Repair of $O^6$-Benzylation of guanine by the *Escherichia coli* Ada and Ogt and the Human $O^6$-Alkylguanine-DNA Alkyltransferases*

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$O^6$-Methylguanine is removed from DNA via the transfer of the methyl group to a cysteine acceptor site present in the DNA repair protein $O^6$-alkylguanine-DNA alkyltransferase. The human alkyltransferase is inactivated by the free base $O^6$-benzylation, raising the possibility that substantially larger alkyl groups could also be accepted as substrates. However, the *Escherichia coli* alkyltransferase, Ada-C, is not inactivated by $O^6$-benzylation. The Ada-C protein was rendered capable of reaction by the incorporation of two site-directed mutations converting Ala~316~ to a proline (A316P) and Trp~336~ to alanine (W336A) or glycine (W336G). These changes increase the space at the active site of the protein where Cys~321~ is buried and thus permit access of the $O^6$-benzylguanine inhibitor. Reaction of the mutant A316P/W336A-Ada-C with $O^6$-benzylguanine was greatly stimulated by the presence of DNA, providing strong support for the concept that binding of DNA to the Ada-C protein activates the protein. The Ada-C protein was able to repair $O^6$-benzylation in a 16-mer oligodeoxyribonucleotide. However, the rate of repair was very slow, whereas the *E. coli* Ogt, the human alkyltransferase, and the mutant A316P/W336A-Ada-C alkyltransferases reacted very rapidly with this 16-mer substrate and preferentially repaired it when incubated with a mixture of the methylated and benzylated 16-mers. These results show that benzyl groups are better substrates than methyl groups for alkyltransferases provided that steric factors do not prevent binding of the substrate in the correct orientation for alkyl group transfer.

The protein $O^6$-alkylguanine-DNA alkyltransferase is an important component of the cellular resistance to the toxic and mutagenic effects of alkylating agents (1–4). This protein has now been reported to be present in a wide range of organisms, and cDNA-derived amino acid sequences are available for at least 13 distinct proteins (2, 4–6). The alkyltransferase repair proteins act by transferring alkyl groups from the $O^6$-position of guanine onto a cysteine acceptor site located in the protein sequence. In all known alkyltransferases, this cysteine residue is contained within the sequence PCHR, and there are many other highly conserved residues. This provides strong evidence that the DNA reactions brought about by these proteins are the same irrespective of species. *Escherichia coli* contains two alkyltransferases, the products of the *osta* gene and the *ada* gene. The content of Ogt protein remains constant, whereas the content of Ada is highly inducible in response to alkylating agents (1, 3).

The *E. coli* Ada protein is the best characterized of all of the alkyltransferases, and a crystal structure at 2.1-Å resolution for the carboxyl-terminal domain of this protein (Ada-C),¹ which is fully active in repair of $O^6$-methylguanine, has been published (6, 7). This structure shows that the cysteine acceptor site is buried, and the authors suggest that the protein must undergo a significant conformational change upon binding a DNA substrate and that this alteration facilitates the transfer reaction.

There has been considerable interest in the development of inactivators of human alkyltransferase (AGT) because it is well established that the presence of this protein in tumor cells imparts resistance to killing by both therapeutic methylating agents such as dacarbazine and temozolomide and chloroethylating agents such as 1,3-bis(2-chloroethyl)1-nitrosourea (8). One of the most promising compounds that has been used as an inactivator is $O^6$-benzylguanine (9). The basis for this inactivation is well understood. The base analog is recognized as a substrate by the human alkyltransferase, and the benzyl group is transferred to the active-site cysteine, forming an S-benzylcysteine adduct and inactivating the protein (10). However, little is known of the means by which $O^6$-benzylguanine binds to the active site, and despite the similarity in amino acid sequence, the Ada-C protein was not inactivated by $O^6$-benzylguanine (10–12). It was suggested that this difference is due to a restriction of the space available at the active site in the Ada protein (13). Such a steric effect could also account for published differences in the relative efficiencies with which alkyltransferases from different species repair substrates containing larger alkyl groups (2, 4, 14, 15). Although methyl groups have been reported to be the best substrate for all of these repair proteins, larger alkyl groups are also repaired at slower rates, and the ability to act on these groups is quite species-specific.

In the present work, we have investigated the ability of the Ada-C, Ogt, and AGT proteins to react with $O^6$-benzylguanine as a free base and when incorporated into a 16-mer oligodeoxyribonucleotide. Site-directed mutation of the Ada-C protein

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¹ The abbreviations used are: Ada-C, the carboxyl-terminal domain of the *E. coli* $O^6$-alkylguanine-DNA alkyltransferase; AGT, human $O^6$-alkylguanine-DNA alkyltransferase; bp, base pair; HPLC, high performance liquid chromatography.
to increase the size of the pocket surrounding the cysteine acceptor produced a protein that was able to react with free Oβ-benzylguanine, and the rate of this reaction was greatly increased when DNA was added. The mutated Ada-C protein and the Ogt were very effective in repairing Oβ-benzylguanine contained within an oligodeoxynucleotide, and both these proteins and the AGT preferentially removed benzyl groups from such substrates rather than methyl groups. These results provide further insight into the alkyltransferase reaction and into the design of therapeutically useful inactivators.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oβ-benzylguanine, 2,4-diamino-6-benzyloxy-5-nitropyrimidine, and 2,4-diamino-6-benzyloxy-5-nitropyrimidine were synthesized as described previously (9, 16). All nonalkylated oligodeoxynucleotides were made in the Macromolecular Core Facility, Hershey Medical Center, by using a Milligen 7500 DNA synthesizer. Restriction enzymes were purchased from Life Technologies, Inc. and New England Biolabs (Beverly, MA). Ampicillin, kanamycin, isopropyl β-D-thiogalactopyranoside, and most other chemicals were purchased from Sigma. Plasmid pGem-3Zf (+) and T4 polynucleotide kinase were purchased from Promega (Madison, WI). Pfu DNA polymerase and the Chamaeleon™ double-stranded mutagenesis kit were purchased from Stratagene (La Jolla, CA). N1-Hemithym-N-nitrosourea (5.9 mCi/μmol) was obtained from Amersham Corp.

Oβ-Benzyl[8-3H]guanine (0.34 mCi/μmol) was prepared by catalytic tritium exchange of Oβ-benzylguanine with tritiated water by Amersham Corp. and was purified as described previously (10).

**Synthesis of the 16-mer oligodeoxynucleotide** 5'-dAACAGCCAATCCGTGCACG-3' in which aG = Oβ-benzylguanine or Oβ-methylguanine was described previously (17). Samples were repurified by HPLC (18) as needed.

**Expression of Ogt and Control and Mutant Ada-C Proteins—**These proteins were expressed in *E. coli* JM109 cells using the pQE30 vector (Qiagen) for the expression of the protein with a small extension [Met-Arg-Gly-Ser-(His)6-Gly-Ser] at the amino terminus. To express the control Ada-C, the region representing the cDNA from pGEMADA (13) by polymerase chain reaction using primers introducing a BamHI site (underlined) at the 5' end (5'-GGCGATCCATGCTGACCTACTGAGTTCTGAAA-3') and a KpnI site (underlined) after the stop codon (italic) at the 3' end (5'-GCTATGGGTACCTACGAAAGATACCC-3') using the same conditions as described above for control Ada-C construct. The 531-bp product was digested with BamHI and KpnI enzymes and ligated into pQE30 vector digested with the same enzymes to form pQE30-Ogt. The ligation mixture was then electroplated into JM109 cells.

**Purification of Ogt and Control and Mutant Ada-C Proteins—**JM109 cells transformed with pQE30-Ogt, pQE30-Ada-C, or one of the mutants were grown in a 1-liter culture inoculated with a grown-overnight culture at 1:50 dilution. At a cell density equivalent to an *A$_{600}$* of 0.5, isopropyl-β-D-thiogalactopyranoside was added to final concentration of 0.3 mM, and the cells were harvested 4 h later. The pellet was suspended in 30 ml of 20 mM Tris-HCl, pH 8.0, 250 mM NaCl and disrupted using a French press. After centrifugation at 17,000 × *g* for 45 min at 4 °C to remove cell debris, the supernatant was applied to a 2-ml column of Talon IMAC resin (Clonetech) equilibrated with 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, and the column was washed with this buffer containing 10 mM imidazole. The Ada-C protein was then eluted using 200 mM imidazole, and the fractions found to contain Ada-C by SDS-polyacrylamide gel electrophoresis were pooled and dialyzed immediately against 50 mM Tris-HCl, pH 7.6, 250 mM NaCl, 5 mM dithiothreitol, and 0.1 mM EDTA. The yield of protein was about 8 mg/liter of culture, and the purity was >90% as judged by SDS-polyacrylamide gel electrophoresis.

**Purification of AGT—**AGT was expressed and purified to homogeneity by ammonium sulfate precipitation, chromatography on Mono-S, and gel-filtration as described previously (10, 20).

**Assay of Dealkylation of Oligodeoxynucleotides Containing Oβ-Methylguanine or Oβ-Benzylguanine—**The purified alkyltransferase proteins were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, and 0.1 mM EDTA with 5'-dAACAGCCAATCCGTGCACG-3' in which aG represents Oβ-benzylguanine or Oβ-methylguanine. The reaction was stopped by the addition of 1% SDS, and the mixture of alkyltransferase and Ogt was very effective in repairing Oβ-benzylguanine oligodeoxynucleotides that were detected by absorbance at 254 nm were 95.9, 15, and 21 min, respectively.

**Assay of Sensitivity to Inactivation by Oβ-Benzylguanine and Related Compounds—**The purified Ada-C protein or mutant Ada-C proteins were incubated with Oβ-benzylguanine or other inhibitors in 0.5 ml of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 5.0 mM dithiothreitol in the presence or absence of calf thymus DNA as shown for 30 min at 37 °C. The residual alkyltransferase activity was then determined by a 30-min incubation with a ^H-methylated DNA substrate that had been methylated by reaction with N1-Hemithym-N-nitrosourea essentially as described (19, 20).

The alkyltransferase samples were incubated with 7 μg of ^H-methylated DNA substrate containing 7,000 cpm of [3H]methylguanine and 0.36 μg of carrier calf thymus DNA in 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, and 0.1 mM EDTA in a total volume of 1 ml at 37 °C for 30 min. The DNA was then precipitated with 0.25% perchloric acid at 4 °C and hydrolyzed by heating in 0.5 ml of 0.1 N HCl at 70 °C for 30 min. This procedure was repeated, and the combined hydrolysates were taken, neutralized by the addition of 0.15 ml of 1 M Tris-HCl, and filtered through a circular 0.45-μm filter (Millipore). The labeled 7-methylguanine and Oβ-methylguanine present were then separated by HPLC on a Beckman Ultrosphere ODS column (25 × 0.46 cm) using isocratic elution at 37 °C with 0.05 mM ammonium formate, pH 4.5, containing 12% methanol. The eluate was monitored for radioactivity by mixing with 3.5 parts of Flow Scint III (Packard Instruments, Meriden, CT) and passing through a Radiomatic Flo-One/Beta A-140A radioactivity monitor (Packard Instruments). The counting efficiency was 35%.

The results were expressed as the percentage of the alkyltransferase remaining. Less than 5% of the activity was lost on incubation in the absence of the inhibitors. The graphs of activity remaining against inhibitor concentration were used to calculate an *IC*$_{50}$ value representing the amount of inhibitor needed to produce a 50% loss of activity.

**Reaction of AGT with Oβ-Benzylguanine—**Measurements of [8-3H]guanine formation from Oβ-benzyl[8-3H]guanine were carried out using an assay mixture consisting of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 5 μM dithiothreitol in a volume of 0.25 ml in the presence
or absence of DNA 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 guanine and 0.2 mM O6-benzylguanine. Aliquots were then separated by reverse-phase-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.

**RESULTS**

Experiments were carried out using the carboxyl-terminal domain of Ada-C, which is responsible for the activity to repair O6-methylguanine. The protein expressed contained residues 179–354 of the full-length Ada protein. For comparison, all amino acid residues are indicated by number according to the full-length sequence. The key region of the protein containing the active-site cysteine residue that is located between Asn313 and Lys341, which are conserved in all known alkyltransferase sequences, is shown in Fig. 1.

Purified Ada-C was totally resistant to inactivation by O6-benzylguanine, and the resistance was not altered when calf thymus DNA was added (Fig. 2a). This confirms previous studies in which crude cell extracts of *E. coli* were used (10–13) and rules out the possibility that the resistance is due to the rapid metabolism of the O6-benzylguanine by bacterial enzymes. The crystal structure of Ada-C suggests that the tryptophan residue at position 336 may limit access to the cysteine acceptor site (6, 21) and that inactivation is accompanied by the formation of a protein–guanine adduct (Fig. 1). This confirms previous studies with the human AGT that show that O6-benzylguanine inactivates the protein by acting as a pseudosubstrate and that inactivation is accompanied by the formation of a protein–guanine adduct (22, 23).

The equivalent position (316) in the Ada-C protein is occupied by an alanine. Therefore, a double mutant of the Ada-C protein was made in which Trp336 was changed to Ala and Ala316 was changed to Pro (13) (see Fig. 1). This protein was purified to homogeneity and found to be definitely sensitive to inactivation by O6-benzylguanine (Fig. 2a), but the inhibition was relatively weak with an ED\textsubscript{50} value of 280 μM (Table I). This compares to a value of about 0.3 μM for the inactivation of human AGT by O6-benzylguanine assayed under the same conditions (22). However, with the A316P/W336A-Ada-C mutant, there was a large stimulation of the rate of inactivation by O6-benzylguanine when DNA was present (Fig. 2a). The ED\textsubscript{50} value was reduced by 28-fold to 10 μM in the presence of DNA.

Benzyl-substituted pyrimidine derivatives have been shown to be even more potent inhibitors of AGT than O6-benzylguanine (16). These inhibitors, 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine (Fig. 2b) and 2,4-diamino-6-benzyloxy-5-nitropyrimidine (Fig. 2c), had little effect on the activity of the Ada-C. However, the A316P/W336A-Ada-C mutant was readily inhibited by these compounds in the presence of DNA, with ED\textsubscript{50} values of 5 and 8 μM (Fig. 2, b and c, respectively). Inactivation was increased in the presence of DNA, but the stimulation was only 5–6-fold.

Although A316P/W336A-Ada-C is clearly sensitive to these inhibitors, it is still 2 orders of magnitude more resistant than the human AGT for which the ED\textsubscript{50} values in the presence of DNA are 0.1 μM for O6-benzylguanine (Table I) and 0.04 μM for 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine and 2,4-diamino-6-benzyloxy-5-nitropyrimidine (16). This difference could not be reduced by changing Trp336 to G1y, which occupies the equivalent position in AGT, or by including a third mutation in which Lys341 was changed to Pro (Table I). (The rationale for introducing a second Pro at this position is that all of the O6-benzylguanine-sensitive mammalian AGT sequences in-
formation of S-benzylcysteine at the active site with the liberation of guanine (10, 24). The rate of formation of guanine can therefore be used to measure the ability of the alkyltransferase to react with O⁶-benzylguanine. The inability of Ada-C alkyltransferase to react with O⁶-benzylguanine was confirmed in this way because no [8-³H]guanine was detected even when 300 μg of protein was incubated for up to 3 h with O⁶-benzyl[8-³H]guanine (Table I). Formation of [8-³H]guanine by A316P/W336A-Ada-C was easily measured, and the rate increased 24-fold in the presence of DNA (Table I). K314P/A316P/W336G-Ada-C was slightly less active in guanine production but was also stimulated 25-fold by DNA. The A316P/W336G-Ada-C and K314P/A316P/W336G-Ada-C mutants were also slightly less active, and guanine production was stimulated only 17-fold. These results agree well with the sensitivity to inactivation by O⁶-benzylguanine. They are also consistent with the ED₅₀ and the level of [8-³H]guanine production by the human AGT. The latter is about 100 times greater than the formation from A316P/W336A-Ada-C (Table I).

The effect of DNA concentration on the stimulation of [8-³H]guanine formation from O⁶-benzyl[8-³H]guanine was measured using a 16-mer oligodeoxyribonucleotide (Fig. 3). Maximal stimulation required the addition of about 10 μg (2 nmol) of the oligodeoxyribonucleotide and amounted to 25-fold and 17-fold, depending on whether residue Trp³⁵⁶ was Ala or Gly (Fig. 3). When the human AGT was used, the maximal stimulation was only 6-fold (Fig. 3), in good agreement with earlier reports (25). Although the experiment shown in Fig. 3 was carried out with a small oligodeoxyribonucleotide, the maximal stimulation values obtained agree well with those found when high molecular weight calf thymus DNA was used (Table I).

The production of guanine from O⁶-benzylguanine in the absence of DNA by A316P/W336A-Ada-C was relatively insensitive to salt, with no decrease at 0.2 M NaCl and only a 22% reduction when 0.5 M NaCl was added (Fig. 4, insert). In contrast, stimulation of the activity by DNA was very salt-sensitive, with an 82% reduction at 0.2 M NaCl and complete abolition of the stimulation at 0.5 M (Fig. 4). This result is consistent with either a strong inhibition of DNA binding by salt or its interference with the activation of the protein structure caused by DNA binding.

The ability of the human and E. coli alkyltransferase preparations to react with O⁶-benzylguanine when it is incorporated into an oligodeoxyribonucleotide was examined by using a 16-mer oligodeoxyribonucleotide that contains O⁶-benzylguanine. The AGT, Ada-C, and A316P/W336A-Ada-C proteins were each incubated with this substrate for 10 min, and the production of the unalkylated form of the 16-mer oligodeoxyribonucleotide was measured after HPLC separation of substrate and product (Fig. 5a). All of the proteins were clearly able to remove benzyl groups from this substrate, but considerably more of the Ada-C protein was needed to achieve the same level of repair, and even with a considerable molar excess of the protein, repair was not complete. This suggests that repair is much slower by this protein than by AGT or A316P/W336A-Ada-C. This was confirmed by measuring the time course of repair at the benzylated 16-mer oligodeoxyribonucleotide (Fig. 5b). Repair by AGT or A316P/W336A-Ada-C was so rapid that the reaction had gone to completion within 1 min. In contrast, even with 5 times the amount of Ada-C protein, about 20 min were required for complete reaction (Fig. 5b).

To compare the abilities of the alkyltransferases to repair O⁶-benzylguanine with the repair of O⁶-methylguanine, the proteins were incubated with a mixture of the 16-mer oligodeoxyribonucleotide containing O⁶-benzylguanine and a 16-mer

![Fig. 3. Effect of an oligodeoxyribonucleotide on the production of guanine from O⁶-benzylguanine by AGT, A316P/W336A-Ada-C, and A316P/W336G-Ada-C. The alkyltransferases were incubated with 0.7 μM O⁶-benzyl[8-³H]guanine in the presence of the 16-mer oligodeoxyribonucleotide, 5'-dTACGTACGTACGTACG-3', as indicated. The assay was carried out with 100 μg of the Ada-C mutants for 1 h and with 2 μg of AGT for 20 min at 37 °C.](image)

![Fig. 4. Effect of NaCl on the production of guanine from O⁶-benzylguanine by AGT, A316P/W336A-Ada-C. The assay was carried out with 0.7 μM O⁶-benzyl[8-³H]guanine and 100 μg of A316P/W336A-Ada-C in the presence and absence of calf thymus DNA (100 μg) and the salt concentration shown for 1 h at 37 °C. Insert, an expanded scale for the assays carried out in the absence of DNA.](image)
oligodeoxyribonucleotide of identical sequence containing O\textsuperscript{6}-methylguanine (Fig. 6). The Ada-C efficiently repaired the O\textsuperscript{6}-methylguanine in this mixture, but repair of O\textsuperscript{6}-benzylguanine was only apparent when all of the methylated substrate had been repaired. In contrast, the AGT and the A316P/W336A-Ada-C mutant proteins repaired both adducts, but these proteins preferentially acted on O\textsuperscript{6}-benzylguanine because the peak corresponding to the 16-mer oligodeoxyribonucleotide containing O\textsuperscript{6}-benzylguanine was reduced to a greater extent than that which represents the 16-mer oligodeoxyribonucleotide containing O\textsuperscript{6}-methylguanine. Thus, benzyl groups are actually preferred substrates for both of these alkyltransferases.

E. coli contains a second alkyltransferase gene (1, 26, 27). Its product, the Ogt protein, is a constitutive alkyltransferase. As shown in Fig. 5, Ogt protein very rapidly repairs O\textsuperscript{6}-benzylguanine contained in the 16-mer oligodeoxyribonucleotide. When tested by incubation with the mixture of the 16-mer oligodeoxyribonucleotides containing O\textsuperscript{6}-benzylguanine and O\textsuperscript{6}-methylguanine, the Ogt protein repaired both adducts at comparable rates (Fig. 6). Previous studies have shown that Ogt shows some sensitivity to inactivation by O\textsuperscript{6}-benzylguanine but is considerably less sensitive than the human AGT (10, 11). Thus, the Ogt protein resembles the A316P/W336A-Ada-C mutant in both a weak ability to react with O\textsuperscript{6}-benzylguanine as a free base and a rapid reaction with this adduct when it is present in an oligodeoxyribonucleotide. As shown in Fig. 1, the Ogt protein has glycine in the position equivalent to Trp\textsuperscript{336} of Ada-C and does contain a proline residue at the position equivalent to Lys\textsuperscript{314}.

DISCUSSION

Preliminary results in model systems consisting of human tumors grown as xenografts in mice have suggested that O\textsuperscript{6}-benzylguanine may be a useful drug to enhance chemotherapy by certain alkylating agents (4, 28–30). Phase I clinical trials to develop this concept are currently underway. However, irrespective of the results of these trials, O\textsuperscript{6}-benzylguanine may
not be an ideal agent for this purpose. It is only sparingly soluble in water, and a polyethylene glycol-based vehicle is being used for administration to achieve plasma concentrations adequate to reduce the human AGT level adequately. The more active pyrimidine analogs of $O_6$-benzylguanine such as 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine are very rapidly degraded and excreted (31, 32). More potent water-soluble and metabolically stable derivatives of $O_6$-benzylguanine may therefore offer advantages, but the lack of a clear understanding of how $O_6$-benzylguanine binds to the active site of the protein and is recognized as a substrate is a handicap to such studies. Molecular modeling approaches would be a useful way to facilitate the design of better inhibitors, but at present the absence of a three-dimensional structure for the AGT protein prevents this approach. Because the crystallization and determination of the structure of Ada-C has already been accomplished (6, 7), the identification of mutant forms of Ada-C that are able to react with $O_6$-benzylguanine should facilitate such modeling studies.

Our results provide strong support for the concept that the inability of certain alkyltransferases to react with $O_6$-benzylguanine is due to steric factors blocking the access of the relatively bulky benzyl group to the cysteine acceptor site (13). Neither the W336A mutation as shown here nor the P316A mutation (13) alone are sufficient to impart significant sensitivity of Ada-C to $O_6$-benzylguanine. This steric hindrance is therefore provided by: (a) the presence of a bulky tryptophan residue at position 336, which in the sensitive mammalian alkyltransferases is occupied by a glycine; and (b) the absence of a proline residue at the position-5 residues on the amino-terminal side of the active-site cysteine. All known mammalian alkyltransferases, which are readily inactivated by $O_6$-benzylguanine, have this proline, and most of the microbial alkyltransferases lack it. The yeast alkyltransferase, which is totally refractory to $O_6$-benzylguanine (10), lacks this proline and has a tryptophan residue at the equivalent position of tryptophan336 in Ada-C. The *E. coli* Ogt protein, which is weakly reactive with $O_6$-benzylguanine (10, 11), lacks both the tryptophan and this proline but has a proline residue in the $2^7$ position. All of the mammalian alkyltransferases have proline residues in both the $2^7$ and $2^5$ positions. Mutation of either of these residues to alanine imparts some resistance of the human alkyltransferase to $O_6$-benzylguanine, but changing both has a much greater effect (22). It is therefore possible that the second proline at the $2^7$ position in Ogt contributes to its ability to repair $O_6$-benzylguanine.

The crystal structure of the Ada-C protein indicates that a change in the conformation of the protein must occur in response to DNA binding, and it was postulated that this activates the protein (6, 7). Our results showing that the conversion of $O_6$-benzylguanine to guanine by the mutant A316P/W336A-Ada-C is stimulated about 25-fold by addition of DNA at low salt concentrations are consistent with this hypothesis. It is noteworthy that the stimulation of the formation of guanine from $O_6$-benzylguanine by the mutant A316P/W336A-Ada-C by the 16-mer oligodeoxyribonucleotide (Fig. 2) was maximal with only about 2 nmol of the 16-mer oligodeoxyribonucleotide, even though the reaction contained 5 nmol of Ada-C.

![Fig. 6. HPLC analysis of the competition for repair of $O_6$-methylguanine and $O_6$-benzylguanine in 16-mer oligodeoxyribonucleotides by AGT, Ada-C, A316P/W336A-Ada-C, or Ogt. A1–A4, results for AGT (0, 20, 60, and 80 µg); B1–B4, results for Ada-C (0, 25, 50, and 200 µg); C1–C4, results for A316P/W336A-Ada-C (0, 30, 65, and 85 µg); D1–D4, results for Ogt (0, 20, 60, and 80 µg). The proteins were incubated with a mixture of 600 pmol each of 5'-dAACAGCCTATm6GGCCC-3' and 5'-dAACAGCCTATb6GGCCC-3' in a total volume of 0.05 ml for 10 min at 37 °C. The arrow marked peak 1 coincides with the elution of an authentic marker of 5'-dAACAGCCTATGCCC-3'. The arrow marked peak 2 coincides with the elution of 5'-dAACAGCCTATm6GGCCC-3', and the arrow marked peak 3 coincides with the elution of 5'-dAACAGCCTATb6GGCCC-3'.](image-url)
protein. This indicates that more than 1 molecule of the protein can bind to 1 molecule of the 16-mer oligodeoxyribonucleotide and is consistent with a recent report based on sedimentation equilibrium analysis that up to 4 molecules of AGT can be accommodated on a 16-nucleotide length of DNA (33).

The reaction of the mutant A316P/W336A-Ada-C and of the Ogt alkyltransferase with O6-benzylguanine when this substrate was contained in an oligodeoxyribonucleotide was very much faster than the reaction with the free base, even when the latter reaction rate was enhanced by the addition of unalkylated DNA. The wild-type Ada-C reacts with O6-benzylguanine only when it is incorporated into an oligodeoxyribonucleotide. This indicates that the binding of the DNA positions the adduct in the correct orientation to facilitate repair. There are now numerous examples of enzymes that repair or methylate DNA that act via base-flipping mechanisms (34–36). These include HhaI and HaeIII methyltransferases (37, 38), T4 endonuclease V (39, 40), uracil DNA glycosylase (41, 42), 3-methyladenine DNA glycosylase II (43, 44), and DNA photolyase (45).

Several authors have suggested that alkyltransferase follows a similar general mechanism (34, 36, 46, 47), and the crystal structure of the Ada-C protein would be consistent with such a model, in which the methylated base is flipped out of the DNA structure and rotated into a binding pocket in the protein (6, 7). Our results show that as long as this pocket in the alkyltransferase can accommodate the benzyl group, this substrate is preferred over methyl. Such a preference could be related to the more facile displacement of benzyl groups over methyl in bimolecular displacement reactions or to the presence of a hydrophobic region at the active site that binds benzyl better than methyl.

Although it is well established that alkyl adducts on the O6-position of guanine that are slightly larger than methyl such as ethyl and chloroethyl are effectively removed from DNA by alkyltransferases from mammalian sources and bacteria (1, 2, 8, 14), there is little information showing repair of more bulky groups. Our results indicate that AGT would be expected to remove benzylic and related groups very effectively and thus could protect against some environmental carcinogens such as N-nitrosomethylbenzylamine and 4-(methylthio)mino)1-(3-pyridyl)-1-butanoate (48, 49). For example, O6-benzyl guanine is produced by such agents and is a preferred substrate for the alkyltransferase (57). Our results showing that benzyl adducts are not only substrates for the human AGT and E. coli Ogt reaction but are actually preferred substrates suggest that there may well be other endogenous alkylating agents. The noninducible Ogt protein is more likely than Ada to be responsible for reducing the rate of spontaneous mutations in E. coli (19). Although this Ogt protein reacts only weakly with O6-benzylguanine as a free base (10, 11), as shown in Figs. 55 and 6, it reacts much more readily with O6-benzylguanine when this is present in DNA, and such adducts are therefore highly likely to be targets of repair in vivo.

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