FOR THE RECORD

Active-site alkylation destabilizes human O^6-alkylguanine DNA alkyltransferase

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(RECEIVED July 20, 2003; FINAL REVISION September 14, 2003; ACCEPTED September 15, 2003)

Abstract

O^6-alkylguanine-DNA alkyltransferase (AGT) repairs pro-mutagenic O^6-alkylguanine and O^4-alkylthymine lesions in DNA. The alkylated form of the protein is not reactivated; instead, it is rapidly ubiquitinated and degraded. Here, we show that alkylation destabilizes the native fold of the protein by 0.5–1.2 kcal/mole and the DNA-binding function by 0.8–1.4 kcal/mole. On this basis, we propose that destabilization of the native conformational ensemble acts as a signal for ubiquitination.

Keywords: O^6-alkylguanine-DNA alkyltransferase; O^6-methylguanine-methyltransferase; DNA binding; denaturation; protein-alkylation

O^6-Alkylguanine-DNA alkyltransferase is a ubiquitous repair protein that plays a vital role in minimizing the mutagenic effects of alkylating agents (Samson 1992; Pegg et al. 1995; Sekiguchi et al. 1996; Pegg 2000). It catalyzes the stoichiometric transfer of a variety of alkyl substituents from the O^6-position of guanine to an active-site cysteine, preventing incorrect base pairing caused by these adducts. More than 100 alkyltransferases are now known, and crystal structures are available for three family members, the Ada-C protein from *Escherichia coli* (Moore et al. 1994); the human alkyltransferase (hAGT; Daniels and Tainer 2000); and the protein from the thermophilic archaeon, *Pyrococcus kodakaraensis* (Hashimoto et al. 1999). All known alkyltransferases lack the ability to dealkylate themselves, and no dealkylation activity has been found in cell extracts to date (Pegg et al. 1995; Daniels and Tainer 2000; Loktionova and Pegg 2002). On this basis, it is widely thought that alkyltransferase participates in a single reaction in which it is irreversibly inactivated.

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Article and publication are at http://www.proteinscience.org/cgi/doi/10.1110/ps.03319404.

Prior to alkylation, human AGT is a relatively long-lived protein (half-life ~24 h in vivo and in cell extracts [Pegg et al. 1991; Xu-Welliver and Pegg 2002]. However, it disappears rapidly from cells treated with DNA-alkylating agents or O^6-benzylguanine (Pegg et al. 1995; Pegg 2000). In eukaryotic cells, alkylated AGT is ubiquitinated and transferred to the cytoplasm, where it is degraded by the 26S proteasome (Srivenugopal et al. 1996; Major et al. 1997; Xu-Welliver and Pegg 2002). It has been proposed that the signal for ubiquitination is a conformational shift resulting from alkylation (Daniels and Tainer 2000; Daniels et al. 2000; Xu-Welliver and Pegg 2002). Consistent with this view, crystal structures show that the DNA-recognition helix (residues 125–136) and the guanine-binding loop (residues 153–160 of the benzylated protein are 0.5–1.5 Å more distant from the amino-terminal domain than they are in the native form [Daniels et al. 2000]). This more-open structure is also detected as an increase in stokes radius (Hora et al. 1983) and an increased susceptibility to proteolysis (Kanugula et al. 1998). Intriguingly, active-site methylated and benzylated AGTs bind DNA with affinities close to those of the native protein, indicating that the conformational shift is not sufficient to inactivate this function (Rasimas et al. 2003). Here, we show that benzylation destabilizes the folded ensemble of human AGT molecules in the presence

and absence of DNA. This raises the possibility that conformational lability may be part of the signal for ubiquitination and degradation.

Results and Discussion

Benzylation destabilizes the native fold of human AGT

The urea-dependent unfolding of AGT and C145-benzyl AGT were monitored by circular dichroism (CD) at 218 nm (Fig. 1) and were analyzed according to a two-state transition model. The reasonable fit indicates that the data are compatible with this model over the range of solution conditions that we have explored. Standard-free energy differences were calculated from apparent equilibrium constants (K_{obs}) using $\Delta G^\circ = -RT \ln K_{obs}$. Extrapolation of $\Delta G^\circ(N \rightarrow U)$ to [urea] = 0 provides estimates of the relative stabilities of the folded species of $\Delta G^\circ(N \rightarrow U$, nonalkylated) = 4.0 ± 0.4 kcal/mole; $\Delta G^\circ(N \rightarrow U$, benzylated) = 3.6 ± 0.8 kcal/mole with respect to the unfolded ensemble. Thus, the difference in stability attributable to benzylation is $\Delta\Delta G^\circ = -0.5 \pm 0.8$ kcal/mole. The large uncertainty in this value reflects the noise in the data and the long extrapolation to [urea] = 0 from concentrations with accurately measurable mole fractions of denatured AGT. As an alternate measure of the effect of benzylation on stability, we determined the value of $\Delta\Delta G^\circ$ at the midpoint urea concentration for the nonbenzylated protein ([urea] = 3.84 M). Here, $\Delta\Delta G^\circ$ was −1.2 ± 0.2 kcal/mole. Because both estimates of $\Delta\Delta G^\circ$ are small, we favor that obtained at the midpoint urea concentration, as mole fractions of native and denatured-

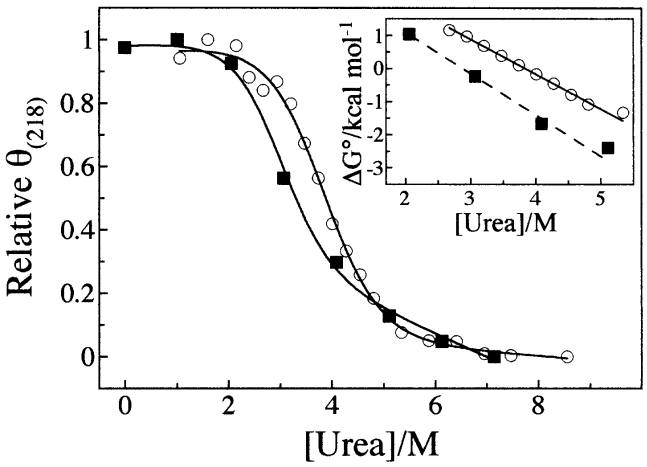

Figure 1. The unfolding of nonalkylated AGT (open circles) and alkylated AGT (closed squares) at equilibrium detected by circular dichroism. The final protein concentration was 110 μg/mL. Data were normalized to a full-scale of 1.0. (*Inset*) A plot of ΔG° vs. urea concentration for 1.5 M ≤ [urea] ≤ 6 M.

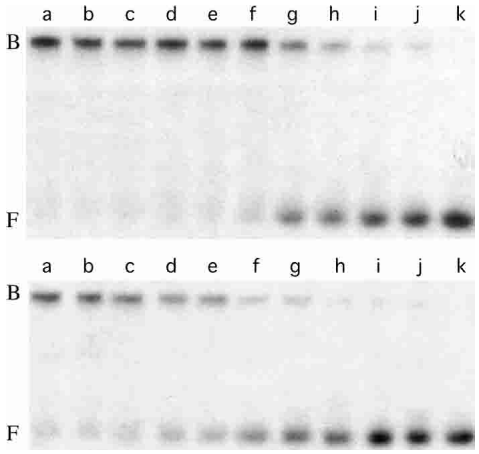

Figure 2. Effect of urea on the stability of an AGT–DNA complex. (*Top* panel) The complex of the nonalkylated AGT with the 16-mer duplex, titrated with urea. Samples contained 0.6×10^{-7} M 16-mer duplex and 1.9×10^{-6}M nonalkylated AGT, in binding buffer modified with 0–7.6 M urea (lanes *a–k*). (*Bottom* panel) The complex of benzylated AGT with the 16-mer duplex titrated with urea. Samples contained 0.6×10^{-7} M 16-mer duplex and 2.1×10^{-6}M benzylated AGT, in binding buffer modified with 0–6.8 M urea (lanes *a–k*).

AGT are measured most accurately in this range, and no extrapolation is needed.

Benzylation destabilizes the AGT–DNA complex

The DNA-binding activities of the nonalkylated protein and the benzylated protein were monitored by EMSA (Fried and Crothers 1981; Garner and Revzin 1981). The complexes shown in Figure 2 were formed with a 16-bp duplex and contain four molecules of AGT per molecule of DNA (Rasimas et al. 2003). The dependence of binding activity on [urea] (Fig. 3), shows that the benzylated complex is less stable than the one formed with nonalkylated protein. Extrapolation of $\Delta G(N \rightarrow U)$ to [urea] = 0 provides estimates of the relative stabilities of the species active in DNA binding, $\Delta G^\circ(N \rightarrow U$, nonalkylated) = 4.1 ± 0.3 kcal/mole; $\Delta G^\circ(N \rightarrow U$, benzylated) = 2.7 ± 0.7 kcal/mole, with respect to the urea-inactivated ensemble. Thus, the difference in stability attributable to benzylation is $\Delta\Delta G^\circ(N \rightarrow U) = -1.4 \pm 0.2$ kcal/mole. We also determined the value of $\Delta\Delta G^\circ$ at the midpoint urea concentration for the nonbenzylated protein ([urea] = 4.49 M). (The value of $\Delta\Delta G^\circ$ in the middle of the transition region is used widely in the comparison of mutant and wild-type proteins [c.f. Serrano et al. 1992 and Matouschek et al. 1994].) Here, $\Delta\Delta G^\circ(N \rightarrow U) = -0.8 \pm 0.2$ kcal/mole. The difference in these $\Delta\Delta G^\circ$ values reflects slight differences in the slopes of the graphs of ΔG° as a function of [urea], amplified by extrapolation to [urea] = 0 (Fig. 3, inset). Accordingly, we favor the value of $\Delta\Delta G^\circ(N \rightarrow U)$ measured at the transition midpoint as a

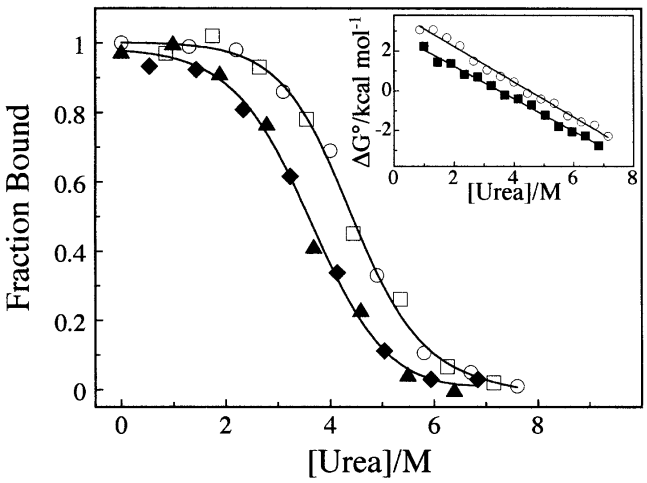

Figure 3. Analysis of the effect of urea on the stability of an AGT–DNA complex. Graph of mole fraction of 16-mer DNA bound as a function of [urea]. (Open symbols) Complex formed with nonalkylated AGT; (solid symbols) complex formed with benzylated AGT. The different symbols within a data set (e.g., open circles, squares) correspond to independent experiments. (*Inset*) Graph of ΔG° as a function of urea concentration.

reflection of the difference in stability of the DNA-binding activity.

The values of $\Delta G°(N \rightarrow U)$ for the nonalkylated protein and its DNA-binding activity are similar. This is understandable, as the DNA-binding reactions were carried out under conditions of protein excess (typically 0.6×10^{-7} M DNA, and 1.9×10^{-6} M AGT). Thus, if binding is stoichiometric, no more than ~12% of the protein population can be stabilized by formation of a 4:1 complex with the available DNA. On the other hand, the DNA-binding activity of the benzylated protein appears to be more sensitive to urea than does the protein folding monitored by CD ($\Delta G°(N \rightarrow U$, benzylated, CD) = 3.6 ± 0.8 kcal/mole; $\Delta G°(N \rightarrow U$, benzylated, EMSA) = 2.7 ± 0.7 kcal/mole). This is supported by the greater free energy difference attributable to benzylation when detected by DNA binding $\Delta\Delta G°(N \rightarrow U$, EMSA) = −(0.8–1.4) kcal/mole; $\Delta\Delta G°(N \rightarrow U$, CD) = −(0.5–1.2) kcal/mole. These results suggest that the structure(s) necessary for DNA binding by the benzylated protein may be more easily disrupted by urea than is the average structure detected by CD. (This observation comes with the caveat that the CD and the EMSA experiments were performed in different buffers, although the pH and the total salt concentrations were similar.)

The results shown here indicate that benzylation causes a modest reduction in the stability of the native fold of AGT. Such destabilization was predicted on the basis of comparisons of the crystal structure of the benzylated protein with that of the nonalkylated form (Daniels et al. 2000). This comparison shows benzyl-Cys145 in close contact with the carbonyl oxygen of Met 134 and with the side chain of Asn 137 in the active site. Steric clash with these residues is proposed to contribute to a 0.5–1.5 Å shift of the adjacent DNA-binding helix (residues 125–136) away from the active site (Daniels et al. 2000). The probable disruption of hydrogen bonding and hydrophobic packing that accompany this shift is a plausible mechanism for destabilization. However, the small magnitude of the experimentally observable destabilization suggests that additional interactions compensate energetically for the alteration of structure near the active site.

Whereas alkylation does not appear to have a dramatic effect on the free-energy difference associated with DNA binding (Rasimas et al. 2003), the linked conformational shift might expose new lysine residues for ubiquitination, and thus hasten the protein's degradation (Daniels et al. 2000; Xu-Welliver and Pegg 2002). Alternatively, the conformational change might act as a signal for factors that catalyze its dissociation from DNA. In either case, it is likely that this conformational change would be facilitated by destabilization of the native fold of AGT, whether the protein was free in solution or DNA-bound.

Materials and methods

AGT protein

Recombinant carboxy-terminal $(His)_6$-tagged human AGT protein was prepared as described previously (Pegg et al. 1993) and appeared homogeneous as judged by SDS-PAGE and analytical ultracentrifuge criteria (Fried et al. 1996). Samples were dialyzed against 50 mM Tris (pH 7.6), 5 mM DTT, and stored frozen at −80°C until needed. AGT protein was benzylated at the active site cysteine (C145) by incubation with 1 mM O^6-benzylguanine at 37°C for 30 min as described (in a few cases, AGT was benzylated by incubation with 0.5 mM O^6-benzylguanine at 25°C for 30 min.; Kanugula et al. 1998). Parallel aliquots subjected to this reaction were completely benzylated, as detected by mass spectrometry and by the elimination of detectable alkyltransferase activity (Rasimas et al. 2003). AGT concentrations were determined spectrophotometrically, using a molar extinction coefficient $\varepsilon_{280} = 3.93 \times 10^4$ $M^{-1}cm^{-1}$, calculated from the data of Roy et al. (1995).

Nucleic acids

Sixteen-residue oligodeoxyribonucleotides, with sequences (oligonucleotide A) 5′-GACTGACTGACTGACT-3′ and (oligonucleotide B) 5′-AGTCAGTCAGTCAGTC-3′ were obtained from Invitrogen. Oligonucleotide A was labeled at the 5′-end with ^{32}P as described (Maxam and Gilbert 1977). Both DNAs were transferred into 10 mM Tris (pH 8.0) using Sephadex G25 centrifuge columns (Amersham Biosciences). Oligonucleotides A and B were combined and annealed as described (Kanugula et al. 1995), using a 1.05-fold molar excess of B to ensure complete hybridization. Stock DNA concentrations were measured spectrophotometrically, using $\varepsilon_{260} = 1.3 \times 10^4$ $M^{-1}cm^{-1}$.

Protein stability studies

Equilibrium unfolding studies were carried out to detect changes in the stability of wild-type hAGT in the presence and absence of

O^6-benzylguanine. Urea (10 M) stock solutions were prepared as described previously (Ropson and Dalessio 1997). On the day of an experiment, a 9-M urea solution was prepared from the frozen stock containing the buffer components listed below. The final urea concentration of this solution was determined by refractive index measurements using a Milton Roy Abbe-3C refractometer at 25°C with an equation relating relative refractive index to concentration (Pace 1986). All experimental buffers contained 20 mM sodium phosphate, 50 mM NaCl, 0.1 mM EDTA (pH 7.6). Immediately before use, DTT was added to all experimental solutions for a final concentration of 1 mM. Samples (0.11 mg/mL protein) were equilibrated at the measurement temperature (25°C) for at least 30 min prior to CD measurements.

Equilibrium unfolding transitions were monitored by circular dichroism (CD). A Jasco J-710 spectropolarimeter was used to follow the loss of secondary structure in the far UV, using a thermostated 0.1-cm cell. All measurements were made at 25°C. Final protein concentrations were 0.11 mg/mL. Each data point was corrected for the background signal of the buffer and urea solutions. Nonlinear least-squares fits of the equilibrium data were obtained using an equation adapted by from Santoro and Bolen (1988) as described previously (Dalessio and Ropson 1998).

Electrophoretic mobility-shift assays

Binding reactions were carried out at 20 ± 1°C in 10 mM Tris (pH 7.6), 1 mM DTT, 100 mM NaCl 10 μg/mL BSA (binding buffer), modified to contain 0–7.6 M urea. Protein–DNA complexes were formed by adding appropriate amounts of AGT to solutions containing ^{32}P-labeled oligodeoxyribonucleotides. Samples were incubated at 20 ± 1°C for 1 h. Duplicate samples incubated for longer periods gave indistinguishable results, indicating that equilibrium had been attained. Electrophoresis was performed in 10% polyacrylamide gels (acrylamide:N,N′methylene bisacrylamide 75:1), cast, and run at 8 V/cm in 20 mM Tris-acetate (pH 7.6), 2 mM EDTA, 100 mM NaCl. Autoradiograms were obtained with Kodak XAR5 film, exposed at 4°C. Gel segments containing individual electrophoretic species were excised from the gel using the film as a guide and counted in a scintillation counter (Fried 1989; Fried and Garner 1998).

Acknowledgments

This work was supported by NIH grants CA-18137 to A.E.P., GM-57906 to I.J.R., and 5 T32 GM-08601 and 1 T32 ES-07312 to J.J.R.

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