DNA Repair 10 (2011) 1193–1202

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

6-Carboxyfluorescein and structurally similar molecules inhibit DNA binding and repair by O^6-alkylguanine DNA alkyltransferase

Manana Melikishvili, David W. Rodgers, Michael G. Fried*

Center for Structural Biology, Department of Molecular and Cellular Biochemistry, University of Kentucky, 741 South Limestone, Lexington, KY 40536-0509, United States

ARTICLE INFO

Article history:
Received 22 April 2011
Received in revised form 6 September 2011
Accepted 9 September 2011
Available online 5 October 2011

Keywords:
DNA repair
DNA alkyltransferase
Substrate analogue
Fluorescein
EMSA
Analytical ultracentrifugation
Fluorescence assay

ABSTRACT

Human O^6-alkylguanine-DNA alkyltransferase (AGT) repairs mutagenic O^6-alkylguanine and O^4-alkylthymine adducts in single-stranded and duplex DNAs. These activities protect normal cells and tumor cells against drugs that alkylate DNA; drugs that inactivate AGT are under test as chemotherapeutic enhancers. In studies using 6-carboxyfluorescein (FAM)-labeled DNAs, AGT reduced the fluorescence intensity by ~40% at binding saturation, whether the FAM was located at the 5′ or the 3′ end of the DNA. AGT protected residual fluorescence from quenching, indicating a solute-inaccessible binding site for FAM. Sedimentation equilibrium analyses showed that saturating AGT-stoichiometries were higher with FAM-labeled DNAs than with unlabeled DNAs, suggesting that the FAM provides a protein binding site that is not present in unlabeled DNAs. Additional fluorescence and sedimentation measurements showed that AGT forms a 1:1 complex with free FAM. Active site benzylation experiments and docking calculations support models in which the primary binding site is located in or near the active site of the enzyme. Electrophoretic analyses show that FAM inhibits DNA binding ($IC_{50} \sim 76\ \mu M$) and repair of DNA containing an O^6-methylguanine residue ($IC_{50} \sim 63\ \mu M$). Similar results were obtained with other polycyclic aromatic compounds. These observations demonstrate the existence of a new class of non-covalent AGT-inhibitors. After optimization for binding-affinity, members of this class might be useful in cancer chemotherapy.

1. Introduction

O^6-alkylguanine and O^4-alkylthymine are mutagenic and cytotoxic residues that occur in DNA exposed to alkylating agents [1,2]. In many organisms, these modified bases are repaired by O^6-alkylguanine-DNA alkyltransferase (AGT, also known as methylguanine methyl-transferase, MGMT), a single-cycle (suicide) enzyme that mediates transfer of an alkyl group from a DNA base to an active site cysteine [3,4]. The alkyl-enzyme does not appear to be re-cycled; in eukaryotic cells it is ubiquitinated and rapidly degraded [5,6]. In addition to protecting normal cells, AGT's repair activities also protect tumor cells against DNA-alkylating drugs. Clinical trials of AGT-inhibitors are underway, in attempts to increase the efficacy of these alkylating drugs in cancer chemotherapy [7–9], however we are only starting to learn how AGT interacts with and repairs DNA adducts.

Human AGT is a small, monomeric protein ($M_r = 21{,}519$) that is expressed constitutively in normal cells [10–12]. It is a member of a large family of DNA-modifying and -repair enzymes that act by flipping a target DNA base out of its stacked location in the duplex and binding it in an active site pocket [12–14]. Intriguingly, AGT binds single stranded and duplex DNAs with similar affinities ($K \times \omega \sim 10^6\ M^{-1}$) [15,16] and repairs single- and double-stranded DNAs with a modest preference for duplex [17,18]. Together these results suggest a mechanism that minimizes differences in the free energy changes associated with the base-flipping transition in single stranded and duplex substrates. Here we describe the interactions of AGT with short oligonucleotides in which the 5′-terminal or 3′-terminal residue is a 6-carboxyfluorescein (FAM) derivative. Our original aim was to use these DNAs in FRET-measurements of the separations between fluorophore-labeled AGT proteins and DNA ends. We were surprised to find that DNA-labeling with FAM perturbed the binding stoichiometries and affinities of target DNAs. The widespread use of fluorophores like FAM in studies of protein–DNA interaction, the potential of compounds like FAM to be useful probes of AGT structure and the possibility that molecules like FAM might become useful chemotherapeutic enhancers justify a more detailed characterization of these effects; the result of that characterization is offered below.

Abbreviations: AGT, O^6 alkylguanine-DNA alkyltransferase; FAM, 6-carboxyfluorescein.

* Corresponding author. Tel.: +1 859 323 1205; fax: +1 859 323 1037.
E-mail address: michael.fried@uky.edu (M.G. Fried).

1568-7864/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2011.09.007

2. Materials and methods

2.1. Enzymes and reagents

Restriction endonuclease NarI and T_4 polynucleotide kinase were purchased from New England Biolabs. 6-Carboxyfluorescein (FAM; CAS Number 3301-79-9), 9-(2,2 dicyanovinyl)julolidine (DCVJ; CAS Number 58293-56-4), guanosine 5′ monophosphate (CAS Number 85-32-5) and acrylamide were from Sigma. 4,4′-Bis(phenylamino)-[1,1′-binaphthalene]-5,5′-disulfonic acid (bis-ANS [47]; CAS Number 65664-81-5) was from Invitrogen. Thioflavin T (ThT; CAS Number 2390-54-7) was the kind gift of Dr. H. Levine (University of Kentucky). [γ-^{32}P]ATP (6000 Ci/mmol) was from NEN Radiochemicals (Perkin Elmer). All other chemicals were reagent grade or better.

2.2. Protein purification

Recombinant human AGT protein in which the six C-terminal residues have been substituted with histidines was purified according to published protocols [4]. The purity of the protein was verified by SDS-gel electrophoresis [19,20]; by this criterion, sample purities of >95% were routine. Protein samples were ∼96% active in a quantitative alkyltransferase assay described below. Samples were stored at −80 °C until needed. AGT concentrations were measured spectrophotometrically using $\varepsilon_{280} = 3.93 \times 10^4\ M^{-1}\ cm^{-1}$ [16].

2.3. DNA samples

Oligonucleotides of 16, 24 and 26 residues (Table 1) were purchased from Invitrogen. They were purified by the supplier using reverse-phase HPLC, and after receipt, by phenol extraction (3×) followed by ether extraction and extensive dialysis against 10 mM Tris (pH 8.0 at 20 °C) buffer. Concentrations were measured spectrophotometrically using extinction coefficients provided by the manufacturers. The 24-mer oligonucleotide E (see Table 1) was labeled at its 5′ hydroxyl with ^{32}P as described [21]. Unincorporated [γ-^{32}P]ATP was removed by buffer exchange using Sephadex G-10 mini-spin columns equilibrated with 10 mM Tris (pH 8.0 at 21 °C), 1 mM EDTA. DNA duplexes were prepared by mixing purified 5′-labeled oligonucleotide with 1.05-fold molar excesses of complementary unlabeled strands (oligonucleotide C or D), heating to 90 °C for 1 min, then slowly cooling to room temperature. After annealing, the purities of duplex DNAs were tested by native polyacrylamide gel electrophoresis [22].

2.4. Mobility shift assays

Binding reactions were carried out at 20 °C in 10 mM Tris (pH 7.6), 50 mM KCl and 5 mM 2-mercaptoethanol. Mixtures were equilibrated for 30 min. Duplicate samples incubated for longer periods gave identical results, indicating that equilibrium had been attained (result not shown). Electrophoresis was carried out in 20% polyacrylamide gels, as described [15]. Autoradiographic images were captured on storage phosphor screens (type GP, GE Healthcare) detected with a Typhoon phosphorimager and quantitated with Image-Quant software (GE Healthcare).

2.5. DNA alkyltransferase assays

The NarI endonuclease is inactive when substrate DNA contains an O^6-methylguanine at position 2 of its cognate sequence [23] (numbering shown in Table 1). Cleavage is restored by DNA alkyltransferase activity provided by AGT. Oligonucleotides C and D containing the NarI sequence (Table 1) were annealed separately with complementary oligo E, as described above. Alkyltransferase reactions were carried out for 10 or 15 min (as indicated) with 1.1 μM AGT and 0.25 μM duplex DNA dissolved in 20 mM Tris–acetate (pH 7.9 at 25 °C), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol. Reactions were stopped by addition of SDS (final concentration 0.2%, w/v) followed by two extractions with water-saturated phenol and three extractions with water-saturated diethylether. Samples were incubated at room temperature under vacuum to evaporate ether before digestion with NarI. Samples were resolved by native gel electrophoresis [22]; electrophoretic distributions were recorded and quantified using a phosphorimager. In reactions carried out in AGT-excess, 98% of the DNA in our preparations was a substrate for both alkyltransferase and NarI activities. When this DNA was titrated with AGT, maximal repair was obtained when [AGT]/[competent DNA] > 1.04, corresponding to an alkyltransferase activity of 96%.

Table 1
Oligodeoxyribonucleotides used in this study.

DNA	Sequence
16-mers	
Oligo A	5′-AGT CAG TCA GTC AGT C-3′
5′-FAM-Oligo A	5′-(FAM)AGT CAG TCA GTC AGT C-3′
3′-FAM-Oligo A	5′-AGT CAG TCA GTC AGT C(FAM)-3′
24-mers	
Oligo C	5′-GGG TCA TTT GGC GCC TTT CGA TCC-3′
Oligo D	5′-GGG TCA TTT G**G**C GCC TTT CGA TCC-3′
Residue positions[a]	1 2 3 4 5 6
Oligo E (complements oligos C and D)	3′-CCC AGT AAA CCG CGG AAA GCT AGG-5′
26-mers	
Oligo F	5′-AGT CAG TCA GTC AGT CAG TCA GTC AG-3′
5′-FAM-Oligo F	5′-(FAM)AGT CAG TCA GTC AGT CAG TCA GTC AG-3′
3′-FAM-Oligo F	5′-AGT CAG TCA GTC AGT CAG TCA GTC AG(FAM)-3′

[a] NarI recognition sequence shown in large type. Residue shown in bold is O^6-methylguanine.