Published in final edited form as:

FEBS Lett. 2014 August 25; 588(17): 3023–3029. doi:10.1016/j.febslet.2014.06.011.

Solution phase dynamics of the DNA repair enzyme spore photoproduct lyase as probed by H/D exchange

Shourjo Ghose, Jonathan K. Hilmer, Brian Bothner, and Joan B. Broderick*

Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, United States

Abstract

Spore photoproduct lyase (SPL) catalyzes the repair of the UV lesion spore photoproduct (SP) in a reaction dependent on S-adenosyl-L-methionine (SAM). We have utilized H/D exchange to show that in the presence of SAM, a significant reduction in H/D exchange is observed upon binding SPTpT or undamaged oligonucleotide, indicating a shift of 20 or 10 amide protons, respectively, from a rapidly-exchangable state to a fully-protected conformation. In the absence of SAM, neither the oligonucleotide nor the SPTpT produce a significant perturbation in H/D exchange, indicating SAM is a requisite binding partner. Performing the same experiments in aerobic conditions reduced the magnitude of ligand-induced structural changes, consistent with the importance of the oxygen-sensitive iron–sulfur cluster for SAM and substrate binding.

Keywords

Radical SAM; DNA repair; H/D exchange; Mass spectrometry

1. Introduction

Bacterial spores exhibit remarkable resistance to various environmental stressors, including wet and dry heat, variations in temperature, and UV radiation [1]. The UV resistance of bacterial spores can be attributed to the unusual photochemistry of spore DNA, coupled with the efficient repair of accumulated damage. On exposure of spores to UV radiation, an unique methylene bridged thymine dimer, 5-thyminyl-5,6-dihydrothymine (spore photoproduct or SP) accumulates as the main photoproduct [2–4]. This accumulated photo-damage is rapidly repaired upon spore germination, thereby giving rise to the unusual UV resistance of the bacterial spore [1,5]. The repair of SP is catalyzed by a specialized enzyme, spore photoproduct lyase (SPL), and involves the direct reversal of SP into two thymines in a light-independent reaction (Fig. 1).

SPL is a member of the radical S-adenosylmethionine (SAM) protein superfamily; this large superfamily includes enzymes that play critical roles in metabolism, and they are found in all kingdoms of life. Radical SAM enzymes have in common the utilization of a site-differentiated $[4\text{Fe}-4\text{S}]^+$ cluster (most commonly coordinated by a $\text{CX}_3\text{CX}_2\text{C}$ motif) to

*Corresponding author. jbroderick@chemistry.montana.edu (J.B. Broderick).

reductively cleave SAM to generate a 5'-deoxyadenosyl radical intermediate, which subsequently abstracts a hydrogen atom from substrate to initiate catalysis [6–9]. A radical mechanism for repair of SP by SPL first suggested by Mehl and Begley in 1999 [10] has received considerable experimental and computational support in recent years [11–13]. It is generally accepted from these studies that SPL, like lysine 2,3-aminomutase (LAM) but unlike most other characterized radical SAM enzymes, utilizes SAM as a cofactor rather than a substrate [12].

The binding of SPL to its substrate has been investigated in a number of ways. DNaseI footprinting has been used to demonstrate that SPL can bind to DNA containing an SP lesion and protect it from digestion [14]. It has been suggested that SPL recognizes the SP lesion in a sequence-independent manner as a result of SP-induced helical distortion of the phosphodiester backbone, and that additional distortion induced by SPL binding helps to initiate repair [14]. Such a model is consistent with the binding mechanisms of a multitude of DNA binding proteins such as Uvr(A)BC exonuclease [15], DNA photolyase [16,17], and phage T4 endonuclease V [18]. Studies using stereochemically-defined synthetic SP substrates have demonstrated that SPL repairs only the 5R(5' → 3')SP, with the presence of a phosphodiester linkage in SP enhancing the rate of repair [19–21]. These results suggest that SPL has an active site that is capable of productive binding of only a single, well-defined diastereomer of SP. A recently published crystal structure of SPL bound to the 5R-SP dinucleoside and SAM reveals a characteristic $\alpha_6\beta_6$ partial triosephosphate isomerase (TIM) barrel consisting of a laterally open active site that could accommodate a large substrate such as DNA [22]. SPL has been proposed to utilize a mechanism of binding similar to other DNA-binding enzymes like DNA endonuclease, and probably employs a base-flipping mechanism to bind and initiate repair of SP [22,23]. Despite the insights provided by this crystal structure, many outstanding questions remain unanswered; among these are the role of the catalytic 4Fe–4S cluster in substrate recognition and binding, the possible role of SAM in ordering the enzyme active site prior to SP binding, and the local and global changes in conformation brought about by cofactor and substrate binding.

In order to obtain insight into the role of conformational changes associated with binding and recognition of damaged DNA by SPL in solution, we have carried out hydrogen–deuterium exchange (HDX) reactions with mass spectrometry detection. The basic mechanism of HDX involves abstraction of an amide proton by solvent followed by its replacement by deuterium from the abstracting solvent, as described in the general Linderstrom-Lang scheme [24]. In this scheme, it is a prerequisite for exchange that the amide proton be available for exchange: exchange can be inhibited due to proton participation in hydrogen bonds, especially in the case of bonds within secondary structural elements and the core of a protein. Strong hydrogen bonding can even prevent exchange on the surface of proteins [25,26].

The net sum of hydrogen bonding and similar effects is to reduce the observed HDX rate, compared to the rate observed for hypothetical unstructured or random coil conformations. In this way, HDX can be a sensitive and specific probe for changes in dynamics and conformation of proteins in solution. When coupled with mass spectrometry (HDX-MS), this approach does not require large quantities or high concentrations of protein, and

experiments can be conducted very rapidly [27]. The additional challenges of working with oxygen-sensitive proteins such as SPL can also be addressed with HDX-MS: rapid analysis minimizes potential for oxidation, and the small sample sizes and automated HPLC equipment aid in practical concerns of maintaining an anaerobic environment.

Our primary objective in this endeavor is to investigate the changes brought about by ligand and/or cofactor binding to SPL, under biologically relevant solution conditions. In addition to collecting novel data on the SP/ligand system, our experiments also demonstrate the validity and utility of the HDX-MS approach for difficult real-world protein studies, where traditional approaches (X-ray crystallography, nuclear magnetic resonance) are unavailable and/or impractical. Moreover, we provide evidence here that our system does indeed possess the sensitivity to differentiate between dinucleoside and dinucleotide forms of the substrates and therefore can be employed as a general screening mechanism for potential ligands in other protein systems.

In order to examine the range of potential conformational changes brought about by cofactor/substrate binding, we have utilized undamaged double-stranded DNA oligonucleotide as well as the synthetic substrates lacking (5R-SP) and possessing (5R-SPTpT) a phosphodiester bridge between the adjacent nucleosides. These stereochemically defined synthetic molecules have been shown to be substrates for SPL in previous studies from our lab [28]. The results reported here provide evidence for a conformational change of SPL upon binding SAM. Further, the protection from HDX is significantly enhanced in the presence of the synthetic substrates, despite their small size. We also demonstrate that SAM binding is a requisite precursor to substrate binding, suggesting that a SAM-induced conformational change enhances the binding affinity for substrate.

2. Materials and methods

2.1. Protein expression and purification

The N-terminal hexa-histidine tagged SPL from *Clostridium acetobutylicum* (Ca) was expressed using *Escherichia coli* Tuner(DE3)-pLysS cells transformed with a pET14b expression vector containing the *splB* gene. The resulting protein was grown in minimal media and purified anaerobically by Ni-HisTrap chromatography and FPLC as previously described [29,30]. The protein was anaerobically dialyzed in 20 mM sodium phosphate, 350 mM NaCl, 5% glycerol, pH 7.5. The protein was then concentrated using an Amicon concentrator fitted with an YM-10 membrane to a final concentration of ~650 μ M. All protein samples used in assays were prepared in the MBRAUN box (O_2 1 ppm) unless mentioned otherwise. The protein and iron concentration were determined by methods previously described [31,32]. SAM was synthesized as previously described [33].

2.2. Preparation of enzyme/ligand mixtures

A 6-mer oligonucleotide (5'-GCAAGT-3' and complement 5'-ACT TGC-3') were obtained from Integrated DNA Technologies (Coralville, IA). Equimolar amounts of each strand were mixed in water and then annealed by heating to boiling, followed by removal of the heat source and slow cooling of the water temperature to 25 °C. Proteins were prepared in advance in a buffer consisting of 40 mM sodium phosphate, 350 mM NaCl, 5% glycerol, pH

7.5 under appropriate anaerobic conditions. Protein solutions were diluted to an identical concentration (250 μ M final concentration) for all assays. A ligand solution or control buffer was added to each of the matched control/experimental protein samples. The ligand solutions and their final concentrations were: 6mer oligo (1.0 or 2.0 mM), synthetic 5R-SPTpT (250–750 μ M), 5R-SP (250–750 μ M) [29,30], and SAM (2.5–54 mM). All anaerobic assays were conducted with buffer and/or D₂O previously degassed on a Schlenk line. The degassed buffer and/or D₂O was brought into an MBRAUN glove box and the assays prepared as mentioned above. All aerobic samples were handled in an identical fashion as their anaerobic counterpart with the exception of the degassing step and were thereafter prepared on benchtop. The preparation of all enzyme/ligand mixtures was conducted identically, with matched timing for solution preparation steps and for the delay (10 min) between preparation and subsequent HDX reactions.

2.3. H/D exchange (HDX) reactions

The 10 μ L aliquots of protein/ligand solutions were transferred to autosampler vials and sealed: the capped tubes were then covered with Teflon tape to minimize O₂ contamination. Two Hamilton gas tight syringes were filled with 40 μ L of anaerobic D₂O-containing buffer (20 mM sodium phosphate, 350 mM NaCl, 5% glycerol, pD 7.9) and their tips covered with Teflon tape. The D₂O and sample were then mixed by puncturing the septum of the MS vial and injecting the D₂O; the samples were then manually mixed with the Hamilton syringe. Following mixing, the vial was placed into the HPLC autosampler and the automated sampling series was begun immediately: the interval between samples was 2 min. The autosampler was not within an anaerobic environment, but the anaerobic reactions remained sealed in the autosampler vials for the duration of the reactions (30 min). Injection of the sample into the HPLC system provided the quenching step, as described below.

2.4. LCMS analysis

The HPLC system was composed of an Agilent 1100 HPLC with binary pump, temperature-controlled autosampler and column compartment, and a C4 reverse phase column (8 $\times$ 1 mm, Microchrom Biosciences). Solvents consisted of H₂O with 0.1% (v/v) formic acid in channel “A” and acetonitrile with 0.1% formic acid in channel “B”. The autosampler and reactions were held at 25 °C, and the column compartment was maintained at 4 °C to maximize reproducibility. Prior to sample injection, the C4 column was equilibrated with 20% “B” at 0.6 mL/min: this flow rate was maintained throughout the run. Concurrent with sample injection ($t = 0.01$ min), the solvent composition was changed via the binary pump to 100% “B”: the residual water in the system and column were sufficient to slightly delay the elution of the protein from the flow-through. The protein elution peak was centered at approximately 0.4 min. Following protein elution, at 1.21 min the solvent composition was changed back to 20% B for column re-equilibration. The mass spectra were obtained on a Bruker microO-TOF Mass Spectrometer equipped with an ESI source. The capillary exit voltage was 120 V and gas temperature was 200 °C. All data were recorded in positive mode between 300 and 3000 m/z in profile mode. The hardware summation time was 1 s with no rolling averaging.

Quenching of the H/D exchange reaction was a consequence of sample injection into the HPLC system. A flow rate of 600 $\mu\text{L}/\text{min}$ carried the sample from the autosampler (maintained at 25 $^{\circ}\text{C}$) to the column compartment and reverse-phase column (maintained at 4 $^{\circ}\text{C}$) in less than 2 s. Although this temperature produces more back-exchange compared to 0 $^{\circ}\text{C}$, it is more stable. The solvents also provide quenching due to the low pH and reduced water composition of the loading solvent: 80/20 $\text{H}_2\text{O}/\text{acetonitrile}$ with 0.1% (v/v) formic acid. A control reaction was run immediately following each experimental reaction to account for hidden variation in the instrumentation, and replicates of control/experiment reaction pairs were conducted on separate days to ensure unbiased results.

2.5. Analysis of H/D exchange (HDX) data

With the described chromatographic system, the protein eluted at 0.4 min during the 2 min run. The resulting mass spectra were then averaged and processed using the Data Analysis 4.0 software suite supplied by Bruker Daltonics. The Maximum Entropy routine was used for charge-state deconvolution of the raw data, which were then exported into text files: these steps were automated via scripts to ensure reproducibility. The deconvoluted spectra were processed using Python and Scipy scripts, and a reference spectrum of a 0% D_2O sample was used to calculate the cross-correlation with the experimental spectra for the true HDX reactions. The cross-correlation function produced a symmetric peak centered at the deuterium uptake of the experimental sample, describing the shift between reference and experimental peaks. These calculations were manually verified against the more common centroid peak assignment method, but the use of cross-correlation was less sensitive to bias permitting use of a fully-automated processing workflow. The shape and symmetry of the cross-correlation shift plot also provided quality control for deviations in peak shapes between the reference and experimental spectra: none were observed. A minimum of three trials were conducted for each experiment. A control was run with every experimental reaction to test system performance, but was not used to apply any correction factor. All experimental replicates were unique and unmodified. Data was collected non-sequentially with delay intervals (up to days) to ensure they were independent measurements. Data were plotted and analyzed using Origin 8.0 (MicroCal, MA). The standard deviation was reported as the variation in deuterium uptake between the independent trials at each time point.

3. Results

3.1. H/D exchange into SPL alone

The enzyme SPL has a mass of 41118.0 Da. On dilution with D_2O , a rapid initial uptake of deuterons was observed as indicated by a mass increase of 186 Da within the first 5 min. The rate of deuterium uptake slowed in subsequent injections, with incorporation reaching a plateau after approximately 30 min with an average mass increase of 228.8 (± 2.2 Da), indicating significant exposure of solvent-exchangeable protons in SPL. The mass change due to deuterium uptake for SPL in the absence of SAM and substrates was the same regardless of whether the exchange was carried out under aerobic or anaerobic conditions (data not shown). Experiments were terminated after 30 min because the rate of H/D exchange had reached a plateau, and in order to minimize any artifacts due to oxidation or loss of the [4Fe-4S] cluster that could occur during more extended experiments.

3.2. Effect of SAM on H/D exchange

In the presence of SAM, deuterium uptake by SPL also reached a plateau within 30 min. This pattern was present in all experimental trials; however, the magnitude of the mass increase in the presence of SAM was smaller than that observed in its absence, indicating that some of the exchangeable protons on SPL transitioned to an effectively unexchangeable conformation upon SAM binding. Under aerobic conditions the average difference in uptake at 30 min between the SPL alone and SPL + SAM samples was 6.3 ± 0.6 , indicating that the binding of SAM results in protection of approximately 6 protons from H/D exchange (Table 1). SAM binding to radical SAM enzymes is known to involve the [4Fe-4S] cluster, [34] which is generally oxygen sensitive, suggesting the possibility that anaerobic conditions would affect SAM binding and thus H/D exchange. H/D exchange was therefore also carried out on anaerobic SPL + SAM samples, resulting in a further decrease in deuterium uptake, with a difference in mass change of 13.0 ± 0.4 relative to SPL alone (Table 1).

3.3. Effect of substrate on H/D exchange

In the absence of SAM, the presence of a minimal substrate dinucleotide 5R-SPTpT provided only minimal perturbation to the H/D exchange of SPL, decreasing the number of exchanged hydrogens by 1.1 ± 0.5 (Table 1). A short 6-mer oligonucleotide provided a similar decrease in exchangeable positions, 2.6 ± 1.3 (Table 1). In the presence of both SAM and the 6-mer oligonucleotide (5'-GCAAGT-3'), the deuterium uptake was comparable to the samples containing only SPL and SAM, with a decrease in deuterium uptake of 6.9 ± 3.4 Da under aerobic conditions, and 10.4 ± 0.8 under anaerobic conditions (Table 1).

Interestingly, a significant decrease in exchangeable sites is observed in SPL samples containing both SAM and the synthetic SP dinucleotide 5R-SPTpT (Fig. 2, Table 1). Compared to the samples with SPL alone, those samples containing SPL + SAM + 5R-SPTpT showed a decrease in deuterium uptake of 12.0 ± 1.2 Da under aerobic conditions, and 20.4 ± 1.4 Da under anaerobic conditions (Fig. 3); the latter number is nearly double that observed for SPL in the presence of SAM alone (Table 1). Given the small sizes of both SAM and the 5R-SPTpT dinucleotide substrate, the change in deuterium uptake most likely reflects a reordering of H-bonding arrangement brought about by a dynamic perturbation in the enzyme, rather than simply steric blocking of exchangeable sites by SAM and substrate. In order to examine the contribution of the substrate phosphodiester linkage to the perturbation in SPL deuterium uptake, as well as to provide a direct comparison between our results and the recent X-ray crystal structure of SPL, [22] we conducted H/D exchange experiments using 5R-SP dinucleoside and SAM. Under anaerobic conditions, the samples containing the dinucleoside substrate and SAM showed an uptake of 17.4 ± 1.5 Da, which is about 3.4 Da less than those with the dinucleotide counterpart (Fig. 3, Table 1).

4. Discussion

Here we have used HDX as a probe to explore the solution phase conformational dynamics in SPL upon binding to undamaged DNA and dinucleotide 5R-SPTpT and dinucleoside 5R-SP substrates. SPL displays initial, rapid deuterium uptake in the first few minutes after mixing with D₂O buffer, followed by an approach to a limiting value. This behavior is

consistent throughout the experiments irrespective of ligand binding and all experiments demonstrate saturation of exchange within the time monitored (30 min). Based on the amino acid sequence of SPL, there are remaining unexchanged protons, but the rate at which they exchange is so slow that they are effectively static and thus silent compared to the observed exchanging protons. Experiments conducted over longer periods (60 min, data not shown), confirmed this behavior. This is a critical observation because it rules out one mechanism of H/D exchange for SPL: global protein exposure (“unfolding”). Global protein exposure is a mechanism that must be examined carefully, since it has the potential to produce misleading results in H/D exchange experiments in the context of ligand binding [35]. Careful analysis of the protein and ligand concentrations used herein indicate that such complications are unlikely for the SPL system. In addition, the observation that the number of “unexchangeable” protons has a ligand dependence is inconsistent with global unfolding; rather, the results presented here are consistent with a model in which a conformational change to one with more extensive hydrogen bonding occurs upon ligand binding.

Based on the structure of SPL, we propose that likely candidates for the rapid H/D exchange include the long β hairpin located between strand $\beta 5$ and $\alpha 6$ [22]. This hairpin motif has been shown to project out of the core domain and its conformation remains unchanged when SPL is free of substrate (Fig. 4A) [22]. Other regions likely to be exhibiting rapid deuterium uptake include the loop regions near the active site of the SPL, including the 8 residue loop region binding the [4Fe–4S] cluster. All these regions would be amenable to exchange reactions in the absence of SAM and substrate due to the wide lateral opening of the SPL TIM barrel.

Our data show that in the absence of SAM, addition of DNA oligonucleotide or the synthetic substrate 5R-SPTpT results in little alteration in deuterium uptake (Table 1). These results suggest either that SPL has low affinity for substrate or DNA in the absence of SAM, or that only minimal conformational changes occur upon binding DNA or substrate in the absence of SAM. Upon addition of SAM alone, however, the protein undergoes a significant structural change, as reflected by the difference in maximal uptake of deuterium between the bound and unbound forms (Fig. 3A). Although this change occurs under aerobic conditions (where the [4Fe–4S] cluster is not intact), it is more pronounced under anaerobic conditions (Fig. 3A). We believe that this change in deuterium uptake is most likely due to an alteration in the H-bonding interactions of the specific amino acids involved in the stabilization of SAM in the active site [22]. Our exchange data indicate that when SPL is bound to the ligand/s, approximately 20 residues are dramatically protected from HDX. We hypothesize that these residues could be participating in direct ligand contact, or they could be stabilizing an induced fold in the protein. In contrast, there is no detectable change in the remaining ~333 residues. This partitioning of residues into two regions, ligand-affected and ligand-independent, sets useful bounds upon the maximum possible “sphere of influence” mediated by ligand binding, regardless of whether this change is induced by direct ligand contact or structural perturbation.

Our data indicates that SAM causes significant structural perturbations regardless of the integrity of the iron–sulfur cluster, consistent with the observation that SAM binds SPL not only via coordination of the iron–sulfur cluster, but also by interactions such as hydrogen

bonding, hydrophobic interactions and salt bridges [22]. The amino group of the methionyl moiety of SAM interacts with Ser142 and Asp143 and the carboxylate moiety interacts with Lys174 and Ser195, while the adenine and ribose groups are held in place via hydrogen bonding interactions with Tyr96, Tyr98, Ala234 and Ala273 [22]. It is likely that these interactions and the associated conformational changes upon SAM binding contribute to the substantially decreased levels of deuterium exchange in the presence of SAM. The increase in H/D exchange protection under anaerobic conditions can be ascribed to the presence of a functional [4Fe–4S] cluster in the anaerobic enzyme that increases SPL stability and also provides a site for SAM coordination.

On the addition of the 6mer oligonucleotide to SPL in the presence of SAM, the deuterium uptake is very similar to that of SPL + SAM, indicating that the principal conformational change is induced by SAM binding, and the contribution of the 6mer is relatively minor. However, the effect of the dinucleotide substrate 5R-SPTpT plus SAM on SPL H/D exchange is significantly greater than that of SAM alone (Fig. 3B). The results suggest that 5R-SPTpT binding induces significant conformational changes in the active site of SPL, as compared to SAM or 5R-SPTpT binding alone. In order to probe the effects of the phosphodiester linker of 5R-SPTpT on binding, we conducted H/D exchange experiments with the 5R-SP dinucleoside substrate. The 5R-SP has been previously characterized through kinetic assays to be a substrate for SPL, though not as good a substrate as the 5R-SPTpT [29]. The phosphodiester bridge between the nucleotide residues involved in the SP lesion is thought to be important in SPL binding to SP, as SPL is likely similar to other DNA repair enzymes in recognizing the damage-induced deformation on the DNA backbone [36,37]. The role of the phosphodiester bridge in binding has been well established in other DNA repair enzymes bound to lesions containing either a phosphate bridge or a formacetal linker [38,39]. In all cases a conserved arginine residue has been shown to interact directly with the phosphodiester bridge to correctly orient the substrate in the active site [38,40]. Although the crystal structure of SPL was solved with a dinucleoside substrate, the location of Lys309 and Arg273 (Fig. 4B) are suggestive of their interaction with the phosphodiester bridge [22,41]. We conclude that our observed difference in uptake of 3.4 Da between the 5R-SPTpT and 5R-SP substrates is largely due to the interactions of these two amino acids with the phosphodiester backbone of the dinucleotide SP (Figs. 3C and 4B). Other possible contributors to the decreased H/D exchange include Tyr98, which undergoes a ~45° rotation to accommodate the lesion, as well as Thr101 and Lys106 which undergo conformational changes upon lesion binding [22]. In addition, the β hairpin, which experiences a conformational change of 2 Å on lesion binding, might also be involved in base flipping interactions commonly observed in DNA repair enzymes such as DNA photolyases [39,42].

Our results suggest HDX protection is highly localized and lacks large-scale allosteric or global characteristics. Combined with the existing structural data on SPL and other radical SAM enzymes, is reasonable to conclude that these highly localized effects can be assigned to the active site, and are involved in substrate recognition and binding. Such localized structural changes have been commonly observed for other DNA repair enzymes such as (6–4) photolyase and CPD photolyases, where the damaged regions are stabilized in the active site through hydrogen bonding [38,40]. We believe that a similar sequence of events follows

on SPL binding to its substrate, where the binding event breaks the existing H-bonding interactions in the active site, leading to a new H-bonding network upon binding the lesion pair. Comparison between reactions conducted anaerobically vs aerobically shows an additional protection of protons (Fig. 3D). This again points to the relevance of the [4Fe–4S] cluster in enhancing protein stability and maintaining the correct conformation for SAM and ligand binding.

5. Summary and conclusions

The data presented herein demonstrates that the number of exchangeable protons on SPL decreases by 20 upon binding SAM and the minimal substrate 5*R*-SPTpT. Our results suggest that binding of SAM and 5*R*-SPTpT effectively seals off the active site, thus protecting a large number of protons which can be rapidly exchanged in the ligand-free state. The presence of the [4Fe–4S] cluster is also important for this interaction, as performing the experiment under aerobic conditions (with associated loss of the cluster) results in decreased protection from H/D exchange. It is interesting to note that even binding of SAM alone results in protection of thirteen exchangeable sites under anaerobic conditions, while binding of substrate alone offers insignificant protection from H/D exchange. The sum of existing structural data and our H/D exchange measurements supports assignment of these protected region to the active site, and not to allosteric effects. Conformational changes associated with cofactor and substrate binding have been implicated in other radical SAM enzymes [43–45], and may serve to provide a solvent inaccessible and protected active site to safely catalyze radical reactions.

Acknowledgments

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number GM54608. We would like to thank the Murdock Charitable Trust and NIH Cobre 5P20RR02437-03 for support of the Mass Spectrometry Facility at MSU.

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Fig. 1.

Repair of SP by SP lyase. SP lyase is a radical *S*-adenosylmethionine (SAM) enzyme that utilizes a site differentiated [4Fe-4S] cluster to reductively cleave a bound SAM to generate a 5'-deoxyadenosyl radical intermediate (5'-dAdo•). The dAdo radical intermediate abstracts the pro-R hydrogen on C6 of SP to initiate repair.

Fig. 2.

Deuterium uptake of SPL (250 μ M) with and without [SAM (27 mM) + SPTpT (375 mM)] under anaerobic conditions. The initial uptake of deuterons was around 151.9 ± 1.0 Da for the ligand bound (■) sample and 180.9 ± 2.6 Da for the unbound (●). Uptake had reached a plateau by 30 min. with the maximum uptake being at 215.6 ± 4.7 Da for ligand bound and 235.2 ± 2.3 Da for unbound protein.

Fig. 3. The influence of the effector molecules on SPL as depicted by the comparative difference in deuterium uptake over 30 min. Panel A, B depict the average difference in deuterium uptake with SAM and SAM + 5RSPTpT respectively. In the both these cases the experiments were repeated under aerobic and anaerobic conditions. Panel C depicts the difference in deuterium uptake by SPL in the presence of SAM + 5RSPTpT and SAM + 5RSP, both under anaerobic conditions. Panel D is an overall comparison of the differential deuterium uptake as a function of different conditions and affecter molecules. In all cases the average difference in

mass (M (Da)) is the difference in measured mass between SPL alone and with the effector molecule at 30 min. The data is indicative of significant difference in deuterium uptake between the aerobic and anaerobic trials of the same effector molecule. The most significant difference in uptake is observed when the enzyme is bound to SAM and 5R-SPTpT concomitantly. The standard error was reported as the difference in deuterium uptake between the trials at each injection point.

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Fig. 4.

Panel A represents the crystal structure of SPL with the β hairpin highlighted in gold and the 8 residue Fe-S binding loop in magenta. Panel B is a close up of the active site pocket with dinucleotide SPTpT modeled in based on the position of dinucleoside SP in the solved structure, illustrating the proximity of R273 and K309 to the expected location of the phosphodiester backbone of the 5*R*-SPTpT substrate.

Table 1

Mass changes in SPL as a result of H/D exchange upon diluting in D₂O. H/D exchange was probed in SPL alone as a control, and then experiments were carried out in the presence of SAM, synthetic dinucleotide spore photoproduct (5*R*-SPTpT), synthetic dinucleoside spore photoproduct (5*R*-SP) a 6-mer oligo, or combinations of these as indicated. The difference in mass (M (Da)) is calculated by subtracting the mass of D labeled SPL with the effector molecule at 30 min from the mass of D labeled SPL only at 30 min. The first column of data shows the mass change of the SPL in each sample as measured 30 min after dilution in D₂O. The second column of data shows the average difference in mass change at 30 min between SPL alone and SPL bound to SAM and/or SP and/or oligo.

Sample	Difference in M (Da) ($M_{\text{SPL}} - M_{\text{sample}}$)
SPL only (control)	N/A
SPL + 5 <i>R</i> -SPTpT	1.1 ± 0.5
SPL + 6mer oligo	2.6 ± 1.3
SPL + SAM (Aerobic)	6.3 ± 0.6
SPL + SAM (Anaerobic)	13.0 ± 0.4
SPL + SAM + 6mer oligo (Aerobic)	6.9 ± 3.4
SPL + SAM + 6mer oligo (Anaerobic)	10.4 ± 0.8
SPL + SAM + 5 <i>R</i> -SPTpT (Aerobic)	12.0 ± 1.2
SPL + SAM + 5 <i>R</i> -SP (Anaerobic)	17.0 ± 1.5
SPL + SAM + 5 <i>R</i> -SPTpT (Anaerobic)	20.4 ± 1.4