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Escherichia coli single-stranded DNA binding protein SSB promotes AlkB-mediated DNA dealkylation repair

Richa Nigam, Roy Anindya*

Department of Biotechnology, Indian Institute of Technology Hyderabad, Kandi, Sangareddy, Hyderabad 502285, Telangana, India

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ABSTRACT

Repair of alkylation damage in DNA is essential for maintaining genome integrity. *Escherichia coli* (*E.coli*) protein AlkB removes various alkyl DNA adducts including *N*1-methyladenine (N^1meA) and *N*3-methylcytosine (N^3meC) by oxidative demethylation. Previous studies showed that AlkB preferentially removes N^1meA and N^3meC from single-stranded DNA (ssDNA). It can also remove N^1meA and N^3meC from double-stranded DNA by base-flipping. Notably, ssDNA produced during DNA replication and recombination, remains bound to *E. coli* single-stranded DNA binding protein SSB and it is not known whether AlkB can repair methyl adduct present in SSB-coated DNA. Here we have studied AlkB-mediated DNA repair using SSB-bound DNA as substrate. *In vitro* repair reaction revealed that AlkB could efficiently remove N^3meC adducts inasmuch as DNA length is shorter than 20 nucleotides. However, when longer N^3meC-containing oligonuleotides were used as the substrate, efficiency of AlkB catalyzed reaction was abated compared to SSB-bound DNA substrate of identical length. Truncated SSB containing only the DNA binding domain could also support the stimulation of AlkB activity, suggesting the importance of SSB-DNA interaction for AlkB function. Using 70-mer oligonucleotide containing single N^3meC we demonstrate that SSB-AlkB interaction promotes faster repair of the methyl DNA adducts.

1. Introduction

DNA can be damaged by many endogenous and exogenous agents [1]. Exposure to alkylating agents results in alkylated DNA adducts. The methylation adducts that are specifically generated by S_N2 methylating agents are N^1-methyladenine (N^1meA) and N^3-methlyctosine (N^3meC). These adducts are cytotoxic and prevent Watson-Crick base-pairing when present in the single-stranded DNA (ssDNA). The enzyme involved in the repair of N^1meA and N^3meC in *E. coli* is known as AlkB (Alkylation protein B). AlkB is a non-heme iron (Fe^{II}) and 2-oxoglutarate (2OG)-dependent dioxygenases [2]. It catalyzes hydroxylation of methyl group present on the ring nitrogen atoms and subsequently the oxidized methyl group is released as formaldehyde, thus restoring the undamaged nucleotides in the DNA [3]. AlkB can repair mehyl adducts found in double-stranded DNA by base-flipping. It also repairs methyl adducts from ssDNA efficiently [4]. However, bacterial ssDNA regions are usually stabilized by protein binding, such as, single-stranded DNA-binding protein SSB. *E. coli* SSB is a tetrameric protein and each monomer has two domains: the characteristic OB-domain (Oligonucleotide/oligosaccharide Binding) [5] that binds ssDNA and structurally disordered C-terminal domain (CTD) [6]. CTD of SSB is known to bind several proteins involved in DNA metabolism [7]. For the *E. coli* SSB, two major DNA-binding states were reported [8], $(SSB)_{65}$, where the tetramer binds to 65 nucleotides at high salt concentration (NaCl concentration >200 mM), and $(SSB)_{35}$, where the SSB binds to 35 nucleotides at lower salt concentration (NaCl concentration <20 mM). Single molecule experiments demonstrated that SSB binding to ssDNA is not static, rather, it diffuses randomly back and forth along ssDNA [9] and the DNA ends undergo spontaneous unwrapping. SSB plays direct role in the DNA replication and recombination [10]. During recombination, SSB helps in heteroduplex formation by binding to the displaced strand and preventing strand annealing [11]. SSB is also involved in DNA repair pathways including base excision repair and recombination repair [12–14]. However, no report exists which directly or

* Corresponding author.
E-mail address: anindya@iith.ac.in (R. Anindya).

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indirectly indicates that AlkB can repair alkyl-adducts present in SSB-bound ssDNA.

2. Materials and methods

Purification of recombinant proteins - Plasmids were transformed into the *E. coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene), and protein expression was induced by the addition of 1 mM IPTG following published procedure [15]. AlkB and SSB genes were cloned in SalI-EcoRI and NcoI-XhoI sites of pTYB3, respectively. pTYB3-SSB-1-115 construct was generated by PCR mediated internal deletion method. For tag-less protein production, AlkB, SSB and SSB-1-115 were expressed as chitin-fusion protein and purification was carried out using chitin agarose, as suggested by the manufacturer (NEB). Proteins were further purified by Superose-12 (GE Healthcare) gel filtration column and analyzed using an AKTA Prime FPLC system (GE Healthcare). The purity of the proteins was analyzed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining and concentrations were determined by Bradford assays (Bio-Rad).

3methyl Cytosine (N^3meC) containing oligonucleotide substrate - 70-mer oligo nucleotide substrate consisting of 69 thymine and single N^3meC at 35th position, 15th position or at 1st position was chemically synthesized (Active™-Imperial Life sciences). Shorter oligonucleotides (10-mer) containing N^3meC at 5th position were also synthesized. M13mp18 ssDNA was purchased from NEB and MMS treated to generate methyl adducts and purified following our previously published protocol [15].

Demethylation assay by direct detection of formaldehyde - AlkB-mediated demethylation was analyzed by repair of different lengths of N^3-meC present in ssDNA. Demethylation repair reactions (50 μl) were carried out as described before [16]. Briefly, AlkB (0.2 μM) was incubated with N^3-meC containing substrate such as M13 ssDNA (1 μg) or N^3-meC containing oligonucleotides (2 μM) at 37 °C for 1 h in reaction buffer containing 20 mM Tris-HCl pH 8.0, 200 μM 2OG, 2 mM L-Ascorbate, 20 μM $Fe(NH_4)_2(SO_4)_2$. The released formaldehyde was directly quantified by reaction with acetoacetanilide and ammonia which form an enamine-type fluorescent intermediate (A_{max} 365 nm, E_{max} 465 nm) [16]. Reaction without DNA was used for blank value correction. Formaldehyde standard curve was prepared by selecting a range of pure formaldehyde concentrations. To detect formaldehyde, a 50 μl sample containing demethylation repair reaction product was mixed with 40 μl of 5 M ammonium acetate and 10 μl of 0.5 M acetoacetanilide (final volume 100 μl). After 15min, fluorescence was measured using a 96-well microplate and Synergy (Biotek Instrument) multimode reader.

Demethylation assay by FDH-coupled indirect detection of formaldehyde- Formaldehyde production was continuously monitored by a Formaldehyde dehydrogenase(FDH)-coupled DNA repair assay as described before [16,17]. Briefly, AlkB (0.2 μM), N^3-meC containing oligonucleotide (2 μM) and SSB (4 μM) were incubated in the presence of reaction buffer (20 mM HEPES, pH 8.0, 200 μM 2OG, 2 mM L-Ascorbate, 20 μM $Fe(NH_4)_2(SO_4)_2$) containing 0.01 U FDH and 1 mM NAD^+ and monitored continuously for the production of NADH (peak absorption at 340 nm) using a 96-well plate reader. Typically, three reactions were monitored concurrently for 60 min, each set containing one control (no DNA).

Enzyme Kinetic analysis - AlkB (0.2 μM) was mixed with the eight different concentration of SSB-bound or free 70-mer internal N^3meC containing oligonucleotide (ranging from 0.2 to 6.3 μM). The amount of formaldehyde released at each time point was quantified as described before [16]. The initial velocity was calculated by the analysis of the slope of these time course reactions. The velocity data were then fit to the Michaelis-Menten equation and Lineweaver-Burk plot to calculate Vmax (maximal velocity), K_M (Michaelis constant) using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. Results and discussion

So far all the *in vitro* studies of AlkB activity have been carried out either in the presence of very short oligonucleotide or completely protein-free ssDNA [4,18–25] but never in the presence of SSB-bound single-stranded DNA. It is estimated that *E. coli* had 1000-2000 tetrameric SSB molecules per cell which is more than sufficient for stoichiometric binding of SSB molecules to all ssDNA formed predominantly during replication and repair [10,26]. We hypothesized that AlkB has to repair the SSB-coated ssDNA *in vivo*. Presented here are results that demonstrate that SSB indeed stimulate AlkB activity.

SSB facilitates AlkB-mediated repair of longer ssDNA - Although repair of methyl adducts on ssDNA substrate by AlkB was reported previously, none of the earlier studies had examined SSB-bound ssDNA as the substrate of AlkB. We were curious to know whether SSB, being an ssDNA binding protein, hinders or promotes AlkB function. To investigate this question, we overexpressed and purified AlkB, SSB and truncated SSB containing only the OB domain (SSB-1-115) without any affinity tag (Fig. 1A). Size exclusion chromatography analysis revealed that both SSB and SSB-1-115 forms tetramer (Fig. 1B) in agreement with the previous report [27]. It was demonstrated before that unstructured C-terminal domain of SSB are located at the periphery of the tetramer and are not involved in the interaction with DNA [5]. We also found that both SSB and SSB-1-115 could bind ssDNA (data not shown). The SSB tetramer binds ssDNA in a variety of binding modes depending on solution conditions, especially salt concentration. At low monovalent salt concentrations (<20 mM NaCl), an SSB tetramer binds to approximately 35 nucleotides of ssDNA, known as $(SSB)_{35}$ mode. However, at higher salt concentrations (>0.2 M NaCl), an SSB tetramer binds to approximately 65 nucleotides of ssDNA, known as $(SSB)_{65}$ mode [28]. Because increasing ionic strength might affect possible AlkB-SSB interaction, we decided to study role of SSB in AlkB-mediated repair at low ionic strength (10 mM NaCl) throughout our study. As the substrate for *in vitro* repair we have used variety of damaged DNA including MMS-damaged M13 ssDNA containing multiple methyl-adducts or chemically-synthesized DNA substrate of 10-mer oligo-dT containing single N^3meC at position 5 and 70-mer oligo-dT consisting of a single N^3meC located either at the 35th position or 15th position. In AlkB-catalyzed oxidative demethylation, oxidized methyl groups are released as formaldehyde which was measured by adding acetoacetanilide and ammonia directly to the repair reaction to generate a fluorescent compound with peak emission of 465 nm, as described previously [16]. Concentration of released formaldehyde was determined from the formaldehyde standard curve [29] (Fig. 1C). In first set of experiment, we have used MMS-damaged M13 ssDNA (1 μg or 0.2 pmol) containing numerous randomly located methyl adducts as the AlkB substrate. As expected, AlkB (0.2 μM) was able to remove the methyl adducts and formaldehyde release was detected (Fig. 1D). Next we incubated purified SSB (4 μM) and methylated M13 DNA (1 μg or 0.2 pmol) to form ssDNA-SSB complex and used this as substrate for AlkB reaction. As shown in Fig. 1D, significantly higher formaldehyde release was observed, suggesting that the repair activity was diminished when free M13 ssDNA was used as substrate. To rule out any stimulation due to molecular crowding effect, equivalent amount of BSA was added instead of SSB in the reaction mixture. As expected, BSA did not alter the amount of formaldehyde release (Fig. 1D). We hypothesized that if the stimulation of AlkB activity is dependent on SSB-DNA interaction, then

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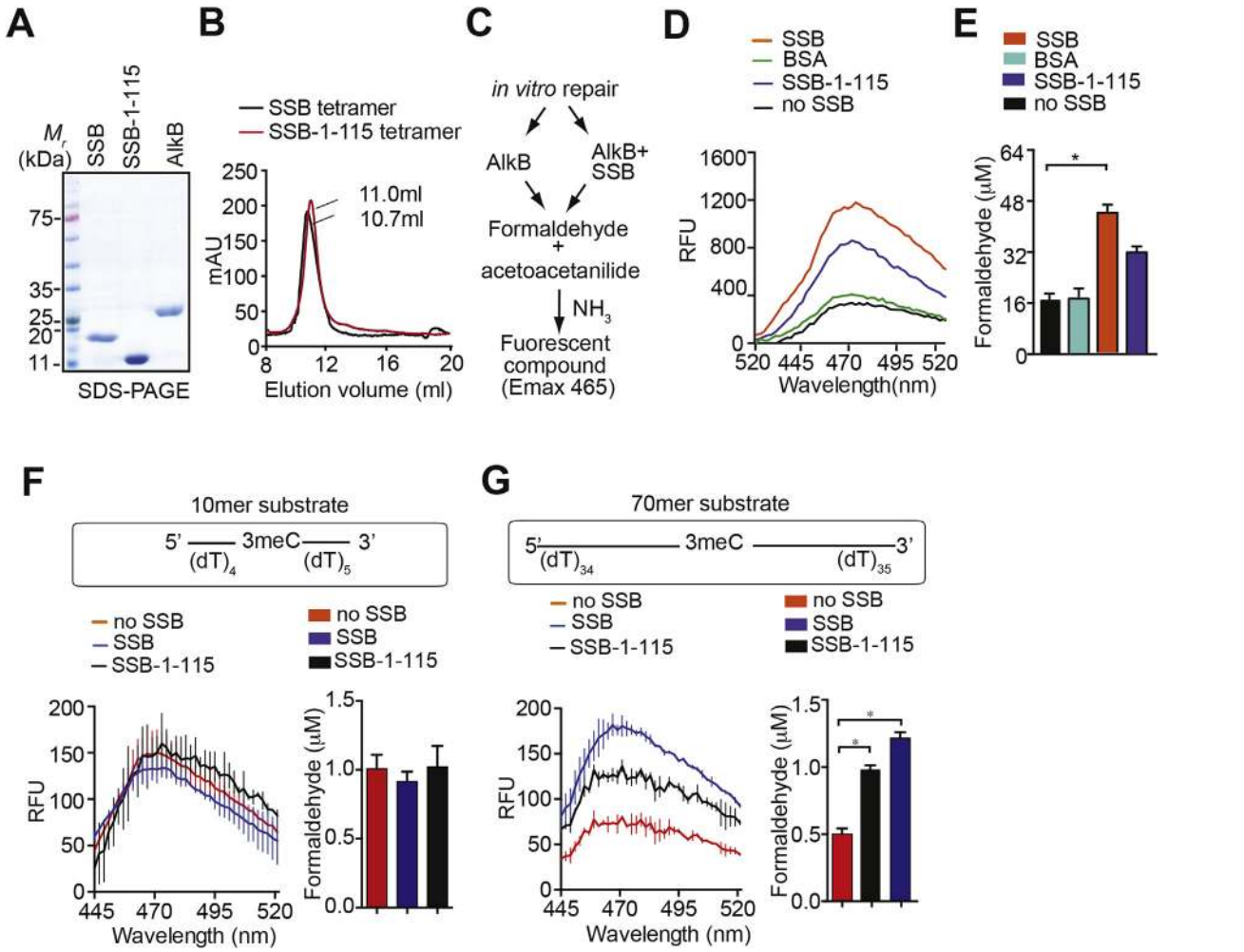

Fig. 1. AlkB removes 3meC lesions from SSB-bound single-stranded DNA. (A) SDS-PAGE analysis of purified tag-less *E. coli* AlkB and SSB. (B) Size exclusion chromatography (SEC) analysis of purified SSB and SSBΔC (100 μg). SEC was performed using Superose-12. Comparison of elution volume of SSB and SSB-1-115 with molecular weight standard indicates both the protein to be tetramer. SSB tetramer eluted (10.7 ml) ahead of SSB-1-115 tetramer (11 ml). (C) Detection of formaldehyde by Acetoacetanilide method. Chemical reaction of formaldehyde with acetoacetanilide in the presence of ammonia leads to fluorescent compound. (D) Comparison of DNA repair by AlkB (0.2 μM) in the presence of SSB (4 μM). BSA (2 μM) was used as control. Methylated M13 ssDNA (1 μg) was used as substrate. Emission spectra depict the product of reaction between acetoacetanilide and formaldehyde, formed due to repair of methyl adducts. Formaldehyde production due to oxidative demethylation of methylated M13 ssDNA. (E) Formaldehyde release was quantified by formaldehyde standard curve and fluorescence emission spectra of formaldehyde released during demethylation (E_{max} 465 nm). (F) Comparison of DNA repair using 10-mer single N^3meC containing oligonucleotide (2 μM) as substrate. (G) Comparison of DNA repair using 70-mer single N^3meC containing oligonucleotide (2 μM) was used as substrate. Error bar depicts the standard error. The *P* value was calculated by two-tailed Student's t-test (* indicates $P < .05$).

the repair will be unaffected if SSB lacking C-terminal disordered region is used. Indeed, when the repair reaction was carried out in the presence of SSB-1-115, formaldehyde released was observed (Fig. 1E), suggesting that binding of ssDNA to SSB itself might promote the AlkB activity. Binding of large M13 ssDNA to full-length or truncated SSB could prevent secondary structure formation and promote AlkB-mediated repair.

Previous *in vitro* DNA binding experiments determined that only oligonucleotides longer than 20 nucleotide could efficiently bind to *E. coli* SSB [30]. We hypothesized that shorter (<20 bases) methylated ssDNA would not be able to bind SSB and, therefore, DNA will be completely accessible to AlkB. Hence, if free SSB protein is able to stimulate AlkB activity in allosteric manner, we might see an enhanced repair. Therefore, we used shorter oligonuleotides (10-mer) containing single N^3meC (2 μM) as substrate of AlkB in the presence or absence of SSB (4 μM). As shown in Fig. 1F, AlkB was able to oxidatively demethylate N^3meC present in 10-mer oligonucleotide and produce formaldehyde. Notably, the amount of formaldehyde release was very small compared to methylated M13 substrate. This could be due the large size of M13 ssDNA which is 6400 base long and methylated by MMS treatment and ought to have large number methylated DNA adducts. The 10-mer and 70-mer substrate used here have single damage lesion and yield very low level of formaldehyde during repair. However, no stimulation of AlkB activity was observed in the presence of SSB. SSB-1-115 also had no effect on AlkB mediated repair of 10-mer N^3meC containing oligonucleotide (Fig. 1F). Similar results were obtained with 20-mer N^3meC containing oligonucleotide (data not shown). This data suggest that when the length of ssDNA was too short for SSB binding, AlkB activity could not be allosterically activated by SSB.

Next, we used longer oligonucleotide as the substrate that can bind SSB. 70-mer oligonucleotide with single N^3meC at 35th position (2 μM) was used as substrate of AlkB (0.2 μM) in the presence or absence of SSB (4 μM). As shown in Fig. 1G, AlkB was able to oxidatively demethylate N^3meC present in 70-mer oligonucleotide and produce formaldehyde, albeit with lower efficiency compared to 10-mer oligonucleotide. However, when SSB was added stimulation of AlkB activity was observed. Truncated SSB lacking C-

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terminal unstructured region (SSB-1-115) also resulted stimulation on AlkB mediated repair of 10-mer N^3meC containing oligonucleotide (Fig. 1G). This result is in agreement with our previous experimental results obtained with M13 ssDNA (Fig. 1D) and indicate that both DNA wrapping by SSB and AlkB-SSB interaction promote AlkB activity.

To further confirm that AlkB is probably inefficient in repairing large ssDNA and the role of SSB could perhaps be to promote AlkB activity to the normal level we used an alternate indirect assay method [16]. Formaldehyde dehydrogenase (FDH) oxidizes formaldehyde to formate using NAD^+ as the electron acceptor whose reduction to NADH can be measured by absorbance at 340 nm and previously used to monitor formaldehyde release including JmjC histone demethylase [31] and AlkB [17]. The reactions were initiated by adding oligonucleotide containing single N^3meC and absorbance at 340 nm at different time points revealed production of formaldehyde. As shown in Fig. 2A, comparable increase of absorbance at 340 nm was observed when N^3meC containing shorter oligonucleotide (10-mer) were used, indicating that the presence of SSB had no influence on the amount of formaldehyde produced in the AlkB-catalyzed demethylation reaction. The fact that similar amount of formaldehyde was generated in the demethylation of 10-mer DNA strongly suggests that the reaction had occurred as proposed in Fig. 2A. Next, we studied the repair reaction with 70-mer oligonucleotide (2 μM) with single N^3meC at 35th position. As before, *E. coli* AlkB (0.2 μM) was added in the presence and absence of SSB (4 μM) and formaldehyde release was detected. We observed stimulation of AlkB activity in the presence of full length SSB (Fig. 2B). Stimulation was also observed with truncated SSB lacking C-terminal unstructured region (SSB-1-115), suggesting that SSB-binding and protein-protein interaction both are responsible for stimulation of AlkB activity. These findings from the indirect FDH-coupled repair assay are in accord with our observations from the direct repair assay (Fig. 1).

To further explore the mechanism of stimulation of AlkB-mediated DNA repair by SSB, we studied the kinetics of AlkB catalyzed reaction using 70-mer oligo-dT with single N^3meC containing oligonucleotides. For all of our experiments, we have studied complexes of SSB under low-salt conditions without Mg^{2+} cation which retains AlkB interaction. Under these conditions, the binding mode of *E. coli* SSB is such that two monomers of the SSB tetramer are in contact with ssDNA and two SSB tetramers together can bind a 70-mer ssDNA [5,28]. *E. coli* SSB is also known to translocate spontaneously and rapidly along the ssDNA via partial unwrapping of one end segment of ssDNA followed by re-wrapping of the other end in its place [9]. We hypothesized that if the terminal nucleotide contains the methyl adducts, it might be mostly accessible to AlkB for repair compared to the damage nucleotide which is located internally. Therefore, while SSB-wrapping and AlkB-SSB interaction might be required to promote the repair of an internally located methyl adduct, it might be redundant for terminally located methyl adduct repair. First, we examined 70-mer oligo-dT with single N^3meC at the 5′-end for repair (Fig. 3A). Evidently, AlkB was able to repair 70-mer terminal N^3meC containing oligonucleotide with a K_M values 2.4 μM. When tetrameric SSB was added to the reaction mixture, the K_M remained almost unchanged (2.7 μM), suggesting SSB had little effect on AlkB activity (Fig. 3B and C). Similar K_M was also calculated by us using a 40-mer N3-me oligo-dC substrate [29], and others with 19-mer oligo containing a single N3meC [17]. This data indicate that N^3meC positioned at the 5′-termini of SSB-bound DNA is as accessible to AlkB as it is in the short oligonucleotide. Next, we examined 70-mer oligo-dT with single N^3meC located internally, either at the 15th position or at the 35th position for repair (Fig. 3D). AlkB was able to repair 70-mer oligonucleotide with internal N^3meC (35th), but with a high K_M (6.7 μM) (Fig. 3E and F). Interestingly, when SSB was added to the reaction mixture, the K_M decreased significantly (2 μM), suggesting strong stimulation of AlkB activity by SSB. Repair 70-mer internal N^3meC (15th) was also observed but as expected with a high K_M (8.2 μM) (Fig. 3G and H). As before, when SSB was added to the reaction mixture, the K_M reduced (1.8 μM) (Fig. 3I). Notably, the K_M of 15th position N^3meC oligonucleotide was higher than that of 35th position oligonucleotide. We assume that 35th nucleotide is located at the interface between the two SSB tetramers and could be exposed; whereas, the methylation site at the 15th position could be wrapped by SSB. The kinetic experiments described here imply that AlkB-mediated repair of internally located damage of 70-mer DNA is indeed augmented by SSB as a result of increased affinity for substrate binding. These results unveil an important role of SSB in AlkB mediated repair of large ssDNA. It appears that the presence of SSB lowers the K_M of AlkB-catalyzed reaction and crucial for efficient repair of longer substrate but may be dispensable for the small oligonucleotide substrate. When the length of the single-stranded DNA increases, the

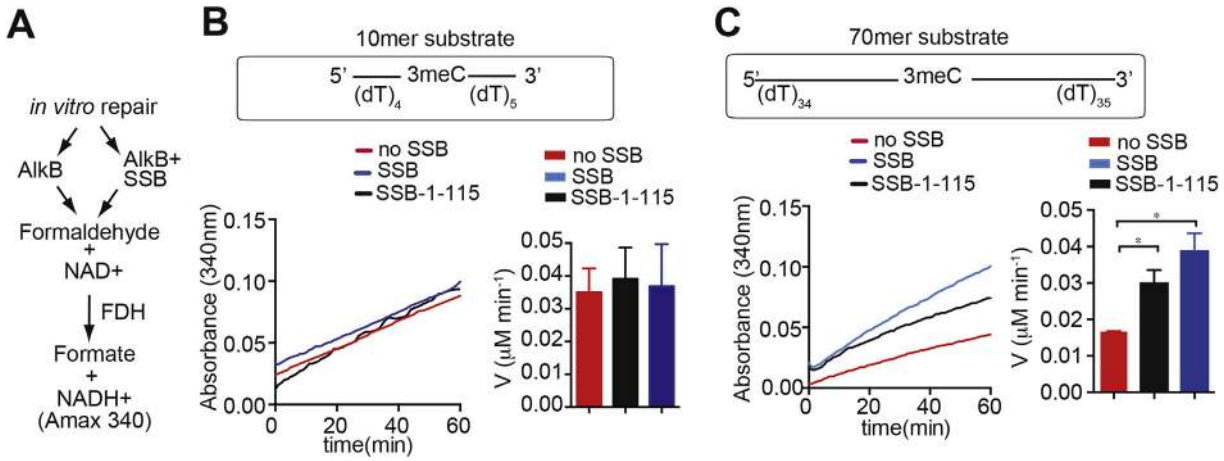

Fig. 2. Formaldehyde dehydrogenase (FDH)-coupled assay to detect formaldehyde produced during AlkB-mediated repair. (A) AlkB-mediated repair reaction for formaldehyde production and its detection by formaldehyde dehydrogenase (FDH)-coupled assay. Demethylation reaction using 10-mer single N^3meC containing oligonucleotide (2 μM) and AlKB (0.2 μM) in the presence or absence of tetrameric SSB (4 μM) 4 μM, 1 mM NAD^+ and 0.01U FDH. (B) 10-mer single N^3meC containing oligonucleotide (2 μM) as substrate. (C) 70-mer single N^3meC containing oligonucleotide (2 μM) was used as substrate. Formaldehyde concentration were determined using a standard plot. Error bar depicts the standard error. The P value was calculated by two-tailed Student's t-test (* indicates $P < .05$).

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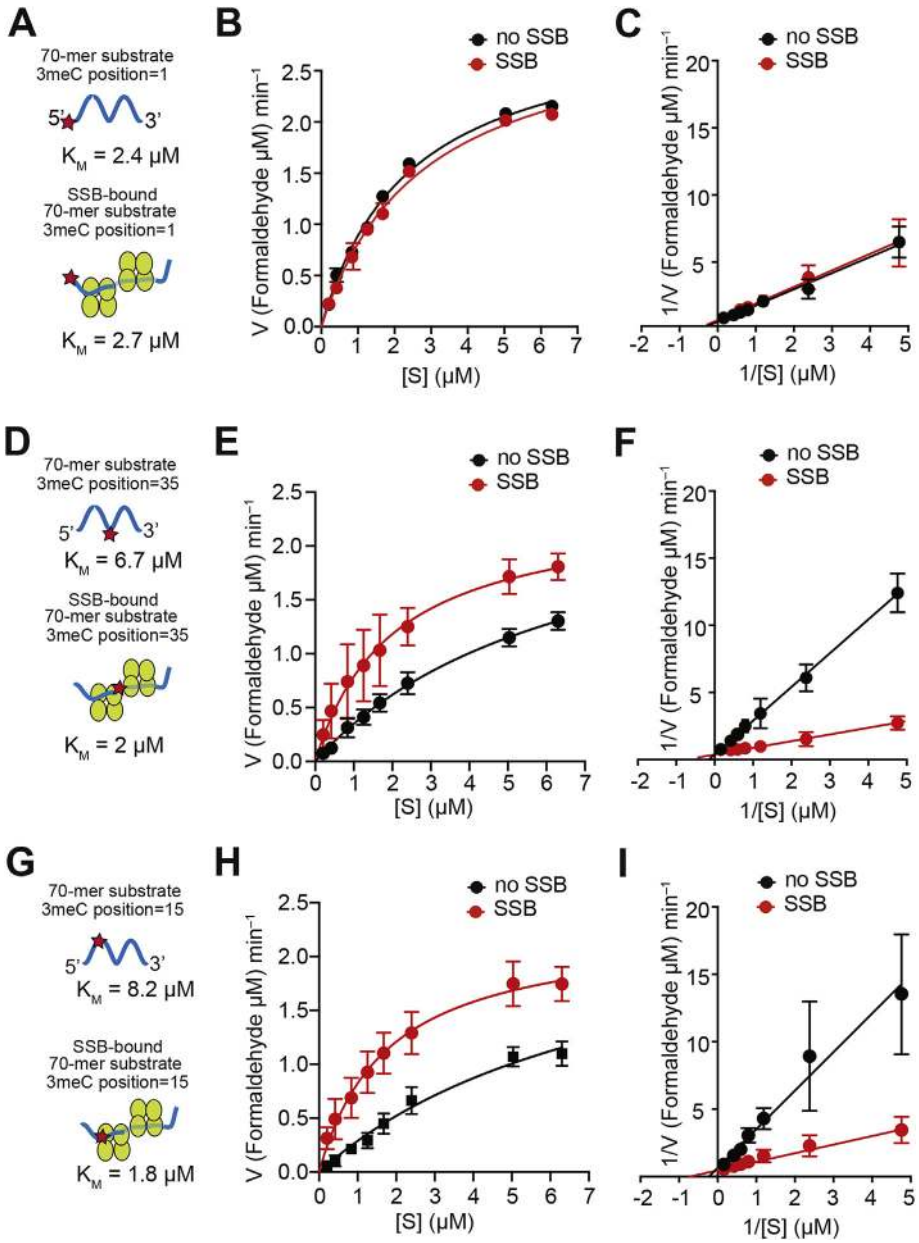

Fig. 3. Kinetics of repair of SSB-bound 3meC containing 70-mer oligonuleotide substrate. (A) 70-mer oligonuleotide containing single N^3meC at the 5′-end as substrate for AlkB reaction. (B) Michaelis-Menten analysis of steady-state kinetics for AlkB-mediated repair of free and SSB-bound 70-mer terminal N^3meC containing oligonucleotide. AlkB (0.2 μM) was mixed with various concentration of free oligonucleotide and SSB-bound oligonucleotide as substrate. (C) Lineweaver-Burk transformation of the steady-state kinetics data. Graphs represent averages of six experiments. Error bar depicts the standard error. (D) 70-mer oligonuleotide containing single N^3meC at the 35th position. (E) Michaelis-Menten analysis of steady-state kinetics for AlkB-mediated repair of free and SSB-bound 70-mer internal N^3meC oligo-dT (35th position). (F) Lineweaver-Burk transformation of the steady-state kinetics data. (G) 70-mer oligonuleotide containing single N^3meC at the 15th position. (H) Michaelis-Menten analysis of steady-state kinetics for AlkB-mediated repair of free and SSB-bound 70-mer internal N^3meC oligo-dT (15th position). (I) Lineweaver-Burk transformation of the steady-state kinetics data. Reactions were quantified by detecting formaldehyde released using formaldehyde standard plot. Curves represent nonlinear regressions to the Michaelis-Menten equation obtained using GraphPad Prism 7.0 software. Error bar depicts the standard error.

catalytic efficiency of AlkB decreases. Since longer stretch of ssDNA is always bound by SSB, AlkB readily repairs such SSB-bound DNA. SSB-AlkB interaction may further promote AlkB-mediated repair. The binding affinity of SSB to ssDNA decreases drastically with decreasing length of the oligonucleotide. However, removal of methyl adducts by AlkB from very short patch of single-stranded region is rather efficient and therefore would be SSB-independent. It is possible to speculate that AlkB-mediated repair of methyl adduct will probably be executed without affecting any cellular process, such as replication and recombination, where the long stretches of ssDNA remains predominantly SSB-bound. Thus, based on the data presented here, it appears that wrapping of ssDNA by SSB could alter the ssDNA structure and melt the secondary structure that is more suitable to AlkB-mediated repair. Together, these might result in reduction of the K_M and alleviate repair.

In conclusion, we found that AlkB inefficiently repairs longer single stranded DNA; however, it readily repairs SSB-bound methylated ssDNA of equal length. Considering the critical role of SSB as a platform for recruitment of DNA repair and recombination

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proteins, our work represents first demonstration of the role of SSB in promoting oxidative demethylation repair. However, at present, it is not clear whether AlkB protein constitutes an integral part of the SSB or remains bound to other regions of the DNA. Despite the efficient repair of SSB-coated ssDNA, the role of SSB protein in *E. coli* demethylation repair remains unknown. More work is needed to understand the importance of SSB with respect to AlkB function under various intracellular stress responses, such as SOS response.

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