Comparison of the Sequence-Selective DNA Binding by Peptide Dimers with Covalent and Noncovalent Dimerization Domains†

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Received July 20, 1998; Revised Manuscript Received October 28, 1998

ABSTRACT: Sequence-specific DNA binding proteins generally consist of more than two DNA-contacting regions to ensure the selectivity of recognition. The multiple DNA binding modules are connected either through the covalent linker or through the noncovalent dimerization domain. We have compared the DNA binding of peptide dimers with covalent and noncovalent dimerization domains to explore the potential advantage of each linkage on the sequence-specific DNA binding. Three sets of head-to-tail peptide dimers were synthesized by using the same basic region peptide to target the same DNA sequence; one dimer was assembled with a bridged biphenyl derivative as a covalent dimerization domain, and two other dimers were assembled with the cyclodextrin guest noncovalent dimerization domains. One of the noncovalent dimers was a heterodimer that consisted of cyclodextrin and guest peptides, while the other was a homodimer that consisted of peptides bearing both cyclodextrin and the guest molecule within the same chain. Both noncovalent dimers formed the specific DNA complexes within narrower ranges of peptide concentrations and showed higher sequence selectivity than the covalent dimer did. Among the three dimers, the noncovalent homodimer that can form an intramolecular inclusion complex showed the highest sequence selectivity. Because the noncovalent homodimer with the higher stability of the circular intramolecular inclusion complex exhibited the higher sequence selectivity, it was concluded that an equilibrium involving a conformational transition of a monomeric peptide effectively reduced the stability of its nonspecific binding complex, hence increasing the efficacy of cooperative dimer formation at the specific DNA sequence.

Sequence-specific DNA binding of proteins, such as transcription factors, is essential to regulation of key processes of gene expression. As seen in the structural studies of the sequence-specific DNA−protein complexes, proteins make networks of stereospecific interactions with bases and phosphate backbone at the surface of the specific DNA sequence (*1*, *2*). These DNA binding proteins generally consist of more than two DNA-contacting regions to ensure the selectivity of recognition (*3*, *4*). A class of proteins binds DNA with multiple DNA binding modules connected through covalent linkages, and the other with the noncovalent formation of homo- and heterodimers. In the former class of proteins, the greatest progress to date has been achieved in studying the sequence-specific DNA binding of the C_2H_2 zinc finger proteins. Phage display techniques have been successfully used to design novel three-finger proteins with desired specificities (*5*). In addition, connecting two three-finger peptides with an appropriately designed linker has provided six-finger proteins with stability and sequence selectivity higher than those of the three-finger protein (*6*). In the latter class of proteins, the protein−protein interactions controlling the dimerization are thought to play significant roles in enhancing the selectivity of specific DNA binding and increasing the sensitivity of equilibrium binding to the change in protein concentrations (*7*−*9*). Among these dimeric proteins, the basic leucine zipper (bZIP) class of eukaryotic transcription factors has been known to employ a small but efficient structural motif for the sequence-selective recognition of DNA (*10*). The dimerization is mediated through a coiled-coil structure of the leucine zipper domain, while the region rich in basic amino acid residues (the basic region) located N-terminal to the leucine zipper domain directly contacts the DNA major groove (*11*−*14*). Despite the current interests in exploring the advantages of these covalent and noncovalent linkages on the sequence-specific DNA binding, it is difficult to compare the DNA binding of covalently linked multimodular proteins and those of noncovalent dimeric proteins directly because the DNA-contacting regions of these native proteins are different from each other. For this purpose, the designed proteins with naturally occurring DNA binding regions and artificial dimerization domains would provide useful systems. Kim and co-workers demonstrated that the basic region of the bZIP protein by itself was sufficient for the sequence-specific DNA binding domain by using disulfide-bonded dimers of the basic region (*15*, *16*). By extending this strategy, several groups reported sequence-selective DNA binding of covalently linked basic region dimers. Disulfide-bonded dimers and trimers of the basic region peptides were reported to target nonpalindromic

† This work was supported by a Grant-in Aid for Scientific Research (09680569 and 0955720) from the Ministry of Education, Science, Sports and Culture, Japan, to T.M. and Y.S. Y.A. is a research fellow of the Japan Society for the Promotion of Science.

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10.1021/bi981743o CCC: $18.00
Published on Web 01/08/1999

DNA sequences (*17*−*19*). Transition metal complexes were successfully used to generate basic region peptide dimers that distinguished between the DNA sequences that differed by the presence or absence of a single G•C base pair (*20*−*22*).

We have utilized dyad symmetric bridged biphenyl derivatives as the covalent dimerization domain for basic region peptide dimers (*23*−*25*). The basic region peptide dimers that differed only in the chirality of the dimerization domains showed remarkably different affinities for nonspecific DNA sequences. To study the DNA binding of noncovalent peptide dimers, we have functionalized short peptides with an artificial dimerization domain consisting of β-cyclodextrin (Cd) and its guest molecule. The heterodimeric system consisting of the basic region peptide with the adamantyl group (Ad) and that with Cd displayed cooperative and specific binding to palindromic or nonpalindromic DNA sequences, depending on the sorts of basic region peptides (*26*, *27*). The homo-oligomer consisted of identical basic region peptides modified with the adamantyl group at the N terminus and Cd at the C terminus (GAdCd) specifically bound to the direct repeat of two 5 bp DNA sequences with little affinity for a single copy of the 5 bp (*28*). CD spectral measurements suggested that the free GAdCd formed a circular intramolecular host−guest inclusion complex, which added an equilibrium to that between the monomer and the oligomer−DNA complex to affect the sequence selectivity of the peptide with both Ad and Cd. As mentioned previously, our systems for assembling the covalent and noncovalent dimers will complement each other in determining whether the cooperative binding enhances the sequence selectivity of peptide dimers. In this report, we have compared DNA binding of peptide dimers with covalent and noncovalent dimerization domains sharing the same DNA-contacting regions (Figure 1). We find that noncovalent dimers form the specific DNA complexes within narrower ranges of peptide concentrations and bind with higher sequence selectivity than the covalent dimer. We also show that the equilibrium involving a conformational transition of a monomeric peptide effectively reduced the stability of its nonspecific binding complex, hence increasing the efficacy of cooperative dimer formation at the specific DNA sequence.

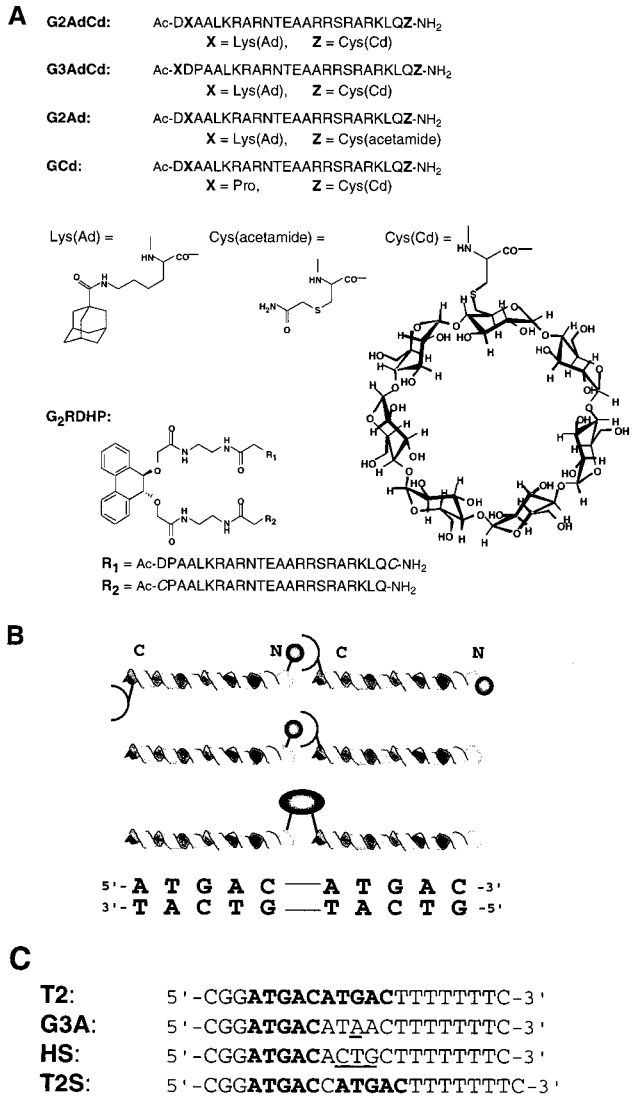

FIGURE 1: (A) Structures and amino acid sequences for the basic region peptide dimers with covalent (G_2RDHP) and noncovalent (G2Ad/GCd, G2AdCd, and G3AdCd) dimerization domains. Abbreviations for the amino acid are as follows: A, Ala; C, Cys; D, Asp; E, Glu; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; and T, Thr. (B) Schematic representations showing the binding orientation of G2AdCd (top), G2Ad/GCd (middle), and G_2RDHP (bottom) dimers to a direct repeat of two GCN4 binding half-sites (T2). The helices denote the basic region peptides, and N and C indicate the N and C termini of the peptide, respectively. Half-circles, filled spheres, and a filled oval represent the artificial dimerization domains: β-cyclodextrin, the adamantyl group, and the 9,10-dihydrophenanthrene derivative, respectively. (C) A specific DNA sequence for peptide dimers consisting of a direct repeat of two GCN4 binding half-sites (T2) and its mutated DNA sequences (G3A, HS, and T2S). G3A and HS contain mutations within the half-site of 1 and 3 bp, respectively. T2S possesses an additional base-pair between two half-sites.

EXPERIMENTAL PROCEDURES

Synthesis of Peptide Dimers. G2AdCd, G2Ad, GCd, and G3AdCd were synthesized as described previously (*28*). G_2-RDHP was synthesized according to the synthesis of the covalent dimers of the GCN4 basic region peptide (*24*, *25*) with slight modifications by using the bridged biphenyl derivatives and two peptides shown in Figure 1B. Data from electrospray mass spectrometry were recorded in the positive ion mode on a Perkin-Elmer Sciex API III. MS (electrospray; 50% acetonitrile and 0.05% formic acid): calcd for [M^+] 5937.84, found 5937.47. Peptide concentrations were determined by quantitative amino acid analyses performed with an AccQ Tag Chemistry Package (Waters) according to the manufacturer's protocol.

Synthesis and 5′-End Labeling of Oligonucleotides HS, G3A, T2, and T2S. Oligonucleotides were synthesized on an Applied Biosystems model 391 synthesizer with the standard phosphoramidite method as described previously (*28*). Following lyophilization, the purified DNA was dissolved in TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. An oligonucleotide (5 pmol) was 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently purified on a Sep-Pak cartridge (Millipore Waters). Following vacuum-drying, the 5′-labeled oligonucleotide was annealed to a 4-fold molar excess of the unlabeled complementary strand in 25 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA (pH 8.0).

Electrophoretic Mobility Shift Assays. All reactions were executed in a total volume of 20 μL with peptide concentrations as indicated in a binding mixture containing ~50 pM ^{32}P-labeled double-stranded DNA, 20 mM Tris-HCl (pH 7.6), 4 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 6% sucrose. DNA binding buffer for G_2RDHP contained additional 0.1% NP40 and 10 mg/mL BSA. After the binding mixture was incubated for 30 min at 4 °C, an aliquot (8−9 μL) was electrophoresed on a 10% polyacrylamide gel in TBE buffer (20 mM Tris, 20 mM boric acid, and 0.1 mM EDTA) at 4 °C. The gels were dried on a Bio-Rad model 583 gel dryer and exposed to X-ray films (Fuji Medical X-ray Films RX) in the dark at −70 °C for 13−15 h.

Determinations of the Stabilities of Dimer−DNA Complexes. ScanJet II and the Deskscan II software were used to obtain data from the X-ray films. The quantification of each band was performed with the NIH-image (version 1.55) software to integrate the intensity of dots of which each band is composed. The fraction of ^{32}P-labeled DNA bound to peptide dimers was obtained by using eq 1:

$$\Theta = [\text{dimer-bound DNA}]/([\text{free DNA}] + [\text{dimer-bound DNA}]) \quad (1)$$

where [free DNA] and [dimer-bound DNA] are the intensities of the bands of the peptide-unbound DNA and peptide dimer-bound DNA, respectively. In the 1:1 dimer−DNA complex formation for G_2RDHP, the experimentally obtained DNA binding data (Θ) were fitted to a theoretical isotherm (eq 2) (*25*) using the equilibrium dissociation constant of the dimer−DNA complex (K_d) as an adjustable parameter.

$$\Theta_{fit} = [pep]_t/(K_d + [pep]_t) \quad (2)$$

where $[pep]_t$ represents a total concentration of the G_2RDHP peptide. In the 2:1 peptide−DNA complex formation, two kinds of equations were used to fit DNA binding data to theoretical isotherms (eq 3 in the case of heterodimer G2Ad/GCd and eq 4 in the case of homodimers G2AdCd and G3AdCd):

$$\Theta_{fit} = 1/\{1 + 2K_{d2}/[K_{d1} + 2[pep]_t - (K_{d1}^2 + 4K_{d1}[pep]_t)^{1/2}]\} \quad (3)$$

$$\Theta_{fit} = 1/\{1 + 8K_{d2}/[K_{d1} + 4[pep]_t - (K_{d1}^2 + 8K_{d1}[pep]_t)^{1/2}]\} \quad (4)$$

where K_{d1} and K_{d2} are the equilibrium dissociation constants of the peptide dimer and the DNA complex of the peptide dimer, respectively. When data sets were fitted using each equation, a value of K_{d1} was fixed to be 1.34×10^{-6} M, an equilibrium dissociation constant of a 1:1 complex with Ad- and Cd-modified basic region peptides (unpublished experiments), and K_{d2} was treated as an adjustable parameter. In eq 3, $[pep]_t$ represents a concentration of G2Ad or GCd and in eq 4 that of G2AdCd or G3AdCd. In this study, concentrations of the G2Ad and GCd peptides were equal. The Igor Pro software (version 2.02; WaveMetrics Inc., Lake Oswego, OR) was used to minimize the difference between experimentally obtained dimer fraction and Θ_{fit} for all data. The apparent equilibrium dissociation constants (K_d) of DNA binding by noncovalent dimer peptides were calculated using eq 5:

$$K_d = K_{d1}K_{d2} \quad (5)$$

ΔG was calculated from the equation $\Delta G = -RT \ln(1/K_d)$, where T is 277 K and R is 0.001987 kcal mol^{-1} K^{-1}.

Measurements of CD Spectra. CD spectra were obtained with a Jasco J-720 CD spectrometer equipped with a PTC-343 temperature controller at 4 °C in a 1 mm cell. Samples contained 20 mM Tris-HCl (pH 7.5), 4 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 4 μM peptide. Spectra were the average of 32 scans and were corrected with a spectrum of buffer alone but not smoothed.

RESULTS

Design of DNA-Binding Peptide Dimers with Covalent or Noncovalent Dimerization Domains. Three sets of peptide dimers that could target the same DNA sequence, but differed in the dimerization domains, were designed to determine whether the sequence selectivity of the covalent dimer could be different from that of the noncovalent dimer. A 23-amino acid peptide derived from the basic region of yeast bZIP protein GCN4 was used as a DNA binding domain for all of the peptide dimers. The noncovalent homodimer G2AdCd possessed both the adamantyl group (Ad) and β-cyclodextrin (Cd) within the same peptide chain (*28*) (Figure 1A). Because the noncovalent homodimerization was regulated by the N-terminal Ad and the C-terminal Cd, G2AdCd bound to the target DNA sequence with a head-to-tail dimer configuration (Figure 1B). The same head-to-tail configuration of a noncovalent heterodimer was achieved by using two basic region peptides: one with the N-terminal adamantyl group (G2Ad) and another with the C-terminal Cd (GCd). A covalent dimer G_2RDHP had a bridged biphenyl derivative (*25*) as a dimerization domain that connected the C terminus of a basic region peptide to the N terminus of another peptide to orient two peptides in the head-to-tail configuration (Figure 1).

DNA Binding of Peptide Dimers with Covalent and Noncovalent Dimerization Domains. On the basis of the facts that a disulfide bonded head-to-tail peptide dimer (*17*, *18*) and G2AdCd (*28*) specifically bound to a DNA sequence consisting of a direct repeat of two GCN4 binding half-sites (T2, 5′-ATGAC-ATGAC-3′), G2Ad/GCd and G_2RDHP were also expected to target the T2 sequence. Two kinds of mutated T2 sequences, HS (5′-ATGACA*CTG*C-3′), which contained three base mutations, and G3A (5′-ATGACAT-*A*AC-3′) with only one base mutation in one of the half-sites, were used to evaluate the sequence selectivity of these head-to-tail dimers (Figure 1C). In addition, reactions with these dimers binding to T2S (5′-ATGAC*C*ATGAC-3′) were carried out to analyze an effect of an additional base pair between the two direct repeat sequences. DNA binding of the dimers to the specific (T2) and nonspecific sequences was tested by electrophoretic mobility shift titrations (*29*, *30*).

Titration of G2AdCd with T2 revealed cooperative formation of a 2:1 peptide−DNA complex (Figure 2A, top). G2AdCd did not bind HS DNA (*28*) or G3A at all even when the peptide concentrations were increased to 200 nM (Figure 2A, bottom). Peptide concentrations that ranged from 4 to 30 nM were required for achieving 10−90% saturations

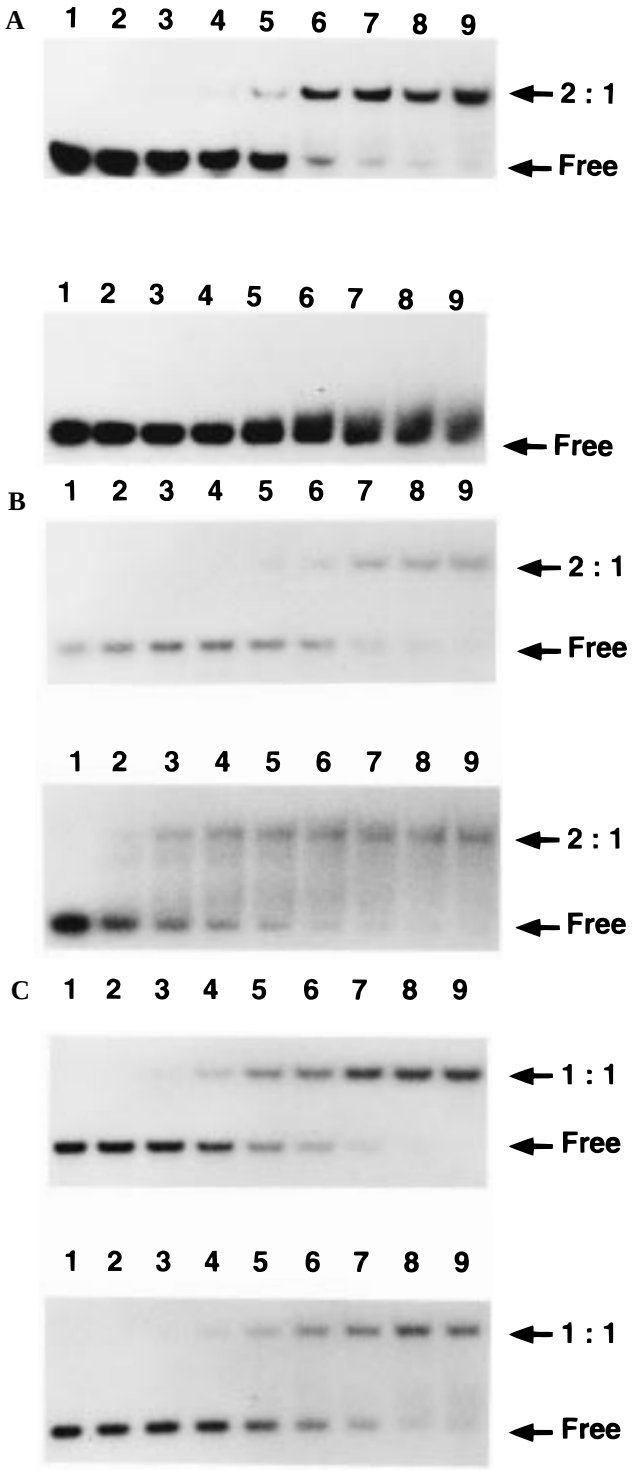

FIGURE 2: Autoradiograms showing the electrophoretic mobility shift titrations of G2AdCd (A), G2Ad/GCd (B), and G_2RDHP (C) to T2 (top) and G3A (bottom). Binding reactions were carried out as described in Experimental Procedures. (A) No G2AdCd was added to the reaction mixture in lane 1. G2AdCd concentrations in lanes 2−9 at the top were 1, 2, 4, 6, 10, 20, 30, and 50 nM for T2 and in lanes 2−9 at the bottom were 5, 10, 20, 30, 50, 75, 100, and 150 nM for G3A, respectively. (B) No peptide was added to the reaction mixture in lane 1. Concentrations of G2Ad/GCd in lanes 2−9 at the top were 0.5, 1, 1.5, 3, 5, 7.5, 10, and 20 nM for T2 and in lanes 2−9 at the bottom were 5, 10, 20, 30, 50, 75, 100, and 150 nM for G3A, respectively. (C) No G_2RDHP was added to the reaction mixture in lane 1. Concentrations of G_2RDHP in lanes 2−9 at the top were 0.025, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM for T2 and in lanes 2−9 at the bottom were 0.1, 0.25, 0.5, 1, 1.5, 2.5, 7.5, and 10 nM for G3A, respectively.

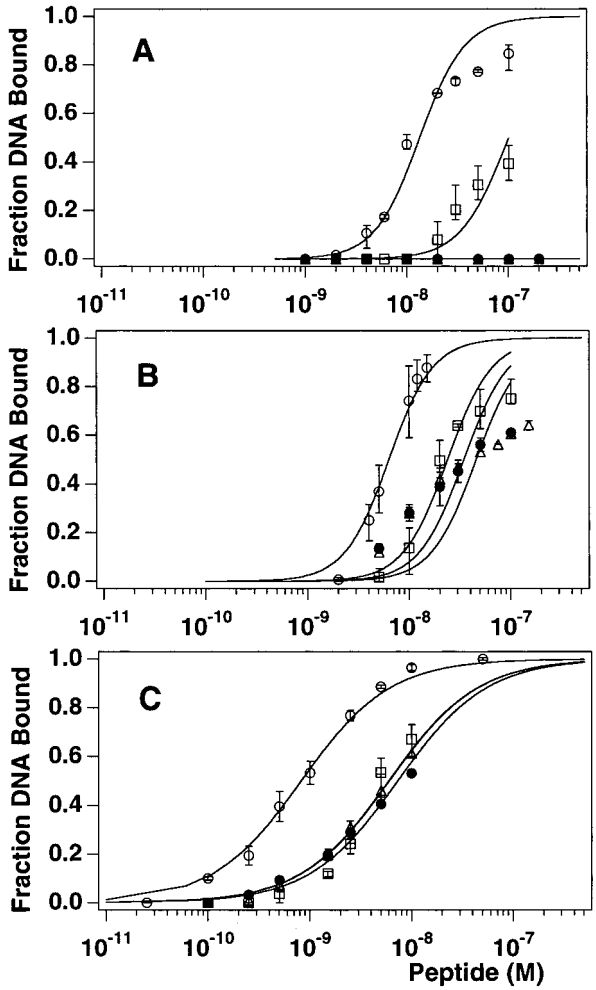

FIGURE 3: Semilogarithmic plots showing the fractions of ^{32}P-labeled T2 (○), G3A (●), HS (△), and T2S (□) bound to the G2AdCd (A), G2Ad/GCd (B), and G_2RDHP (C) dimers as a function of peptide concentration. The solid curves represent the best fit for the titration data obtained as described in Experimental Procedures.

of the T2 site (Figure 3A). G2AdCd formed a dimer−DNA complex with T2S with moderate affinity, but did not achieve full saturation even at 200 nM (Figure 3A). G2Ad/GCd avidly bound to T2 (Figure 2B, top), but also formed dimer−DNA complexes with HS, G3A (Figure 2B, bottom), and T2S with the affinities in the following decreasing order: T2 > T2S > HS > G3A (Figure 3B). Levels of saturation of the T2 site from 10 to 90% were obtained with peptide concentrations that ranged from 2 to 20 nM. G2Ad/GCd bound to HS, G3A, and T2S sites to a saturation level of 10% at ∼5 nM, but did not bind to a saturation level of 90% even at 200 nM. G_2RDHP also formed a 1:1 dimer−DNA complex with T2 (Figure 2C, top), G3A (Figure 2C, bottom), HS, and T2S with stabilities in the following decreasing order: T2 > T2S ≈ HS ≈ G3A (Figure 3C). Concentrations of the peptide required for achieving 10−90% saturations ranged from 0.1 to 7 nM and from 1 to ∼60 nM for T2 and other sequences, respectively (Figure 3C).

Comparison of Sequence Selectivity of Peptide Dimers with Covalent or Noncovalent Dimerization Domains. Free energy changes for the complex formation of the dimers with specific (ΔG_{T2}) and nonspecific DNA sequences (ΔG_{HS}, ΔG_{G3A}, and ΔG_{T2S}) were calculated at 277 K from the

Table 1: Free Energy Changes Associated with Head-to-Tail Dimer Complex Formation for G2AdCd, G3AdCd, G2Ad/GCd, and R_2DHP with T2, HS, and G3A

	ΔG (kcal mol^{-1})[a]				selectivity (kcal mol^{-1})[b]		
peptide	T2	HS	G3A	T2S	$\Delta\Delta G_{HS}$	$\Delta\Delta G_{G3A}$	$\Delta\Delta G_{T2S}$
G2AdCd	−20.0 (±0.2)	> −17.2	> −17.2	−17.9 (±0.1)	>2.8	>2.8	2.1
G3AdCd	−20.6 (±0.2)	−17.6 (±0.4)	−17.7 (±0.3)	−17.5 (±0.2)	3.0	2.9	3.1
G2Ad/GCd	−20.8 (±0.1)	−18.5 (±0.4)	−18.9 (±0.3)	−19.3 (±0.2)	2.3	1.9	1.5
R_2DHP	−11.5 (±0.1)	−10.4 (±0.1)	−10.3 (±0.1)	−10.4 (±0.1)	1.1	1.2	1.1

[a] Determined as described in Experimental Procedures. [b] $\Delta\Delta G_{HS}$, $\Delta\Delta G_{G3A}$, and $\Delta\Delta G_{T2S}$ were calculated from the relationships $\Delta\Delta G_{HS} = \Delta G_{HS} - \Delta G_{T2}$, $\Delta\Delta G_{G3A} = \Delta G_{G3A} - \Delta G_{T2}$, and $\Delta\Delta G_{T2S} = \Delta G_{T2S} - \Delta G_{T2}$, respectively.

equilibrium dissociation constants determined by electrophoretic mobility shift assays (Table 1). By using these values, the sequence selectivity of each dimer was estimated as differences between ΔG_{T2} and ΔG_{HS} ($\Delta\Delta G_{HS}$), between ΔG_{T2} and ΔG_{G3A} ($\Delta\Delta G_{G3A}$), and between ΔG_{T2} and ΔG_{T2S} ($\Delta\Delta G_{T2S}$). The G2AdCd dimer showed significantly higher selectivities for T2 than for HS and G3A compared to the other two dimers, G_2RDHP and G2Ad/GCd, even though the $\Delta\Delta G$ values for G2AdCd were the lowest estimate owing to its lack of binding to HS or G3A below 200 nM. Although the cooperative binding of G2AdCd was very sensitive to the single base pair substitution within the half-site ($\Delta\Delta G_{G3A}$ > 2.8 kcal mol^{-1}), it was relatively tolerant of the spacing mutant ($\Delta\Delta G_{T2S}$ = 2.1 kcal mol^{-1}). G2Ad/GCd discriminated among these sequences with a selectivity higher than that of G_2RDHP. Comparison of the stabilities of G2AdCd dimer−DNA complexes and those of G2Ad/GCd indicated a mechanism that was employed for the high selectivity of G2AdCd. G2AdCd and G2Ad/GCd formed specific T2 complexes with comparable stabilities. On the other hand, the stabilities of the nonspecific DNA complexes of these two dimers differed by more than 1.7 and 1.3 kcal mol^{-1} for G3A and HS, respectively. It appeared that the high sequence selectivity of G2AdCd did not result from the increasing stability of the specific T2 complex, but from decreasing stabilities of the nonspecific complexes. Because both G2AdCd and G2Ad/GCd employed noncovalent dimerization, the highly sequence-selective DNA binding of G2AdCd was not simply explained by the cooperative binding of two monomers to the target DNA sequence.

Stability of the Intramolecular Inclusion Complex Affects the Sequence Selectivity of GAdCd. G2AdCd and G2Ad/GCd share the same DNA binding regions and the same noncovalent dimerization mechanisms. However, free G2AdCd forms the circular intramolecular host−guest inclusion complex that is not formed with G2Ad/GCd (*28*). We next analyzed the role of intramolecular inclusion complex formation in the sequence-selective binding of G2AdCd. The stability of the intramolecular inclusion complex of G2AdCd could be controlled either by substituting Ad with a different guest molecule or by changing its location. G3AdCd possessed the Lys residue with the Ad group at position −1 of the basic region peptide (the aspartic acid is referred to as position 1) instead of at position 2 for G2AdCd (Figure 1A). The CD spectral analyses of these two peptides in the absence of DNA revealed that the [θ] value at 222 nm, a measure of α-helicity, for G3AdCd was lower than that for G2AdCd, indicating that the helical content of G2AdCd was lower than that of G3AdCd (Figure 4A,B). The helical contents of the G2AdCd and G3AdCd variants lacking β-cyclodextrin, G2Ad and G3Ad, respectively, were similar. Changing the position of the adamantyl group did not affect the helix propensity but did alter the helical content of G3AdCd. Because formation of the intramolecular complexes of G2AdCd and G3AdCd peptides necessarily decreased the helical contents of the peptides, the reduction of helical contents paralleled the increase in the population of the intramolecular complex. This in turn implied that the stability of the intramolecular inclusion complex for G2AdCd was greater than that of G3AdCd. The G3AdCd dimer bound to T2 with approximately the same affinity as that for G2AdCd (Figure 5), but bound as dimers to both HS and G3A. Thus, the decreasing stability of the intramolecular inclusion complex resulted in an increasing affinity of the G3AdCd dimer for the nonspecific sequences HS and G3A without affecting its affinity for T2.

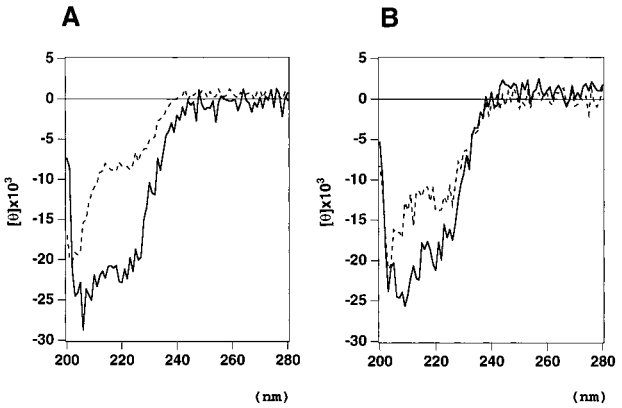

FIGURE 4: Circular dichroism spectra of (A) G2Ad and G2AdCd and (B) G3Ad and G3AdCd indicate that the helical content of G3AdCd is higher than that of G2AdCd. Spectra of the basic region peptides possessing only the adamantyl group (G2Ad and G3Ad, solid lines) and both β-cyclodextrin and the adamantyl group (G2AdCd and G3AdCd, dashed lines).

DISCUSSION

Cooperativity in DNA binding is considered to play significant roles in (i) increasing the sensitivity of equilibrium binding to the change in protein concentrations and (ii) enhancing the selectivity of specific DNA binding (*3*, *8*). The noncovalent peptide dimers G2AdCd and G2Ad/GCd clearly exhibited the former characteristic. As obviously shown by the titration curves in Figure 3A−C, the noncovalent dimers G2AdCd and G2Ad/GCd formed specific dimer−DNA complexes within much narrower concentration ranges as compared to the covalent dimer G_2RDHP. This characteristic also played a part in increasing the selectivity of the noncovalent dimers at the peptide concentrations required for saturating the specific DNA sequence. At the peptide concentration for 90% saturation of the specific T2

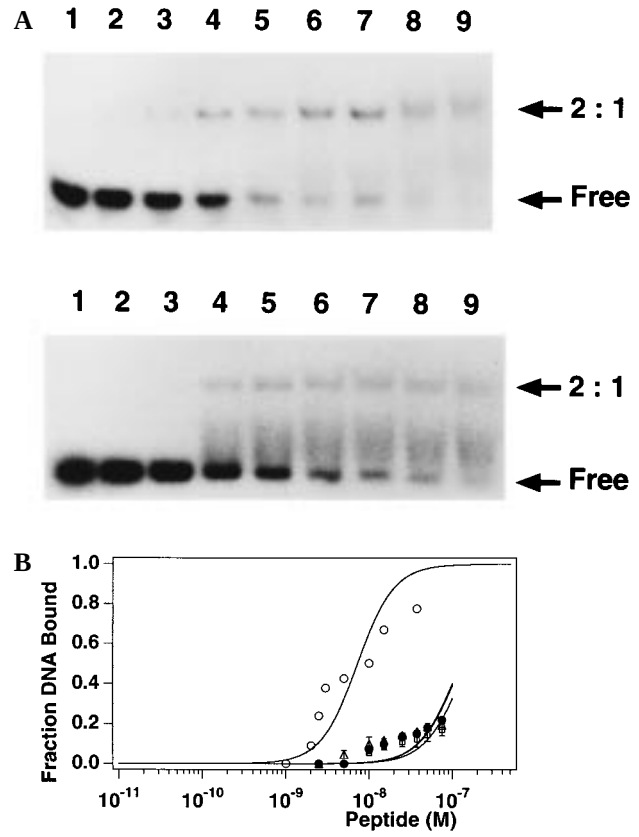

FIGURE 5: (A) Autoradiograms showing the electrophoretic mobility shift titrations of G3AdCd to T2 (top) and G3A (bottom). Binding reactions were carried out as described in Experimental Procedures. No G3AdCd was added to the reaction mixture in lane 1. G3AdCd concentrations in lanes 2−9 at the top were 2, 4, 6, 10, 20, 30, 50, and 100 nM in the case of binding to T2 and in lanes 2−9 at the bottom were 5, 10, 20, 30, 50, 75, 100, and 150 nM in the case of binding to G3A, respectively. (B) Semilogarithmic plots showing the fractions of ^{32}P-labeled T2 (○), G3A (●), HS (△), and T2S (□) bound to the G3AdCd dimer as a function of peptide concentration. The solid curves represent the best fit for the titration data obtained as described in Experimental Procedures.

sequence, G_2RDHP bound the mutated sequence HS or G3A with a saturation level of 50%. The noncovalent heterodimer G2Ad/GCd bound with a saturation level of <40%, and the homodimer G2AdCd virtually did not bind to the mutated DNA sequences when the specific sequence was occupied at a saturation level of 90%. Such comparisons and the $\Delta\Delta G$ values listed in Table 1 clearly indicated that the noncovalent dimers G2Ad/GCd and G2AdCd were more sequence-selective than the covalent dimer G_2RDHP. However, the conclusion that the cooperative binding dimers are more sequence-selective than the covalent dimer is relevant only when the steric factors resulting from the β-cyclodextrin/adamantane and G_2RDHP dimerization domains are equivalent. Previous studies have suggested that structures of the linker region or the covalent dimerization domain connecting each DNA-contacting region are critical for the overall stabilities of DNA binding for complexes of proteins with multiple DNA binding modules (*6*), peptide dimers (*20*, *21*, *22*, *25*), and synthetic polyamides (*31*). The free energy change for G_2RDHP and T2 complex formation was −11.5 kcal mol^{-1}, which was only 2.3 kcal mol^{-1} more favorable than the free energy change for formation of the complex of the G2Ad monomer with HS containing a single copy of the GCN4 binding half-site under the same condition (ΔG = −9.2 kcal mol^{-1}) (*28*). It is possible that the bridged biphenyl group in G_2RDHP is not optimized enough to ensure the effective binding of two monomers. Further studies for evaluating the steric effect of the dimerization domain are necessary to clarify these points.

Does the cooperative interaction energy modulate the sequence-selective DNA binding of the noncovalent dimer? If it is assumed that the stability of a specific complex between a GCd *monomer* and the half-site ($\Delta G_{GCd/half-site}$) is equal to that of the specific complex between the G2Ad *monomer* and the half-site ($\Delta G_{G2Ad/half-site}$ = −9.2 kcal mol^{-1}), the free energy responsible for the cooperative interaction of G2Ad and GCd at the T2 sequence, $\Delta G_{coop(G2Ad/GCd/T2)} = \Delta G_{G2Ad/GCd/T2} - \Delta G_{G2Ad/half-site} - \Delta G_{GCd/half-site}$, is deduced to be −2.4 kcal mol^{-1}. The value of cooperative interaction energy is similar to that obtained for the cooperative binding of oligonucleotides to DNA by triple-helix formation (*32*−*37*) and the cooperative dimer binding of the phage λ repressor (*3*, *38*). G2AdCd did not form a 1:1 complex with HS, indicating that the affinity of the G2AdCd monomer for the 5′-ATGAC-3′ half-site was smaller than that of the G2Ad monomer. This implies that the cooperative interaction energies are gained for the dimer binding of G2AdCd and G3AdCd to T2 more than 1.6 and 2.2 kcal mol^{-1}, respectively. The lower stability of the complex between the G2AdCd monomer and the half-site as compared to that of the G2Ad monomer is likely compensated by the cooperative interaction energy for the binding of the G2AdCd dimer to the T2 sequence larger than that of G2Ad/GCd.

G2Ad/GCd cooperatively formed dimer complexes with HS and G3A, which G2AdCd did not. Such a cooperative enhancement in the affinity of the nonspecifically bound dimer was also observed for the binding of G3AdCd to G3A and HS. In these cases, the bound dimer consists of one specifically bound monomer and one nonspecifically bound monomer, with Cd−Ad complexation apparently stabilizing the complex. Taken together with the results that G2AdCd formed the dimer−T2 complex with the stability similar to that of G2Ad/GCd and G3AdCd, the cooperative interaction energy and the affinity of the monomer for nonspecific DNA rather than the affinity of the monomer for the specific half-site control the stabilities of these half-specific dimer complexes. In fact, G2AdCd formed a dimer−DNA complex with T2S, which only differs from T2 in the spacing between two direct repeats, with a much higher stability than that with HS or G3A. The energetic difference between the G2AdCd dimer−T2 and G2AdCd dimer−T2S complexes (2.1 kcal mol^{-1}) would result from the cooperative interaction energy between two monomers, in case both monomers form specific monomer−DNA complexes with two half-sites of T2S. If this is the case, the selectivity of G2AdCd for T2 over T2S is mainly controlled by the cooperative interaction energy that varies with the DNA sequence.

Then what decreases the affinities of the G2AdCd monomer for the half-site and nonspecific DNA sequences? Even if they shared the same noncovalent dimerization mechanism, formation of the intramolecular host−guest inclusion complex is the key feature for differentiating the DNA binding of G2AdCd from that of G2Ad/GCd. Because the basic region peptide derived from GCN4 binds its target DNA sequence in the α-helical structure (*11*−*13*, *15*, *16*,

28), the G2AdCd peptide necessarily dissociates its intramolecular inclusion complex to bind the target DNA sequence in the helical structure. If the fact that G3AdCd, the G2AdCd derivative with the less stable intramolecular inclusion complex, binds to T2, HS, and G3A with a selectivity between those for G2AdCd and G2Ad/GCd is considered, it seems reasonable to conclude that the equilibrium favoring intramolecular inclusion complex formation reduces the affinities of monomeric G2AdCd for the specific and, possibly to a greater extent, for the nonspecific DNA sequences. The observed high selectivity of G2AdCd for T2 over other sequences is due to (i) decreasing the affinities of monomeric G2AdCd for the specific half-site and for nonspecific DNA sequences and (ii) increasing the cooperative interaction energy of dimer−T2 complex formation.

It has been suggested that modest dimerization might cause cooperative DNA binding only upon the specific DNA site (*39*). In fact, Dervan and co-workers have demonstrated that the cooperative system is more sequence-specific than the noncooperative one for triple helix formation (*36*). Such a DNA binding mode was observed for the noncovalent homodimer G2AdCd. However, the half-specific complexes are stable enough to be observed in the cases of other noncovalent dimers G2Ad/GCd and G3AdCd as reported for some native and non-native proteins with the noncovalent dimerization mechanism (*27*, *39*−*41*). The cooperative formation of the dimer−DNA complex through noncovalent interactions of monomers does not necessarily warrant the high sequence selectivity. The affinity of the monomer for nonspecific sites and the structure of the dimerization domain, which could modulate the cooperative interaction energies, are important factors for increasing the selectivity in the cooperative DNA binding systems. The mechanism involving an equilibrium that leads to formation of less active DNA binding species, such as a conformationally altered monomer, would be quite important for increasing the selectivity and for exerting the cooperative nature of DNA−protein complex formation.

SUPPORTING INFORMATION AVAILABLE

Equilibrium dissociation constants for G2AdCd, G3AdCd, G2Ad/GCd, and R_2DHP with specific and nonspecific DNA sequences (Table S1) and the derivations of eqs 3 and 4 (5 pages). Ordering information is given on any current masthead page.

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BI981743O