Temperature-sensitive protein–DNA dimerizers

Karl E. Hauschild*, Renee E. Metzler*, Hans-Dieter Arndt†, Rocco Moretti*, Marni Raffaelle*, Peter B. Dervan†‡, and Aseem Z. Ansari*‡

*Department of Biochemistry and the Genome Center, University of Wisconsin, Madison, WI 53706; and †Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

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Programmable DNA-binding polyamides coupled to short peptides have led to the creation of synthetic artificial transcription factors. A hairpin polyamide–YPWM tetrapeptide conjugate facilitates the binding of a natural transcription factor Exd to an adjacent DNA site. Such small molecules function as protein–DNA dimerizers that stabilize complexes at composite DNA binding sites. Here we investigate the role of the linker that connects the polyamide to the peptide. We find that a substantial degree of variability in the linker length is tolerated at lower temperatures. At physiological temperatures, the longest linker tested confers a ''switch''-like property on the protein–DNA dimerizer, in that it abolishes the ability of the YPWM moiety to recruit the natural transcription factor to DNA. These observations provide design principles for future artificial transcription factors that can be externally regulated and can function in concert with the cellular regulatory circuitry.

DNA recognition | extradenticle | HOX proteins | polyamide

Multivalent interactions are frequently encountered in biological systems (1–3). Typically, the monovalent elements that mediate these molecular interactions use a small surface area and the resulting binding is weak (2). These monovalent elements are reiterated, often in a modular fashion, and the resulting multivalency greatly improves the association between the interacting molecules (1–3). Although the multivalent association may or may not be cooperative, it significantly improves association between interacting partners (2, 4). This principle of multivalent binding has also been applied in drug design. For example, small molecule fragments that bind weakly to a target protein have been identified by NMR and linked to each other to generate multivalent ligands that associate strongly with the target protein (5). In a more recent approach, small molecules that bind weakly to a particular surface of a target protein are tethered by disulfide exchange to an engineered proximal cysteine residue (6). Subsequently, these small molecule fragments are used to identify additional fragments that bind adjacent surfaces. The resulting composite molecule displays an improved affinity for the target protein, in part due to multivalent interactions (6–8). In modular assembly of a composite molecule, the properties of linkers connecting various fragments can greatly influence the binding of a multivalent ligand to its target molecule (1–3).

Recently, we described the creation of bifunctional molecules, where one module binds a specific DNA sequence while the other interacts weakly with a specific DNA binding protein (Fig. 1) (9). This bifunctional molecule greatly improves the affinity of the targeted DNA-binding protein for its specific DNA sequence, whereas it is ineffective at sites where the DNA sequence does not match the preferred site of the target protein. In contrast to strategies that target adjacent surfaces of a single protein to create bivalent ligands, we use a DNA site as well as a protein interaction module to generate a bidentate surface that enhances the association of the targeted protein with its cognate DNA site (Fig. 1). These molecules therefore function as protein–DNA dimerizers (PDDs) and could potentially serve as artificial regulators of gene expression.

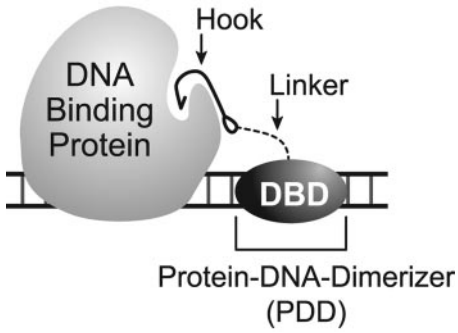

Fig. 1. Schematic of a protein–DNA dimerizer (PDD) recruiting a natural transcription factor. A general depiction of a bifunctional molecule comprising a DNA-binding domain (DBD) conjugated by means of a linker to a protein-specific ''hook'' moiety. This synthetic PDD should specifically stabilize the DNA binding of a natural transcription factor for which the hook is designed. In this study, the DNA-binding module is a minor groove binding polyamide, and the hook is a short YPWM peptide that specifically binds to the developmental factor Exd.

Malfunctioning transcriptional networks are often causally correlated with onset of diseases as diverse as cancer and diabetes (10–12). Often a break down in the regulatory networks is a consequence of a mutated or an aberrant transcription factor (10–12). A molecule that can substitute for an inactive transcription factor or counteract the deleterious effects of a malfunctioning regulator would have considerable value. Thus far, a modular approach of tethering a DNA binding module to a regulatory module has been used to generate artificial transcription factors (ATFs). The DNA module mediates gene-specific targeting, whereas the tethered regulatory module controls the extent of gene expression. Small molecules, such as polyamides, as well as protein-based zinc fingers have been successfully used as DNA-binding modules in the design of ATFs (13–17). Polyamides are comprised of heterocyclic amino acids that are capable of binding the DNA minor groove with affinities and specificities paralleling natural transcription factors (17). Rules have been identified that relate each Watson–Crick base pair to a particular pairing of heterocycles, so that high-affinity ligands can be generated for most given DNA sequences (17).

In our initial design, we used a short linker to conjugate the DNA-binding module to the protein-interaction module (compound **4** in Fig. 2*B*) (9). The protein-interaction module (also referred to as the ''hook'' in Fig. 1) was a conserved peptide (**FYPWM**) derived from the Hox-family of transcription factors (18). This Hox peptide interacts with Extradenticle (Exd), a DNA-binding protein, and stabilizes the assembly of a ternary Hox–Exd–DNA complex (19–21). The crystal structure of the Hox–Exd–DNA complex (18) was used to design a polyamide

Abbreviations: PDD, protein–DNA dimerizer; ATF, artificial transcription factor; ts, temperature-sensitive; Exd, Extradenticle.

‡To whom correspondence may be addressed. E-mail: dervan@caltech.edu or ansari@biochem.wisc.edu.

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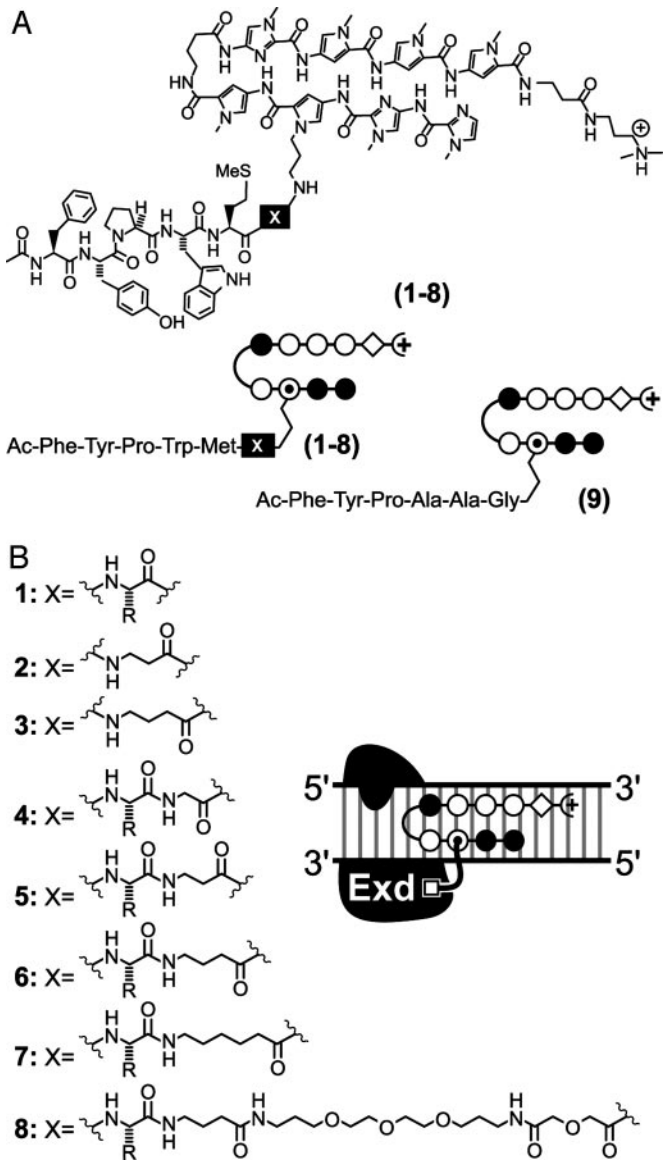

Fig. 2. Polyamide conjugates examined in this study. (*A*) Chemical structure of parental polyamide–peptide conjugate. The position marked X is where the linker is varied. Open circles represent *N*-methylpyrrole (Py), filled circles represent *N*-methylimidazole (Im), the diamond represents β-alanine, and the pyrrole with the peptide attached is depicted. (*B*) The composition of different linkers in compounds **1**-**8**. In the displayed format, the C terminus of peptide is attached to the amine of the linker that is then attached to the polyamide at the position marked by an ''X'' in the compounds shown in Fig. 2*A*. R in compounds **1** and **4**-**8** is the lysine side chain. The adjacent cartoon illustrates the DNA binding orientation of the polyamide–peptide (YPWM) conjugate with respect to the Exd in the ternary complex. The YPWM ''hook'' is represented as a white box embedded in Exd.

that would display the YPWM peptide adjacent to the cognate DNA-binding site for Exd. This bifunctional small molecule efficiently mimicked the ability of natural Hox proteins to stabilize Exd binding to DNA (9).

Toward our goal of defining a general design strategy for PDDs, we tested the role of the linker in determining the effectiveness of these compounds to recruit Exd. The goals of this study were to determine (*i*) the optimal linker length for maximum effectiveness of the PDD, (*ii*) the maximal distance a hook can be positioned without a significant loss in effectiveness, and (*iii*) the effect of various linkers on PDD function at physiological temperatures.

Here we describe a series of conjugates with linkers of different length. We find that, at low temperatures, the YPWM motif attached to a linker that is ≈29 Å longer than the optimal length (≈4 Å) effectively recruits the DNA, binding protein to an adjacent site. This tolerance to linker variability may help overcome a key stumbling block encountered by structure-based design, in that it permits flexibility in the design of PDDs and also offers access to a much larger surface of the DNA-binding protein in the selection of highly specific small molecules that bind proteins of interest.

Not surprisingly, increasing the linker length inflicts an energetic penalty on the ability of the YPWM moiety to stabilize binding of Exd to an adjacent DNA site. However, we find that this penalty can be tuned such that a bifunctional molecule capable of functioning at lower temperatures is rendered inactive at physiological temperatures. This finding suggests that linker-based destabilization could provide a key parameter in the design of temperature-sensitive (ts) ''chemical switches.'' Temperature sensitivity permits rapid spatio-temporal control of the activity of a molecule. The ability to generate ts proteins has been of value in dissecting biological systems (22–25). Similarly, ts chemical switches would allow externally regulateable chemical tools to explore new biological space. Beyond the generation of ts PDDs and ATFs, our general strategy of linker-based destabilization may be used to confer a ts property on composite molecules that were designed from the assembly of distinct chemical fragments.

Materials and Methods

Synthesis of PDDs with Varying Linker Length. *t*Bu-protected peptide acids were assembled on SASRIN resin by using manual SPPS Fmoc protocols, and purified by flash column chromatography. Compounds **1**-**9** were synthesized by using HBTU/HOBt-mediated solution phase couplings of respective *t*Bu-protected peptide acids with the polyamide amine core according to described procedures (9). Global deprotection (TFA/DCM/EDT/Et_3SiH, 90:5:2.5:2.5) and preparative RP-HPLC (C18) purification delivered the homogeneous conjugates **1**-**9** in 25–50% yield after lyophilization. ESI-MS (average mass, $[M + 2H]^{2+}$); for **1**: calculated, 1,081.7, found, 1,081.5; for **2**: calculated, 1,052.7, found, 1,052.6; for **3**: calculated, 1,059.7, found, 1,059.7; for **4**: calculated, 1,110.2, found, 1,109.8; for **5**: calculated, 1,117.2, found, 1,117.0; for **7**: calculated, 1,138.3, found, 1,137.8; for **8**: calculated, 1,283.4, found, 1,283.0. Conjugates **6** and **9** were reported in ref. 9.

Purification of Exd. The *Drosophila* protein Exd (residues 236–320) was expressed by using standard methods. Purification consisted of ammonium sulfate precipitation followed by cation exchange and size exclusion chromatography as previously described (9, 18). After purification, the concentration of Exd was determined by using an extinction coefficient of 12,090 $cm^{-1}\cdot M^{-1}$ at 280 nm.

EMSA. A 40-mer DNA radiolabeled at the 5′ end with ^{32}P (following standard methods) was used in all reactions. Reactions were performed in a buffer composed of 150 mM K-glutamate, 50 mM Hepes (pH 8.0), 2 mM DTT, 100 ng/μl BSA, 10% DMSO, and 10% glycerol. Polyamides were kept in subdued lighting whenever possible. For the initial binding tests, polyamide–peptide conjugates (50 nM final concentration) were mixed with ^{32}P-DNA and incubated for 30 min at 4°C. Exd was then added to bring the reaction volume to 20 μl at the appropriate final concentration (see Fig. 3). These reactions were incubated at 4°C for 1 h, then 15 μl was loaded onto a prerun 10% acrylamide/3% glycerol gel, and the gels were run at 200 V for 2.5 h and were processed as described below.

For the EMSA experiments conducted at different tempera-

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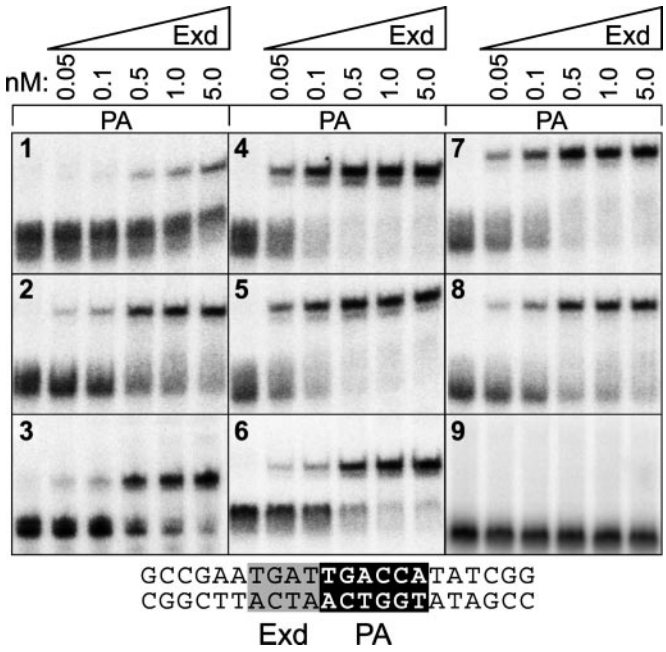

Fig. 3. Ability of polyamide conjugates **1**-**9** to recruit Exd at 4°C. Saturating levels (50 nM) of the polyamide–peptide conjugates (**1**-**9**) were preincubated with ^{32}P-labeled DNA (lanes 1–6), and Exd was titrated in the reactions (lanes 2–6). On gel electrophoresis, a reduction in the mobility of the labeled DNA upon ternary complex formation is observed for compounds **1**-**8**. DNA sequence bound by Exd and the polyamide (PA) conjugates is highlighted by gray and black boxes, respectively.

tures, DNA was mixed with the appropriate polyamide (50 nM final) and first incubated at 4°C for 30 min; Exd was then added to bring the final reaction volume to 60 μl, with final Exd concentrations of 0.01, 0.03, 0.1, 1, 3, 10, 30, or 100 nM. This reaction was then mixed thoroughly and separated into four 15-μl aliquots, which were then incubated separately at 4°C, 14°C, 23°C, 30°C, or 37°C for 1 h. Ten microliters of these reactions were then run for 2.5 h on 10% acrylamide/3% glycerol gels that were equilibrated to the appropriate reaction temperatures. Before loading, all gels were prerun for 30 min at voltages that maintained a starting current between 36 and 38 mA. These voltages also did not detectably increase the temperature of the gels. Experiments at 14°C and 23°C were performed in triplicate, whereas data from six experiments was used for the other three temperatures. To minimize variability, a gel-loading regimen was cycled to account for differences in incubation times. The gels were dried, exposed to a PhosphorImager screen overnight, and visualized on a Typhoon 9410 PhosphorImager. Binding constant determinations were performed by using IMAGEQUANT TL to quantify the EMSA gel data and SIGMAPLOT 6.0 for nonlinear regression as well as the van't Hoff analysis.

The top strand of the DNA duplex used in these experiments is 5′-TCC CGG CGA GGA TGA TTG ACC ATA TCG GCG CCA CCC GAT C-3′.

Quantitative DNase I Footprint Titrations. Footprinting experiments were performed as described (9). For raw data, protocols, and experimental details, see supporting information, which is published on the PNAS web site.

Results

Small Molecule PDD Design. The composition and structure of the PDDs investigated here are shown in Fig. 2. A specific DNA-binding hairpin polyamide was linked to the YPWM peptide motif by using different linkers (Fig. 2*B*). The point of attachment is chosen such that it positions the YPWM hook over a thymine residue in the minor groove. This thymine is closest to the Exd-binding pocket as observed in three Hox–Exd crystal structures, and it is common to all natural Hox–Exd-binding sites (18, 26, 27). Eight compounds were synthesized, each bearing the same polyamide and the F**YPWM** hook (Fig. 2). The eight linkers range from ≈2.5 Å in the case of compound **1** to ≈33 Å in the case of the polyethylene glycol linker of compound **8**. Conjugates **1** and **4**-**8** have a lysine residue at the C terminus of their YPWM hook, which occurs in various Hox proteins. The lysine also improves the solubility of these rather hydrophobic compounds. Conjugates **2** and **3**, which do not bear the lysine, are less soluble. Control compound **9** carries a double alanine substitution in the binding motif, which was shown to be sufficient to fully abrogate ability of natural Hox proteins, as well as our artificial dimerizer, to stabilize Exd binding to DNA.

Table 1. Affinity of conjugates for DNA

Conjugate	K_D, nM
1	0.15 ± 0.02
2	0.25 ± 0.04
3	0.21 ± 0.04
4	0.29 ± 0.05
5	0.12 ± 0.02
6	0.27 ± 0.05
7	0.30 ± 0.05
8	0.32 ± 0.06

Affinity of the Conjugates for DNA. Two different assays were used to determine whether the linker altered the affinity or specificity of the PDDs for their target site. First, we performed EMSAs where increasing amounts of each PDD conjugate were incubated with a 47-bp duplex DNA molecule bearing a single optimal polyamide-binding site. The compounds reduced the mobility of the radioactively labeled DNA and, at 20 nM, each of the compounds saturated their binding site (data not shown). Quantitative DNase I footprinting (28) was then performed to more precisely determine the affinity of each of the conjugates for their site (Table 1; also see supporting information). The conjugates bound with subnanomolar affinity to the 96-bp fragment bearing a single cognate site (Table 1). Interestingly, the presence of the cationic lysine residue did not detectably alter DNA affinity of the conjugates (Table 1, compare **4**–**8** to **2** and **3**).

Ability to Stabilize Exd Binding to an Adjacent DNA Site. EMSAs (Fig. 3) reveal that conjugates **1**-**8** were able to stabilize Exd binding to the adjacent cognate DNA site (5′-TGAT-3′). Neither the polyamide lacking the YPWM moiety nor the PDD conjugate bearing an inactive FYPAAK moiety (conjugate **9**) stabilized Exd binding. The affinity of Exd for this PDD–DNA complex can be readily monitored by the formation of a ternary Exd–PDD–DNA complex with increasing concentration. The data indicate that compound **1** with a short linker (≈2.5 Å when fully extended) does not optimally position the hook with respect to its hydrophobic docking site on the surface of Exd (Fig. 3). The absence of a lysine residue in compounds **2** or **3** did not appreciably alter its ability to recruit Exd in comparison to compound **4**, which does bear the lysine and is ≈1 Å longer. Importantly, the data suggest that, at 4°C, the linker range can vary from ≈4 to ≈33 Å (extended) with a minimal cost to Exd binding. This result encourages the use of longer linkers in the initial screens for hooks whose docking sites on target proteins have not been determined structurally (see *Discussion*).

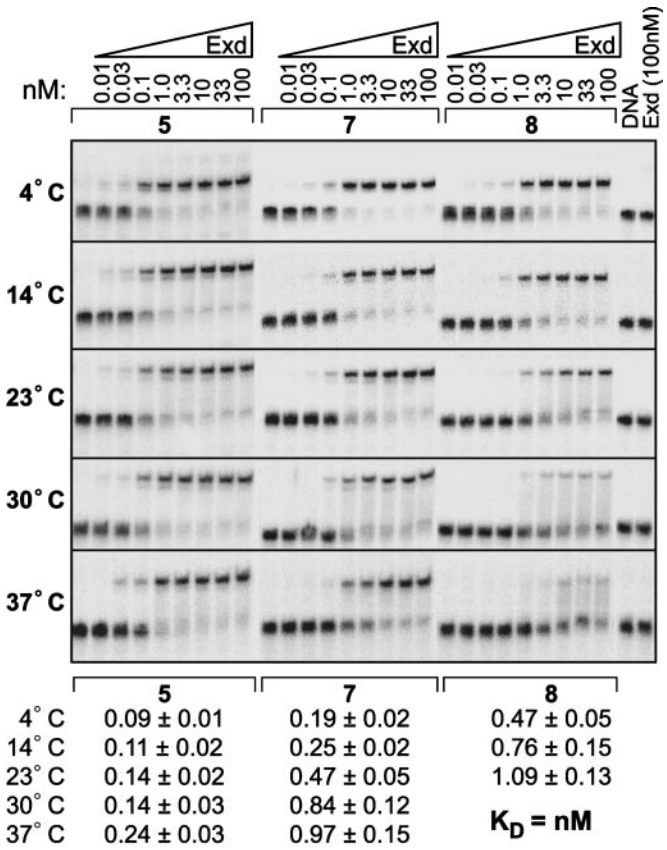

Fig. 4. Temperature effects on the ability of **5**, **7**, and **8** to form a ternary complex. The equilibrium dissociation constants of Exd for the DNA–polyamide complexes were determined at increasing temperatures. Conjugates **5** and **7** maintained their ability to recruit Exd at each of the temperatures tested, whereas **8** displayed a dramatic temperature sensitivity in its ability to recruit Exd to the composite site.

Thermal Effects on Binding of Exd. Although binding at 4°C displayed a small effect of linker length on the ability of seven compounds (**2**-**8**) to recruit Exd, we reasoned that the energetic penalty of a longer linker would be apparent at higher temperatures. To determine the effects of a longer linker on binding, we measured the ability of compounds **5**, **7**, and **8** to recruit Exd at five different temperatures. To minimize the inherent limitations of EMSA, we performed the experiments under carefully controlled conditions (see *Materials and Methods*). As shown in Fig. 4, at 4°C, compounds **5**, **7**, and **8**, with linkers that maximally project ≈7, ≈11, and ≈33 Å, respectively, recruit Exd with less than an order of magnitude difference in their apparent equilibrium dissociation constants (K_D = 0.1 nM for **5** vs. 0.5 nM for **8**). However, compound **8**, bearing a defined length 28-atom polyethylene glycol linker, shows a dramatically reduced binding affinity at 30°C and at physiological temperatures (37°C). Compound **5** with a seven-atom linker is least affected by the thermal variation, with a <3-fold decrease in the ability to recruit Exd over a 33-degree change in temperature (4°-37°C).

Predicted Entropic Penalty. In our studies, the interacting components of the reaction remain unaltered, with the variable linker providing the only source of difference between the eight compounds (Fig. 2). Because conformational/rotational entropy of linkers is often evoked to explain the decrease in multivalent binding (2), we explored two theoretical treatments for predicting the energetic penalties of restraining the mobility of straight chain linkers upon binding.

Table 2. Predicted entropic penalty of Exd binding

Conjugate	No. of bonds	Extended length, Å	Jencks ΔS,* cal/mol·K	WMS ΔS,* cal/mol·K
1	2	2.46	9.0	4.54
2	3	3.81	13.5	6.09
3	4	4.97	18.0	7.64
4	5	6.14	22.5	9.27
5	6	7.42	27.0	10.8
6	7	8.65	31.5	12.4
7	9	11.14	40.5	15.5
8	27	32.59	121.5	43.9

*Calculated ΔS for the portion of the linker shown in Fig. 2*B*. Each model predicts an additional constant contribution because of the restriction of rotation of other bonds in the constant portion of the system.

The first treatment by Page and Jencks (29) is based on the empirical analysis of entropy changes associated with the cyclization of small aliphatic hydrocarbons in the gas phase. The loss in entropy caused by cyclization ranges from 3.7 to 4.9 cal/mol·K per internal bond rotation, with 4.5 cal/mol·K being a more generally representative value. As shown in Table 2, this estimation suggests that the penalty for limiting bond rotation in our linkers would range from 9 to 122 cal/mol·K (in addition to the constant entropic cost associated with the interactions formed by the nonvariable features of the complex).

The second treatment by Whitesides and coworkers (30) (the WMS model) suggests that the Jencks model overestimates entropic changes, because the bond rotations in typical chemical linkers are more hindered and less flexible than the aliphatic hydrocarbon chains of corresponding length. In this model, the values for rotational entropy for a model compound were calculated by using the Boltzman distribution and a probability-based definition of entropy. Entropic changes limiting bond rotation are given for various bond types, and range from 0.2 cal/mol·K for an amide bond to 2.9 cal/mol·K for carbonyl–C_α bond. The average per bond values predicted by the WMS model for the compounds under study are nearly half of those predicted by Page and Jencks (Table 2). However, this analysis focuses entirely on the conformational contribution to entropy, assuming that the kinetic contribution will be the same in the free and frozen forms. Ercolani (31) has argued that this assumption may not apply for a quantum mechanical system and that a frozen bond cannot be handled classically.

Nevertheless, both treatments assume that differences in binding energy result primarily from differences in conformational entropy. If this were the case, van't Hoff analysis of the equilibrium dissociation constants (K_D) should show a linear response over temperature with parallel slopes. The binding data in Fig. 4 and the van't Hoff analysis in Fig. 5 indicate that the compounds under study do not show this type of linear response.

Thermodynamic Analysis. The temperature dependence of the binding constant was used to approximate the entropic and enthalpic contributions to the binding free energy. As shown in Fig. 5, a van't Hoff analysis of Exd binding to the **5**–DNA complex surprisingly indicates that binding is entropically favored (29.8 cal/mol·K). At room temperature (296 K), the entropic contribution to the free energy of binding ($\Delta G° = -13.3$ kcal/mol) is nearly −8.8 kcal/mol. Previous NMR studies suggest that both the YPWM moiety as well as Exd homeodomain are preordered and the binding does not induce appreciable change in the structure of either partner (18, 32, 33). Therefore, the entropic contribution may arise from the release of water and counterions from the Exd–DNA interface as well

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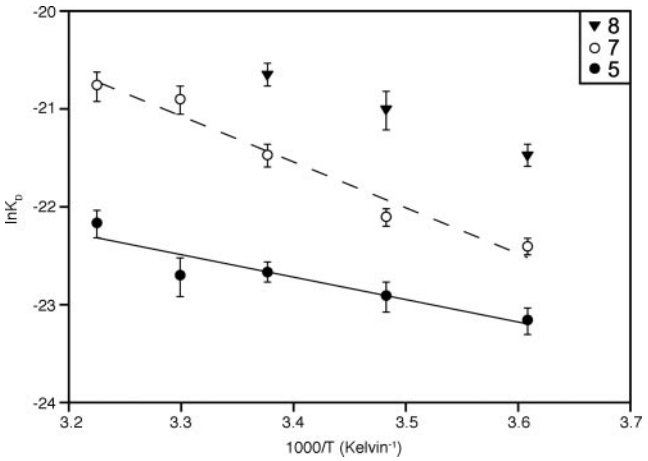

Fig. 5. van't Hoff analysis of the ternary complex. The natural log of the K_D of Exd for polyamide–peptide/DNA complexes was plotted as a function of change in temperature. The solid line for **5** represents the best fit to the data. A dashed line for **7** represents a less than linear relationship, and the data for **8** were not fitted because the K_D drops off dramatically at 30°C. A linear relationship between the dissociation constant and an increase in temperature permits the extraction of entropic and enthalpic contributions to the free energy of binding. The van't Hoff analysis assumes that $\ln K_D = -\Delta H/RT + \Delta S/R$ provided $\Delta Cp = 0$. In the linear regime slope $= -\Delta H/R$ and the y-intercept $= \Delta S/R$. A nonlinear profile cannot be treated by using the van't Hoff analysis.

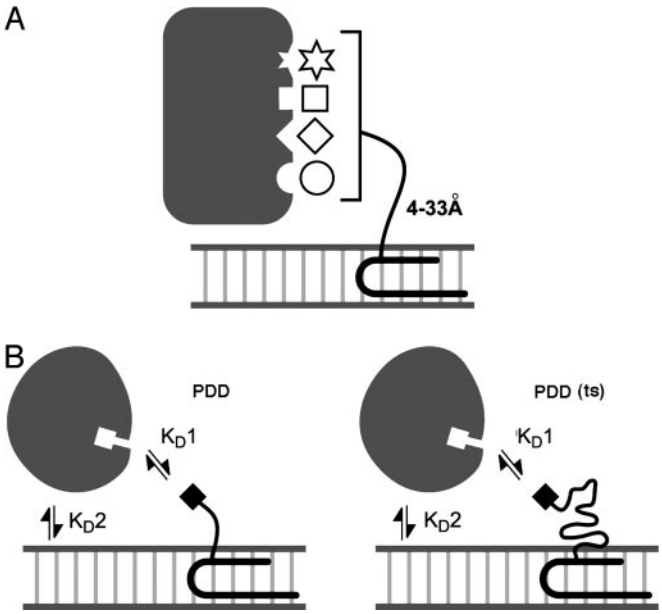

Fig. 6. Models summarizing the findings reported here. (*A*) The model suggests that, at lower temperatures, linkers ranging from 4 to 33 Å may successfully display a hook and recruit the targeted natural transcription factor. The ability to use a long flexible linker permits the screening of small molecule hooks that may bind to surfaces of natural transcription factors that are distal from the DNA surface and may not have been defined at atomic resolution. The model also predicts that hooks that display high specificity for a given DNA binding protein can, due to multivalent interactions, overcome low affinity for the target to form a very stable ternary complex. (*B*) The energetic penalty of a longer linker at higher temperatures can be harnessed. Thus, two fragments that are connected by an optimal linker can be made ''conditional mutants'' by simply replacing a linker with a sufficiently long linker that destabilizes binding at higher temperatures. Thus, even though the affinity of the hook for the target (K_D1) or the affinity of the target for a specific DNA sequence (K_D2) does not change, the complex is less stable at higher temperatures because of the energetic penalty of restricting a long linker. This property can be harnessed to make a PDD, or, in principle, any multivalent ligand, into a ts mutant.

as the release of the ordered solvent cage around the hydrophobic YPWM peptide on binding to Exd. The burial of the Trp residue of the hook into the hydrophobic pocket of Exd probably contributes significantly to the enthalpy of binding.

The ability of **7** and **8** to stabilize Exd binding almost as well as **5** at 4°C is consistent with the reduced conformational entropy of the linker at lower temperatures. The affinity of Exd for the **8**–DNA complex when examined at 4°C and 23°C reveals that the decrease in the binding of Exd to the **8**–DNA complex results from disfavorable entropic changes (the intercept on *y*-axis is $\Delta S/R$). Within the limitations of the EMSA assay, the data for **8**, and to a lesser extent **7**, suggest that factors other than simple conformational entropy of the linker contribute to the switch-like loss in the ability to recruit Exd at higher temperatures. van't Hoff analysis for **8**–DNA–Exd complex is not possible because **8** is incapable of recruiting Exd stably at 30°C or 37°C. However, if the data for compound **7** were fit to the van't Hoff equation (dashed line in Fig. 5, $\Delta G° = -12.6$ kcal/mol), van't Hoff entropy would be 11.0 cal/mol·K, ≈19 cal/mol·K less than compound **5**. This result does not compare well with the $\Delta\Delta S$ of 4.7 cal/mol·K predicted by the WMS model and 13.5 cal/mol·K by the Jencks model. Additionally, there is a change in van't Hoff enthalpy comparing **5** with **7**, which manifests itself as a difference in slope. This effect is not predicted by either of the models, which assume that the enthalpy of binding should not change.

Discussion

Our data are consistent with the expectation that longer linkers inflict an energetic penalty on the ability of a bivalent ligand to bind to its target. Beyond this immediate observation, our studies provide several general insights into designing a PDD class of ATFs.

The first design principle that emerges from these studies is that, at lower temperatures, the linkers that are longer than the optimal length do not significantly reduce the ability of a ''hook'' to recruit a DNA-binding protein to its cognate DNA site (Fig. 1). This unanticipated observation permits the screening of libraries of biopolymer or small molecule hooks that bind different DNA-binding proteins without *a priori* knowledge of the structure or the surface of the target protein to which the hook binds (Fig. 6*A*). Thus, by using a 33-Å linker to present a hook, one can sample the surface of a DNA-binding proteins within this radius, thereby significantly improving the probability of identifying a ligand for a desired DNA-binding protein. Once a sufficiently specific hook/ligand is identified, its function as a PDD can be optimized by systematic analysis. Moreover, the ability to design polyamides to target different DNA sequences provides a rapid means of targeting unique sequences in the context of an entire genome, thus greatly improving the specificity of the hooks as well as the specificity of the PDD class of ATFs.

This approach may overcome the limitations inherent to methods where the binding event has to be monitored by NMR with all its attendant restrictions on the solubility and size of the binding proteins (5). In addition to structure–activity relationships by NMR, the "ligand-assembly" method described by Ellman and coworkers (34) and the "protein capture" approach of Bachhawat-Sikder and Kodadek (35) both use multivalent interactions to generate ligands that bind tightly to a desired target protein (34, 35). More recently, Wells and coworkers (6) described an approach wherein a weakly interacting ligand is tethered to a cysteine thiol engineered on the surface of a target protein. An extension of that approach was used to generate multivalent ligands that bind with high affinity to adaptable or

mobile surfaces (7, 8). Our method also utilizes multivalency to improve binding; however, in our case, DNA sequence provides one level of specificity and the hook provides the protein targeting specificity (Fig. 6). The resulting bivalent surface (DNA-binding site and the hook), in principle, should stably recruit the target protein, improving its binding by several orders of magnitude, as in the case of Exd.

The second general design principle that emerges from this study is the ability to use linkers to confer temperature sensitivity on a bivalent or multivalent compound. Because the use of suboptimally long linkers can destabilize multivalent binding at physiological temperatures (Fig. 6*B*), one can generate a ts compound that, in a nonlinear manner, switches off its ability to function as a PDD. This principle can be readily extended to convert other multivalent ligands into ts switches. The ability to generate a conditional switch provides a powerful means to gain rapid temporal control over the function of a ligand or phenomenon of interest. These ts mutations in proteins have yielded a wealth of information in identifying the functional roles of proteins without significantly perturbing cellular regulatory pathways at permissive conditions (22–25). The generation of ts chemical switches similarly offers a level of control on the function of the compound, and by carefully balancing the linker choice with the desired temperature sensitivity, one could differentially regulate the function of the compound in different organisms or even different tissues in an organism. Moreover, a linker designed as an aptamer that can self-assemble around a second small molecule of interest would allow for the creation of ligand-gated chemical switches. A similar conceptual approach would serve to generate ATFs that function as sensitive "chemosensors" in other applications (36, 37).

Nature often uses such modular design to generate highly sophisticated molecular switches. One of the well studied examples of genetic switches is that of C_I repressors from bacteriophage λ. In this case, a weak oligomerization module attached to the DNA binding domain permits the dimers to stably bind to adjacent binding sites (38). This cooperative binding generates a sensitive switch that activates the lysogenic decision by phage after bacterial infection (38). In metazoan systems, ligand binding to cell surface receptors induces dimerization of the receptor, which in turn switches on a signaling cascade (1, 12, 39, 40). In an elegant demonstration of this principle, Schreiber and coworkers (39) designed a bivalent molecule that would induce the dimerization of receptor molecules. These protein–protein dimerizers were instrumental in dissecting the mechanism of signal transduction. Our work describes an extension of this principle of multivalent assembly to create modular PDDs (9). The results reported here enhance our understanding of the parameters to design effective PDDs. Broadening the applicability of PDDs also serves a longer-term goal of generating ATFs that function in concert natural transcription factors and respond to cellular signals with high fidelity.

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