Analytical Biochemistry 414 (2011) 261–265

Contents lists available at ScienceDirect

Analytical Biochemistry

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

DNA aptamers for as analytical tools for the quantitative analysis of DNA-dealkylating enzymes

Svetlana M. Krylova [a], Anna A. Karkhanina [a], Michael U. Musheev [a], Eleanor A.L. Bagg [b], Christopher J. Schofield [b], Sergey N. Krylov [a,*]

[a] Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario, Canada M3J 1P3
[b] Department of Chemistry and the Oxford Centre for Integrative Systems Biology, University of Oxford, Oxford OX1 3TA, UK

ARTICLE INFO

Article history:
Received 2 February 2011
Received in revised form 6 March 2011
Accepted 8 March 2011
Available online 12 March 2011

Keywords:
DNA aptamers
Enzymatic demethylation
AlkB protein
Nonequilibrium capillary electrophoresis of equilibrium mixtures

ABSTRACT

The AlkB family of oxygenases catalyze the removal of alkyl groups from nucleic acid substrates in an iron and 2-oxoglutarate-dependent manner and have roles including in DNA repair. To understand the biological functions of these DNA-dealkylating enzymes it is desirable to measure their expression levels *in vitro* and *in vivo* in complex biological matrixes. Quantitative analyses of the enzymes require affinity probes capable of binding AlkB family members selectively and with high affinity. Here we report that DNA aptamers can serve as efficient affinity probes for quantitative detection of such enzymes *in vitro*. Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) was applied as a general tool for: (i) selection of DNA aptamers, (ii) characterization of binding parameters for the aptamers, and (iii) quantitative detection of the target in an aptamer-based affinity analysis. The selected aptamers have a range of K_d values between 20 and 240 nM. The aptamers enabled accurate quantitative analysis of AlkB even in the presence of the *Escherichia coli* cell lysate. Aptamers can likely be developed for other nucleic acid repair enzymes. They may also be developed for use in *in vitro* and potentially *in vivo* studies of known nucleic acid-modifying enzymes including for functional analysis.

Alkylation of nucleobases is a common type of DNA damage, which is linked to diseases including cancer, neurological disorders, and developmental defects. One cellular defense mechanism involves oxidative demethylation, catalyzed by nonheme iron (II) and enzymes from the subfamily of 2-oxoglutarate-dependent oxygenases, which are conserved from bacteria to humans [1]. The *Escherichia coli* AlkB protein and its functional human homologues oxidize the methyl group in 1-methyladenine (1-meA)[1] or 3-methylcytosine (3-meC) in DNA/RNA to give a labile hydroxymethyl group, resulting in release of formaldehyde and the normal base, A or C, respectively. AlkB also catalyses demethylation of 3-methylthymine, 1-methylguanine, 1,N^6-ethenoadenine, and 3,N^4-ethenocytosine [2]. AlkB is able to repair alkylation damage in dsDNA and RNA; however, it works better on single-stranded DNA substrates [3]. Eight human homologues of *E. coli* AlkB (hABH1–8) have been identified, two of which, ABH2 and ABH3, also have a similar DNA/RNA demethylation function [4]. It is reported that oxidative RNA damage inhibiting translation and leading to the formation of aggregated nonfunctional proteins is increased in neurological disorders, e.g., Alzheimer disease [5]. Because RNA damage repair is a proposed function of ABH3 *in vivo*, it was suggested that the biological significance of ABH3 may be linked to various neurological diseases [6]. Recently, ABH8 was found to modify wobble uridines in tRNA, catalyzing both methylation of cm^5U to form mcm^5U [7,8], and subsequent hydroxylation producing $mchm^5U$ [9]. High expression of ABH8 was observed to contribute to the progression of urothelial carcinomas [10]. Another human homolog of AlkB, FTO (fat mass and obesity associated protein) is linked to obesity development in mammals [11].

Reported analytical methods developed to characterize the enzymatic activity of DNA-demethylating enzymes have included gel electrophoresis using radiolabeled DNA, HPLC–mass spectrometry or indirect analysis via detection of reaction coproducts [12,13,2]. Mostly, enzyme-catalyzed DNA demethylation of biologically relevant substrates has been studied *in vitro* by indirect approaches, e.g., analysis of the succinate or the coproducts of catalysis. Coproduct formation, however, does not necessarily represent quantitative demethylation. Furthermore, the formation of coproducts depends on the demethylation mechanism of a particular enzyme; therefore, monitoring coproduct turnover is not applicable to the discovery of new demethylases operating by unknown mechanisms [14,15]. Recently, we reported a new direct method for monitoring enzymatic activity using biologically relevant substrates for *in vitro* study [16]. In this method, a fluores-

* Corresponding author. Fax: +1 416 736 2100x22345.
E-mail address: skrylov@yorku.ca (S.N. Krylov).

[1] *Abbreviations used:* DTT, dithiothreitol; LIF, laser-induced fluorescence; NECEEM, nonequilibrium capillary electrophoresis of equilibrium mixtures; PCR, polymerase chain reaction.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.03.010

cently labeled single-base methylated substrate and its demethylated product are separated by capillary electrophoresis (CE), resolving the challenge of quantitatively distinguishing the substrate and the product, which differ only by a single methyl group.

The oxidative reaction of AlkB, utilizing a nonheme iron (II), 2-oxoglutarate (2OG), and dioxygen as cofactors, is difficult to quantitatively control especially in cells/crude cell lysates; hence enzyme activity cannot be easily used as a means for their quantitative detection. As an alternative, affinity probes can be used to assay target proteins in a complex biological matrix. Antibodies are traditionally used for studying the regulation of DNA/RNA repair enzymes [17]. Over the last two decades, a new type of affinity probe, i.e., aptamers (single-stranded oligonucleotides of typically 20 to 100 nucleotides in length) have been developed for use in highly sensitive and specific assays [18]. Aptamers are more stable than antibodies, and their synthesis is reproducible and inexpensive. Aptamers can be easily tagged with fluorescent labels without compromising their affinity and thus allowing a variety of analytical applications in diagnostic devises and assays. A general approach for aptamer selection, termed SELEX, was first suggested two decades ago [19]. The discovery of new nanosensors containing aptamers embedded in nanoparticles, protecting aptamers from nucleases, opens new opportunities for *in vivo* usage of aptamers [20]. Despite substantial efforts toward aptamer selection, aptamers have been identified for fewer than 200 proteins [21] and only a few have been used for diagnostics or biomedical applications.

To this point, no aptamers have been determined for AlkB or its analogs. The overall goal of our effort is to establish the use of aptamers for analyses of AlkB subfamily proteins. The first step toward this was demonstration of the "aptamerogenecity" of AlkB that can be used as a precedent for aptamer development for other proteins of this subfamily. Another objective of this work was to further test nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) as a versatile method for aptamer selection, characterization, and analytical utilization [22].

Here, we report the successful selection of aptamers for AlkB. A similar procedure can be used for the selection of RNA aptamers. The selected aptamers will be potentially useful for the discovery and study of DNA-modifying enzymes and the development of assays for AlkB *in vitro* and *in vivo*. In addition to aptamer selection, we report their first analytical application as affinity probes in NECEEM-based quantitative analysis of AlkB.

Materials and methods

Materials

The following materials were used: Microcon 30 kDa MW cutoff filters (Fisher, Whitby, ON, Canada), an uncoated fused-silica capillary (Polymicro, Phoenix, AZ, USA), real-time PCR master mix with SYBR Green, iQ SYBR Green Supermix; (Bio-Rad, Mississauga, ON, Canada), RNase AWAY solution for removing nucleases and nucleic acid contamination (Molecular Bio-Products, San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich (Toronto, ON, Canada). AlkB from *E. coli* was purified according to a published procedure [2]. A 1 mM stock solution of AlkB in 50 mM Tris–HCl, 500 mM NaCl, 1 mM DTT at pH 7.5 was stored at −80 °C. A synthetic fluorescently labeled DNA library had a random region of 40 nucleotides flanked by constant regions of 20 nucleotides each (for PCR amplification); a fluorescein label was at the 5′-end. The library was stored at −20 °C. PCR primers were designed using the published software [23] to eliminate self-dimers and hetero-dimers. Labeled with the Alexa 488 fluorophore at the 5′-end, the primers were used at a concentration of 10 μM and stored at −20 °C. All solutions were made using Milli-Q-quality deionized water filtered through a 0.22-μm filter (Millipore, Nepean, ON, Canada).

Instrumentation

CE procedures were performed using a PACE MDQ instrument purchased from Beckman Coulter (Fullerton, CA, USA). Two detection types were used: laser-induced fluorescence (LIF) detection with excitation at 488 nm and emission at 520 nm and light absorption detection at 280 nm. Uncoated fused-silica capillaries with total lengths of 87 and 50 cm (77 and 40 cm to the detection window) and inner and outer diameters of 75 and 365 μm, respectively, were used. The longer capillary was used for bulk affinity analysis and for aptamer selection while the shorter capillary was used for affinity analyses of enriched libraries and for analyses of the PCR products. A sample was introduced into the capillary by a pressure pulse of 1 psi × 28 s. Electrophoresis was carried out for a total of 30 min by an electric field of 375 V/cm with a positive electrode at the injection end of the capillary. The capillary temperature was maintained at 15 °C. Fractions were collected in an automatic mode by replacing the regular outlet tube with a vial containing 5 μl of water. Concentrations of Alexa-labeled oligonucleotides were measured fluorescently at 520 nm using a NanoDrop 3300 fluorometer.

Determination of bulk affinity

The mixture containing 45 nM annealed (DNA melted at 95 °C for 5 min then cooled to 20 °C at a rate of 7.5 °C/min) naïve DNA library (a library with a random combination of the 4 basic nucleotides in its "random" region), 5 μM AlkB, 50 mM NaCl, and 10 nM fluorescein, in 50 mM Hepes (pH 7.5), was incubated for 15 min to approach equilibrium. A plug of 150 nl of this equilibrium mixture was then injected into the capillary. The NECEEM method was run in 50 mM Tris-acetate buffer (pH 8.2) at 30 kV (345 V/cm) with LIF detection. EC_{50} values represent the calculated effective concentrations of the protein at which a half of the DNA library is bound to the protein target.

Selection of aptamers for AlkB

The equilibrium mixture for NECEEM-based selection of aptamers contained 21.5 μM naïve DNA library, 100 nM AlkB, 50 mM NaCl, and 100 nM fluorescein. The mixture was incubated for 15 min at room temperature to approach equilibrium. NECEEM was run in CE run buffer for aptamer selection (50 mM Tris-acetate, pH 8.2). Fractions were collected in the outlet vial containing the selection buffer (50 mM Hepes, pH 7.5, supplemented with 50 mM NaCl). AlkB–DNA complexes were separated from unbound DNA by capillary electrophoresis at 30 kV (345 V/cm) and quantified with LIF detection.

DNA in the collected fractions was amplified by PCR. Real-time analytical PCR using an intercalating fluorescent agent, SYBR Green, was performed to determine the number of cycles for preparative PCR. The amplification reaction mixture contained 20 μl of the fractionated DNA (Pool 1) with the following controls: (i) positive control, 10–100 pM DNA library; (ii) negative control, ddH_2O as a template; (iii) Pool 1. Then, 1–2 μl of each of the DNA templates was added to the prealiquoted real-time PCR SYBR Green master mixes. DNA was amplified using a first 3-min cycle at 95 °C followed by 39 additional cycles consisting of denaturing DNA at 95 °C for 10 s, annealing DNA at 56 °C for 10 s, and polymerizing DNA at 72 °C for 10 s. An S-shaped amplification curve was plotted to determine the number of cycles required for the

reaction mixture to generate the product at a level of 50–60% of the maximum determined as 15 cycles.

Symmetric PCR was used for the amplification of a fluorescently labeled AlkB aptamer. The DNA template (2 μM from the collected fractions of Pool 1) was mixed with the PCR master mixture, 1 μl of *Taq* DNA polymerase and HPLC-purified primers (a fluorescently labeled forward primer and biotin-labeled reverse primer) at concentrations of 300 nM each. To run the asymmetric PCR, 3 μl symmetric PCR product from Pool 1 was mixed with 1 μl of *Taq* DNA polymerase, forward primer at a concentration of 1 μM, and the reverse primer at a concentration of 50 nM. Fifteen cycles of PCR were then run. The desired products were separated from the reaction by-products as follows. Undesired products containing the biotin label were bound to streptavidin-coated superparamagnetic iron oxide particles which were collected using a magnet, then discarded.

Each PCR sample was analyzed by injecting 50 nl (0.5 psi × 10 s injection) into a 50-cm-long capillary and CE run in 25 mM sodium tetra-borate buffer (pH 9.3) at 30 kV (600 V/cm) with LIF detection to verify the presence/absence of the undesirable (for asymmetric PCR) double-stranded DNA product.

Affinity analysis

To evaluate binding affinity of the aptamer pools, a 50 nl plug of the equilibrium mixture containing 250 nM AlkB and 50 nM aptamer in 50 mM Hepes, pH 7.5, supplemented with 50 mM NaCl was injected into a 50-cm-long capillary by a 0.5 psi × 10 s pressure pulse. An electric field of 400 V/cm was applied to the capillary with the positive electrode being at the inlet. The electrophoresis run buffer was 50 mM Tris-acetate, pH 8.2.

Cloning and screening clones

Aptamer Pool 5 was amplified by PCR using unlabeled primers. The formation of dsDNA was assessed using a 4% agarose gel. Double-stranded DNA was precipitated using ice-cold (stored at −20 °C) 33;1 ethanol/3 M NaOAc solution, which was added to 45 μl of the symmetric PCR product of the aptamer Pool, and kept at −20 °C for 30 min. Then, after spinning the solution at a rate of ~13,000 rpm for 20 min at 9 °C, the pellet was dissolved in 20 μl of 10 mM Tris–HCl, pH 7.5, and the solution was stored at −20 °C. Cloning was performed using the pT7 Blue-3 Perfectly Blunt cloning kit (VWR, Mississauga, ON, Canada). Each clone was tested for its affinity to AlkB as described above.

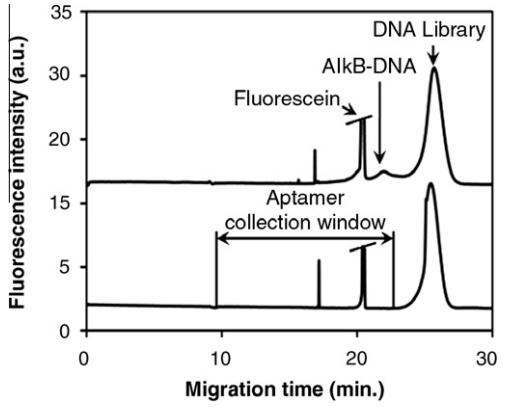

Fig.1. Determination of the aptamer-collection window for NECEEM-based aptamer selection for AlkB protein. The bulk affinity assay was used to determine the position of a complex of DNA with 5 μM AlkB (upper trace). For aptamer selection the equilibrium mixture contained 100 nM AlkB and 50 nM naïve DNA library. The aptamer-collection window for blind fraction collection (lower trace) spanned from 9.5 to 23 min, to include the AlkB–DNA complex.

Aptamer specificity

To validate the aptamer selectivity, the reaction between 50 nM aptamer and proteins of *E. coli* cell lysate (containing 100 mg L^{-1} of the total protein) was tested under conditions identical to those used in the affinity analyses.

E. coli BL21(DE3) were grown at 37 °C to a density corresponding to an absorbance of 1.6 at 600 nm measured in a cuvette with an optical path length of 1 cm. Cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C. Pelleted cells were resuspended in sonication buffer: 50 mM Tris–HCl, 2.5 mM $MgCl_2$, 5 mM KCl at pH 8.3 containing a protease inhibitor (PI) cocktail as instructed by the manufacturer (Sigma Aldrich, Oakville, ON, Canada). Bacterial lysates were prepared by sonication on ice with 5 s "on"/15 s "off" intervals for a total of 10 min. Cell debris was pelleted by centrifugation at 15,000*g* for 20 min at 4 °C and removed. Cell lysates were aliquoted and stored at −80 °C. The concentration of total protein in the cell lysate was measured by the bicinchoninic acid (BCA) assay, using bovine serum albumin as a standard.

Aptamer-based assay of AlkB protein

Standard solutions containing AlkB at concentrations between 1 and 1000 nM were mixed with 42 nM DNA aptamer and *E. coli* cell lysate (containing 34 mg L^{-1} of the total protein) in 50 mM Hepes, 50 mM NaCl, pH 7.5, incubated for 1 h at room temperature, and injected into a 50-cm-long capillary by a pressure pulse of 0.5 psi × 5 s. NECEEM was then run in 50 mM Tris-acetate (pH 8.2) supplemented with 2.5 mM $MgCl_2$ at 30 kV with LIF detection. Fluorescein was used as an internal standard. The presence of $MgCl_2$ in the running buffer was required to facilitate the separation of the internal standard from the components of the equilibrium mixture. A calibration curve was plotted as the function of the fluorescence corresponding to the AlkB–DNA complex (which is proportional to the fraction of AlkB-bound aptamer) versus the concentration of AlkB protein.

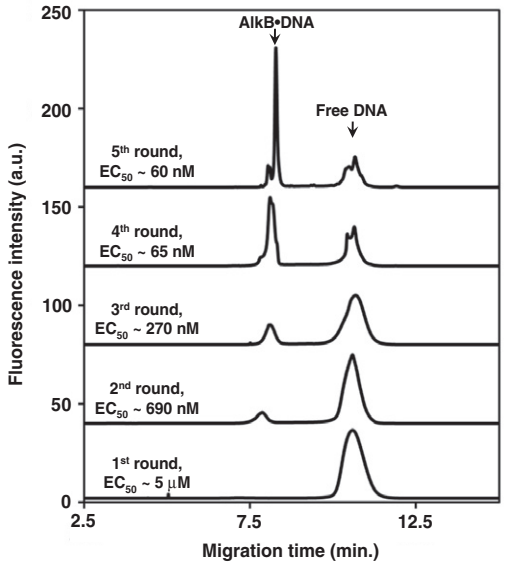

Fig.2. Progress of library enrichment in rounds 1–5 of aptamer selection for AlkB studied by the NECEEM method. EC_{50} values represent the calculated effective concentrations of the protein at which a half of the library is bound to the protein. The equilibrium mixtures contained 250 nM AlkB and 50 nM of the library incubated together for 15 min in 50 mM Hepes, 50 mM NaCl, pH 7.5.

Results and discussion

The selection of aptamers for AlkB protein relied on the separation of DNA–AlkB complexes from the unbound DNA library using CE. The DNA library contained single-stranded oligonucleotides labeled with the Alexa fluorescent label, which made DNA and its complexes with the AlkB protein "visible" in CE with LIF detection. Given the considerable difference between the molecular weights of the AlkB protein and DNA in the library, the protein–DNA complex could be efficiently partitioned from the unbound library by gel-free CE (Fig. 1). The equilibrium mixture containing the fluorescently labeled DNA library, AlkB, and fluorescein (used as an internal standard) was injected in an uncoated capillary with the positive electrode at the inlet, subjecting the mixture to free-zone electrophoresis. An electroosmotic flow in the direction from the positive to the negative electrode is generated in an uncoated capillary and propels negatively charged DNA to the negative electrode despite the electrostatic force exerted in the opposite direction. DNA, with a large negative charge due to its sugar-phosphate backbone, migrated slower than AlkB. The DNA–AlkB complex was expected to migrate between the unbound protein and the unbound DNA. To determine the aptamer collection window, a bulk affinity assay was performed using AlkB solutions at a relatively high concentration (5 μM). A complex of DNA with 5 μM AlkB protein was detectable in a bulk affinity assay (Fig. 1, upper trace). The aptamer collection window was determined as the time period corresponding to the elution of the fraction of DNA migrating faster than the unbound DNA library. The right-hand boundary of the aptamer collection window was thus established as being between the peaks corresponding to fluorescein (the internal standard) and the unbound DNA library. In the first round of aptamer selection, the equilibrium mixture contained AlkB at a concentration of 100 nM. Undetectable AlkB–DNA complexes were collected within the aptamer collection window (Fig. 1, lower trace).

The aptamer selection, production, and purification procedures were repeated in five rounds resulting in 5 pools (Pools 1–5) of enriched DNA libraries. During rounds 2 and 3, the DNA-enriched library obtained from the previous round of selection was mixed with 100 nM AlkB. During the forth round of aptamer selection the procedure was repeated using the same (100 nM) and reduced (10 nM) concentrations of AlkB. The last, fifth, round was performed to select aptamers for 100, 10, and 1 nM AlkB.

In order to validate aptamer selection, affinity analysis of the obtained enriched library was performed under conditions identical to those used in the selection step. A gradual increase in the fraction of AlkB-bound DNA was observed in the comparative binding analysis of Pools 1–5 with 250 nM AlkB (Fig. 2). EC_{50} values determined using the NECEEM procedure [22] decreased from 5 μM for the naïve library to 60 nM for Pool 5, selected after the fifth round of aptamer selection.

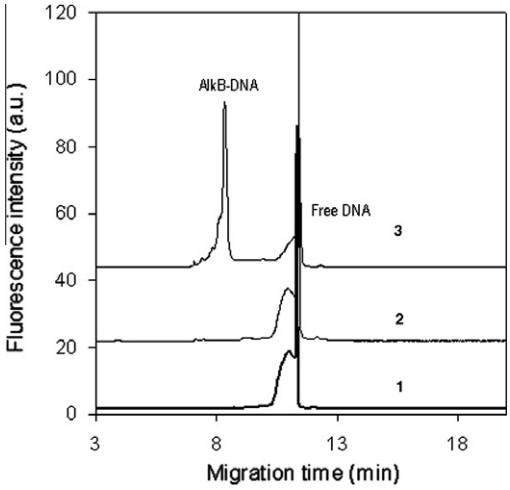

Fig.3. Cross-reactivity of Pool 5 aptamers with *E. coli* lysate proteins tested by the NECEEM method. Trace 1 corresponds to DNA aptamers (Pool 5). Trace 2 corresponds to DNA aptamers (Pool 5) mixed with cell lysate, containing 100 mg L^{-1} of the total protein. Trace 3 corresponds to DNA aptamers (Pool 5) mixed with 250 nM AlkB in the presence of cell lysate, containing 100 mg L^{-1} of the total protein. Equilibrium mixtures were prepared by incubating the aptamers with lysate/protein samples for 15 min in 50 mM Hepes, 50 mM NaCl, pH 7.5. The electrophoresis run buffer was 50 mM Tris-acetate, pH 8.2.

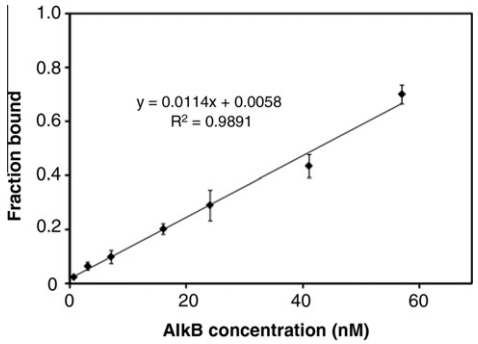

Fig.4. Calibration curve for a synthetic AlkB aptamer No. 2 (Table 1) in the presence of cell lysate measured by the NECEEM method. The equilibrium mixtures were prepared by incubating 42 nM aptamer with varying protein concentrations for 1 h in 50 mM Hepes, 50 mM NaCl, pH 7.5. The electrophoresis run buffer was 50 mM Tris-acetate, pH 8.2, supplemented with 2.5 mM $MgCl_2$. Measurements were recorded in duplicate for every AlkB concentration; the presented values are averages with standard deviations as errors.

Table 1
Sequences and K_d values determined for individual clones of AlkB aptamers.

Clone ID	K_d (nM)	Sequence (5′ → 3′)
1	58	F-GTATAAACCTAGTACGAAGCGTATCTTTACGGGTGCCTGA-R
2[a]	40	F-TGCCTAGCGTTTCATTGTCCCTTCTTATTAGGTGATAATA-R
3	20	F-CCCATATCGGTGAATGCACGAGCAACCGATTGACACGGG-R
7	159	F-CAGGCCAATAACTAGCAGGTTACTGTTCATCATTTCGCAC-R
8	70	F-CCCATAGCGGTGAATGCATGAGCAACCCGATTGACACGGG-R
10	80	F-CCGAAGAGCCTTTTTAGCGTACTGAAAAGGAGTTACTCTC-R
11	240	F-AGAAATTGGTACTGTATGAAACGGCAGCTGCACGTCGCG-R
12	45	F-CAGCACCAGGAAGATGAATGCTCGAGCAACTCGACGGTCG-R
13[a]	88	F-AGTTACTCTGCAAGAAGTTGTCAAGAGCGTGGGAGGGCGG-R
16[a]	50	F-CCCATATCGGTGAATGCACGAGCAACCCGATTGACACGGG-R
19	47	F-CAGCCACCAGGAAGATGAATGCTCGAGCAACTCGACGGTCG-R
22	136	F-CCGAAGACCTTTTTAGCGTACTGAAAAGGAGTTACTCTC-R
24	128	F-GACTGCTGATGAGTCACTTTAACGTGGAGCAAAGATTAAA-R

Constant primer sequences: F, CTCCTCTGACTGTAACCACG; R, GCATAGGTAGTCCAGAAGCC.
[a] Reported K_d values are averages of those for up to three clones with identical sequences.

After five rounds of aptamer selection the final, and most active, Pool 5 was inserted into bacterial plasmid vectors, which were transformed into *E. coli* to obtain bacterial colonies containing plasmids with individual aptamer sequences. Twenty four colonies with proven presence of the inserts were chosen for the sequencing and binding analysis. Sequencing of the clones revealed that only 13 sequences were unique. Affinity studies of the individual clones showed that K_d values of aptamer–AlkB complexes ranged between 20 and 240 nM (Table 1).

In order to study the specificity of aptamers selected for AlkB by the NECEEM method, we tested their potential cross-reactivity with other proteins. Nonspecific binding to proteins present in an *E. coli* cell lysate was investigated, because AlkB is an *E. coli* protein. Fig. 3 shows the affinity assay for aptamers of Pool 5 with *E. coli* lysate. Cell lysate was present in the equilibrium mixtures at a cumulative concentration 20 times exceeding that of recombinant AlkB. We found that the aptamers did not bind nonspecific proteins of the *E. coli* lysate to a significant extent. The level of cross-reactivity did not exceed 0.15% of the total protein–aptamer complexes formed.

The absence of cross-reactivity with other components of the cell lysate proved that the aptamers developed were highly specific to AlkB. In addition, we found that the AlkB–aptamer complex could be detected in the presence of *E. coli* cell lysate (Fig. 3, trace C). In general, even if significant cross-reactivity were found, the NECEEM method could be further used as a technique to eliminate sequences with cross-reactvity for the undesired targets, contaminants, or other homologues through a negative selection approach.

In this study we have further tested an analytical application for the AlkB aptamers, through their use as an affinity assay for the determination of AlkB protein concentration in the presence of nonspecific proteins in the relevent biological matrix, i.e., an *E. coli* cell lysate. A binding curve was obtained to detect AlkB in the range of concentrations from 1 to 1000 nM in the presence of *E. coli* cell lysate. It was found that the binding curve had a linear region between 1 and 60 nM (Fig. 4). To develop the assay method we used a synthetic AlkB aptamer with the sequence of clone 2 (Table 1), which showed one of the highest affinities to AlkB. The experimental conditions were similar to those used in the affinity study; however, the presence of cell lysate proteins apparently slowed the kinetics of aptamer–protein interactions. Therefore, the incubation time was increased to 1 h. Saturation of protein–aptamer complex formation was observed at protein concentrations greater than 100 nM. The detection range can be increased either by sample dilution or by using a combination of aptamers with different affinities to the protein [24].

Concluding remarks

To conclude, we have introduced a method for selection of aptamers to the AlkB protein from a highly diverse DNA library, with a low number of consecutive steps of partitioning. The advantages of the selection technique include homogeneous phase separation, which minimizes the possibility of the native AlkB protein undergoing conformational changes. The selection process was fast and after the fourth round of selection revealed DNA aptamers with affinities in the 10 nM range. The selected aptamers were used for AlkB analysis over a wide range of protein concentrations from 1 to 1000 nM. Aptamers to AlkB protein are important bioanalytical tools for studying the regulation of DNA/RNA repair enzymes. They can be used in affinity assays, Western blotting, and histochemistry. Potentially, aptamers may serve as modifiers of enzymatic activity and could be used as either drug delivery tools or potential drug candidates. We foresee that this work will stimulate selection of aptamers to the human homologues of AlkB and development of the use of aptamers in bimolecular screening applications.

Acknowledgment

The work was funded by NSERC Canada and the BBSRC.

References

[1] S.C. Trewick, T.F. Henshaw, R.P. Hausinger, T. Lindahl, B. Sedgwick, Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage, Nature 419 (2002) 174–178.
[2] R.W.D. Welford, I. Schlemminger, L.A. McNeil, K.S. Hewitson, C.J. Schofield, The selectivity and inhibition of AlkB, J. Biol. Chem. 278 (2003) 10157–10161.
[3] P.O. Falnes, R.F. Johansen, E. Seeberg, AlkB-mediated oxidative demethylation reverses DNA damage in *Escherichia coli*, Nature 419 (2002) 178–182.
[4] C.Q. Yi, C.G. Yang, C.A. He, A non-heme iron-mediated chemical demethylation in DNA and RNA, Acc. Chem. Res. 42 (2009) 519–529.
[5] X. Shan, H. Tashiro, C.L. Lin, The identification and characterization of oxidized RNAs in Alzaheimer's disease, Oncogene 21 (2003) 8886–8894.
[6] P.O. Falnes, A. Klungland, I. Alseth, Repair of methyl lesions in DNA and RNA by oxidative demethylation, Neuroscience 145 (2007) 1222–1232.
[7] L. Songe-Moller, E. van den Born, V. Leihne, C.B. Vagbo, T. Kristoffersen, H.E. Krokan, F. Kirpekar, P.O. Falnes, A. Klungland, Mammalian ALKBH8 possesses tRNA methyltransferase activity required for the biogenesis of multiple wobble uridine modifications implicated in translational decoding, Mol. Cell. Biol. 30 (2010) 1814–1827.
[8] D. Fu, J.A. Brophy, C.T. Chan, K.A. Atmore, U. Begley, R.S. Paules, P.C. Dedon, T.J. Begley, L.D. Samson, Human AlkB homolog ABH8 Is a tRNA methyltransferase required for wobble uridine modification and DNA damage survival, Mol. Cell. Biol. 30 (2010) 2449–2459.
[9] Y. Fu, Q. Dai, W. Zhang, J. Ren, T. Pan, C. He, The AlkB domain of mammalian ABH8 catalyzes hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA, Angew. Chem. Int. Ed. Engl. 49 (2010) 8885–8888.
[10] K. Shimada, M. Nakamura, S. Anai, M. De Velasco, M. Tanaka, O.Y. Tsujikawa, N. Konishi, A novel human analogue ABH8, contributes to bladder cancer progression, Cancer Res. 69 (2009) 3157–3164.
[11] T. Gerken, C.A. Girard, Y.C.L. Tung, C.L. Webby, V. Saudek, K.S. Hewitson, G.S.H. Yeo, M.A. McDonough, S. Cunliffe, L.A. McNeill, J. Galvanovskis, P. Rorsman, P. Robins, X. Prieur, A.P. Coll, M. Ma, Z. Jovanovic, I.S. Farooqi, B. Sedgwick, L. Barroso, T. Lindahl, C.P. Ponting, F.M. Ashcroft, S. O'Rahilly, C.J. Schofield, The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase, Science 318 (2007) 1469–1472.
[12] T.W. Roy, A.S. Bhagwat, Kinetic studies of *Escherichia coli* AlkB using a new fluorescence-based assay for DNA demethylation, Nucleic Acids Res. 35 (2007) e147.
[13] D.H. Lee, S.G. Jin, S. Cai, Y. Chen, G.P. Pfeifer, T.R. O'Connor, Repair of methylation damage in DNA and RNA by mammalian AlkB homologues, J. Biol. Chem. 280 (2005) 39448–39459.
[14] Y. Mishina, E.M. Duguid, C. He, Direct reversal of DNA alkylation damage, Chem. Rev. 106 (2006) 215–232.
[15] L. Liu, S.L. Gerson, Targeted modulation of MGMT: clinical implications, Clin. Cancer Res. 12 (2006) 328–331.
[16] A.A. Karkhanina, J. Mecinovic, M.U. Musheev, S.M. Krylova, A.P. Petrov, K.S. Hewitson, E. Flashman, C.J. Schofield, S.N. Krylov, Direct analysis of enzyme-catalyzed DNA demethylation, Anal. Chem. 81 (2009) 5871–5875.
[17] M.P. Westbye, E. Feyzi, P.A. Aas, C.B. Vagbo, V.A. Talstad, B. Kavli, L. Hagen, O. Sundheim, M. Akbari, N.B. Liabakk, G. Slupphaug, M. Otterlei, H.E. Krokan, Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA, J. Biol. Chem. 283 (2008) 25046–25056.
[18] A.A. Goulko, F. Li, C. Le, Bioanalytical applications of aptamer and molecular-beacon probes in fluorescence-affinity assays, Trends Anal. Chem. 28 (2009) 878–892.
[19] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.
[20] L.J. Nielsen, L.F. Olsen, V.C. Ozalp, Aptamers embedded in polyacrylamide nanoparticles: a tool for in vivo metabolite sensing, ACS Nano 4 (2010) 4361–4370.
[21] http://aptamer.icmb.utexas.edu/.
[22] M.V. Berezovski, M.U. Musheev, A.P. Drabovich, J.V. Jitkova, S.N. Krylov, Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides, Nat. Protoc. 1 (2006) 1359–1368.
[23] http://scitools.idtdna.com/Primerquest.
[24] A.P. Drabovich, V. Okhonin, M. Berezovski, S.N. Krylov, Smart aptamers facilitate multi-probe affinity analysis of proteins with ultra-wide dynamic range, J. Am. Chem. Soc. 129 (2007) 7260–7261.