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The radical SAM enzyme spore photoproduct lyase employs a tyrosyl radical for DNA repair†

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The spore photoproduct lyase is a radical SAM enzyme, which repairs 5-(α-thyminyl)-5,6-dihydrothymidine. Here we show that the enzyme establishes a complex radical transfer cascade and creates a cysteine and a tyrosyl radical dyade to establish repair. This allows the enzyme to solve topological and energetic problems associated with the radical based repair reaction.

UV irradiation causes the formation of a variety of dinucleotide lesions, which are typically formed between two pyrimidines.[1,2] In cellular DNA, UV irradiation causes the formation of the well studied cyclobutane pyrimidine dimers (CPDs), pyrimidine (6-4)-pyrimidone photoproducts (6-4PPs), and their Dewar-valence isomers.[1] In bacterial endospores, in contrast, these bipyrimidine photoproducts are only formed in low amounts. Due to the unusual packing of the DNA in spores, UV irradiation was found to create selectively the (5*R*)-5-(α-thyminyl)-5,6-dihydrothymidine lesion (spore photoproduct or SP-lesion).[3–5] This dimeric thymidine-photoproduct is repaired during germination by the spore photoproduct lyase (SPL), which is a radical SAM enzyme.[6]

The SPL catalyzes the direct repair of the SP-lesion, utilizing a radical mediated mechanism (Fig. 1A).[7,8] SPL needs for initiation of the repair reaction a reduced $[4Fe4S]^{1+}$ cluster and an *S*-adenosyl-methionine (SAM).[7] It is currently not clear whether the SAM functions as a co-substrate, which is consumed during the repair reaction, or as a cofactor, which is regenerated after each catalytic cycle. Recent isotope studies performed by Broderick and Li laboratories[9–11] have underpinned earlier mechanistic proposals[8] suggesting that the repair reaction is initiated by an electron transfer from the iron sulfur cluster to SAM to generate a 5′-deoxyadenosyl radical (5′-dAdo•). This abstracts the C(6)H hydrogen atom from the 5,6-dihydrothymine moiety on the 5′-side of the lesion. Following fragmentation of the radical, a 3′-allyl type thymine radical is formed. This allyl radical needs to be reduced. Recent studies by the Atta[12] and Li laboratories[13] showed that the allyl reducing moiety is a conserved cysteine. A recent crystal structure of the SPL from *Geobacillus thermodenitrificans*[14] in complex with the lesion[15] (Fig. 1B) shows the topological problem associated with closing the catalytic cycle. While the allyl radical is situated and reduced at the 3′-side, transfer of the radical center back to the 5′-dAdoH requires moving the radical back to the 5′-part in the active side over a distance of roughly 10 Å. This creates next to a topological problem also an energetic obstacle, because regeneration of the adenosyl radical by the thiyl radical would be endothermic by 62 kJ mol^{-1}.[16]

Here we provide first evidence that the enzyme uses a further tyrosyl radical intermediate to solve the energetic and topological problem. In the crystal structure the tyrosine bridges the conserved cysteine and the 5′-dAdoH. The structure shows that Tyr98 is located 3.6 Å and 5.1 Å away from both centers, respectively. In order to clarify the role of Cys140 and Tyr98 we prepared two mutants C140A and Y98F (Fig. 2A) and determined their catalytic activity. To this end we quantified product formation after 3 h and after reaction overnight. The measured K_m and V_{max} values are listed in Table 1. The data show that both mutants have a strongly reduced catalytic activity. The Y98F mutant shows in addition a reduced substrate binding affinity, which indicates that the phenolic hydroxyl group is important to organize the substrate in the active site. Direct interactions with the lesion are, however, not observed in the crystal structure. For the catalytically competent wild type (wt) enzyme we measured a ratio of product/5′-dAdoH of greater than two indicating that the cleavage of one SAM molecule repairs more than one lesion (turnover). The mutants in contrast use more than one SAM per product showing that here the turnover is smaller than one product molecule (0.7 and 0.25, Fig. 2B) formed per SAM. This result shows that Cys140 and Tyr98 are important for establishing catalytic turnover. Interestingly, we were able to rescue catalytic turnover when we replaced C140 by a glycine residue. Although speculative at this point we believe that we are able to replace the thiyl radical by a thermodynamically comparable glycyl radical[16] in the catalytic cycle. This hypothesis is supported by the analysis of the catalytic turnover, which shows that the C140G mutant features a product/5′-dAdoH ratio

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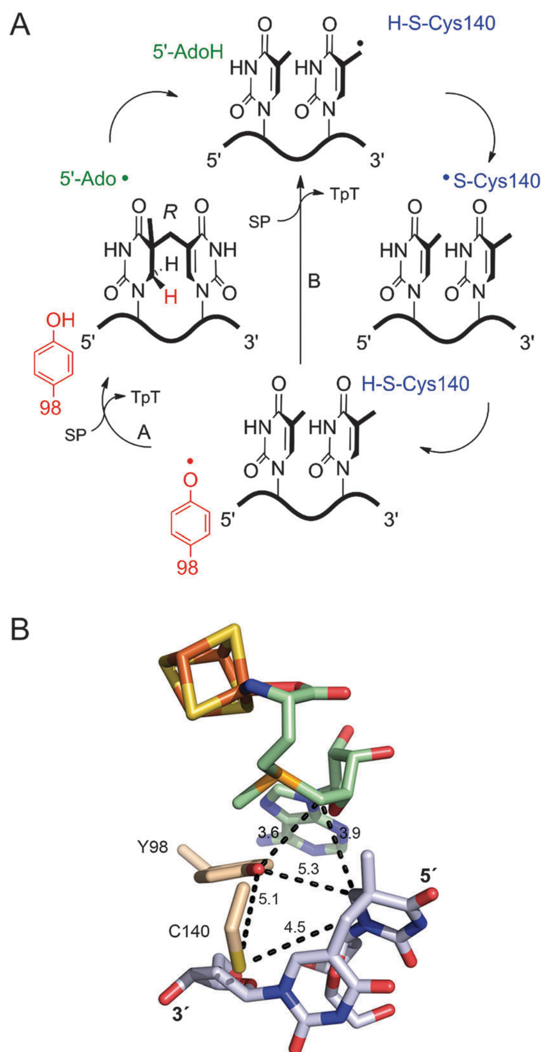

Fig. 1 (A) Proposed repair mechanism of the spore photoproduct lyase. Route A describes the regeneration of the initial 5′-dAdoH. In route B the tyrosyl radical propagates the catalytic cycle. (B) Active side of the spore photoproduct lyase (pdp: 4FHD), with the $[4Fe4S]^{1+}$ cluster in orange, SAM in green, the critical amino acids in light orange, and the SP dinucleoside lesion in blue. Distances are given in Ångstrom and are shown as dotted lines.

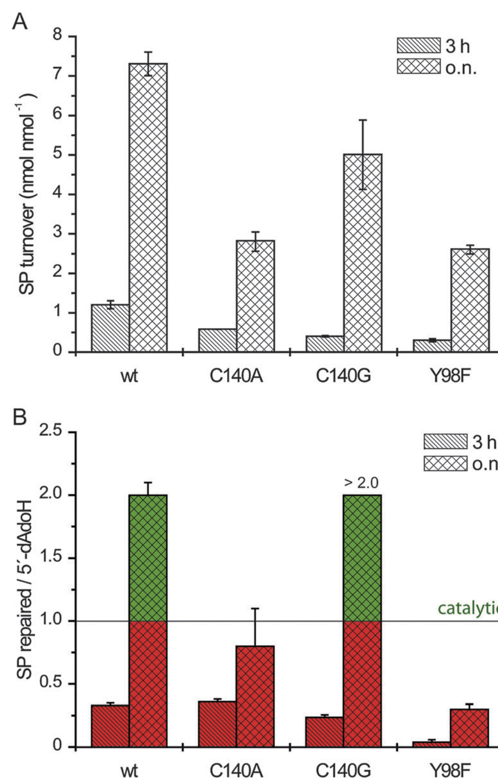

Fig. 2 (A) Bar graphs showing the activity of the wild type (wt) and mutant SPL enzymes. (B) Ratio of repaired SP to produced 5′-dAdoH. Green shading stands for catalytic usage of SAM. o.n. = over night.

Table 1 Kinetic data of SPL enzymes discussed in this study

SPL	K_M (μM)	V_{max} estimated (10^{-3} min^{-1})	V_{max} at 10wtK_M (10^{-3} min^{-1})
wt	0.9 ± 0.2	6.7 ± 0.5	12.5 ± 0.5
C140A	1.1 ± 0.8	35.2 ± 15.2	4.1 ± 0.2
C140G	n.a.	n.a.	3.5 ± 0.0
Y98F	11.3 ± 32.8	25.3 ± 66.7	2.3 ± 0.4

n.a. = not available.

of >2. In addition, SPL from *clostridium* species lack the Cys but feature a Gly-residue close by (S6, ESI†).

Whatever the reason for the high activity of the C140G mutant may be, the combined data indicate that the enzyme uses next to the conserved Cys140 residue additionally the side chain of Tyr98 to establish catalytic DNA repair. This idea is supported by sequence analysis, which proves that the tyrosine residue is highly conserved in all SPL enzymes (S6, ESI†).

In order to obtain direct proof for the involvement of a tyrosyl radical during the repair reaction we next exploited the strong UV-visible absorbance of the Tyr radical at λ = 410 nm.[17] We measured the absorbance spectrum of the wild type enzyme during the repair reaction and of the Y98F mutant under exactly the same conditions. The obtained UV-visible spectra (S5, ESI†) were subtracted from each other. This subtraction is needed because the absorption of the expected tyrosyl radical overlaps with the absorption of the [4Fe4S] cluster. The obtained difference spectrum is depicted in Fig. 3. Clearly evident is the presence of an additional absorption band with a maximum of λ = 410 nm in the wt enzyme that is absent in the Y98F mutant. The shape of the curve and in particular the absorption maximum is in perfect agreement with reported UV-visible spectra of tyrosyl radicals within active sites of proteins.[17,18]

Based on our mutagenesis studies but in particular based on the UV-visible difference spectrum we postulate that the SPL enzyme uses two redox active residues for the repair reaction as depicted in Fig. 1. While the Cys140 reduces as suggested by the initially formed allyl radical, it is tempting to speculate that it is Tyr98 that reduces the thiyl radical in the next step, which would transfer the radical from the 3′-side of the lesion back to the 5′-area in the active site. This solves the topological problem. Regeneration of the 5′-adenosyl radical by the tyrosyl radical would be possible based on the distance but it would be energetically equally inefficient.[16] We therefore believe that it is the tyrosyl radical which initiates the next catalytic cycle after

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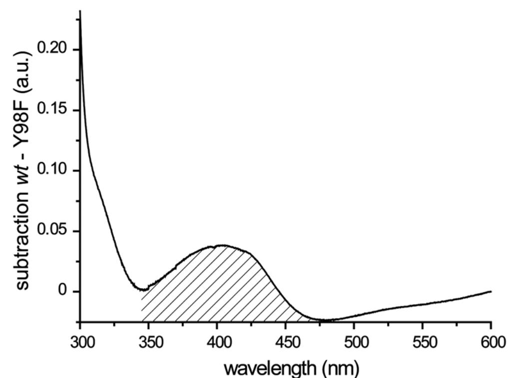

Fig. 3 UV-visible difference spectrum of wt SPL and of the Y98F mutant under assay conditions. The grey part emphasizes the signal that we attribute to the Tyr98 radical with its maximum at 410 nm. Conditions were 80 μM SPL, 150 μM SAM, 460 μM SP dinucleoside, 3 mM dithionite in 50 mM HEPES, 210 mM NaCl, and 5% glycerol, pH 7.5 at 35 °C.

exchange of the product by the substrate (route B in Fig. 1). In this scenario, the 5′-adenosyl radical would act as a radical that starts the radical chain reaction, while Cys140 and Tyr98 would carry the radical chain reaction on. This scenario also explains why the catalytic turnover numbers of the SPL enzyme, although significantly above 1 (*ca.* 7–10), are relatively low. Even though the strong oxygen sensitivity of the protein disturbs accurate determination of turnover numbers, we certainly expect higher numbers when the SAM would carry the catalytic cycle alone. Our mechanistic model solves the energetic and topological problems associated with the SPL repair mechanism.

In conclusion, the arrangement of Cys140 and Tyr98 in the active site[14] and the here proposed radical cascade established by these two residues allows to transfer the radical after the repair reaction from the 3′-side back to the 5′-side so that it can abstract the pro-*R* C(6)H-atom[9,11] from the next substrate. When the next catalytic cycle is initiated by the waiting Tyr98 radical the energetically problematic regeneration of the adenosyl radical can be avoided. While this manuscript was under review, Hioe and Zipse reported theoretical data of potential thermochemical profiles.[19] They also suggest that the SPL enzyme can circumvent the endothermic formation of the adenosyl radical when the radical chain reaction is propagated by an intermediate tyrosyl radical. Although formation of the Tyr98 radical is based on our data very likely, this conclusion needs further substantiation particularly based on EPR spectroscopic data. EPR would enable us not only to characterize the radical in more detail but would give in addition information about the environment in which the radical operates. Our data suggest that the SPL enzyme is the first example of a radical SAM enzyme in which the radical reaction is propagated by a tyrosine residue.

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