Affinity purification and characterization of human O⁶-alkylguanine-DNA alkyltransferase complexed with BCNU-treated, synthetic oligonucleotide

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ABSTRACT

Tumor cells resistant to chloroethylnitrosourea (CENU) therapy contain high levels of O⁶-alkylguanine DNA-alkyltransferase (GATase), a DNA repair enzyme that aborts DNA interstrand cross-linking by removing CENU-induced O⁶-alkylguanine adducts. Because the transferase binds covalently to CENU-treated oligonucleotides, we reacted partially purified GATase from cultured human lymphoblasts with a BCNU-treated, ³⁵S-5'-end-labeled, synthetic oligonucleotide designed to have a polyadenylated 3' terminus. Immunoprobable Western blots of this reaction mixture with GATase-specific monoclonal antibody indicated that 25–30% of the transferase became complexed. We purified this complex by affinity chromatography with oligo(dT) cellulose, recovering homogenous material that appeared as a discrete 35-kDa Coomassie blue or silver-stained band after SDS-polyacrylamide gel electrophoresis. Autoradiography and Western immunoblotting confirmed that this band contained both the radiolabeled oligonucleotide and the GATase protein.

INTRODUCTION

The chloroethylnitrosoureas (CENUs), which include carmustine (BCNU) and lomustine (CCNU) are effective drugs in cancer chemotherapy (1–2). Although the cytotoxicity of these bifunctional alkylating agents has been shown to correlate with their ability to induce DNA interstrand cross-links, the sequence of reactions leading to the formation of these cross-links has not been fully elucidated (3–4).

Studies on the response of tumor cells to alkylating agents indicated the presence of an O⁶-alkylguanine repair system in DNA (5–10). Later this was found to involve O⁶-alkylguanine-DNA alkyltransferase (GATase) (11–17), an enzyme that repairs O⁶-alkylguanine in DNA by irreversibly accepting the alkyl group (6,8). Investigations implicating GATase in cellular responses to CENUs led to the proposal (12) that a chloroethyl species generated by these alkylating agents initially reacts with guanine in DNA to form O⁶-chloroethylguanine (Fig. 1), and that this monoadduct then slowly rearranges to form an intrabase exocyclic adduct, O⁶,N¹-ethanoguanine that can eventually form a –CH₂–CH₂– bridge with the complementary cytosine on the opposite strand (15). The two intermediates, O⁶-chloroethylguanine and O⁶,N¹-ethanoguanine, may both be substrates for GATase (14). Most recently, studies in our laboratory (17) have demonstrated the formation of a covalent DNA-GATase complex which is consistent with repair of O⁶,N¹-ethanoguanine (Fig. 1).

To characterize the structure of this complex, and thus gain insight into the molecular mechanism involved in DNA cross-linking by CENUs, we sought to purify a complex containing a polyadenylated oligodeoxynucleotide using oligo(dT) cellulose affinity

Fig. 1. Proposed repair reactions of GATase with BCNU-induced adducts in DNA.

chromatography. The results indicate that we have obtained a homogeneous preparation adequate for characterization of the DNA-GATase linkage and for amino acid sequencing of the transferase.

MATERIALS AND METHODS

Materials

Oligo(dT) cellulose was obtained from Bethesda Research Laboratories; BCNU from Bristol Laboratories; an immunogold silver staining kit was from Janssen; nitrocellulose, BA 83, was from Schleicher and Schuell; polyvinylidene difluoride membranes (Immobilon) were from Millipore; ultrafiltration membrane filters (YM10) and Centricon and Centriprep concentrators (10,000 or 30,000 MW cut-off) were from Amicon; and Quick-spin Sephadex G-25 columns were from Boehringer-Mannheim. Adenosine 5'-(γ -[^{35}S]thio)-triphosphate, triethyl ammonium salt (600 Ci/mmol) and N-[^3H]-methyl-N-nitrosourea (10 Ci/mmol)

were purchased from Amersham; T₄ polynucleotide kinase was from Bethesda Research Laboratories or United States Biochemical Corp.

Oligonucleotide Preparation

A 30-mer oligodeoxynucleotide was designed with the sequence 5'-dGGGGGGCCCCC(A)₁₈-3' so as to have a self-complementary sequence at the 5' end, and a single-stranded poly(dA) tail at the 3' end for affinity purification on oligo(dT) adsorbents. The oligodeoxynucleotide was prepared with an Applied Biosystems 380A DNA synthesizer by a cyanoethyl phosphoramidite procedure. After synthesis it was deprotected by treatment with ammonium hydroxide and extracted with butanol as described previously (17). The final concentration of the 30-mer was estimated by measuring UV absorbance given that 1 A₂₆₀ unit corresponds to 25 µg 30-mer per ml.

BCNU was dissolved in anhydrous ethanol at a concentration of 5 M immediately before use. This solution was added to 0.1 µg/µl of the 30-mer dissolved in 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA to yield a final BCNU concentration of 500 mM. The mixture was incubated at 37°C for 90 min, after which the drug was removed by gel filtration through Quick-spin Sephadex G-25 columns.

The alkylated 30-mer was 5' end-labeled with [³⁵S]ATP_γS using T₄ polynucleotide kinase. Typically, the 30-mer (~4 µg) was incubated for 60 min at 37°C with 250 µCi [³⁵S]ATP_γS and 400 units of T₄ polynucleotide kinase in buffer containing 60 mM Tris-HCl (pH 7.6), 10 mM MgCl₂ and 33 nM ATP in a total volume of 200 µl. The reaction was stopped by chilling to 0°C and removing unincorporated ATP by gel filtration through Quick-spin Sephadex G-25 columns.

Oligodeoxynucleotide-GATase Complex Formation and Isolation

Human GATase partially purified from cultured lymphoblasts (CEM-CCRF) as described previously (14) was incubated at 37°C for 60 min with the alkylated, radiolabeled 30-mer. Enzyme-to-substrate molar ratio was 1:2. Before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the reaction mixture was concentrated by ultrafiltration using Centrprep 30. For affinity purification of the complex, the reaction mixture was precipitated by SDS/KCl according to Trask et al. (18), with omission of carrier calf thymus DNA and BSA, and the pellet recovered by centrifugation was resuspended in distilled water and dialyzed extensively against distilled water before concentration to ~10 ml by pressure filtration using a YM 10 membrane.

Oligo(dT) Cellulose Chromatography

The procedure of Maniatis et al. (19) was followed with slight modifications. Oligo(dT) cellulose (~0.1 g) was treated with 2 mls of 0.1 M NaOH, and washed extensively with distilled water, before pouring into a low pressure column (4×0.7 cm diam.; Bio-Rad), which then was equilibrated with 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% SDS, and 0.5 M LiCl (loading buffer). About 10 ml of solution containing ~100 pmol of the complex was adjusted to the composition of the loading buffer before being applied to the column. The initial flow-through was collected and reapplied to the column. This step was repeated, after which the column was washed with 10 volumes of loading buffer followed by 10 volumes of buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% SDS and 0.2 M LiCl (wash buffer) before elution of the bound material with 10 mM Tris-HCl (pH 7.6) (elution buffer). The entire process was monitored by collecting 1 ml fractions and measuring the UV absorbance at 280 nm and radioactivity by scintillation counting. Fractions corresponding to the A₂₈₀ peak (unbound) and the radioactivity peak (bound) were collected and concentrated.

Fig. 2. Western blots probed with Mab 19.2 of free [^3H -methyl]GATase (left) and GATase reacted with BCNU-treated, ^{35}S -labeled 30-mer (right). The corresponding autoradiograph is shown on the right of each blot.

SDS-PAGE and Western Blotting

Reaction mixture containing the oligo-GATase complex or affinity purified fractions were analyzed by electrophoresis through 0.75 mm thick 12.5% SDS-polyacrylamide gels by the method of Laemmli (20) using a Mini-PROTEAN II apparatus (Bio-Rad). Protein bands were either stained directly with Coomassie blue R-250 or silver nitrate (Bio-Rad), or were transferred to nitrocellulose or Immobilon membranes for immunoprobings or staining with Coomassie blue.

Electrophoretic transfer of proteins to nitrocellulose or to Immobilon was performed with a Bio-Rad Mini Trans-Blot cell at 70V for 2 hr in 25 mM Tris, 192 mM glycine, and 20% v/v methanol. For immuno-staining, the nitrocellulose membrane was blocked with 5% BSA for 2 hr and then probed with human GATase-specific monoclonal antibody (Mab 19.2, 10 $\mu\text{g}/\text{ml}$) (21) dissolved in 20 mM Tris-HCl (pH 8.2), 0.1% BSA, 0.9% NaCl, and 1% normal goat serum (Gibco). Antibody-GATase binding was visualized with an immunogold-linked goat antimouse secondary antibody followed by silver enhancing stain (Janssen) according to the manufacturer's instructions. Proteins on Immobilon membrane were stained with Coomassie blue following the technical protocol from Millipore.

Partially purified human GATase, reacted with N-[^3H]-methyl-N-nitrosourea-treated

Fig. 3. (A) Distribution of free GATase at 25 kDa ($\triangle$ — $\triangle$) and complexed GATase at 35 kDa (---) in reaction mixtures containing GATase and ^{35}S -labeled, BCNU-treated 30-mer. Indicated volumes of reaction mixture were analyzed by SDS-PAGE, transferred to nitrocellulose by Western blotting and probed with Mab 19.2. Immunostained band intensities were quantified by densitometric scanning of photographic negatives of the blots. (B) Intensities of radiolabeled 35-kDa bands in autoradiographs of the blots in panel A. Band intensities were quantified by densitometric scanning.

calf-thymus DNA as described previously (14), was used as a radioactive marker in SDS-PAGE and Western blot analyses.

Stained gels, after treatment with En-Hance (Dupont) were dried onto Whatman filter paper and exposed to Cronex films (Dupont) using Cronex Lighting-Plus intensifying screens for various times at -70°C . Stained membranes, after drying, were similarly subjected to autoradiography. The intensities of bands observed in autoradiographs or photographic negatives of immunoprobed blots were measured with a densitometer (E-C Apparatus Corp.) interfaced with a Shimadzu C-R3A integrating recorder.

RESULTS

When human GATase, purified 10-fold, was reacted with BCNU-treated, ^{35}S -labeled 30-mer oligodeoxynucleotide, and the reaction products were analyzed by SDS-PAGE, autoradiography revealed a radiolabeled 35-kDa band corresponding to the complex formed

Fig. 4. Oligo(dT) cellulose chromatography of poly(dA)-tailed complex. Complex (~ 100 pmol in 10 ml of loading buffer) was applied to the column, and the flow-through was collected and reappplied to the column twice, followed by 10 ml each of loading buffer and of wash buffer. The complex was eluted with salt-free buffer. Radioactivity due to ^{35}S -labeled complex (—) was determined by scintillation counting. Protein was monitored by measuring the $\text{OD}_{280\text{nm}}$ (.....).

between the 25-kDa transferase and the 10-kDa 30-mer. A human GATase-specific monoclonal antibody, 19.2, used to estimate the distribution of complexed and uncomplexed transferase in a Western blot of such SDS-PAGE revealed two bands. One band represented free GATase at 25 kDa and the other complexed GATase at 35 kDa (Fig.2). Autoradiography of this blot confirmed that the 35 kDa band also contained the ^{35}S -labeled oligomer. To determine if the intensity of these immunostained bands reliably represented the relative amounts of free and complexed GATase protein, we analyzed different amounts of reaction mixture by SDS-PAGE and quantified the photographed immunostained bands by densitometry. The amount of ^{35}S -labeled oligomer at 35 kDa in the corresponding autoradiographs was also quantified by densitometry. The intensities of the immunostained bands plotted in Figure 3A increased until a plateau was reached at approximately $5\ \mu\text{l}$ of reaction mixture. As shown in Figure 3B, the intensity of the radiolabeled 35-kDa complex band increased linearly with reaction mixture volume throughout the range examined indicating that the electroblot transfer was linear and that the immunoassay shown in Figure 3A became saturated. If one assumes that the intensities of the immunostained bands comprising the linear portion of the graph in Figure 3A (up to $2\ \mu\text{l}$) reflect the relative amounts of free and complexed GATase, then it appears that as much as 25–30% of the transferase was complexed.

Thus, since a significant fraction of GATase became complexed with the 30-mer in these reactions, we proceeded to purify the complex by affinity chromatography on an oligo(dT) cellulose column. Unreacted free polyadenylated oligomer that might compete with the complex for oligo(dT) binding sites was separated from the complex by SDS/KCl precipitation of the reaction mixture before loading the column. Figure 4 shows a typical

Fig. 5. SDS-PAGE of the pooled fractions from oligo(dT) cellulose chromatography. Silver-stained gel lanes marked Ag are shown on the left of each pair with the corresponding autoradiograph on the right (designated ^{35}S or ^3H). The position of the non-complexed transferase at 25 kDa is shown in the lanes on the left labeled ^3H -methyl GATase. The precolumn sample lane (load) and the unbound fraction lane contained the equivalent of 1 pmol of GATase. The lane with the bound fraction was equivalent to 70 pmol of GATase; however, its autoradiograph was exposed for one-tenth as long as the other fractions.

elution profile of this chromatography. The majority of proteins applied were not retained in the column under the high salt conditions imposed by the loading buffer. However, the major ^{35}S -labeled component, representing the complex, bound to the column at 0.5 M LiCl, remained there at 0.2 M LiCl, and was eluted only when the LiCl concentration was reduced to zero.

The extent of purification achieved by affinity chromatography was assessed by SDS-PAGE analysis of the material loaded onto the column, the unbound fraction, and the bound fraction. Figure 5 shows that the unbound fraction contains almost the same complement of proteins as the prechromatography preparation. However, it contains virtually no radiolabeled material except for a faint band at about 30-kDa that may represent some other DNA binding protein. By contrast, the bound fraction comprises only two stained bands; the one at 35 kDa corresponds to the complex, while the second, at about 10 kDa, corresponds to residual free 30-mer. The autoradiograph of these gel lanes indicates that the ^{35}S -labeled, 35-kDa complex was recovered almost entirely in the bound fraction. If one compares the virtual absence of proteins associated with a very intense ^{35}S -labeled band in the bound fraction with the abundant protein content and a less intense ^{35}S -labeled band in the unbound fraction, it is clear that the complex was highly purified.

The yield of purified complex was estimated from the intensity of the radiolabeled band at 35 kDa in autoradiographs of the bound fraction in comparison with the preparation before chromatography. Twenty percent of the radiolabeled complex applied to the column was recovered in the purified bound fraction (Table 1). Recovery of residual free oligomer

Table 1. Recovery of Free and Complexed Oligonucleotide from Oligo(dT) Affinity Columns

	35-kDa band ^a (complexed oligomer)	Complex recovery (%)	10-kDa band ^a (free oligomer)	Free oligomer recovery (%)
<i>Exp. 1</i>				
Load	179.2		39.1	
Bound ^b	38.8 ± 7.3	21.6 ± 4.0	14.0 ± 4.2	35.8 ± 10.7
<i>Exp. 2</i>				
Load	37.6		6.8	
Bound ^b	7.4 ± 0.6	19.7 ± 1.6	2.6 ± 0.5	38.2 ± 7.4

^aDensitometer peak areas (arbitrary units) of bands in autoradiographs of SDS-polyacrylamide gels.

^bValues are means and standard deviations of 2 or 3 determinations.

(10 kDa) indicated that over 30% was bound, suggesting that it binds to the column more efficiently than does the complex (Table 1).

The 35-kDa species in the bound fraction was further characterized by Coomassie blue staining and Mab 19.2 probing of Western blots. Figure 6 shows that this fraction consists of a single Coomassie blue-stained band, indicating the preparation was homogeneous for protein. The immunostained band at 35 kDa corresponds exactly to the radiolabeled band

Fig. 6. Western blot of the complex purified by oligo(dT) cellulose chromatography. Blotted strips were probed with Mab 19.2 (left) or stained with Coomassie blue (R-250) (right); the corresponding autoradiograph (³⁵S) is shown on the right of each strip.

detected by autoradiography, confirming that this species contains both human GATase and ^{35}S -labeled 30-mer.

DISCUSSION

GATase becomes covalently bound to BCNU-treated DNA during the DNA repair process, and we exploited this reaction to purify the protein. Use of alkylated, synthetic oligodeoxynucleotide with a polyadenylated 3' terminus as a substrate for GATase enabled us to purify to homogeneity the resulting oligo-GATase complex by its affinity to an oligo(dT) matrix. The purified complex was identified as a 35-kDa species, which was shown by autoradiography and antibody-antigen reactivity to comprise the 10-kDa ^{35}S -labeled oligomer and the 25-kDa protein. The estimated overall recovery of GATase protein was 5%, based on the fraction of the initial transferase that became complexed (25–30%) and the yield of complex recovered in the affinity chromatography (20%).

The 5' end of the 30-mer used in the study was designed to have both a series of 6 adjacent guanines and a self-complementary sequence of 6 cytosines. This construction was based on the preference of GATase for double-stranded DNA (22,23) and the greater susceptibility of oligomers with several consecutive guanines to alkylation by chloroethylating agents (24–26).

Because this method of purification relies on complementary base interactions between the matrix-bound oligo(dT) and the polyadenylated species, the free oligomer also bound to the matrix and coeluted with the complex, as expected. Under the chromatographic conditions used, the recovered material contained three times as much complex as free oligomer; however, preferential binding of the uncomplexed 30-mer can be inferred from the apparent enrichment of this component during the elution process. It may be possible in the future to further reduce the free oligomer component by more extensive washing of the SDS/KCl precipitate (18). However, for projected studies on the purified complex that will involve selective oligonucleotide digestion, co-purification of the unbound 30-mer may not pose a problem.

Quantitation of the stoichiometry of the reaction leading to complex formation has proved difficult. Consistent recovery of only a low percentage of radiolabeled oligomers in the purified complex (27) suggests that only a small fraction of oligomers becomes alkylated or that the precursors leading to the complex constitute only a minor fraction of the total pool of adducts. The latter possibility is most likely since monofunctional nitrosoureas, such as methylnitrosourea or ethylnitrosourea, produce many more adducts at base nitrogens and phosphates than at O^6 -guanine in DNA. Estimates of the amount of GATase forming the complex have not been possible previously owing to the lack of availability of an assay able to detect both the free and complexed forms of the enzyme. The development of a GATase-specific antibody, Mab 19.2 (21), has satisfied this need, and indicates that as much as 25–30% of the transferase protein can become complexed, an appreciably greater amount than we previously speculated (27). This value of 25% may reflect the fraction of O^6 -guanine diadducts (e.g. $\text{N}'\text{O}^6$ -ethanoguanine) that form complexes with GATase, relative to the fraction (75%) of O^6 -guanine monoadducts (e.g. O^6 -chloroethylguanine) that would be substrate for GATase but not change its molecular size. Alternatively it is possible that only 25–30% of the GATase protein was enzymatically active.

The availability of a purified, homogeneous oligo-GATase complex will now enable us to characterize the transferase protein. Currently we are using it to determine the amino acid sequence. The complex can also be used to raise additional monoclonal and polyclonal

antibodies against the human transferase GATase and to screen such antibodies. Finally, we are characterizing the protein-oligodeoxynucleotide linkage by enzymatic and chemical digestions to obtain the mononucleotide-amino acid ethano diadduct. Analysis of this structure should help elucidate the adduct from which it was formed.

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