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Rescuing DNA repair activity by rewiring the H-atom transfer pathway in the radical SAM enzyme, spore photoproduct lyase†

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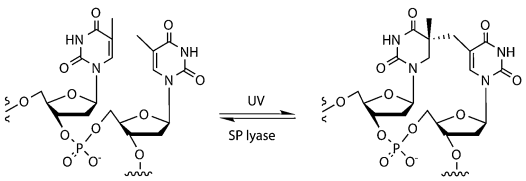
The radical SAM enzyme, spore photoproduct lyase, requires an H-atom transfer (HAT) pathway to catalyze DNA repair. By rational engineering, we demonstrate that it is possible to rewire its HAT pathway, a first step toward the development of novel catalysts based on the radical SAM enzyme scaffold.

Spore photoproduct lyase (SP lyase) is a radical SAM enzyme catalyzing the repair of a unique thymidine dimer: 5-(α-thyminyl)-5,6-dihydrothymidine, a DNA damage encountered specifically in bacterial spores and commonly called the spore photoproduct (SP) (Scheme 1).[1,2]

In contrast to DNA photolyases (CPD and 6-4 photolyases), which use light energy and a flavin cofactor to catalyze the radical-based repair of thymidine dimers, SP lyase requires an iron–sulfur cluster and *S*-adenosyl-L-methionine (SAM).[1,3] SP lyase generates the highly reactive 5′-deoxyadenosyl (5′-dA) radical which has been demonstrated to abstract the C6 pro*R*-hydrogen atom of SP[4,5] inducing the formation of the 5-thyminyl-5,6-dihydrothymin-6-yl radical. After radical migration, the methylene bridge between the two nucleobase residues is cleaved (similar to what happens with DNA photolyases)[1] and a 3′-thymine allylic radical intermediate is likely formed.[6] Though, in contrast to DNA photolyases in which an electron from the repaired lesion is given back to the flavine cofactor to complete the catalytic cycle, SP lyase uses a complex and still unclear mechanism to regenerate the SAM cofactor.[1]



Scheme 1 Formation and repair of the spore photoproduct.

We and others have established that SP lyase requires a critical cysteine residue to conclude the repair reaction.[5–8] The importance of this conserved residue (C141 in *Bacillus subtilis*) was established by mutagenesis studies showing that *in vivo*, C141 is critical for the viability of bacterial spores exposed to UV radiation,[8] while *in vitro*, substitution of C141 invariably led to the formation of DNA adducts (*i.e.* a sulfinic acid adduct).[6]

We solved the crystal structure of SP lyase from *Geobacillus thermodenitrificans* (*Gt*) and discovered that this crucial cysteine residue (C140 in *Gt*) is located in close proximity to the SP lesion.[7] The position and the distance of this cysteine residue, relative to the α-methylene carbon atom of the 3′-thymine moiety (4.5 Å), led us to propose a function as ultimate H-atom donor to the DNA lesion and a key role in the ill-defined migration and control of radicals inside the enzyme active site.[7]

C141 is strictly conserved among *Bacilli* species. However, despite the very high sequence homologies between SP lyases from *Bacilli* and *Clostridia* species (Fig. S1, ESI†), clostridial SP lyases lack this functionally critical residue, forming thus a distinct sub-class of enzymes (Fig. S1, ESI†). To investigate mechanistic and structural differences between these two sub-classes of SP lyases, we built a structural model of the SP lyase from *Clostridium acetobutilicum* (*Ca*) which has been previously biochemically characterized.[9] The comparison of the active sites of the modeled clostridial SP lyase with the *Gt* enzyme showed that the architecture of the active sites, including the spatial orientation of residues involved in the binding of the DNA lesion, is conserved (Fig. S2, ESI†). However, no cysteine residue in the clostridial SP lyase model superposed with the

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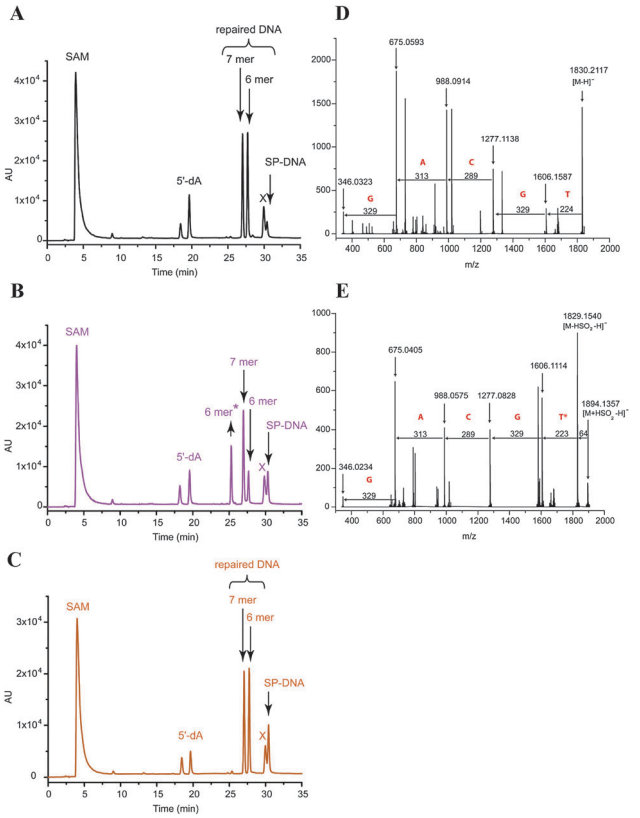

Fig. 1 Repair activity of the wild-type and mutant proteins incubated for 1 hour with a SP-containing DNA (13-mer). HPLC analysis of the reaction performed with (A) the wild-type SP lyase, (B) the C140A mutant and (C) the C140A/S76C mutant. A new compound, named 6-mer*, is identified as a sulfinic adduct eluting at 25.3 min. X indicates an impurity. (D) MS/MS analysis of the 6-mer and (E) the 6-mer* purified from reactions performed with the wild-type enzyme or the C140A mutant, respectively (for full annotations see Fig. S6, ESI†). 40 μM of reconstituted protein were incubated with 3 mM SAM (RT at 4 min), 40 μM SP-containing DNA (RT at 30.4 min), 5 mM dithiothreitol (DTT), 3 mM sodium dithionite in Tris buffer pH 8, under anaerobic conditions.

critical cysteine C140 in *Gt*. Instead, an alanine residue (A138) occupies this position, a substitution known to preclude DNA repair in *Gt* SP lyase.[6,7] Interestingly, we identified in the clostridial structural model, a cysteine residue (C74) in close proximity to the methylene bridge of the SP lesion suggesting mechanistic adaptation among SP lyases. Exploiting the differences between clostridial and bacilli SP lyases, we aimed to reorient the H-atom transfer pathway in *Gt* SP lyase and investigate functional and structural diversities in this enzyme family.

We assayed *in vitro* the DNA repair activity of the purified wild-type and mutant *Gt* SP lyases with a synthetic 13-mer DNA containing the SP lesion.[10] The assay with the wild-type enzyme enabled us to identify and quantify all substrates and products. As shown in Fig. 1A, the incubation of *Gt* SP lyase with this 13-mer substrate, lacking a phosphodiester bond in the SP lesion, led to the formation of two DNA fragments, a 7- and a 6-mers (retention time (RT) at 27 min, m/z = 2119.2 and RT at 27.7 min, m/z = 1830.1, respectively) indicating the repair of SP back into two thymidines (Fig. 1A and Fig. S3–S5, ESI†). The 5′-dA resulting from the homolytic SAM cleavage could also be identified (RT at 19.6 min, m/z = 252).

Analyses of the reaction performed with the C140A SP lyase mutant exhibited a different profile. In addition to the 6- and 7-mers which demonstrated the repair of SP, we monitored the formation of a new DNA species eluting at 25.3 min (Fig. 1B). LC-MS/MS analysis allowed the identification of this new product as a 6-mer containing a sulfinate group on the 5′-thymine residue, named 6-mer* (Fig. 1D and E, Fig. S6 and Table S1, ESI†). The formation of the 6-mer* and the 6-mer products in the absence of C140 is explained by the quenching of the allylic radical intermediate by a $SO_2^{-\bullet}$ radical (originating from sodium dithionite)[6] or its reaction with DTT used as a reductant, respectively.[11]

Since in the wild-type enzyme C140 fulfills the function of radical quenching, we hypothesized that positioning another cysteine residue in the vicinity of the DNA lesion should rescue the repair activity of the C140A mutant. Superimposition of the clostridial model with the *Gt* SP lyase structure indicated that the clostridial C74, located close to the SP lesion, occupies the same position as serine 76 in *Gt* SP lyase (Fig. S2, ESI†). We hence carried out site-directed mutagenesis of S76 in the C140A mutant.

Interestingly, contrary to the single mutant, functional analysis of the double mutated (C140A/S76C) enzyme showed that no more sulfinic DNA adducts were produced (Fig. 1C). Indeed, we exclusively observed the formation of the 6- and 7-mer DNA fragments, demonstrating complete repair of SP, as shown with the wild-type enzyme (Fig. 1A). The insertion of a cysteine residue inside the mutated enzyme active-site thus completely restored the SP lyase repair activity. Furthermore, kinetic experiments revealed that, while the wild-type SP lyase had a DNA repair rate of 1.66 nmol min^{-1} mg^{-1} (in the range of the reported values for SP lyases[5,12]), the double mutant proved to be more than two-times faster (3.48 nmol min^{-1} mg^{-1}) (Fig. 2).

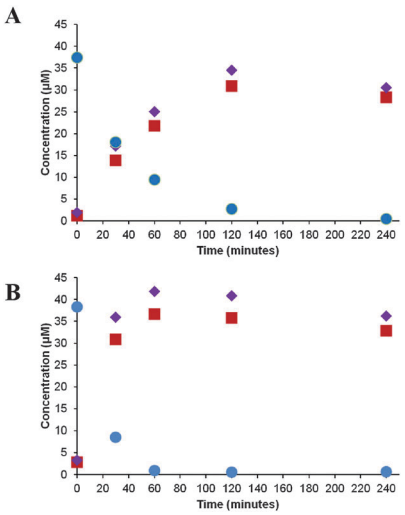

Fig. 2 Repair activity of the wild-type (A) and double mutant SP lyase (B). Time-dependent production of 6-mer (diamond) and 7-mer (square). The remaining substrate (13-mer) is depicted by circles. Assays included the reconstituted protein (8 μM), SAM (3 mM), SP-containing DNA (40 μM), DTT (5 mM) and sodium dithionite (3 mM) in Tris buffer pH 8 under anaerobic conditions.

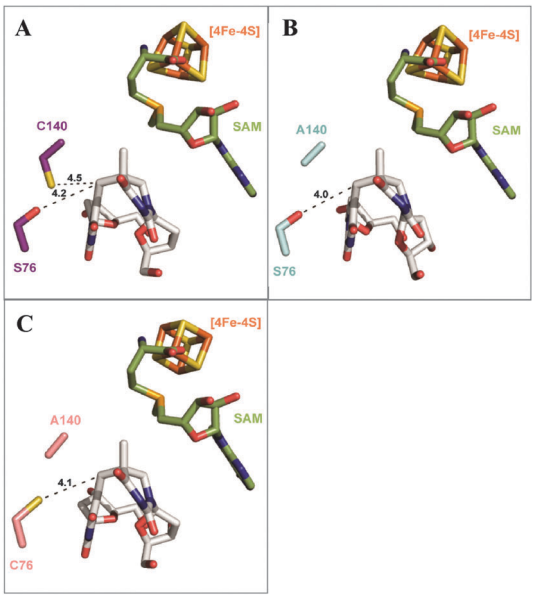

Fig. 3 Structures of (A) the wild-type SP lyase (PDB code 4FHD), (B) the C140A mutant (PDB code 4FHF) and (C) the double mutant active sites. Residues are depicted in the stick format and colored in purple (wild-type), cyan (C140A mutant), and salmon (double mutant). SP is shown in white. Distances are indicated in Å.

Since no structure from clostridial SP lyases is available, we crystallized and solved the structure of the double mutant whose active site mimics this sub-class of SP lyases. We obtained the structures of this mutant in the absence and presence of SP (Table S2, ESI†) at high resolutions (2 and 2.6 Å, respectively). The SP-free and -bound structures of the double mutant overlaid perfectly with the wild-type enzyme (r.m.s.d is 0.16 Å and 0.21 Å, respectively), revealing that the double mutation did not affect the overall fold of the enzyme as well as the SP binding (Fig. S7–S9, ESI†). Remarkably, in the double mutant, the newly inserted cysteine (C76) is closer to the methylene group of SP (4.1 Å) than C140 which is in the wild-type enzyme (4.5 Å) (Fig. 3), both distances being in perfect agreement with direct H-atom abstraction.

The recently solved structure of SP-containing DNA shows that conformational changes occur during the transition of SP to the repaired DNA,[13] likely bringing the 3′-thymine allylic radical intermediate close to the cysteine H-atom donor. In the C140A/S76C mutant, not only the distance between the H-atom donor (*i.e.* the SH group of C76) and the SP methylene bridge is shortened, but the orientation of the cysteine side-chain in the active site is also modified (Fig. 3). Both parameters likely influence the HAT pathway in the double mutated enzyme and may thus explain the increased repair rate.[14] Further studies notably regarding the interaction between SP lyase and its product will be required to definitively support this hypothesis.

Several SP lyases from *Bacilli*[7,12,15,16] and one from *Clostridia*[9] have been biochemically characterized. However, it was not clear so far how the repair activity takes place in *Clostridia* as the crucial cysteine residue found in *Bacilli* (C140 in *Gt*) is not conserved among clostridial SP lyases. In this study, we were able to rescue SP lyase activity by generating a "clostridia-like" SP lyase from the *Gt* enzyme. We established that a cysteine residue in close proximity to the SP lesion is required for catalysis by all SP lyases independently of their origin (*i.e. Bacilli* or *Clostridia*). The spatial position of this cysteine residue in the active site appears to be not crucial, while the distance between the side-chain of the cysteine residue and the 3′-thymidine residue of SP has to be in a range compatible with direct H-atom transfer (4–4.5 Å). Interestingly, we monitored an increased repair rate for the rescued enzyme concomitant with the decrease of the distance between the methylene bridge and the H-atom donor. If distance and orientation changes between the H-atom donor and acceptor could be responsible for this increased repair rate,[14] the following catalytic steps, notably those implicated in SAM regeneration, may also be involved in this process. Indeed, once the H-atom is transferred from the thiol group to the radical substrate, a thiyl radical is likely formed on the protein, which has to be reduced for further catalytic turnovers. It was recently proposed that a tyrosine residue (Y98 in *Gt* SP lyase), also located in the vicinity of C76 (6.9 Å in the double mutant), is involved in reducing the thiyl radical (Fig. S10, ESI†).[7,17,18] Interestingly, this tyrosine residue is strictly conserved among *Bacilli* and *Clostridia* SP lyases (Fig. S1, ESI†) and our structural model predicts an identical position within the active-site.

To conclude, our study (i) shows that SP lyases use a common mechanism to repair DNA independently of their origin and (ii) highlights how radical SAM enzymes can control radicals in their active sites. We anticipate that similar HAT pathways are widespread among radical SAM enzymes and required to control these highly reactive species formed during catalysis. Finally, we demonstrate for the first time that it is possible to rewire the HAT pathway in radical SAM enzymes, paving the way for the rational design of new catalysts for synthetic biology.

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