Characterization of an Active Spore Photoproduct Lyase, a DNA Repair Enzyme in the Radical S-Adenosylmethionine Superfamily*

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Jeffrey M. Buis1§, Jennifer Cheek†1, Efthalia Kallili‡, and Joan B. Broderick1‡

From the 1Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana 59717 and the 2Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

The major photoproduct in UV-irradiated Bacillus spore DNA is a unique thymine dimer called spore photoproduct (SP, 5-thyminyl-5,6-dihydrothymine). The enzyme spore photoproduct lyase (SP lyase) has been found to catalyze the repair of SP dimers to thymine monomers in a reaction that requires S-adenosylmethionine. We present here the first detailed characterization of catalytically active SP lyase, which has been anaerobically purified from overexpressing Escherichia coli. Anaerobically purified SP lyase is monomeric and is red-brown in color. The purified enzyme contains ~3.1 iron and 3.0 acid-labile S2− per protein and has a UV-visible spectrum characteristic of iron-sulfur proteins (410 nm (11.9 μM−1 cm−1) and 450 nm (10.5 μM−1 cm−1)). The X-band EPR spectrum of the purified enzyme shows a nearly isotropic signal (g = 2.02) characteristic of a [3Fe-4S]1+ cluster; reduction of SP lyase with dithionite results in the appearance of a new EPR signal (g = 2.03, 1.93, and 1.89) with temperature dependence and g values consistent with its assignment to a [4Fe-4S]1+ cluster. The reduced purified enzyme is active in SP repair, with a specific activity of 0.33 μmol/min/mg. Only a catalytic amount of S-adenosylmethionine is required for DNA repair, and no irreversible cleavage of S-adenosylmethionine into methionine and 5′-deoxyadenosine is observed during the reaction. Label transfer from [5′-3H]S-adenosylmethionine to repaired thymine is observed, providing evidence to support a mechanism in which a 5′-deoxyadenosyl radical intermediate directly abstracts a hydrogen from SP C-6 to generate a substrate radical, and subsequent to radical-mediated β-scission, a product thymine radical abstracts a hydrogen from 5′-deoxyadenosine to regenerate the 5′-deoxyadenosyl radical. Together, our results support a mechanism in which S-adenosylmethionine acts as a catalytic cofactor, not a substrate, in the DNA repair reaction.

The unusual resistance of bacterial spores to UV light and ionizing radiation has been of long-standing interest. In 1965, Donnellan and Setlow (1) showed that UV-irradiated bacterial spores did not contain the thymine dimers typically found in other irradiated cells but instead contained another type of thymine photoproduct. This dimer was later determined to be spore photoproduct (SP, 5-thyminyl-5,6-dihydrothymine; see Fig. 1), a thymine dimer that appears to be unique to Bacillus subtilis and other spore-forming microorganisms (2). The generation of these unique thymine dimers upon UV irradiation is thought to result from the binding of small, acid-soluble proteins to the spore DNA (3–7). During dormancy spores are unable to repair this damage to their DNA from UV exposure. Early in the germination cycle, however, two distinct mechanisms of DNA repair are active, presumably giving rise to the high level of UV resistance of bacterial spores. One of these mechanisms is the nucleotide excision repair pathway that detects and removes lesions from the DNA (including SP and cyclobutane-type pyrimidine dimers) in a manner similar to that characterized in Escherichia coli (8, 9). The second repair mechanism involves the specific reversal of SP to two thymines catalyzed by the enzyme SP lyase in B. subtilis (10, 11).

Pyrimidine dimers such as the spore photoproduct of Bacillus 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 pyrimidine dimer. Repair of these dimers, therefore, is critical in order to avoid mutations. The only well characterized means of pyrimidine dimer repair is photoreactivation, which is catalyzed by the enzyme DNA photolyase (12, 13). The photolyase family of enzymes are activated by visible light and contain a flavin (FAD) and either folate or deazaflavin. It has been shown that DNA photolyases cleave cyclobutane pyrimidine dimers to their monomers through a reaction mechanism that involves a radical dimer species (12, 13). Photoreactivation has been shown to be absent in many species, including B. subtilis, suggesting that an alternative means of pyrimidine dimer repair might be found (14–16). SP lyase is the first identified nonphotoactivatable pyrimidine dimer reversal enzyme, and thus it is of significant interest to probe the detailed mechanism of thymine dimer repair by this enzyme.

SP lyase shares some sequence homology in its C-terminal region with members of the DNA photolyase/(6-4) photolyase/adenosine deaminase superfamilies. The unusual resistance of bacterial spores to UV light and ionizing radiation has been of long-standing interest. In 1965, Donnellan and Setlow (1) showed that UV-irradiated bacterial spores did not contain the thymine dimers typically found in other irradiated cells but instead contained another type of thymine photoproduct. This dimer was later determined to be spore photoproduct (SP, 5-thyminyl-5,6-dihydrothymine; see Fig. 1), a thymine dimer that appears to be unique to Bacillus subtilis and other spore-forming microorganisms (2). The generation of these unique thymine dimers upon UV irradiation is thought to result from the binding of small, acid-soluble proteins to the spore DNA (3–7). During dormancy spores are unable to repair this damage to their DNA from UV exposure. Early in the germination cycle, however, two distinct mechanisms of DNA repair are active, presumably giving rise to the high level of UV resistance of bacterial spores. One of these mechanisms is the nucleotide excision repair pathway that detects and removes lesions from the DNA (including SP and cyclobutane-type pyrimidine dimers) in a manner similar to that characterized in Escherichia coli (8, 9). The second repair mechanism involves the specific reversal of SP to two thymines catalyzed by the enzyme SP lyase in B. subtilis (10, 11).

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blue light photoreceptor protein family (14, 17, 18); structural studies of DNA photolyase reveal a helix-turn-helix DNA-binding site in this region. The N-terminal region of SP lyase includes a three cysteine motif, CXCXCX_{3}C, characteristic of the radical AdoMet superfamily (14, 19, 20). This superfamily uses a site-differentiated [4Fe-4S] cluster and S-adenosylmethionine (AdoMet) to initiate diverse radical reactions, including the activation of glycol radical enzymes and the biosynthesis of cofactors (21–25). AdoMet coordinates an iron of the [4Fe-4S] cluster in radical AdoMet enzymes through the amino and carboxylate groups of AdoMet, as first demonstrated through spectroscopic studies of pyruvate formate-lyase-activating enzyme and subsequently observed in crystal structures of four other members of the superfamily (biotin synthase, HemN, MoaA, and lysine 2,3-aminomutase) (26–33). Thus it appears that a site-differentiated cluster is conserved throughout the radical AdoMet superfamily because of an essential role for the unique iron of the cluster as a structural element, coordinating and anchoring AdoMet for subsequent radical chemistry (28, 29).

The presence of the radical AdoMet motif in SP lyase suggested that it also would contain an iron-sulfur cluster and utilize AdoMet in catalysis (34). In support of this hypothesis, Nicholson and co-workers (34) showed that SP lyase purified from overexpressing E. coli contained iron and acid-labile sulfide and had a visible chromophore that decreased upon addition of dithionite. Although the SP lyase purified from E. coli showed no SP repair activity, SP lyase purified from overexpressing B. subtilis did catalyze AdoMet-dependent SP repair (34). The active B. subtilis enzyme, however, was isolated in small quantities insufficient for iron and sulfur or spectroscopic analysis (34). More recent work provided evidence that SP lyase is a dimer in the [4Fe-4S] form, possibly with a cluster-bridged dimer architecture, and that SP lyase converts AdoMet to methionine and 5’-deoxyadenosine (35).

SP lyase, like many of the radical AdoMet enzymes, has been difficult to isolate and characterize in its catalytically active state due to problems with both cluster lability and protein instability; this is reflected in the fact that previous studies of SP lyase have been done on catalytically inactive protein, protein with a low cluster content, and/or protein that required artificial reconstitution of the iron-sulfur cluster. Our interest in understanding the properties of the iron-sulfur cluster in the native enzyme, as well as the detailed mechanism of catalysis, have led us to pursue the isolation of catalytically active enzyme containing an intact iron-sulfur cluster. We report here the first detailed characterization of catalytically active SP lyase isolated from overexpressing E. coli under strictly anaerobic conditions.

EXPERIMENTAL PROCEDURES

Materials—B. subtilis strain KI1152 chromosomal DNA was a generous gift from Prof. Lee Kroos (Michigan State University) (36). B. subtilis strain 168 was obtained from the ATCC (ATCC 23857D). E. coli NovaBlue and Tuner(DE3)pLysS strains and the pET-14b expression vector were obtained from Novagen, and plasmid pUC18 was from Stratagene. The Co-Sepharose™ high performance resin was purchased from Amersham Biosciences. Some bacterial cultures were grown in a defined MOPS medium (MM medium) that has been previously described (37, 38). S-Adenosylmethionine was synthesized from methionine and ATP using AdoMet synthetase and subsequently purified by cation exchange chromatography as described previously (26). All other chemicals were obtained commercially and used as received.

Subcloning, Growth, and Expression of SP Lyase—Amplification of the splB gene was accomplished with the synthetic oligonucleotide primers 5′-GAGGCCGCGCCATATGCAGAGCCCAATTGTG-3′ (containing an Ndel site) and 5′-GCGCGCGGATCCGGAATCGGAAAATGGCTGAT-3′ (containing a BamHI site), which were synthesized by the Michigan State Macromolecular Structure, Sequencing, and Synthesis Facility. The coding sequence was amplified using standard PCR techniques with B. subtilis chromosomal DNA (strain KI1152) as the template. The Ndel/BamHI-digested PCR product was cloned into the same sites in pET-14b, in-frame with an N-terminal hexahistidine tag. The resulting construct (pET-14b/spl17) was transformed into NovelBlue E. coli for isolation and purification of the plasmid DNA. pET14b/spl17 was then transformed into Tuner(DE3)pLysS E. coli for protein overexpression. The fidelity of the PCR product was verified by dideoxynucleotide sequencing.

A single colony of the resulting overexpression strain was used to inoculate 50 ml of a defined MOPS medium (MM) containing 50 μg/ml ampicillin (37). This culture was grown to saturation at 37 °C and used to inoculate a 10-liter flask of MM/ampicillin. The 10-liter culture was grown at 37 °C in a New Brunswick Scientific fermentor (250 rpm, 5 p.s.i. O₂). When the culture reached an A600 of 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration, and the medium was supplemented with 750 mg of Fe(NH₄)₂(SO₄)₂. The culture was grown for an additional 2 h, and then was cooled to 25 °C and placed under nitrogen (5 p.s.i.). The culture was further cooled to 4 °C and left under nitrogen for 12 h. The cells were then harvested by centrifugation and stored under nitrogen at −80 °C until used for purification.

Purification of SP Lyase—SP lyase was purified from E. coli Tuner(DE3)pLysS transformed with pET14b-SPL17, prepared as described above. All steps in the purification were performed in a single day under strictly anaerobic conditions in a Coy anaerobic chamber (Coy Laboratories, Grass Lake, MI) at 4 °C.
Spore Photo product Lyase

except where noted. Solutions and buffers used in the purification were thoroughly degassed by sparging with nitrogen or by repeated pump/purge cycles on a Schlenk line prior to bringing them into the Coy chamber. The pelleted cells (13–19 g) were brought into the anaerobic chamber and resuspended in 20–30 ml of pH 8.0 lysis buffer containing 20 mM sodium phosphate, 500 mM NaCl, 1% Triton X-100, 5% glycerol, 10 mM MgCl₂, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1 mg of lysozyme/g of cells, and 0.2 mg of DNase I and RNase A. This suspension was agitated for 1 h and then centrifuged at 27,000 × g for 30 min at 4 °C. The resulting crude extract was loaded directly onto a Co-Sepharose™ High Performance affinity column (0.7 × 2.5 cm, 1 ml) that had been previously equilibrated with buffer A (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 5% glycerol, pH 8.0). The column was washed with 15 ml of buffer A, and then a 25-ml step gradient (5-ml steps at 10, 20, 50, 70, and 100% buffer B) from buffer A to buffer B (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, 5% glycerol, pH 8.0) was run to elute the adsorbed proteins. SP lyase eluted as a sharp brownish band at 50% buffer B. The fractions were analyzed by SDS-PAGE, and those judged to be ≥95% pure were pooled and concentrated at 4 °C using an Amicon concentrator equipped with a YM-10 membrane. The protein was placed in o-ring sealed tubes, flash-frozen, and stored at −80 °C.

Protein, Iron, and Sulfide Assays—Routine determinations of protein concentrations were done by the method of Bradford (39), using a kit sold by Bio-Rad and bovine serum albumin as a standard. Iron assays were performed using the methods of Fish (39), using a kit sold by Bio-Rad and bovine serum albumin as a molecular weight standard. SP lyase eluted as a sharp brownish band at 50% buffer B. The fractions were analyzed by SDS-PAGE, and those judged to be ≥95% pure were pooled and concentrated at 4 °C using an Amicon concentrator equipped with a YM-10 membrane. The protein was placed in o-ring sealed tubes, flash-frozen, and stored at −80 °C.

Preparation of SP-containing DNA—Labeling of pUC18 DNA was carried out as follows. NovaBlue E. coli (Novagen) carrying pUC18 was grown overnight at 37 °C in 50 ml of 2× YT medium containing 50 μg/ml ampicillin, 0.45 mM deoxyadenosine, and 10 μM [methyl-3H]thymidine (0.74 MBq/ml) (Amersham Biosciences). Labeled DNA was extracted from the overnight culture using a Promega Wizard mini-prep kit. The specific activity of the purified DNA was typically 1.5 × 10⁷ cpm/μmol. To form a complex between the [3H]DNA and the SspC, the two were mixed in a 5:1 SspC:DNA ratio in 70 mM Tris acetate, 125 mM NaCl, pH 7.0, and incubated at 37 °C for 2 h in a custom-made quartz vacuum hydrolysis tube. This complex was irradiated with 30 kJ/m² UV light at 254 nm in order to form spore photoprodut on the [3H]DNA. To quantify the SP formed, the resulting irradiated DNA was hydrolyzed by the addition of 500 μl of trifluoroacetic acid, sealed under vacuum, and heated at 165 °C for 3 h. The hydrolyzed DNA was lyophilized and redissolved in 100 μl of Milli-Q H₂O. The amount of spore photoprodut was quantified by running the irradiated hydrolyzed [3H]DNA on a Waters Spherisorb S5 4.0 × 250-mm analytical column, run at a flow rate of 1.8 ml/min in degassed Milli-Q H₂O for 25 min with fractions collected every 0.5 or 1 min (1, 34, 44, 45). Liquid scintillation counting was performed on each fraction using a Wallac 1414-001 or a Beckman LS 6500 liquid scintillation counter. Labeled thymine elutes at ~3 min, and the spore photoprodut elutes at 10–11 min. The percentage of thymine in the spore photoprodut form was determined by dividing the counts/min in the SP peak by the total counts/min of the sample.
Assay of SP Lyase Activity—SP lyase activity was determined using a modified version of the assay developed by Nicholson and co-workers (34). All solutions except DNA and protein solutions were prepared anaerobically in an Mbraun glove box just prior to use. Protein and DNA solutions were made anaerobic by repeated vacuum-purge cycles prior to bringing them into the glove box. Assays with and without SP lyase (1 μM) were set up in parallel in custom-made vacuum hydrolysis tubes in the glove box. Reaction mixtures (150–200 μl total volume) contained 0.2 nmol of SP lyase, 2 mM AdoMet, 25,000–50,000 cpm of 3H-pUC18 DNA (3H at the thymine methyl and containing 200–435 nmol SP), 4 mM dithiothreitol, 3 mM dithionite, and 30 mM KCl, all in 25 mM Tris acetate, pH 7.0. Identical experiments were set up in which the mixture was supplemented with 0.2 mM ferrous ammonium sulfate or in which holo-SP lyase was replaced with apo-SP lyase. All reaction mixtures were incubated under anaerobic conditions at 37 °C for 15 min to 24 h and terminated with the addition of 0.5 ml of trifluoroacetic acid. Hydrolysis tubes containing the quenched reactions were sealed under vacuum and heated to 165 °C for 2 h. The trifluoroacetic acid was evaporated, and the dried residue was resuspended in 100 μl of Milli-Q H2O. A portion of the solution (15 μl) was loaded onto a Waters Spherisorb S5P 4.0 × 250 mm analytical column and run at a flow rate of 1.8 ml/min in degassed Milli-Q H2O for 25 min with fractions collected every 0.5 or 1 min (1, 34, 44, 45). Liquid scintillation counting was performed on each fraction using a Wallac 1414–001 or a Beckman LS 6500 liquid scintillation counter.

HPLC Detection of AdoMet Cleavage during DNA Repair—Standards of 3 mM AdoMet and 3 mM 5′-deoxyadenosine were run over a C18 Waters Spherisorb 5-μm ODS2 4.6 × 150-mm column at 1 ml/min with a step gradient (4 ml of Milli-Q H2O, 0.1% trifluoroacetic acid, 13 ml of 82% Milli-Q H2O, 0.1% trifluoroacetic acid, 18% acetonitrile, 0.1% trifluoroacetic acid, 14 ml of 100% acetonitrile, 0.1% trifluoroacetic acid) used to elute. Samples (200 μl) containing 3 mM dithiothreitol, 4 mM sodium dithionite, 30 mM KCl, 200 μg of SP-containing pUC18 DNA, and a 1:1 ratio of AdoMet to SP lyase (7 nmol each) in 25 mM Tris acetate buffer, pH 7.0, were prepared in an anaerobic glovebox and incubated at 37 °C for time periods of 1, 40, 90, 130 min and 24 h. Samples were divided in half, and one part was used as above to test for repair activity. The other half was ultracentrifuged through a YM-10 filter to remove protein and DNA; a 15-μl aliquot of the resulting filtrate was loaded onto the C18 column and run with the above step gradient. Control samples were prepared without SP lyase to determine AdoMet stability over time and without AdoMet to check for background signals.

Label Transfer from AdoMet to Thymine—AdoMet labeled with 3H at the 5′-position was synthesized by using the following procedure. A 5-ml reaction containing 100 mM Tris-HCl, pH 8.0, 50 mM KCl, 26 mM MgCl2, 5.2 mM adenosine triphosphate, 8% β-mercaptoethanol, 1 mM EDTA, 6.8 mM methionine, 2.5 μl of inorganic pyrophosphatase, 500 μl of AdoMet synthetase crude lysate (prepared as previously described (26)), and 1 mCi of 30–60 Ci/mmol of [2′,8′,5′-3H]ATP (Amersham Biosciences) was stirred at room temperature for 16 h and then quenched with 500 μl of 1 M HCl. The reaction was monitored by TLC and purified by loading onto a Source 15s.6 cationic exchange column. A linear gradient of Milli-Q H2O to 1 M HCl was used to elute the 3H-labeled AdoMet. The fractions containing AdoMet were lyophilized and redissolved in 50 mM HEPES, 200 mM NaCl, pH 7.5. Repair assays were carried out using the labeled AdoMet (5 × 106 cpm/μmol) and unlabeled pUC18 DNA under the assay conditions described previously, except that DNA was purified with a Wizard mini-prep purification kit prior to DNA hydrolysis. The ratio of labeled AdoMet to SP was also altered to 10:1. Samples were prepared in duplicate, and control samples were prepared in the absence of SP lyase. Controls were also prepared in the absence of DNA to make sure all of the labeled AdoMet was removed prior to HPLC analysis.

Spectroscopic Measurements—UV-visible spectra were recorded on a HP8453 diode-array spectrophotometer. EPR measurements were obtained at X-band and 12 K on a Bruker ESP300E spectrometer equipped with a liquid helium cryostat and a temperature controller from Oxford Instruments.

RESULTS

Overexpression and Purification of SP Lyase Containing an N-terminal Hexahistidine Tag—The spIB gene was subcloned into pET-14b in order to provide an IPTG-inducible expression system as well as an in-frame N-terminal hexahistidine affinity tag to ease the purification of SP lyase. The resulting SP lyase expression vector, pET14b-SPL17, was used to transform E. coli Tuner(DE3)pLysS for overproduction of histidine-tagged SP lyase (SP lyase-His6), which migrates at ~43 kDa on SDS-PAGE (Fig. 2). In contrast to previous reports (35), our purified SP lyase appeared as a single band on SDS-PAGE, with no evidence of C-terminal cleavage of ~7 kDa. The
Tuner(DE3)pLysS/pET14b-SPL17 cells were lysed using an enzymatic lysis procedure. The crude extract was then passed through a Co-Sepharose™ High Performance affinity column, and pure fractions (>95%) were identified by SDS-PAGE, pooled, concentrated, and stored under nitrogen at −80 °C. If the SP lyase-His₆ was not sufficiently pure after a single run over the affinity column, the protein was dialyzed and repurified over the same column. Typically, ~25 mg of pure protein is obtained from 10 liters of growth media.

Iron-Sulfur Cluster of Purified SP Lyase—SP lyase elutes as a dark brown band from the Co-Sepharose™ High Performance column, consistent with the presence of an iron-sulfur cluster in the protein. The UV-visible spectrum of the purified enzyme (Fig. 3) is characteristic of the presence of an iron-sulfur cluster, although it is not particularly definitive of a specific cluster type. The spectrum exhibits a broad shoulder with maxima at 410 (11.9 mM cm⁻¹) and 450 nm (10.5 mM cm⁻¹), similar to what has been observed for anaerobically purified pyruvate formate-lyase-activating enzyme (42) and lipoyl synthase (46). The anaerobically purified SP lyase has been found to contain iron (3.1 ± 0.3 mol of iron per mol of SP lyase) and acid-labile sulfide (3.0 ± 0.3 mol of S⁻ per mol of SP lyase). The amount of iron present in the purified SP lyase is strongly dependent on the precise growth and purification conditions. The purified SP lyase exhibited instability at higher concentrations and precipitated above ~250 μM. Addition of a 5-fold excess of AdoMet to the protein resulted in increased solubility (concentrations of ~750 μM could be achieved) and increased protein stability at room temperature. These results suggest a stabilizing structural role for AdoMet in SP lyase.

SP lyase exhibits a strong, nearly isotropic EPR signal that is centered at g = 2.02 and observable only below 35 K (Fig. 4). The g value, the low anisotropy, and the temperature dependence are consistent with the assignment to a [3Fe-4S]¹⁺ cluster being present in the as-isolated form of the enzyme. Spin quantification of the [3Fe-4S]¹⁺ shows that it accounts for between 25 and 35% of the total iron. SP lyase can be reduced anaerobically by titration with sodium dithionite, as is evidenced by a decrease in the visible absorption to produce a spectrum more characteristic of a [4Fe-4S] cluster (Fig. 3). This reduction results in a dramatic change in the EPR spectral properties (Fig. 4). Rather than the intense, nearly isotropic signal observed for the as-isolated enzyme, the reduced enzyme has a nearly axial signal that is characteristic of a [4Fe-4S]¹⁺ cluster, with gₓ = 2.03, gᵧ = 1.93, and gₐ = 1.89 (based on simulations; data not shown). The relaxation properties of this signal are also consistent with its assignment as a [4Fe-4S]¹⁺ cluster, as the signal broadens above 20 K and is unobservable above 40 K. Spin quantification of the [4Fe-4S]¹⁺ cluster shows that it accounts for between 22 and 31% of the total iron present in the protein. Addition of AdoMet to dithionite-reduced SP lyase results in a [4Fe-4S]¹⁺ EPR signal essentially identical to that in the absence of AdoMet, albeit with a lower intensity (Fig. 4).

Subunit Structure—SP lyase has been reported previously to be dimeric under conditions favoring reconstitution of iron-sulfur clusters, and to likely contain a subunit-bridging iron-sulfur cluster (35). As our SP lyase is purified with a significant proportion of iron-sulfur clusters intact, we have been able to re-investigate the question of subunit structure using protein that has not been subjected to artificial reconstitution. Analytical gel filtration chromatography shows that SP lyase migrates...
It has been shown previously by Setlow and co-workers (44) that spore photoproduct can be produced from overexpressing E. coli DNA in the presence of Bacillus small, acid-soluble proteins. We have used this technique to generate spore photoproduct on [methyl-3H]thymine-labeled pUC18 E. coli DNA, with typical preparations yielding spore photoproduct constituting between 4 and 7% of total thymine. Spore photoproduct production and repair were monitored by HPLC analysis and scintillation counting after acid hydrolysis of the DNA. In this study, hexahistidine-tagged SP lyase anaerobically purified from overexpressing E. coli was found to have a specific activity of 0.33 μmol/min/mg. Time course assays were linear up to 60 min (Fig. 6), after which the degree of repair was variable, most likely due to protein instability under the assay conditions. On

with an apparent molecular mass of 46 kDa (Fig. 5). Simultaneous detection at 426 and 280 nm shows that the visible chromophore elutes with this 46-kDa peak. A small shoulder on this peak (~85 kDa) is visible with both 280 and 426 nm detection and may represent a small amount of SP lyase dimer, which could be an artifact of the high protein concentrations used. These results, together with the iron-protein ratios we obtain for purified SP lyase, provide support for SP lyase as a monomer binding a single [4Fe-4S] cluster.

Enzymatic Activity—It has been shown previously by Setlow and co-workers (44) that spore photoproduct can be produced on E. coli DNA in the presence of Bacillus small, acid-soluble proteins. We have used this technique to generate spore photoproduct on [methyl-3H]thymine-labeled pUC18 E. coli DNA, with typical preparations yielding spore photoproduct constituting between 4 and 7% of total thymine. Spore photoproduct production and repair were monitored by HPLC analysis and scintillation counting after acid hydrolysis of the DNA. In this study, hexahistidine-tagged SP lyase anaerobically purified from overexpressing E. coli was found to have a specific activity of 0.33 μmol/min/mg. Time course assays were linear up to 60 min (Fig. 6), 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 published work (34, 45, 47) would not provide a reliable estimate of SP lyase activity. It should be noted that our specific activity cannot be directly compared with previous literature values, because previous reports of SP lyase activity have provided numbers in terms of percentage of total SP repaired in a given time, a number that is not directly comparable from assay to assay because the amount of SP present initially can vary. We have, however, been able to convert one of these literature values of “% SP repaired” (47) to an apparent specific activity; the calculated specific activity in this case was 0.0002 μmol/min/mg, nearly 1000-fold lower than the activity we report here.

AdoMet Is a Catalytic Cofactor—AdoMet can be used as either a cofactor (e.g. lysine 2,3-aminomutase) or cosubstrate (e.g. PFL-AE, BioB, and LipA) in the radical AdoMet superfamily. Previously published reports indicated that SP lyase uses AdoMet as a cosubstrate (35, 48); however, results from our laboratory demonstrate that SP lyase can repair SP using only catalytic amounts of AdoMet. The data in Table 1 illustrate this point. Assays were run for 1 h and contained 0.23 nmol of SP lyase, 2 mM AdoMet, 3 mM sodium dithionite, 4 mM dithiothreitol, 30 mM KCl, and 25 mM Tris acetate, pH 7.0. Linear repair is observed up to 60 min with a specific activity of 0.33 μmol of SP repaired per min/mg of SP lyase. An apparent lag period may result from the need for reduction of SP lyase prior to initiation of DNA repair activity.

| nmol of AdoMet | nmol of SP repaired |
|----------------|---------------------|
| 0.23           | 130 ± 10            |
| 0.46           | 130 ± 10            |
| 2.3            | 180 ± 10            |
Spore Photoprodut Lyase

FIGURE 7. HPLC analysis of AdoMet cleavage to 5′-deoxyadenosine by SP lyase. HPLCs of (A) standard sample containing AdoMet (2.5 min) and 5′-deoxyadenosine (8 min); (B) control sample containing AdoMet under assay conditions with no SP lyase; and assay mixes containing SP lyase after 90 min (C and overnight (D)). Samples B–D contained 3 mM sodium dithionite, 4 mM dithiothreitol, 30 mM KCl, and 25 mM Tris acetate, pH 7.0. Samples C and D also contained 200 μg of SP-containing pUC18 DNA, 36 μM AdoMet, and 36 μM SP lyase.

substrate, then 5′-deoxyadenosine should be present as a stoichiometric product of the reaction. In contrast, if AdoMet is being used as a catalytic cofactor, then the 5′-deoxyadenosine is an intermediate, not a product, of the reaction. It has been reported previously that 5′-deoxyadenosine is generated during repair of SP by SP lyase (35, 48); however, such an observation is not consistent with our data supporting a catalytic role for AdoMet in SP repair. We have therefore re-investigated AdoMet cleavage by SP lyase. SP repair reactions containing a 1:1 mixture of AdoMet to SP lyase, in addition to SP and other reaction components, were carried out and divided in half. One-half of the reaction was quenched and checked for SP repair by the methods described above. The other half of each sample was filtered through a Millipore Centricon centrifugal device with a YM-10 membrane to remove protein and DNA; the filtrate was then run over a C18 ODS2 column. The resulting chromatograms (Fig. 7) show that essentially no 5′-deoxyadenosine is produced under the conditions employed. In the reaction run for 90 min (Fig. 7C), our standard repair assay revealed that 146 nmol of SP had been repaired, and yet the HPLC shows that less than 0.1 nmol of 5′-deoxyadenosine is present; >95% of the 7 nmol of AdoMet present in the reaction remains in the AdoMet form. Similar results are obtained after overnight incubation (Fig. 7D). The results clearly indicate that AdoMet is not cleaved stoichiometrically to methionine and 5′-deoxyadenosine during SP repair. The lack of AdoMet cleavage observed here differentiates our results from two previously published reports, which show 5′-deoxyadenosine production by SP lyase (35, 48); the reasons for these differences are unclear at this time.

Direct H Atom Transfers Accompany DNA Repair—We have previously shown that SP repair is initiated by direct H atom abstraction from the C-6 position of SP by an AdoMet-derived 5′-deoxyadenosyl radical intermediate (49). These results support a mechanism in which the resulting substrate radical undergoes a radical-mediated β-scission to generate the product thymine radical. The product thymine radical is then proposed to re-abstract an H atom from the 5′-deoxyadenosine formed in the first step, thereby regenerating the 5′-deoxyadenosyl radical intermediate. The 5′-deoxyadenosyl radical intermediate could then recombine with methionine, with loss of an electron to the iron-sulfur cluster, to regenerate AdoMet (Fig. 9). To further investigate this mechanism, we have carried out repair assays in which the AdoMet is labeled at the 5′-position with tritium. The mechanism shown in Fig. 9 would predict label transfer from this position into the repaired thymine. Repair assays were carried out using labeled AdoMet and unlabeled irradiated DNA. Control experiments were conducted in the absence of SP lyase to check for erroneous label transfer or other experimental problems. In all cases, repaired DNA was isolated from other reaction components, hydrolyzed, and then subjected to chromatography on a Waters Spherisorb SSP column. Analysis of fractions by scintillation counting reveals a peak at 3 min (Fig. 8), the elution time of thymine under these conditions. This peak is observed reproducibly in experimental samples containing SP lyase and is absent in samples containing no SP lyase. Furthermore, the amount of label appearing in the thymine peak is consistent with what we would predict (115 cpm) based on the specific activity of the starting AdoMet and using 15.8 as an estimate of the tritium kinetic isotope effect. The transfer of the expected amount of tritium from AdoMet to thymine during SP repair by SP lyase provides further support...
for a mechanism in which AdoMet is a catalytic cofactor, and in which there is direct H atom transfer between AdoMet-derived deoxyadenosyl radicals and substrate/product (Fig. 9).

**DISCUSSION**

We provide here the first detailed characterization of a catalytically active form of spore photoproduct lyase, a novel DNA repair enzyme that utilizes an iron-sulfur cluster and S-adenosylmethionine to initiate repair of thymine dimers. We have been able to express this *B. subtilis* protein in *E. coli*, and upon affinity purification under anaerobic conditions, an active enzyme (0.33 μmol/min/mg) containing iron and acid-labile sulfide (at a ratio of ~3 each per protein monomer) is obtained. All of our studies are done on this “as-isolated” protein either as-is or after reduction with dithionite; no artificial cluster reconstitution has been required to achieve any of the results described herein. This differentiates this study from one published previously (35), in which the enzyme being investigated required cluster reconstitution and was done on a catalytically inactive His₉₋₁₀-tagged form, and may in part explain the differences between our results and those published previously (35).

We have constructed a clone of SP lyase that includes an N-terminal six-histidine tag for ease of purification and provides readily visible overexpression in *E. coli* Tuner cells, as indicated by SDS-PAGE of whole cell extracts (Fig. 2). Although the cells are grown under aerobic conditions, we have purified the SP lyase-His₆ under strictly anaerobic conditions to minimize oxidative cluster degradation during purification. Such anaerobic purification conditions have proved useful in isolating the holo-forms of other members of the radical AdoMet family of enzymes, particularly the pyruvate formate-lyase-activating enzyme (42) and lipoyl synthase (46, 50).

The anaerobically purified SP lyase-His₆ contains ~3 iron and 3 S²⁻ per protein monomer, is reddish-brown in color, and has a visible chromophore similar to that observed for PFL-AE (42). Like several other members of the radical AdoMet family of enzymes, SP lyase-His₆ is found to contain a [3Fe-4S]³⁺ cluster when isolated under anaerobic conditions. This [3Fe-4S]³⁺ cluster, however, accounts for only approximately one-third of the total iron in the sample, with the remaining iron being in an EPR-silent form. The EPR-silent iron is likely also present as part of an iron-sulfur cluster, because the overall Fe:S ratio is 1:1; Mössbauer spectroscopic studies are currently underway to identify the EPR-silent cluster in the purified enzyme.

SP lyase gains both a [4Fe-4S]⁺ cluster and catalytic activity upon reduction, consistent with previous work demonstrating the presence of catalytically relevant [4Fe-4S]⁺ clusters in the radical AdoMet enzymes PFL-AE (51), LAM (52), and aRNR (53). Our data suggests that this [4Fe-4S]⁺ cluster is generated by reductive cluster conversion of the [3Fe-4S]³⁺ cluster, because addition of dithionite causes loss of the [3Fe-4S]³⁺ EPR signal and gain of a signal assigned to a [4Fe-4S]⁺ cluster; such facile reductive cluster conversions are observed for several members of the radical AdoMet superfamily.

In contrast to other members of the radical AdoMet superfamily, addition of S-adenosylmethionine to the reduced SP lyase does not alter the line shape or g values of the [4Fe-4S]⁺ EPR signal; however, it does result in a decrease in intensity of the signal (Fig. 4). This reduction in intensity of the [4Fe-4S]⁺ EPR signal in the presence of AdoMet has been observed previously for SP lyase (35); one interpretation of this observation could be that there is nonproductive reductive cleavage of AdoMet (and corresponding oxidation of the cluster). We have however found no evidence for AdoMet cleavage in the SP lyase [4Fe-4S]⁺-AdoMet complex (Fig. 7), indicating that other explanations, such as a change in spin state of some population of the cluster, must be considered. Further studies are underway in our laboratory to address this perplexing observation.

DNA-binding proteins are often dimeric, with the resulting 2-fold symmetry presumably important for binding the palindromic sequences typically recognized by sequence-specific DNA-binding proteins. Among the notable exceptions is DNA photolyase, which is monomeric and recognizes a specific DNA structure, rather than a specific sequence (13). Because SP lyase has some sequence homology to DNA photolyase and presumably also recognizes a specific structure rather than a sequence, it might also be expected to be monomeric rather than dimeric; this idea is supported by the results presented here, in which SP lyase migrates primarily as a monomer by gel filtration chromatography (Fig. 5). Previous work, however, has provided evidence for a dimeric form of SP lyase after incubation under cluster reconstitution conditions (35). Although the latter observation could be an artifact of cluster reconstitution, it could also be functionally relevant, and thus further investigation of the oligomeric state of SP lyase under a range of conditions is warranted.

A mechanism for SP lyase originally proposed by Mehl and Begley (54), and subsequently supported by
work done in our laboratory (49), involves 5'-adenosylmethionine as a catalytic cofactor that is not consumed during turnover (Fig. 9). Other workers, however, have reported the generation of 5’-deoxyadenosine as a product when AdoMet is incubated with SP lyase either in the presence or absence of substrate DNA, with enhanced 5’-deoxyadenosine production in the presence of substrate DNA (35, 48). We have reinvestigated the question of AdoMet cleavage using our purified SP lyase and find no evidence for AdoMet cleavage to 5’-deoxyadenosine and methionine, regardless of whether substrate DNA is present (Fig. 7). Our evidence for the absence of 5’-deoxyadenosine product when AdoMet is incubated with SP lyase is consistent with a mechanism in which AdoMet is used as a catalytic cofactor rather than a substrate in the reaction, as shown in Fig. 9.

To further investigate the role of AdoMet as a cofactor or substrate in the SP lyase reaction, we performed a series of SP lyase assays, including varying ratios of AdoMet to SP lyase, with substrate DNA present in excess. As shown in Table 1, the results clearly support the catalytic involvement of AdoMet in the repair of spore photoproduct by SP lyase. Indeed, a single molecule of AdoMet can participate in the repair of at least hundreds of SP lesions.

We reported previously the first experimental evidence for the mechanism shown in Fig. 9, by showing that tritium at C-6 in spore photoproduct is incorporated into AdoMet during the SP lyase-catalyzed reaction (49). The results reported here provide additional evidence for this mechanism by demonstrating that when the reaction is carried out with 5’-tritiated AdoMet, the tritium label is incorporated into repaired thymine residues.

Together, the results reported herein and previously by our laboratory provide strong experimental support for the mechanism shown in Fig. 9, in which the reduced iron-sulfur cluster of SP lyase reductively cleaves AdoMet to generate a 5’-deoxyadenosyl radical intermediate and methionine. The 5’-deoxyadenosyl radical abstracts a hydrogen atom from C-6 of spore photoproduct. The resulting substrate radical undergoes a radical-mediated β-scission to produce a product radical, which then abstracts a hydrogen atom from 5’-deoxyadenosine to form product and a 5’-deoxyadenosyl radical. AdoMet is then re-formed upon transfer of an electron back to the iron-sulfur cluster. SP lyase is one of only two characterized members of the radical AdoMet superfamily (the other being lysine 2,3-aminomutase) to use AdoMet as a catalytic cofactor.

In addition to being consistent with our current understanding of SP lyase, the mechanistic proposal in Fig. 9 displays intriguing parallels to the proposed mechanism for DNA photolyase. In both cases, radical chemistry is used to cleave a covalent pyrimidine dimer, with the DNA photolyase using radical anion chemistry and SP lyase using neutral radical chemistry. These two enzymes, however, use very different mechanisms by which to initiate the radical chemistry as follows: NADH, a secondary cofactor, and light in the case of DNA photolyase (13), and an iron-sulfur cluster and AdoMet in the case of SP lyase. Thus, radical chemistry is not only a significant source of DNA damage but is also an important means of repairing DNA damage in vivo. It is intriguing that such vastly different cofactors are utilized to perform what is quite similar chemistry. By analogy to what Beintert (55) has proposed for other enzyme systems, perhaps the Fe-S/AdoMet cofactor combination found in SP lyase is a primitive “holdover” from more anaerobic times, and the NADH and light-driven reaction is a more modern adaptation.
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