Gt) mutant repair SP TpT at reaction rates of 0.32 and 0.04 min⁻¹ respectively, both of which are comparable to those exhibited by the corresponding *Bs* enzymes (Table 1). Moreover, these *Gt* enzymes display identical apparent KIEs to their *Bs* counterparts. Considering the ~80% sequence identity between these two enzymes, we conclude that the structural information obtained for *Gt* SPL also reflects the *Bs* enzyme.

Both enzyme kinetics and the structural data suggest that the two tyrosine residues are involved in the SPL catalytic cycle. They must play a role downstream of C141_(Bs), the intrinsic H atom donor, in the radical relay chain. We tentatively assign Y99_(Bs) to be directly involved in the radical transfer to mediate SAM regeneration (Figure 6). Such a functional assignment is supported by the crystal structures of SPL_(Gt). Y98_(Gt) (equivalent

of Y99_(BS)) is located between C140 and SAM; its hydroxyl group is 5.1 Å away from the SH moiety of C140, and 3.6 Å away from the methylene carbon of SAM (Figure 2). Thus, it is potentially close to the 5'-dA moiety and well positioned for the radical relay process to mediate SAM regeneration. Y99_(BS) also helps stabilize the transient protein conformation right after the SAM cleavage reaction and thus enables a tightly coupled enzymatic reaction. The C5'-S bond cleavage likely induces a subtle protein conformational change before the 5'-dA• attacks the H_{6proR} atom to initiate the SP repair process. This conformational change is disturbed in the Y99F_(BS) mutant, as indicated by the much enhanced uncoupled SAM cleavage (Figure 4). A similar impact on the protein transient conformation is also expected for the Y97_(BS) residue, as reflected by the enhanced uncoupled SAM cleavage in Y97F_(BS) mutant reaction.

The tightly coupled radical relay mechanism is consistent with our experimental finding that no organic radical intermediate was observed by EPR spectroscopy in the “steady state” of the WT SPL_(BS) catalyzed SP TpT repair reaction. A similar observation was reported by Broderick *et al.* using SP containing plasmid DNA as the enzyme substrate (6). These observations contradict with a recent report, where a UV-visible difference spectrum was suggested to correspond to the •Y98_(Gt) formed during the SPL_(Gt) “steady state” reaction (15). However, that spectrum did not resemble the sharp-peak absorbance exhibited by a typical •Y (41–44). We thus tentatively ascribe the cause of the spectral difference to the FeS chromophores, rather than to any tyrosyl radical. Among the possible reaction intermediates shown in Figure 6, the tyrosyl radical on Y99_(BS) is likely the most stable one (29, 45). Stable tyrosyl radicals have been observed in a number of radical enzymes, including Class I ribonucleotide reductase (RNR) (29, 45, 46), monoamine oxidase A (47), photosystem II (48), and prostaglandin H synthase (PGHS) (49, 50). In addition, the thymine methyl radical must be reasonably stable as well, due to its position α to the thymine ring. The fact that no radical can be observed by EPR during the SPL “steady state” suggests these putative radical intermediates in Figure 6 exist as transient species. SPL catalysis likely occurs in a carefully regulated fashion: once the radical intermediate is generated via SAM reductive cleavage, it will be passed quickly through the catalytic chain until the end of the reaction and no radical species is accumulating during catalysis. This process is facilitated by transient protein conformational changes, preventing any of the intermediates from accumulating.

Although we have proposed SAM to be regenerated after each catalytic cycle, the obtained TpT/5'-dA ratios in the SPL_(BS) reactions are not fully in line with this hypothesis. As shown in Table 1, a ~ 1:1 ratio was observed between the consumed SP TpT and the formed 5'-dA for both C141A_(BS) (14) and Y99F_(BS) reactions, which is consistent with the assumption that these residues are components of the HAT chain for SAM regeneration. In contrast, the ratio is ~ 1.5:1 in both WT SPL_(BS) (13) and the Y97F_(BS) mutant, suggesting that only one third of the SAM is regenerated here. We tentatively ascribe this discrepancy to the much weaker binding affinity of the dinucleotide SP TpT compared to the SP containing duplex DNA. As indicated by DNA foot-printing studies, the SPL_(BS) protects at least a 9-bp region surrounding SP; all negatively charged phosphodiester moieties within this region are likely to contribute to the binding interaction with SPL (51). A similar example can be found in DNA photolyase, which repairs the cyclobutane dimer under visible light. The photolyase recognizes 6 bps surrounding the dimer (52). The binding affinity of the dinucleotide thymine dimer to photolyase is 10⁴-fold lower than that of the dimer containing oligonucleotide (53), suggesting that loss of the extra electrostatic interactions by removing the phosphodiester moieties upstream and downstream of the dimer drastically weakens substrate binding to the enzyme. Considering that SPL_(BS) recognizes 3 more phosphodiester groups than photolyase, the binding affinity of dinucleotide SP TpT is likely to be much weaker than that of SP containing duplex

oligonucleotide. Therefore, d_4 -SP containing oligonucleotides will be needed to re-examine the KIEs reported here before the mechanism in Figure 6 can be fully established. Such experiments are not trivial considering the synthetic challenge to prepare enough deuterium labeled SP containing oligomers. On the other hand, despite the imperfect substrates used in our current studies, the obviously different reaction rates as well as the apparent and competitive KIEs among the WT and mutant SPL proteins still provide strong supports for our proposed mechanism. The similar SAM regeneration pattern of the Y97F_(Bs) mutant and WT SPL_(Bs) also supports our hypothesis that Y97_(Bs) may not be a direct component of the HAT pathway.

Another potential challenge to establish our mechanism above is the reasonable stability for the putative $\bullet$ Y99_(Bs) species. The redox potential for a tyrosyl radical is suggested to be 0.4 V lower than a cysteinyl radical (0.94V vs 1.33V) at pH 7 (29, 45). Moreover, the bond dissociation energy (BDE) is found to be 360 kJ/mol for the O-H bond in tyrosine and 368–381 kJ/mol for the S-H bond in cysteine (29, 45), although some recent calculations suggest that the BDEs for the tyrosyl O-H and cysteinyl S-H are very similar at 365±5 kJ/mol (32). Nevertheless, considering the strong C-H bond in 5'-dA, which possesses a BDE of 433 kJ/mol (54, 55), the last H-abstraction step (step 4 in Figure 6) to regenerate the 5'-dA $\bullet$ in our mechanism is therefore strongly uphill. Among the enzymatic reactions studied to date, the most similar case to our mechanistic proposal is probably the adenosylcob(II)alamin regeneration in class II RNR. Class II RNR utilizes an adenosylcob(II)alamin coupled by a conserved cysteine for catalysis (45, 56). The catalytically essential cysteinyl radical is suggested to take an H atom from the methyl moiety of 5'-dA, producing the 5'-dA $\bullet$ radical that recombines with cob(III)alamin to regenerate the cofactor at the end of each catalytic cycle. This thermodynamically unfavorable H-abstraction is considered to be coupled with the favorable adenosylcob(II)alamin regeneration step. SAM regeneration is even more favored thermodynamically (57). The C5'-S bond in SAM possesses a BDE of ~ 251 kJ/mol (36), indicating that it is much stronger than the C-Co bond in adenosylcobalamin, which exhibits a BDE of 134 kJ/mol (58). Therefore, the highly favorable C-S bond formation in SAM may compensate the unfavorable H-atom abstraction by the tyrosyl radical and enable the SAM regeneration step to proceed.

Although thermodynamically the regeneration of SAM is not an issue, the 70~80 kJ/mol BDE difference between the O-H bond in tyrosine and the C-H bond in 5'-dA suggests that kinetically this process is still slow. As shown by Frey *et al.*, ligation of SAM to the [4Fe-4S] cluster in lysine-2,3-aminomutase (LAM) can lower the energy barrier for the SAM reductive cleavage step by almost 1 volt (~ 80 kJ/mol) (59). It is reasonable to assume that transition state stabilization via protein environment may be available in SPL as well, making the energy barrier for this H-abstraction step surmountable. Additionally, SAM regeneration may also be assisted by the Y97_(Bs) residue. As discussed above, Y97_(Bs) likely plays a key role for SAM/5'-dA $\bullet$ binding. However, we cannot rule out its further interaction with the 5'-dA $\bullet$, leading to $\bullet$ Y97_(Bs). Such a hypothesis implies both Y97_(Bs) and Y99_(Bs) to be involved in the radical propagation process, which may justify the similar high KIEs as well as the similarly enhanced uncoupled SAM cleavage reactions observed in Y99F_(Bs) and Y97F_(Bs) mutants. An example of this can be found in the enzyme PGHS, which catalyzes prostaglandin synthesis (49, 50). The cyclooxygenase reaction in PGHS employs a catalytically essential tyrosine radical, $\bullet$ Y385. However, another tyrosine, Y504, has also been identified as a radical carrier (60). The $\bullet$ Y385 and $\bullet$ Y504 radicals are formed independently and appear to be under equilibrium after formation (49). The Y504 is proposed to serve as a reservoir of oxidizing equivalent, and the Y504F mutant decreases the cyclooxygenase activation efficiency by 50% for PGHS-1 and 200% for PGHS-2 (60). Recently, using 3-nitrotyrosyl radical in place of $\bullet$ Y122 in *E. coli* Class 1a RNR, Stubbe *et*

al. showed that the radical is delocalized onto the three tyrosines of the long-range proton-coupled electron transfer pathway (61). Y356 is the predominant location (85~90%) of the radical, with the remaining 10~15% delocalized onto Y731 and Y730 in the $\alpha 2$ subunit (61). Such an observation also suggests that the relative redox potential between Y356 and Y731/730 could vary by ~ 100 mV. Similarly, the involvement of the Y97_(Bs) may result in a partially populated •Y at this residue, which can interact and fine-tune the redox potential of the •Y99_(Bs) and/or the 5'-dA•. Such a radical delocalization likely reduces the energy barrier for the H-abstraction step, making it kinetically competent. Further work to reveal the biological function of Y97_(Bs) using X-ray crystallography and biochemical means is currently in progress.

In conclusion, our report strongly implicates SPL to be the first member of the radical SAM superfamily to possibly bear a HAT chain. This transfer chain is likely to be essential for SAM regeneration in SPL catalysis. For the past decade, only SPL and LAM were considered to use SAM catalytically (58, 62). Very recently, another radical SAM enzyme, 7-carboxy-7-deazaguanine synthase (QueE) was found to recycle SAM after each turnover (63). Considering the large number of radical SAM enzymes discovered to date (more than > 44,000 (64)) a catalytic role of SAM can be expected among additional members of this superfamily. Our studies thus provide much-needed insight in understanding the SAM regeneration process not only in SPL, but potentially to other radical SAM enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Setlow P. Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol. 2006; 101:514–525. [PubMed: 16907802]
2. Desnous CL, Guillaume D, Clivio P. Spore photoproduct: A key to bacterial eternal life. Chem Rev. 2010; 110:1213–1232. [PubMed: 19891426]
3. Li L. Mechanistic studies of the radical SAM enzyme spore photoproduct lyase (SPL). BBA - Proteins and Proteomics. 2012; 1824:1264–1277. [PubMed: 22197590]
4. Rebeil R, Nicholson WL. The subunit structure and catalytic mechanism of the *Bacillus subtilis* DNA repair enzyme spore photoproduct lyase. Proc Natl Acad Sci USA. 2001; 98:9038–9043. [PubMed: 11470912]
5. Fajardo-Cavazos P, Rebeil R, Nicholson W. Essential cysteine residues in *Bacillus subtilis* spore photoproduct lyase identified by alanine scanning mutagenesis. Curr Microbiol. 2005; 51:331–335. [PubMed: 16163454]

6. Cheek J, Broderick J. Direct H atom abstraction from spore photoproduct C-6 initiates DNA repair in the reaction catalyzed by spore photoproduct lyase: Evidence for a reversibly generated adenosyl radical intermediate. *J Am Chem Soc.* 2002; 124:2860–2861. [PubMed: 11902862]
7. Buis J, Cheek J, Kalliri E, Broderick J. Characterization of an active spore photoproduct lyase, a DNA repair enzyme in the radical *S*-adenosylmethionine superfamily. *J Biol Chem.* 2006; 281:25994–26003. [PubMed: 16829680]
8. Chandra T, Silver SC, Zilinskas E, Shepard EM, Broderick WE, Broderick JB. Spore photoproduct lyase catalyzes specific repair of the 5*R* but not the 5*S* spore photoproduct. *J Am Chem Soc.* 2009; 131:2420–2421. [PubMed: 19178276]
9. Silver S, Chandra T, Zilinskas E, Ghose S, Broderick W, Broderick J. Complete stereospecific repair of a synthetic dinucleotide spore photoproduct by spore photoproduct lyase. *J Biol Inorg Chem.* 2010; 15:943–955. [PubMed: 20405152]
10. Pieck J, Hennecke U, Pierik A, Friedel M, Carell T. Characterization of a new thermophilic spore photoproduct lyase from *Geobacillus stearothermophilus* (SPLg) with defined lesion containing DNA substrates. *J Biol Chem.* 2006; 281:36317–36326. [PubMed: 16968710]
11. Chandor A, Berteau O, Douki T, Gasparutto D, Sanakis Y, Ollagnier-De-Choudens S, Atta M, Fontecave M. Dinucleotide spore photoproduct, a minimal substrate of the DNA repair spore photoproduct lyase enzyme from *Bacillus subtilis*. *J Biol Chem.* 2006; 281:26922–26931. [PubMed: 16829676]
12. Chandor-Proust A, Berteau O, Douki T, Gasparutto D, Ollagnier-De-Choudens S, Fontecave M, Atta M. DNA repair and free radicals, new insights into the mechanism of spore photoproduct lyase revealed by single amino acid substitution. *J Biol Chem.* 2008; 283:36361–36368. [PubMed: 18957420]
13. Yang L, Lin G, Liu D, Dria KJ, Telser J, Li L. Probing the reaction mechanism of spore photoproduct lyase (SPL) via diastereoselectively labeled dinucleotide SP TpT substrates. *J Am Chem Soc.* 2011; 133:10434–10447. [PubMed: 21671623]
14. Yang L, Lin G, Nelson RS, Jian Y, Telser J, Li L. Mechanistic studies of the spore photoproduct lyase via a single cysteine mutation. *Biochemistry.* 2012; 51:7173–7188. [PubMed: 22906093]
15. Kneutinger AC, Heil K, Kashiwazaki G, Carell T. The radical SAM enzyme spore photoproduct lyase employs a tyrosyl radical for DNA repair. *Chem Commun.* 2013; 49:722–724.
16. Mehl RA, Begley TP. Mechanistic studies on the repair of a novel DNA photolesion: The spore photoproduct. *Org Lett.* 1999; 1:1065–1066. [PubMed: 10825958]
17. Benjdia A, Heil K, Barends TRM, Carell T, Schlichting I. Structural insights into recognition and repair of UV-DNA damage by spore photoproduct lyase, a radical SAM enzyme. *Nucleic Acids Res.* 2012; 40:9308–9318. [PubMed: 22761404]
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
19. Gill SC, Von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem.* 1989; 182:319–326. [PubMed: 2610349]
20. Fish WW. Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Methods Enzymol.* 1988; 158:357–364. [PubMed: 3374387]
21. Beinert H. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal Biochem.* 1983; 131:373–378. [PubMed: 6614472]
22. See supporting information.
23. Cleland WW. The use of isotope effects to determine enzyme mechanisms. *Arch Biochem Biophys.* 2005; 433:2–12. [PubMed: 15581561]
24. Cleland, WW. Enzyme mechanisms from isotope effects. In: Kohen, A.; Limbach, H-H., editors. *Isotope effects in chemistry and biology*. Vol. Chap 37. Taylor & Francis; Boca Raton: 2006. p. 915-930.
25. Fersht, A. Structure and mechanism in protein science : A guide to enzyme catalysis and protein folding. Vol. Chap 3. W.H. Freeman; New York: 1999. p. 103-131.

26. Li L, Marsh ENG. Deuterium isotope effects in the unusual addition of toluene to fumarate catalyzed by benzylsuccinate synthase. *Biochemistry*. 2006; 45:13932–13938. [PubMed: 17105211]
27. Werst MM, Davoust CE, Hoffman BM. Ligand spin densities in blue copper proteins by Q-band ^1H and ^{14}N endor spectroscopy. *J Am Chem Soc*. 1991; 113:1533–1538.
28. Belford, RL.; Nilges, MJ. Computer simulations of powder spectra. EPR Symposium, 21st Rocky Mountain Conference; Denver, Colorado. Aug. 1979
29. Stubbe J, Nocera DG, Yee CS, Chang MCY. Radical initiation in the class I ribonucleotide reductase: Long-range proton-coupled electron transfer? *Chem Rev*. 2003; 103:2167–2201. [PubMed: 12797828]
30. Jordan A, Reichard P. Ribonucleotide reductases. *Ann Rev Biochem*. 1998; 67:71–98. [PubMed: 9759483]
31. Guo J, Luo Y, Himo F. DNA repair by spore photoproduct lyase: A density functional theory study. *J Phys Chem B*. 2003; 107:11188–11192.
32. Hioe J, Zipse H. Radicals in enzymatic catalysis-a thermodynamic perspective. *Faraday Discuss*. 2010; 145:301–313.
33. Yagisawa S. Two types of rate-determining step in chemical and biochemical processes. *Biochem J*. 1989; 263:985–988. [PubMed: 2597141]
34. Northrop DB. Steady-state analysis of kinetic isotope effects in enzymic reactions. *Biochemistry*. 1975; 14:2644–2651. [PubMed: 1148173]
35. Vey JL, Drennan CL. Structural insights into radical generation by the radical SAM superfamily. *Chem Rev*. 2011; 111:2487–2506. [PubMed: 21370834]
36. Frey P, Magnusson O. S-adenosylmethionine: A wolf in sheep's clothing, or a rich man's adenosylcobalamin? *Chem Rev*. 2003; 103:2129–2148. [PubMed: 12797826]
37. Kohen A, Klinman JP. Enzyme catalysis: Beyond classical paradigms. *Acc Chem Res*. 1998; 31:397–404.
38. Kohen A, Klinman JP. Hydrogen tunneling in biology. *Chem Biol*. 1999; 6:R191–198. [PubMed: 10381408]
39. Yokoyama K, Numakura M, Kudo F, Ohmori D, Eguchi T. Characterization and mechanistic study of a radical SAM dehydrogenase in the biosynthesis of butirosin. *J Am Chem Soc*. 2007; 129:15147–15155. [PubMed: 18001019]
40. Szu PH, Ruszczycky MW, Choi SH, Yan F, Liu HW. Characterization and mechanistic studies of DesII: A radical S-adenosyl-L-methionine enzyme involved in the biosynthesis of TDP-D-desosamine. *J Am Chem Soc*. 2009; 131:14030–14042. [PubMed: 19746907]
41. Cotruvo JA, Stich TA, Britt RD, Stubbe J. Mechanism of assembly of the dimanganese-tyrosyl radical cofactor of class Ib ribonucleotide reductase: Enzymatic generation of superoxide is required for tyrosine oxidation via a Mn(III)Mn(IV) intermediate. *J Am Chem Soc*. 2013; 135:4027–4039. [PubMed: 23402532]
42. Torrents E, Sahlin M, Biglino D, Gräslund A, Sjöberg BM. Efficient growth inhibition of *Bacillus anthracis* by knocking out the ribonucleotide reductase tyrosyl radical. *Proc Natl Acad Sci USA*. 2005; 102:17946–17951. [PubMed: 16322104]
43. Cotruvo JA, Stubbe J. *Escherichia coli* class Ib ribonucleotide reductase contains a dimanganese(III)-tyrosyl radical cofactor *in vivo*. *Biochemistry*. 2011; 50:1672–1681. [PubMed: 21250660]
44. Bollinger JM Jr, Edmondson DE, Huynh BH, Filley J, Norton JR, Stubbe J. Mechanism of assembly of the tyrosyl radical-dinuclear iron cluster cofactor of ribonucleotide reductase. *Science*. 1991; 253:292–298. [PubMed: 1650033]
45. Stubbe J, Van Der Donk WA. Protein radicals in enzyme catalysis. *Chem Rev*. 1998; 98:705–762. [PubMed: 11848913]
46. Cotruvo JA, Stubbe J. Class I ribonucleotide reductases: Metallocofactor assembly and repair *in vitro* and *in vivo*. *Annu Rev Biochem*. 2011; 80:733–767. [PubMed: 21456967]
47. Rigby SEJ, Hynson RMG, Ramsay RR, Munro AW, Scrutton NS. A stable tyrosyl radical in monoamine oxidase a. *J Biol Chem*. 2005; 280:4627–4631. [PubMed: 15556933]

48. Faller P, Debus RJ, Brettel K, Sugiura M, Rutherford AW, Boussac A. Rapid formation of the stable tyrosyl radical in photosystem II. *Proc Natl Acad Sci*. 2001; 98:14368–14373. [PubMed: 11762431]
49. Tsai AL, Kulmacz RJ. Prostaglandin H synthase: Resolved and unresolved mechanistic issues. *Arch Biochem Biophys*. 2010; 493:103–124. [PubMed: 19728984]
50. Lü JM, Rogge CE, Wu G, Kulmacz RJ, Van Der Donk WA, Tsai AL. Cyclooxygenase reaction mechanism of PGHS — evidence for a reversible transition between a pentadienyl radical and a new tyrosyl radical by nitric oxide trapping. *J Inorg Biochem*. 2011; 105:356–365. [PubMed: 21403766]
51. Slieman TA, Rebeil R, Nicholson WL. Spore photoproduct (SP) lyase from *Bacillus subtilis* specifically binds to and cleaves SP (5-thymine-5,6-dihydrothymine) but not cyclobutane pyrimidine dimers in UV-irradiated DNA. *J Bacteriol*. 2000; 182:6412–6417. [PubMed: 11053385]
52. Sancar A. Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem Rev*. 2003; 103:2203–2238. [PubMed: 12797829]
53. Kim ST, Sancar A. Effect of base, pentose, and phosphodiester backbone structures on binding and repair of pyrimidine dimers by *Escherichia coli* DNA photolyase. *Biochemistry*. 1991; 30:8623–8630. [PubMed: 1716150]
54. Hioe J, Savasci G, Brand H, Zipse H. The stability of Ca peptide radicals: Why glycyl radical enzymes? *Chem Eur J*. 2011; 17:3781–3789. [PubMed: 21341321]
55. Hioe J, Zipse H. Hydrogen transfer in SAM-mediated enzymatic radical reactions. *Chem Eur J*. 2012; 18:16463–16472. [PubMed: 23139189]
56. Licht S, Gerfen GJ, Stubbe J. Thiyl radicals in ribonucleotide reductases. *Science*. 1996; 271:477–481. [PubMed: 8560260]
57. Hinckley GT, Frey PA. Cofactor dependence of reduction potentials for $[4\text{Fe-4S}]^{2+/1+}$ in lysine 2,3-aminomutase. *Biochemistry*. 2006; 45:3219–3225. [PubMed: 16519516]
58. Marsh ENG, Patterson DP, Li L. Adenosyl radical: Reagent and catalyst in enzyme reactions. *ChemBioChem*. 2010; 11:604–621. [PubMed: 20191656]
59. Wang SC, Frey PA. Binding energy in the one-electron reductive cleavage of *S*-adenosylmethionine in lysine 2,3-aminomutase, a radical SAM enzyme. *Biochemistry*. 2007; 46:12889–12895. [PubMed: 17944492]
60. Rogge CE, Liu W, Kulmacz RJ, Tsai AL. Peroxide-induced radical formation at tyr385 and tyr504 in human PGHS-1. *J Inorg Biochem*. 2009; 103:912–922. [PubMed: 19433337]
61. Yokoyama K, Smith AA, Corzilius B, Griffin RG, Stubbe J. Equilibration of tyrosyl radicals (Y356•, Y731•, Y730•) in the radical propagation pathway of the *Escherichia coli* class Ia ribonucleotide reductase. *J Am Chem Soc*. 2011; 133:18420–18432. [PubMed: 21967342]
62. Frey PA, Hegeman AD, Ruzicka FJ. The radical SAM superfamily. *Crit Rev Biochem Mol Biol*. 2008; 43:63–88. [PubMed: 18307109]
63. McCarty RM, Krebs C, Bandarian V. Spectroscopic, steady-state kinetic, and mechanistic characterization of the radical sam enzyme QueE, which catalyzes a complex cyclization reaction in the biosynthesis of 7-deazapurines. *Biochemistry*. 2013; 52:188–198. [PubMed: 23194065]
64. Booker SJ. Radical SAM enzymes and radical enzymology. *BBA - Proteins and Proteomics*. 2012; 1824:1151–1153. [PubMed: 22850428]
65. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D*. 2004; 60:2256–2268. [PubMed: 15572779]

Figure 1.

SP formation and enzymatic repair mediated by SPL. In this study, we also employed a d_4 -SP TpT species, where the H_{proR} and the three hydrogen atoms of the methyl group on the 5'-T are replaced by deuterium atoms.

Figure 2.

Active site of *Gt* SPL in complex with SP (in white), the [4Fe-4S] cluster and SAM. The C140_(Gt), Y96_(Gt) and Y98_(Gt) residues correspond to the C141_(Bs), Y97_(Bs) and Y99_(Bs) residues in *Bs* SPL respectively. The distances between protein residues, SP and SAM are indicated by dashed lines (PDB code 4FHD) (17).

Figure 3. HPLC chromatograph of the SP TpT repair mediated by the SPL Y99F_(Bs) mutant with 30 μ M enzyme, 150 μ M SAM and 1 mM dithionite. SP TpT eluted at 5.4 min, 5'-dA at 8.9 min, and TpT at 14.7 min. See SI for HPLC chromatograph of the Y97F_(Bs) mutant reaction.

Figure 4.

Mass spectrometry analysis of 5'-dA isolated from *Bs* SPL reactions conducted with d_4 SP TpT as the substrate. The d_1 -5'-dA forms *via* H atom abstraction from the d_4 SP TpT and the unlabeled 5'-dA forms *via* uncoupled SAM cleavage reaction. The exact mass for each 5'-dA species is shown in SI.

Figure 5. Secondary structure of substrate-free WT SPL_(Gt) (cyan) and the Y98F_(Gt) mutant (purple, equivalent to Y99F_(Bs) mutant). The superposition of both structures was performed with Coot using the SSM program and all residues (65). The iron-sulfur cluster is depicted in orange (Fe) and yellow (S). SAM is colored by atom type (green C, blue N, red O and yellow S).

Figure 6.

Hypothesized reaction mechanism for SPL (The residues are numbered according to the protein sequence in *Bs* SPL). This mechanism implies that SPL uses a minimum of four H atom transfer processes (labeled in blue numbers) in each catalytic cycle. One of the four processes occurs between a tyrosine and a cysteine, suggesting that SPL uses a novel HAT pathway for SAM regeneration. The role of Y97(_{Bs}) in SPL catalysis needs further elucidation and is thus not shown here.

Table 1Summary of the *BS* SPL reactions

SPL enzyme	ν (min^{-1})	Apparent KIE	Competitive KIE	SP repaired/5'-dA
W.T.	0.41 ± 0.03	2.8 ± 0.3	3.4 ± 0.3	1.5 ± 0.2
C141A _(Bs)	0.14 ± 0.02	1.7 ± 0.2	3.0 ± 0.3	1.08 ± 0.1
Y97,99A _(Bs)	N.A.	N.A.	N.A.	N.A.
Y97F _(Bs)	0.12 ± 0.01	16 ± 1.5	11.5 ± 1.5	1.6 ± 0.2
Y99F _(Bs)	0.06 ± 0.005	10.5 ± 1	9 ± 1	1.0 ± 0.1
Y97,99F _(Bs)	< 0.004	N.A.	N.A.	0.92 ± 0.1

Note: (1) N.A. = not available. (2) As shown by Figures S5 and S6, both KIEs were determined within the first 3 turnovers, where the steady state kinetics may not be achieved.