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The Recognition of Nucleotides with Model β-Hairpin Receptors: Investigation of Critical Contacts and Nucleotide Selectivity

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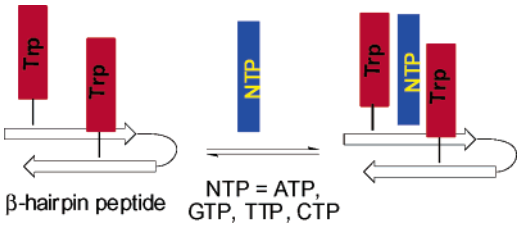

We have investigated the factors that contribute to binding of ATP by a designed 12-residue β-hairpin peptide, **WKWK**, and have determined its selectivity for binding to the naturally occurring nucleotide triphosphates. We have previously shown that **WKWK** creates an ATP binding pocket on one face of the β-hairpin consisting of two Trp and two Lys residues. Mutation of the two Lys residues on the binding face of the β-hairpin resulted in a lower affinity, indicating that each is involved in ATP binding and that each residue contributes approximately −1.5 kcal/mol to the energy of complexation. Replacement of either Trp residue of the ATP binding pocket with Phe or Leu destabilizes the complex formed with ATP by approximately 1 kcal/mol, indicating that both Trp residues participate in interactions with ATP. For binding to the nucleotide triphosphates, the order of binding affinity was shown to follow dTTP > GTP > ATP > CTP, with differences in binding energies spanning as much as 1.6 kcal/mol. NMR analysis demonstrates that both aromatic interactions with the Trp side chains and CH−π interactions between the ribose protons and the Trp residues may contribute significantly to binding. The results from our model system provide useful thermodynamic information regarding protein−nucleic acid interactions that occur at the surface of a β-sheet.

Introduction

The interaction between nucleotides and proteins is a fundamental process occurring in nature, and is central to molecular biology. The recognition of adenosine 5′-triphosphate (ATP) by enzymes, for example, is essential for many signal transduction and energy transfer processes in the cell.[1] ATP binding sites in proteins generally consist of aromatic, aliphatic, cationic, and hydrogen bonding residues.[1] The adenine moiety of ATP is typically found stacked with aromatic side chains of proteins such as Phe and Tyr, while the nucleotide phosphates interact with the side chains of Lys or Arg in a number of protein−nucleotide crystal structures.[2] The aromatic stacking interactions are believed to provide a substantial energetic contribution to the recognition of ATP. Mutation studies have indicated that a single conserved

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stacking interaction between ATP and a Tyr residue of an aminoglycoside transferase contributes approximately −2 kcal/mol to ATP recognition, which is a third of the overall binding energy.[3] Similar interactions regulate the recognition of single-stranded oligonucleotides by proteins, but the interaction typically occurs at the surface of a unique protein architecture. Proteins bind single-stranded nucleic acids on the surface of a conserved five-stranded β-barrel characterized as the OB-fold (oligonucleotide/oligosaccharide-binding fold).[4] Aromatic Trp, Tyr, and Phe side chains protruding from the solvent-exposed β-sheet surface stack with exposed nucleotides, while cationic side chains interact with the phosphate backbone. These interactions have been observed in solution structures, crystal structures, and mutagenesis studies of human replication protein A,[5] *E. coli* single-stranded DNA binding protein (SSB),[6] the U1A protein,[7] the cold shock proteins,[8] and the telomere-binding proteins.[9] For example, mutations of single Phe residues on the binding face of the *E. coli* cold shock protein A (CspA) to Leu side chains decreased the stability of the complex formed with single-stranded DNA by as much as 1.3 kcal/mol.[8c]

The recognition of nucleobases and nucleotides by synthetic receptors is a common strategy to mimic biological recognition. Earlier examples of designed nucleobase receptors have anchored aromatic bases using complementary hydrogen bonding interactions, and an aromatic substituent to simultaneously stack with the base in organic media.[10] Such synthetic receptors have demonstrated stronger nucleobase binding with increased π-surface area of the aromatic substituent on the receptor, and have emphasized the role of ring electrostatics on the observed stacking geometry.[11] Cationic hydrophobic cavities, such as calixarenes and cyclophanes, or cleft-like structures, have achieved the recognition of nucleotides in aqueous media through hydrophobic and electrostatic contacts.[12]

While these bio-inspired synthetic systems have clearly shown that effective nucleotide recognition is possible in the absence of a macromolecular protein receptor, the use of a structured peptide receptor may be more appropriate to probe the interactions between proteins and nucleic acids. Unstructured oligolysine or oligoarginine-based peptides, incorporating one to a few Trp residues, have already been shown to bind single-stranded oligonucleotides through aromatic and electrostatic interactions with some success in water.[13] Additionally, miniature α-helical proteins have been used to bind to an RNA-loop[14] and double-stranded DNA[15] with impressive affinities. A peptide that binds ATP with appreciable affinity has also been reported, although the mode of binding was not determined.[16] With recent advances in de novo protein design,[17] it is now possible to construct a desired protein fold by using a relatively short peptide sequence to study the factors that contribute to protein folding and recognition in an isolated, controlled system. Monomeric β-hairpin peptides are models for antiparallel β-sheets, in which two antiparallel β-strands are connected by a turn nucleator, such as Asn-Gly.[18] Because the first β-hairpins were reported only about a decade ago, most studies of β-hairpins to date have focused on determining the factors that influence β-sheet stability.[19] We have recently found that β-hairpin peptides also present novel molecular receptors for biologically relevant aromatic compounds in water.[20]

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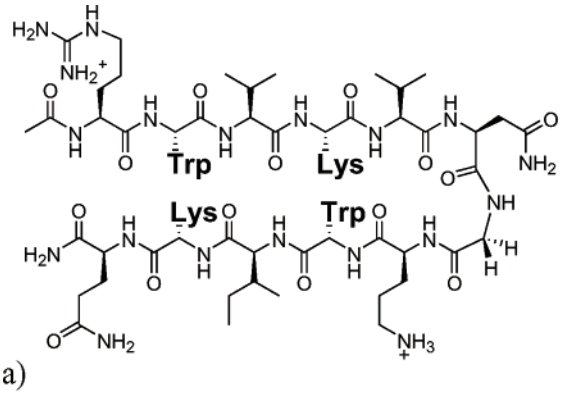

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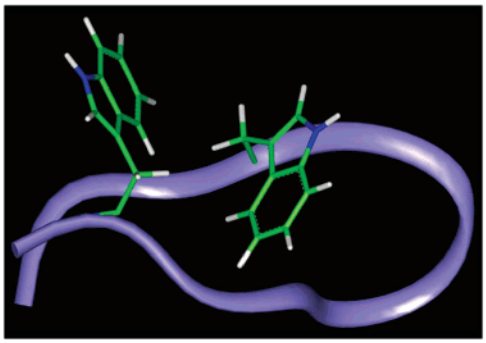

b)

FIGURE 1. (a) Structure of peptide **WKWK** with the sequence acetyl-Arg-**Trp**-Val-**Lys**-Val-Asn-Gly-Orn-**Trp**-Ile-**Lys**-Gln-NH_2. Residues in bold present the binding face for ATP. (b) Solution structure of the backbone of the tryptophan zipper hairpin containing an Asn-Gly turn sequence reported by Cochran et al., with the diagonal Trp side chains shown (pdb code: 1LE1).[23]

In a previous report, we have introduced the designed β-hairpin peptide **WKWK** (Figure 1a) that binds a molecule of ATP strongly in water via aromatic and electrostatic interactions with the diagonal Trp and Lys residues on one face of the β-hairpin.[20a] Intercalation of the adenine ring between the Trp side chains was shown to provide −1.8 kcal/mol to binding, while electrostatic interactions between the ATP phosphates and the Lys side chains contribute about −3 kcal/mol to recognition, leading to a total binding energy of approximately −5 kcal/mol.[20a] The basic β-hairpin sequence was derived from a hairpin reported by Gellman and co-workers[21] and incorporates an Asn-Gly type I′ turn motif.[22] The diagonal relationship of the Trp-Trp pair was chosen based on the solution structure of the Trp zipper β-hairpin reported by Cochran and co-workers, where the diagonal Trp residues in the non-hydrogen bonding sites of the hairpin appear to form a binding cleft for aromatic guests (Figure 1b).[23] The structure shown in Figure 1b illustrates the intrinsic right-handed twist of the β-hairpin, which brings the diagonal Trps at positions 2 and 9 of the 12-residue peptide closer together than expected from a flat projection of the β-hairpin, while the diagonal Lys residues at positions 4 and 11 of **WKWK** are further apart.[24,25] In the present investigation, we have determined the influence of mutation of critical side chains in **WKWK** on ATP binding affinity, and have used cyclic derivatives of the mutated β-hairpins to control for changes in β-hairpin population in the mutant peptides which may effect binding. Additionally, we have investigated the selectivity of nucleotide recognition by **WKWK**. These results provide insight into the energetics of protein−nucleic acid interactions that occur at a solvent-exposed β-sheet surface.

Experimental Section

Peptide Synthesis and Purification. Peptides were synthesized by automated solid-phase peptide synthesis on an Applied Biosystems Pioneer Peptide Synthesizer, using Fmoc protected amino acids on a PEG-PAL-PS resin. The amino acid residues were activated for coupling with HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′,-tetramethyluronium hexafluorophosphate) and HOBT (*N*-hydroxybenzotriazole) in the presence of DIPEA (diisopropylethylamine) in solvent DMF (*N*,*N*-dimethylformamide). Deprotections were carried out in 2% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), 2% piperidine in DMF for approximately 10 min. Extended cycles (75 min) were used for each amino acid coupling step. The N-terminus was acetylated with 5% acetic anhydride, 6% lutidine in DMF for 30 min. Cleavage of the peptide from the resin was performed in 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane (TIPS):water for 3−4 h. TFA was evaporated and cleavage products were precipitated with cold ether. The water-soluble peptides were extracted with water and lyophilized. Cyclization to form the cyclic peptides was performed by air-oxidation of the cysteine residues in methanol over a period of 5 days, or by stirring in 10 mM phosphate buffer, pH 7.5, in 1% DMSO for 3−7 h at room temperature.[26] Peptides were purified by reversed-phase HPLC, using a Vydac C-18 semipreparative column and a gradient of 0−60% B in 50 min, where solvent A was 95:5 water:acetonitrile, 0.1% TFA, and solvent B was 95:5 acetonitrile:water, 0.1% TFA. Once purified, the peptides were lyophilized to powder and peptide identity was confirmed by MALDI mass spectrometry. Peptides were then desalted on polyacrylamide desalting columns and lyophilized to powder.

^{1}H NMR Measurements: Folding Characterization. A 3−5 mg sample of the purified, desalted peptides was dissolved in 10 mM d_3-acetate buffer, pH 5.0 (uncorrected), along with 50 mM 3-(trimethylsilyl)-1-propane sulfonic acid sodium salt (DSS) external standard. ^{1}H NMR scans were obtained on a 600 MHz Varian spectrometer at 25 °C. 1D NMR measurements were collected using water presaturation. 2D NMR measurements used pulse sequences from the Chempack software, including TOCSY, COSY, and ROESY measurements. Peptide proton assignments were made following standard methods.[27] Deviations in α-hydrogen chemical shifts from random coil values, $\Delta\delta H_\alpha$, were calculated according to

$$\Delta\delta H_\alpha = \delta H_{\alpha,obs} - \delta H_{\alpha,RC}$$

where $\delta H_{\alpha,obs}$ is the observed chemical shift of a given α-proton (H_α) in the peptide, and $\delta H_{\alpha,RC}$ is the random coil chemical

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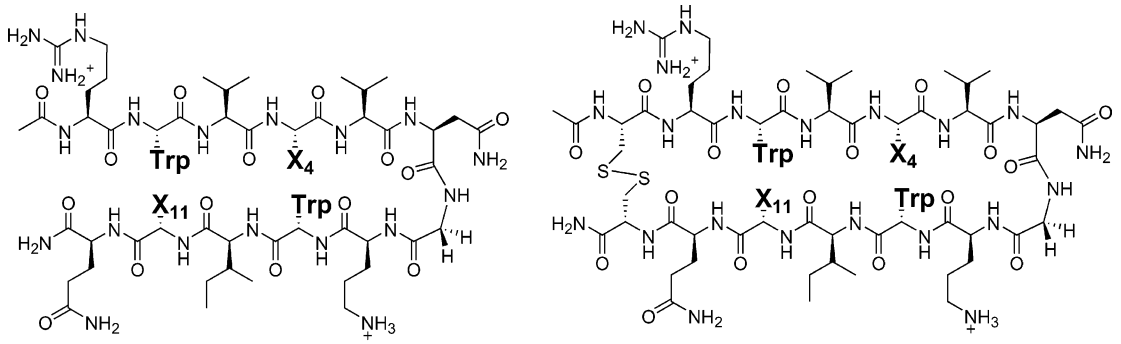

a) **WKWK**: X_4 = Lys; X_{11} = Lys
WTWK: X_4 = Thr; X_{11} = Lys
WKWT: X_4 = Lys; X_{11} = Thr
WRWR: X_4 = Arg; X_{11} = Arg

b) **cycWKWK**: X_4 = Lys; X_{11} = Lys
cycWTWK: X_4 = Thr; X_{11} = Lys
cycWKWT: X_4 = Lys; X_{11} = Thr
cycWRWR: X_4 = Arg; X_{11} = Arg

FIGURE 2. (a) Lys mutants of the ATP binding β-hairpin peptide **WKWK**, referred to as **WX_4WX_{11}**. (b) Corresponding cyclic peptides of the Lys mutants constrained by a disulfide bond, referred to as **cycWX_4WX_{11}**. The residues in bold create the binding face for ATP recognition.

shift of the corresponding proton determined from unstructured 7-mers of the peptide. For example, the random coil references for peptide **WKWK** were taken from sequences Ac-Arg-Trp-Val-Lys-Val-Asn-Gly-NH_2 and Ac-Asn-Gly-Orn-Trp-Ile-Lys-Gln-NH_2. Random coil references were made for each peptide under investigation. Quantification of hairpin folding was determined using the glycine spitting method[19a] and was calculated from the following equation

$$\text{fraction folded} = \Delta\delta\text{Gly}_{\text{obs}}/\Delta\delta\text{Gly}_{100}$$

where $\Delta\delta\text{Gly}_{\text{obs}}$ is the observed splitting of the glycine diastereotopic protons of the peptide and $\Delta\delta\text{Gly}_{100}$ is the glycine splitting for a sequence presumed to take on a 100% fold. $\Delta\delta\text{Gly}_{100}$ was determined from the corresponding cyclic peptide of each sequence.

^{1}H NMR Titrations. Samples of peptide **WKWK** were prepared in 10 mM d_3-acetate buffer, pH 5.0 (uncorrected), using 4,4-dimethyl-4-silapentane-1-ammonium trifluorate (DSA) as an external standard.[28] The concentration of peptide was held at 100−150 μM for titrations with the nucleotide triphosphates. Nucleotides were added in 1−5 μL increments from a 1−1.5 M stock in D_2O and 1D proton spectra were acquired. The concentrations of the nucleotide stock solutions were determined by UV, using the molar extinction coefficients for the nucleotides ($\epsilon_{259,\text{ATP}}$ = 15 400 M^{-1} cm^{-1}; $\epsilon_{253,\text{GTP}}$ = 13 700 M^{-1} cm^{-1}; $\epsilon_{267,\text{dTTP}}$ = 10 200 M^{-1} cm^{-1}; $\epsilon_{271,\text{CTP}}$ = 9 100 M^{-1} cm^{-1}).[29] Binding was measured by fitting the upfield shifting of the tryptophan H-5 aromatic protons, which were completely overlapped in the 1D spectra, to the following equation for 1:1binding on KaleidaGraph, using nonlinear least-squares fitting,[30]

$$\delta_{\text{obs}} = \delta_{\text{R}} + \Delta_{\text{o}}/2[\text{R}_{\text{o}}]\{[\text{R}_{\text{o}}] + [\text{S}_{\text{o}}] + 1/K - \sqrt{(([\text{R}_{\text{o}}] + [\text{S}_{\text{o}}] + 1/K)^2 - 4[\text{R}_{\text{o}}][\text{S}_{\text{o}}])}\}$$

where δ_{obs} is the observed chemical shift of a single tryptophan proton, δ_{R} is the chemical shift of the tryptophan proton in the absence of nucleotide, Δ_{o} is the maximum change in chemical shift at 100% complexation, $[\text{R}_{\text{o}}]$ is the concentration of the peptide receptor, $[\text{S}_{\text{o}}]$ is the concentration of nucleotide substrate, and K is the binding constant. All titrations were carried out at 25 °C.

Fluorescence Titrations. Peptide samples were prepared in 10 mM acetate buffer, pH 5.0. Peptide concentrations were determined in 5 M guanidine hydrochloride, using the absorbance of the tryptophan residues at 280 nm (ϵ = 5 690 M^{-1} cm^{-1}).[31] The concentration of **NalKNalK** was determined using the calculated molar extinction coefficient of the Nal residues at 284 nm in 5 M guanidine hydrochloride (ϵ_{284} = 12 530 M^{-1} cm^{-1}). ATP stock solutions were prepared in 10 mM acetate buffer, pH 5.0, and concentrations were determined by UV/vis spectroscopy. Fluorescence scans were obtained at 25 °C, using an excitation wavelength of 297 nm for Trp or Nal, and the fluorescence at the emission maximum was monitored. The emission intensities of Trp near 345 nm or Nal near 340 nm were fit as a function of ATP concentration to the following 1:1 binding equation on KaleidaGraph, using nonlinear least-squares fitting,[32]

$$I = [I_{\text{o}} + I_{\infty}([\text{L}]/K_{\text{d}})]/[1 + ([\text{L}]/K_{\text{d}})]$$

where I is the observed fluorescence intensity, I_{o} is the initial fluorescence intensity of the peptide, I_{∞} is the fluorescence intensity at binding saturation, [L] is the concentration of added nucleotide ligand, and K_{d} is the dissociation constant.

Aggregation Studies. Peptides were analyzed for aggregation by proton NMR and circular dichroism (CD) over concentrations in the low micromolar to low millimolar range. The CD and proton NMR spectra of the peptides were invariant with changes in concentration, with the exception of peptide **NalKNalK**, which exhibited concentration-dependent shifting in the NMR spectra in the range of 500 μM to 5 mM. Aggregation in hairpin **NalKNalK** was also demonstrated in the low micromolar range by fluorescence; the fluorescence of the Nal side chains quenched with increasing peptide concentration in the concentration range of 10−250 μM, indicating that **NalKNalK** aggregates in the concentration range of the binding studies and folding measurements.

Results and Discussion

Mutation Studies. Evidence for electrostatic interactions between the Lys residues of **WKWK** and the ATP

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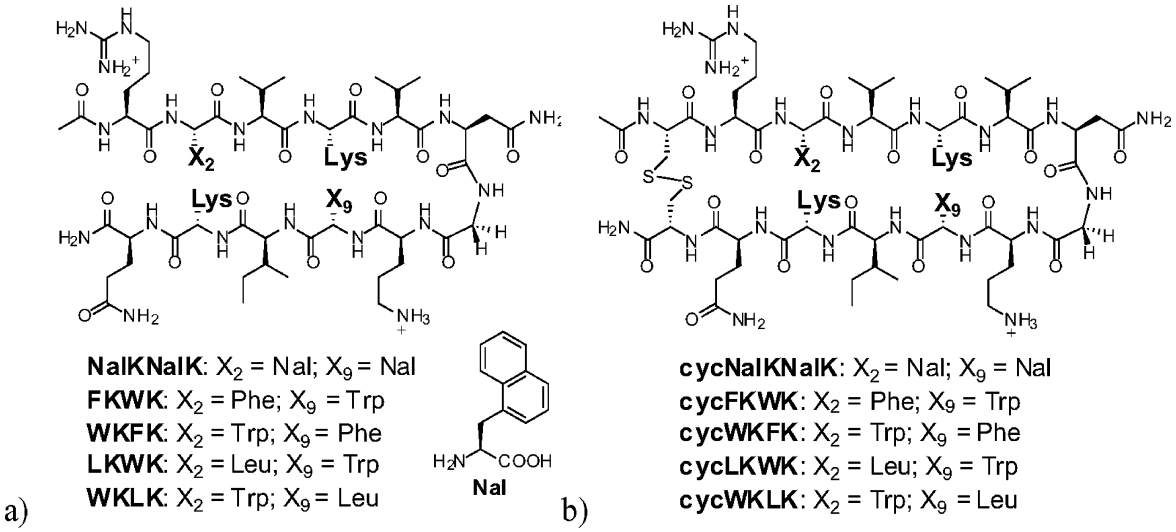

FIGURE 3. (a) Trp mutations of the ATP binding β-hairpin **WKWK**, referred to as $\mathbf{X_2KX_9K}$. The structure of 1-naphthylalanine (Nal) is also shown. (b) Corresponding cyclic peptides of the Trp mutants, referred to as $\mathbf{cycX_2KX_9K}$.

phosphates was previously demonstrated by a salt dependence for ATP binding.[20a] To further investigate the role of the Lys residues of **WKWK** in the binding of ATP, Lys 4 and Lys 11 were mutated individually to neutral Thr residues, generating hairpins **WTWK** and **WKWT**, respectively (Figure 2a). Thr residues were chosen to replace the Lys residues based on the high β-sheet propensity of Thr,[33] and because Thr was assumed to not participate in interaction with ATP, although hydrogen bonding is possible. Both Lys residues were also mutated to Arg side chains generating peptide **WRWR**, to determine if the guanidinium side chain of Arg forms a stronger interaction with the phosphates of ATP. The replacement of a Lys side chain with an Arg residue in cationic polypeptides has led to an increase in binding affinity for single-stranded DNA due to the unique hydrogen bonding capabilities of the guanidinium of Arg.[13b] Furthermore, significant binding enhancements have been observed by replacing Lys residues with Arg in peptides that bind to the TAR RNA loop.[34] A cyclic version of each Trp mutant peptide was also investigated to determine whether changes in the extent of folding of the mutants influenced the observed binding constants (Figure 2b). The cyclic derivatives of the Lys mutant hairpins were constrained into a β-hairpin conformation by a disulfide bond between N- and C-terminal Cys residues.

We have also used mutation studies to probe the contribution of each Trp residue to ATP binding (Figure 3). Trp 2 and Trp 9 were replaced by 1-naphthylalanine (Nal) residues, generating mutant hairpin **NalKNalK** (Figure 3a), to investigate the differences in Trp−adenine and Nal−adenine interactions. The Nal side chain closely resembles the Trp side chain, but has a slightly larger π-surface area and is less electron rich on the face of the ring, which may influence its stacking properties (Figure 4).[35] Furthermore, the mutation of the Trp side chains to Nal allows us to determine if the indole NH of Trp participates in an H-bonding interaction with ATP, since Nal lacks a ring NH functionality. Trp 2 and Trp 9 were each mutated to a Phe residue generating β-hairpin

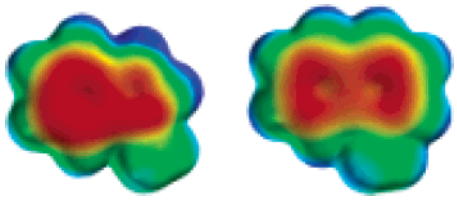

FIGURE 4. Electrostatic potential maps of (a) 3-methylindole and (b) 1-methylnaphthalene, calculated at the HF/6-31G* level, normalized to −20 to 20 kcal/mol (red = electron rich; blue = electron poor).

receptors **FKWK** and **WKFK**, respectively (Figure 3). Similarly, Trp 2 and Trp 9 were mutated to aliphatic Leu residues, giving hairpins **LKWK** and **WKLK**, respectively (Figure 3). The Trp to Phe mutations were performed to investigate the difference in adenine stacking interactions with Trp and Phe aromatic residues in proteins, as both residues are prevalent on the binding face of the OB-fold. The Trp to Leu mutations were performed to determine the necessity of an aromatic residue, as compared to a hydrophobic residue, at the ATP binding site. To account for the differences in the hairpin populations of the Trp mutant hairpins, we also investigated ATP binding to the cyclic derivative of each mutant peptide (Figure 3b).

A β-hairpin structure was demonstrated for each mutant peptide by cross-strand NOEs (see Supporting Information), and the downfield shifting of the α protons (H_α) of the peptides relative to random coil reference sequences (Figure 5).[36] The extent of folding of each hairpin was quantified using the glycine splitting method, in which the magnitude of the splitting of the glycine diastereotopic protons of the turn ($\Delta\delta$Gly) is directly correlated with the fraction fold of the β-hairpin (see the Experimental Section).[19a−c,37] The $\Delta\delta$Gly value for the

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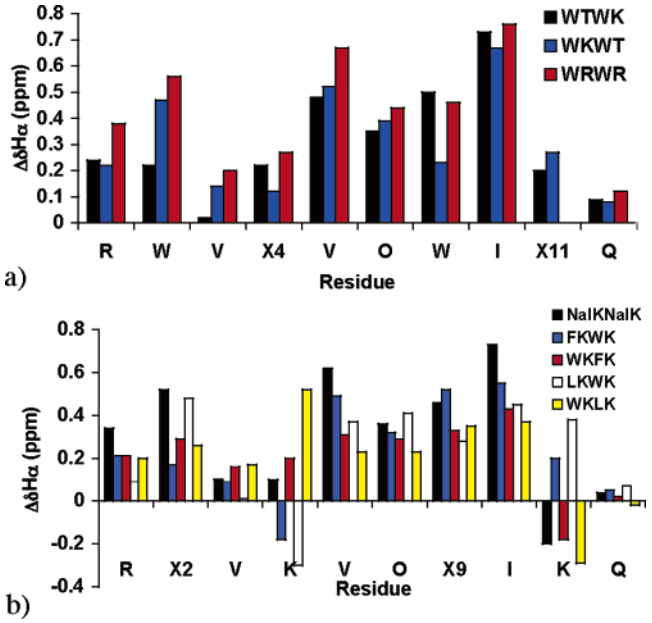

FIGURE 5. H_α chemical shift deviations from random coil values for (a) Lys mutant β-hairpins **WX_4WX_{11}**, where X_4 and X_{11} designate the positions of the Lys mutations, and (b) Trp mutant β-hairpins **X_2KX_9K**, where X_2 and X_9 designate the position of the Trp mutations.

TABLE 1. Folded Populations of β-Hairpins[a]

peptide	% folded
WKWK	96
WTWK	98
WKWT	81
WRWR	93
NalKNalK	100
FKWK	79
WKFK	67
LKWK	85
WKLK	71

[a] The folded populations were determined relative to the corresponding cyclic peptide.

fully folded state was derived from the cyclic derivative of each mutant β-hairpin.[38] The mutated peptides showed folded populations in the range of 70−100% (Table 1).

(a) ATP Binding to Lys Mutants. The binding of the Lys mutant peptides to ATP was determined by fluorescence quenching studies. ATP binding led to quenching of the Trp fluorescence of the β-hairpins in the range of 345 nm, and was fit as a function of ATP concentration to a 1:1 binding isotherm, using nonlinear least-squares fitting (Figure 6).[32] Table 2 gives the affinity constants for ATP binding to **WKWK**, the Lys mutant hairpins, and the cyclic derivatives of each hairpin. It is noteworthy that ATP forms a measurably weaker complex with the cyclic derivative **cycWKWK** relative to **WKWK** (Table 2). This may reflect subtle conformational restrictions of the side chains that form the ATP binding site in the constrained derivative of **WKWK**.

TABLE 2. Affinity Constants for ATP Recognition by Lys Mutant β-Hairpins WX_4WX_{11} and $CycWX_4WX_{11}$[a]

peptide	K_{assoc}, M^{-1}	K_d, mM	ΔG, kcal/mol (error)[b]	$\Delta\Delta G$,[c] kcal/mol
WKWK[d]	5800	0.17	−5.1 (0.2)	-
cycWKWK	2700	0.37	−4.7 (0.1)	0.4
WTWK	280	3.6	−3.3 (0.1)	1.8
cycWTWK	470	2.1	−3.6 (0.1)	1.5
WKWT	320	3.1	−3.4 (0.1)	1.7
cycWKWT	530	1.9	−3.7 (0.1)	1.4
WRWR	2000	0.5	−4.5 (0.1)	0.6
cycWRWR	1600	0.63	−4.4 (0.1)	0.7

[a] Determined in 10 mM acetate buffer, pH 5.0 at 298 K. [b] Errors determined from the average deviation between 2 and 4 separate titration experiments. [c] Change in complex stability with ATP relative to peptide **WKWK**. [d] Data reproduced from ref 20a.

Upon replacing Lys 4 or Lys 11 with Thr, the binding affinity for ATP was decreased by 1.7 to 1.8 kcal/mol in hairpins **WTWK** and **WKWT** relative to β-hairpin peptide **WKWK** (Table 2). This demonstrates the significant contribution of the Lys 4 and Lys 11 residues to ATP recognition by **WKWK**. Covalent constraint of peptides **WTWK** and **WKWT** into a fully folded state by a disulfide bond led to a recovery of −0.3 kcal/mol in ATP binding affinity (Table 2). Thus, it is reasonable to estimate that each Lys residue in peptide **WKWK** contributes approximately −1.5 kcal/mol to ATP binding due to electrostatic interactions with the ATP phosphates. This is in good agreement with our original estimate of −3 kcal/mol for the contribution of electrostatic interactions to ATP recognition by **WKWK**, and supports the fact that only Lys 4 and Lys 11 form electrostatic interactions with the ATP phosphates.[20a]

WRWR formed a complex with ATP that was 0.6 kcal/mol less stable than the **WKWK**−ATP complex (Table 2). The cyclic derivative **cycWRWR** (Figure 2b) bound ATP as well as the unconstrained peptide **WRWR** (Table 2). This was surprising since the guanidinium group of Arg could allow the side chain to participate in a bifurcated hydrogen bond, while providing electrostatic stabilization to the complex,[34] and short oligo-Arg peptides have been shown to bind to single-stranded and duplex DNA more strongly than oligo-Lys peptides.[13b] It may be that there are one or two favorable *intra*molecular cross-strand cation−π interactions between the Arg and Trp side chains of **WRWR** that must be disrupted to form an interaction with ATP, resulting in an unfavorable contribution to the overall binding free energy relative to peptide **WKWK**. Indeed, Trp···Arg interactions have been shown to be stronger than Trp···Lys interaction in β-hairpins[19c] and α-helices.[39] Relative to random coil chemical shift values, the δ-protons of the Arg side chains in **WRWR** were more upfield shifted than the ϵ-protons of the Lys side chains in **WKWK** by approximately −0.2 ppm, suggesting a stronger Trp−Arg interaction. These chemical shift data are in agreement with results reported by Tatko and Waters, where a Trp−Arg interaction was found to be more stabilizing than a Trp−Lys interaction by −0.2 kcal/mol in a β-hairpin peptide.[19c]

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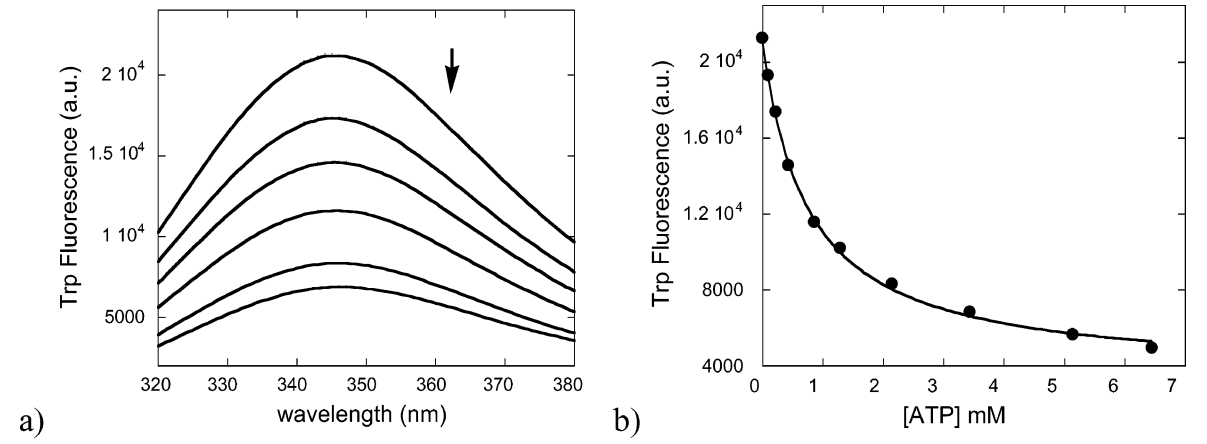

FIGURE 6. (a) Tryptophan fluorescence quenching of **WRWR** in the presence of increasing concentrations of ATP in 10 mM acetate buffer, pH 5.0 at 25 °C ([**WRWR**] = 40 μM, [ATP] = 0−5 mM). The arrow indicates the direction of change in fluorescence intensity with increasing ATP. (b) Fit of fluorescence quenching data to a 1:1 binding isotherm (see Experimental Section).

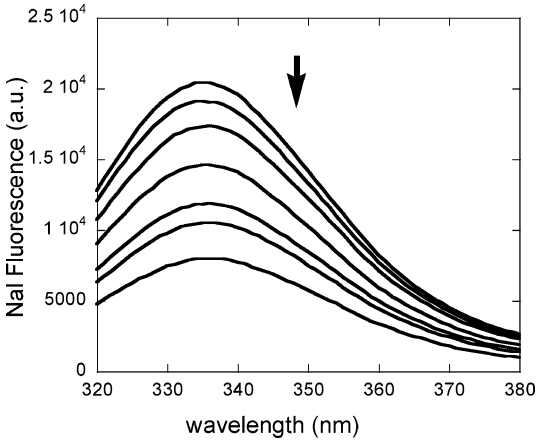

FIGURE 7. Fluorescence quenching of peptide **NalKNalK** in the presence of increasing concentrations of ATP in 10 mM acetate buffer, pH 5 at 298 K ([**NalKNalK**] = 32 μM, [ATP] = 1−1.5 mM). The arrow indicates the direction of change in Nal fluorescence with increasing [ATP].

Alternatively, the Lys or Arg residues may not be positioned in such a way that hydrogen bonding to the phosphate is possible. Rather, the intensity of the charge of the cationic side chain may be more important for the strength of the electrostatic interaction with the phosphates. The Lys side chain more closely resembles a point charge and has been shown to form a stronger interaction with phosphate than the more diffuse electrostatic charge of Arg by theoretical calculations.[40]

(b) ATP Binding to Trp Mutants. The binding of ATP to the Trp mutant hairpins was determined by monitoring the quenching of the Trp or Nal fluorescence of the hairpin with increasing ATP concentration (Figure 7). The mutation of Trp 2 and Trp 9 to Nal residues had a negligible influence on the binding affinity for ATP (Table 3), suggesting that the Nal side chains of **NalKNalK** form a similar stacking interaction with adenine. However, we have found that NalKNalK aggregates in the concentration range studied, which may attenuate its observed association with ATP. The cyclic derivative **cycNalKNalK** binds ATP as well as the unconstrained hairpin **NalKNalK** (Table 3), as expected given the high folded population of **NalKNalK** (Table 1).

TABLE 3. Affinity Constants for ATP Recognition by Trp Mutant β-Hairpins X_2KX_9K and cycX_2KX_9K[a]

peptide	K_{assoc}, M^{-1}	K_d, mM	ΔG, kcal/mol (error)[b]	$\Delta\Delta G$, kcal/mol[c]
WKWK	5800	0.17	−5.1 (0.2)	
cycWKWK	2700	0.37	−4.7 (0.1)	0.4
NalKNalK	4100	0.24	−4.9 (0.1)	0.2
cycNalKNalK	3300	0.30	−4.8 (0.1)	0.3
FKWK	1300	0.77	−4.2 (0.1)	0.9
cycFKWK	490	2.0	−3.7 (0.1)	1.4
WKFK	660	1.5	−3.8 (0.1)	1.3
cycWKFK	1500	0.67	−4.3 (0.1)	0.8
LKWK	720	1.4	−3.9 (0.2)	1.2
cycLKWK	200	5.0	−3.1 (0.2)	2.0
WKLK	1500	0.67	−4.3 (0.1)	0.8
cycWKLW	400	2.5	−3.5 (0.2)	1.6

[a] Determined in 10 mM acetate buffer, pH 5.0 at 298 K. [b] Errors determined by the average deviation between 2 and 4 titration experiments. [c] Change in ATP binding affinity relative to **WKWK**.

The mutation of Trp 2 to Phe (peptide **FKWK**) led to a 0.9 kcal/mol destabilization in the ATP−hairpin complex, while mutation of Trp 9 to Phe (peptide **WKFK**) led to a 1.3 kcal/mol destabilization (Table 3). These results provide additional evidence that both Trp residues of **WKWK** are involved in the binding of ATP. In addition, these results indicate that adenine forms a stronger interaction with the indole side chains of Trp over Phe, as expected due to the larger π-surface area of Trp. Cyclization of peptide **FKWK** further decreased the complex stability with ATP by 0.5 kcal/mol (Table 3). This may be due to unfavorable conformational restrictions of the side chains in the ATP binding site in **cycFKWK**, as proposed with the cyclization of **WKWK** (Table 3). As a result, we attribute the loss in ATP binding affinity that occurs when Trp 2 is mutated to a Phe residue to the loss in a favorable interaction between ATP and Trp 2. In contrast, **cycWKFK** binds to ATP more strongly than the unconstrained peptide **WKFK** by −0.5 kcal/mol (Table 3), suggesting that at least some of the complex destabilization in mutant hairpin **WKFK** results from β-hairpin destabilization. This is reasonable since **WKFK** displays a lower folded population than **FKWK** (Table 1).

(40) Mavri, J.; Vogel, H. J. *Proteins* **1996**, *24*, 495−501.

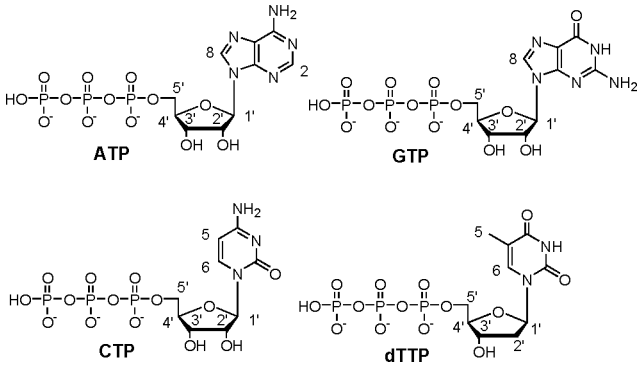

FIGURE 8. Structure of nucleotide substrates ATP, GTP, CTP, and dTTP with proton numbering.

We also found that between 1.2 and 0.8 kcal/mol in complex stability is lost by the substitution of Trp 2 or Trp 9 with an aliphatic Leu residue in peptides **LKWK** and **WKLK**, respectively (Table 3). The cyclization of both **LKWK** and **WKLK** led to a further 0.8 kcal/mol decrease in ATP binding affinity, likely due to side chain restrictions in the ATP binding site of the cyclic derivatives (Table 3). Thus, the loss in complex stability that results from the Trp to Leu mutations is likely a reflection of the loss in favorable ATP interactions with Trp 2 and Trp 9 of the hairpin, rather than β-hairpin destabilization. The mutations of Trp to Leu provide additional evidence that each Trp residue of **WKWK** contributes approximately −1 kcal/mol to ATP binding. Comparison of the cyclic Phe and Leu mutants suggests that the presence of a Phe side chain may be more favorable for ATP binding than a Leu side chain, since **cycFKWK** and **cycWKFK** bind to ATP with 0.6 and 0.8 kcal/mol greater affinity than **cycLKWK** and **cycWKLK**. However, the same trend is only observed for one set of uncyclized peptides, **FKWK** and **LKWK**, for which the difference in binding affinity is 0.3 kcal/mol. Thus, it is difficult to draw any definitive conclusions regarding effects of Phe versus Leu on binding energy.

Nucleotide Selectivity. The selectivity of nucleotide recognition by peptide **WKWK** was investigated by proton NMR titrations to avoid inner filter effects in fluorescence titrations that result from the large absorbance of cytosine, thymine, and guanine bases at the Trp excitation wavelength. The recognition of nucleotides ATP, guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), and thymidine 5′-triphosphate (dTTP) by peptide **WKWK** was investigated (Figure 8). The binding studies were performed in the presence of 10 mM sodium chloride to permit the accurate determination of binding constants in the NMR concentration range.[41] These conditions did not affect the extent of folding of the β-hairpin. Titration of the nucleotide triphosphates into a solution of **WKWK** each produced upfield shifting of the Trp H-5 protons of both Trp residues of the β-hairpin (Figure 9).[42]

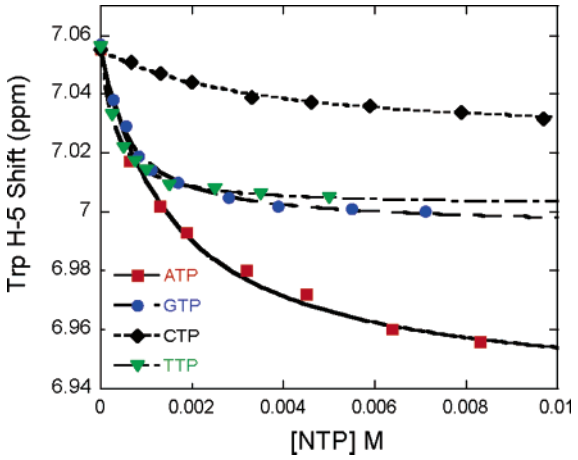

FIGURE 9. Trp H-5 aromatic proton shift of hairpin **WKWK** as a function of nucleotide triphosphate (NTP) concentrations in 10 mM d_3-acetate buffer, pH 5.0 (uncorrected) at 298 K ([**WKWK**] = 98−149 μM). Red squares = ATP; blue circles = GTP; black diamonds = CTP; green triangles = dTTP. The line through the data represents the best fit to a 1:1 binding equation (see Experimental Section). The error in the chemical shift measurements is ±0.002 ppm, which is about the size of the markers.

TABLE 4. Affinity Constants for WKWK Binding to Nucleotide Triphosphates[a]

nucleotide	K_{assoc}, M^{-1}	K_d, mM	ΔG, kcal/mol	Δ_o,[b] ppm
ATP	700	1.4	−3.9 (0.1)	−0.12
GTP	2200	0.45	−4.6 (0.1)	−0.06
dTTP	3700	0.27	−4.9 (0.1)	−0.05
CTP[c]	270	3.7	−3.3 (0.1)	−0.03

[a] In 10 mM d_3-acetate buffer, 10 mM NaCl, pH 5.0 (uncorrected) at 298 K. [b] Δ_o is the maximum change in chemical shift of the Trp H-5 proton obtained by the data fit. [c] N3 of cytidine has a pK_a of 4.2, indicating that the CTP ring may be partially protonated under the solvent conditions of the binding studies.[43]

Upfield shifting of the aromatic protons of both Trp 2 and Trp 9 residues indicates binding of the nucleotide bases between the indole side chains of the β-hairpin. A 1:1 binding stoichiometry for the substrates was demonstrated from Job plots by NMR (see the Supporting Information). Binding saturation was apparent at substantially lower concentrations of dTTP and GTP compared to ATP and CTP binding, demonstrating a higher affinity for these substrates. Fitting of the shifting of the H-5 proton as a function of nucleotide concentration to a 1:1 binding equation provided the binding constants for the nucleotide triphosphates (Table 4).

The association constant for **WKWK** binding to ATP was reduced by approximately 8-fold in the presence of 10 mM sodium chloride, due to screening of favorable electrostatic interactions (Table 4). Peptide **WKWK** demonstrates measurable selectivity for binding to dTTP and GTP nucleotides (Table 4). For **WKWK** binding to nucleotide triphosphates, the order of binding affinity follows dTTP > GTP > ATP > CTP, with differences in binding energies spanning as much as 1.6 kcal/mol. Given

(41) The best binding data results when the host concentration is held at less than the K_d for binding. See: Schneider, H.-J.; Dürr, H. In *Frontiers in Supramolecular Organic Chemistry and Photochemistry*; VCH Publishers: Weinheim, Germany, 1991; pp 129−139. The lowest peptide concentration detectable by NMR was approximately 100 μM.

(42) The Trp H-5 triplets of peptide **WKWK** are overlapped in the 1D proton spectra. These are the only well-resolved resonances in the aromatic region of the 1D spectra.

(43) Bloomfield, V. A.; Crothers, D. M.; Tinoco, I., Jr. In *Physical Chemisty of Nucleic Acids*; Harper & Row Publishers: New York, 1974; p 69.

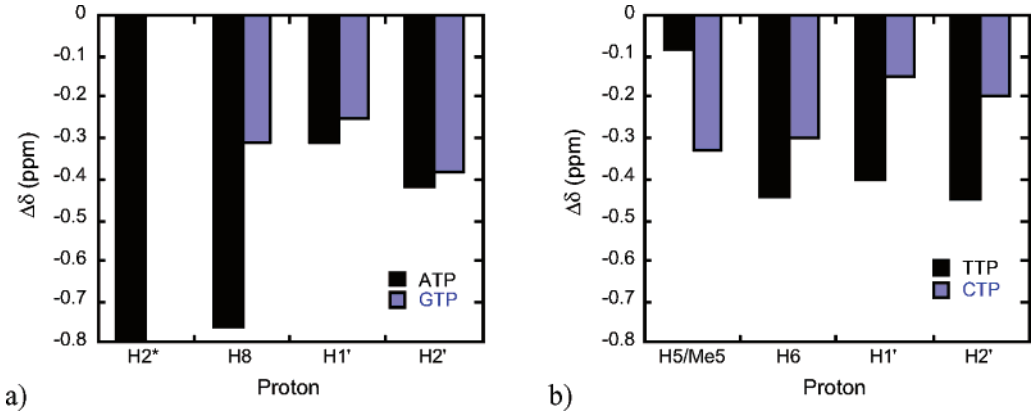

FIGURE 10. Changes in chemical shifts of nucleotide protons in the presence of **WKWK**. (a) Shifting of ATP protons in 3 equiv of peptide, and shifting of GTP protons in 4 equiv of peptide. Proton H2* of ATP was completely overlapped with the Trp protons. The Δδ value reported for ATP H2 was taken from the chemical shift of the most downfield Trp proton and is therefore a lower bound estimate. (b) Shifting of dTTP and CTP each in 3 equiv of peptide.

that adenine has demonstrated the strongest stacking ability in model systems,[12c,44] and even with indoles,[45] our observed selectivity for dTTP and GTP is surprising. Because ATP is known to self-associate, we corrected the observed K_d for self-association[46] using reported literature values for the self-association constant.[47] However, the magnitude in the change in K_d upon correction was within error of our measurements. The weak association with CTP is in agreement with previous model studies that have shown relatively weak stacking interactions with cytidine compared with the other nucleobases in DNA.[44,45] Because CTP has a pK_a of 4.2, we repeated the binding study of CTP at pH 7. The K_d at the higher pH was within error of that measured at pH 4.2, suggesting that protonation of CTP is not the reason for its weak binding affinity. The selectivity for GTP and dTTP may be related to the presence of the carbonyl at position 4 in both rings, which could hydrogen bond with the flanking Lys residues. However, the presence of such a hydrogen bond was not determined.

The change in chemical shift of the Trp H-5 proton of **WKWK** at binding saturation, Δ_o, is the greatest for binding to ATP, suggesting that the face of the adenine ring approaches the Trp H-5 proton in the bound state. Although the shifting at the H-5 protons is small for binding of dTTP, GTP, and CTP (Table 4), the magnitude of the shifting is well outside the experimental error in chemical shift measurements.[48] The modest Δ_o values for GTP, dTTP, and CTP binding may reflect a lesser degree of overlap between the aromatic ring faces of the nucleobases with the Trp H-5 protons.

The change in chemical shifts of the aromatic protons of the nucleotides when bound to **WKWK** provides additional evidence for the occurrence of aromatic interactions in binding. Upfield shifting of aromatic base protons was observed for all nucleotides bound to **WKWK**, supporting a stacking interaction with the Trp residues of the β-hairpin (Figure 10). Upfield shifting of the aromatic base protons was especially pronounced for ATP binding to **WKWK**, in which the magnitude of shifting was 2−3 times greater than the shifting that occurred at the aromatic protons of dTTP, GTP, and CTP (Figure 10). Although this suggests that ATP binding is associated with the greatest π−π overlap in the bound state, it may also reflect differences in the conformation of the aromatic interaction, which may result in different extents of upfield shifting. Interestingly, upfield shifting of the ribose protons was observed as well, indicating that they are also in close proximity to the face of the Trp aromatic rings, and may also contribute to binding via favorable CH−π interactions (Figure 10). For ATP and CTP, upfield shifting of the nucleobase protons is greater than that of the ribose protons, but for GTP and dTTP, the upfield shifting of the nucleobase and ribose protons is similar in magnitude. This may suggest a larger relative contribution of CH−π interactions for binding of GTP and dTTP.

Conclusions

The designed β-hairpin receptor **WKWK** presents a useful scaffold for investigating interactions that contribute to nucleotide recognition. With cationic and aromatic Trp residues displayed on the surface of the hairpin, **WKWK** represents a minimalist model system for studying the noncovalent interactions that contribute to binding of ssDNA by the OB-fold motif. Through mutation studies, we have confirmed the participation of Lys−phosphate salt-bridges in mediating nucleotide recognition and have demonstrated the potential of this interaction to provide a large contribution to the overall binding energy, even at a solvent-exposed surface. Mutation studies of the critical Trp side chains of the binding cleft indicate that the Trp residues interact with ATP in a noncooperative fashion, such that each Trp residue forms individual transient stacking interactions with adenine worth approximately −1 kcal/mol each. The mutation studies corroborate our original energy estimates for the contributions of the Lys and Trp residues of **WKWK** to ATP recognition. Moreover, we find stronger binding with the uncyclized peptide **WKWK** than with

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(45) Mutai, K.; Gruber, B. A.; Leonard, N. J. *J. Am. Chem. Soc.* **1975**, *97*, 4095−4104.
(46) Deans, R.; Cooke, G.; Rotello, V. M. *J. Org. Chem.* **1997**, *62*, 836−839.
(47) Peral, F.; Gallego, E. *Biophys. Chem.* **2000**, *85*, 79−92.
(48) The error in chemical shift measurements is ±0.002 ppm.

its cyclized counterpart, indicating a well formed binding site in **WKWK**. Upon investigating nucleotide selectivity, we have found that peptide **WKWK** demonstrates a preference for binding to dTTP and GTP nucleotides. NMR chemical shift measurements indicate that aromatic interactions are present between **WKWK** and each nucleotide triphosphate substrate investigated. Upfield shifting of the ribose protons of all four nucleotides suggests that CH$-\pi$ interactions with the ribose ring may also contribute to binding. These results contribute to the general thermodynamic understanding of protein–nucleic acid interactions that occur at a protein β-sheet surface and highlights the potential of using de novo protein design to create functional miniature proteins.

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Supporting Information Available: Proton assignments for all peptides, observed cross-strand NOEs in β-hairpins and cyclic peptides, and Job plots. This material is available free of charge via the Internet at http://pubs.acsorg.

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