Dinucleotide Spore Photoproduct, a Minimal Substrate of the DNA Repair Spore Photoproduct Lyase Enzyme from *Bacillus subtilis*®

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The overwhelming majority of DNA photoproducts in UV-irradiated spores is a unique thymine dimer called spore photoproduct (SP, 5-thymine-5,6-dihydrothymine). This lesion is repaired by the spore photoproduct lyase (SP lyase) enzyme that directly reverts SP to two unmodified thymines. The SP lyase is an S-adenosylmethionine-dependent iron-sulfur protein that belongs to the radical S-adenosylmethionine superfamily. In this study, by using a well characterized preparation of the SP lyase enzyme from *Bacillus subtilis*, we show that SP in the form of a dinucleoside monophosphate (spore photoproduct of thymidilyl-(3′→5′)-thymidine) is efficiently repaired, allowing a kinetic characterization of the enzyme. The preparation of this new substrate is described, and its identity is confirmed by mass spectrometry and comparison with authentic spore photoprod-

The DNA of all organisms is subject to modifications upon exposure to a wide variety of chemical and physical agents. Among them, solar ultraviolet radiation is known to induce dimerization reactions between adjacent pyrimidines (1). In the vast majority of living systems, the resulting photoproducts are cyclobutane pyrimidine dimers (CPDs)\(^5\) and pyrimidine (6-4) pyrimidone photoproducts (Scheme 1\(^A\)) that can be generated at any of the four bipyrimidine doublets (TT, CT, TC and CC), although the yields of the lesions depend on the bases involved (2). These lesions induce mutations and can be lethal because of blocking of the replication machinery. The photochemistry in bacterial spores is quite different. Indeed, in this dormant form produced by some bacteria such as *Bacillus subtilis*, the only photoproduct produced upon exposure to UV light corresponds to two thymines linked by the methyl group of one of the bases (3, 4). The formation of this specific lesion, 5-thyminyl-5,6-dihydrothymine (spore photoproduct, SP) (Scheme 1A), is explained by specific features of the spores, including DNA conformation (A form), dehydration, the presence of dicoplicenic acid in the core, and binding of small acid-soluble proteins to DNA (5–8). The formation of SP as the unique DNA lesion in irradiated spores is proposed to account for their extreme resistance to UV radiation. Indeed, spores express a specific repair enzyme, the spore photoproduct lyase (SP lyase) that directly reverts SP to two unmodified thymines upon germination (9, 10), much more efficiently than dimeric photoproducts are removed from other cell types by the classical nucleotide excision repair pathway. The specific photochemistry of DNA in spores combined with the action of SP lyase appears to be a major evolutionary advantage for spore-forming bacteria in resistance to UV radiation.

In their N-terminal half, all SP lyase enzymes contain a strictly conserved amino acid sequence containing three cysteines CXXCCXXC that have been shown to be essential for activity by site-directed mutagenesis (11). These cysteines have therefore been proposed to provide protein ligands for a catalytically essential [4Fe-4S]\(^{+2/+1}\) cluster (11). This CXXCCXXC sequence is indeed the signature for a superfamily of [4Fe-4S] iron-sulfur enzymes, named “radical SAM” (12), involved in a variety of biosynthetic pathways and metabolic reactions that proceed via radical mechanisms (13–15). Spectroscopic and biochemical studies from Nicholson and co-workers (16) and Broderick and co-workers (17) have shown the following: (i) the protein carries a single iron-sulfur cluster (16); (ii) the reaction is absolutely dependent on S-adenosylmethionine (AdoMet) (16, 17); and (iii) the repair mechanism is likely to involve a 5′-deoxyadenosyl radical (Ado\(^r\)) generated through reductive cleavage of AdoMet (16, 17). Labeling experiments indicate
that the reaction is initiated by direct C-6 hydrogen atom abstraction by AdoH, with the resulting substrate radical undergoing scission to re-generate the two initial thymines (Scheme 2) (17).

The SP lyase enzyme demonstrates a novel aspect of the diversity of DNA repair mechanisms in living organisms. In particular, it is remarkable that in the case of this enzyme, DNA repair is achieved through nucleotide radical intermediates, generally considered as precursors of DNA damage. To better understand the chemistry of this unique enzyme, we undertook this study. The first issues we address concern the substrate specificity of SP lyase. The following still are not known: (i) whether a dinucleotide SP lesion is a substrate; (ii) whether the enzyme is stereoselective. The following still are not known: (i) whether the enzyme is stereoselective; and (iii) whether the enzyme is stereoselective. To be able to answer these issues, it is absolutely required that well defined compounds rather than UV-irradiated plasmid DNA are used as substrates. Here we report an original approach for further enzymatic studies. Combined with a previous study addressing the question of the stereoselectivity of the enzyme (18), this work provides new insights into the chemistry of SP lyase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following strains were used in this study. *Escherichia coli* DH5α was used for routine DNA manipulations. *E. coli* Tuner (DE3) (Stratagene) was used for SP lyase overexpression. Enzymes, oligonucleotides, and culture media were purchased from Invitrogen. T4 DNA ligase was from Promega. Bacterial alkaline phosphatase and plasmid DNA purification kit, Flexiprep™, were from Amersham Biosciences. DNA fragments were extracted from agarose gel and purified with High Pure PCR product purification kit (Roche Applied Science), and DNA sequencing was performed by Genome Express (Grenoble, France). TpT was prepared as described previously (19).

**Cloning and Construction of SP Lyase-overexpressing Plasmids**—The SplB gene, encoding SP lyase, was amplified by a PCR-based method using *B. subtilis* genomic DNA as a template. The following primers were used: 5’-tgtcgccatATGcagacacctgtgctg-3’ (Ndel site underlined and ATG codon in uppercase) hybridized to the noncoding strand at the 5’ terminus of the gene and 5’-tttataaaaaagctttgctgttgatcacaac-3’ (HindIII site underlined) hybridized to the coding strand. PCR was run on a Stratagene RoboCycler Gradient 40 machine. The PCR product was digested with Ndel and HindIII and then ligated with T4 DNA ligase into the pT7-7 plasmid, which had been digested previously with the same restriction enzymes. The cloned gene was entirely sequenced to ensure that no error was introduced during the PCR. The plasmid was then named pT7-7-SPL. The hexahistidine linker sequence was introduced into pT7-7-SPL as described previously (20). The new plasmid was named pT7-7-SPL6H.

**Protein Expression**—*E. coli* Tuner (DE3) were transformed by pT7-7-SPL6H and then grown overnight at 37 °C in LB medium (100 ml) supplemented with ampicillin (100 μg/ml). The overnight culture was used to inoculate fresh LB medium (10 liters) supplemented with the same antibiotic, and bacterial growth was allowed to proceed at 37 °C until A600 reached 0.9. To reduce formation of inclusion bodies, protein expression was performed at 18 °C and induced by adding 500 μM of iso- propyl 1-thio-β-D-galactopyranoside. Cells were collected after 18 h of culture by centrifugation at 4000 × g at 4 °C for 30 min and resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 200 mM KCl). The cells were disrupted by sonication and centrifuged at 220,000 × g at 4 °C for 1 h and 30 min. The solution obtained was then loaded onto a nickel-nitriol triacetic acid-Sepharose superflow column that had been equilibrated previously with buffer A. The column was washed extensively with the same buffer. Nonspecifically adsorbed proteins were eluted by a wash step with buffer A containing 30 mM imidazole, and then the SP lyase was eluted with buffer A containing 0.5 M imidazole. Fractions containing SP lyase were immediately concentrated in an Amicon cell fitted with a YM30 (Spectrum) membrane, and 3 mM DTT was added before freezing.

**Aggregation State Analysis**—Fast protein liquid chromatography gel filtration with an analytical Superdex-75 (Amersham Biosciences) at a flow rate of 0.5 ml/min, equilibrated with 0.1 mM phosphate buffer, pH 7, containing 200 mM KCl, was used for size determination and was performed under strict anaerobic conditions. A gel filtration calibration kit (calibration protein II; Roche Applied Science) was used as molecular weight standards.
Iron and Sulfide Binding to SP Lyase — The following procedure was carried out anaerobically in a glove box (Jacomex B553 (NMT)). ApoSP lyase (100 μM monomer) was treated with 5 mM DTT and incubated overnight with a 5-fold molar excess of both Na2S (Fluka) and (NH4)2Fe(SO4)2 (Aldrich) at 6 °C. The protein was desalted on Sephadex G-25 (80 ml, same buffer), and the colored fractions were concentrated on Nanosep 10 (Amicon).

Production of SPTpT Substrates — The spore photoproduct of the dinucleoside monophosphate thymidylyl-(3’–5’)-thymidine was prepared in two different ways. It was first obtained by hydrolysis of UVC-irradiated DNA, as described previously (21). Briefly, dry films of calf thymus DNA (Sigma) were prepared by freeze-drying an aqueous solution and subsequently exposing them to the UVC light emitted by a germicidal lamp. Irradiated DNA was then resuspended in water and digested by sequential incubation with nuclease P1 and phosphodiesterase II, pH 5, and alkaline phosphatase and phosphodiesterase I, pH 8. Normal bases were released as nucleosides, whereas bipyrimidine photoproducts were found as modified dinucleoside monophosphates. SPTpT was purified by HPLC. The concentration of the final solution was determined after acidic hydrolysis of an aliquot and comparison of the intensity of the HPLC signal of the released thymine spore photoproduct with that of authentic SPTpT isolated from DNA. In addition, an aliquot fraction (100 μl) of the purified SPTpT was mixed with pure formic acid. The resulting solution was heated at 120 °C for 2 h and then dried under vacuum. The residue was made soluble in 100 μl of water and characterized by HPLC-MS/MS. Irradiation conditions were optimized by irradiating 1 × 1-cm dry films prepared from 1 ml of solutions containing 0.3 mM TpT and increasing concentration of either the Ca2+ or the Na+ salt of DPA.

SP Lyase Activity — Typically, the reaction mixture contained wild-type SP lyase (1 μM), SPTpT substrate (10 μM), AdoMet (1 mM), and DTT (2 mM) in a final volume of 50 μl of 100 mM Tris-HCl, pH 8, containing 200 mM KCl. In addition, either dithionite (3 mM) or the physiological reducing system (Fdx/Fdx red/NADPH, 4:1:1 μM) was added as a reductant. The reactions were carried out under anaerobic conditions at 37 °C for various periods of time. At each time point (0, 10, 30, 60, 120, and 240 min) 5 μl of the solution was transferred to an Eppendorf tub, and the reaction stopped by flash-freezing in liquid nitrogen. Each sample was then diluted in 45 μl of 2 mM triethyl ammonium acetate and analyzed by HPLC coupled to tandem mass spectrometry for their TpT and SPTpT content.

HPLC-Mass Spectrometry Analysis — Conversion of the spore photoproduct (SPTpT) into the unmodified dinucleoside monophosphate (TpT) in SP lyase–treated samples was quantified by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS). Samples (20 μl from the initial 50 μl) were injected onto an Uptisphere ODB (particle size 3 μm, 150 × 2 mm inner diameter) octadeucysil silica gel column (Interchim, Montluçon, France) connected to a series 1100 Agilent chromatogram.
THE FOLLOWING PROVIDES THE FIRST CHARACTERIZATION OF THE SP LYASE ENZYMES

RESULTS

**Characterization of the SP Lyase Enzyme**—The enzyme was an N-terminal His-tagged protein purified from extracts of an *E. coli* strain overexpressing the *splB* gene from *B. subtilis* as described under “Experimental Procedures.” Analysis by denaturing gel electrophoresis (SDS-PAGE) showed that SP lyase was obtained as nearly pure protein with an apparent molecular mass of 41 kDa, as expected from the amino acid sequence (data not shown). The as-isolated pure protein, which lacked the light absorption bands in the visible spectrum characteristic for [Fe-S] clusters and did not contain measurable amounts of iron and sulfur, had a marked propensity to precipitate that was partly prevented by using buffers containing large concentrations of salt (200 mM KCl). Addition of DTT and glycerol also helped in this matter. Nevertheless, even under these conditions, only 30% of the SP lyase remained soluble after 48 h at 4 °C.

We prepared the holo-form of the enzyme by reconstituting the [Fe-S] cluster by treatment of the as-isolated apoform with iron and sulfide salts under anaerobic conditions in accordance with procedures described previously (26). After reconstitution, the enzyme was found to contain ~3.5–3.9 iron and sulfur atoms per protein monomer. In the holo-form, and under anaerobic conditions, the protein no longer precipitated and could resist several cycles of freezing-thawing without precipitation, loss of the cluster, or inactivation. Size exclusion chromatography analysis of the reconstituted enzyme under anaerobic conditions showed that SP lyase exists predominantly as a dimer in solution, as shown previously (16).

The light absorption spectrum of the reconstituted protein, shown in Fig. 1A, is very different from that reported previously (20) and is most similar to spectra of other [Fe-S] proteins of the radical SAM enzyme superfamily (ribonucleotide reductase, MiaB, and HemN) (26–28). It is thus consistent with the presence of a large proportion of polypeptides carrying a [4Fe-4S] cluster in solution. If [2Fe-2S] clusters are present, the UV-visible spectrum indicates that they account for a minor proportion of total iron. Exposure of the protein to air resulted in a rapid degradation of the cluster, as measured by the decrease of the absorption band at 420 nm (data not shown). This explained why the as-isolated protein was in the apo-form. During anaerobic reduction of the reconstituted protein with an excess of sodium dithionite, the solution bleached with a concomitant decrease of the absorption band at 420 nm. The resulting reduced solution displayed an axial EPR spectrum with g values of 2.03 and 1.93 (Fig. 1B). The temperature dependence and microwave power saturation properties of the EPR signals were characteristic for the $S = 1/2$ ground state of a [4Fe-4S]$^{1+}$ cluster (data not shown). Integration of the signal indicated that only 10% of total iron present in the sample was in the form of a reduced [4Fe-4S]$^{1+}$ cluster. This likely results from incomplete reduction of the cluster because of a low redox potential, as generally observed in the radical SAM family of enzymes, and may also be related to partial degradation of the protein under reducing conditions.

The following provides the first characterization of the SP lyase iron centers by Mössbauer spectroscopy, using a protein
sample that had been anaerobically reconstituted with $^{57}$Fe and sulfide. This study was carried out because we are aware of examples for which Mössbauer spectroscopy contradicted UV-visible spectroscopy regarding the nature and diversity of protein-bound clusters. The Mössbauer spectra, obtained at 78 and 4.2 K, are displayed in Fig. 2. At both temperatures, quadrupole doublets were observed. Inspection of the spectra indicates that the protein sample was not homogeneous and contained at least four different species labeled A–D, respectively. In Fig. 2 the solid lines are simulations obtained with four different doublets using the parameters and relative amounts listed in Table 1. Because the left line of the four quadruple doublets coalesces at approximately $+0$ mm/s, the determination of the hyperfine parameters of these species suffers from a rather large degree of uncertainty. Moreover, there is a significant overlap between doublets A and B, and therefore their relative area ratios cannot be determined accurately. For C and D, the determination of the area is more reliable because the high energy absorption peaks are well resolved. The 4.2 and 78 K spectra are almost identical except for an apparent small decrease of the area attributed to doublet B. The ratios quoted in Table 1 are derived from the spectrum at 4.2 K.

Doublet D is attributed to octahedral Fe$^{2+}$ ($S = 2$) impurities accounting for $\sim20\%$ of total iron. Doublet C can also be assigned to ferrous iron (about 10\% of total iron), but whether it belongs to an [Fe-S] complex cannot be established. The hyperfine parameters of the major doublet A (Table 1) are consistent with a $[4\text{Fe}-4\text{S}]^{2+}$ ($S = 0$) cluster, accounting for 40\% of total iron, and are comparable with parameters reported for clusters of other radical SAM enzymes (29–32). As is often the case with such enzymes, $[2\text{Fe}-2\text{S}]^{2+}$ ($S = 0$) clusters are also present in the SP lyase preparations. The parameters of doublet B are consistent with such a cluster, and they are in the range of those

| Site | $\delta$ (mm/s) | $\Delta E_Q$ (mm/s) | Area (%) |
|------|----------------|-------------------|--------|
| A    | 0.44 (4)       | 1.06 (12)         | 40 (5) |
| B    | 0.33 (5)       | 0.66 (10)         | 27 (5) |
| C    | 0.70 (10)      | 1.98 (20)         | 12 (2) |
| D    | 1.26 (10)      | 2.88 (20)         | 21 (2) |
obtained for $[2\text{Fe}-2\text{S}]^{2+}$ clusters found in ribonucleotide reductase-activating enzyme and in biotin synthase (31, 32). Furthermore, we exclude the presence of $[3\text{Fe}-4\text{S}]^{2+}$ clusters in SP lyase preparations on the basis of the absence of characteristic $S = 1/2$ signals in the EPR spectrum (data not shown).

As a conclusion the Mössbauer spectra from preparations anaerobically reconstituted SP lyase indicate a large degree of inhomogeneity regarding the nature of the $[\text{Fe-S}]$ clusters, despite a well defined UV-visible spectrum. Apart from the presence of high spin ferrous impurities, the results are consistent with the presence of $[2\text{Fe}-2\text{S}]^{2+}$ and $[4\text{Fe}-4\text{S}]^{2+}$ clusters. The minor species, B, C, and D, revealed by Mössbauer spectroscopy, could represent either intermediate forms on the way to the $[4\text{Fe}-4\text{S}]^{2+}$ cluster or derive from the $[4\text{Fe}-4\text{S}]^{2+}$ cluster by degradation, as observed with many radical SAM enzymes (31, 33).

Substrate Specificity of the Reconstituted SP Lyase—SP lyase was tested for its ability to repair a mixture of DNA lesions. For this purpose, samples of calf thymus DNA were exposed to UV-C radiation in the dry state as described under “Experimental Procedures.” These substrates are interesting for specificity studies because they contain all types of lesions (Scheme 1A) as follows: cyclobutane dimers, (6-4) photoproducts, and the spore photoproduct (22). B. subtilis reconstituted SP lyase (10 μM) was incubated with 0.5 mg/ml of irradiated DNA in the presence of 1 mM AdoMet, an excess of sodium dithionite (2 mM), and 2 mM DTT under anaerobic conditions. The DNA was then enzymatically hydrolyzed and analyzed by HPLC-MS/MS to establish its content in each of the bipyrimidine photoproducts at $t = 0$ min and after 30 min of incubation. Comparison of the two samples showed that no significant decrease in the level of cyclobutane dimers and (6-4) photoproducts (both thymine-thymine and thymine-cytosine derivatives) could be observed (Fig. 3). In contrast, almost all SP lesions initially present were repaired after 30 min of incubation. This established that our reconstituent SP lyase was active and confirmed its remarkable substrate selectivity with respect to photolesions of DNA, as reported previously (10). As a complementary assay for the activity of our preparations, we showed that reconstituted SP lyase in the absence of DNA substrate was able to catalyze the reductive cleavage of AdoMet to 5′-deoxyadenosine by dithionite (data not shown), as reported previously (16).

Preparation of SPTpT, the Dinucleoside Monophosphate SP Lesion—To date, SP lyase activity has been assayed using either SP-containing [methyl-3H]DNA extracted from irradiated spores or tritiated pUC18 plasmid DNA exposed to UV radiation in the presence of a small acid-soluble protein from B. subtilis, as a substrate (17). The repair activity was defined in terms of remaining SP after a given reaction time, determined after hydrolysis of DNA, isolation of the released SP, and scintillation counting (10, 17, 20). To study the repair reaction with a better defined substrate, available in larger quantities, we decided to use instead the modified dinucleoside monophosphate SPTpT in a pure form (Scheme 1B). This compound was first prepared by UV irradiation of dry films of DNA followed by hydrolysis and purification (see “Experimental Procedures”). It thus represents the natural dinucleotide substrate of the enzyme. In particular, the presence of the phosphodiester linkage between the two thymidine bases locks the photoproduct in a conformation similar to that occurring within DNA. To prepare larger amounts of SPTpT, a strategy was designed for using the dinucleoside monophosphate (TpT) rather than DNA as the starting material (19). Unfortunately, irradiation of TpT in the dry state yielded only trace amounts of SPTpT, whereas the (6-4) photoproduct together with the cis,syn and trans,syn cyclobutane dimers were the major photoproducts (data not shown). This problem was overcome by adding DPA into the dry film to be irradiated (Scheme 1B). Indeed, it is now well established that DPA in spores behaves as a photosensitizer that favors the formation of SP at the expense of the other bipyrimidine photoproducts (8). Addition of DPA had the same effect within dry films of TpT (Fig. 4). DPA is associated with Ca$^{2+}$ in spores, and DPA-Ca$^{2+}$ was thus used initially in our experiments. However, DPA-Ca$^{2+}$ solutions are difficult to prepare and are quite unstable. We thus repeated the experiments with the sodium salt. Both DPA salts were found to exhibit the same photochemical properties (Fig. 4). It is inter-
interesting to note that addition of DPA to the irradiated dry films had an effect on both the absolute and the relative amount of SPTpT produced because the latter was greatly favored with regard to the other photoproducts (Fig. 5), thereby facilitating the subsequent purification steps. SPTpT produced upon irradiation of TpT was extensively characterized by HPLC coupled to mass spectrometry. The molecular weight of the purified compound was found to be 546, as shown by a pseudo-molecular ion observed at \( m/z = 545 \) on the mass spectrum of the corresponding HPLC peak (Fig. 6). Identification of the product as the spore photoproduct of TpT was then inferred from its fragmentation mass spectrum (Fig. 7A) that was absolutely identical (both in terms of mass and relative intensity of the fragments) to that of SP obtained either from isolated DNA (21) or, more interestingly, from irradiated spores (7). A last piece of evidence for the identity of SPTpT was its ability to release the spore photoproduct of thymine upon acidic hydrolysis as the result of the cleavage of the two \( N \)-glycosidic bonds. The assessment of the hydrolysis product was based on its chromatographic and mass spectrometry features (Fig. 7B) that were identical to those of authentic thymine spore photoproduct (34).

**SPTpT Is a Substrate of SP Lyase**—The use of SPTpT as a substrate facilitated analysis of the repair reaction because samples could be directly submitted to HPLC-MS/MS at the end of the incubation. This procedure allows easy monitoring and quantification of both substrate conversion and TpT (repaired dinucleotide) product formation. Indeed, these compounds can be easily separated by HPLC and identified, as they display characteristic well defined mass spectrometric features. Typical chromatograms obtained with the reconstituted SP lyase are presented in Fig. 8. The reaction was carried out anaerobically under the following standard conditions: 1 \( \mu \)M enzyme, 10 \( \mu \)M SPTpT substrate, 1 \( \mu \)M AdoMet, and either 2 \( \mu \)M dithionite or 1 \( \mu \)M NADPH (in the presence of catalytic amounts of flavodoxin and flavodoxin reductase) as the reducing agent. The results presented in Fig. 8 clearly showed that SPTpT, corre-
sponding to the peak eluted at 19 min, could be totally converted by the enzymatic reaction into the expected repaired TpT product (retention time, 25 min) after 60 min. The identity of the reaction product, not present initially in the reaction mixture, was assessed from the comparison of its chromatographic and mass spectrometry features with those of a standard sample. No TpT could be detected when protein or AdoMet was omitted from the reaction mixture (control). A typical time curve for SP lyase-dependent conversion of SPTpT to TpT is shown in Fig. 9. Complete conversion of SPTpT into TpT (100% yield; 10 turnovers) was observed after about 40–50 min, under the given assay conditions. These results demonstrate that SPTpT is a substrate for SP lyase and that SP lyase functions catalytically with such a substrate. Furthermore, Fig. 9 shows, for the first time, that an enzymatic system based on NADPH/flavodoxin/flavodoxin reductase can function as a reducing agent as efficiently as dithionite. Previously reported assays indeed were based on dithionite only (16–18, 20). It is interesting to note that similar results were obtained whether the SPTpT substrate was obtained from irradiated DNA or from irradiated TpT. As shown in Fig. 10, the initial reaction rate displayed a saturation behavior with respect to SPTpT concentration. The resulting double-reciprocal plot shown in Fig. 10B allowed the determination of a $V_{\text{max}}$-specific activity value of 0.24 mol/mol SP lyase/min and a $K_m$ value for the SPTpT substrate of 6 $\mu$M.

**DISCUSSION**

To study the chemistry of spore photoproduct (SP) repair, we prepared the SP lyase enzyme from *B. subtilis* in pure form. As frequently observed with some radical SAM enzymes, reconstituted SP lyase preparations were not homogeneous because they contained polypeptides with either a [4Fe–4S]$^{2+}$ or a [2Fe–2S]$^{2+}$ cluster, as shown by Mössbauer spectroscopy. In the case...
of biotin synthase or the activating component of ribonucleotide reductase, readily \([4\text{Fe}-4\text{S}]^{2+}\) to \([2\text{Fe}-2\text{S}]^{2+}\) cluster interconversion has been demonstrated \textit{in vitro} (31, 33, 35). It is likely that this interconversion also occurs \textit{in vitro} of SP lyase enzyme demonstrates that the clusters in this protein are very similar to those in biotin synthase or in ribonucleotide reductase activase, because comparable Mössbauer parameters were observed for all these proteins. There is no doubt that the \([4\text{Fe}-4\text{S}]^{2+}\) cluster in SP lyase is very labile and oxygen-sensitive, and this explains why a cluster-free protein was obtained after purification. Although all clusters of reconstituted SP lyase were in the \(S = 0\) state, in agreement with the absence of an EPR signal, reduction by sodium dithionite generated an EPR signal with \(g\) values and properties characteristic of an \(S = 1/2\) \([4\text{Fe}-4\text{S}]^{+1}\) species. Despite this inhomogeneity, our preparation proved to be active and highly selective for \textit{in vitro} repair of the SP lesion, as shown from the exclusive and total repair of the SP lesion within a DNA sample containing a variety of lesions as a consequence of UV irradiation: SP, cyclobutane dimers, and (6-4) photoproducts (Fig. 3).

Assaying DNA repair by SP lyase is not a trivial issue because of the difficulty of access to the substrate. Indeed, DNA containing the SP lesion cannot be readily extracted from bacterial spores, whereas irradiation of DNA \textit{in vitro} generally gives rise to a variety of base modifications, with minor production of the SP lesion. Consequently, assays are generally based on irradiated radioactively labeled DNA (17). There are also few reports on the synthesis of the SP lesion in the form of the corresponding dinucleoside. The currently available methods involve an important number of steps, because of the need for protection and deprotection of both the sugar and the base, and provide the product with very low yields (36). More importantly, the product obtained is the dinucleoside, lacking the phosphodiester bridge. Even though such a compound might serve as a mimic of an interstrand SP lesion, it is important to recall that the most abundant, almost unique, SP produced during spore DNA irradiation is the intrastrand one, resulting from coupling of two adjacent thymines on the same strand (8). The results reported here demonstrate for the first time that SPTpT, the well defined dinucleotide SP lesion (Scheme 1B) in which the two bases are linked by the methylene group and the two 2-deoxyriboses by a phosphodiester bridge, behaves as a good substrate of SP lyase enzyme that catalyzes its conversion to the repaired TpT dinucleotide. Together with the TpT dinucleotide product, this substrate also provides the great advantage that it can be monitored and quantified accurately by HPLC-MS/MS providing a very simple assay for the repair reaction. So far, very little information is available regarding the kinetic parameters associated with SP lyase. There is only one reported value for the specific enzyme activity using an SP-containing DNA, even though there is no information regarding saturation of the enzyme and no reported \(K_m\) value. The well defined SPTpT substrate, studied here, provides a tool to establish those parameters.

As mentioned above, total synthesis of SPTpT is not straightforward. Therefore, it was first isolated, following enzymatic digestion, from isolated DNA exposed to UVC as a dry film. The major drawback of this approach is the low amount of photoproducts obtained. To increase the availability of this substrate, SPTpT was then prepared by exposure of TpT to UVC in the presence of DPA. The latter compound is present in large amounts in spores and is partly responsible for the specific formation of SP within DNA (8). The observation that DPA favors the formation of SP from the dinucleoside monophosphate TpT provides additional support that its impact on the photosensitizing properties of spore DNA is mainly due to its photosensitizing properties. Interestingly, the product obtained upon irradiation of TpT cannot be distinguished from SPTpT isolated from irradiated DNA as follows. (i) They both exhibit the same chromatographic properties. (ii) Their fragmentation mass spectra are identical. (iii) They are repaired with the same efficiency by SP lyase. The latter result is of importance because, by using separated \(5R\) and \(5S\) isomers of the synthetically prepared SP thymidine derivative, we recently demonstrated that only the \(5S\)-configured lesion was repaired, showing that SP lyase is highly stereoselective (18). The present series of observations thus strongly suggest that the same diastereoisomer was produced upon irradiation of TpT and DNA, even though further spectroscopic data are needed to definitively establish its absolute configuration (see Scheme 1A for the structure of the two stereoisomers).

Finally, the fact that the SPTpT dimer is a substrate indicates that the SP lesion does not absolutely need to be contained within a single- or double-stranded DNA for recognition and repair by the SP lyase enzyme. The fact that SPTpT is recognized \((K_m = 6 \ \mu\text{M})\) and repaired strongly suggests that the active site of the enzyme is finely designed to flip the thymine dimer to be repaired out of the duplex DNA target, in order to bind it specifically. A similar mechanism has been observed in the case of the DNA photolyase-CPD complex, which recently has been structurally characterized (37). A model is presented in Scheme 3. On the other hand, the nucleic acid environment, in which the thymine dimer is present, might play some role as well, as revealed by the comparison of the enzyme activities obtained \textit{in vitro} when the assayed substrate was a DNA containing the SP lesion or the SPTpT dimer. In the former case an

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**Scheme 3.** Schematic diagram showing the interactions between the DNA lesion and the enzyme. SAM is AdoMet.
activity of about 12 mol/mol/min was reported (17), whereas in the experiments reported here, repair was achieved with a $V_{m}$ value of 0.24 mol/mol/min, indicating a 50-fold increase because of the presence of a double-stranded DNA in the SP environment. However, the comparison might not be totally relevant because the two substrates were assayed under slightly different conditions. In particular, the former value has been obtained at 37°C, as compared with 20°C for the assays reported here. Enzymatic activity with SPTpT can also be compared with that obtained with an interstrand synthetic SP dinucleoside. With the same enzyme preparations and comparable assay conditions as those used in this study, we reported a specific activity of about 0.004 mol/mol/min using the dinucleoside substrate (18). This 60-fold difference in repair activity demonstrates the importance of the phosphodiester bridge, probably because it limits the conformational freedom of the substrate dimer and suggests that the enzyme is not effective in repairing interstrand lesions. As indicated above, this is not a major problem for sporulating bacteria because irradiation of DNA in spores almost exclusively generates the intrastrand SP (8).

Future experiments should focus on the incorporation of the SPTpT dimer into oligonucleotides with different sequences, in order to further analyze the substrate selectivity properties of this fascinating enzyme.

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