Differential effects of single-stranded DNA binding proteins (SSBs) on uracil DNA glycosylases (UDGs) from *Escherichia coli* and mycobacteria

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ABSTRACT

Deamination of cytosines results in accumulation of uracil residues in DNA, which unless repaired lead to GC→AT transition mutations. Uracil DNA glycosylase excises uracil residues from DNA and initiates the base excision repair pathway to safeguard the genomic integrity. In this study, we have investigated the effect of single-stranded DNA binding proteins (SSBs) from *Escherichia coli* (EcoSSB) and *Mycobacterium tuberculosis* (MtuSSB) on uracil excision from synthetic substrates by uracil DNA glycosylases (UDGs) from *E.coli*, *Mycobacterium smegmatis* and *M.tuberculosis* (referred to as Eco-, Msm- and MtuUDGs respectively). Presence of SSBs with all the three UDGs resulted in decreased efficiency of uracil excision from a single-stranded ‘unstructured’ oligonucleotide, SS-U9. On the other hand, addition of EcoSSB to EcoUDG, or MtuSSB to MtuUDG reactions resulted in increased efficiency of uracil excision from a hairpin oligonucleotide containing dU at the second position in a tetraloop (Loop-U2). Interestingly, the efficiency of uracil excision by MsmUDG from the same substrate was decreased in the presence of either Eco- or MtuSSBs. Furthermore, MtuSSB also decreased uracil excision from Loop-U2 by EcoUDG. Our studies using surface plasmon resonance technique demonstrated interactions between the homologous combinations of SSBs and UDGs. Heterologous combinations either did not show detectable interaction (EcoSSB with MtuUDG) or showed a relatively weaker interaction (MtuSSB with EcoUDG). Taken together, our studies suggest differential interactions between the two groups (SSBs and UDGs) of the highly conserved proteins. Such studies may provide important clues to design selective inhibitors against this important class of DNA repair enzymes.

INTRODUCTION

Uracil can be found in the genome as a result of its incorporation by DNA polymerases or by deamination of cytosine residues. Unless repaired, the product of cytosine deamination would lead to GC→AT transition mutations. Uracil DNA glycosylase (UDG) excises uracil residues and initiates the base excision repair pathway to keep the mutation rate to a minimum. Recent studies on crystal structures of UDGs from various sources (1–4) and the enzyme kinetics studies using synthetic substrates (5–8) have highlighted the structural and mechanistic aspects of substrate recognition and interaction of this class of the enzymes.

UDGs excise uracil from various structural contexts in DNA with varying efficiencies. UDG from *Escherichia coli* (EcoUDG) utilizes double-stranded DNA 3-fold less efficiently than single-stranded substrates (7,9). However, uracil is excised extremely poorly from the second position in the tetraloop of a hairpin oligomer, Loop-U2 (7). Highly inefficient excision of uracil from Loop-U2 (~0.3% compared to the ‘unstructured’ substrates) suggested that destabilization of these loop structures may be required for efficient repair. Single-stranded DNA binding protein (SSB) was thought to be involved in melting such structures. As expected, addition of SSB from *E.coli* (EcoSSB) resulted in increased efficiency of uracil excision (~30% compared to the ‘unstructured’ substrates) from Loop-U2 by EcoUDG (8).

Mycobacteria, a group of bacteria with G+C rich genomes, are responsible for serious human health problems such as tuberculosis and leprosy. Because of the high G+C contents and the stressful habitat of the host macrophages, cytosine deamination may constitute a major form of DNA damage in these organisms, making UDG a crucial DNA repair enzyme. Our earlier studies with UDG from *Mycobacterium smegmatis* (MsmUDG) demonstrated that, unlike EcoUDG, MsmUDG excises uracil from Loop-U2 with an efficiency of ~20% when compared with single-stranded ‘unstructured’ substrates (10). It was therefore of interest to us to determine the effect of SSB on uracil excision by MsmUDG. In this study, we have determined the effect of EcoSSB and SSB from *Mycobacterium tuberculosis* (MtuSSB) on uracil excision by EcoUDG and UDGs from *M.smegmatis* and *M.tuberculosis* (Msm- and MtuUDG respectively). The differential effects of SSBs on UDGs that we have observed in this study have allowed us to discuss the aspects of SSB–UDG interaction.

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MATERIALS AND METHODS

UDG reactions

Uracil containing synthetic DNA, 5'-ctcaagtgUaggcatgcaagagct-3' (SS-U9) and 5'-ctagaggcatUttggatcct-3' (Loop-U2) were used. The 5'-32P-labeled oligonucleotides (1 pmol) were treated with UDG in 15 µl reactions, containing 1× UDG buffer (50 mM Tris–HCl, pH 8.0, 1 mM Na2EDTA, 1 mM DTT and 25 µg/ml BSA) incubated at 37°C for 10 min, mixed with an equal volume of 0.1 M NaOH, heated at 90°C for 10 min, dried in a speed vac, taken up in 10 µl formamide dye and analyzed on 18% polyacrylamide/8 M urea gels (10).

Range finding reactions

UDG reactions were performed as above with various dilutions of enzyme in the presence or absence of 5 pmol of SSB tetramer. To follow the kinetics of SSB effect, UDG reactions were carried out wherein 1 pmol of 5'-end-labeled oligomer was preincubated with or without various concentrations of SSB (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5 or 10 pmol). UDG reactions were carried out using an appropriate dilution of the enzyme.

Melting temperature (Tm) determination

Melting temperatures (Tm) were measured using Beckman DU600 spectrophotometer in a buffer consisting of 50 mM Tris–HCl, pH 7.4, 5 mM Na2EDTA and 100 mM NaCl. Absorbance changes were measured at 260 nm for 0.68 µM Loop-U2 oligomer, with or without 1 µM Eco- or MtuSSB.

Purification of SSBs and UDGs

E.coil overexpression plasmid (pTL119) was transformed into E.coil BW310 (ung+) and the protein purified as described previously (8). MtuSSB was cloned in a T7 RNA-polymerase-based expression system (pETMtuSSB) and overexpressed in E.coil BW310 (ung+), harboring T7 RNA polymerase gene on a ColE1 compatible plasmid pACT7. The MtuSSB was purified as described previously (11). Native form of MsmUDG was purified from M.smegmatis SN2 (10). Eco- and MtuUDGs were purified from E.coil BW310 (ung+) using pTrc99C/pET11d-based overexpression constructs (8; unpublished data).

Surface plasmon resonance (SPR) studies

Equilibrium and the kinetic constants that govern the SSB–UDG interaction were determined by SPR (12) using BIACore 2000 (LKB-Pharmacia Biotech). An aliquot (40 µl, 15 pmol) of a 24mer (5'-biotin-GATCAGATTATGCCCCAATAACCAC-3') was immobilized on a streptavidin (SA5) sensor chip in HBS200 (10 mM HEPES, pH 7.4, 200 mM NaCl, 3.4 mM Na2EDTA and 0.005% Tween-20) to the extent of ~1000 response units (RU). Following a 300 s wash, SSB was injected to obtain an increase of ~450–1600 specific RU. The binary complex of DNA–SSB was washed with HBS30 (10 mM HEPES, pH 7.4, 50 mM NaCl, 3.4 mM Na2EDTA and 0.005% Tween-20) for 300 s. Under the conditions used, SSB did not dissociate from the oligo. Therefore, it was suitable to study the interaction of UDGs (as a DNA–SSB–UDG ternary complex). Aliquots of UDGs (400–6000 nM in HBS30) were injected at a flow rate of 5 µl/min over the immobilized single-stranded DNA at a constant temperature of 25°C. Whenever required, the DNA surface was regenerated by a short pulse (10 µl) of 0.1% SDS. This procedure did not alter the ability of the immobilized DNA to interact with SSBs. The association rates (kobs), the dissociation rates (kobs) and the equilibrium constants (Ks) were calculated according to the manufacturer’s instructions using the BIACore evaluation software.

RESULTS

Effect of Eco- and MtuSSB on uracil excision from ‘unstructured’ substrates by different UDGs

Figure 1 shows uracil excision from SS-U9, an ‘unstructured’ substrate with the sequence 5'-ctcaagtgUaggcatgcaagagct-3'. SSB has been shown to form a stable complex with this oligomer (8). Preincubation of SS-U9 with EcoSSB decreased the uracil excision by all three UDGs (Eco-, Msm- and Mtu-UDG, Fig. 1A–C respectively). This decrease is most likely a consequence of binding of SS-U9 to SSB. A similar decrease was also observed in the presence of MtuSSB. However, the extent of decrease with MtuSSB was more when compared to that observed in the presence of EcoSSB (Fig. 1A–C, compare lanes 2–4 with lanes 5–7 and 8–10).

Effect of Eco- and MtuSSB on uracil excision from Loop-U2 by different UDGs

In order to determine the effect of SSB on the structured substrates, we used a hairpin oligonucleotide, Loop-U2 (5'-ctagaggcatUttggatcct-3') containing uracil in the second position of the tetraloop. As reported earlier, preincubation of Loop-U2 with EcoSSB resulted in enhanced excision of uracil by EcoUDG (8) (Fig. 2A, compare lanes 2–4 with lanes 5–7). Although the EcoSSB does not form a stable complex with Loop-U2, based on the susceptibility of the loop nucleotides to KMnO4, it was suggested that the EcoSSB-mediated increase in the rate of uracil excision was primarily due to opening of the loop structure (8). However, under similar conditions, preincubation of Loop-U2 with MtuSSB resulted in a slight decrease in uracil excision by EcoUDG (Fig. 2A, compare lanes 2–4 with lanes 8–10). Furthermore, the MsmUDG-mediated uracil excision from Loop-U2 was inhibited by both the Eco- and MtuSSB (Fig. 2B, compare lanes 2–4 with lanes 5–7 or 8–10). On the other hand, preincubation of Loop-U2 with Eco- or MtuSSB showed enhanced uracil excision by MtuUDG (Fig. 2C, compare lanes 2–4 with lanes 5–7 and 8–10). Thus, both Eco- and MtuSSB exhibit differential effects on uracil excision by UDGs from the structured substrates.

Effect of Eco- and MtuSSB on the kinetics of uracil excision from Loop-U2

To gain an insight into the mechanism of differential effects of Eco- and MtuSSB on the three different UDGs, the effect of increasing concentration of SSBs on uracil excision from Loop-U2 was analyzed. As shown in Figure 3, with the increasing concentration of EcoSSB, uracil excision from Loop-U2 was enhanced remarkably by EcoUDG. Similarly, uracil excision by MtuUDG was also increased. However, under the same conditions the rate of uracil excision by MsmUDG was decreased.

Figure 4 shows the kinetics of the effect of MtuSSB on UDGs. Uracil excision from Loop-U2 by MtuUDG was enhanced. However, under the same conditions, the rate of uracil excision by both the Eco- and MsmUDGs was enhanced.
decreased. On the other hand, at the lower substoichiometric ratios, $Mtu$ SSB resulted in enhanced uracil excision by both $Eco$- and $Msm$ UDGs (Fig. 5).

$T_m$ determination of Loop-U2 in the presence or absence of $Eco$- and $Mtu$ SSBs

In order to determine whether $Mtu$- and $Eco$ SSB have similar potential to melt hairpin structures, we determined the $T_m$ of Loop-U2 in the absence or presence of $Eco$- or $Mtu$ SSB. In the absence of SSB, the $T_m$ for Loop-U2 was 59°C. In the presence of either $Eco$- or the $Mtu$ SSB, the $T_m$ values were 30 and 27°C respectively (Fig. 6). Both the SSBs decreased the $T_m$ of oligomer Loop-U2 to a similar extent and thus have a similar potential to melt these structures.

SSB–UDG interaction

To understand the mechanism of the differential effects of SSBs on uracil excision from Loop-U2, we examined the possibility of protein–protein interactions between the UDGs and the SSBs by the SPR technique. The experiments were performed with UDGs and SSBs from $E. coli$ and $M. tuberculosis$, which were purified as recombinant proteins from $E. coli$. Initially, we immobilized SSBs ($Eco$- or $Mtu$-) on a carboxymethyl-dextran (CM5) sensor chip surface and passed UDGs as the analytes. However, these studies failed to show significant responses (data not shown). Subsequently, we devised a novel approach to study the SSB–UDG interaction. A 24mer DNA (5'-biotinylated) was immobilized on the streptavidin (SA5)
sensor chip surface and used to bind various UDGs or SSBs. Under the buffer conditions used (HBS−/SSB), UDGs did not show any interaction with the immobilized DNA. However, the SSBs interacted with the immobilized DNA to form a binary complex (DNA−SSB). More importantly, under the conditions used, this binary complex did not dissociate and provided a surface to study interactions with UDGs. In fact, such ternary interactions may also be physiologically relevant for uracil excision repair during various DNA transactions involving SSB.

The results of these experiments are shown in Table 1. It is clear that the homologous SSBs and UDGs (EcoSSB with EcoUDG and MtuSSB with MtuUDG) interact with one another. On the other hand, the heterologous combinations either did not show a detectable interaction (EcoSSB with MtuUDG) or showed a poor interaction (MtuSSB with EcoUDG). A relatively stronger interaction of EcoSSB with EcoUDG is a result of rapid association rate \( k_{\text{ass}} \) \( \times 10^2 \) M\(^{-1}\)s\(^{-1}\) and slower dissociation rate \( k_{\text{diss}} \) \( 1 \times 10^{-2} \) s\(^{-1}\). In comparison, although the association rate of interaction of MtuSSB with MtuUDG is \( 5\)-fold lower \( k_{\text{ass}} \) \( 1.16 \times 10^3 \) M\(^{-1}\)s\(^{-1}\), it has been compensated for by a proportionate decrease in the dissociation rate \( k_{\text{diss}} \) \( 1.56 \times 10^{-3} \) s\(^{-1}\), and the resulting \( K_d \) values of the two interactions are comparable \( (1.7 \times 10^{-7} \) M\) for EcoSSB with EcoUDG, and \( 1.4 \times 10^{-7} \) M for MtuSSB with MtuUDG). Among the heterologous combinations, only MtuSSB showed an interaction with EcoUDG \( (K_p \) \( 0.85 \times 10^{-5} \) M\) which was more than two orders of magnitude less than that of the homologous proteins. It is not clear if the poor interaction in the case of the heterologous proteins is a consequence of an alternative mode of protein–protein interaction which is different from that of the homologous proteins.

**Table 1.** Kinetic and equilibrium constants of SSB and UDG interactions

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<tr>
<th>Kinetic parameter</th>
<th>SSBs</th>
<th>UDGs</th>
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<tr>
<td>( k_{\text{ass}} ) (M(^{-1})s(^{-1}))</td>
<td>EcoSSB</td>
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<tr>
<td>( k_{\text{diss}} ) (s(^{-1}))</td>
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<td>( K_d ) (M)</td>
<td>EcoSSB</td>
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n. d., not detectable.

SSBs (450–1600 RU) were bound to the biotinylated oligo immobilized onto the SA-5 sensor chip surface, and the UDGs (400–6000 nM) were used as analytes to determine the parameters of their interaction by the BIAcore evaluation software (Materials and Methods).

**DISCUSSION**

SSB interacts with DNA and modulates several key processes such as replication, transcription, repair and recombination (13–17). Although the SSBs bind to DNA with high affinity, the outcome of these interactions can be very different (14). Most of the SSBs such as EcoSSB, T4 gp32, T7 gene 2.5 protein and RPA activate DNA replication. However, many others e.g., the SSBs from filamentous phage M13, fd or Pf3 block
DNA synthesis by preventing viral DNA strands from going into the replicative form (18). SSBs are also involved in interactions with various proteins in vivo. The EcoSSB interacts with DNA polymerases, exonuclease I, RecA, UvrD, MucA and MucB (19–22). It has been suggested that the interactions of SSB with various proteins may be mediated through its C-terminal domain (13,23).

In the present study, we have analyzed the effects of Eco- and MtuSSBs on uracil excision by three different UDGs, Eco-, Msm- and MtuUDG. Of these, the first one serves as a prototype for the UDGs and the latter ones represent UDGs from a fast- and a slow-growing mycobacteria. Our studies show that both the SSBs resulted in decreased efficiency of UDG-mediated uracil excision from SS-U9, an 'unstructured' substrate of uracil as the ninth base. As observed earlier (8), this decrease in uracil excision is likely to be a consequence of binding of the SSB to the oligomer through interaction of the nucleotide bases with SSB such that binding of uracil into the active site pocket of UDG becomes a rate limiting step.

The crystal structure of an engineered human UDG with its factor, why then does EcoSSB show contrasting effects on the efficiency of uracil excision from Loop-U2, in that it stimulates EcoUDG but inhibits MsmUDG?

We propose that the enhanced uracil excision from structured oligomers could be a consequence of at least two events. The transient opening of the loop structure by SSB (i.e., SSB–DNA interaction) is one of them, and the possible interaction of UDGs with the SSBs in a binary (SSB–UDG) or a ternary (DNA–SSB–UDG) complex constitutes the other (Table 1). Contributions from each of these interactions could vary. For instance, a weak or transient SSB–DNA interaction which increases the probability of capturing the target uracil by UDG, is positive and best seen when the SSB amounts are substoichiometric to DNA (Fig. 5). However, in the stable SSB–DNA complexes (such as those with 'unstructured' DNA, or with the structured substrates at high SSB:DNA ratios) binding of uracil into the active site pocket of UDG will be more difficult leading to the decrease in efficiency of uracil excision by UDG. The interactions between SSB and UDG (or DNA–SSB–UDG) may be relevant under the latter condition. Based on the data in Table 1 and the observation that for the homologous combinations (EcoUDG with EcoSSB, and MtuSSB with MtuUDG) SSBs promote uracil excision from Loop-U2, it is tempting to propose that in the homologous systems, the
effects of such protein–protein interactions are stimulatory. Interestingly, we have observed that all our SSB preparations from *E. coli* (ung') cells contained UDG activity in spite of the fact that the purification schemes for both the proteins utilize different chromatographic steps (9,26). In fact, this observation necessitated the use of *E. coli* BW310 (ung') for overexpression and purification of SSBs for this and an earlier (8) study. The SSB–UDG interaction would also be relevant from the physiological considerations, as this could facilitate the recruitment of UDG for uracil excision repair during various DNA transactions involving SSB. Earlier also, using the yeast two-hybrid system, the N-terminal domain (amino acids 28–79) of human UDG was found to interact with the C-terminus of replication protein A (RPA2, a subunit of heterotrimeric human SSB) (27).

In the present study, we have also studied four heterologous combinations of SSBs and UDGs. Among these, except for the combination of *Mtu*UDG with *Eco*SSB, the other three, i.e., *Msm*UDG with *Eco*SSB, *Msm*UDG with *Mtu*SSB and *Eco*UDG with *Mtu*SSB resulted in inhibition of uracil DNA glycosylase activity. While the interpretation for the general dominance of a decrease in uracil excision in heterologous combinations remains largely unclear, it could be that the modes of interaction between the heterologous proteins (e.g., *Mtu*SSB and *Eco*UDG) are different from those of the homologous proteins.

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