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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

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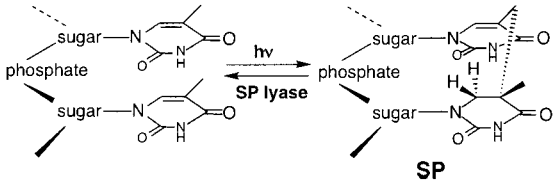
The major photoproduct in UV-irradiated *Bacillus* spore DNA is a unique thymine dimer called spore photoproduct (SP, 5-thyminyl-5,6-dihydrothymine).[1,2] Pyrimidine dimers such as SP are a major component of UV-induced DNA damage. These dimers can block replication and transcription, or can result in mutations if transcription does proceed past the region of the dimer. Repair of these dimers, therefore, is critical to avoid mutations. Although pyrimidine dimers can be excised and replaced, the only well-characterized example of direct pyrimidine dimer reversal is the photoreactivation catalyzed by DNA photolyase.[3,4] However photoreactivation has been shown to be absent in many species, suggesting that alternate means of pyrimidine dimer repair might be found.[3,5,6]

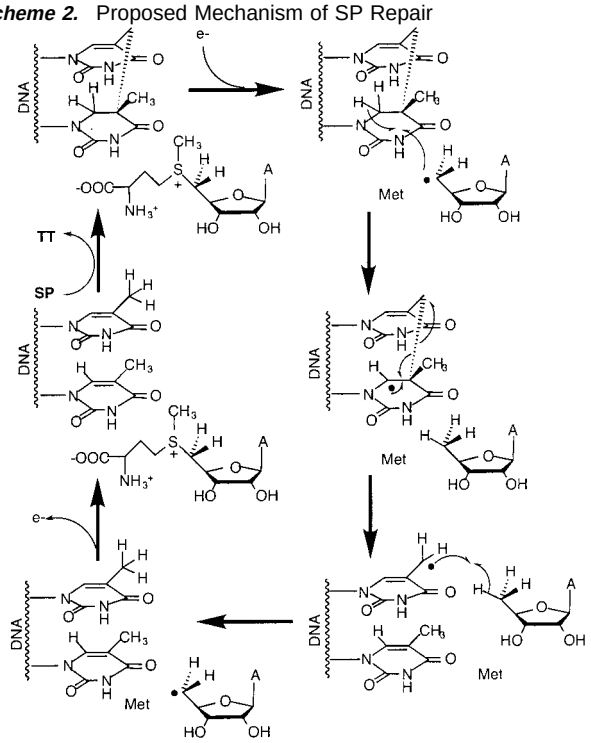
The enzyme SP lyase is the first identified *nonphotoactivatable* pyrimidine dimer lyase, catalyzing the repair of SP dimers to thymine monomers (Scheme 1) in a reaction that requires *S*-adenosylmethionine (AdoMet).[7−9] Chemical and preliminary spectroscopic studies have suggested that SP lyase is a member of the family of Fe-S/AdoMet enzymes that catalyze radical-mediated reactions.[9−11] The Fe-S/AdoMet enzymes appear to operate by common initial steps involving the generation of a 5′-deoxyadenosyl (5′dAdo) radical intermediate.[12] Here we provide evidence for the involvement of a 5′dAdo radical in the SP lyase mechanism by demonstrating that during repair, label is transferred to AdoMet from SP specifically ^{3}H-labeled at C-6. This label transfer also supports the proposal that SP repair is initiated by C-6 H atom abstraction, with the resulting substrate radical presumably undergoing β-scission to provide the monomeric products, as shown in Scheme 2 and previously suggested by Mehl and Begley.[13] Furthermore, we demonstrate that AdoMet acts as a catalytic cofactor during repair to *reversibly* generate the putative adenosyl radical intermediate, as is required by the mechanism in Scheme 2.

SP lyase was cloned, overexpressed, and purified by using procedures to be reported in detail elsewhere.[11,14] The purified enzyme contains an iron−sulfur cluster, as determined by UV−visible, EPR, and Mössbauer spectroscopy.[11] Preparation of the DNA substrate for SP lyase was accomplished by using modifications of previously reported procedures.[15,16] DNA tritiated at the methyl or C-6 position of thymine was produced by growing cultures of NovaBlue *Escherichia coli* (Novagen) carrying pUC18 in medium enriched with methyl- or C-6-tritiated thymidine. The isolated pUC18 DNA was complexed with SASP-C (a small, acid-soluble protein from *Bacillus*) and then UV-irradiated to produce spore photoproduct.[15,16] The amount of spore photoproduct generated was determined by HPLC analysis and scintillation counting after acid hydrolysis of the DNA.[17] SP lyase activity assays were

Scheme 1



Scheme 2. Proposed Mechanism of SP Repair



conducted by using modifications of previously published methods.[18] The degree of SP repair was analyzed by HPLC and scintillation counting of reaction mixtures and controls after DNA hydrolysis.[17]

Our purified SP lyase has a specific activity of 0.31 μmol SP repaired/min/mg SP lyase.[19] Time course assays showed linear SP repair up to 60 min, after which the degree of repair was variable, most likely due to protein instability under the assay conditions. On the basis of these results, it would seem that the overnight incubations used in previous work[9] would not provide a reliable estimate of SP lyase activity.

To probe the involvement of a 5′dAdo radical intermediate in the SP lyase mechanism, we investigated the possibility of label transfer from SP to AdoMet or 5′dAdo during SP repair. After running a repair reaction for a defined period of time,[20] the reaction

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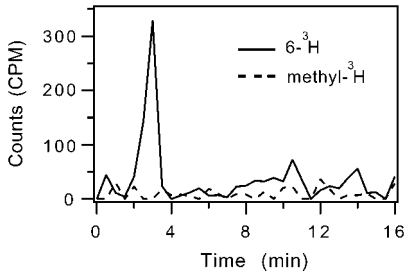

Figure 1. Tritium is incorporated into AdoMet during SP repair. Assays were done as described in the text. DNA tritiated at either the C-6 or methyl group of thymine (and SP) was used as substrate. Control assays were run in which no SP lyase was present. The sample components were then separated on a C18 column. Shown above are difference chromatograms (assay minus control) for the C-6-tritiated and methyl-tritiated samples. Elution times: AdoMet, 2.6 min; 5′-dAdo, 7 min.

mixture was injected onto a Spherisorb C18 column and eluted with H_2O (3 min) and then 18% CH_3CN in H_2O (13 min) while collecting fractions for scintillation counting. Under these conditions, AdoMet elutes at 2.6 min, 5′dAdo elutes at 7.0 min, and the unhydrolyzed DNA elutes in the final 100% CH_3CN wash. The resulting difference chromatograms (assay mix minus control mix containing no SP lyase) are shown in Figure 1. When the substrate DNA contains ^{3}H at the methyl position of thymine (and SP), no label transfer from substrate into either AdoMet or 5′dAdo is observed (Figure 1, dashed line). However, when DNA tritiated at the C-6 of thymine (and SP) is used as the substrate, significant incorporation of ^{3}H into AdoMet is observed (Figure 1, solid line). These results provide evidence not only for an intermediate 5′dAdo radical in the SP repair mechanism, but also for C-6 H atom abstraction as the initial step of the repair reaction, consistent with the mechanistic proposal shown in Scheme 2. The label transfer results are most consistent with a mechanism in which the 5′-dAdo radical intermediate abstracts H• directly from C-6 of SP. Although it is conceivable that the 5′-dAdo radical generates an intermediate protein radical, which then abstracts H• from C-6 of SP, such a mechanism would be difficult to reconcile with our label transfer results. In addition, EPR spectra of reactions in progress show no evidence for an intermediate protein radical.

The mechanism shown in Scheme 2 would also predict label transfer from 5′-^{3}H-AdoMet into the thymine monomers resulting from SP repair due to the abstraction of a 5′dAdo hydrogen by the product thyminyl radical. We have synthesized 5′-^{3}H-AdoMet[21] and incubated it with SP lyase and nontritiated SP-containing DNA. Preliminary results show incorporation of this label into thymine monomers, providing further evidence for the proposed mechanism in Scheme 2.

Our observation that the C-6 tritium label from SP ends up in AdoMet, and not in 5′dAdo, suggests that AdoMet is used as a catalytic cofactor to *reversibly* generate the putative 5′dAdo radical intermediate. Use of AdoMet as a catalytic cofactor has been observed previously for lysine aminomutase, one of the Fe-S/AdoMet enzymes.[22] In the case of SP lyase, however, Rebeil and Nicholson report the formation of 5′dAdo during turnover,[10] suggesting that AdoMet is being consumed, similar to what has been observed in the cases of the activating enzymes of pyruvate formate-lyase[23] and anaerobic ribonucleotide reductase.[24] To further investigate these possibilities, we have performed SP lyase activity assays under conditions of limiting AdoMet, with 1, 2, and 10 equiv of AdoMet relative to SP lyase.[25] With 1 or 2 equiv of AdoMet relative to SP lyase, 130(±10) nmol of SP was repaired, while with 10 equiv 180(±10) nmol of SP was repaired.[26] In the case of the 1-equiv assay, one AdoMet is mediating the repair of >500 SP lesions. Furthermore, no appreciable amount of 5′dAdo is formed during a typical SP lyase activity assay. These results clearly demonstrate that AdoMet acts as a catalytic cofactor and is not consumed stoichiometrically during turnover.

The results presented here provide the first experimental evidence for the involvement of a 5′dAdo radical intermediate in the DNA repair reaction catalyzed by SP lyase. The putative 5′dAdo radical intermediate, which is generated *reversibly* by SP lyase, interacts *directly* with the damaged DNA, abstracting a hydrogen atom from C-6 of SP to initiate repair. This radical-based mechanism and the use of AdoMet as a reversible radical initiator represent a new paradigm for repair of UV-induced DNA damage.

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(18) Assay mixtures contained 0.2 nmol SP lyase, 2 mM AdoMet, 25−50000 cpm of ^{3}H-pUC18 DNA (^{3}H at the thymine methyl and containing 200−435 nmol SP), 5 mM sodium formate, 4 mM dithiothreitol, 3 mM dithionite, and 155 mM KCl, all in 25 mM Tris-acetate buffer, pH 7.0. Control assays contained everything except SP lyase. Reactions were carried out under anaerobic conditions at 37 °C for varying periods of time and terminated by flash-freezing in liquid nitrogen.
(19) This is the first report of a specific activity for SP lyase. Previously, SP lyase activity has been defined as the percent of the total SP present that is repaired in a given time.[9] Since the total amount of SP present in DNA samples can vary, these numbers are not directly comparable if different batches or quantities of DNA are used. We therefore use here the more conventional units of micromoles of SP repaired per min.
(20) Assays were done as in ref 18, except that 0.47 nmol of SP lyase was used. SP-containing DNA (7 nmol) with ^{3}H label either at the methyl or C-6 position of thymine was used as a substrate.
(21) 5′-^{3}H-AdoMet was synthesized enzymatically from methionine and 2,8,5′-^{3}H-ATP by literature methods (Walsby et al. *J. Am. Chem. Soc.* In press.)
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(25) Assays were run for 1 h as described in ref 18 and included 0.23 nmol of SP lyase and 17.6 nmol of DNA containing 435 nmol of SP.
(26) No SP was repaired when no AdoMet was present.

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