Release of normal bases from intact DNA by a native DNA repair enzyme

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Base excision repair is initiated by DNA glycosylases removing inappropriate bases from DNA. One group of these enzymes, comprising 3-methyladenine DNA glycosylase II (AlkA) from *Escherichia coli* and related enzymes from other organisms, has been found to have an unusual broad specificity towards quite different base structures. We tested whether such enzymes might also be capable of removing normal base residues from DNA. The native enzymes from *E.coli*, *Saccharomyces cerevisiae* and human cells promoted release of intact guanines with significant frequencies, and further analysis of AlkA showed that all the normal bases can be removed. Transformation of *E.coli* with plasmids expressing different levels of AlkA produced an increased spontaneous mutation frequency correlated with the expression levels, indicating that excision of normal bases occurs at biologically significant rates. We propose that the broad specificity 3-methyladenine DNA glycosylases represent a general type of repair enzyme 'pulling' bases in DNA largely at random, without much preference for a specific structure. The specificity for release of damaged bases occurs because base structure alterations cause instability of the base-sugar bonds. Damaged bases are therefore released more readily than normal bases once the bond activation energy is reduced further by the enzyme. Qualitatively, the model correlates quite well with the relative rate of excision observed for most, if not all, of the substrates described for AlkA and analogues.

Keywords: base excision/DNA alkylation damage/DNA damage/DNA glycosylase/DNA repair

Introduction

Base excision repair (BER) of DNA damage is initiated by DNA glycosylases that release inappropriate base structures from DNA. Base release results in the formation of an AP-site which is acted upon by an AP-endonuclease or an AP-lyase cleaving the DNA strand 5' or 3' to the baseless sugar site, respectively. Further tailoring of the ends by deoxyribophosphodiesterase and/or other nuclease activities is then required to allow for correct resynthesis and strand ligation (Seeberg *et al.*, 1995). In mammalian cells, at least two different pathways exist for AP-site processing involving the phosphodiesterase activity of

DNA polymerase β or the FEN1 endonuclease for the 5'-end tailoring, yielding repair patches of 1–2 or ~7 nucleotides, respectively (Frosina *et al.*, 1996; Sobol *et al.*, 1996; Klungland and Lindahl, 1997). However, for both pathways, the resynthesis and ligation steps are carried out by the consecutive action of DNA pol β and DNA ligase III.

The important step in the BER pathway of DNA base damage recognition is part of the DNA glycosylase reaction mechanism. Structural analysis of the enzyme–DNA complex of the uracil DNA glycosylase has shown that the base and sugar moieties of the nucleotide are flipped out from the helix and placed into a pocket of the enzyme structure (Slupphaug *et al.*, 1996). A nucleophilic attack on the C1 sugar carbon then causes base release. Structural analyses of other DNA glycosylases have indicated a similar mechanism of action for all these enzymes with a flexible cleft or pocket that may accommodate flipped out nucleosides. Some enzymes are specific for the removal of a single or a few closely related base residues, such as the uracil DNA glycosylase and the 8-oxoG DNA glycosylases (Seeberg *et al.*, 1995). Another group of enzymes, comprising 3-methyladenine DNA glycosylase II (AlkA) from *Escherichia coli* and related enzymes from other organisms, have been found to possess an unusual broad specificity towards quite unrelated base structures including several types of chemically methylated purine and pyrimidines (Karran *et al.*, 1982; McCarthy *et al.*, 1984); ethano- (Habraken *et al.*, 1991), etheno- (Hang *et al.*, 1996; Dosanjh *et al.*, 1994) and mustard- (Mattes *et al.*, 1996) modified purines; 5-methyloxidized thymines (Bjelland *et al.*, 1994), hypoxanthine (Saparbaev and Laval, 1994), 8-oxoguanine (Bessho *et al.*, 1993) as well as several other DNA lesions produced by anti-tumour cytostatics (Matijasevic *et al.*, 1993) (e.g. chloroethylguanine). These enzymes normally are referred to as 3-methyladenine DNA glycosylases since the *N*-alkylation product 3-methyladenine is removed with high efficiency. However, in view of the wide range of lesions removed, it may seem that these enzymes have a more general repair function in the cell and might be important for dealing with a large range of structurally different, but quantitatively minor lesions. Although the structure of one enzyme in this group, 3-methyladenine DNA glycosylase II from *E.coli*, has been solved, it still remains unclear what the actual structural determinant is for damage recognition except for the aromatic and hydrophobic nature of nucleic acid bases in general (Labahn *et al.*, 1996; Yamagata *et al.*, 1996). In this study, we have asked whether this group of enzymes might also be capable of removing normal bases from DNA and report that such excision occurs with biologically significant frequencies. It is proposed that the primary function of the broad specificity enzymes is to lower the activation energy for *N*-glycosylic

bonds in general, largely without specificity for the base involved. Excision efficiency will then be a function of the stability of the base attachment to the DNA such that chemically modified base residues with a reduced sugar bond stability will be released rapidly whereas normal bases with stable base–sugar bonds will only be removed occasionally.

Results

Excision of guanine from intact DNA by 3-methyladenine DNA glycosylases from *E.coli*, yeast and human cells

In order to assay for the release of normal base residues from DNA by AlkA and analogues, we amplified DNA by PCR in the presence of nucleotides radiolabelled in the base moiety and analysed for base release by HPLC after incubation with different enzymes. The results showed that guanine was removed by AlkA from *E.coli*, MAG from *Saccharomyces cerevisiae* and MPG from human cells (Figure 1). Other DNA glycosylases tested: the alkylation repair-specific 3-methyladenine DNA glycosylase I (Tag) from *E.coli* (Figure 1), uracil DNA glycosylase from human cells and a 3-methylpurine-specific enzyme from *Bacillus cereus* (data not shown), all tested negative for guanine removal. At pH 7.5, guanines were removed by AlkA at a rate of 0.47 fmol/U which is 2100 times slower than for the removal of *N*³-methylA and 42 times slower than for *N*⁷-methylG. In comparison, the chemical half-life of *N*³-methylA in DNA is ~25 h and that of *N*⁷-methylG is 150 h (Lawley and Brookes, 1963), whereas that of guanine is several orders of magnitude higher. However, these results suggest the possibility that excision efficiency could be correlated with the inherent chemical stability of the base attachment to the phosphodiester chain.

The AlkA enzyme releases all the normal bases from intact DNA

The reactions of 3-methyladenine DNA glycosylases with intact DNA were tested further with AlkA from *E.coli*. Experiments with DNA radiolabelled at other base moieties showed that adenine was removed at a rate ~5-fold slower than guanine, and pyrimidines were released with ~20-fold lower frequency (Table I). These results show that normal base release can occur for all the normal bases; however, pyrimidines are removed more slowly than purines. Again, it might be speculated that excision efficiency is correlated with base–sugar bond stability since pyrimidines are less prone to spontaneous release than purines. Other DNA glycosylases tested, i.e. the 3-methylpurine-specific DNA glycosylases from *E.coli* and *B.cereus* and the human uracil DNA glycosylase, did not produce any normal base release from these substrates (data not shown)

AlkA expression induces spontaneous mutations in *E.coli*

Release of base residues from DNA, enzymatic or non-enzymatic, results in the formation of AP-sites which are known to be strong pre-mutagenic lesions. To investigate whether the normal base release by AlkA would occur at biologically significant rates, we analysed the spontaneous

Fig. 1. Excision of unmodified G by different 3-methyladenine DNA glycosylases. Incubation was carried out for 300 min under standard conditions using 25 U of AlkA from *E.coli*, 3 U of MAG from *S.cerevisiae*, 69 units of MPG from human cells and 74 U of 3-methyladenine DNA glycosylase I (Tag) from *E.coli*. Excised bases were quantified by HPLC analysis. In separate experiments, excision of guanine by AlkA was shown to be linearly dependent on time of incubation and amount of enzyme. Relative to *N*³-methylA excision, MAG excises guanine 3.9 times faster than AlkA and 27 times faster than MPG.

Table I. Excision of normal purines and pyrimidines from DNA by AlkA from *E.coli*

Base	fmol base excised/ U of enzyme	Rate of excision relative to G
Guanine	0.470	1
Adenine	0.077	0.17
Cytosine	0.019	0.04
Thymine	0.013	0.03

Fig. 2. Spontaneous mutation frequency of *E. coli* as a function of AlkA expression levels. *Escherichia coli* MS23 (*alkA1*) was transformed by different *alkA*⁺ plasmids expressing AlkA constitutively at different levels as previously described (Kaasen *et al.*, 1986). Numbers on the x-axis represent expression levels relative to those found in unadapted (uninduced) wild-type cells.

mutation frequency in *E. coli* transformed by different AlkA plasmids yielding different levels of expression without exposing the cells to any alkylating agents (Figure 2). It was found that the spontaneous mutation frequency increased with increasing AlkA expression levels, consistent with the formation of pre-mutagenic AP-sites in DNA from normal base release. It was inferred that such release would be biologically significant since the expression levels of AlkA used were comparable with those obtained by inducing wild-type cells under alkylation adaptation conditions (10–20 on the scale).

Increased spontaneous mutation frequency from excess glycosylase action has been observed previously from overproducing the MAG enzyme in yeast (Xiao and Samson, 1993). This was ascribed to the presence of endogenous alkylation exceeding the level of normal repair capacity thus causing more alkylation repair when the enzyme is being overproduced. However, in view of our results, we rather believe that the effect of overproducing MAG can be ascribed to increased non-specific 'repair'.

Guanine, but not *N*³-methylA excision, is strongly dependent on pH within the physiological range

The experiments presented above suggest that the efficiency of base excision by AlkA would depend on the stability of the base–sugar bond. It is known that protonation of the *N*⁷ groups of purines will impart positive charges on the base residue, causing destabilization of the *N*-glycosylic bond (Loeb and Preston, 1986). We therefore investigated the effect of pH on the AlkA-induced release of guanines as compared with the release of *N*³-methylA (Figure 3). AlkA activity is normally monitored by *N*³-methylA removal, and previous experiments have shown that AlkA has a broad pH optimum (Bjelland *et al.* 1994). A 10-fold increase in the enzymatic guanine removal is observed by lowering the pH of the reaction from 7.9 to 6.5. Within the same pH range, there is no effect upon the rate of *N*³-methylA removal whose attachment to the deoxyribose moiety is not affected by small changes in the pH. However, there is a distinct pH dependence of

Fig. 3. Excision of unmodified G and *N*³-methylA by AlkA as a function of pH. Conditions for guanine excision (●) were the same as described in Materials and methods except that the pH was adjusted as indicated. The reaction mixtures for *N*³-methylA excision (○) contained 1.0 µg (4000 d.p.m.) of [³H]dimethylsulfate-treated calf thymus DNA with a specific activity of 1.2 Ci/mmol. Bases released were quantified by HPLC. The values plotted represent the amount of base excised in 30 min.

enzymatic guanine excision almost proportional to the concentration of free proton ions, indicating that the less stable protonated form is the most prone to excision by AlkA. This means that at the physiological pH of 6.5, there is only an ~5-fold difference in the efficiency of guanine versus *N*⁷-methylG removal.

Discussion

The AlkA enzyme from *E. coli* originally was identified as an enzyme specifically involved in the repair of alkylation damage, and functional homologues of AlkA have been characterized from many different organisms including yeast and mammals. More recently, these enzymes have been shown to have a wide substrate specificity for many different damaged bases that appear to share little obvious similarity. We show here that these enzymes are also capable of removing normal base residues from DNA although with lower frequencies than for damaged residues. However, such removal appears to be biologically significant as suggested by the increased spontaneous mutation frequency induced by moderately increased AlkA levels in *E. coli*.

Based on the data presented here and previous studies of substrate specificity, we now suggest a novel model for how bases are excised by AlkA and analogues which does not depend on the precise chemical structure of the excised base. It is proposed that the enzyme reduces the activation energy of base–sugar bonds largely without specificity for a given base structure. The enzymatic reduction in the bond energy adds to the instability caused by any previous chemical modification of the base. The rate of excision will then be a function of bond stability and will only occur if the bond energy in total is reduced below a threshold value (Figure 4). The stability of the base–sugar bond depends on several different parameters, such as

Fig. 4. Schematic representation of non-specific base-sugar bond destabilization model for AlkA type activity on guanines and methylated purines in DNA. Solid lines represent the activation energy for spontaneous base release and broken lines represent the lowered activation energy in the presence of AlkA.

charge, steric interactions between the sugar and the damaged base, and charge distribution and mobility of the π electrons of the aromatic base structure. It has been suggested previously that the specificity of AlkA is base charge dependent. However, whereas several of the 'good' substrate bases carry a positive charge, charge alone is not sufficient to account for specificity. Both O^2 -methylT and O^4 -methylT are positively charged, but only O^2 -methylT is excised (McCarthy *et al.*, 1984). However, all of the substrate bases reported appear to have a reduced stability of the *N*-glycosylic bond to deoxyribose in DNA. $N^2,3$ -ethenoG, $1,N^6$ -ethenoA and $1,N^2$ -ethenoG are all labile and good substrates, whereas $3,N^4$ -ethenoC is stable and is not excised (Dosanjh *et al.*, 1994; Hang *et al.*, 1996). There is also a qualitative correlation between the efficiency of removal and bond stability. For example, in the case of methylated purines, O^6 -methylG is stable, N^7 -methylpurines are unstable and the N^3 -methyl derivatives even more unstable (Lawley and Brookes, 1963), corresponding to no apparent removal, slow removal and rapid removal, respectively (Thomas *et al.*, 1982). Of the pyrimidines, O^2 -methylC is removed more rapidly than O^2 -methylT, whereas O^4 -methylT is not removed (McCarthy *et al.*, 1984), again correlated with the spontaneous rate of release of these base residues (Singer *et al.*, 1978).

The crystal structure of AlkA recently has been solved (Labahn *et al.*, 1996; Yamagata *et al.*, 1996). The active site comprises a large flexible sized hydrophobic cleft lined with aromatic side chains that may accommodate many different types of aromatic base residues. There are no charged amino acid residues inside the pocket to make specific contacts with the base, consistent with the model proposed. The cleft is deep enough to accommodate flipping of the entire nucleoside residue, suggesting that bond destabilization will occur from the flipped out state of the nucleoside. It is only for bases with the lowest activation energy, such as N^3 -methylA, that excision will take place once the base is flipped out. Other substrates such as N^7 -methylG will only occasionally be removed. The active site of AlkA contrasts with the tight-fitting active site of the uracil DNA glycosylase that makes specific contacts with the side groups of the pyrimidine ring (Slupphaug *et al.*, 1996). Site-specific mutations have been made to accommodate cytosine or thymine in the active site of the uracil DNA glycosylase which then

result in removal of normal pyrimidines from the DNA (Kavli *et al.*, 1996).

In a previous report, we have shown that overexpression of AlkA from multicopy plasmids sensitizes cells to alkylation exposure (Kaasen *et al.*, 1986). We now believe that this effect is simply caused by excess AlkA being induced from the alkylation exposure. It must be essential for the cells to regulate the levels of such enzymes in order to deal with ambient genetic stress, but otherwise to minimize expression to limit removal of normal bases as much as possible. It may be of relevance in this respect that both the *alkA* gene in *E.coli* (Evensen and Seeberg, 1982) and the *MAG* gene function in yeast (Chen *et al.*, 1990) are strictly controlled under normal conditions, but are up-regulated under conditions of alkylation exposure.

The bond destabilization model explains the broad substrate specificity observed for AlkA and related enzymes. Functionally, AlkA and its analogues appear to be the counterpart or complement of the nucleotide excision repair pathway in terms of broad specificity towards even unknown base structures in DNA. However, much less energy is expended using base excision rather than nucleotide excision repair, and the base destabilization/excision pathway may be of particular importance for genome maintenance in stationary phase bacteria or in the G_1 phase of eukaryotes. It has been proposed that accumulation of rare lesions may to some extent be responsible for cellular aging (Fraga *et al.*, 1990; Lindahl, 1993). The action of enzymes such as AlkA and similar enzymes in higher organisms may function to prevent accumulation of rare lesions in DNA and could therefore operate to prolong the life-span of living cells.

Materials and methods

Radiolabelling of DNA

DNAs containing radiolabelled G, A, C and T were produced by PCR (30 cycles, 2 kb fragment with 100 pmol of each primer). The labelled nucleotides [$8\text{-}^3\text{H}$]dGTP (11.7 Ci/mmol), [$8\text{-}^3\text{H}$]dATP (7.8 Ci/mmol), [$5\text{-}^3\text{H}$]dCTP (18.9 Ci/mmol) and [$\text{methyl-}^3\text{H}$]dTTP (48 Ci/mmol) were used in amounts of 8.5, 7.7, 1.5 and 4.2 nmol, respectively, with 5 U of Vent polymerase, 2.5 nmol of unlabelled triphosphate and 20 nmol of other triphosphates in a reaction volume of 100 μl . The amplified DNA was purified by polyacrylamide gel electrophoresis and electroelution followed by ethanol precipitation.

Enzymes and assay conditions

All glycosylases were purified to homogeneity. Uracil DNA glycosylase was purchased from Boehringer Mannheim. Human MPG enzyme was a generous gift from T.O'Connor (O'Connor, 1993). Purification of *E.coli* Tag (Bjelland and Seeberg, 1987), *E.coli* AlkA (Bjelland *et al.*, 1994) and *S.cerevisiae* MAG (Björås *et al.*, 1995) was as described, and purification and characterization of the 3-methyladenine-specific DNA glycosylase of *B.cereus* will be published elsewhere (M.Björås, I.Alseth and E.Seeberg, in preparation). All enzyme reactions were carried out in 70 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.5/1 mM dithiothreitol/1 mM EDTA/5% glycerol for 30 min at 37°C unless otherwise indicated. One enzyme unit has been defined as the amount of enzyme that excises 1 pmol of N^3 -methylA in 30 min. For the pH dependence activity measurements, the reaction buffers were as given above except that the pH of the 3-(*N*-morpholino)propanesulfonic acid was adjusted as indicated.

HPLC analysis

HPLC analysis was performed as described by Eide *et al.* (1996) with the following modifications: for G separation, the mobile phase [50 mM (NH_4) H_2PO_4 , 5% methanol] was adjusted to pH 2.0, with a retention time of 4.4 min for G. For A, 0.1 M triethylamine acetate (TEAAc), pH 5.4, with 3% methanol was used; A eluting at 6.4 min. For T, the mobile

phase was 0.1 M TEAAc, pH 7.0, 1% methanol; T retention of 9.7 min. For C, 0.1 M acetic acid with 3% 0.1 M Na acetate were used and fractions eluting between 4 and 7 min were lyophilized and rerun on the same column using the same conditions; C eluting at 6.2 min. Other HPLC systems were also used to verify co-migration with the corresponding native base, and to ensure adequate separation from the nucleosides and monophosphates. In separate controls, it was shown that AlkA did not release any free base from the unincorporated radiolabelled dNTPs.

Mutation analysis

The *E. coli* strain MS23 (*alkA1*) was transformed by different plasmid constructs carrying *AlkA*⁺ in different sequence contexts, expressing AlkA constitutively at different levels as indicated (Kaasen *et al.*, 1986). Approximately 10⁹ stationary phase cells were plated on nutrient agar (LB plates) containing 100 µg of rifampicin/ml (to measure forward mutation to rifampicin resistance). Total cell number was measured by plating appropriate dilutions on nutrient agar without rifampicin. The data represent the average of four different experiments with three independent cultures, and error bars represent standard deviation. Enzyme levels were measured as described (Evensen and Seeberg, 1982).

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