

Recombinant human O^6 -alkylguanine-DNA alkyltransferase induces conformational change in bound DNA

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Received 9 December 1996; revised version received 20 March 1997

Abstract Circular dichroism, and steady-state and time-resolved fluorescence spectroscopy were used to compare the native recombinant human DNA repair protein O^6 -alkylguanine-DNA alkyltransferase (AGT) with AGT bound to ds-DNA. Contrary to fluorescence, analysis of the far-UV CD spectra indicated a conformational change of AGT upon binding to DNA: its α -helical content is increased by $\sim 12\%$. Analysis of near-UV CD spectra revealed that DNA was also affected, probably being separated into single strands locally.

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Key words: O^6 -Alkylguanine-DNA alkyltransferase; Protein-DNA interaction; Circular dichroism; Time-resolved fluorescence

1. Introduction

The DNA repair protein O^6 -alkylguanine-DNA alkyltransferase (AGT; EC 2.1.1.63) removes covalently bound alkyl groups from the mutagenic and toxic DNA lesion O^6 -alkylguanine (O^6 -AlkGua) by a stoichiometric one-step 'suicide' reaction (for review see [1,2]). Apparently, AGT is active in the absence of other proteins or cofactors [3,4]. Binding of AGT to DNA, a prerequisite for DNA repair, is non-specific and mediated mainly by ionic interactions [4].

The crystal structure of a 19 kDa C-terminal fragment of bacterial AGT (Ada) was published by Moore et al. [5]. This fragment contains the active center and is homologous to human AGT. The active site thiol of Cys¹⁴⁶C, corresponding to Cys³²¹ in the full-length Ada protein, is buried [5]. A conformational change is, therefore, required to activate AGT. Simple swiveling of the C-terminal helix (165C-175C) about the loop residues 150C-160C would expose a potential DNA binding surface and simultaneously render the thiol of Cys¹⁴⁶C accessible to duplex DNA [5]. Analysis of the protein-DNA complex by circular dichroism (CD) and steady-state fluorescence suggests that the interaction of human AGT with DNA induces a conformational change in the protein [6]. The question remains whether binding of AGT to DNA also induces a conformational change in DNA; e.g. it

has been suggested that AGT rotates the target alkylnucleotide out of the DNA double helix [7].

CD is highly sensitive to restrained dynamics and asymmetry in the environment of optically active chromophores (near-UV), and represents a measure for the secondary structure of proteins (far-UV [8]) and DNA (near-UV [9,10]). The decay of *Trp* fluorescence intensity reflects the local environment of *Trp* residues. Decay of fluorescence anisotropy provides independent information on changes of protein shape and on *Trp* side-chain dynamics.

We have investigated the interaction of recombinant human AGT with native ds-DNA, using CD and time-resolved fluorescence spectroscopy.

2. Materials and methods

Native recombinant human AGT was prepared by a combination of Ni²⁺-nitrilo-triacetic acid (NTA)-agarose affinity chromatography and ion exchange chromatography, as described previously [4].

2.1. Fluorescence spectroscopy

For fluorescence measurements the same solutions were used as for CD. Optical density was ≤ 0.1 (0.4 cm pathlength) at the wavelength used for excitation.

Steady-state fluorescence spectra were recorded on a Spex Fluorolog 211 photon counting spectrofluorimeter (Spex Industries, New York, USA [11]).

2.2. Time-resolved fluorescence spectroscopy

Fluorescence lifetimes and fluorescence anisotropy decays (FAD) were measured in the single photon counting mode, using a model 199 Edinburgh Instruments Ltd. Spectrometer (Edinburgh, UK), as outlined previously [11,12].

2.3. CD

Prior to measurements AGT was transferred to 20 mM NaH₂PO₄/Na₂HPO₄, pH 7.6; 50 mM NaF; 10% glycerol; 50 μ M EDTA; 1 mM β -mercaptoethanol, by gel exclusion chromatography (PD-10, Pharmacia-LKB). NaCl was replaced by NaF for better transparency in the far-UV. High molecular weight calf thymus DNA (Boehringer Mannheim, Mannheim, Germany) was dialyzed against 20 mM NaH₂PO₄/Na₂HPO₄, pH 7.6; 10% glycerol; 50 μ M EDTA overnight. Protein and DNA concentrations were determined spectrophotometrically (PU 8800, Philips), using $\epsilon[280] = 28.900$ [M⁻¹ cm⁻¹] for AGT [11] and $\epsilon[260] = 20.0$ [l/g-cm] for DNA.

CD measurements were carried out on an AVIV 62DS CD spectrometer (Lakewood, NJ, USA) and a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan), both calibrated with a 0.1% aqueous solution of d-10-camphorsulfonic acid [13]. The spectral bandwidth was 1.5 nm. In order to prevent artefacts varying the pathlength of the cell (0.01–10 mm) limited the absorption of the sample and, thus, the voltage of the photomultiplier. To determine secondary structural composition, spectra were analyzed with the CONTIN program package [14]. Further details have previously been described [15].

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Abbreviations: AGT, O^6 -alkylguanine-DNA alkyltransferase; DTT, 1,4-dithiothreitol; FAD, fluorescence anisotropy decay; FWHM, full width at half maximum; NTA, nitrilo-triacetic acid

Table 1
Fluorescence lifetime (A) and anisotropy decay data (B)

A	B ₁	B ₂	B ₃	τ ₁ [ns]	τ ₂ [ns]	τ ₃ [ns]	χ ²	<τ> [ns]
AGT ^a	0.07	0.37	0.56	0.76	3.21	8.24	1.18	5.9
AGT, DNA ^b	0.08	0.38	0.54	0.79	3.23	8.35	1.18	5.8
B	r ₀	r ₁	r _∞	∅ [ns]	χ ²			
AGT ^a	0.166	0.102	0.064	13.6	0.72			
AGT, DNA ^b	0.169	0.072	0.097	15.1	1.16			

Excitation wavelength, 295 nm; bandwidth, 12 nm. The fluorescence emission was passed through filters DUG 11 and WG 320. At least 80 000 counts were accumulated in the peak channel of the total fluorescence intensity. The lamp pulse was recorded at 345 nm using a diluted suspension of 'Ludox' (Du Pont, Wilmington, DE, USA). All measurements were carried out at 4°C. ^a0.98 g/l AGT, ^b1.0 g/l AGT and 0.15 g/l calf thymus DNA.

(A) Total fluorescence intensity decays were fitted to the equation $s(t) = b_0 + \sum b_i \exp(-t/\tau_i)$ with $i = 1, 2, \dots, n$. b_i and τ_i are the amplitude and lifetime of the i th excited state, respectively; χ^2 , reduced chi-squared. B_i and the mean lifetime, $\langle \tau \rangle$, were calculated according to: $\langle \tau \rangle = \sum B_i \tau_i = \sum [b_i \tau_i / \sum b_i \tau_i]$.

(B) Anisotropy decays were fitted to $r(t) = r_1 \exp(-t/\emptyset) + r_\infty$; r_i , anisotropies; \emptyset , the rotational correlation time. The limiting anisotropies are: $r(t \rightarrow 0) = r_0$, and $r(t \rightarrow \infty) = r_\infty$, $r_0 = r_1 + r_\infty$.

3. Results and discussion

3.1. Steady-state fluorescence

The fluorescence emission spectra of native and DNA-bound AGT peaked at $\lambda = 342.5$ nm, with a full width at half maximum (FWHM) of 58.5 nm. Hence, the fluorescence emission properties of the *Trp* residues do not reflect DNA binding nor the conformational changes induced in AGT upon binding (see below). This may be explained by *Trp*⁶⁵ being located in the N-terminal domain which is probably not involved in DNA binding [5]. *Trp*¹⁰⁰ is not essential for DNA binding [16]. *Trp*¹⁶⁷ is part of the C-terminal helix which has been proposed to fit into the major groove of DNA mediating the binding to DNA [5]. *Trp*¹⁶⁷ should, therefore, be a good reporter group. Unfortunately its fluorescence signal appears to be quenched by the neighboring *Glu*¹⁶⁶ [17,18]. Removal of 31 C-terminal amino acids from human AGT does not affect its repair activity [19]. *Trp*¹⁹¹ is, therefore, not involved in DNA binding. The proposed elongation of the helices (see below) would not affect any of the *Trp* residues.

3.2. Time-resolved fluorescence spectroscopy

The results of time-resolved fluorescence measurements are presented in Table 1. In parallel to the steady-state data the mean fluorescence lifetime, $\langle \tau \rangle$, of AGT was not significantly changed upon addition of DNA. Binding of AGT to DNA was only reflected by an increase of the limiting anisotropy, r_∞ , because – compared to $\langle \tau \rangle$ of *Trp* residues – the AGT-DNA complex has a much longer rotational correlation time, \emptyset . Assuming that the residual r_∞ is only due to the mismatch of $\langle \tau \rangle$ and \emptyset in the case of pure AGT [11], the fraction of AGT bound to DNA can be estimated from the ratio of $r_\infty/r_0 = 0.097/0.169$ to be $\sim 57\%$ (Table 1B). This val-

ue is in reasonable agreement with the prediction based on the law of mass action (equation (1) in [11]). The respective values from Fig. 1 (47.5 μ M AGT; and 231 μ M base pairs = 23.1 μ M binding sites, association constant 7.1×10^5 M⁻¹ [4]) resulted in a concentration of occupied binding sites of 21.9 μ M. Hence, almost all of the DNA and $\sim 50\%$ of the AGT molecules were bound.

3.3. CD spectroscopy

Upon addition of calf thymus DNA, both the magnitude of the far-UV CD signal and the α -helical content were increased ($\sim 6\%$ in the case of 0.15 g/l DNA; Fig. 1A; Table 2). Only a limited amount of DNA could be added, beyond which precipitation started. The turbidity of the solutions was carefully examined and all measurements were carried out with clear solutions. The spectra were measured at $\lambda \geq 200$ nm only, due to the high absorption of the DNA. Nevertheless the determination of the α -helical content is reliable [8]. The secondary structural composition of AGT determined here is in reasonable agreement with the values calculated from the homology model of the three-dimensional structure of human AGT based on the crystal structure of the C-terminal domain of Ada [11]. The CD signal of DNA was subtracted, although it was negligible in the far-UV when compared to the AGT signal.

Since only $\sim 50\%$ of AGT molecules are bound to DNA, the α -helical content is actually increased by $\sim 12\%$, corresponding to 25 amino acids or almost seven turns. The increase in α -helical content is at variance with the data of Chan et al. [6] who observed a decrease of $\sim 7\%$ while the content of β -turns was increased by $\sim 7\%$. This discrepancy may perhaps be attributed to partial precipitation of the protein. Both alkylation of AGT and binding to DNA result in similar conformational changes of AGT but in the latter case they

Table 2
Secondary structural composition of AGT and AGT bound to DNA

Protein	α -helix [%]	β -sheet [%]		β -turn [%]	remainder [%]
		anti-par.	par.		
(1) AGT	24.9	18.2	5.1	18.1	33.7
(2) AGT, 0.073 g/l DNA	27.7	17.2	4.7	17.9	32.5
(3) AGT, 0.15 g/l DNA	31.6	15.1	5.2	16.7	31.4

Secondary structural composition of AGT was determined from far-UV CD spectra using the CONTIN program [14]. Spectra were measured from 200 to 245 nm at room temperature. AGT concentrations are given in Fig. 1A.

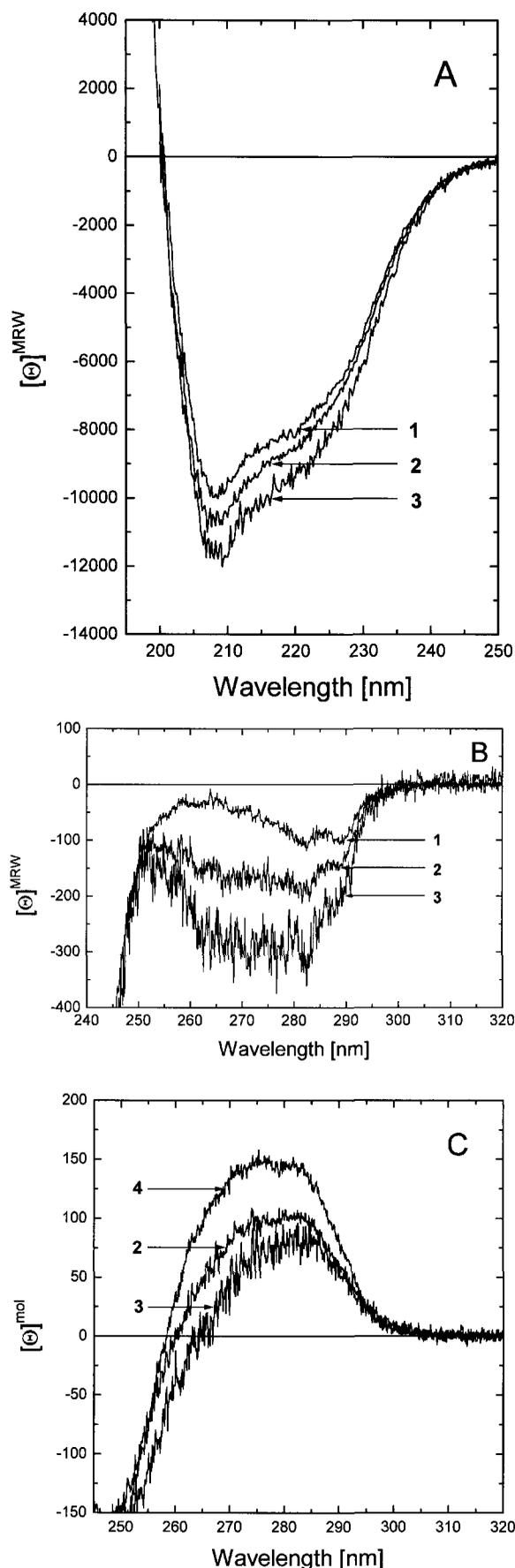


Fig. 1. CD spectra of native human AGT incubated with DNA. A: Far-UV; B, C: near-UV. Samples were prepared as described in Section 2 and measured at room temperature (25°C). In the case of A and B, the spectra of pure calf thymus DNA were subtracted. In C, the spectra of the pure protein solution were subtracted. A, B: $[\theta]^{MRW}$ is given in degrees \times cm² \times decimol⁻¹. C: The spectra are normalized to the molar concentration of DNA bases. The spectra shown are original tracings including noise. A: (1) 1.02 g/l AGT; (2) 1.02 g/l AGT, 0.073 g/l calf thymus DNA; (3) 1.0 g/l AGT, 0.15 g/l DNA. B, C: (1) 1.03 g/l AGT; (2) 1.02 g/l AGT, 0.073 g/l DNA; (3) 1.03 g/l AGT, 0.3 g/l DNA; (4) 0.073 g/l DNA.

are more pronounced [11]. Alkylation may ‘freeze’ some of the conformational changes induced by DNA binding (see below).

For the binding of Ada to DNA, Moore et al. [5] proposed a generalized binding to DNA mediated by a variant of the helix-turn-helix motif comprising the helices three, four and five, as well as fitting of the C-terminal helix into the major groove of DNA. There is experimental evidence for both. (i) In human AGT *Arg*¹²⁸ is required in order to interact with the DNA substrate [20,21]. Actually the protein conformation in the vicinity of *Arg*¹²⁸ exhibits close homology to the helix-hairpin-helix motif [22] (h is a hydrophobic residue):



(codons 115–130 of human AGT, amino acids in accordance with the motif are typed in bold, fully conserved residues are underlined). This motif provides non-sequence-specific interactions of proteins and DNA and can bind either ss-DNA or ds-DNA. Conformational stabilization and elongation of helices upon binding to DNA are common features of helix-turn-helix proteins [23]. Hence, upon binding to DNA the loop between helices four and five probably becomes almost helical, and helix four may be extended at its N-terminus. (ii) The G156A mutant was stimulated only very slightly by addition of DNA [19], indicating the involvement of parts of AGT positioned C-terminally of the active site, especially the C-terminal helix which may be involved in DNA binding similar to the ‘hinge’ helix of the PurR repressor [24]. DNA binding may cause helix six to be extended N-terminally because the C-terminal the residues are not conserved in different species. In favor of two binding sites are also the results of Kanugula et al. [20] obtained with the R128A mutant AGT: addition of ss-DNA increased the activity of this mutant in cleaving *O*⁶-benzylguanine considerably less than ds-DNA while both ss- and ds-DNA are effective in causing stimulation in the case of native AGT. ss-DNA will occupy only one site in the case of R128A which is insufficient to fully stimulate AGT. ds-DNA, combining the residual affinity of the R128A site and of the other site, binds to both sites and fully stimulates AGT.

In the near-UV, the CD spectra of pure protein and DNA were of similar magnitude. The spectrum of calf thymus DNA measured in the present study was very similar to that published by Fairall et al. [25]. Spectral changes in the near-UV may, in principle, be due to conformational changes either in AGT or DNA, or in both molecules. When the undisturbed spectrum of DNA is subtracted (Fig. 1B), all of the changes are attributed to AGT. In this case the magnitude of the near-UV CD spectrum is increased (Fig. 1B), in accordance with

the data of Chan et al. [6]. Pure AGT exhibits a relatively pronounced near-UV CD spectrum, probably because of its rather rigid structure [11]. Therefore, the increase in the signal is unlikely to originate exclusively from alterations within the AGT molecule, in particular since only ~50% of AGT molecules were bound to DNA.

Attributing all of the conformational changes to DNA results in a decreased CD signal at 275 nm while its shape does not change (Fig. 1C). As the winding angle of the DNA helix increases, the twist angle of the base pairs, and thus the CD signal decrease [26]. When DNA is bent, winding angle and base-pair twist change in a similar way, also resulting in a decreased CD signal [27]. Hence, DNA may be either overwound or bent by AGT; or in association with bending the DNA may be overwound similar to the situation in some protein-DNA complexes involving proteins which contain a helix-turn-helix DNA-binding motif [28,29]. Overwinding obstructs formation of single-stranded regions. Locally induced ss-DNA which has been suggested by Spratt and Campbell [30] would also be in accordance with the near-UV CD spectra [31]. Since this would facilitate rotation of the target alkyl-nucleotide out of the DNA double helix [7], it is the more probable conformational change induced by bound AGT.

Acknowledgements: This work was supported by the Dr. Mildred Scheel Stiftung für Krebsforschung. We thank Konstanze Thiemann and Jürgen Stahl for carrying out the CD measurements, Dipl.-Chem. Günther Kurapkat for determining the secondary structural composition of proteins by numerical analyses of far-UV CD spectra, and Dr. Mary Lucey for helpful discussions.

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