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Articles

Activation of Human O^6-Alkylguanine–DNA Alkyltransferase by DNA†

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ABSTRACT: The effect of DNA on the activity of human O^6-alkylguanine–DNA alkyltransferase was investigated by using O^6-benzylguanine as a substrate or inhibitor. The sensitivity of the alkyltransferase to inactivation by O^6-benzylguanine was increased by addition of calf thymus DNA. In order to investigate this phenomenon in more detail, the ability of the alkyltransferase to convert O^6-benzyl[8-^{3}H]guanine to [8-^{3}H]guanine was measured. The rate of guanine production was increased about 6-fold by addition of DNA. The effect of DNA was completely abolished by addition of 0.2 M NaCl, which had no effect on the reaction in the absence of DNA. When a mutant P140A alkyltransferase, which is known to be less sensitive to inactivation by O^6-benzylguanine presumably as a result of steric hindrance, was used, the rate of reaction was increased by a considerably larger amount, about 16-fold. Oligodeoxynucleotides were able to stimulate the production of guanine from O^6-benzylguanine. Single-stranded oligodeoxynucleotides were as effective as double-stranded, and a maximal stimulation was obtained with a 12-mer. These results demonstrate that the alkyltransferase binds to a region of DNA covering at most 12 bases and undergoes a conformational change which facilitates the reaction of adducts at the O^6-position of guanine with the cysteine acceptor site on the protein. When O^6-benzyl[8-^{3}H]deoxyguanosine was used as a substrate, the addition of DNA decreased the rate of formation of 2′-deoxy[8-^{3}H]guanosine. Inactivation of the alkyltransferase by O^6-benzyldeoxyguanosine was also inhibited by DNA addition. This suggests that binding of the deoxynucleoside alone is not sufficient to cause the conformational change needed for activation and that the presence of DNA either interferes with the ability to bind O^6-benzyldeoxyguanosine or does not favor the reaction with this substrate. These results may explain why O^6-benzylguanine is a better inactivator of cellular alkyltransferase than its deoxynucleoside.

The protein O^6-alkylguanine–DNA alkyltransferase (EC 2.1.1.63) (alkyltransferase)[1] repairs O^6-methylguanine and related lesions in alkylated DNA by removing the alkyl group from the O^6-position of guanine. O^6-Alkylguanine has been implicated in the toxic, mutagenic, and carcinogenic effects of exposure to alkylating agents, and the presence of the alkyltransferase protects against these consequences (Yarosh, 1985; Lindahl et al., 1988; Pegg, 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993). Alkyltransferase protein has been cloned from a variety of sources including bacteria, yeast, and mammalian cells. Studies using the protein purified from cells or from recombinant sources have shown that it acts by transferring the alkyl group from the DNA to a cysteine acceptor site located in the protein sequence. The resulting *S*-alkylcysteine is not converted back to cysteine, and the protein therefore acts only once (Lindahl et al., 1988; Demple, 1990; Pegg & Byers, 1992). All known O^6-alkylguanine–DNA alkyltransferases contain the cysteine acceptor site in

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[1] Abbreviations: alkyltransferase, O^6-alkylguanine–DNA alkyltransferase (EC 2.1.1. 63); PCA, perchloric acid; RP-HPLC, reversed-phase high-pressure liquid chromatography.

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a sequence -PCHR-, and it has been suggested that the reaction is brought about by a thiolate anion formed at the cysteine and stabilized by the adjacent basic amino acid residues (Demple, 1990; Ling-Ling et al., 1992; Mitra & Kaina, 1993; Moore et al., 1994). Protonation of a heteroatom on the guanine may facilitate this reaction (Spratt & de los Santos, 1992). Direct evidence for these hypotheses is lacking, and there is little other information on the mechanism of the alkyltransferase reaction. It is well-known that the protein binds to DNA, a property that has been exploited in its purification (Mitra & Kaina, 1993; Bhattacharyya et al., 1988; Lee et al., 1992; Gonzaga et al., 1992; Chan et al., 1993; Potter et al., 1994). Studies using circular dichroism and fluorescence analysis have shown that the interaction with DNA brings about a conformational change in the protein (Takahasi et al., 1990; Chan et al., 1993), but there was no information on the effects of this conformational change on the reaction.

In previous studies, we have shown that O^6-benzylguanine is a powerful inactivator of the human alkyltransferase that acts by serving as a substrate for the protein, forming *S*-benzylcysteine at the active site and liberating guanine (Dolan et al., 1990; Moschel et al., 1992; Pegg et al., 1993). The availability of a low molecular weight substrate for the alkyltransferase reaction has enabled us to study the effects of DNA binding on its rate. The results show clearly that the conformational change undergone by the alkyltransferase facilitates the reaction. This system was also used to study the requirements for oligodeoxynucleotides for this change to occur. Mutations affecting the ability of DNA to bring about this change were identified. In contrast to the inactivation of the alkyltransferase by O^6-benzylguanine which was enhanced by DNA, the inactivation by O^6-benzyldeoxyguanosine was inhibited, suggesting that the binding of the deoxynucleoside involves the same site as DNA.

MATERIALS AND METHODS

Purification and Assay of Alkyltransferase. The recombinant human alkyltransferase and its P140A and W100A mutants were expressed in *Escherichia coli* and purified as previously described (Pegg et al., 1993; Crone & Pegg, 1993). All preparations had specific activities of 30–35 pmol/μg of protein, and the theoretical maximum is 46 pmol/μg of protein (Pegg et al., 1993). Enzyme activity against a DNA substrate containing O^6-methylguanine was assayed using calf thymus DNA which had been methylated by reaction with *N*-[^{3}H]-methyl-N-nitrosourea as described (Dolan et al., 1990).

Effect of DNA on the Ability of O^6-Benzylguanine To Inactivate the Alkyltransferase. Inactivation was studied essentially as previously described for crude extracts from HT29 cells (Moschel et al., 1992) by incubating the purified alkyltransferase with various concentrations of O^6-benzylguanine for 30 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 0.1 mM EDTA in a volume of 0.5 mL. A further 0.5 mL of the same buffer containing the methylated DNA substrate was then added and the remaining alkyltransferase activity measured over a further 30-min incubation period.

Reaction of Alkyltransferase with O^6-Benzylguanine or O^6-Benzyldeoxyguanosine. O^6-Benzyl[8-^{3}H]guanine (0.34 Ci/mmol) was produced by catalytic tritium exchange and purified as previously described (Pegg et al., 1993). The separation of guanine, which elutes at 3 min, from O^6-benzylguanine, which elutes at 12 min, was carried out by RP-HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm) using isocratic elution at a temperature of 36 °C with a buffer of equal parts methanol and 0.05 M ammonium formate, pH 4.5. The eluate from the HPLC was monitored for radioactivity by mixing with 3.5 volumes of Flow Scint III and passing through a Radiomatic Flo-One\Beta A-140A radioactivity monitor (Packard Instruments). The efficiency of counting was 38%. In some cases, 2-mL fractions of the eluate were collected and assayed for radioactivity in a Beckman scintillation counter.

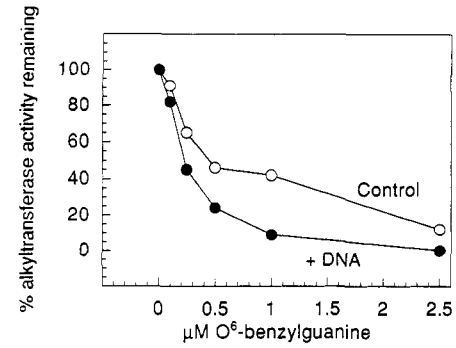

FIGURE 1: Effect of DNA on inactivation of human alkyltransferase by O^6-benzylguanine. The alkyltransferase protein was incubated for 30 min in the presence or absence of 200 μg of DNA in a total volume of 0.5 mL containing the concentration of O^6-benzylguanine shown. The remaining alkyltransferase activity was then measured by incubation with the standard [^{3}H]-methylated DNA substrate as described under Materials and Methods. Results are the means of triplicate estimations which agreed within ±10%.

Labeled O^6-benzyldeoxyguanosine (1.27 Ci/mmol) was produced by Amersham by catalytic tritium exchange (method TR8/600 in the manufacturer's catalog) from the parent deoxynucleoside (Moschel et al., 1992) and purified by RP-HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm) using isocratic elution at a temperature of 36 °C with a buffer of methanol/0.05 M ammonium formate, pH 4.5 (35:65). About 7% of the labeled material was the required product. Analysis of the products after acid hydrolysis of the labeled O^6-benzyldeoxyguanosine indicated that the ^{3}H was located on the 8-position of the purine. The separation of 2′-deoxyguanosine, which elutes at 4 min, from O^6-benzyldeoxyguanosine, which elutes at 33 min, was carried out using the same HPLC system used for purification. O^6-Benzylguanine elutes at 26 min in this system. Radioactivity was monitored as described above.

Measurements of guanine formation from O^6-benzylguanine or deoxyguanosine from O^6-benzyldeoxyguanosine were carried out using various amounts of the [^{3}H]O^6-benzylguanine or O^6-benzyldeoxyguanosine and alkyltransferase protein in an assay mixture consisting of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 5 mM dithiothreitol in a volume of 0.25 or 0.5 mL as indicated. The formation of labeled product was stopped by the addition of 0.6–0.8 mL of the same buffer containing 0.2 mM of guanine and 0.2 mM of O^6-benzylguanine. Aliquots were then separated by HPLC, and radioactivity was determined as described above.

RESULTS

Addition of calf thymus DNA led to an increase in the effectiveness of O^6-benzylguanine as an inactivator of the purified human alkyltransferase (Figure 1). This suggests that the presence of DNA enhances the reaction with this substrate, but this system is not well suited for further analysis of this effect since it involves a preliminary incubation of the drug with the protein in the presence or absence of DNA

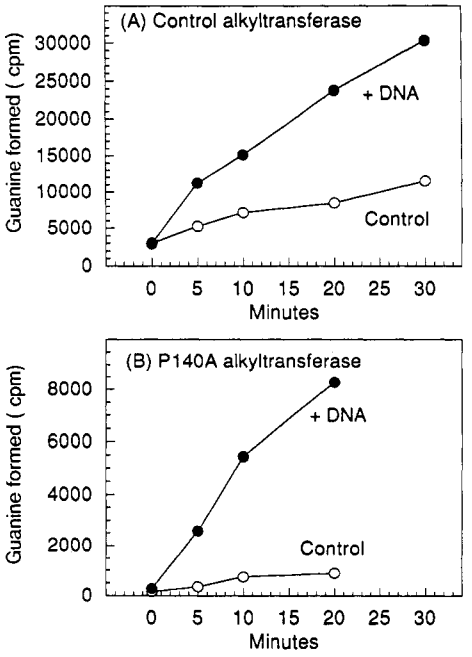

FIGURE 2: Effect of DNA on the rate of conversion of O^6-benzylguanine to guanine by human alkyltransferase. Assay buffer containing 0.7 μM O^6-benzyl[^{3}H]guanine was incubated at 37 °C with 5 μg of human alkyltransferase in 0. 5 mL (panel A) or with 40 μg of the P140A alkyltransferase in 0.25 mL (panel B) in the presence (filled circles) or absence (open circles) of calf thymus DNA as indicated. The formation of guanine in the absence of alkyltransferase amounted to less than 5% of the value in its presence and was subtracted.

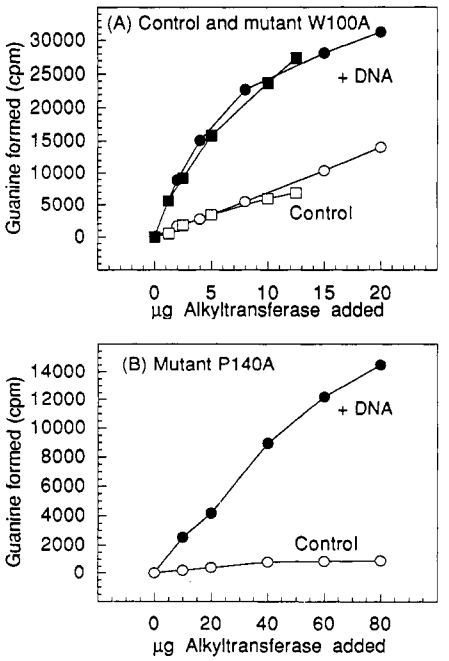

FIGURE 3: Comparison of wild-type, W100A, and P140A alkyltransferase in stimulation by DNA of conversion of O^6-benzylguanine to guanine. A total volume of 0.25 mL containing 0.7 μM O^6-benzyl-[^{3}H]guanine was incubated at 37 °C with the amount of alkyltransferase shown for 10 min in the presence (filled symbols) or absence (open symbols) of 200 μg of calf thymus DNA as indicated. Results in panel A show the guanine formed by control alkyltransferase (circles) or the W100A mutant alkyltransferase (squares). Results in panel B show the guanine formed by the P140A mutant alkyltransferase.

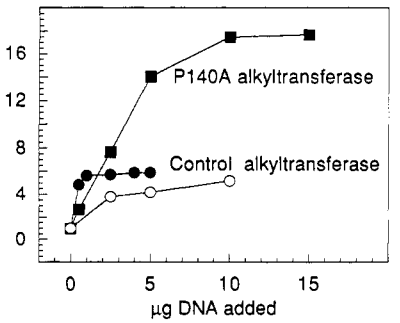

FIGURE 4: Effect of DNA concentration on conversion of O^6-benzylguanine to guanine. The effect of varying amounts of DNA as indicated on the production of guanine was measured as in Figure 3 using 6 μg of control alkyltransferase (circles) and 40 μg of P140A alkyltransferase (squares). The open circles show results using a closed circular plasmid DNA (pSAM320), and the filled circles and squares show results using calf thymus DNA. Results are expressed as the ratio of guanine production in the presence of DNA to guanine production in the absence of DNA.

followed by a second incubation in the presence of a labeled DNA substrate to assay the amount of activity remaining. Therefore, the effect of DNA on the production of guanine from O^6-benzylguanine was measured (Figure 2). We have previously shown that the formation of guanine is time dependent following second-order kinetics and ceases when all of the alkyltransferase has been inhibited (Pegg et al., 1993). Addition of DNA clearly increased the rate of guanine formation by both the control alkyltransferase (Figure 2A) and its P140A mutant (Figure 2B).

As previously reported (Crone & Pegg, 1993), the P140A alkyltransferase mutant was much less active than the wild-type AGT in producing guanine from O^6-benzylguanine (Figure 3). It was necessary to put in about 40 times more protein from this mutant compared to the wild-type in order to get the same amount of guanine released. However, the formation of guanine by this mutant was increased even more dramatically by DNA (Figures 2 and 3). The stimulation of the W100A mutant alkyltransferase by DNA was similar to that of the wild-type (Figure 3).

The effect of DNA concentration on the reaction was studied (Figure 4). Only 2–3 μg of calf thymus DNA was need to give a maximal stimulation of the control alkyltransferase, which amounted to about a 6-fold increase. About 10 μg of DNA was needed to give a maximal stimulation of the P140 mutant, but this amounted to a 16-fold increase. [It should be noted that, because of the lower ability of the mutant to react with O^6-benzylguanine, a much higher amount of protein (40 μg of the P140A as compared to 6 μg of the control protein) was used in this experiment.] A closed circular, double-stranded plasmid DNA also stimulated the control alkyltransferase and produced the same maximal stimulation, but 10 μg was needed.

In order to examine the requirements for DNA to bring about the activation of the alkyltransferase, various oligodeoxynucleotides were tested (Figure 5). Single-stranded oligodeoxynucleotides of 34–36 bases were equally, or more, effective than calf thymus DNA, giving increases of 4–7-fold with the control alkyltransferase and 15–28-fold with the P140A mutant. When complementary sequences were used and hybridized together, the effect was not increased, confirming that either single-stranded or double-stranded oligodeoxynucleotides can bring about the stimulation. The size of the oligodeoxynucleotide producing a maximal effect was investigated by using molecules of increasing size consisting of repeats of the sequence, $(TCAG)_n$ (Figure 6). The results show that an optimal effect was obtained with sequences of 12 nucleotides. RNA was also able to activate the human alkyltransferase. The production of guanine from O^6-benzylguanine was increased about 3-fold in the presence

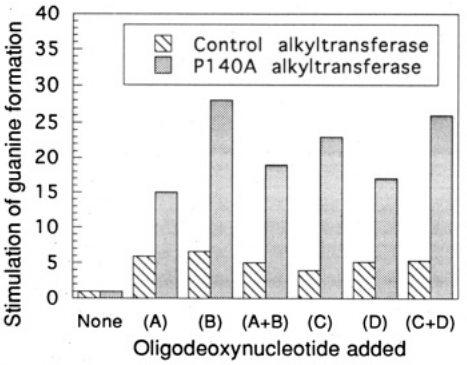

FIGURE 5: Effect of oligodeoxynucleotides on conversion of O^6-benzylguanine to guanine. Either the control (5 μg) or the P140A (40 μg) alkyltransferase was incubated with O^6-benzyl[^{3}H]guanine as described in Figure 3 in the presence of no addition or 5 μg of oligodeoxynucleotides (A, B, C, or D) as indicated and the amount of guanine formed determined. Results are given as the ratio of guanine produced in the presence of the oligodeoxynucleotide divided by that produced in its absence. The oligodeoxynucleotides wereas follows: A, 5′-CTAGAGGGTATTAATAATGGAAGCTGCACATTTCTT-3′; B, 5′-CGAAGAAATGTGCAGCTTCCATTATTAATACCCT-3′; C, 5′-CGAAAAAATGTGCAGCTTCCATCACCGTGAGACT-3′; and D, 5′-CTAGAGTCTCACGGTGATGGAAGCTGCACA-TTTTTT-3′. A and B are complementary, as are C and D, and when added together were annealed to ensure the formation of double-stranded DNA. Results are expressed as the ratio of guanine production in the presence of the oligodeoxynucleotide to guanine production with no addition.

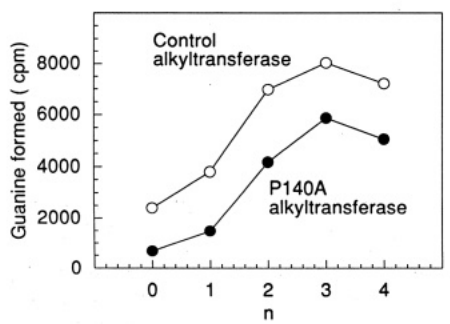

FIGURE 6: Effect of oligodeoxynucleotide length on conversion of O^6-benzylguanine to guanine. The oligodeoxynucleotide (5′-TCAG-3′)$_n$, where n = 1–4 as shown, was added to incubation mixture containing the control (5 μg) or P140A alkyltransferase (40 μg) as indicated. The production of guanine was measured after 10-min incubation as in Figure 3.

of 5 μg of tRNA (results not shown). This stimulation was not abolished by treatment of the tRNA preparation with DNase.

The rate of repair of methylated DNA by the mammalian alkyltransferases is reduced substantially by increasing ionic strength (Bhattacharyya et al., 1990). The effect of addition of NaCl on the production of guanine from O^6-benzylguanine was therefore tested in the presence or absence of DNA. The reaction in the absence of DNA was not affected at all by NaCl, but the stimulation by DNA was very sensitive to salt and was totally abolished by 0.2 M NaCl (Figure 7).

Human alkyltransferase is also inactivated by O^6-benzyldeoxyguanosine although this deoxynucleoside is less effective than the free base, O^6-benzylguanine (Moschel et al., 1992). We tested whether O^6-benzyldeoxyguanosine was converted into 2′-deoxyguanosine by the alkyltransferase, and as shown in Figure 8, this was the case. However, the production of 2′-deoxyguanosine from O^6-benzyldeoxyguanosine was not stimulated by DNA or oligodeoxynucleotides and was actually significantly reduced (Table 1). An experiment was carried out to test whether the presence of

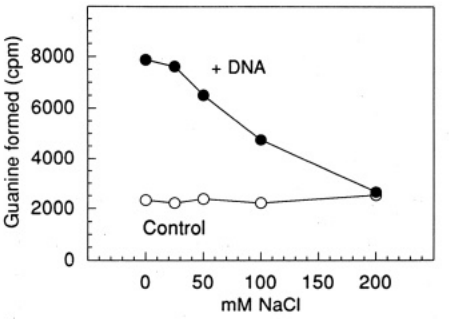

FIGURE 7: Effect of NaCl on production of guanine from O^6-benzylguanine. The effects of the concentration of NaCl shown on the conversion of O^6-benzylguanine into guanine by the control alkyltransferase were measured as in Figure 3.

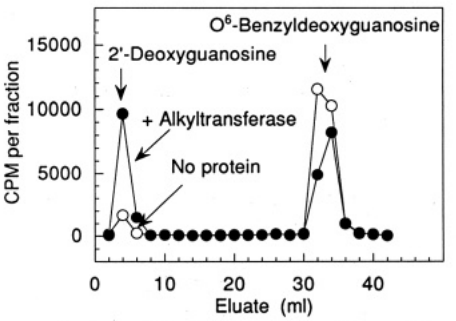

FIGURE 8: Production of 2′-deoxyguanosine from O^6-benzyldeoxyguanosine by human alkyltransferase. Assay buffer containing 0.23 μM [^{3}H]-labeled O^6-benzyldeoxyguanosine was incubated with no protein (open circles) or 30 μg of control alkyltransferase (closed circles) in a total volume of 0.25 mL for 20 min at 37 °C. The 2′-deoxyguanosine formed and remaining O^6-benzyldeoxyguanosine were then separated by HPLC, and 2-min fractions were collected and assayed for radioactivity as shown.

Table 1: Effect of DNA or Oligodeoxynucleotide on the Formation of Deoxyguanosine from O^6-Benzyldeoxyguanosine by Alkyltransferase[a]

alkyltransferase used	addition	deoxyguanosine formation (cpm)
control (30 μg)	none	13511
control (30 μg)	25 μg of DNA	7158
control (30 μg)	50 μg of DNA	5216
control (30 μg)	50 μg of (5′-TCAG-3′)$_4$	10032
P140A (200 μg)	none	4948
P140A (200 μg)	50 μg of DNA	2464
P140A (200 μg)	150 μg of DNA	1274
P140A (200 μg)	100 μg of (5′-TCAG-3′)$_4$	3939

[a] Assays were carried as described in the legend to Figure 8 with the addition of calf thymus DNA or the 16-mer, (5′-TCAG-3′)$_4$, as shown.

calf thymus DNA inhibited the inactivation of the control alkyltransferase by O^6-benzyldeoxyguanosine. The ED_{50} value [defined as in previous studies (Moschel et al., 1992) as the concentration needed to produce 50% inhibition in a 30-min exposure] was increased by at least an order of magnitude to more than 20 μM (results not shown).

DISCUSSION

These experimental results show the value of a low molecular weight pseudosubstrate to probe the mechanism of the alkyltransferase protein. In addition to allowing the independent analysis of the effects of DNA on the reaction, O^6-benzylguanine is acted upon relatively slowly by the alkyltransferase. The second-order rate constant was found to be about 600 $M^{-1}\cdot s^{-1}$ in previous studies using the purified human alkyltransferase in the absence of DNA (Pegg et al., 1993).

This is much less than that for O^6-methylguanine in double-stranded DNA substrates where values of (4–17) × 10^6 $M^{-1}s^{-1}$ have been reported for the mammalian protein and even greater values of up to 10^9 $M^{-1}s^{-1}$ were estimated for the *E. coli* Ogt and Ada carboxyl domain alkyltransferases (Yarosh, 1985; Dolan et al., 1988; Pegg & Dolan, 1989; Graves et al., 1989; Wilkinson et al., 1989; Demple, 1990; Bhattacharyya et al. 1990; Georgiadis et al., 1991; Mitra & Kaina, 1993; Chan et al., 1993; Liem et al., 1993). The high rate constant and the cumbersome assays for activity with methylated DNA substrates make it very difficult to measure initial rates of reaction even when the assay is carried out at 4 °C. Also, it is impossible to assay the effect of DNA independently when these substrates are used.

Our results show clearly that the conformational changes in alkyltransferase brought about by the binding of DNA, which have previously been detected by measurements of fluorescence or circular dichroism (Takahashi et al., 1990; Chan et al., 1993), facilitate the alkyltransferase reaction. It is probable that the conformational change in the protein allows the correct orientation of the O^6-alkylguanine for reaction with the cysteine acceptor. Recent studies of the crystal structure of the *E. coli* Ada carboxyl domain alkyltransferase indicate that such a change must occur since there is insufficient space to accommodate the substrate in the structure of the protein as crystallized in the absence of DNA (Moore et al., 1994). Furthermore, the mechanism of the alkyltransferase reaction suggested by the crystal structure involves a conformational change in the protein brought about by binding the DNA substrate which breaks the bond between a conserved glutamic acid residue and the histidine adjacent to the cysteine acceptor site, allowing the histidine to aid in the generation of the thiolate anion which acts as the nucleophile needed for the alkyl transfer (Moore et al., 1994). Although the Ada alkyltransferase does not react with O^6-benzylguanine, presumably due to steric hindrance (Dolan et al., 1991; Pegg et al., 1993), our results are consistent with this type of mechanism.

Previous studies have shown that the mutation of proline-140 to alanine in the alkyltransferase renders it less sensitive to inactivation by O^6-benzylguanine (Crone & Pegg, 1993). The amount of O^6-benzylguanine needed to give 50% inactivation of the alkyltransferase activity in a 30-min preincubation prior to assay of residual activity using a methylated DNA substrate was increased 40-fold by this mutation. When the assay was conducted in a competitive manner by adding the O^6-benzylguanine at the same time as the methylated DNA substrate, the difference was reduced to 12-fold (Crone & Pegg, 1993). These results are consistent with the findings reported here that the P140A mutant forms guanine from O^6-benzylguanine more slowly than the control alkyltransferase in the presence or absence of DNA but that the reaction of P140A is stimulated to a greater extent by DNA addition than the control (Figure 4). When the results were expressed in terms of the amount of protein added, the rate of formation of guanine from O^6-benzylguanine by control alkyltransferase occurred at 43 times the rate for the P140A mutant when assays were conducted in the absence of DNA and at 16 times the rate for the P140A mutant when DNA was present.

It was suggested that the resistance of the P140A alkyltransferase mutant to inactivation by O^6-benzylguanine occurs because of steric factors such that the access of the drug to the active site is limited when this proline is substituted (Crone & Pegg, 1993). The greater effect of DNA addition on this mutant could therefore be due to the conformational change brought about by DNA increasing the size of the space available for substrate binding. It is noteworthy that the *E. coli* Ada carboxyl domain alkyltransferase, which clearly must undergo a conformational change before even an O^6-methylguanine can be accommodated (Moore et al., 1994), is totally resistant to O^6-benzylguanine (Dolan et al., 1991; Pegg et al., 1993) and contains an alanine residue in the equivalent position to P140 in the human alkyltransferase (Lindahl et al., 1988; Mitra & Kaina, 1993; Moore et al., 1994).

The strong inhibitory effect of salt on the rate of repair of methylated DNA substrates by the alkyltransferase (Bhattacharyya et al., 1990) appears to be due to the interference with the DNA activation. As shown in Figure 7, there was no effect of increasing NaCl up to 0.2 M on the conversion of O^6-benzylguanine to guanine, but the ability of DNA to stimulate this reaction was completely abolished by the added salt. This conclusion is consistent with studies measuring protein fluorescence which showed that the binding constant for DNA was reduced by 2 orders of magnitude on increasing NaCl from 0.1 to 0.2 M for the Ada protein (Takahashi et al., 1990) and that DNA binding was abolished by adding 0.3 M NaCl to the mammalian alkyltransferase (Chan et al., 1993). Furthermore, the approximately 5-fold reduction in repair rate for methylated DNA seen at 0.2 M NaCl (Mitra & Kaina, 1993) is close to the extent of stimulation by DNA of the reaction with O^6-benzylguanine. As pointed out by Mitra and Kaina (1993), the intracellular ionic strength is likely to be sufficient to slow the alkyltransferase reaction down significantly *in vivo*. This may account for the fact that the disappearance of O^6-methylguanine from DNA after exposure to methylating agents takes a few hours whereas the repair of methylated DNA *in vitro* is accomplished in minutes (Yarosh, 1985; Pegg, 1990).

Significant alterations in the rate of alkyltransferase activity with various substrates and assay conditions have been observed when recombinant fusion proteins of glutathione *S*-transferase at the amino terminus of the human alkyltransferase protein sequence were used (Liem et al., 1993; Morgan et al., 1993). An explanation for these anomalous results may be related to the inability of these fusion proteins to undergo the normal change in configuration on binding DNA. Results with such fusion proteins should be interpreted with caution until experimental proof is available that they do accurately reflect the structure of the native alkyltransferase.

Our results indicate clearly that the activation of the alkyltransferase reaction toward O^6-benzylguanine is not greater when double-stranded DNA is used. Single-stranded DNA is clearly adequate to bring about a maximal stimulation. This contrasts to the actual repair of methylated DNA substrates, which is 2 orders of magnitude greater with double-stranded substrates (Scicchitano et al., 1986; Demple, 1990; Bhattachyyra et al., 1990; Pegg & Byers, 1992; Liem et al., 1993). It is quite possible that it is the binding of the opposite strand that activates the protein to repair O^6-methylguanine in the other strand. The lack of correlation between the ability to stimulate the alkyltransferase reaction on O^6-benzylguanine and the ability to serve as substrates is emphasized by the finding that tRNA also stimulates the reaction. Although O^6-methylguanine in tRNA was reported to be repaired by the *E. coli* Ada alkyltransferase (Karran, 1985), the rate of repair was found to be at most 4 orders of magnitude lower than the rate of O^6-methylguanine repair in double-stranded DNA substrates (Pegg et al., 1988).

Our results also indicate that a length of 12 or more deoxynucleotides is needed to get a maximal activation. This is only slightly more than the values of 7 or 8 that were calculated on the basis of fluorescence measurements as being needed to occupy the binding site (Takahashi et al., 1990; Chan et al., 1993) and agrees well with studies of the minimum size needed for optimal repair of oligodeoxynucleotides containing O^6-methylguanine (Scicchitano et al., 1986; Dolan et al., 1988; Liem et al., 1993). Our results suggest that attempts to cocrystallize the alkyltransferase protein with oligodeoxynucleotides of 8–12 bases may be needed to obtain crystals indicating the active configuration of the protein.

At present, there is little information on the location of the alkyltransferase DNA binding site. The assay using the stimulation of the conversion of O^6-benzylguanine to guanine provides a useful tool to identify this site. Since W100A and P140A do not reduce the stimulation by DNA, it is clear that these residues are not essential for DNA binding. The fact that W100A does not influence the response to DNA is particularly interesting since this residue is conserved in all known alkyltransferases and is located in a helix–turn–helix region that may form a DNA binding motif (Moore et al. 1994).

Finally, our results provide at least a partial explanation for the fact that O^6-benzyldeoxyguanosine was found to be a less potent inactivator of human alkyltransferase than O^6-benzylguanine both in crude HT29 cell extracts and in HT29 cell cultures (Moschel et al., 1992). At first sight, this finding is surprising since the deoxynucleoside more closely resembles the physiological DNA substrate than the free base. The demonstration that 2′-deoxyguanosine is formed by the alkyltransferase acting on O^6-benzyldeoxyguanosine (Figure 8) confirms that this deoxynucleoside does act as a substrate for the protein. However, our results show clearly that not only is the reaction of the alkyltransferase with O^6-benzyldeoxyguanosine not stimulated by DNA but it is, in fact, inhibited (Table 1). These results suggest that the binding of O^6-benzyldeoxyguanosine alone is not sufficient to trigger the activation of the alkyltransferase brought about by oligodeoxynucleosides. Furthermore, the inhibition of the reaction by DNA may result from competition between added DNA and the O^6-benzyldeoxyguanosine for the binding site or be due to the conformational change produced by DNA disfavoring the reaction of the O^6-benzyldeoxyguanosine. The free base, O^6-benzylguanine, reacts more rapidly because it can be bound in the correct orientation to the activated form of the alkyltransferase which also contains bound DNA.

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REFERENCES

Bhattacharyya, D., Tano, K., Bunick, G. J., Uberbacher, E. C., Bhenke, W. D., & Mitra, S. (1988) *Nucleic Acids Res. 16*, 6397–6410.

Bhattacharyya, D., Foote, R. S., Boulden, A. M., & Mitra, S. (1990) *Eur. J. Biochem. 193*, 337–343.

Chan, C., Wui Z., Ciardelli, T., Eastman, A., & Bresnick, E. (1993) *Arch. Biochem. Biophys. 300*, 193–200.

Crone, T. M., & Pegg, A. E. (1993) *Cancer Res. 53*, 4750–4753.

Demple, B. (1990) in *Protein Methylation* (Paik, W. K., & Kim, S., Eds.) Chapter 17, pp 285–304, CRC Press, Boca Raton, FL.

Dolan, M. E., Scicchitano, D., & Pegg, A. E. (1988) *Cancer Res. 48*, 1184–1188.

Dolan, M. E., Moschel, R. C., & Pegg, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5368–5372.

Dolan, M. E., Pegg, A. E., Dumenco, L. L., Moschel, R. C., & Gerson, S. L. (1991) *Carcinogenesis 12*, 2305–2310.

Georgiadis, P., Smith, C. A., & Swann, P. F. (1991) *Cancer Res. 51*, 5843–5850.

Gonzaga, P. E., Potter, P. M., Niu, T., Yu, D., Ludlum, D. B., Rafferty, J. A., Margison, G. P., & Brent, T. P. (1992) *Cancer Res. 52*, 6052–6058.

Graves, R. J., Li, B. F. L., & Swann, P. F. (1989) *Carcinogenesis 10*, 661–666.

Karran, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5285–5289.

Lee, S. M., Rafferty, J. A., Elder, R. H., Fan, C. Y., Bronley, M., Harris, M., Thatcher, N., Potter, P. M., Altermatt, H. J., Perinat-Frey, T., Cerny, T., O'Connor, P. J., & Marginson, G. P. (1992) *Br. J. Cancer 66*, 355–360.

Liem, L. K., Wong, C. W., Lim, A., & Li, B. F. L. (1993) *J. Mol. Biol. 231*, 950–959.

Lindahl, T., Sedgwick, B., Sekiguchi, M., & Nakabeppu, Y. (1988) *Annu. Rev. Biochem. 57*, 133–1575.

Ling-Ling, C., Nakamura, T., Nakatsu, Y., Sakumi, K., Hayakawa, H., & Sekiguchi, M. (1992) *Carcinogenesis 13*, 837–843.

Mitra, S., & Kaina, B. (1993) *Prog. Nucleic Acid Res. Mol. Biol. 44*, 109–142.

Moore, M. H., Gulbus, J. M., Dodson, E. J., Demple, B., & Moody, P. C. E. (1994) *EMBO J. 13*, 1495–1501.

Morgan, S. E., Kelley, M. R., & Pieper, R. O. (1993) *J. Biol. Chem. 268*, 19802–19809.

Moschel, R. C., McDougall, M. G., Dolan, M. E., Stine, L., & Pegg, A. E. (1992) *J. Med. Chem. 35*, 4486–4491.

Pegg, A. E. (1990) *Cancer Res. 50*, 6119–6129.

Pegg, A. E., & Dolan, M. E. (1989) in *DNA Repair Mechanisms and Their Biological Implications in Mammalian Cells* (Lambert, M. W., & Laval, J., Eds.) pp 45–59, Plenum Press, New York.

Pegg, A. E., & Byers, T. L. (1992) *FASEB J. 6*, 2302–2310.

Pegg, A. E., Morimoto, K., & Dolan, M. E. (1988) *Chem.-Biol. Interact. 65*, 275–281.

Pegg, A. E., Boosalis, M., Samson, L., Moschel, R. C., Byers, T. L., Swenn, K., & Dolan, M. E. (1993) *Biochemistry 32*, 11998–12006.

Potter, P. M., Lassiter, A., & Brent, T. P. (1994) *Mol. Cell. Biol.* (in press).

Scicchitano, D., Jones, R. A., Kuzmich, S., Gaffney, B., Lasko, D. D., Essigmann, J. M., & Pegg, A. E. (1986) *Carcinogenesis 7*, 1383–1386.

Spratt, T. E., & de los Santos, H. (1992) *Biochemistry 31*, 3688–3694.

Takahashi, M., Sakumi, K., & Sekiguchi, M. (1990) *Biochemistry 29*, 3431–3436.

Wilkinson, M. C., Potter, P. M., Cawkwell, L., Georgiadis, P., Patel, D., Swann, P. F., & Margison, G. P. (1989) *Nucleic Acids Res. 17*, 8475–8484.

Yarosh, D. B. (1985) *Mutat. Res. 145*, 1–16.