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Research paper

Promoter scanning of the human COX-2 gene with 8-ring polyamides: Unexpected weakening of polyamide–DNA binding and selectivity by replacing an internal N-Me-pyrrole with β-alanine

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ABSTRACT

Rules for polyamide–DNA recognition have proved invaluable for the design of sequence-selective DNA binding agents in cell-free systems. However, these rules are not fully transferrable to predicting activity in cells, tissues or animals, and additional refinements to our understanding of DNA recognition would help biomedical studies. Similar complexities are encountered when using internal β-alanines as polyamide building blocks in place of N-methylpyrrole; β-alanines were introduced in polyamide designs to maintain good hydrogen bonding registry with the target DNA, especially for long polyamides or those with several GC bp (P.B. Dervan, A.R. Urbach, Essays Contemp. Chem. (2001) 327–339). Thus, to clarify important subtleties of molecular recognition, we studied the effects of replacing a single pyrrole with β-alanine in 8-ring polyamides designed against the Ets-1 transcription factor. Replacement of a single internal N-methylpyrrole with β-alanine to generate a β/Im pairing in two 8-ring polyamides causes a decrease in DNA binding affinity by two orders of magnitude and decreases DNA binding selectivity, contrary to expectations based on the literature. Measurements were made by fluorescence spectroscopy, quantitative DNA footprinting and surface plasmon resonance, with these vastly different techniques showing excellent agreement. Furthermore, results were validated for a range of DNA substrates from small hairpins to long dsDNA sequences. Docking studies helped show that β-alanine does not make efficient hydrophobic contacts with the rest of the polyamide or nearby DNA, in contrast to pyrrole. These results help refine design principles and expectations for polyamide–DNA recognition.

1. Introduction

N-methylpyrrole and -imidazole based polyamides (PA) are being used with increasing frequency in fundamental and applied biomedical research programs [1–5]. Although the field owes much to work on Distamycin A and other lexitropsins or their analogs [6], most of the recent improvements in polyamide utility derive from the DNA recognition rules developed by Dervan [7]. These rules in principle allow one to build a polyamide to recognize a DNA sequence of interest, for example to influence transcription factor–DNA binding and control gene expression [8–10]. Dervan and Sugiyama have also published extensively on refinements to the understanding of polyamide–DNA binding, for example looking at the details of preferred and less-favored DNA–polyamide interactions and orientations [11–15]. These refinements indicate the importance of DNA sequence context and structural details of the polyamides for obtaining maximal DNA–PA binding strength.

Even with this extensive knowledge at hand and other important additions to the field [16–22], there is much that is unknown about PA design and modes of PA action in living cells. Our recent work has focused on the polyamide-based process called promoter scanning [23]: the method can identify hotspots for DNA–PA interactions that lead to improved control of gene expression. If these hotspots are near each other, it is tempting to construct larger, more specific PA molecules from the smaller active molecules identified in the promoter scanning process. This extension would take advantage of improved DNA binding strength and selectivity that can be found with larger polyamides. This approach is particularly relevant in light of recent reports that PAs over a wide range in size (400–4000) can be biologically effective [5,16].

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To capture the activity of PA1 (Fig. 1) in more selective molecules, we previously increased PA length, adding β-alanine (β) springs every 4–5 heterocycles to keep good registry between the hydrogen bonding groups of DNA and polyamides [24], keeping the lessons of Im/G alignment in mind [26]. In a promoter scanning study to control gene expression of COX-2, we used PA1 in combination with other PA sequences. We observed a complete reversal in COX-2 expression, depending on which other polyamides with which PA1 was combined [25]. However, activity of the larger molecules did not follow trends observed with the shorter PA from which they were derived [5], and the role of β appeared to be more complex than anticipated.

In order to better understand the incorporation of β in the construction of longer, more selective PAs based on PA1, we have used the PA1 framework to carry out the present biophysical study of the role of β/Im pairs vs. Py/Im pairs for DNA base pair recognition and binding. The literature provides systematic studies of β/β pairs vs. Py/Py pairs [24,26], but not as much quantitative information is available on any changes to DNA binding that might occur by replacing a single Py residue with β. We report that, in contrast to the manner in which β helps maintain tight and selective DNA binding for some PA designs, especially when used as β/β pairs for G-rich targets, β/Im pairs can greatly decrease the binding affinity and specificity of PA molecules for DNA.

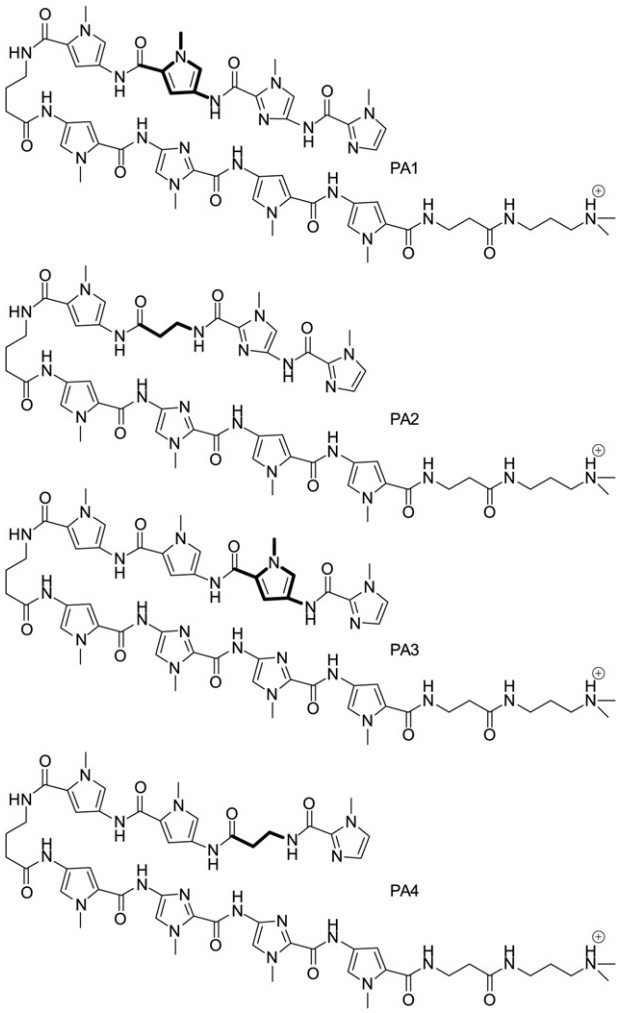

Fig. 1. Structures of compounds PA1–PA4. They are shown as monocations for clarity; they are isolated as tetracations. Bold bonds indicate the Py and β pairs compared in this study.

In order to validate our findings, we used orthogonal assay methods for measuring DNA binding thermodynamics and kinetics, including a fluorescence assay [27], BIAcore using biotin labeled DNA hairpins [28–30], and quantitative footprinting [31]. To test the generality of our results, we studied a variety of DNA substrates from hairpins with 10 or 20 bp of duplex DNA derived from the COX-2 promoter to a linear, 120-mer dsDNA with one PA binding site and a 524-mer dsDNA with multiple cognate and off-target PA binding sites. Quantitative footprinting was done using fluorescent labels and capillary electrophoresis (CE) [32] rather than more traditional radiolabeling and PAGE methods. The unusual breadth of techniques in one study provides valuable perspective on the validities of various methods.

2. Materials and methods

2.1. Synthesis

Polyamides were prepared by solid phase synthesis using Boc methodology [33] and were characterized by HPLC/MS (ESI^+, Figs. S1–S3), ^1H NMR, high resolution mass spectrometry (ESI^+ MS^n) and combustion analysis (C, H, N, or C, H, N, F; see Supporting information). Of note, isolation by RP-HPLC using mobile phases containing 0.1%TFA led to isolated material with all basic nitrogens protonated, including imidazole; this contrasts with a previous report that used similar isolation procedures but indicated that imidazole groups were not protonated.

2.2. Fluorescence spectroscopy

Changes in fluorescence intensity of a TAMRA-labeled, hairpin-forming oligonucleotide as a function of PA concentration were used to quantitate DNA binding [27]. 5′-CCT GGA GAG GAA GCC AAG TGT TTT CAC TTG GCT TCC TCT CCA GG-3′ was purchased from IDT HPLC pure (Coralville, IA) either unlabeled or labeled with T-TAMRA at either T_{34} or T_{37} as noted by the underlined T positions in the above sequence and as shown by asterisks in Fig. 2. Using a Centricon unit, the DNA was rinsed twice with Milli-Q water, subsequently annealed from boiling water, quantitated using vendor extinction coefficient, and either aliquoted, lyophilized and stored at −20 °C or used directly.

The experiments were performed using quartz cuvettes at 10 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.4, 25 °C on a Fluorolog-3 (SPEX) spectrofluorimeter. All samples were stirred continuously. TAMRA-labeled oligonucleotides were excited at 559 nm and the resulting emission observed through a monochromator set at 580 nm or a 592 nm bandpass filter (Edmund

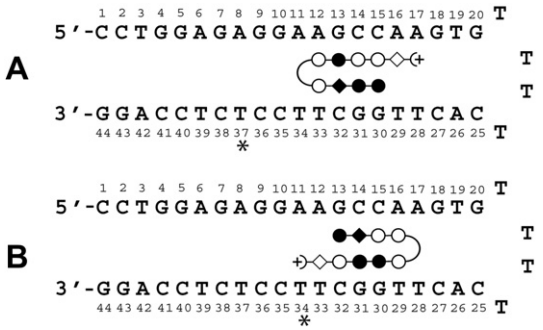

Fig. 2. Map of predicted polyamide–DNA interactions. (A) PA1/2 and (B) PA3/PA4 bound to hairpin DNA. Open circle, Py; filled circle, Im; open diamond, β; filled diamond, Py (PA1, PA3) or β (PA2, PA4); +, Dp; curved line, γ; *, position of TAMRA dye when used. A 5′-biotin conjugate of the DNA without dye labels was used for SPR.