Accelerated Publications

Kinetic Consequences of Covalent Linkage of DNA Binding Polyamides†

Ramesh Baliga,‡ Eldon E. Baird,§ David M. Herman,§ Christian Melander,§ Peter B. Dervan,§ and Donald M. Crothers*,‡

Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107, and Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

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ABSTRACT: Polyamides composed of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) subunits can bind in the minor groove of DNA at predetermined sequences with subnanomolar affinity and high specificity. Covalent linkage of polymer subunits using a γ-aminobutyric acid linker has been shown to increase both the affinity and specificity of polyamides. Using a fluorescence detected stopped-flow assay, we have studied the differences in association and dissociation kinetics of a series of polyamides representing unlinked, hairpin and cyclic analogues of the four ring polyamide ImPyPyPy-β-Dp. Whereas the large differences seen in the equilibrium association constants between the unlinked and covalently linked polyamides are primarily due to higher association rate constants, discrimination between matched and mismatched sites by each polyamide can be ascribed in large part to differences in their dissociation rate constants. The consequences of this kinetic behavior for future design are discussed.

The availability of large amounts of DNA sequence data from a variety of organisms has motivated the design of modular ligands that can bind with high affinity and specificity at predetermined sequences and thereby potentially regulate the expression of any gene of interest. One of the most promising families of such small molecules is synthetic polyamides composed of *N*-methylpyrrole (Py)[1] and *N*-methyl imidazole (Im) amino acids, which bind sequence specifically in the minor groove of DNA as side-by-side stacked antiparallel dimers (*1*). Efforts over the course of the last 20 years have resulted in development of compounds that can bind sites ranging in size from 5 to 13 base pairs (*2*) using a simple set of "pairing rules" (*3*) based on knowledge gained from several thermodynamic and structural studies (*4*−*11*). Significantly, polyamides have recently been shown to be cell permeable and able to regulate gene expression using repression (*12*), anti-repression (*13*) or activation (*14*). In light of these striking demonstrations of biological activity, a detailed study of the kinetics of polyamide−DNA interactions is an imperative for evaluating their future biological applications.

As a starting point toward understanding polyamide kinetics, we have recently described the use of fluorescence-based stopped-flow techniques for determining the macroscopic kinetic parameters of binding of Distamycin A (Dst) to its target sites (*15*). The next step toward understanding the kinetics of polyamides is a study of how covalent links between polyamides affect their binding kinetics. Linkage of two polyamides using a γ-aminobutyric acid linker (γ) turn to give a "hairpin" polyamide was first shown to increase the affinity of three ring polyamides by over 100-fold (*16*). Further constraining the polyamide by addition of a γ-turn at the opposite end increases the affinity by an additional factor of 40 (*17*). A similar effect was also observed for 4-ring polyamides, where a >3600-fold enhancement in affinity was observed for the covalently linked polyamides relative to the unlinked (*18*, *19*). Here we describe the use of a modified florescence-based assay to study three polyamides representing the unlinked, hairpin and cyclic versions of the four ring ImPyPyPy-β-Dp. Our studies reveal the dynamic basis for the observed enhancement in affinity and the kinetics underlying the specificity.

MATERIALS AND METHODS

Oligonucleotide Target Sites and Polyamides. Oligonucleotides were purchased from the Keck oligonucleotide synthesis facility at Yale University and purified using standard protocols. Oligonucleotide duplexes were made by annealing stoichiometric amounts of each strand with its complement. For duplexes containing 2-aminopurine (2-AP), the extinction coefficient at 260 nm for 2-AP was assumed to be 1000 M^{-1} cm^{-1}. Target duplexes were prepared by mixing equimolar amounts of each strand followed by melting and slow annealing. Serial dilutions of each duplex were made from the same stock into TKMC buffer (*16*). Stock solutions of duplexes were stored frozen at −20 °C until use. The sequences of oligonucleotides used in these studies are illustrated in Figure 1. Three polyamides, ImPy-

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* To whom correspondence should be addressed. Phone: (203) 432-5204. Fax: (203) 432-6144. E-mail: donald.crothers@yale.edu.

‡ Yale University.

§ California Institute of Technology.

[1] Abbreviations: Im, *N*-methyl imidazole-2-carboxamide, Py, *N*-methyl pyrrole-2-carboxamide; β, β-alanine; γ, γ-aminobutyric acid; Dp, dimethylamino propylamide; Dst, Distamycin A.

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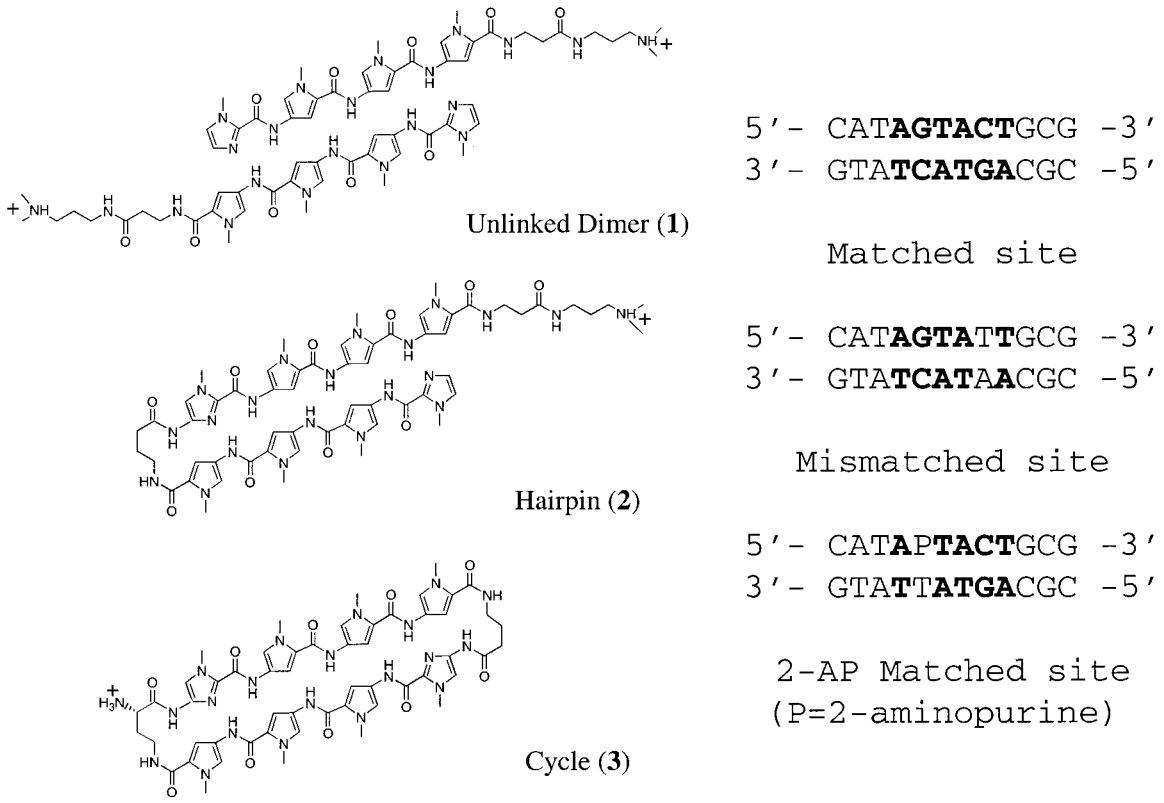

FIGURE 1: Sequences of the three polyamides, the matched and mismatched DNA duplex and the 2-aminopurine labeled competitor duplex.

PyPy-β-Dp, ImPyPyPy-γ-ImPyPyPy-β-Dp, and cyclo-[γ-ImPyPyPy-(R)$^{NH2}\gamma$-ImPyPyPy-], were synthesized using solid-phase synthesis protocols as described previously (*20*). The structures of the three polyamides (**1**−**3**) used in this work are shown in Figure 1, and their characterization has been reported previously (*19*, *20*).

Equilibrium Fluorescence Measurements. Equilibrium fluorescence measurements were obtained on a Hitachi F4500 fluorescence spectrophotometer. For measuring fluorescence enhancement of polyamides, samples were irradiated at the absorbance maximum of the polyamide (310 nm) with excitation slit width at 5 nm and emission slit width at 10 nm. Each spectrum in Figures 2 and 3 represents an average of at least three separate measurements. For detection of polyamide binding to 2-AP containing duplexes, samples were irradiated at the excitation maximum (260 nm). Note that this is different from the excitation maximum of free 2-AP (310 nm) and is attributed to energy transfer from other bases to 2-AP (*21*). Irradiation at 260 nm provides an approximately 3-fold increase in signal (Figure 1, Supporting Information).

Fluorescence-Detected Stopped-Flow Kinetics. Stopped-flow measurements were performed in an Applied Photophysics DX 17 MV stopped-flow microvolume reaction analyzer. Observations were conducted in the fluorescence mode using excitation light set at 310 or 260 nm and both slits of the excitation monochromator set to 1 mm (∼5 nm band-pass). A 320 nm long-pass filter (Schott) was used to prevent any excitation light from reaching the emission photomultiplier. Instrument parameters for stopped-flow measurements were as described before (*15*). Data from stopped-flow experiments were processed using the software Origin (Microcal) and fitted to appropriate equations using the Levenberg Marquardt nonlinear least-squares fitting subroutine.

RESULTS AND DISCUSSION

Fluorescence Enhancement of Polyamides upon Binding Native Duplexes. Binding of the three ring natural product Distamycin A (PyPyPy) at its target site causes an enhancement in its fluorescence intensity in a sequence dependent fashion (*22*). Each polyamide in this study is more fluorescent in the unbound state than Dst because of the addition of more Py or Im moieties to the molecules. Analogous to the Dst case, binding of each polyamide at its target site causes a significant increase in the steady state fluorescence spectrum (Figure 2). However the enhancement of fluorescence for the linked polyamides upon binding the matched site is small relative to the unlinked version. At the concentrations where fluorescence is detectable for these complexes, we were unable to detect any difference in fluorescence enhancement for binding of the hairpin and cyclic polyamides to matched vs mismatched sites. This is clearly due to the much higher binding constants of these polyamides in comparison to the unlinked polyamide (*19*).

Fluorescence Quenching of 2-AP Containing Target Duplex upon Binding of Polyamides. To enhance the sensitivity of fluorescence detection of polyamide binding, we investigated the use of 2-aminopurine (2-AP) containing duplexes. The fluorescence of a 2-AP base in a duplex is exceedingly sensitive to its helical microenvironment. There is approximately a 100-fold increase in fluorescence when a 2-AP base within a single strand is incorporated into a double helix (*23*). Patel et al. have described the use of a 2-AP containing duplex to monitor the binding of netropsin and even spermine in the minor groove of DNA (*24*). Since

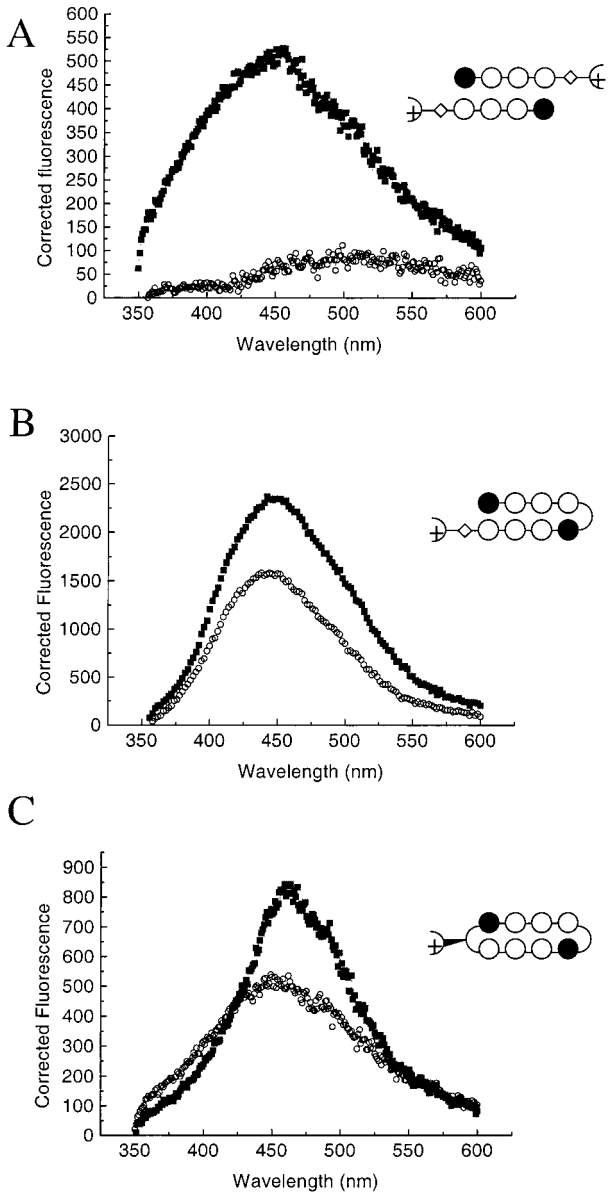

FIGURE 2: Fluorescence enhancement of polyamides upon binding to the matched site. Samples were irradiated at 310 nm with excitation slit at 5 nm and emission slit at 10 nm. Open circles represent spectrum from polyamide alone and filled squares represent spectrum of complex. The concentrations of the matched site and the polyamides for the experiments were (A) [DNA] = 4 μM, [Unlinked] = 4 μM; (B) [DNA] = 4 μM, [Hairpin] = 4 μM; (C) [DNA] = 2 μM [Cycle] = 2 μM. Ball and stick representations (*19*) indicate the polyamide used. Open balls represent pyroles, filled balls represent imidazoles and the diamond represents β-alanine.

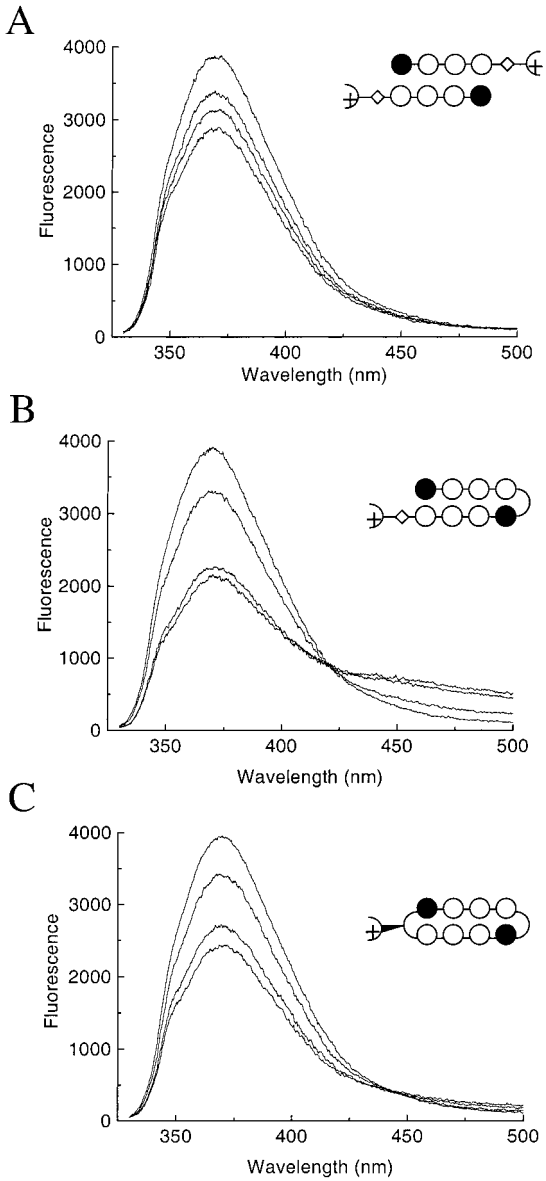

FIGURE 3: Fluorescence decrease upon polyamides binding to 2-AP substituted matched site. Samples were irradiated at 260 nm with excitation slit at 5 nm and emission slit at 10 nm with PMT at 950 V. The concentration of duplex was 1 μM and polyamide concentration was 250 nM to 1 μM in steps of 250 nM. Ball and stick representations indicate the polyamide used.

incorporation of the 2-AP residue does not change the functional groups displayed in the minor groove in comparison to a GC base pair, we incorporated a 2-AP base within the sequence corresponding to the matched site (Figure 1). As shown in Figure 3, binding of each of the polyamides shows a saturable quenching of 2-AP fluorescence in addition to the fluorescence enhancement expected for the polyamides. The quenching of 2-AP fluorescence is greater in magnitude than the increase in fluorescence of each polyamide and should provide a more sensitive assay for measuring the binding of polyamides.

Association Kinetics. Increase in fluorescence of the ligand upon binding DNA was used to measure the binding of polyamides to their matched or mismatched sites as a function of time. The observed rates for binding of unlinked polyamide to the matched target site vary as square of the concentration of the polyamide (Figure 4). This indicates that binding of the second monomer is the rate limiting step in the reaction. This behavior is notably different from that observed for binding of Dst to its 2:1 site where the apparent association rate varies linearly with Dst concentration (*6*). Association rates of the covalently linked 4-ring polyamides were too fast to measure. However, based on the dead time of the instrument ($\sim$2 ms) and the concentration of polyamide used, our studies enable us to put a lower limit on these fast association rate constants of about 10^8 M^{-1} s^{-1}. Back calculation of the on rates from reported values of equilibrium association constants and measured off-rates (see below) confirms this estimate (Table 1).

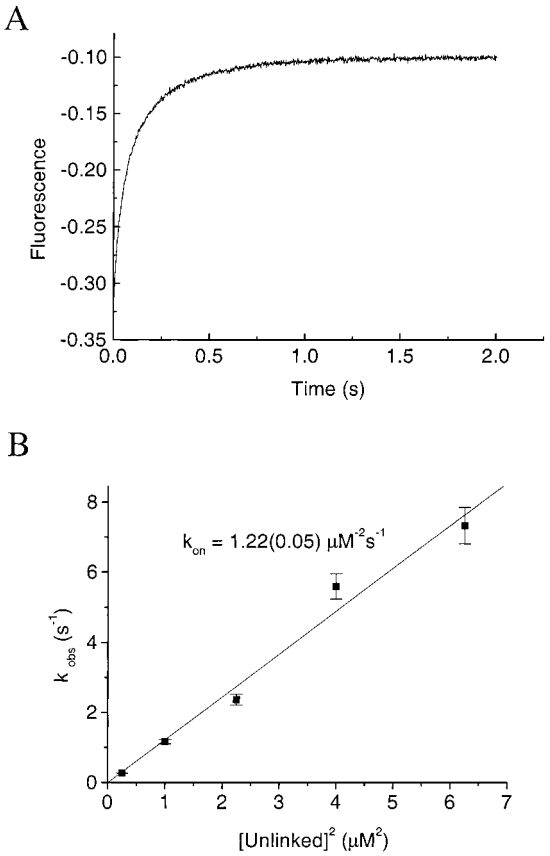

FIGURE 4: (A) Representative fluorescence detected stopped-flow trace of ImPyPyPy-β-Dp binding to its matched site under excess polyamide conditions. The trace shown here corresponds to a reaction in which the final concentration of polyamide and matched site was 1 μM and 50 nM, respectively. (B) Concentration dependence of trimolecular association rate.

Dissociation Kinetics. Sequestration of free drug using a detergent such as SDS allows the measurement of off rates of DNA binding drugs (*25*). However, in the case of the covalently linked polyamides under study here, time-dependent photobleaching behavior was observed for free polyamide. As shown in Figure 5, at the slit widths used in the experiment, the process is fairly fast with a $t_{1/2}$ of around 10 s. Interruption of the beam by closing the shutter for a brief period followed by reopening was used to confirm a light dependent process. Interestingly, complexation of the polyamide with DNA prevents the photobleaching process (Figure 2, Supporting Information). Although the precise nature of this photobleaching process remains to be investigated, the signal from this event is sufficient to obscure or distort the fluorescence decrease due to dissociation of hairpin and cyclic polyamides from DNA. Therefore, we devised an alternate method for observing the slow kinetics of dissociation for these polyamides. Mixing a preequilibrated ligand−DNA complex with an excess of competitor DNA containing a single 2-AP substitution allows observation of dissociation of the drug by simply recording the decrease in fluorescence of 2-AP at 370 nm (Figure 6A). The off rate of each polyamide can be obtained in this way (Figures 3−5, Supporting Information). In a separate experiment, we compared the off rate obtained for the complex of unlinked polyamide at the matched site using the SDS sequestration method vs mixing with 2-AP containing competitor and the results were in good agreement (Figure 6B). An examination of the dissociation rates thus obtained (Table 1) shows that the dissociation of covalently linked polyamides is indeed slower than that of the unlinked version. And more importantly, the reported sequence specificity (*18, 19*) of each polyamide can be largely explained on the basis of differences in off-rates between the matched and mismatched sites.

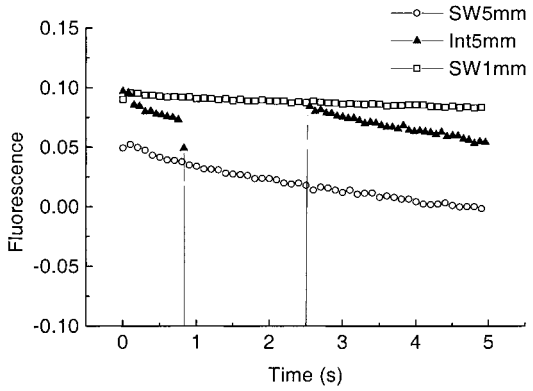

FIGURE 5: Photobleaching of hairpin polyamide. Shown here for 5 μM hairpin polyamide. Two different slit widths, 1 and 5 mm, are shown to demonstrate the effect of intensity of incident light. Also, the middle trace (Int-5 mm) shows a case where illumination is interrupted for 1.5 s and resumed thereafter.

Kinetic Origins of Enhanced Affinity in Linked Polyamides. Since the linked and unlinked polyamides have different reaction rate laws, a calculation of the ratio of formation rates by linked and unlinked forms must specify a concentration, which we choose to be the unlinked polyamide concentration that yields half-saturation of its binding site. By combining the measured association and dissociation rate constants, we calculate the half-saturation concentration to

Table 1: Dissociation Rate Constants of Each Polyamide from the Matched and Mismatched Sites[a]

polyamide	site	k_{off} (s^{-1})	k_{off} mismatched/ matched	K_{eq} matched/ mismatch	k_{on}(calc) (M^{-1} s^{-1})
unlinked	match	0.025 (0.003)	15	15	5×10^5
	mismatch	0.396 (0.021)			5×10^5
hairpin	match	0.002 (0.0001)	75	90	7×10^7
	mismatch	0.151 (0.005)			6×10^7
cycle	match	0.002 (0.0002)	33	55	15×10^7
	mismatch	0.065 (0.003)			8×10^7

[a] A preequilibrated solution made by mixing equimolar (1 μM) amounts of polyamide and target duplex was mixed with a 10-fold excess of 2-AP containing competitor duplex. Values of equilibrium association constants measured at the same target sequence embedded in a longer duplex (*18, 19*) were used to calculate specificity [K_{eq}(matched)/K_{eq} (mismatched)] and bimolecular on-rate constants ($K_{eq}k_{off}$) for linked and cyclic polyamides. The (apparent) bimolecular rate constant for the unlinked polyamide was calculated from the reported apparent monomer association equilibrium constant (*19*).

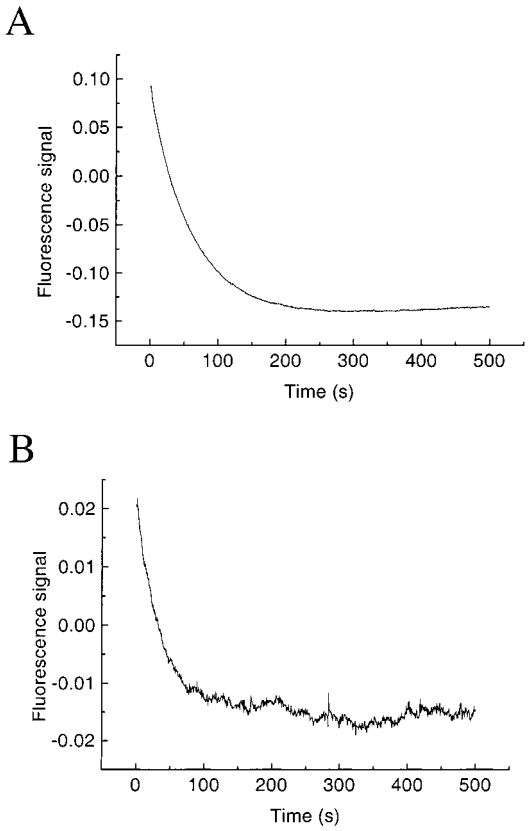

FIGURE 6: (A) Stopped flow traces for SDS assisted dissociation of ImPyPyPy-β-Dp from the matched site. Dissociation experiments carried out here used equilibrated complex formed with [DNA] = 1 μM, [ImPyPyPy-β-Dp] = 1 μM and mixed with an equal volume of 2% SDS. B) Stopped flow traces for 2-AP labeled competitor assisted dissociation of ImPyPyPy-β-Dp from the matched site. Dissociation experiments carried out here used equilibrated complex formed with [DNA] = 1 μM, [ImPyPyPy-β-Dp] = 1 μM and mixed with an equal volume of 10 μM 2-AP labeled competitor. The off-rates obtained were 0.021 (0.003) s^{-1} and 0.025 (0.003) s^{-1}, respectively.

be 1.3×10^{-7} M, in reasonable agreement with the value of 0.5×10^{-7} M obtained from DNAse I footprinting of DNA (*11*). The half-saturation concentration for the hairpin polyamide is 2.9×10^{-11} M (*10*). At 1.3×10^{-7} M concentration, the hairpin polyamide complex would be formed 500-fold faster than the unlinked; the ratio is much larger at the half saturation concentration of the hairpin. Thus, the 1 order of magnitude faster dissociation rate of the unlinked polyamide accounts for only a small part of the more than 3 orders of magnitude larger concentration required for half saturation of the DNA binding sites, compared to the hairpin (*18*, *19*).

Implications for Gene Regulation. Several studies of the kinetics of binding of transcription factors to DNA are now available. Typical association rate constants vary from 10^6 M^{-1} s^{-1} measured for yeast TBP (*26*) and *E. coli* Met repressor (*27*), to the 10^4 M^{-1} s^{-1} measured in the case of a series of zinc fingers derived from *zif*268 (*28*) and high mobility group protein HMG-1 (*29*). The covalently linked polyamides will clearly be able to reach their target sites as fast as or faster than these cellular proteins, if present within the cell at comparable concentrations. In the absence of accessory factors, TBP dissociates from its target sites at a rate of 7.1×10^{-4} s^{-1} (*26*). Similarly, zinc fingers (*28*) and HMG-I (*29*) were found to dissociate from DNA with rates in the order of 10^{-4} s^{-1}. Since the dissociation rates measured for the covalently linked polyamides in this study approach these rates, we expect that these and longer polyamides will be able to occupy their target sites as long as, or longer than, the cellular factors mentioned above.

A significant kinetic problem for use of artificial DNA-binding agents as gene regulators is the time required to reach the target site in the presence of one or more competing mismatched sites. In general, reaching equilibrium requires a time long compared with the half-life of the mismatched sites. The linked polyamides have favorable properties in this regard. If they had association rate constants of 10^6 to 10^7 M^{-1} s^{-1}, values more typical of drug−DNA complexes, equilibration would be 100 to 10 times slower for compounds of equal affinity and specificity. Thus, the affinity can be large, as needed to displace proteins, without requiring slow equilibration.

CONCLUSIONS

Our results from stopped-flow kinetic studies of this series of polyamides lead to two main conclusions. First, covalent linkage of the subunits results in polyamides with dramatically *enhanced affinity* primarily due to faster *association* rates. Second, the basis for *discrimination* between matched and mismatched sites for each polyamide is in large part the difference in *dissociation* rates from these sites. Since the association rates of longer covalently linked polyamides are essentially diffusion limited, and sequence discrimination arises primarily from the dissociation rates, measurement of dissociation rates using 2-AP containing competitor sequences can be used to substantially accelerate the pace at which sequence specificity of these polyamides is studied. Also, since linked polyamides containing four rings already have association rates that are close to diffusion limited, gains in affinity for longer polyamides can only derive from slower dissociation rates. At the same time, gains in specificity at the level of association will, in all likelihood, only be possible for polyamides with smaller numbers of contiguous rings. These observations on kinetics of covalently linked polyamides should provide new avenues for optimizing the *specificity* of polyamides.

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SUPPORTING INFORMATION AVAILABLE

Excitation and emission spectra of 2-AP containing duplex complexed with hairpin polyamide, fluorescence of pre-equilibrated complex of 2.5 μM hairpin + 2.5 μM matched duplex mixed with 1× TKMC, and stopped-flow traces for dissociation of indicated polyamides from matched or single mismatch sites. This material is available free of charge via the Internet at http://pubs.acs.org.

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