Characterization of a new Thermophilic Spore Photoproduct Lyase from *Geobacillus Stearothermophilus* (splG) with Defined Lesion Containing DNA Substrates

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Running Title: Thermophilic Spore Photoproduct Lyase of *Geobacillus stearothermophilus*

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The *Geobacillus stearothermophilus* splG gene encodes a thermophilic spore photoproduct lyase (splG) which belongs to the family of radical SAM enzymes. The aerobically purified apo-splG forms a homodimer, which contains one [4Fe-4S] cluster per monomer unit after reconstitution to the holo form. Formation of the [4Fe-4S] cluster was proven by quantification of the amount of iron and sulfur per homodimer and by UV and EPR spectroscopy. The UV-spectrum features a characteristic absorbance at 420 nm typical for [4Fe-4S] clusters and the EPR data were found to be identical to those of other proteins containing an [4Fe-4S]⁺ center. Probing of the activity of the holo-splG with oligonucleotides containing one spore photoproduct (SP) lesion at a defined site proved that the enzyme is able to turn over substrate. In addition to repair we observed cleavage of SAM to generate 5'-deoxyadenosine. In the presence of azaSAM the splG is completely inhibited, which provides direct support for the repair mechanism.

Spores of various *Bacillus* and *Clostridium* species are extremely resistant to harsh physical, chemical and biological conditions allowing them to survive even under extreme conditions (1,2). The oldest known viable spore was discovered from a *Bacillus* species, designated 2-9-3, in a 250 million-year-old salt crystal from the Permian Salado Formation (3). The resistance of spores from *Geobacillus stearothermophilus* towards heat is even so high, that the survival of the organism during heat sterilization is used as a bioindicator for insufficient heat treatment (4).

Particularly noteworthy is the unusually high stability of spores in the presence of UV-light. For example under typical UV-sterilization conditions only about 70% of thermophilic *Geobacillus stearothermophilus* spores are inactivated. Under the same conditions typical pathogens such as *Herpes simplex* or Polio viruses are fully destroyed (5,6). In addition, UV irradiation of spores gives rise to different DNA lesions (7). Whereas in normal cells mostly cyclobutane pyrimidine dimers and (6-4) lesions (8) are formed, in spores the unusual photoproduct 5-thyminyl-5,6-dihydrothymine, SP, depicted in Scheme 1 (7,9), is exclusively generated (10). These differences in the photoreactivity may be due to an unusual packing of the DNA in spores (1,11,12), and the high amounts of dipicolinic acid (DPA) present in spores (13).

During germination, the SP lesion is repaired either by the general nucleotide excision repair (NER) pathway (14,15) or by a single enzyme, called spore photoproduct lyase, which is able to split the SP lesions directly back into two thymidines. Recent studies by the groups of Nicholsen (16) and Broderick (17) performed with the SP-lyase from *B. subtilis* showed that the enzyme requires S-adenosylmethionine (SAM) as a cofactor for repair. A detailed sequence comparison, spectroscopic studies (18,19), and a recent labelling experiment (17) all
provide evidence that the spl is a member of the radical-SAM enzyme superfamily which also includes anaerobic ribonucleotide reductase (rNR), pyruvate formate lyase (PFL), lysine-2,3-aminomutase (KAM), benzylsuccinate synthase activating enzyme (BssDAE) and biotin synthase (BioB) (20). The DNA repair mechanism postulated by Begley is depicted in Figure 1. It is assumed that the 5'-dAdo radical (17,18), formed after electron transfer from the [4Fe-4S]-cluster to the SAM coenzyme, initiates the repair by abstracting the C6 hydrogen of the SP lesion. The C-C bond linking the two pyrimidines undergoes β-fragmentation to give an allyl-type radical. The thermodynamically problematic last step is the transfer of the hydrogen atom back from the 5'-dAdo to the thymine monomer radical, which completes the repair process (21).

HPLC and ESI-MS mass spectrometric analyses showed that the spl cleaves SAM under anaerobic and reductive conditions to generate approximately 2 molecules of 5'-deoxyadenosine per homodimer spl in the absence of the SP lesion substrate (18). In the presence of the SP lesion substrate and with small DNA single strands, which was possible under strict anaerobic conditions. Using a novel assay with a synthetic dinucleotide SP lesion substrate and with small DNA single strands, which contain one SP lesion at a defined site we show that the fully reconstituted protein is active in the presence of SAM. We observed a specific activity of 2.6 µmol SP repaired/min/mg splG. In the presence of azaSAM the splG is completely inhibited, which provides direct support for the mentioned mechanism. An interesting feature of the enzyme is that it forms an extremely stable, catalytically active homodimer.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** S-adenosylmethionine was purchased from Sigma-Aldrich and further purified by HPLC to remove additional S-adenosylhomocysteine. All bacteria media were purchased from Roth and all other chemicals were obtained from commercial suppliers and used without further purification.

**Bacterial strains and growth conditions.** *Geobacillus stearothermophilus* strain 10 (DSM No. 13240) was grown in nutrient broth medium (meat peptone 5.00 g/l and meat extract 3.00 g/l, pH 7.0) under aerobic conditions in a shaker (300 rpm) at 60°C. *E. coli* strain Tuner™ pLysS (DE3) (Novagen) was used for overexpression of splG.

**Construction of plasmid.** Genomic DNA of *G. stearothermophilus* was isolated using the DNeasy® Tissue Kit (Qiagen). The splG gene was obtained by PCR amplification from the genomic *G. stearothermophilus* DNA using *Accu Prime™ Pfx* DNA polymerase (Invitrogen) with the two primers, 5’-CACCATGCATCACCATCACCATTACCATGAAACCGTTTGTGCCAAAACT-3’ and 5’-TTACGTAAAAATACGTCACTTGGG-3’. The resulting 1.0 kb splG gene PCR product was cloned into the vector pET101-D (Invitrogen) by the Topo reaction.

**Overproduction and purification of recombinant protein.** *E. coli* strain Tuner™ pLysS (DE3), which is a lacYZ deletion mutant of BL21, enables the adjustment of the levels of protein expression throughout all cells in a culture, because the lac permease (lacY) mutation allows uniform entry of IPTG into all cells in the population. Recombinant splG was expressed with an N-terminal His₆-tag. Cells were cultivated with shaking at 150 rpm in 1 liter of LB medium (22) containing 100 µM Fe(III)·Citrate at 37°C to an OD₆₀₀ = 0.6. IPTG was added to 1 mM and incubation was continued at 22°C for 12 h. Cells were harvested (10000 x g, 8 min, 4°C) and stored at -20°C. Subsequently the cell pellet was resuspended in 30 mL of 60 mM Na₂HPO₄ (pH 8.0) / 300 mM NaCl/ 10 mM imidazole / 5 mM L-cysteine/ 20 mM ammonium ferrous sulphate/ 10 mM ATP, and 1 tablet protease inhibitor mixture (Roche Applied Science). The crude cell extract was solubilised in a French Press at 1000 psi. The cell debris was removed by centrifugation (38000 x g, 35 min, 4°C) and the supernatant applied to a Ni-NTA-agarose column (Qiagen) equilibrated with 50 mM Tris-HCl (pH 8.0) / 300 mM NaCl and 10 mM imidazole. SplG was eluted from the column using the same buffer containing 500 mM imidazole.
In a second purification step apo-splG dimer was separated by a gel permeation chromatography with 50 mM Tris-HCl (pH 8.0) / 300 mM NaCl. The purified splG, up to 2 mg/L expression, was concentrated and transferred into a glovebox (MBrain, unilab) containing a 5% H₂: 95% N₂ atmosphere, and less than 2 ppm O₂. Inside the anaerobic chamber the aerobic buffer containing splG apoprotein was exchanged by gel filtration to the reconstitution buffer.

[
4Fe-4S] cluster reconstitution The apo-splG was dissolved in 800 µL reconstitution buffer, 50 mM Tris-HCl (pH 8.0), with 5 mM DTT for 0.5 h, followed by the addition of FeCl₂ and Na₂S to a final concentration of 1 µM. The splG was then incubated anaerobically for 16 h at 4°C. The holo-protein solution was finally concentrated in an Ultrafree®-0.5 centrifugal filter device (Millipore) up to a volume of 200 µL and the buffer was exchanged.

Synthesis of 5S- and 5R-configured spore lesion.

Both diastereomers of the spore photoproduct were synthesized as described previously (23).

Synthesis of the azaSAM. The synthesis and purification was similar to previously described procedures (24-26).

Synthesis of ss-DNA containing one SP lesion.

500 nmol of a 6 mer ssDNA (5'-GGTGGG-3') was resuspended in 4 ml buffer, containing 50 mM NaCl, 5 mM pyridine-2,6-dicarboxylic acid and 5 mM CaCl₂ (pH 7.0). The solution was lyophilized in a 6 well cell culture plate. The obtained dry film was subsequently irradiated at 254 nm under anaerobic conditions. The SP containing DNA was separated by rp-HPLC from residual undamaged DNA. Oligonucleotides were concentrated in vacuo using a Savant Speed Vac and were desalted with Sep-Pak® cartridges (Waters) before use.

Analyses of the SP in ss-DNA. To prove that the SP was formed in the oligonucleotide, the oligonucleotide with the sequence 5'-GGTspTGG-3' was enzymatically digested and analyzed by rpHPLC-MS/MS. The results were compared with an analysis of previous studies (27). To 100 µL of a 20 µM solution of the oligonucleotide to be digested, 10 µM of a buffer containing 300 mM ammonium acetate, 100 mM CaCl₂ and 1 mM ZnSO₄ (pH=5.7) was added, followed by addition of 22 units of nuclease P1 (penicilium citrinum) and 0.05 units of calf spleen phosphodiesterase. The solution was incubated at 37°C for 3 h. To the resulting solution 12 µL of a buffer containing 500 mM Tris-HCl, 1 mM EDTA (pH = 8.0), 10 units alkaline phosphatase (calf intestinal phosphatase) and 9.1 units snake venom phosphodiesterase were added sequentially followed by incubation at 37°C for another 3 h. The solution thus obtained was added to 6 µL of 0.1 M HCl heat denatured for 5 min. The solution was then centrifuged at 3000 rpm for 5 min. 30 µL of the solution was transferred into an HPLC vial and the sample was injected into an HPLC-MS/MS system.

Assay for ss-DNA repair, cleavage of SAM, inhibition with azaSAM and the 5S- and 5R-configured spore lesion. To prove enzyme activity the amount of 5'-deoxyadenosine and repaired SP was determined and analysed by rp-HPLC. Each reaction mixture contains 100 mM Tris-HCl (pH 7.0), 200 mM KCl, 3 mM sodium dithionite, 5 mM DTT and 0.45 mM SAM. All solutions were degassed and the chemicals were transferred into the glove box as solids. One sample contained only the components described above. To a second sample 50 µM holo-splG was added. The third sample contained 50 µM splG and the 5S-configured spore lesion (1 mM). The fourth sample was identical to sample three but instead of the 5S-configured spore lesion contained the 5R-configured spore lesion (1 mM). The reaction conditions for the ss-DNA assay are identical to those described above. The reaction vial contained additionally 15 µM of the purified SP DNA strand and a 10 fold decreased splG concentration. A sample was taken every 20 min for the kinetic measurement. All other reaction mixtures were incubated for 12 h at 4°C. For the inhibition assay azaSAM was used instead of SAM at the same concentration. After incubation, the reaction mix was frozen in liquid nitrogen and stored at -80°C. All samples were thawed and centrifuged to remove precipitated protein before HPLC purification.

rp-HPLC Analyses. For rp-HPLC analysis, the samples were injected directly after centrifugation into the rp-HPLC system in order to decrease the amount of SAM cleaved to S-adenosylhomocysteine. Up to 50 µL of the enzyme reaction mixture were injected onto the rp-HPLC column (Nucleosil C18, 250 mm, 5 µm, 300 Å, Macherey&Nagel) equilibrated with 0.1% TFA in H₂O. The products were separated using a 20 mL linear gradient 30% solvent B (50 % CH₃CN with 0.1% TFA) (flow rate 0.7 mL/min and temp. 22 °C). The HPLC gradient was chosen to allow rapid separation and hence easy detection of
thymidine in the assay solution, which is the only expected product of the repair reaction. The peaks were assigned by co-injection of thymidine and \( 5' \)-deoxyadenosine, and also by further analysis of the newly formed peak by rpHPLC coupled to a FTICR spectrometer to get a high resolution mass of the newly formed product.

**LC-FTICR Analysis.** Fractions of the rp-HPLC analyses were lyophilized and resuspended with dd H\( _2 \)O. All measurements were run on a Thermo Finnigan LTQ FT (Thermo Finnigan, Bremen, Germany). The resolution was attuned to 100,000 at \( m/z = 400 \). The mass range was adjusted up to 1000. The measurements were conducted using an IonMax ion source with an ESI-head (Thermo Finnigan, Bremen, Germany). The spray capillary voltage was 3 kV, the heating capillary temperature 300 °C and the flow rate 200 \( \mu \)L/min.

**UV/VIS spectroscopy.** UV/VIS spectroscopy was performed in 150 mM Tris-HCl (pH8.0). The holo-splG (0.8 mg/mL) was transferred from the glove box with a UV-cuvette (precision cell made of quartz suprasil\textregistered, Helma) air tight with a plug. The cluster reduction was performed beforehand in the glovebox with 3.6 mM sodium dithionite. All measurements were performed with a UV-visible spectrometer (Varian 100 Bio, Cary). The average value of the iron and sulfur concentration, determined from a standard curve, was divided by the protein concentration in the assay to determine the moles of iron and sulfur bound per mole of protein monomer.

**RESULTS**

Based on the sequence from *G. stearothermophilus*, oligonucleotides were designed to amplify the splG gene by PCR with chromosomal DNA from *G. stearothermophilus*. The obtained 1054 bp fragment was cloned into the pET101-D vector and the expression of the gene was successfully performed in *E. coli*.

We first wanted to clarify if the enzyme exists in a monomeric or dimeric form (18). To this end, the sp lyase from *G. stearothermophilus* was isolated and purified under aerobic and anaerobic conditions. In both cases the protein had a dark brown colour indicative for the presence of an intact Fe/S cluster. This result provided the first evidence that the cluster survives aerobic handling of the protein. Fig. 1 shows the SDS-PAGE results. In the second lane of the gel, a new band (marked with an arrow) was detected, which corresponded to the splG monomer after induction with IPTG and also stained in Western blot experiments. Subsequent affinity chromatography to purify apo-splG was performed. However, only a protein mixture as shown in Fig. 1, lane 3 was obtained.
The molecular weight marker indicated for the major band a molecular weight around 64 kDa, already indicating that the splG protein dimerizes under aerobic and anaerobic conditions. In order to clarify the molecular weights in more detail, MALDI-MS analysis (data not shown) of this band was performed. This MS analysis revealed molecular weight values of 74 kDa and 81 kDa for the isolated proteins. The 81 kDa protein possesses the correct molecular weight for the dimerized form of apo-splG, while the molecular weight at 74 kDa is a proteolytically cleaved apo-splG dimer.

The second set of bands (Fig. 1 lane 3) between 36 and 50 kDa on the gel were also further analyzed by MALDI-MS mass spectrometry (data not shown). The two analyzed protein fractions featured molecular weights of 37 kDa and 40.5 kDa, which are the correct molecular weights for the apo-splG monomer (40.5 kDa) and again of a proteolytically shortened version of the protein (37 kDa). The last band on the SDS-PAGE gel (Fig. 1, lane 3) corresponded to a small, N-terminally His6–tagged 27 kDa fragment of the SDS-PAGE gel (Fig. 1, lane 3) corresponded to a version of the protein (37 kDa). The last band on the SDS-PAGE gel (Fig. 1, lane 3) corresponded to a small, N-terminally His6–tagged 27 kDa fragment of the splG. All the proteolytically shortened fragments with molecular weights smaller than those calculated from the DNA sequence are clearly C-terminal deletion fragments because all fragments are positively recognized by the anti-His6-tag monoclonal antibody in Western blotting experiments.

In order to convert the functionally inactive apo-splG into the catalytically competent form, the [4Fe-4S] cluster needed to be reconstituted. To this end, the apo-splG was treated with FeCl₂, Na₂S and DTT in the glove box. The fully reconstituted form was as expected highly oxygen sensitive. Handling of the reconstituted enzyme required strict anaerobic conditions. Figure 1, lane 5 shows the fully reconstituted holo-splG dimer after purification by affinity chromatography of the apo-protein. Interestingly, holo-splG showed the same behavior as in the gel electrophoresis experiments (Fig. 1, lane 4). The protein exists as a full length and a C-terminally truncated dimer and monomer. A more detailed SDS-PAGE analysis (Fig. 2) of the protein monomers and dimers showed that the bands are in all cases rather broad. In order to investigate if this effect is caused by potential residual secondary structure elements, maybe induced by the Fe/S cluster, we added increasing amounts of TCEP to the protein solution (up to 250 mM). Indeed, this procedure significantly sharpened the bands indicating that residual secondary structural elements were resolved. Most surprisingly, however, is the observation that the ratio between the protein in the monomeric and dimeric state did not change. This shows that the splG protein dimer is extremely stable.

We next started to investigate the presence of the [4Fe-4S]¹⁺²⁺ cluster in more detail using UV-VIS spectroscopy. The absorption spectra of the reconstituted splG dimer exhibited a broad absorption peak around 420 nm (Fig. 3A), characteristic for a [4Fe-4S]²⁺ cluster. Based on the UV-VIS spectrum we can fully rule out that the protein contains an [2Fe-2S]²⁺ cluster as such a cluster would possess absorption peaks/shoulders around 330, 420, 460, and 560 nm (31,32). In addition, the molar extinction coefficient (ε₄₁₀ ≈ 15000 M⁻¹ cm⁻¹) for the [4Fe-4S]²⁺ cluster fits nicely to the calculated value of ε₄₁₀ ≈ 13500 M⁻¹ cm⁻¹ per enzyme monomer. Based on the extinction coefficient we concluded that the holo-splG dimer contains one [4Fe-4S]²⁺ cluster per monomer. Further support for this stoichiometry stems from an analysis of the iron content using either the method of Fischer and Price, whilst ICP-AES. A value of 3.8 ± 0.3 mole of Fe per mole of holo-splG monomer was found by the method of Fischer and Price. ICP-AES revealed a value of 4.5 ± 0.4 mole of Fe per mole of holo-splG. In addition, we determined 3.7 ± 0.3 mole of acid-labile sulfur per mole of enzyme using the method of King and Morris. The iron sulfur cluster is likely bound to the protein via the well established motif C₉₁-X-X-X-C₉₅-X-X-C₉₈.

In order to reduce the [4Fe-4S]²⁺ to the catalytically active [4Fe-4S]¹⁺ form, 3.6 mM sodium dithionite was added to the protein solution in the UV-cuvette. This resulted immediately in a strong reduction of the absorbance in the entire visible region (Fig. 4B), demonstrating that the reconstituted iron sulfur cluster is indeed redox-active. Addition of the dithionite solution caused an almost full loss of all absorption bands λ > 350 nm within 30 min (Fig. 4B) indicating full conversion of the cluster into the active [4Fe-4S]¹⁺ redox state.

We next performed EPR spectroscopy to obtain a deeper insight into the [4Fe-4S] cluster. The obtained data are in full agreement with the presence of two [4Fe-4S]¹⁺ clusters per splG homodimer. The axial EPR spectrum with g values of 2.04, 1.93 and 1.89 (Fig. 4A) are typical of [4Fe-4S]¹⁺ clusters and are very similar to spectra obtained for aRNR and splB (33,34). Addition of the coenzyme SAM to the reduced splG showed a strong decrease of the signals (Fig. 4B) indicating an interaction of SAM with the cluster. Further reduction of the signal intensity was obtained by addition of the synthetic 5S-configured SP (Fig. 4C). SplG treated with oxygen after reconstitution shows an extreme change of the spectrum (Fig.
fractions were pooled, lyophilized and resuspended. About the newly formed compound, the enzymatic repair reaction. In order to get further information about the cleavage ceases with time indicating that the formed methionine is able to bind competitively to the Fe/S cluster (36). Since 5′-dAdoH formation proceeded in our hands even in the absence of any SP substrate, an increased tendency to form a stable dimer. All these features show that splG is very similar to splB. SplG is however more stable and has a strongly increased tendency to form a stable dimer.

The main objective was the characterization of the catalytic potential of the spore photoprodut lyase using for the first time well defined substrates. To this end we analyzed formation of the reduced 5′-deoxyadenosine (5′-dAdoH) because this compound is readily detectable by rp-HPLC at 260 nm and formation of 5′-dAdoH is a typical indicator used to monitor spore photoprodut lyase activity (35). When we added SAM to the reconstituted splG and analyzed the solution by rp-HPLC we indeed detected cleavage of SAM to 5′-dAdoH (Scheme 2A). SAM was cleaved even in the absence of any SP substrate in a time dependent manner (data not shown). However, the detection of the 5′-dAdoH formation proceeded in our hands even in the absence of substrate. We therefore studied the ability of holo-splG to repair the spore photoprodut. We investigated the activity with monomeric substrate analogs that were recently synthesized in our group (23). Addition of the 5S-configured lesion substrate to a solution containing the enzyme splG resulted in a clear increase in 5′-dAdo formation (Scheme 2D). In addition, the appearance of a new peak in the rpHPLC at 12.5 min was observed. Co-injection of thymidine, which co-eluted with the new peak showed formation of the expected product of the enzymatic repair reaction. In order to get further information about the newly formed compound, the fractions were pooled, lyophilized and resuspended in ddH2O. The fractions were then analyzed by rp-HPLC coupled to MS/MS using a Finnigan LC-FTICR system (Fig. 5). For the peak eluting at 13.5 min, the molecular ion [5′-dAdoH+TFA]+ at 364.09 was clearly detect. The peak which eluted at 12.5 min gave a mass of m/z = 355.08, corresponding to [dT+TFA]+. These MS results prove the formation of 5′-dAdoH and production of deoxythymidine in the presence of the 5S-configured spore lesion analog. In accord with earlier work using the enzyme splB (23), no increase in 5′-AdoH formation and no deoxythymidine production is observed in the presence of the 5R-configured substrate showing that splG as well as splB is a highly stereospecific enzyme, which recognizes and repairs only one of the two potential diastereomers of the lesion namely the 5S-configured lesion.

In order to analyze the activity of splG in more detail and to determine if the enzyme capable of turnover we next investigated how the protein repairs small DNA strands containing a defined SP lesion at a defined site. In order to generate the substrate we irradiated DNA with UV-light. Based on the observation that irradiation of calf thymus DNA in the presence of DPA generates SP lesions (13), we prepared a solution of the single strand 5′-GGTTGG-3′ and DPA, which was subsequently concentrated to dryness. The obtained DNA film was irradiated under strictly anaerobic conditions with UV-light (λ = 254 nm). Analysis of the DNA after defined intervals by rpHPLC showed that the UV light converted the DNA into several unknown products. After about 300 min of irradiation, product formation ceased. The DNA was dissolved in water and the product DNA strands were isolated by semi-preparative rpHPLC. The largest fraction was enzymatically digested and the digest was further analyzed by rpHPLC-MS (Fig. 6). We indeed obtained the signal with the correct molecular mass for the SP lesion. In addition the fragmentation pattern of the spore photoprodut lesion was exactly as previously described (27). Our irradiation procedure therefore produced a small single stranded DNA strand containing one SP lesion in the middle (5′-GGTspTGG-3′). This pure substrate was finally used in kinetic measurements. The reconstituted holo-splG together with SAM was added under strictly anaerobic conditions to the solution containing 5′-GGTspTGG-3′. The obtained mixture was analyzed by rpHPLC every 20 min. Indeed, addition of the enzyme and SAM slowly converted the SP lesion containing strand (blue) into a new DNA strand (red). Co-injection proved that the new signal (red) was caused by the ssDNA strand 5′-GGTTGG-3′ proving efficient repair (Fig. 7). Based on these data we calculated a specific enzyme activity of 2.6 µmol of repaired SP lesion containing ssDNA per min and per mg splG giving a turn-over number of approximately 100 showing that splG is catalytically competent. Indeed, using azaSAM instead of SAM in the ssDNA repair assay (Fig.8) fully blocked the repair reaction providing strong support for the repair mechanism proposed by Begley, Nicholsen and Broderick. Most interesting is the fact that the repair is an absolutely clean process. No side reactions such as DNA cleavage were observed, showing that the enzyme has the ability to tightly control the reactive radical so close to the DNA.
DISCUSSION

The repair of the spore DNA lesion (SP) is performed in nature by an unusual repair enzyme called DNA spore photoprodut lyase. So far the repair mechanism has been studied with the enzyme isolated from Bacillus subtilis. It was found that the enzyme belongs to the class of radical SAM enzymes, which use the 5’-adenosyl radical to initiate a radical reaction. Labeling studies by Broderick (17) provided recently support for the repair mechanism proposed by Begley. More detailed studies of the repair mechanism and particularly of the lesion recognition step are needed in order to answer the question of how the enzyme controls radical processes so close to a DNA duplex. Here, radical induced DNA damage is always a possible and likely side reaction. In order to address these questions we isolated a spore photoprodud lyase enzyme from the thermophilic Geobacillus stearothermophilus organism. This enzyme turned out to be stable enough for detailed analysis. We also developed a new procedure that allows preparation of small oligonucleotides containing one SP lesion at a defined site for detailed repair and binding studies. Using this substrate we could provide direct evidence that the enzyme is sufficiently stable to turn-over the lesion substrate.

SplG dimerizes during the aerobic purification process. The protein is inherently stable as a dimer under anaerobic conditions after cluster reconstitution (Fig. 2, Fig. 3). The dimeric state was found to be so stable that even the addition of 250 mM TCEP does not induce monomerization. SplG shares these properties with several other well characterized members of the radical SAM protein family. Proteins such as bioB, aRNR-AE and PFL (37-39) also form protein dimers. The preferred dimer state and unusual stability of secondary intra- and intermolecular structures of the splG in comparison to splB is probably a consequence of the thermophilic origin of G. stearothermophilus strain whose temperature optimum is around 60°C. The dimerization of the full length SplG monomer is presumably facilitated by the fifth cysteine at position 326, which is lacking in the SplB protein. This would indicate that the dimerization involves formation of at least two intermolecular disulfide bridges. This dual linkage of the monomers together, maybe with other intramolecular forces, seems to be responsible for the unusual stability of the homodimer.

Comparison of the deduced amino acid sequence of SplB and SplG reveals four conserved cysteine residues (C91, C95, C98 and C141). Three cysteines are needed to coordinate the [4Fe-4S] cluster. The function of the fourth conserved cysteine is unknown. Recently published results, however, indicate that this conserved cysteine is vital for the function of the protein. Site directed mutagenesis showed that a mutation at this position in the splB gene (C141A) is associated with a loss of enzyme activity in vivo (40). Potentially, the conserved fourth cysteine is required for dimer formation.

The [4Fe-4S] cluster formation of splB and splG is coordinated by the first three cysteines, which demonstrates a high degree of similarity to other proteins belonging to the radical SAM super family (41,42). We could confirm the existence of a [4Fe-4S] cluster after quantification of the sulfur and iron content, which was measured using two independent methods. We measured the Fe content to be approximately 4 mol of iron per mol for the holo-splG monomer. In addition a value of 4 mol of acid-labile sulfide per mol of enzyme was measured and is characteristic for a [4Fe-4S] cluster (43). The presence of such a cluster is supported by UV- and EPR-spectroscopic data. We could observe an intense absorption at 420 nm in the UV-spectra, typical for [4Fe-4S] clusters (44). Moreover, we obtained no spectroscopic evidence for the presence of a [2Fe-2S] cluster after reduction with dithionite (45). Dithionite reduction proceeds very slowly over a period of 30 min.

The EPR spectra concur with the results of UV spectroscopy and the quantification of the iron and sulfur content in the splG. Here we obtained clear evidence for the presence of the [4Fe-4S] cluster in the reconstituted splG homodimer. The dramatic decrease of the EPR signals after adding the cofactor SAM and the 5S-configured substrate is evidence for the binding of SAM and the 5S-configured SP to the [4Fe-4S] cluster.

Regarding the functional analysis of splG, we could show that the purified homodimer is able to cleave SAM to 5’-deoxyadenosine and methionine using rp-HPLC-MS. This feature was frequently used to characterize the activity of the enzyme. (17-19,23). However, 5’-deoxyadenosine formation is not a reliable indicator for the repair mechanism. For the B. subtilis spl, formation of about 2 molecules of 5’-deoxyadenosine per molecule of (His10) splB dimer was observed. Comparably, Ollagnier et al. (35) observed that approximately 3 molecules of SAM were cleaved per dimer by the aRNR-AE of E. coli. The SAM cleavage of the holo-splG is half compared to splB. Based on these data we are currently not sure if SAM cleavage is indeed a proper indicator for spl en-
zyme activity. Cheek \textit{et al.} (17) used a SP specifically $^3$H-labeled at C-6. They observed that the C-6 tritium label from SP is finally found in AdoMet, and not in 5$'$-deoxyadenosine. This suggests that the SAM is indeed needed as a catalytic cofactor to reversibly generate the putative 5$'$-deoxyadenosine radical intermediate and not as a co-substrate. It could well be that the background 5$'$-deoxyadenosine formation proceeds via a shunt pathway.

We therefore decided to use synthetic SP substrates to confirm the activity of splG. We firstly studied enzyme activity using synthetic SP lesions. With these substrates, formation of thymidine, and hence repair, was clearly observed. However, only with the 5$S$-configured substrate was product formation detected. No repair could be seen if the 5$R$-substrate was used, similar to a recent study that we performed with splB. Most interesting are the studies with a defined DNA single strand containing an SP lesion at a defined site. This ssDNA substrate was prepared in a novel way by irradiation of a dry film of ssDNA in the presence of DPA under strict anaerobic conditions at 254 nm. The DNA containing the SP lesion was isolated by rpHPLC and the presence of the lesion was confirmed by rpHPLC-MS and rpHPLC-MS/MS after a total digest of the ssDNA. This novel substrate was added to a buffered solution of splG in the presence of SAM. The ssDNA substrate was readily accepted by the repair enzyme, which converted the lesion-containing DNA with a turn over number of about 100 into the repaired ssDNA. The usage of azaSAM instead of SAM blocked the repair reaction as expected.

In conclusion, we show that the purified holo-splG dimer of \textit{Geobacillus stearothermophilus} repairs the 5$S$-configured SP DNA lesions in the dimeric state. We were able to generate a pure 6mer ssDNA strand containing a SP lesion at a defined site, which can be use to determine the enzymatic activity. The calculated specific enzyme activity is 2.6 \textmu mol SP repaired/min/mg splG. This enzyme activity is completely inhibited by the cofactor mimic azaSAM. The thermophilic enzyme apo-splG was purified under aerobic conditions and fully reconstituted under conditions favouring the cluster formation to the holo-splG. This holo-splG contains the correct amount of iron and sulfur for two [4Fe-4S] cluster per homodimer holo-splG. The presence of this cluster was confirmed by EPR and UV-spectroscopy. Transcriptional regulation of splG is not performed by a homolog of splA in \textit{G. stearothermophilus}. The presented results provide a more stable spl enzyme and a new method that allows synthesis of defined SP lesion containing substrates. Both achievements should now allow a detailed biochemical and structural analysis of the repair reaction similar to what was recently achieved in the case of the CPD DNA photolyase.
References:


Footnotes:

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1ABBREVIATIONS: ATP, adenosine 5′-triphosphate disodium salt solution; a.u., absorption units; CPD, cyclobutane pyrimidine dimer; dd H2O, double distilled water; IPTG, isopropyl-β-D-thiogalactopyranoside; (6-4)PP, (6-4) pyrimidone photoproduct; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SP, spore photoproduct (5-thyminyl-5,6-dihydrothymine); splB, spore photoproduct lyase B. subtilis; splG, spore photoproduct lyase G. stearothermophilus; TCA, trichloroacetic acid; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid

Scheme legends:

Scheme 1:
Proposed repair mechanism of the spore photoproduct. The repair reaction is proposed to proceed by hydrogen abstraction from C6 of the spore photoproduct followed by β-scission of the bond linking the two pyrimidines and back transfer of the hydrogen atom.
Scheme 2: 
Repair of the 5S- and 5R-configured spore photoproducts by splG. A repair of 5S-configured spore photoproducts to thymidine was observed. No repair of the 5R-configured spore photoproducts was detected.
HPLC analysis of SAM conversion to 5'-deoxyadenosine (AdoH) and repair of SP to thymidine by splG. The enzyme reaction and HPLC analysis were performed as described in Experimental Procedures. (A) Reaction contained 50 µM splG and SAM. (B) Reaction containing 50 µM splG, SAM and the 5R-configured SP. (C) Negative control with only SAM and the 5S-configured SP. (D) Reaction containing 5 nmol splG, SAM and the 5S-configured SP. In all four traces, the y-axes showed the absorbance at 260 nm.

Figure legends:

Figure 1:
Analysis of purified and reconstituted splG in different purification steps by SDS-PAGE. The SplG solutions were heated up to 95°C for 10 min in SDS buffer containing 5 % (v/v) β-mercaptoethanol, and electrophoresed through 10% SDS-PAGE. The line marked M contains molecular weight markers. Lanes 1 and 2 contain the crude cell extract before (1) and after induction (2) with IPTG. The arrow indicates the splG monomer. Lane 3 shows the Ni-NTA purified apo-splG before reconstitution, lane 4 shows the holo-splG after reconstitution. Lane 5 shows the holo-splG dimer after size exclusion purification.

Figure 2:
Analysis of secondary splG structure. The splG monomer and dimer were treated as described in experimental procedures and electrophoresed through 12 % SDS-PAGE. (A) SplG dimer treated with increased TCEP concentrations. (B) SplG monomer treated with increase TCEP concentrations. The first lanes in (A) and (B) contain molecular weight markers.

Figure 3:
UV/VIS absorption spectra of reconstituted splG in 150 mM Tris-HCl (pH 8.0). (A) Spectrum of splG (0.8 mg/ml) recorded after reconstitution of the splG dimer. (B) Spectrum of splG recorded i) prior to dithionite addition and ii) after 5 min and 30 min after dithionite addition.

Figure 4:
EPR spectra of splG. EPR spectra were measured under the following conditions: microwave frequency 9.459 GHz; modulation frequency 100 kHz; modulation amplitude 1.25 mT; microwave power 20 mW; temperature 10K. Figure 5 A shows the splG protein reduced with dithionite (10mM) and the simulation of this spectrum (above) performed with an EPR simulation program (46). In Figure 4 B the spectrum of splG after SAM addition is shown. (C) Spectra for splG after addition of SAM and 5S-configured substrate. Figure 5 D shows the reconstituted splG after exposure to air (amplitude 2.5-fold reduced).
Figure 5:
LC-FTICR analysis of the peaks (retention time = 13.5 min, 12.5 min) from Fig. 2B. Displayed are the molecular weights of thymidine associated with TFA [M+TFA]⁺ = 356.08 (A) and 5'-deoxyadenosine associated with TFA [M+TFA]⁺ = 364.09 (B).

Figure 6:
LC-FTICR analysis of the spore photoproduct after enzymatic total digestion. Displayed is the molecular weight of spore photoproduct [M+H]⁻ = 545,12 (A) as the parent ion which is further fragmented to give the daughter ions shown in B. Here the ions were detected in a molecular weight range between 160 and 555 u (dR: 2-deoxyribose; P: phosphate and the spore photoproduct without dR and P, shown as structure).

Figure 7:
Repair of the ssDNA containing spore photoproducts by splG. A repair of the 6mer ssDNA containing spore photoproducts to undamaged DNA was observed. The enzyme reaction and HPLC analysis were performed as described in Experimental Procedures. The red peak series shows the decreasing of the amount of DNA containing the spore photoproduct. The blue peak series shows the growing amount of repaired DNA. The peaks marked with an x are from the buffer.

Figure 8:
The enzyme assay with azaSAM instead of SAM shows no spore photoproduct repair. The x marked peaks are from the buffer.
SCHEMES AND FIGURES

Scheme 1

Scheme 2
Figure 2
Figure 3

A

B

[Graph showing absorbance vs. wavelength with annotations for different conditions: prior sodium dithionite addition, 3 mM sodium dithionite after 5 min, and 3.6 mM sodium dithionite after 30 min.]
Figure 4
Figure 5
Figure 6

A Spore photo product

\[ \text{[M-H]}^+ \text{ calc.: 545.1290} \]

B M-dR

\[ \text{dRP} \]

Relative Intensity

m/z (a.m.u.)
Figure 8
Characterization of a new thermophilic spore photoproduct lyase from geobacillus stearothermophilus (splG) with defined lesion containing DNA substrates
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