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Binary and ternary binding affinities between exonuclease-deficient Klenow fragment (Kf-exo⁻) and various arylamine DNA lesions characterized by surface plasmon resonance

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Abstract

We used surface plasmon resonance (SPR) to characterize the binding interactions between exonuclease-free Klenow fragment (Kf-exo⁻) and unmodified and modified dG adducts derived from arylamine carcinogens: fluorinated 2 aminofluorene (FAF), 2-acetylaminofluorene (FAAF), and 4-aminobiphenyl (FABP). Tight polymerase binding was detected with unmodified dG and the correct dCTP. The discrimination of correct versus incorrect nucleotides was pronounced with K_D values in order of dCTP $\ll$ dTTP $<$ dATP $<$ dGTP. In contrast, minimal selectivity was observed for the modified templates with Kf-exo⁻ binding tighter to the FAAF (k_{off} : 0.02s⁻¹) and FABP (k_{off} : 0.01s⁻¹) lesions than to FAF (k_{off} : 0.04s⁻¹).

DNA is under constant assault by various endogenous and exogenous pathways, which result in different types of DNA damage. When a polymerase encounters a lesion, it can bypass by replicative polymerase, either inserting the correct base (error-free) or incorrect base (error-prone)¹. The environmental arylamine carcinogens are known to form C8-substituted dG adducts *in vivo*. We have shown that these lesions exist in a mixture of the base displaced stacked, major groove B-type, and wedge conformers, with each leading to potentially unique sequence-dependent mutation and nucleotide excision repair outcomes².

It is important to understand the nature of interactions between polymerase and DNA lesions. Crystal structure and kinetic analyses have been used to elucidate details of polymerase action at an atomic resolution¹. However, similar structural details of bulky DNA lesions have been challenging due to difficulties with obtaining crystals^{3,4}. Consequently, various alternative techniques such as fluorescence, circular dichroism spectroscopy, gel mobility shift assays, and footprinting assays have been used^{5,6}. However, these techniques are either qualitative or semi-quantitative, non-compatible with fast dissociation rates, and require labeling of at least one of the components of interest. Although gel-based assays are relatively simple and robust, samples of interest will not be in chemical equilibrium and the system's components are not amenable to testing across temperatures or salt concentrations⁶. Surface plasmon resonance (SPR) is a chip based, label free solution technique that allows real-time monitoring of binding interactions between DNA and proteins⁵⁻⁸.

In this report, a SPR study was conducted to examine-polymerase interactions of dG lesions derived from three fluorinated prototype arylamine carcinogens: 2-aminofluorene (FAF), 2-

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Supporting Information. The synthesis and mass spectrum of adducts, immobilization procedures, binding profiles, and simulated data are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

acetylaminofluorene (FAAF), and 4-aminobiphenyl (FABP) (Fig. 1c). We employed exonuclease-free *E. coli* DNA polymerase I Klenow fragment (Kf-exo⁻) as it avoids complication of proofreading activity. The features of fluorinated arylamines as effective conformational probes are well documented^{2,9}. The present study takes advantage of the sensitivity of Biacore T200 to conduct SPR analysis of the binary and ternary polymerase complexes of bulky carcinogen-DNA adducts.

Figure 1a and S1a show the construction scheme for a biotinylated hairpin-based template-primer strand on a gold sensor chip. The hairpin-DNA was used to improve stability of oligonucleotides during performance of kinetics experiments. Arylamine-modified 31-mer oligonucleotides were purified by HPLC and characterized by mass spectrometry (Fig. S2). The biotin-hairpin-template/primer strands were annealed, ligated, and purified by denaturing polyacrylamide gel (Fig. S1b). The incorporation of dideoxythymidine (ddT) was carried out using Kf-exo⁻ and the 3' terminal ddT allowed capture of the ternary polymerase/template-primer/dNTP complex without primer extension.

The kinetic assays were optimized with respect regeneration buffer, surface density, and surface testing, as described elsewhere¹⁰ (Fig. S3). The binding kinetics analysis was performed by injecting varying amounts of Kf-exo⁻ to cover the hairpin template-primer DNA (Fig. 1b) coated on streptavidin surface in the absence (binary) and presence (ternary) of dNTPs (100 μ M). The injections were repeated three times for each concentration in random, and the resulting data were fitted to the Langmuir model (1:1) (Fig. 2a). From the fitting, binding constants (k_{on} , k_{off} and K_D) were calculated (Table 1 and S1) using Biacore's BIA simulation software. The Chi-squared values for the 1:1 fitting were less than 1% of R_{max} (0.002–0.003 for all experiments with R_{max} in the range of 0.7–3.5RU) (Figs. S4 and S5). The K_D values for ternary systems were determined using affinity analysis as the association rate (k_{on}) reaches the near-diffusion limit. This procedure allowed the monitoring of interactions between unmodified or adducted DNA with different polymerases on a single chip. Furthermore, DNA over the chip surface was found to be stable for at least 7–10 days, without loss in binding activity under buffered reaction conditions.

The results from the binding assay (Fig. S6) are summarized in Table 1. The Kf-exo⁻ bound tightly to unmodified DNA in the presence of a correct incoming dCTP opposite the templating dG. However, relative to dCTP binding, binding tightness was reduced by 30-, 60-, 34-, and 264-fold in binary, dATP, dTTP, and dGTP, respectively (Fig. 2b and Table 1). The discrimination ability of correct versus incorrect nucleotides was significant, as the Watson-Crick base pair dCTP bound tightly and dGTP does not bind significantly. In contrast, the discrimination effect on Kf-exo⁻ binding was weaker for binding to FAF than for binding to unmodified DNA. The specificity of binding between the correct dCTP and incorrect nucleotides, as well as for the binary system, differed by only 2- to 16-fold. The tightness of Kf-exo⁻ binding in the presence of dCTP was reduced by 4-fold, as compared to that of the unmodified control.

Moreover, the difference in binding affinity between dCTP and dATP was less for FAF (10-fold), as compared to that of unmodified DNA (60-fold) (Fig. 2b). The Kf-exo⁻ bound more tightly to FAAF ($k_{off}=0.02s^{-1}$) and FABP ($k_{off}=0.01s^{-1}$) lesion sites than to the unmodified control ($k_{off}=0.13s^{-1}$) while k_{on} values are similar (Table S1). However, discrimination between correct and incorrect nucleotides was not maintained with FAAF or FABP, for which binding affinities differed by only 1- to 3-fold (Fig. 2b).

Highly specific binding of Kf-exo⁻ to unmodified DNA in the presence of dCTP opposite a dG templating base is in line with the polymerase undergoing conformational change from

an open to a closed system to form Watson-Crick base pairs¹¹. However, Kf-exo⁻ does bind weakly with incorrect nucleotides, probably retaining the open polymerase conformation. In particular, the binding of dGTP is very poor compared to other nucleotides.

To further confirm that the binding of polymerase to DNA is 1:1, theoretical $R_{\max}$ values were calculated and compared with experimental values. The data presented here are consistent with data from sedimentation studies in which polymerase was shown to bind template-primer junction in a 1:1 ratio¹². Interestingly, the K_D value for Kf-exo⁻ binding to FAF adducts was higher in the presence of dCTP than with unmodified DNA (Table 1), indicating that the lesion prevents the nucleotide-induced, catalytically-favored closed conformation. Previous studies have shown that the carcinogenic aminofluorene orients into the energetically favorable solvent-exposed major groove, which causes less disruption at the replication fork, but may perturb the groove structures and the geometry in the active site of the polymerase³.

The aforementioned crystal structure of AF on T7 DNA polymerase showed fuzzy electron densities around the carcinogenic aminofluorene moiety in line with sequence-dependent conformational heterogeneity in solution⁴. The present kinetics data also fit with previously published findings from a single nucleotide insertion assay study in which dATP was the next preferred nucleotide after dCTP¹³.

The higher binding affinity of Kf-exo⁻ to the bulky *N*-acetylated FAAF lesion, compared to unmodified DNA, could be due to the adduct perturbing the template-primer junction while maintaining some specific interactions with amino acids on the active site of the polymerase. It has been shown that the AAF lesion has two hydrogen bond interactions between the N²-amino group of the modified guanine and Asp-534, as well as between the N⁷-guanine and Arg-566⁴. In addition, the lesion adopts a *syn*-glycosidic conformation wherein the fluorene moiety is inserted between the hydrophobic pocket of the O-helix finger subdomain. These changes also keep the polymerase in the open and maintain a distorted conformation of the subdomain fingers, causing the Tyr-530 residue to occupy the binding region of the nucleotide and preventing interaction between the incoming nucleotide and polymerase⁴. The present data are also in agreement with previous results from tryptic digestion studies, in which the polymerase was shown to bind very tightly to unmodified DNA in the presence of the correct nucleotide and to be insensitive to digestion; FAAF did not exhibit any additional stability in relation to the incoming nucleotide¹⁴. FAF adducts are known to exist in a sequence-dependent equilibrium of B-type and base-displaced stacked conformers^{2,9}. FABP is similarly *N*-deacetylated; however, its biphenyl moiety is not as coplanar as fluorene, thereby resulting in a lesser stacked conformer population¹⁵. Consequently, FABP may behave similar to FAAF at the replication fork in the active site of a polymerase.

In summary, tight binding of Kf-exo⁻ was observed with unmodified dG in the presence of a correct dCTP in this study. Nucleotide selectivity was pronounced with K_D values in the order of dCTP $\ll$ dTTP $<$ dATP $<$ dGTP. In contrast, minimal selectivity was observed for the modified templates: Kf-exo⁻ bound tightly to FAAF and FABP lesions as compared to FAF. The SPR results for FAF and FAAF agreed with those obtained from gel-based assays,¹⁶ demonstrating SPR as a powerful and superior tool for studying protein/DNA interactions with bulky DNA lesions as it provides k_{on} and k_{off} rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SPR	surface plasmon resonance
Kf-exo⁻	Klenow fragment exonuclease deficient
FAF	<i>N</i> -(2'-deoxyguanosin-8-yl)-7-fluoro-2-aminofluorene
FAAF	<i>N</i> -(2'-deoxyguanosin-8-yl)-7-fluoro-2-acetyl-aminofluorene
FABP	<i>N</i> -(2'-deoxyguano-sin-8-yl)-4'-fluoro-4-aminobiphenyl

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Figure 1.

(a) Schematic representation of template-primer DNA constructs (b) oligonucleotide sequence used in the SPR assay and (c) the structures of arylamine-dG adducts

Figure 2.

(a) Sensorgrams of Kf-exo⁻ binding with unmodified and arylamine-modified DNA adducts (fitted curves were overlaid as red lines). (b) Plot of specificity ratio of binary or wrong nucleotide to correct dCTP vs. unmodified DNA and modified adducts

Table 1

Dissociation constants (K_D) for dG and dG-arylamine adducts with Kf-exo- using steady-state affinity analysis

Template	Binary	dCTP	dATP	dGTP	dTTP
-G-	1.51±0.50	0.05±0.12	3.10±2.22	13.21 ±12.43	1.72±0.53
-G[FAF]-	0.42±0.31	0.23±0.13	1.98±1.72	3.24±1.44	1.83±1.82
-G[FAAF]-	0.21±0.05	0.19±0.11	0.33±0.05	0.67±0.07	0.43±0.08
-G[FABP]-	0.14±0.09	0.29±0.12	0.31±0.13	0.63±0.11	0.54±0.17

[†] K_D values are in nM