Overexpression of endonuclease III protects *Escherichia coli* mutants defective in alkylation repair against lethal effects of methylmethanesulphonate

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Abstract Endonuclease III of *Escherichia coli* is normally involved in the repair of oxidative DNA damage. Here, we have investigated a possible role of EndoIII in the repair of alkylation damage because of its structural similarity to the alkylation repair enzyme 3-methyladenine DNA glycosylase II. It was found that overproduction of EndoIII partially relieved the alkylation sensitivity of *alkA* mutant cells. Site-directed mutagenesis to make the active site of EndoIII more similar to AlkA (K120W) had an adverse effect on the complementation and the mutant protein apparently inhibited repair by competing for the substrate without base release. These results suggest that EndoIII might replace AlkA in some aspect of alkylation repair, although high expression levels are needed to produce this effect.

Key words: DNA repair; Endonuclease III; Alkylation DNA damage; Helix-hairpin-helix; 5-Hydroxycytosine

1. Introduction

DNA glycosylases catalyse the first step of the base excision repair pathway by hydrolysing the *N*-glycosylic bond between the modified base and the sugar moiety of nucleotides in the DNA [1]. DNA glycosylases can be classified into two separate groups based on the presence or absence of an associated apurinic/apyrimidinic (AP)-lyase activity. In *Escherichia coli*, endonuclease III (EndoIII) represents a bifunctional enzyme with combined DNA glycosylase/AP-lyase activities, whereas the alkylation DNA glycosylase AlkA is monofunctional without any associated AP-lyase activity. During the AP-lyase reaction, the sugar ring of the damaged nucleotide is converted to an unsaturated aldehyde leaving a strand break on the 3′-side of the remaining sugar-phosphate residue.

Typical substrates for AlkA are 3-methyladenine and 7-methylguanine, both of which are major lesions induced in DNA by simple methylating agents [2]. Bacterial mutants carrying *alkA1* are moderately sensitive to alkylating agents [3]. *E. coli* also possesses a second 3-methyladenine DNA glycosylase encoded by the *tag* gene. The double mutant *alkA1 tag* lacking both of these enzymes is extremely sensitive to alkylation exposure, emphasising the importance of this type of enzyme for repair of alkylation damage.

EndoIII recognises ring-saturated pyrimidines, thymine glycols, cytosine hydrates and rearranged pyrimidines such as urea and hydantoins in the DNA [4]. These lesions are induced by reactive oxygen species and are potentially cytotoxic. However, *E. coli* mutants lacking EndoIII (*nth*) are not hypersensitive to oxidising agents like H_2O_2 or γ-radiation [5]. The lack of sensitivity can be explained by the presence of a back-up enzyme, referred to as endonuclease VIII (EndoVIII), with enzymatic properties similar to EndoIII [6,7]. However, EndoIII has nevertheless been shown to possess strong activities for removal of the major premutagenic pyrimidine lesions such as uracil glycol, 5-hydroxycytosine and 5-hydroxyuracil, and *nth* mutants exhibit increased spontaneous mutation frequencies [8–10].

The three-dimensional structures of both EndoIII and AlkA have been determined [11–14], and it appears that these enzymes have an overall structural similarity except for the N-terminal part of AlkA. In both cases, the hydrolysis of the *N*-glycosylic bond seems to require flip out of the base involved. It is proposed that the base is stacked in the active site that is formed between two α-helical domains present in both AlkA and EndoIII. The helix-hairpin-helix (HhH) motif, known from many proteins involved in DNA repair and recombination, is similarly positioned in both proteins, at the entrance of the active site cleft. In spite of apparent differences in specificity, the mechanism for *N*-glycolytic cleavage appears to be similar for AlkA and EndoIII. Hydrolysis of the *N*-glycosylic bond proceeds by a nucleophilic attack of the anomeric sugar carbon. EndoIII is assumed to use lysine at position 120 as a nucleophile, whereas AlkA possibly uses a water molecule [12,13,15]. Conserved aspartate residues in EndoIII and AlkA (D138 and D238, respectively) are essential for enzyme activity and presumably serve to activate the nucleophiles.

The aim of the present study was to investigate a possible role for EndoIII in DNA repair of alkylation damage. EndoIII was overexpressed in alkylation repair defective mutants and the active site of EndoIII was altered to make it more similar to AlkA. The results support a minor role for EndoIII in repair of some type of alkylation damage normally effected by AlkA.

2. Materials and methods

2.1. Bacterial strains

The strains used were AB1157 (wild-type), BK2118 (*tag alkA1*) (from our own collection), MS23 (*alkA1*), BW415 (Δ*nth*), BK3002

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(*alkA1 nth*::*kan*) and BK3035 (*alkA1 nth umuDC*). The BW415 and BW372 strains were kindly provided by B. Weiss, strain GW8024 (originally constructed by R. Woodgate) was kindly provided by G. Walker. Strain BK3002 (*alkA1 nth*) was constructed by bacteriophage T4 transduction [16] of MS23 with phage lysates prepared from BW372 (*nth*::*kan*). The BK3035 (*alkA1 nth umuDC*) strain was constructed in a similar manner by transducing BK3002 with phage lysate from strain GW8023 (*umuDC*::*tn5*). The *nth* mutation of BK3002 was verified by PCR and *umuDC* mutation in strain BK3035 was verified by its loss of mutability to UV light.

2.2. Expression plasmids, site-directed mutagenesis

The DNA fragment encoding *nth* was amplified from W3110 chromosomal DNA by PCR subsequently cloned into the *Nco*I/*Hin*dIII restriction sites of pKK233-2 (Pharmacia) for use in cell survival studies. EndoIII protein was purified using an expression clone obtained from Richard Cunningham. Site-directed mutagenesis was performed by the Quick-Change method (Stratagene), using the two complementary oligonucleotides: (only coding sequence shown) 5′-CTGCCCGGCGTAGGTCGTTGGACAGCCAACGTC-3′. The mutation was verified by DNA sequencing.

2.3. Cell survival measurements

Methylmethanesulphonate (MMS) sensitivity was monitored during exponential growth ($OD_{600nm} = 1$) by making appropriate cell dilutions in ice-cold M9 buffer and plating on LB agar containing different concentrations of MMS as indicated. For pKK233-2 constructs, 1 mM IPTG was added at a cell density of 0.3 (OD_{600nm}) and was also included in the agar plates.

2.4. Mutagenesis

Overnight cultures were diluted 1:20 in fresh medium (LB) and allowed to grow to an $OD_{600nm} \sim 0.7$–1 prior to the addition of MMS (5 mM). After 30 min exposure at 37°C, the cell suspension was diluted 1:50 in LB and incubated with shaking at 37°C for 8 h. Samples were plated on LB agar to determine the number of viable cells and on rifampicin-containing plates (100 μg/ml) to determine the number of MMS-induced rifampicin resistant mutants (Rif^R). For mutation frequency determinations following exposure to H_2O_2, exponentially growing cells were pelleted, washed and resuspended in M9 buffer and subsequently left on ice for 15 min prior to incubation with 5 mM H_2O_2 for 30 min at 37°C.

2.5. EndoIII protein purification

The EndoIII construct and strain (BL21 de3) for expression were kindly provided by Richard Cunningham. EndoIII and the K120W mutant protein were purified using a modified procedure from that described by Asahara et al. [17]. Briefly, the polyethyleneimine supernatant was dialysed against 0.1 M K_2HPO_4 pH 6.6, 10 mM β-mercaptoethanol, 20% glycerol (buffer A) and loaded onto Sephadex CM column and eluted batchwise with 0.5 and 1.0 M NaCl in buffer A. The 0.5 M eluant was dialysed against buffer A and loaded on a Resource S column (FPLC, Pharmacia). The EndoIII and the K120W protein eluted between 0.2 and 0.23 M NaCl. The most concentrated fractions were purified further on a Superdex 75 column (SMART; Pharmacia).

2.6. 5-Hydroxycytosine substrate preparation and activity assay

Duplex oligonucleotide containing one 5-hydroxycytosine (5OHC) at a defined position 5′-gctcatgcgcag-5OHC-ggaaagggagg-3′ was prepared and used in assays as described previously [18].

3. Results

3.1. Overexpression of EndoIII confers increased MMS resistance of alkA mutants

Possible EndoIII alkylation repair activity was analysed by overproducing EndoIII in bacterial mutants defective in the alkyl-DNA glycosylase functions AlkA and Tag. Overexpression of EndoIII significantly increased the MMS survival of *alkA1* mutants indicating that some alkylation repair activity could be an inherent property of EndoIII. The complementation effect was observed both in the *tag alkA1* double mutant as well as in the *alkA1* single mutant strain (Fig. 1A and B).

3.2. Characterisation of an alkA1 nth double mutant strain

In view of the possible role of EndoIII in alkylation repair, we constructed a double mutant carrying both *alkA1* and *nth* mutations (BK3002) and tested for alkylation survival. However, introduction of *nth* in the *alkA1* mutant did not render the cells more sensitive to MMS (Fig. 1C). Also the single *nth* mutant was found to be wild-type resistant to MMS (data not shown). It is possible that EndoIII will recognise some lesion(s) induced by MMS, but with low affinity such that only overexpression of EndoIII may have a significant effect on survival.

We also measured the mutation frequencies in single and double mutant cells exposed to MMS and H_2O_2 (Fig. 2). A deficiency of AlkA caused an increase in the mutation frequency induced by MMS (Fig. 2A), similarly as has previously been observed for the alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [19]. H_2O_2 was found to induce mutations more frequently in *nth* strains than in wild-type cells. Hence, H_2O_2 was chosen as a mutagenic agent to evaluate possible substrate overlap between AlkA and EndoIII. H_2O_2-induced mutations are formed more frequently in both BK3002 (*alkA1 nth*) and BW415 (*nth*) than in MS23 (*alkA1*)

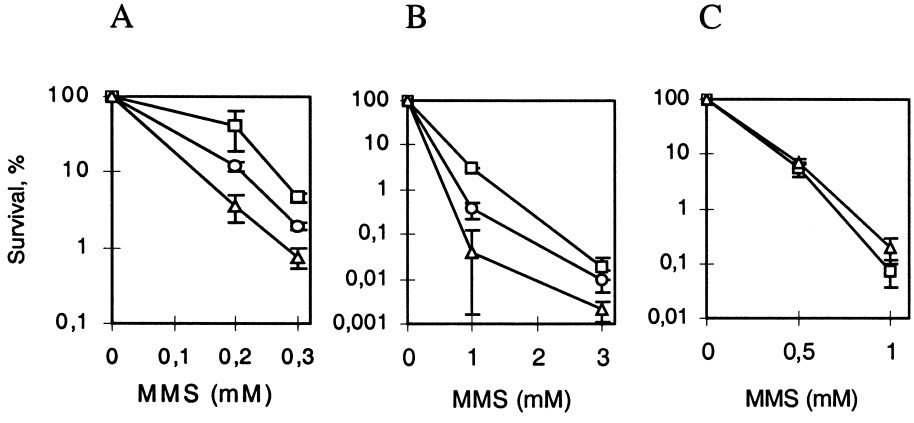

Fig. 1. MMS survival of BK2118 (*tag alkA1*) (A) and MS23 (*alkA1*) (B) cells transformed with pKK233-2 (○), *nth* (□) or *nth*(*K120W*) (△). Survival responses of untransformed MS23 (*alkA1*) (□) and BK3002 (*alkA1 nth*) (△) to MMS are shown in C. Each point represents the average of two measurements obtained from two to three independent experiments. Error bars include the full range of the data point variation.

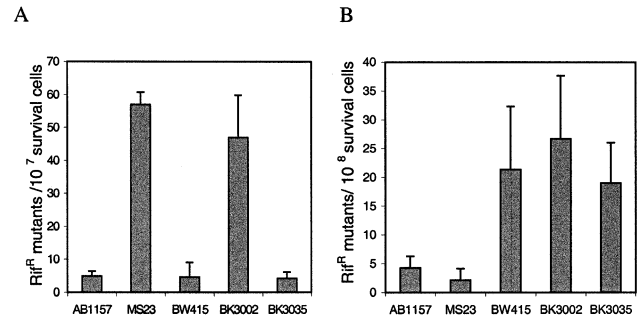

Fig. 2. Induced mutation frequency in different strains exposed to MMS (A) or H_2O_2 (B). Strains used were AB1157 (wild-type), MS23 (*alkA1*), BW415 (*nth*), BK3002 (*alkA1 nth*) and BK3035 (*alkA1 nth umuDC*). Mutation rates were calculated from three independent experiments in which 10 separate cultures were independently exposed and plated. Error bars indicate standard deviation.

or wild-type cells (Fig. 2B). The spontaneously formed mutation rates were from 2.5 ± 1 (lowest; AB1157) to 7 ± 4 (highest; BK3002) Rif^R mutants per 10^8 survival cells, based on the same number of experiments. The UmuDC errorprone bypass function was inactivated in the *alkA1* and *nth* strains to further evaluate the nature of the formed mutations. Most of the mutations induced by MMS in *alkA1* strains were *umuDC* dependent, consistent with methylated adenines representing blocks to DNA replication. Also, the strain BK3035 (*alkA1 nth umuDC*) is slightly more sensitive to MMS exposure than BK3002 (*alkA1 nth*) (data not shown). In contrast, the mutations induced by H_2O_2 in the *nth* background were independent of *umuDC*, indicating that the induced damage is directly premutagenic rather than an obstacle to DNA replication. These results are consistent with the previous findings that AlkA is important in protecting the cell against the lethal damages by alkylating agents, whereas the specific role of EndoIII appears to be attributed to protect the cell against the mutagenic effect of oxidative stress.

3.3. Generation of mutant EndoIII K120W protein with similarity to AlkA

A lysine residue is present at the C-terminal side of the helix-hairpin-helix motif in many different DNA glycosylases, however, all proteins of the AlkA type have a tryptophan at this position [1,20]. K^{120} in EndoIII was changed to tryptophan to make EndoIII more similar to AlkA. The K120W mutant protein was expressed in *alkA1* and *tag alkA1* mutant cells, and the cells were plated on agar plates containing MMS. Surprisingly, it was found that the K120W mutant protein made the *alkA1* mutants even more sensitive to MMS (Fig. 1A and B). The mutant protein was also expressed in wild-type cells, with no effect on the MMS sensitivity (data not shown). However, overexpression of K120W markedly diminished the colony size of wild-type cells exposed to MMS (Table 1) consistent with some effects of K120W on the growth rate during MMS exposure. One interpretation of these results might be that K120W interferes with the repair of MMS-induced DNA damage in the absence of AlkA. When present, AlkA seems to counteract the detrimental effects of K120W with respect to colony forming ability.

Table 1
Average colony diameter (mm) of AB1157 transformants plated on LB agar containing MMS

MMS (mM)	pKK233-2	*Nth*	*nth(K120W)*
0	2.6 ± 0.1	2.4 ± 0.2	2.4 ± 0.1
4	1.3 ± 0.2	1.9 ± 0.2	0.92 ± 0.08

Colony diameter was measured after 20 h incubation at 37°C. All colonies on the same plate were uniformly sized. The values represent the averages of sizes determined from five colonies and their standard deviations. No killing effect was observed at 4 mM MMS for AB1157.

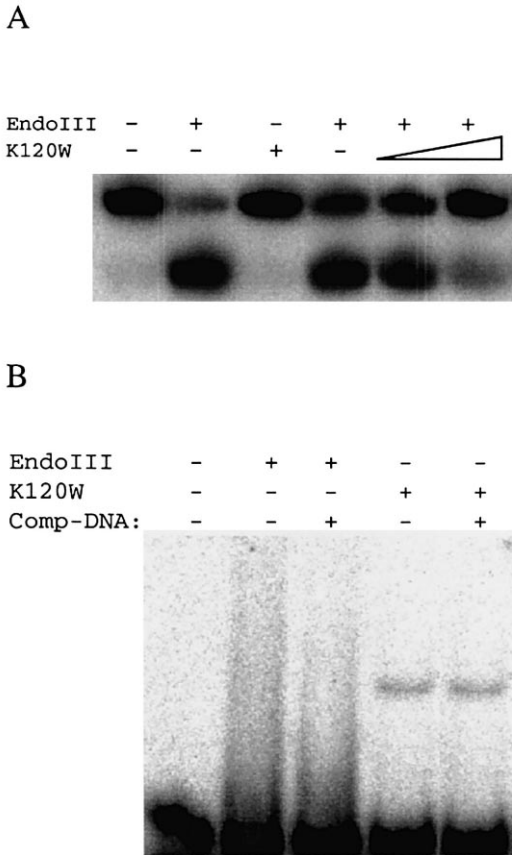

Fig. 3. Interactions with 5-hydroxycytosine-containing DNA duplex by EndoIII proteins. Incision (A) and binding (B) assays were carried out with 100 pM of ^{32}P-endlabelled duplex DNA in reaction buffer. Incision assays were performed for 30 min at 37°C with: no enzyme (lane 1), 4 nM EndoIII protein (lane 2), 4 nM K120W protein (lane 3) and 40 pM EndoIII (lanes 4–6). In lanes 4, 5 and 6 the substrate was preincubated with 0, 0.04 and 4 nM of K120W protein, respectively. In the binding assays, the reaction mixture was incubated on ice for 15 min as follows: lane 1, no enzyme; lanes 2 and 3, 40 nM EndoIII; lanes 4 and 5, 40 nM K120W. In lanes 3 and 5, 100 nM of non-modified competitor DNA duplex was added.

3.4. Characterisation of the EndoIII K120W mutant protein

The K120W mutant protein and EndoIII were purified to near homogeneity ($>99\%$ pure), and tested for their ability to release alkylated base residues from ^3H-methyl nitrosourea-treated DNA. However, neither EndoIII nor K120W were found to excise methyl lesions to any significant extent (data not shown). Hence, the complementing effect of EndoIII in *alkA1* mutants cannot readily be ascribed to excision of 3-methyladenine or 7-methylguanine.

Possible enzymatic activity of K120W protein was analysed by its ability to cleave an oligonucleotide containing 5-hydroxycytosine. As shown in Fig. 3A, lanes 1–3, wild-type En-

doIII protein efficiently cleaved the oligonucleotide, whereas the K120W mutant protein did not produce any nicking activity. By electromobility shift assays, we could demonstrate a stable binding to 5-hydroxycytosine-containing DNA for K120W but not for EndoIII (Fig. 3B). A complex formed between damaged DNA and K120W could prevent excision by the endogenous repair enzymes. As shown in Fig. 3A, lanes 4–6, preincubation of the 5-hydroxycytosine-containing DNA with the K120W mutation protein reduced the extent of nicking produced by EndoIII. Hence, the negative effects of K120W could be ascribed to repair inhibition by binding without base release, preventing access by the active DNA glycosylases.

4. Discussion

The potential role of EndoIII in alkylation repair was investigated. It appears that EndoIII by itself is capable of partially suppressing the MMS sensitive phenotype of the *tag alkA1* and *alkA1* mutants. This could imply that EndoIII is capable of removing some alkylated bases with low affinity, or alternatively that EndoIII is responsible for repair of some other type of lesion induced by exposure to MMS. A major methylation product, 7-methylguanine, spontaneously converts to formamidopyrimidine, which is recently shown to be repaired by EndoIII [21,22]. It has also been found that overexpression of EndoIII in yeast defective in the AP-endonuclease *APN1*, reduces sensitivity to MMS [23]. The stimulatory effect of EndoIII expression was ascribed to the AP-lyase activity of EndoIII. It is possible that the 3′-incised AP-site is processed more efficiently and therefore is less toxic to the cells than 5′-incised AP-sites.

When K^{120} was changed to W, to make EndoIII more similar to AlkA, it was observed that the mutant EndoIII actually interfered with the repair catalysed by AlkA in vivo as judged from the reduced growth rate observed during MMS exposure in wild-type bacteria (Table 1). Moreover, the *alkA1* mutants became even more MMS sensitive upon expression of K120W (Fig. 1). The in vitro data show that the K120W protein interacts with damaged DNA and prevents repair by EndoIII (Fig. 3). Since the *alkA1 nth* double mutant was as sensitive to MMS exposure as the *alkA1* single mutant, the negative effect of K120W expression in MMS-exposed cells cannot be ascribed simply to the inactivation of the EndoIII function. The role of the conserved tryptophan in the HhH motif for alkylation repair has been proposed to involve stacking with π-electrobond-containing bases [13]. Hence, K120W may interfere with innocuous alkylated bases like 7-methylguanine and accentuate the damaging effects of such lesions.

Recently, EndoIII homologues from *Thermotoga maritima* and *Aquifex aeolicus* have been characterised and shown to have 3-methyladenine DNA glycosylase activities [24]. This suggests that changes of a limited number of residues in proteins with a similar scaffold can significantly alter the substrate specificity. The residue in the *T. maritima* enzyme corresponding to K^{120} in EndoIII is a glutamate residue and the enzyme was found to be monofunctional without any associated AP-lyase activity similarly as is the case for all other alkylation repair DNA glycosylases. Engineering of EndoIII to introduce a glutamate residue rather than a tryptophan at position 120 might possibly have produced an enzyme with enhanced activity for alkylation repair.

The damaging reactions of MMS are not fully understood. Apart from methylating DNA, MMS also induces AP-sites and strand breaks [25,26]. Moreover, cell exposure to MMS may result in depletion of the glutathione levels, which in turn increases oxidative stress [27]. Consequently, cellular sensitivity to MMS might to some extent be ascribed to effects other than alkylation, such as for instance oxidative stress for which overproduction of EndoIII could be beneficial.

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