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Brief Communication

The substrate binding interface of alkylpurine DNA glycosylase AlkD

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ABSTRACT

Tandem helical repeats have emerged as an important DNA binding architecture. DNA glycosylase AlkD, which excises *N*3- and *N*7-alkylated nucleobases, uses repeating helical motifs to bind duplex DNA and to selectively pause at non-Watson–Crick base pairs. Remodeling of the DNA backbone promotes nucleotide flipping of the lesion and the complementary base into the solvent and toward the protein surface, respectively. The important features of this new DNA binding architecture that allow AlkD to distinguish between damaged and normal DNA without contacting the lesion are poorly understood. Here, we show through extensive mutational analysis that DNA binding and *N*3-methyladenine (3mA) and *N*7-methylguanine (7mG) excision are dependent upon each residue lining the DNA binding interface. Disrupting electrostatic or hydrophobic interactions with the DNA backbone substantially reduced binding affinity and catalytic activity. These results demonstrate that residues seemingly only involved in general DNA binding are important for catalytic activity and imply that base excision is driven by binding energy provided by the entire substrate interface of this novel DNA binding architecture.

1. Introduction

Genomic integrity is continuously threatened by chemical modifications caused by endogenous metabolites and environmental toxins. Alkylation, oxidation, and deamination produce a variety of single-base lesions with mutagenic and cytotoxic effects [1]. Each type of chemical alteration, however, is recognized by a DNA glycosylase that cleaves the *N*-glycosidic bond to liberate the damaged base and initiate base excision repair [2]. Recognition by most DNA glycosylases is achieved by penetration of the protein into the DNA duplex to exploit structural and/or energetic differences between normal and damaged bases pairs, and by flipping the damaged nucleobase into the enzyme active site [3].

The glycosylase AlkD selectively excises positively charged *N*3- and *N*7-methylpurines by a mechanism distinct from other monofunctional glycosylases [4–7]. AlkD is structurally unique, composed of six tandem helical ALK repeats that bear resemblance to HEAT motifs normally associated with protein interactions [8]. Crystal structures of AlkD in complex with DNA containing a 3mA mimetic and an abasic site analog revealed that, unlike other glycosylases, AlkD does not contact the damaged nucleotide, but rather anchors itself to the DNA duplex through interactions with the strand directly opposite the lesion and with regions of the damaged strand several nucleotides away from the lesion [7]. The enzyme-bound DNA is trapped in a distorted conformation in which the lesion is flipped toward the solvent and away from the protein, and the complementary nucleotide is flipped toward the protein surface. This arrangement is inconsistent with the typical catalytic mechanism of monofunctional glycosylases because it precludes a side-chain carboxylate from stabilizing the oxocarbenium intermediate formed during *N*-glycosidic bond cleavage or activating a water for nucleophilic attack at the anomeric C1′ carbon (Fig. 1) [9–14].

Alternatively, inhibition of AlkD catalyzed 7mG excision by methylphosphonate substitution suggests that the distorted DNA backbone conformation may allow a phosphate in close proximity to the lesion to provide electrostatic stabilization or nucleophilic activation through a substrate-assisted mechanism of catalysis [15], as previously observed for uracil excision from DNA by UDG [11,16,17]. Such a mechanism would suggest that without contacts to the lesion, AlkD must rely on enzyme–substrate binding energy to distort the DNA backbone into an autocatalytic conformation to promote base excision. Here, we demonstrate through mutational analysis that residues throughout the concave DNA binding surface, including residues distant from the lesion, affect binding and catalysis. These required, concerted interactions with multiple sites on both damaged and undamaged strands are consistent with a mechanism in which the DNA backbone must be remodeled into a catalytic conformation.

Abbreviations: 3d3mA, 3-deaza-*N*3-methyladenine; 3mA, *N*3-methyladenine; 7mG, *N*7-methylguanine; FAM, 6-carboxyfluorescein; THF, tetrahydrofuran.

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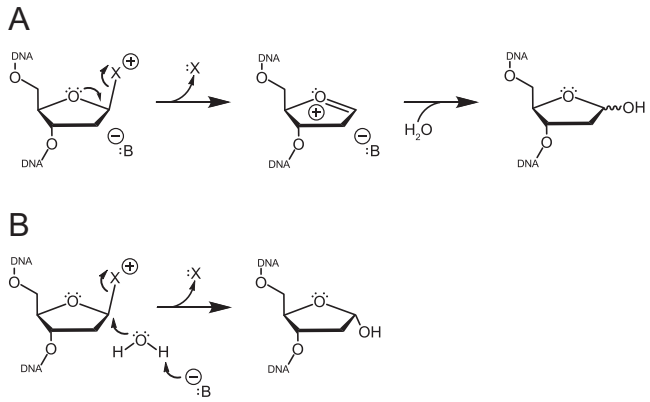

Fig. 1. Mechanisms of cationic lesion excision by monofunctional DNA glycosylases. Cleavage of the *N*-glycosidic bond liberates the damaged nucleobase ("X") and generates an abasic site. (A) Dissociative mechanisms utilize anionic protein side chains or DNA phosphate groups ("B") to directly assist bond breakage and to stabilize the resulting oxocarbenium intermediate. (B) Associative mechanisms employ a general base ("B") to activate a water for nucleophilic attack at the anomeric C1′ carbon.

2. Materials and methods

2.1. Protein purification

AlkD was purified by immobilized metal affinity, heparin affinity, and size exclusion chromatography steps as previously described [6]. AlkD mutants were generated by site-directed mutagenesis using a Quik-Change kit (Stratagene) and purified in the same manner as wild-type AlkD (Fig. S1).

2.2. Thermal melting

Structural integrity of AlkD mutants was verified by far-UV circular dichroism spectroscopy using a J-810 spectropolarimeter (Jasco) and a Peltier temperature controller (Jasco). Molar ellipticity was monitored at 222 nm as mixtures containing 10 μM enzyme, 20 mM Bis-Tris propane (pH 6.5), 100 mM NaCl, and 0.1 mM EDTA were heated at 2 °C/min in a 0.1-cm cell. Melting temperatures (T_m) were derived by fitting the data to the equation $\theta = 1/(1 + e^{(T_m - T)/k})$, where θ is molar ellipticity, T is temperature, T_m corresponds to the temperature at 50% denaturation, and k describes the cooperativity of the transition (Fig. S2).

2.3. DNA binding

DNA binding was monitored by changes in fluorescence anisotropy as enzyme was added to a 25-mer oligonucleotide duplex [d(GACCACTACACCXATTCCTAACAAC)/d(GTTGTTAGGAATYGGTGTAGTGGTC)-FAM] labeled with 6-carboxyfluorescein (FAM). Unmodified G·C-DNA contained X = G and Y = C, mismatched G·T DNA contained X = G and Y = T, and abasic THF·C-DNA contained X = THF and Y = C. Enzyme (0–30 μM AlkD-D113N and AlkD-R148A; 0–75 μM wild-type AlkD and all other mutants) was added to 50 nM FAM-DNA, 20 mM Bis-Tris propane (pH 6.5), 100 mM NaCl, 2 mM DTT, and 0.1 mM EDTA and incubated at 25 °C for 10 min. Fluorescence anisotropy measurements were recorded as previously described [6]. Equilibrium dissociation constants (K_d) were derived by fitting a two-state binding model to the data (Fig. S3).

2.4. Base excision from oligonucleotide substrate

Excision of 7mG from a 25-mer oligonucleotide duplex [d(GACCACTACACC(7mG)ATTCCTTACAAC)/d(GTTGTAAGGAATCGGTGTAGTGGTC)] was measured by autoradiography as previously described [6]. Reactions were performed at 37 °C and contained 20 μM enzyme (5 μM AlkD-D113N and AlkD-R148A), 2 nM DNA, and glycosylase buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM DTT, and 2 mM EDTA]. Due to thermal instability, reactions containing AlkD-D113N (T_m = 30.7 °C) were also carried out at 25 °C to rule out inactivity due to protein unfolding. Second-order rate constants (k_{obs}) were obtained from single-exponential fits to the data (Fig. S4).

2.5. Base excision from genomic DNA substrate

Excision of 3mA and 7mG from methylated calf thymus DNA was measured by HPLC–MS/MS as previously described [18]. Reactions were performed at 37 °C for 1 h and contained 5 μM enzyme, 10 μg DNA, glycosylase buffer, and 0.1 mg/mL BSA. Due to thermal instability, reactions containing AlkD-D113N (T_m = 30.7 °C) were also carried out at 25 °C to rule out inactivity due to protein unfolding. In lieu of enzyme, controls contained 5 N HCl or 2 mM Bis-Tris propane (pH 6.5), 10 mM NaCl, and 0.01 mM EDTA.

3. Results and discussion

3.1. DNA binding architecture

Tandem helical repeats have emerged as an important and widespread structural feature among DNA binding proteins [8]. AlkD is composed of six antiparallel two-helix ALK motifs that stack into a short left-handed solenoid with a positively charged concave binding surface created by basic residues on each C-terminal helix (Fig. 2). Unlike other tandem helical repeats that bind nucleic acids, ALK motifs contact the backbone but not the nucleobases [8]. Sixteen residues on the concave surface of AlkD form electrostatic or hydrophobic contacts with phosphate or deoxyribose groups in substrate- and product-like complexes with DNA containing 3-deaza-*N*3-methyladenine (3d3mA) and tetrahydrofuran (THF), respectively (Fig. 2). The DNA in both complexes is markedly distorted. In the substrate-like complex, the 3d3mA·T base pair is sheared due to rotation of the thymine into the minor groove and toward the protein surface (Fig. 2). A nearly identical conformation is present in a complex containing DNA with a mismatched G·T base pair (PDB: 3JXY) [7]. In the product-like complex, both the thymine and the THF are fully extruded from the duplex, creating a single-base bulge in which base stacking is maintained by the flanking bases (Fig. 2).

In order to understand how this unique nucleic acid binding surface recognizes DNA damage, we mutated 10 of the 16 residues that contact the DNA in the crystal structures and measured DNA binding to 25-mer oligonucleotides containing a centrally located Watson–Crick G·C base pair, a G·T mismatch, or a THF·C abasic site; 7mG excision from the same 25-mer oligonucleotide; and 3mA and 7mG release from methylated genomic DNA. Wild-type AlkD binds G·C-, G·T-, and THF·C-DNA with weak (low micromolar) affinity typical of protein–DNA complexes involving only nonspecific backbone contacts (Fig. 3A and Table S1) [6]. Cleavage of 7mG from the same 25-mer oligonucleotide occurs at $1.2 \times 10^3\ M^{-1}\ s^{-1}$ (Fig. 3B and Table S2), while excision of 7mG and 3mA from methylated genomic DNA occurs 5-fold more slowly ($2.2 \times 10^2\ M^{-1}\ s^{-1}$) and 7-fold more rapidly ($8.0 \times 10^3\ M^{-1}\ s^{-1}$), respectively [18]. These rates of lesion removal are comparable to some 3mA glycosylases that extrude the damaged base into a nucleobase binding pocket during catalysis [19–22].

3.2. Damaged strand interactions

Six basic or neutral hydrophilic residues interact with the modified DNA strand. Three of these residues (Gln38, Thr39,