Spore Photoproduct Lyase: The Known, the Controversial, and the Unknown*

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Spore photoproduct lyase (SPL) repairs 5-thyminyl-5,6-dihydrothymine, a thymine dimer that is also called the spore photoproduct (SP), in germinating endospores. SPL is a radical S-adenosylmethionine (SAM) enzyme, utilizing the 5′-deoxyadenosyl radical generated by SAM reductive cleavage reaction to revert SP to two thymine residues. Here we review the current progress in SPL mechanistic studies. Protein radicals are known to be involved in SPL catalysis; however, how these radicals are quenched to close the catalytic cycle is under debate.

UV light induces intrastand cross-linking reactions in DNA at bipyrimidine sites, which are mutagenic as they alter the DNA structure, inhibit polymerases, and arrest replication (1). Among the four DNA nucleobases, thymine (T) is the most sensitive to UV irradiation followed by cytosine (C) (1). In typical cells after photochemical excitation, a T residue dimerizes with an adjacent T or C, generating either the cyclobutane pyrimidine dimers (CPDs)2 or the pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) as the major photolesions (Fig. 1). In contrast, in bacterial endospores, the dominant DNA photoproduct is 5-thyminyl-5,6-dihydrothymine, a unique thymine dimer, which is also called the spore photoproduct or SP (2–4).

Formation of SP in vivo is largely determined by the unique spore DNA conformation. The spore genomic DNA is saturated by a group of DNA-binding proteins named small acid soluble proteins, as they are readily soluble in 0.5 M acetic acid (5). The small acid soluble protein-DNA interaction, coupled with other factors such as the low spore hydration level, changes the DNA from B-like to A-like conformation (6–9), which subsequently alters the outcome of the thymine photoreaction, making SP the dominant DNA photoproduct (3, 4, 6, 9–11). SPs accumulate in dormant spores and are repaired rapid-

SPL Mechanism: The Known

SPL studies to date have established several important facts regarding the mechanism used by this intriguing DNA repair enzyme.

SPL Repairs SP via Radical SAM Chemistry

Rebel et al. (25) found that SPL is an iron-sulfur enzyme, whose activity depends on reducing conditions and the addition of SAM. They later established that the enzyme utilizes the 5′-deoxyadenosyl radical (5′-daF) generated by SAM reductive cleavage to catalyze the SP reversal to two thymine residues (29) and proposed an SPL mechanism, which was later proved to be largely correct. SPL is now known as a member of the radical SAM superfamily, which is defined by the characteristic CXXC motif (30), although other tri-cysteine motifs may also facilitate this radical chemistry (31–34). The three C residues in this motif bind to three iron in the [4Fe-4S] cluster; the fourth iron is coordinated by SAM (35). The cluster at its +1 oxidation state donates an electron to SAM to cleave its C5′-S bond, generating a 5′-daF. This 5′-daF catalyzes a number of highly diverse biochemical reactions in animals, plants, and microorganisms, including steps in metabolism, DNA/RNA modifica-

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2 The abbreviations used are: CPD, cyclobutane pyrimidine dimer; SAM, S-adenosylmethionine; SP, spore photoproduct; SPL, spore photoproduct lyase; 6-4PP, 6-4 pyrimidone photoproduct; NER, nucleotide excision repair pathway; 5′-dA, 5′-deoxyadenosyl radical; TPt, thymidyl-(3′–5′)-thymidine; HAT, hydrogen atom transfer; Bs, B. subtilis; Gt, G. thermmodenitrificans.

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tion, and the biosyntheses of vitamins, coenzymes, and many antibiotics (22, 32–59).

SPL Repairs the 5R-Isomer of SP

As shown in Fig. 1, SP contains a chiral center at the C5 of 5′-T. Its chirality was predicted to be R by Kim et al. (60) based on the right-handed DNA helical structure. This prediction was confirmed by Mantel et al. (61) using NMR spectroscopy coupled with density functional theory calculations and dinucleotide SP TpT. Later, a dinucleotide SP structure containing a formacetal linker solved by Lin et al. (62) further confirmed this conclusion. After incorporating dinucleoside 5R- and 5S-SP, respectively, which lack the phosphodiester linker between the two thymine residues, into a 12-mer oligonucleotide via solid phase DNA synthesis, subsequent structural studies found that the 5R-SP fits in the topology of the right-handed helix well, whereas the 5S-SP results in a severe strand distortion (63). Jian and co-workers (64) prepared the biologically relevant dinucleotide 5R-SP TpT phosphoramidite and incorporated SP TpT into an oligonucleotide. The latest structure in a 16-mer duplex oligonucleotide revealed that the 5R-SP TpT results in little structural distortion except widening the DNA minor groove by 2.5 Å. Such a small conformational change suggests that SP may be difficult to be recognized by SPL and the NER enzymes.

The biologically relevant SP stereoisomer has thus been established. Some earlier studies suggested that SPL recognizes and repairs the 5S-isomer (65, 66), which has been corrected (67). In a recent SPL structure solved by Benjdia et al. (68), the enzyme contains a dinucleoside 5R-SP, further confirming that the 5R-isomer is the SPL substrate.

SPL Abstracts the H6pro Atom from SP

The first mechanistic insight of the SPL reaction was provided by Mehl and Begley (69) from a clever small molecule model study using a bipyrimidine complex. Their data suggest that SP repair is initiated by hydrogen abstraction at C6 followed by β-scission at the methylene bridge and back hydrogen atom transfer. Cheek et al. (70) utilized tritium to label the C6 of thymine and generated SPs via photoreaction under the assumption that the tritium will remain at C6 in the formed SP. Analysis of SP repair by SPL revealed that tritium entered the catalytic cycle, agreeing with the proposal of Mehl and Begley (69).

Analysis of the SP structure shows that the C6 has two hydrogen atoms and is pro-chiral. To reveal which hydrogen atom is abstracted during SPL catalysis, Lin et al. (71) utilized deuterium-labeled dinucleotide TpTs to generate two SP species via photoreaction and proved that a hydrogen atom from the 3′-CH3 is transferred to the H6pros of SP. Using SP with either the H6pro or the H6pro position labeled by a deuterium, Yang et al. (72) proved that it is the H6pro that is abstracted by the 5′-dA in SP repair (Fig. 2). These results provide the chemical basis for the previous labeling studies, which tritiated the -CH3 moiety of thymine (Fig. 3) (18, 26). The tritium label was transferred to the H6pro in photochemically produced SP and retained in DNA after the SPL reaction. Thus, no radioactivity loss in DNA was observed, leading to the hypothesis that SPL directly reverts SP. If the researchers chose to label thymine C6, the label at the resulting H6pro of SP would leak into medium after SP repair (72), potentially complicating the SPL mechanistic elucidation.

A Conserved Cysteine as the Intrinsic Hydrogen Atom Donor

The β-elimination reaction induced by the SP C6 radical generates a thymine allylic radical, which was suggested to abstract a hydrogen atom from the methyl group of 5′-dA (70). However, although no role was implied for the conserved cysteine 141, spores carrying the C141A mutation are sensitive to UV irradiation (73). A later in vitro study by Chandor-Proust et al. (74) found that the thymine allylic radical was quenched by the dithionite supplemented as a reductant to form a T-SO2 adduct, leading to a hypothesis that Cys-141 may be the hydrogen atom donor. Yang et al. (72) found that the hydrogen donor is able to exchange protons with the aqueous solution. They showed that Cys-141 is solvent-accessible via the iodoacetamide-labeling assay; their enzyme kinetics data further indicate that the Cys-141 in Bacillus subtilis (Bs) SPL is likely the intrinsic hydrogen donor to the thymine radical (Fig. 2) (75). The conclusion is supported by the parallel SPL structural studies by Benjdia et al. (68) using Geobacillus thermocatenulatus (Gt) SPL containing a dinucleoside SP and a SAM bound to the Fe-S cluster (Fig. 4). The conserved cysteine was implied to have no structural role. It is close to the methylene bridge of SP, supporting the assumption that it is the intrinsic hydrogen donor.

Involvement of Tyrosines

Besides this cysteine, two conserved tyrosine residues, Tyr-97 and Tyr-99 in Bs SPL, may also be involved in enzyme catalysis. As shown in Fig. 4, both tyrosines are close to SAM, suggesting that they can interact with the 5′-dA/5′-dA pair. Tyr-98(Gt), equivalent to Tyr-99(Bs), is located between the conserved cysteine and SAM in the SPL structure (68), implying that it can be involved in the radical propagation process (Fig.
2). The Y98F(Gt) mutant was found to reduce the SPL activity by ~4-fold (76), which is consistent with the 6-fold reduction by the Y99F(Bs) mutant found in a separate study (77). Tyr-96(Gt) seems to play a structural role to immobilize SAM at the SPL active site. However, the Y97F(Bs) mutation, which still enables SAM binding with the aromatic ring in phenylalanine, results in a 3-fold reduction of enzyme activity (77). Mutating both tyrosine residues almost completely abolishes the SPL activity (77). Such results, coupled with the drastically altered kinetic isotope effects between the wild-type SPL and the Tyr/Phe mutants, indicate that the Tyr-97(Bs) may also be involved in the radical transfer process, to facilitate the hydrogen abstraction in putative SAM regeneration (77). Although the possibility that these tyrosines play structural roles to maintain the protein H-bonding network cannot be ruled out, they are more likely to be involved in the SPL radical transfer network as discussed above.

SPL Mechanism: The Controversial

To accommodate these results, an SPL mechanism that utilizes four hydrogen atom transfer (HAT) steps has been proposed (Fig. 2). The first two HAT processes are well established, and the last two HAT processes are under debate. SAM is shown to be regenerated at the end of the catalytic cycle, which is also controversial. (The figure is modified with permission from Ref. 77. Copyright (2013) American Chemical Society.)

FIGURE 2. The hypothesized reaction mechanism for SPL (the residues are numbered according to the protein sequence in B. subtilis SPL). This mechanism implies that SPL uses a minimum of four hydrogen atom transfer processes (labeled in blue numbers) in each catalytic cycle. The first two HAT processes are well established, and the last two HAT processes are under debate. SAM is shown to be regenerated at the end of the catalytic cycle, which is also controversial. (The figure is modified with permission from Ref. 77. Copyright (2013) American Chemical Society.)
that from SAM to thymine, may be due to reversible SAM cleavage and the subsequent hydrogen abstraction steps, as observed in radical SAM enzymes BtrN (79) and DesII (50). This possibility needs to be tested in the future.

Studies using SP-containing pUC18 plasmid as substrate for SPL reported that one molecule of SAM catalyzed >500 turnovers with a turnover number of 12.4 min⁻¹ (70, 78), indicating that SAM is used in a truly catalytic manner. However, other studies using smaller but chemically better-defined substrates have found much lower turnover numbers (72, 75–77, 80). Most other in vitro SPL studies utilized dinucleotide or dinucleoside SP as substrate and found that the ratio between 5’-dA generated and SP repaired ranges between 1 and 2, suggesting that SAM is partially regenerated. SPL activity using dinucleoside SP TpT as substrate and found that the ratio between 4006

FIGURE 4. The active site of G. thermodenitrificans (Gt) SPL in complex with SP and SAM. Cys-140(Gt), Tyr-96(Gt), and Tyr-98(Gt) equal to Cys-141(Bs), Tyr-97(Bs), and Tyr-99(Bs) in B. subtilis SPL, respectively. The distances (Å) between selected residues, SP, and SAM are indicated by the black numbers near the dashed lines (Protein Data Bank (PDB) code 4FHD).

for the next turnover. Considering the lack of other redox cofactors as revealed by the SPL structure (68), to abstract a hydrogen atom from 5’-dA and subsequently regenerate SAM is still the most reasonable hypothesis, as shown by Fig. 2.

Despite the attempts to trap and characterize the putative radical species involved in SPL catalysis during steady-state turnover, no radical species were observed by EPR spectroscopy (70, 77). Based on the UV-visible difference spectrum after an overnight reaction, Kneuttinger et al. (28, 76) reported the presence of a tyrosyl radical in SPL. However, the spectrum did not resemble the sharp peak absorbance exhibited by a typical Tyr’ (82–84), indicating that it may be due to the spectral difference between the Fe-S chromophores, rather than Tyr’. It is possible that the putative radical transfer pathway is tightly coupled like that in the Class I ribonucleotide reductase (85, 86), where its existence is not fully established until the transient tyrosyl radicals in the middle of the pathway were observed using unnatural tyrosines to fine-tune the redox potential and disturb the radical transfer process (87).

SPL Mechanism: The Unknown

The differing results from these in vitro studies question whether the current enzyme kinetic data truly reflect the SP repair in vivo. To clarify the controversy, some key questions need to be addressed in future SPL studies.

SPL Efficiency in Vivo

It has been found that >75% of SPs are repaired during the first hour of spore germination (11). However, the absolute quantity of repaired SPs is unclear. The B. subtilis genome has 4.2 million base pairs (88). As each spore may contain up to 200 copies of SPL (25), if 1% of total Ts are converted to SPs (6, 89), each SPL has to repair ~80 SPs in 60 min in germinating spores. As the rate-limiting step for in vivo repair is likely to be damage recognition (90), the enzyme repair should be faster. However, the reported repair rate for dinucleotide SP TpT is only ~0.35 min⁻¹ (24, 72, 80); the rate in duplex DNA may be even slower. Such slow rates cannot explain the fact that SPL plays a major role in SP repair in germinating spores. However, if only 0.1% of Ts are converted to SPs, the reported rates may be sufficient.

DNA Conformation during in Vivo Repair

Besides the abnormally slow repair rate, the fact that the repair of dinucleotide SP TpT is 3–4-fold faster than that in a duplex DNA is also perplexing. SPL is found to bind 9 nucleotides (23) via electrostatic interaction between the negatively charged phosphates on DNA and the positively charged protein surface. Thus, its binding affinity toward an oligonucleotide duplex prepared via DNA synthesis (81) at only 0.08 ± 0.01 min⁻¹. This implies that the use of dinucleotide substrates is not the reason for the much slower kinetics of SPL as compared with the reported values for the enzyme reacting with plasmid DNA (70, 78).

The reason for these discrepancies is unclear as the methodologies used by the various laboratories to analyze the products of the reaction do not differ that greatly. Collectively, it is safe to state that the in vitro data so far are insufficient to support a full SAM regeneration in SPL catalysis.

Despite the controversy, SAM regeneration is still the most reasonable route to close the catalytic cycle. As the conserved cysteine is known as the hydrogen donor, a thiol radical will be generated on this residue. As a tyrosine (Tyr-99 in Bs SPL) is next to the cysteine, its oxidation to a tyrosyl radical is possible. These radicals have to be reduced before the enzyme is ready

3 L. Yang and L. Li, unpublished results.
appear to be strengthened in duplex DNA, as shown by an x-ray crystallographic study (64).

It is thus of interest to reveal how the SP-flipping process is achieved in vivo. One solution could be the involvement of other enzymes. As shown by the NER enzymes, UvrA recognizes the DNA damage, and UvrB bends the DNA before catalyzing the DNA hydrolysis reaction with UvrC (91, 92). Given the minor DNA conformational change induced by SP (64), it is an intriguing possibility that SPL may have to team with other protein(s) for efficient damage recognition and repair. Alternatively, as the genomic DNA changes conformation from A- to B-form during spore germination (20), the DNA may adopt some transient conformation to facilitate the SP flip-out and repair by SPL.

Condition for SAM Regeneration

The lack of reactive DNA conformation may also explain why SAM regeneration cannot be observed in the in vitro studies so far. The reactive DNA conformer likely helps SP flip out, accelerates the enzyme reaction, and induces the required protein conformation for SAM regeneration. Another possibility is that the appropriate experimental conditions for SAM regeneration have not been adopted in in vitro studies. As shown by the investigations of lysine 2,3-aminomutase (93) and 7-carboxy-7-deazaguanine synthase (57), the two radical SAM enzymes known to use SAM catalytically, the in vitro experiments are sensitive to the reducing conditions adopted. Although great care has been taken in SPL studies (72, 75, 77), we cannot completely exclude the possibility that the in vitro condition, which potentially mimics the in vivo SP repair and enables SAM regeneration, has not been identified.

Summary and Perspective

After the past more than 40 years of studies, the first several steps in SPL catalysis have been well established. The remaining issues are mainly about how to close the catalytic cycle. To clarify the controversy in SPL catalysis, especially in a duplex DNA environment, the interaction between SPL and SP-containing oligonucleotide needs to be revealed. This will allow us to identify a biologically relevant system for mechanistic enzymology studies. In addition, the improved enzyme efficiency will enable us to trap and characterize the putative radical species via fast enzyme kinetic means and answer questions such as whether SAM is recycled and whether the recycling requires the radical transfer pathway in the protein.

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