Evidence that covalent complex formation between BCNU-treated oligonucleotides and *E.coli* alkyltransferases requires the O⁶-alkylguanine function

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ABSTRACT

Chloroethylnitrosoureas (CENUs) are thought to induce cytotoxic DNA interstrand cross-links via an initial reaction at O⁶-position of guanine, yielding a rearranged intermediate, O⁶,N¹-ethanoguanine. Repair of these adducts by mammalian and bacterial DNA alkyltransferases blocks the formation of cross-links. Human alkyltransferase can form a covalent complex with DNA containing BCNU-induced cross-link precursors, but the nature of the DNA-protein linkage remains unknown. Using *E. coli* alkyltransferases expressed by the *ada* and *ogt* genes, we now demonstrate that both enzymes can form such complexes with CENU-treated DNA. We attribute this reaction to the O⁶-alkylguanine repair function, because an N-terminal fragment of the *ada* protein, which has only alkylphosphotriester repair activity, failed to form a similar complex. This result is consistent with the idea that complex formation requires an alkyltransferase reaction with a guanine adduct, such as O⁶,N¹-ethanoguanine. It tends to exclude the possibility that such reactions simply involve alkylation of the enzyme by reactive DNA adducts such as chloroethylphosphate or chloroethylguanine.

INTRODUCTION

DNA alkyltransferases (ATases) responsible for the repair of DNA that contains the highly mutagenic lesion O⁶-alkylguanine are present in both bacteria and mammalian cells (1–3). These enzymes transfer and accept the alkyl group of O⁶-alkylguanine in alkylated DNA and become irreversibly inactivated in the process. Thus, this repair capacity depends on the number of available acceptor ATase molecules.

E. coli contains two genes that encode ATases. The *ada* gene encodes a 39 kDa protein that has two ATase functional domains, one acting on O⁶-alkylguanine and O⁴-alkylthymine (4,5) and the other acting on alkylphosphotriesters (6,7): these activities are conserved in respectively, 19 kDa and 20 kDa proteolytic cleavage fragments of the 39 kDa protein. The *ada* gene is expressed constitutively at a very low level (8) but is induced

by exposure to low doses of methylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine, a phenomenon known as the adaptive response (1). The *ogt* gene (9) encodes a 19 kDa protein that acts on longer chain O⁶-alkylguanines and O⁴-methylthymine much more rapidly than the *ada* O⁶-alkylguanine ATase but on O⁶-methylguanine at the same rate (5). The *B. subtilis* *Dat 1* gene that encodes a related non-inducible O⁶-alkylguanine ATase has recently been cloned (10).

Mammalian ATases, in contrast to those of *E. coli* appear to be absolutely specific for O⁶-alkylguanine (11). Although expression can be induced in certain cells and tissues by exposure to genotoxic and non-genotoxic agents the extent of induction is very small in comparison with that observed in *E. coli* during adaptation (12).

Chloroethylnitrosoureas (CENUs), such as the antitumor drug BCNU (carmustine), are alkylating agents whose cytotoxicity is believed to arise from DNA interstrand cross-link formation (13). Both *E. coli* and mammalian ATases can abort this cytotoxic process (14–17), presumably by repairing cross-link precursor adducts at O⁶-guanine, thus conferring resistance to the drugs. Our previous studies showed that the human ATase forms a covalent complex with DNA containing the cross-link precursors induced by CENUs (18–20). A proposed mechanism for formation of this complex is based on a scheme (Fig. 1, Reactions 1, 2 and 3) for CENU-induced cross-linking originally proposed by Ludlum (17,21).

In this article, we report that the ATase activities of the *E. coli* *ada* and *ogt* proteins, but not that of an *ada* protein fragment containing only phosphotriester repair activity, form complexes with CENU-treated DNA. This indicates a requirement for O⁶-alkylguanine adducts in complex formation by DNA alkyltransferases.

MATERIALS AND METHODS

E. coli ATase Preparation

ada protein preparations

E. coli strain JM83 harbouring the plasmid pO62HX (7) grown to stationary phase were used as source of the entire *ada* protein. Extracts, prepared as described (7) and containing around 2%

Figure 1. Reactions proposed for CENU-induced interstrand cross-link formation and for formation of complexes between alkyltransferases and CENU-induced DNA adducts.

of total protein as ATase were subjected to affinity purification using double stranded DNA cellulose as described (9).

In order to produce extracts containing only alkylphosphotriester ATase activity, plasmids containing truncated versions of *ada* were employed. In pO62HSR, the *ada* gene was truncated to an *Rsa* 1 site upstream of the O⁶-alkylguanine ATase active site coding region (8). In pO62PTMT, a short oligonucleotide containing an in frame termination codon was ligated onto the *Rsa* 1 site. Both constructs encoded amino acids 1–302 of the *ada* protein. These constructs were transformed into *E. coli* BS73 and extracts of stationary phase bacteria were used as a source of the alkylphosphotriester ATase.

ogt protein preparation

E. coli strain BS73 harbouring the plasmid pO61M (5) grown to stationary phase were used as a source of the *ogt* protein. Extracts of these contained around 0.3% of total protein as ATase and were subjected to purification as outlined above.

Human ATase Preparation

Human ATase was purified approximately 10-to-100-fold from cultured human lymphoblasts (CEM-CCRF line) to fractions 3 and 4, as previously described (16).

Protein Assay

The protein content of the ATase preparations was determined by using a dye binding assay (Bio-Rad) with bovine serum albumin (BSA) as a standard (22), and by absorbance at 280 nm and 260 nm.

Alkyltransferase Assay

ATase activity was estimated by determining the transfer of ³H-labeled methyl groups from methylated DNA to the transferase protein, essentially as described previously (19). The substrate for the enzyme assay was prepared by reacting calf thymus DNA (Sigma Type 1) with [³H]-methylnitrosourea (10 Ci/mmol; Amersham) as described before (19). ATase in 200 μ l of TEDG buffer [10 mM Tris HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol and 10% glycerol] was incubated with 1 pmol of ³H-labeled methylated DNA for 30 min at 37°C. The reaction was stopped by adding 2.5 volumes of 5% trichloroacetic acid (TCA). The reaction mixture was then heated at 80°C for 30 min to hydrolyze DNA. After cooling to 0°C carrier protein (100 μ g BSA) was added to samples with low protein content (< 100 μ g protein). The precipitate was collected on glass fiber filters (Whatman GF/F), washed twice with 5% TCA and then with 95% ethanol. The filters were transferred to scintillation vials, and the protein was solubilized with NCS (Amersham).

Radioactivity was measured after addition of 5 ml of PCS (Amersham). ATase was determined as a function of increasing amounts of protein in each preparation and the specific activity derived from the linear region of the response.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the complete reaction mixture was applied directly to the gel after incubation with [³H] methylnitrosourea-treated DNA.

Oligonucleotide Preparation

Two substrates, a 20-mer (6.5 kDa) and a 30-mer (10 kDa) with the sequences 5'-dGGGGGGGGGGCCCCCCCCC-3' and 5'-dGGGGGGGGGGGGCCCCCCCCC(A)₁₈, respectively, designed to have a series of adjacent guanines and to be self-annealing, were prepared with an Applied Biosystems 380A DNA synthesizer. The oligomers were alkylated with 0.5M BCNU (Bristol-Myers) for 90 min at 37°C, and then labeled at the 5' end with [³⁵S]ATP_γS (Amersham) using T₄ polynucleotide kinase (US Biochemicals Corp.) as described previously (23). The oligonucleotide was separated from excess drug and ATP by gel filtration through Quick-spin Sephadex G-25 columns (Boehringer Mannheim Biochemicals).

Formation of Oligonucleotide-ATase Complex

ATase was incubated at 37°C for 60 min with the BCNU-treated, ³⁵S-labeled oligomers. The enzyme:substrate ratio was usually 1:2. The complete reaction mixture was subjected to SDS-PAGE analysis.

Inhibition of Nuclease Activity in Crude Extracts

In some reactions with crude *E. coli* ATase preparations (HSR and PTMT), 2.5 nmol of ATP, an additional 50 nmol of EDTA and 1 μg of nonalkylated calf thymus DNA were added to inhibit interfering nucleases (24).

SDS-PAGE Analysis

Extracts that reacted with ³H-labeled methylated DNA or with ³⁵S-labeled, BCNU-treated oligodeoxynucleotide were analyzed by electrophoresis through 0.75 mm-thick, 10% or 12.5% SDS-polyacrylamide gels by the method of Laemmli (25) using a Mini-PROTEAN II apparatus (Bio-Rad). Proteins were stained with Coomassie blue R-250.

Stained gels, after soaking in the scintillator En-Hance (Dupont), were dried onto Whatman filter paper and exposed to film (Kodak XAR-5) using Cronex Lightning-Plus intensifying screens for various times at -70°C.

Alkyltransferase Depletion Assay

ATase was incubated at 37°C for 60 min with increasing concentrations of unlabeled, BCNU-treated oligomers. Residual enzyme activity was determined by the alkyltransferase assay described above. Control experiments were performed with nonalkylated oligomers.

RESULTS

Table 1 indicates the relative purity of the different ATase preparations used in the study. The *E. coli* *ada* and *ogt* proteins had the highest specific activities—50-to-500-fold greater than the HSR and PTMT preparations. The values for specific activity of *ada* and *ogt* ATases indicated that they contained 5 percent and 7 percent, respectively, of active protein reflecting the lability

Table 1. Characteristics of alkyltransferase (ATase) preparations

Preparation	Substrate Specificity	Ref.	Molecular size (kDa)	Specific activity (pmoles/mg protein)
<i>E. coli</i>	<i>ada</i>	4-7	39	~2500
	O ⁶ -alkylguanine O ⁴ -alkylthymine phosphotriester			
	<i>ogt</i>	9	19	~3500
	HSR	7	13	48
PTMT	phosphotriester		~34	7
Human ATase	O ⁶ -alkylguanine	15	25	
Fraction 3				15
Fraction 4				400

of the activities as well as the fact that these preparations were not completely homogeneous. The absorbance ratio at 280 nm and at 260 nm indicated that the HSR and PTMT extracts contained ≥20% nucleic acids (data not shown). Human ATase (fraction 4) included in these studies for comparison, was not as pure as the *ada* and *ogt* preparations while fraction 3 was as crude as the HSR and PTMT preparations.

The size of the ATase protein in each preparation was determined by SDS-PAGE and autoradiography of the ATases that had been labeled by reaction with ³H-labeled methylated DNA. Figure 2 shows that each preparation except PTMT contained a single ³H-labeled band whose mobility corresponded to the expected molecular size for that enzyme (see Table 1). Coomassie blue staining of the gel indicated that the *ada* and *ogt* protein preparations approached homogeneity for the ATase protein, whereas the HSR and PTMT preparations contained a large number of protein bands (data not shown). These latter two preparations particularly PTMT, also appeared to contain nuclease activity, as indicated by the loss of the radiolabeled high-molecular-weight DNA substrate in lanes 4 and 5 compared with the purer preparations in lanes 1, 2 and 3. Lanes 6-12 in Figure 2 show the products of ATase reaction with ³⁵S-labeled, BCNU-treated 20-mer. The *ada* and *ogt* proteins (and human ATase, shown for comparison) were seen as distinct radiolabeled bands, with mobilities corresponding to the combined sizes of the protein and the 6.5 kDa oligomer. There were no clear radiolabeled bands associated with HSR and PTMT proteins (lanes 9-12). Because contaminating nucleases in these preparations might degrade the alkylated 20-mer in the complex-forming reactions, nuclease inhibitors were included in the reaction mixtures analyzed in lanes 10 and 12. PTMT appeared to contain substantially more nuclease activity than did HSR; hence, further studies were focused on the HSR ATase.

Faint diffuse ³⁵S-labeled bands at ~22 kDa, which is close to the 19.5 kDa size expected for complex formed by HSR, were consistently observed with this phosphotriester-specific ATase. Therefore, two additional experiments were performed to exclude the possibility that this faint band represented a complex. First, the HSR ATase was inactivated by heating (50°C, 20 hr). SDS-PAGE analysis showed that the diffuse ³⁵S-labeled banding pattern was the same for both the active and heat-inactivated HSR protein (Fig. 3), whereas with the *ogt* protein, a strong ³⁵S-labeled band corresponding to a complex with the active protein was absent with inactivated protein. Also shown in Figure 3 are

Figure 2. SDS-PAGE and fluorography of reaction products of ATases with ^3H -labeled methylated DNA (left panel) and with ^{35}S -labeled, BCNU-treated oligodeoxynucleotide (right panel). Lanes 1, 6 = human ATase; lanes 2, 7 = *ada* protein; lanes 3, 8 = *ogt* protein; lanes 4, 9 and 10 = HSR protein and lanes 5, 11 and 12 = PTMT protein. Nuclease inhibitors were included in the reactions shown in lanes 10 and 12. The lines connecting the two panels show the band shift expected for complex formed by each ATase.

Figure 3. SDS-PAGE and fluorography of active (a) and heat-inactivated (b) *ogt* and HSR proteins reacted with ^3H -labeled methylated DNA and with ^{35}S -labeled, BCNU-treated oligodeoxynucleotide.

the reactions of *ogt* and HSR proteins with ^3H -labeled methylated DNA, confirming the inactivation of the ATase in each preparation.

In a second experiment, to confirm that phosphotriester ATase activity of the HSR protein does not form a complex, HSR and *ogt* proteins were mixed, and the reaction products obtained with ^{35}S -labeled, BCNU-treated oligomer or ^3H -labeled methylated DNA were analyzed by SDS-PAGE (Fig. 4). The combination of HSR with *ogt* protein did not affect the ^3H -methyl transfer activity seen with either protein alone. The slightly greater intensity of the *ogt* labeled band at 19 kDa in the mixed reaction may be the result of stabilization of the highly purified *ogt* protein by the larger amount of protein in the crude HSR preparation. The formation of ^{35}S -labeled complex by the *ogt* protein in the

Figure 4. SDS-PAGE and fluorography of reaction products of *ogt* and HSR protein separately and together (*ogt* + HSR) with ^3H -labeled methylated DNA and with ^{35}S -labeled, BCNU-treated oligodeoxynucleotide.

mixture was clearly not inhibited by the HSR protein. Thus the absence of complex formation by the HSR ATase is not the result of an inhibitory or degradation factor.

Another reason for the absence of complex formation by the HSR protein might be that BCNU did not produce appreciable levels of phosphotriester adducts. To obtain an estimate of such adducts, we measured the capacity of BCNU-treated oligomer to deplete the HSR ATase activity. Figure 5 shows that 50% of HSR ATase (0.25 pmol) was depleted by less than 0.5 pmol of the BCNU-treated oligomer. Human ATase was similarly depleted indicating that this alkylated oligomer contained

Figure 5. Depletion of ATase by reaction with BCNU-treated 30-mer. Residual activity, assayed as described in **Methods** after incubation of 0.25 pmol ATase with increasing concentration of BCNU-treated 30-mer, is expressed relative to ATase incubated with equivalent amounts of untreated 30-mer. *E. coli* HSR ATase (●); Human ATase, fraction 3 (△).

comparable levels of O⁶-guanine and phosphate adducts. Complete depletion was not attained using the relatively crude HSR and human ATases. This is probably due to non-specific binding of radioactivity to the large amounts of protein present, since the apparent residual activity was observed with heat-inactivated extracts or with bovine serum albumen.

DISCUSSION

The ability to segregate the O⁶-alkylguanine and phosphotriester repair activities of *E. coli* DNA ATases enabled us to investigate these functions as they relate to the formation of a covalent complex with CENU-treated oligodeoxynucleotides. The CENUs reportedly produce as much alkylation of the phosphates as of the bases in DNA (26). Carter et al. (27) have also demonstrated that chloroethyl phosphotriesters produced by CENUs are relatively stable and one of the stereo-isomers is efficiently repaired by *E. coli* extracts. We designed oligomer substrates with a sequence of six to ten guanines and the potential to form a double-stranded structure, features favoring efficient CENU alkylation (28,29) and optimal transferase action (30). The ATase depletion experiment (Fig. 5) confirmed that this substrate contained alkylated phosphate and guanine adducts.

Our results show that *E. coli* *ada* and *ogt* gene proteins, like human ATase, are able to form complexes with CENU-treated oligodeoxynucleotide. Moreover, the data indicate that ATases acting at O⁶-guanine form complexes, whereas phosphotriester ATase does not.

The HSR and PTMT preparations were crude and may have contained factors that prevented or masked the complex-forming reactions. One possibility is that nucleases degraded the oligodeoxynucleotide substrate; however, these crude preparations did not form any complex even after the addition of nuclease inhibitors. The question of whether complex formation was inhibited or masked by factors in the HSR preparation was resolved by mixing experiments in which HSR

did not prevent complex formation by *ogt* protein. We conclude that CENU adducts of phosphotriesters cannot give rise to an oligonucleotide-ATase complex as do the O⁶-guanine adducts.

Previously, we suggested (16,20) that covalent binding of DNA to the enzyme is the result of ATase action on the rearranged O⁶,N¹-ethanoguanine intermediate of interstrand cross-link formation (Fig. 1, Reaction 6). However, the ATase might bind to the alkylated substrate simply as a result of the elimination of Cl from an O-chloroethylated adduct with subsequent alkylation of a nucleophilic site in the enzyme. If so, an oligonucleotide-phosphotriester ATase complex (Fig. 1, complex B) as well as an oligonucleotide-O⁶-alkylguanine ATase complex (Fig. 1, complex A) would be expected to form. However, the fact that complex formation absolutely required an O⁶-guanine adduct is consistent with the scheme (Fig. 1) in which the complex precursor is the cross-link precursor, O⁶,N¹-ethanoguanine (20,31).

The recombinant *ada* and *ogt* proteins can efficiently form oligonucleotide-ATase complexes; hence, they afford the opportunity for large-scale production of a complex that can be purified by an affinity method previously used on a small scale for human ATase (23). The availability of large amounts of the purified complex will make it possible to determine the structure of the putative guanine-cysteine linkage.

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